COMMERCIAL PROSPECTS FOR GENE THERAPY AND THE NEWLY EMERGING HUMAN GENE ESTATE INDUSTRY

by Nevin M. Summers

Master in Architecture (1980)
Harvard University Graduate School of Design

B.S., Natural Sciences (1971)
The Johns Hopkins University

Submitted to
The Alfred P. Sloan School of Management and The School of Engineering
in partial fulfillment of the requirements for the

Degree of Master of Science in the Management of Technology
at the Massachussetts Institute of Technology
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Signature of Author____
Alfred P. Sloan School of Management
May 7, 1993

Certified by____
Charles L. Cooney, Thesis Supervisor
Professor of Chemical and Biochemical Engineering

Certified by____
Edward B. Roberts, Thesis Reader
David Sarnoff Professor of Management of Technology

Accepted by____
Rochelle Weichmann, Director
Management of Technology Program

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ABSTRACT

Biotechnology began an enabling technology that allowed the isolation and transfer of fully functional genes from humans into non-human organisms amenable to industrial processing. This enabling technology has advanced and is now being successfully applied to 1) precisely map and sequence the human genome, the so-called "blueprint of life" 2) identify genes causing inherited metabolic disorders, certain behavioral disorders, cancers, aging, and susceptibility to infection and 3) treat and, in some cases, permanently cure diseases by the direct transfer of genes with corrective capability into the bodies of patients. Such transfer of corrective genes is termed "gene therapy".

In addition to this restoration of normal gene function and health, it is also possible to create genes which confer enhanced or totally novel capabilities. By exploiting the extraordinarily vast and untapped potential diversity present in the nucleotide sequences of genes, advances in molecular evolutionairy technology offer the possibility of designing new genes with desired functions not normally found in Nature. The era of elective human gene design, already on the horizon, will change the pharmaceutical industry and indeed all of healthcare in ways that today cannot be adequately predicted.

As a result of these genetic repair and genetic enhancement technologies, a new commercial activity is emerging, referred to here as the "Human Gene Estate Industry". Like the Real Estate Industry, which uses socially defined/enforced instruments such as deeds and leases to convey land use rights, the Human Gene Estate Industry similarly uses patents and licenses to convey gene use rights. These naive similarities between land- and gene-based ventures halt abruptly, however, when one considers that Humankind has not had the benefit of thousands of years to reflect and act thoughtfully upon the ethical, legal and social implications and consequences of treating the most basic elements that constitute the human species as "commercial property".

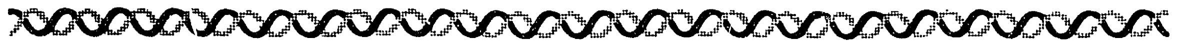
Yet technical advances and global economic competition relentlessly push wider the envelope of human genetic possibility, not only revolutionizing the quality of life and healthcare but also challenging social institutions as well as each of us individually to reassess fundamental ethical values. This thesis explores the current status of gene therapy as an emerging industry and assesses the barriers, both social and technical, which limit its acceptance and commercial diffusion.

Thesis Supervisor: Charles L. Cooney
Professor of Chemical and Biochemical Engineering

Thesis Reader: Edward B. Roberts
David Sarnoff Professor of Management of Technology

DEDICATION

To the memory of Mrs. Walter Gropius, who introduced me to the theory of the Bauhaus and who encouraged me to explore architectural design opportunities in new, uncharted domains.



ACKNOWLEDGEMENTS

Molecular biology has blossomed since I left the academic research community 20 years ago to pursue a career in architecture and real estate development. My recent decision to return to molecular biology, specifically biotechnology, has given me the opportunity to rekindle some old friendships as well as make many new ones. I thank my fellow Westinghouse Science Talent Search Scholars Ellie Ehrenfeld of UC Irvine, Wally Gilbert of Harvard, Eric Lander of the Whitehead Institute and Tom Schneider of the National Cancer Institute and my former Professors Kirby Smith of Johns Hopkins, Bob Schimke of Stanford and Don Brown of the Carnegie Institution of Embryology for helping me understand and grasp the significance of the achievements of the last 20 years of biology.

I also thank Jim Becker, President/CEO of Beacon Construction Company and Senior Lecturer at the MIT Center for Real Estate Development, my friend, business associate and mentor over the last dozen years, for supporting me in my career transition from building highrises, constructs made of glass and steel, to building cells, constructs made of genes and proteins.

Buildings and cells have many properties in common. Both are complex assemblies, each having their own structural language or "architecture". Both have great social utility, one providing shelter, the other health. Both can be thought of as environments, one being outer, the other inner. Both can also be designed and constructed to human specifications using advanced technology. Until recently, the technology to modify cell function did not exist. It now does. The societal implications of our new capability compel us to advance our concerns beyond the simple technical question "how can we make our tool work better?" to include the more socially complex question "now that we have our tool, what *should* we build with it?". It is the latter question which interests me as a formally trained architect. Perhaps my double background as a molecular biologist and as an architect will allow me some useful insight into this topic.

I thank the faculty members of the Whitehead Institute and the MIT Biology Department for allowing me to attend their lectures. The following courses were invaluable in preparing this thesis:

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  by David Housman, Lewis Holmes and Clifford Tabin
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  by David Page and Eric Lander
- *Molecular and Engineering Aspects of Biotechnology (7.37J)*
  by Tony Sinskey, Dan Wang and Harvey Lodish
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  by Paul Schimmel and Alex Rich
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  by Alex Varshavsky
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  by Tom Allen, Charlie Cooney, Stan Finkelstein and Tony Sinskey

I thank David Page of the Whitehead Institute for letting me perform some experiments in his lab in order to re-aquaint myself with the practice of science. I thank Jon Beckwith, Lisa Geller and Marvin Natowicz of the Genetic Screening Study Group at Harvard Medical School for sensitizing me to some of the social aspects of genetic practice. I thank Michael Grodin, Charles DeLisi and Charles Cantor of Boston University for their insights and their seminars on the patenting of genes and on the ethics of gene therapy.

I thank Florence Sender and Lester Thurow of the MIT Sloan School of Management for inviting me to work with them on the formation of a MIT Biotech CEO Roundtable to discuss industry policy and global competitiveness. I am especially grateful to Florence for expressing her vision of socially responsible business in her course, *Maverick Entrepreneurs (15.977)*, and to Lester for writing <u>Head to Head</u> and for teaching *Applied Macroeconomics for Managers (15.966)*. Much thanks also goes to Mike Rappa for *Assessment of Emerging Technology (15.374)* and Shoji Shiba for *Total Quality Management (15.766)*. MIT has been a wonderful mid-career experience for me. I recommend it highly to anyone wishing to expand their professional skills and their horizons.

I wish to express my gratitude to all of the experts (listed in the appendix) who generously gave their time and freely shared their insights with me. Thanks also goes to my faculty advisors, Charlie Cooney and Ed Roberts, for their sincere interest in my academic and professional development.

Lastly, I wish to acknowledge the patience and support of my wife, Barbara, and my daughter, Nova, who stood by me during all the challenges that come with an academic sabbatical at MIT.

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CHAPTER 1
INTRODUCTION

"Just as our present knowledge and practice of medicine relies on a sophisticated knowledge of human anatomy, physiology and biochemistry, so will dealing with disease in the future demand a detailed understanding of the molecular anatomy, physiology and biochemistry of the human genome.

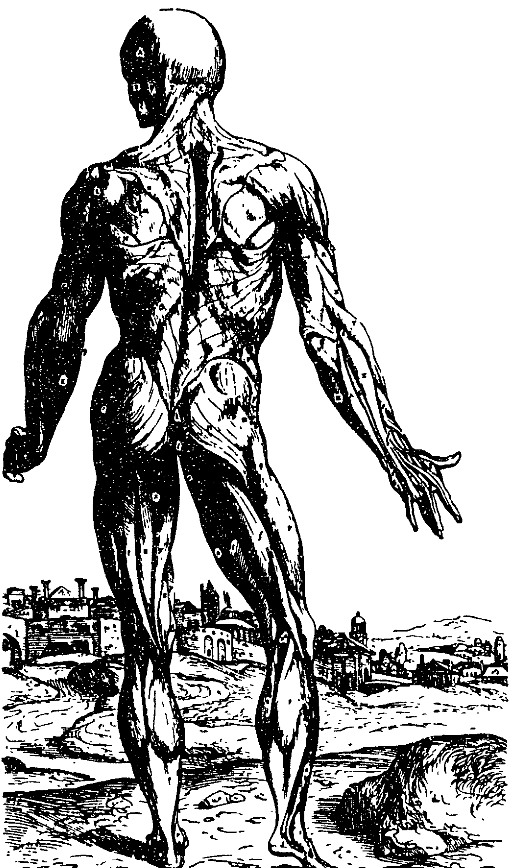
We shall have to have physicians who are as conversant with the molecular anatomy of chromosomes and genes as the cardiac surgeon is with the structure and workings of the heart and circulatory tree."

Paul Berg, M. D.
Nobel Lecture, 1981

1.1 Gene Therapy as Advanced Biotechnology

Gene therapy is the logical and inevitable result of centuries of tradition in Western medicine, science and technology. We have advanced our understanding of human physiology and disease to ever smaller and more fundamemtal domains, from the gross anatomical description of the body (**Figure 1.1**), to the organs and body systems, to the cell and the organnelles within, and ultimately to the molecules that confer structure, provide energy, and store the cumulative evolutionairy history of billions of years in a form that is passed on from generation to generation.

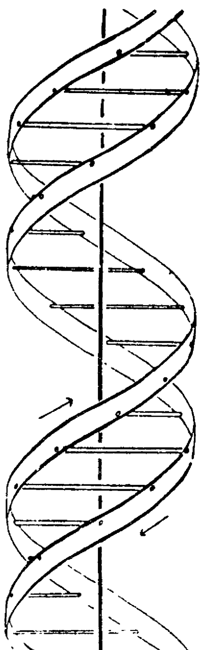
The studies of the mechanisms of inheritance have led us to the remarkable realization that all living things, all micro-organisms, plants and animals alike, use the same storage medium, DNA, and that they organize it into the same basic unit of inheritance, the gene. Moreover, all organisms convert this information into proteins, the key structural and functional elements of the body, using the same genetic code and the same molecular machinery.



1543

De Humani Corporis Fabrica

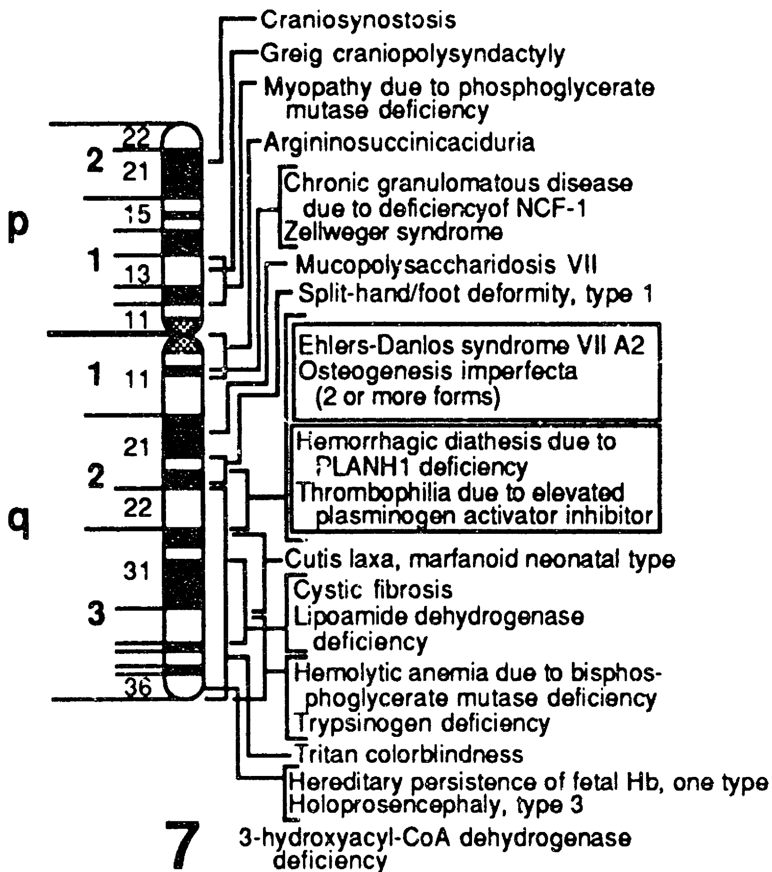
Vesalius



1953

Structure of DNA

Watson and Crick



The Present

Morbid Anatomy of the Chromosome

McKusick & Human Genome Project

Figure 1.1 Major milestones in human anatomy.

The promise of using this universal genetic code and assembly mechanism to make useful protein products, human insulin for instance, in culturable organisms such as bacteria remained unfullfilled until the advent of so-called gene splicing technology. Working in the early 1970's at Stanford and the University of California at San Francisco, Stanley Cohen and Herbert Boyer developed the basic techniques of cutting and joining DNA and of transferring fully functional genes from any organism into bacteria. With their patented invention, the entire industry of biotechnology was begun.

As a result, we now have reliable sources of highly purified products with life saving therapeutic value. For example, hemophiliacs, who suffer from a severe inherited inability to clot their blood, now have pure sources of the protein factors that they need to live. Formerly they had to rely on human blood products that were unknowingly contaminated with HIV, the virus that causes AIDS or with hepatitus virus. Many hemophiliacs contracted these diseases as a result of impure blood transfusions. The number of products made with recombinant DNA technology is impressive.

Biotechnology has certainly revolutionized the means of manufacturing. With the exception of diagnostics, which offers fundamentally new information about disease (discussed later in Chapter 3), biotechnology has not yet however made any major fundamentally new contribution to therapeutic medicine. The industry's flagship products have been known and used for years, albeit produced from less consistent biological sources. For example, insulin extracted from porcine pancreas was used as a therapy for diabetes for many years before the recombinant form arrived on the market.[1]

Gene therapy is about to become the first major new advance in the treatment of disease. The addition of a foreign gene into the cells of a patient is made possible by advances in recombinant DNA technology.

An unsuccessful attempt to carry out gene therapy for the blood disorder beta-thalassemia was made in 1980 in Italy and Israel by Dr. Martin Cline, Chairman of Hematology at UCLA (Larrick and Burck 1991). Dr. Cline did not have prior

[1] Even today, many products of animal origin such as bovine hemoglobin and baboon hearts are under consideration for human use.

approval from the UCLA Human Subjects Protection Committee and, while he had received approval from the Israeli ethics review board for a protocol involving native genes, at the last minute he substituted genes made by recombinant methods. The attempted therapy neither caused harm to the patients, nor improved their clinical condition. As a result of this ethics violation, lack of sufficient animal model work, inadequate safety testing and disregard for certain objections by American review boards, Dr. Cline resigned his chairmanship and had his federal grants terminated for four years. This episode catalyzed a consensus in the scientific community that much more research on the methods of gene transfer was necessary before entering the clinic. Public sensitivity to recombinant DNA technology was heightened, resulting in the establishment of extensive formal review procedures for any experimentation in humans (see Chapter 6). Human gene therapy got off to an unfortunate rocky start and there was a moratorium on the field until 1989.

After years of extensive ethical, scientific and medical review, the first federally approved transfer of a gene into the cells of a human patient took place on May 22, 1989 (Anderson 1992). The gene was used simply as a marker to follow the fate of cells in a patient with advanced cancer. It had no corrective or therapeutic function. The first use of a gene for therapeutic purposes occurred on September 14, 1990 when patients suffering from an inherited deficiency for the enzyme, adenosine deaminase (ADA), which is necessary for the proper functioning of the immune system, received injections of white blood cells to which the gene coding for the enzyme had been added. As of June, 1993 there are a total of 23 gene transfer (marker) protocols and 29 gene therapy protocols approved for human clinical testing by the Recombinant DNA Advisory Committee of the National Institutes of Health (NIH-RAC), one of the two federal bodies that reviews such procedures. The other is the Food and Drug Administration, but, as discussed later in Chapter 6, their review is kept confidential until a manufacturing license issues. A graph of the number of protocols approved by the NIH-RAC is given in **Figure 1.2** and a listing of the titles, investigators and institutions is given in **Appendix B**. As will be discussed in later chapters, gene therapy is being applied to many different diseases, both inherited and acquired. Not only are the rare genetic diseases being targeted, but also the more frequent ones such as heart disease and cancers of many types. Infectious diseases such as AIDS, hepatitus, influenza and malaria are also on the gene therapy hit list.

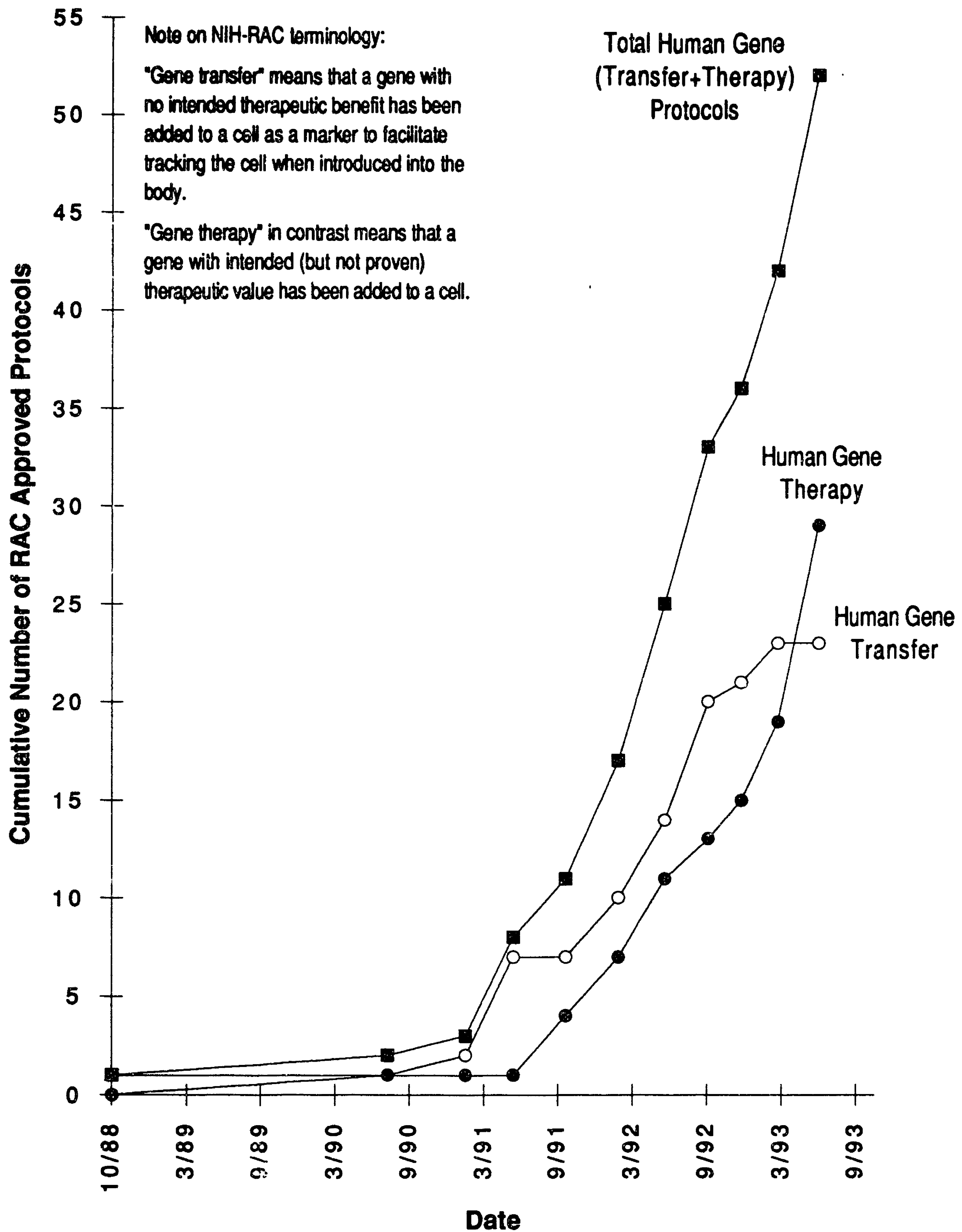


Figure 1.2 Human gene transfer and gene therapy protocols approved by the National Institutes of Health Recombinant DNA Advisory Committee.

1.2 Scientific, Technological and Commercial Factors Contributing to Advances in Gene Therapy

Before exploring the current status of the field, it is worthwhile to look backward at recent scientific and technological advances which have made gene therapy a possibility. A graph of the major medical breakthoughs based on DNA is given in **Figure 1.3.** The basic data, taken from an article by Caskey, have been supplemented with key technological advances such as the Polymerase Chain Reaction, DNA sequencing and DNA separation. While such technologies are themselves not of medical benefit, they are frequently the rate-limiting steps in the development of knowledge. In a field such as modern biology where technology, the pursuit of "know-how", and science, the pursuit of "know-why" are both growing in exponential leaps and bounds, it is difficult to precisely map cause and effect. What is clear, however is that there is a pronounced synergy between the two.

Another major contributing factor to advances in molecular biology in general and gene therapy in particular is the development of a robust commercial sector providing essential goods and services.

Innovations made in the research laboratory setting find their way into the marketplace with astonishing speed. What was the responsibility of the graduate student or the lab assistant one year, may be effectively out-sourced the next year to companies positioned to achieve the economies of scale possible through specialization. Not only are new commercial opportunities created, but, more importantly, individuals are freed from responsibilities which make inefficient use of their time. A well-developed service industry allows researchers to focus on what they do best, and leave preparation and ancillary work to others.

For instance, Wally Gilbert, a Harvard molecular biologist who won the Nobel Prize in part for his co-invention of the first DNA sequencing technology, has noted that students thirty years ago were expected to blow their own lab glassware. Today, disposable pre-sterilized flasks and pipettes are commonplace. Fifteen years ago students were expected to purify their own enzyme reagents, even isolate their own bacterial strains. Now there are companies who commercially provide these materials and who invest their own

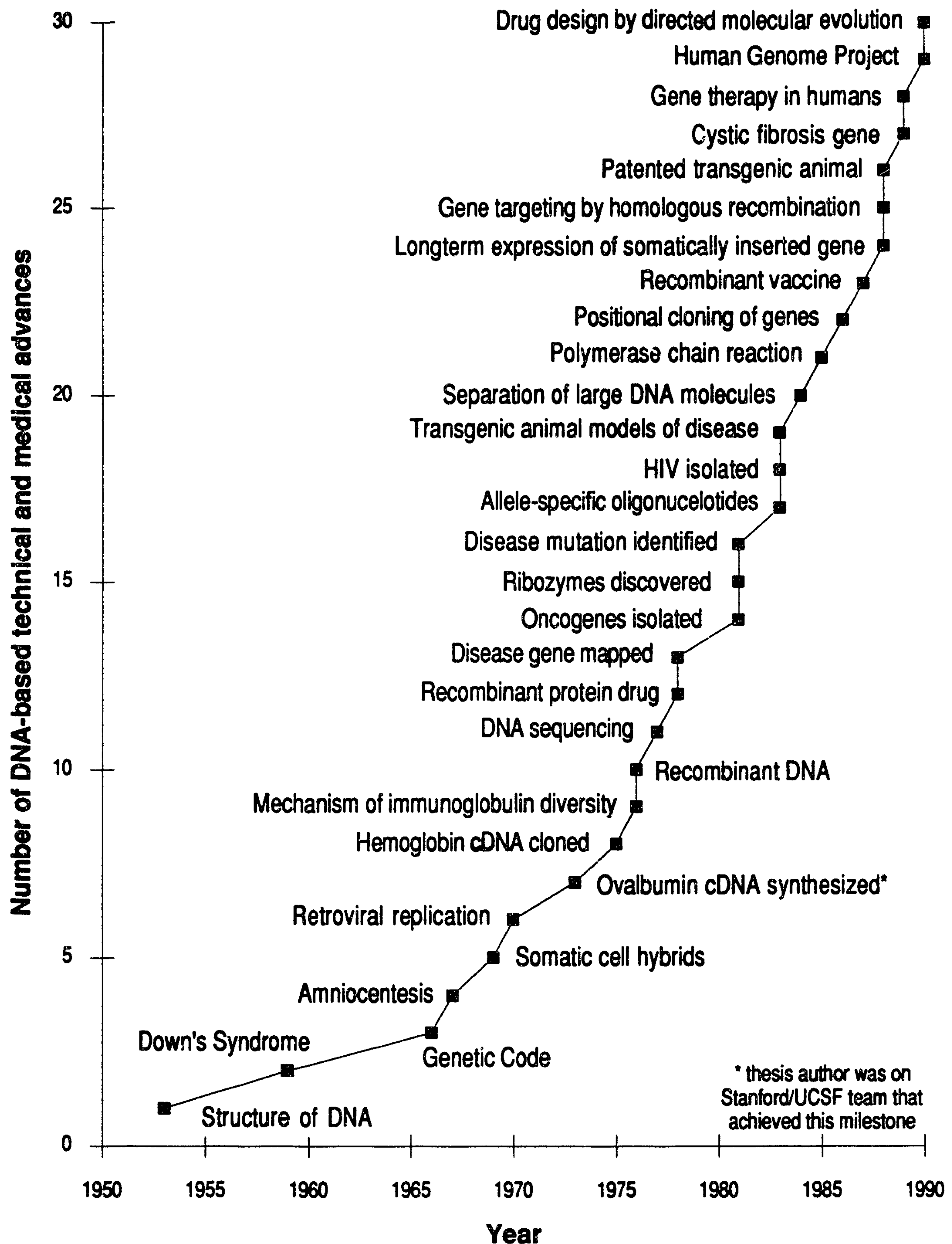


Figure 1.3 Major DNA-based technical and medical advances over the last 40 years (redrawn and modified from Caskey 1992).

profits to develop newer products with even greater functionality. Today's students are expected to clone and sequence their own research genes-of-interest. Gilbert predicts that, in the next decade, many routine procedures such as DNA sequencing, which today are labor intensive and extremely boring for human operators[2], will move out of laboratories altogether and into large-scale service organizations who are economically incented to develop automated robotic technologies (Gilbert 1992).

Indeed there are already firms such as SEQ, Ltd. (Princeton, NJ) who are developing advanced sequencing techniques aimed at orders-of-magnitude reductions in time and cost per base pair (bp) of sequence (Anderson 1993a). The cost of DNA sequencing is currently 10-50 ¢/bp (Charles Cantor, personal communication). SEQ President Richard Horan projects technologies several years into the future that can sequence DNA at a cost of only 0.1 ¢/bp (Richard Horan, personal communication). (This figure does not include the cost of preparing the DNA so that is suitable for sequencing.) A plot showing the growth of the DNA sequencing field in terms of personpower and productivity is given in **Figure 1.4**. The rate of sequencing of nucleotides during the period of 1970 to 1990 has approximately doubled every ten months.

A major impetus to the development of gene knowledge and technology is the Human Genome Project, a federally funded program started in 1988 by the National Institutes of Health and the Department of Energy. The goal of the Project, which includes international collaboration, is to place sequence markers or "road signs" every few million base pairs on all 23 pairs of human chromosomes and eventually sequence all 6 billion base pairs by 2005 (U.S. Dept. of Health and Human Services and U.S Dept. of Energy 1990). The budget has been estimated at approximately $200 million per year, adjusted for inflation, for 15 years. The Genome Project can be thought of as having an infrastructural mission to facilitate the identification and location of genes. **Figure 1.5** is a graph showing the rate of progress in mapping human genes. As of 1992, only about 2700 of the estimated 50,000 to 100,000 genes have been mapped. Each research group has had to independently perform laborious repetitive techniques to subfractionate the DNA and test it with markers, hoping to find patterns in the

[2] Molecular biologist Sydney Brenner has jokingly suggested that a penal colony be set up where prisoners are assigned a sentence of sequencing 25 kilobases with no time off for good behavior.

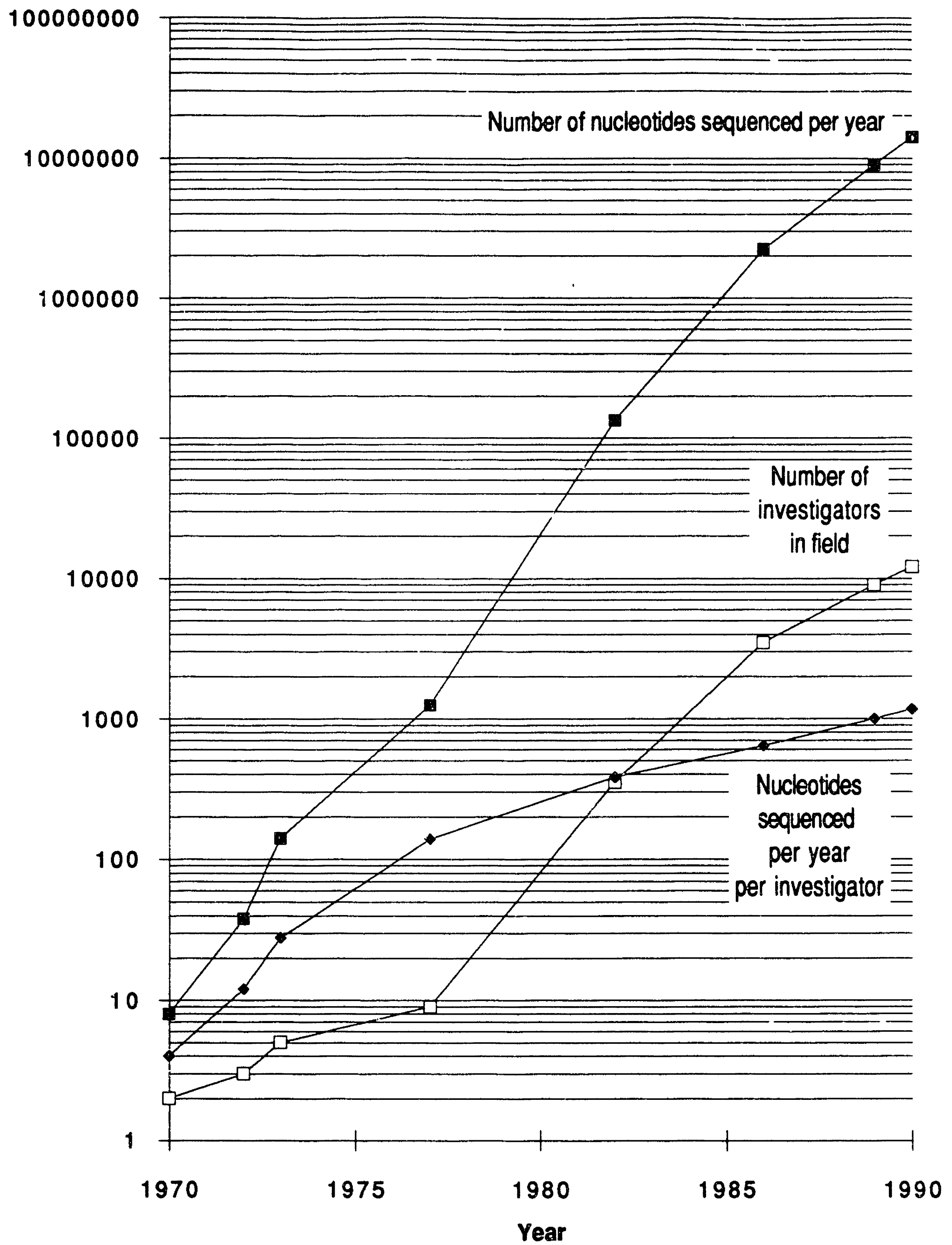


Figure 1.4 Graph showing the growth of the DNA sequencing field (all organisms included) (data from Wu 1993).

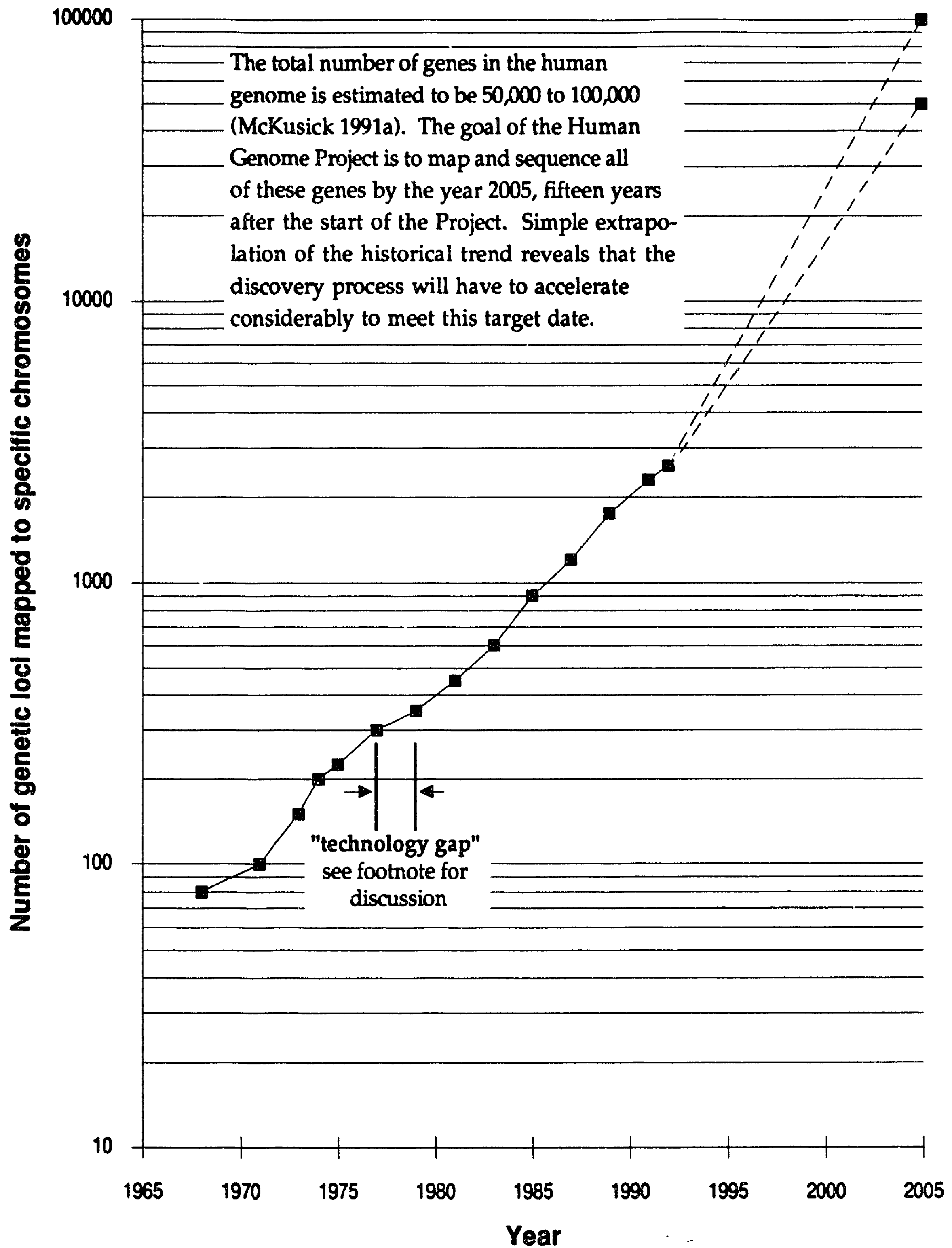


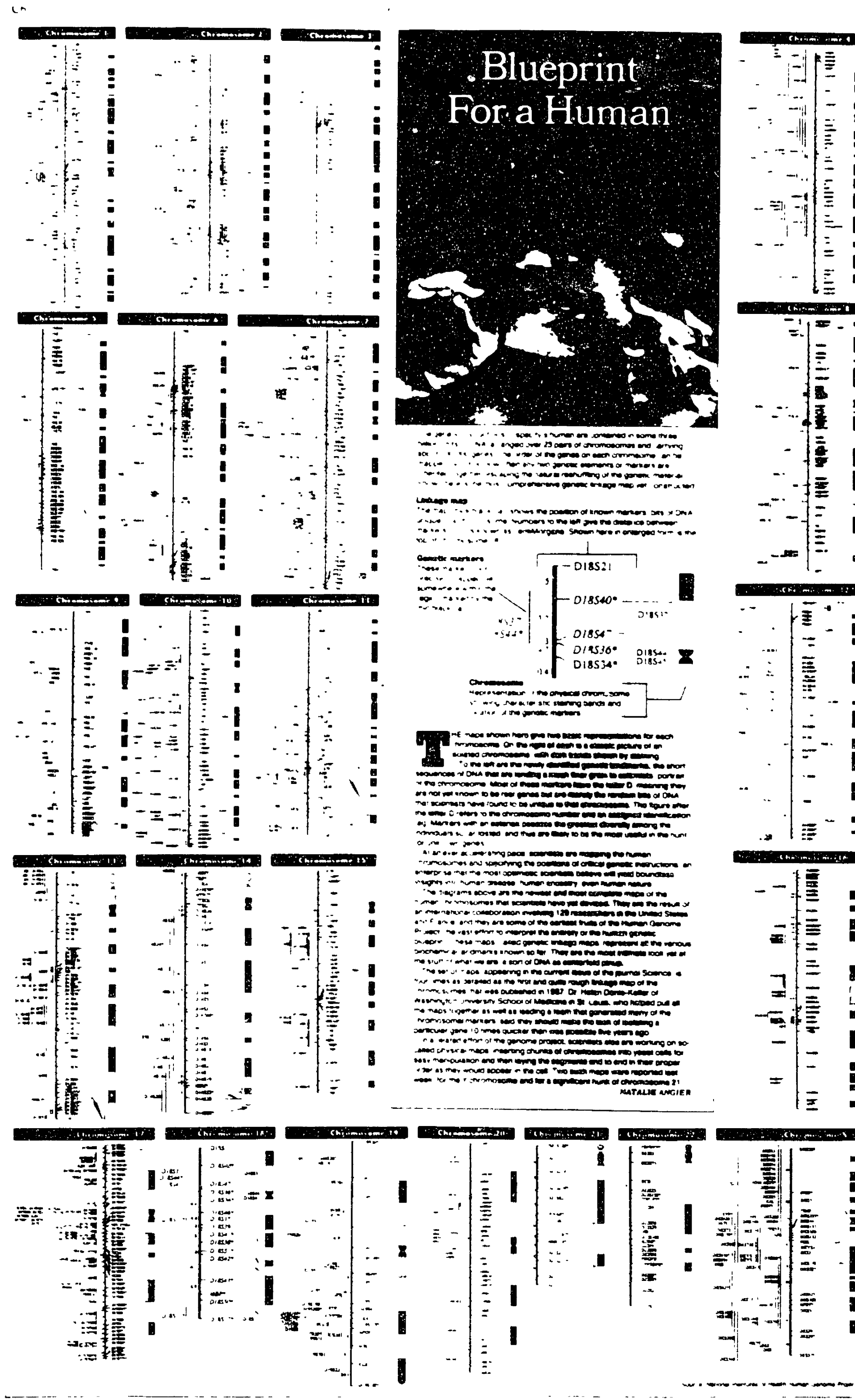
Figure 1.5 Total number of genetic loci mapped to specific chromosomes (data from McKusick 1992).

DNA that tracked with the disease trait. Francis Collins, co-discoverer of the gene for cystic fibrosis in 1989 and newly tapped head of the Project, estimates that it took 10 years, 2 dozen laboratories and $50 to $100 million dollars to find the cystic fibrosis gene, which he regards as one of the more straightforward and solvable genetic problems because the disease is caused by only one gene and occurs with high frequency in the population (Collins 1990). The rationale of the Project is that a large scale investment is required in advanced sequencing technology development and in the mapping of sequence markers up and down all the chromosomes so that future researchers can proceed much more quickly and economically in their pursuit of their particular genes-of-interest.[3] An example of such a marker map is given in **Figure 1.6**. The right panel of **Figure 1.1** is a map indicating the location of all known diseases on Chromosome 7. Note the location of the cystic fibrosis gene about one fifth the way up from the bottom. Sequence marker maps and gene location maps are maintained as an online data base, allowing researchers to share new data as well as access existing data with analytic software (McKusick 1991b). The function of a gene can often be deduced by its homology to genes of known function.

Just as Vesalius's classic anatomical text, *De Humani Corporis Fabrica* (1543) formed the basis for the advances in physiology by William Harvey (1628) and morbid anatomy by Morgagni (1761), the discovery of the structure of DNA (Watson and Crick 1953) and the mapping and sequencing of genes by the

---

[3] According to Dr. Victor McKusick, the period 1977-1980 experienced a real slowdown in the rate of gene mapping due to a "technology gap". In 1970, somatic cell hybridization was introduced. In this method, genes are mapped to specific chromosomes by fusing cultured human cells with rodent cells, forming viable but somewhat unstable cell chimerae. Karyotypic examination of these cloned hybrid cells reveals that human chromosomes are sometimes lost during cell culture, resulting in a cell line that contains, for example, only human Chromosome 5. Any human enzymes expressed in that cell (which can be distinguished from those of the rodent background by standard biochemical means) must have their genes located on human Chromosome 5. According to Dr. McKusick, by 1977 all of the "easy" genes had been mapped by this method. It was not until 1980, the birth of modern molecular genetics, that the rate of gene mapping accelerated. This was due to three technological advances: 1) oligonucleotide probes which allowed direct examination of the gene sequences in the somatic cell hybrids without the previous requirement for expression and biochemical detection of gene product; 2) fluorescently labelled oligonucleotide probes which made it possible to map genes directly to chromosomes of human cells, bypassing the cell hybrids altogether; and 3) the use of restriction enzymes to create Restriction Fragment Length Polymorphisms (RFLPs) from DNA collected from family members, thereby allowing direct observation of gene inheritance patterns in family lineages. The future advances will come from mapping of reference sequence markers and from automated sequencing of libraries of overlapping contiguous DNA fragments (contigs) cloned into yeast artificial chromosomes (YACs) and analysis by high-speed "informatic" computers and software.



Blueprint For a Human

D18S21

D18S40*

D18S4

D18S36*

D18S34*

NATALIE ANGIER

Figure 1.6 "Blueprint for a Human" showing the current status of the human chromosomal maps (The New York Times, Tuesday, October 6, 1992, page C6).

Human Genome Project are transforming contemporary medicine (please refer to **Figure 1.1**) (McKusick 1991a).

We are on the verge of a new theoretical biology and a new theoretical medicine (Gilbert 1992). It can be argued that we do not know enough basic information about the molecular workings of our cells and our bodies to ask the really meaningful questions. Today, for instance, we still do not know all of the genes that are expressed in the brain and in the heart. We know that the body possesses mechanisms to express different sets of genes in different tissues, but we lack the basic data to de-code the molecular addresses that make a brain a brain and a heart a heart. We need a large infrastructural investment, a "genetic information highway", to borrow the Vice President's telecommunications metaphor, before we can even begin to ask these types of questions.

"Language" is a metaphor which is frequently used when describing the genetic information encoded in DNA sequences (Schrage 1993). But when it comes to DNA, maybe language is more than just a metaphor- maybe *DNA is a language,* just like Latin or Chinese, with its own grammar and syntax. The Genetic Code gives us the meaning of DNA sequences in groups of three letters at a time, call them "words", but we need to be able as well to diagram or parse larger groupings of sequences, that is, many "words", to find the higher levels of meaning expressed in these "genetic grammatical constructs". The ability to read DNA, not just as a sequence of letters, but as " biological sentences, paragraphs, chapters, volumes, and libraries" that convey specific high order meaning, would allow us to "read" the genome of any living thing with the same facility that we now enjoy with printed media such as newspapers.

Somewhere, written in the three billion basepairs of our genome, an amount of information that is equivalent to a million page telephone directory (Gilbert 1992), is a script, an epic poem far greater in scope than Homer's Iliad or any other human work, that recounts our descent from unicellular beings through the phyla of the animal kingdom to our current precarious position on the cusp of the evolution of life on planet Earth. In our genetic constitution, we are part worm, part fish and part mouse. Somewhere, also written in the three billion basepairs of our genome, are the detailed instructions that direct the fusion of two sex cells, an egg and a sperm, and the subsequent cellular division and

differentiation into the ten million million (10^{13}) cells that make up each of our bodies (Alberts and others 1989).

In addition to the structural specifications for each gene product, our genome must also contain at least three types of regulatory instructions for each of the estimated 50,000-100,000 human genes (Hood 1992):

- temporal- when in development is the gene turned "on"
- spatial- where in the body or in what tissue is the gene turned "on"
- amplitudinal- how much gene product is to be produced.

The ability to understand DNA, the language of life, means that we can also "write" and "edit" it as well. This is *the paradigm* for the molecular medicine of the future. The following are but three of many examples where the critical future medical skill that is needed can be expressed quite simply by asking, *"Parlez vous DNA?"*.

1. *Human Immunodeficiency Virus (HIV)* is a RNA virus that infects and kills helper T cells, a critical component of the immune system (Alberts and others 1989). Initially, the virus exists in a latent state, that is, the immune system recognizes the invading virus, produces neutralizing antibodies and there are no symptoms. But after a period of several years, the virus "escapes" the defenses of the immune system, killing the essential helper T cells and leaving the patient susceptible to infection by opportunistic microorganisms that rarely infect normal individuals. This sequence of events results in the lethal disease known as Acquired Immune Deficiency Syndrome (AIDS). The HIV virus escapes the immune system by mutating its viral protein sequences (known as antigen epitopes) which identify the virus as a foreign body. Within the span of several years, the viral strain which initially infected the patient mutates or evolves *in vivo* in the patient faster than the patient's immune system can respond (Phillips and others 1991; Zinkernagel and Hengartner 1991). This phenomenon can be thought of as Darwinian evolution of a predator-prey relationship occurring on a molecular scale. Like the antelope which developed over millions of years the capability to outpace its predator, the lion, the HIV virus can develop over only a few years the capability to outpace its prey, the human. The HIV virus, which converts its RNA genome into a complementary DNA copy so that it can incorporate itself into the genome of the cells of its prey, is speaking the ancient

tongue of life. We want to know what it is saying, and we want to be able to effectively engage in a combative debate. But before we can even step up to the podium, we must first ask of ourselves whether we are sufficiently fluent in the official language of the debate. *Parlez vous DNA?*

2. *Aging*. There is a rare genetic disorder, progeria, that afflicts one child in every million to ten million born. Progeria (also known as Hutchinson-Guilford syndrome) is characterized by extreme precocious senility. Children of normal intelligence develop early the customary symptoms of aging such as baldness, loss of weight, wrinkling of the skin, and severe arteriosclerosis. They die, usually of a heart attack, at an average age of 13 years (McKusick 1992). In marked contrast, certain pine trees have a lifespan of over 5000 years (Finch 1990). Scattered throughout the animal and plant kingdoms are sexually reproducing species that show no signs of senescence, have indefinite lifespans, exhibit continuing growth, and exhibit no known dysfunctions or increase in mortality with age. Evolutionairy biologists have noted that the forces of natural selection diminish in importance after an individual has reached sexual maturity and passed on his or her genes to the next generation (Rose 1991). In simple terms, an organism which has passed on its genes through procreation is "spent" and not of further value in evolution. An argument could even be made that the early death of post-reproductive elders in a nomadic tribe, as our earliest ancestors most probably were, would free up scarce resources for others and would facilitate mobility, both of which would improve the chances for survival of the tribe. Of course, there also were essential intergenerational factors such as nurturing, wisdom, experience and social stability which most certainly were necessary for the survival of our primitive ancestors. But the basic questions remain: Must we humans age? Is our senescence obligatory? Are there *intrinsic* limitations to our longevity? What causes children with progeria to have their lives fastforwarded at such a cruel pace? Why do some plants and animals live so long? Could we? The answers can be found in only one language. *Parlez vous DNA?*

3. *Huntington's disease* is a genetic disorder that strikes one out of every 20,000 seemingly normal adults in their later years. The disease is characterized by severe personality changes, memory loss, and "dancelike movements of the head, body, and arms, [which] interfere with speech, muscle control, balance,

coordination, and posture" until eventual death (Milunsky 1992b). As discussed later in this thesis, there is a diagnostic test available to identify individuals with a high likelihood of developing the symptoms at some undefinable point in their remaining life. Patients, who are known from their family history to be at risk and who voluntarily take the test (many simply choose not to know, for there is currently no cure) and who test positive, experience a tortuous process called "symptom searching," that is, "scrutinizing themselves at the slightest twitch or misspoken word for a sign of the disease- when in fact, the illness may be decades away." (Morell 1993). Huntington's disease is doubly cruel, because by the time patients develop the symptoms, they often have families, and must therefore cope with not only their own illness but the inherited fate of their children as well. In March 1993, a tremendous advance toward understanding the disease was reported. On the tip of Chromosome 4 there is a gene that normally has the DNA sequence "CAG" repeated in a row about 11 to 24 times. In Huntington's patients however, the CAG sequence is repeated *42 to 86* times, the longest repeat discovered so far. The length of the repeat seems to correllate with the time of onset and severity of the symptoms. Researchers do not yet know the function of the gene, only the chromosomal position and the sequence of the aberration. What causes such a repeat? What is the gene product? Why do the symptoms occur so late in life? How would one develop a strategy to correct the disease? The answers can be found in only one language. *Parlez vous DNA?*

1.3 Gene Therapy as Preventive Medicine

Many diseases are treatable if detected early in life. Newborn screening, for instance, has been used since the early 1960's to identify children with phenylketonuria (PKU), an amino acid metabolism disorder that causes severe mental retardation if not detected early in life. PKU screening is mandatory in most industrialized nations. The cost to society of such screening programs is more than compensated by the savings in institutionalization, health care cost and lost productivity of severely disabled children (Nichols 1988). In the case of PKU, the retardation can be prevented by dietary restriction of phenylalanine. PKU can be tested using a biochemically based assay. Until recently, many genetic disorders could not be detected for lack of a suitable

assay. As will be discussed later in Chapter 3, many inherited disorders can now be diagnosed at the level of the DNA, either prenatally or after birth but, in both cases, before symptoms start to appear. If diagnosis confirms the high likelihood of a severe disorder early in pregnancy, elective termination (abortion) is an option. If the diagnosis of a severe disorder is made late in pregnancy, or if the parents are personally opposed to abortion, then treatment is the only option.

Since most disorders worsen over time, the earlier the diagnosis, the greater the options for therapy, including that while still in the uterus (Evans and others 1992). While there is discussion of the possibility of fetal gene therapy, it is more likely that such an approach would be applied shortly after birth, provided the symptoms have not yet developed.

In addition to the infancy phase, gene therapy may provide preventive therapeutic benefit later in life. As will be discussed in later chapters, cancer, infectious disease, heart disease and hypercholesterolemia are but a few of the targets identified by gene therapy companies working today.

Preventive therapy has not been a popular paradigm for the majority of drug discovery firms. For them, the ideal market is a chronically ill population requiring a lifetime of treatment with a monopolistic drug. Understandably, no single firm wants to undergo the risk and expense to develop a one-hit therapy where successful diffusion only assures a speedier exit from the business. However, for society as a whole, because it can spread the cost over a greater base and endure greater risk, such a proposition may be totally justifiable. Like the Orphan Drug Act, which provides an economic incentive for firms pursuing therapies for small population sizes (under 200,000), some form of public incentive or "sweeteneer" may be appropriate for therapies whose raison d'etre is prevention. At such an early stage of development, it is not clear what the treatment frequency of gene therapy will be. If the technique can be applied safely and efficaciously to somatic stem cells, from which are regenerated all cells of an organ, then the frequency will be low, possibly once in a lifetime. If, however, differentiated cells are the target, then the therapy would have to be performed at a frequency that reflects the cell turnover rate.

In any case, it is reasonable to expect that commercial proponents of gene

therapy will be required by the payers of health care to demonstrate cost effectiveness before any reimbursement occurs. Reduction of production costs and of distribution and patient delivery costs relative to existing therapies are going to be requirements in addition to the historical safety and efficacy criteria. Life cycle cost-benefit analysis of new therapies will have to demonstrate a marked improvement over older therapies to gain acceptance.

1.4 Gene Therapy as Technology with Ethical Impacts

An early criticism of the field of biotechnology was that it was not Humankind's place to consciously manipulate the genes of living organisms. Gene splicing technology was considered to be fundamentally different than the techniques of selective breeding, which have been applied to plant and animal livestock ever since the establishment of human agrarian society. As public education has increased, this concern has diminished, despite the efforts of certain individuals such as Jeremy Rifkin to keep it alive. The public is making its own assessment of the potential risks relative to the potential benefits of the new technology.

The application of biotechnology to manipulate the genes in humans raises the issue to a level of even higher concern. Is it Humankind's place to alter the genes of humans? Several points should be made on this matter. One is that humans have been altering their genes (albeit indirectly) ever since the species began. Every non-random mating is a small, but conscious alteration in the gene pool of the population. An individual's selection of a mate or mates, a tribal society's laws regarding marriage outside the tribe as well as that regarding consanguinity (the allowance or prohibition of marriage within the extended family group) all constitute an indirect manipulation of human genes by humans.

Any change in the environment by civilization, for better or worse, also has a potential effect on the survival of individuals and thus the passage of their genes into the population. The development of sanitary engineering practices greatly relaxed the severe selective pressures that infectious diseases such as cholera and malaria placed on humans. The diet, particularly that of pregnant women and children, and the buildup of pesticides and other toxic industrial substances in food, air and water are all environmental factors controllable by humans which

affect survival, reproduction and ultimately gene composition in the population.

Lastly, improved medical care can be viewed as prolonging the life of many individuals who otherwise would have died before reaching child-bearing age. As a result, there is an accumulation of "bad genes" that would have been eliminated in absence of medical attention. This is a humanitarian decision which an ethical society such as ours choses to make.[4]

In contrast to the rather blunt or indirect methods mentioned above, gene transfer technology represents the sharpest tool we have developed to date to alter our genetic composition. Are there any ethical concerns unique to the technology? The issue has been extensively discussed (Walters 1986; Walters 1991). A distinction is generally made between the somatic (meaning "body") cells and the germ line. Somatic cells affect only the health of an individual whereas germ line cells affect the health of all progeny who descend from them.

An ethical precedent exists for the transfer of a gene into the somatic cells of a patient, and that is transplantation of either cells such as bone marrow or whole organs such as heart and kidney. Somatic cell gene therapy is generally regarded as presenting no new ethical problems that are not already present in tissue or organ transplantation, both of which are acceptable procedures provided alternate therapies with less risk have been tried or do not exist. Issues such as fairness in the selection of patients, informed consent (particularly as it applies to minors) and privacy/confidentiality exist for any somatic therapy, be it transfer of a gene or of an organ.

Genetic modification of the germ line, however, is regarded as fundamentally different and ethically clouded. The concern centers on the rights of future generations to inherit unmanipulated genomes and their obvious inability to give informed consent. From a medical perspective, there is also the concern that

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[4] Eugenic practices such as sterilization of the mentally retarded have occurred in the past history of the United States. By the late 1920s, about two dozen states had passed eugenic sterilization laws, which were held to be constitutional in 1927 by the U.S. Supreme Court decision in *Buck v. Bell*, where Justice Oliver Wendell Holmes delivered the opinion that "three generations of imbeciles were enough" (Kevles 1992). The adoption of eugenic practices by the Nazis in Germany and the atrocities that ensued resulted in a discrediting of eugenics in the States. The world unfortunately has not seen the last of genocide or "ethnic cleansing", the sinister phrase used to describe the current events in Bosnia.

genetic manipulation of the germ line could cause unforeseen damage to future generations. The technology as applied to lab animals has a very low success rate. It is clear that considerable improvement in germ line manipulation in animals as well as extensive experience with somatic gene therapy in humans will be necessary before there can be any consideration of germ line manipulation in humans. The U.S. federal authorities have placed a moratorium on human germ line gene therapy proposals.

Another area of ethical concern is gene therapy of somatic cells not for the purpose of repair, but for the purpose of enhancement of function, in other words to "improve" desired characteristics. This approach is analogous to elective cosmetic surgery, and runs the risk of being highly arbitrary, frivolous and potentially dangerous. For instance, addition of a gene for the human growth hormone to the cells of a child with short stature is arguably "repair", meaning restoration of normality, but a similar treatment of an already normal child to "enhance" size and possibly athletic ability seems questionable.

It is also important to mention that gene therapy is already an international phenomenon. Human clinical trials of gene therapy began in China and in France in 1991, in Italy in 1992, and are expected to begin in Japan later this year (Anderson 1992; Swinbanks 1993). Even Germany, the country with the most stringent genetic engineering regulations and perhaps with the greatest sensitivity to ethical matters, has approved a human trial for the treatment of cancer (Abbott 1992; Grindley 1993).

Human gene therapy has an important geopolitical aspect to it. As an advanced technology that is at the leading edge or boundary of social acceptability, it is clear that different cultures with different *mores* will have different aspirations for the technology. A culture, for instance, that routinely practices infanticide merely on the basis of gender, placing newborn babies outside to die from exposure simply because they are girls, is going to develop gene therapy very differently from one which values every birth as worth saving and nurturing.

Largely as a result of the success of the Marshall Plan, initiated by the U.S. after World War II to rebuild the ravaged societies of Europe and Japan, the world is evolving economically as a three-way competition between Europe led by

Germany, the Pacific Rim led by Japan, and North America led by the United States (Thurow 1992). With the apparent dissolution of the Cold War, the hope is that the superpowers of the world will engage in economic competition rather than militaristic competition.

The race to develop civilian technology as a source of global economic comparative advantage is becoming much faster. The stakes are also much higher. Each superpower has identified the same seven industries[5] as being critical to their economic success and increased standard of living. The economic pressure to achieve or maintain pre-eminence in each of these seven industries, of which biotechnology is one, can only be expected to increase in the coming years.

This economic acceleration is, more than likely, going to aggravate any differences between cultures that exist regarding the uses or perceived abuses of a socially sensitive technology such as gene therapy. The United States is not in a position to be the moral policeman for the world. If any country wishes to extend the envelope of human genetic possibility, perhaps even into germ line manipulation for the eugenic purpose of creating a so-called "super race", the Unites States' ability to influence that decision may be limited.

We can only express our hope that caution, thoughtfulness, compassion and reason will prevail over any ignoble urges. It has been suggested that the newly acquired capability of one species to consciously and directly manipulate its genome, as well as of all other species, is itself a critical milestone in the evolution of life on the planet (Charles Cantor, personal communication). If that is so, then cooperativity must coexist with competitiveness if we all are to survive. Our species is in a most fragile transition phase as we collectively come to understand the meaning of the new powers we possess.

[5] They are microelectronics, new materials, biotechnology, telecommunications, civilian aircraft manufacturing, robots plus machine tools, and computers plus software.

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CHAPTER 2
MOLECULAR BASIS OF INHERITED AND ACQUIRED DISEASE

"Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the usual law of Nature by careful investigation of cases of rare forms of diseases.

For it has been found in almost all things, that what they contain of useful or applicable nature is hardly perceived unless we are deprived of them, or they become deranged in some way."

William Harvey
1657

2.1 The Central Dogma of Molecular Biology

The elucidation of the molecular workings common to all living things represents one of the greatest achievements of modern science. It is not possible in the context of this thesis to adequately summarize the wealth of information that is known. An excellent introduction entitled Dealing with Genes, The Language of Heredity by Paul Berg and Maxine Singer (1992) is recommended to anyone interested in a clear, jargon-free presentation of the subject.

A key finding of molecular biology which is relevant to the discussion of gene therapy is that each gene, which is composed of a linear sequence of deoxyribonucleotides (DNA), codes for a unique protein, which is composed of a linear sequence of amino acids. Proteins, the workhorses of the body, are responsible for all structural and metabolic activities of the cell . A list of the ten most common classes of proteins is given in **Table 2.1**. Between the gene and its corresponding protein is an intermediate molecule known as messenger ribonucleic acid (mRNA), which is copied or *transcribed* off the gene in the nucleus, edited by a cut-and-paste process and then transported to the cytoplasm

Proteins	Example
• Structural	Collagen, the human body's most abundant protein, is found in various kinds of connective tissues that hold the body together.
• Storage	Ovalbumin, found in eggwhite, is a major source of materials and energy during embryonic development.
• Transport	Hemoglobin transports oxygen from areas of high concentration in the lungs to areas of lower concentration in the tissues.
• Channel	Ion Channel Proteins create electric action potentials in nerve cells by pumping sodium & potassium ions across cell membrane boundaries.
• Receptor	Insulin Receptors, found on the surface of cells, become activated when they bind insulin and trigger a series of chemical reactions in the cell.
• Hormone	Human Growth Hormone, released by the pituitary gland, stimulates the growth of most body parts and has widespread metabolic effects.
• Protective	Antibodies are produced in response to the presence of foreign substances, organisms, or tissues. They bind to and help inactivate these materials.
• Contractile	Actin & Myosin arranged in orderly arrays in muscle cells produce shortening by sliding past each other in a controlled manner.
• Regulatory	Regulatory proteins influence gene expression by binding to DNA.
• Catalytic	Amylase, an enzyme found in saliva, digests starch into sugar molecules. Enzymes are the largest and most diverse class of proteins.

Ribonucleic Acids	Description
• Pre-Messenger (pre-mRNA)	A transcript of the gene containing alternating intron & exon sequences. The introns are removed & the exons spliced together to make the mRNA.
• Messenger (mRNA)	The RNA which codes for the protein. mRNA is transported from the nucleus to the cytoplasm where protein synthesis occurs.
• Transfer (tRNA)	A small RNA which pairs with the corresponding triplet codon of the mRNA & transfers a covalently linked amino acid to the nascent peptide chain.
• Ribosomal (rRNA)	A structural component of the ribosomes, which bring together the mRNA and tRNA during protein synthesis.
• Ribozyme	A RNA with enzyme-like catalytic activity capable of splicing itself or other RNAs.

Table 2.1 The classification of naturally occurring gene products (modified from Singer 1985).

where it is *translated* into protein. This flow of information, often referred to as the "Central Dogma of Molecular Biology" on account of its importance, can be summarized as follows:

Gene (DNA) → mRNA → Protein → Resulting Body Function.

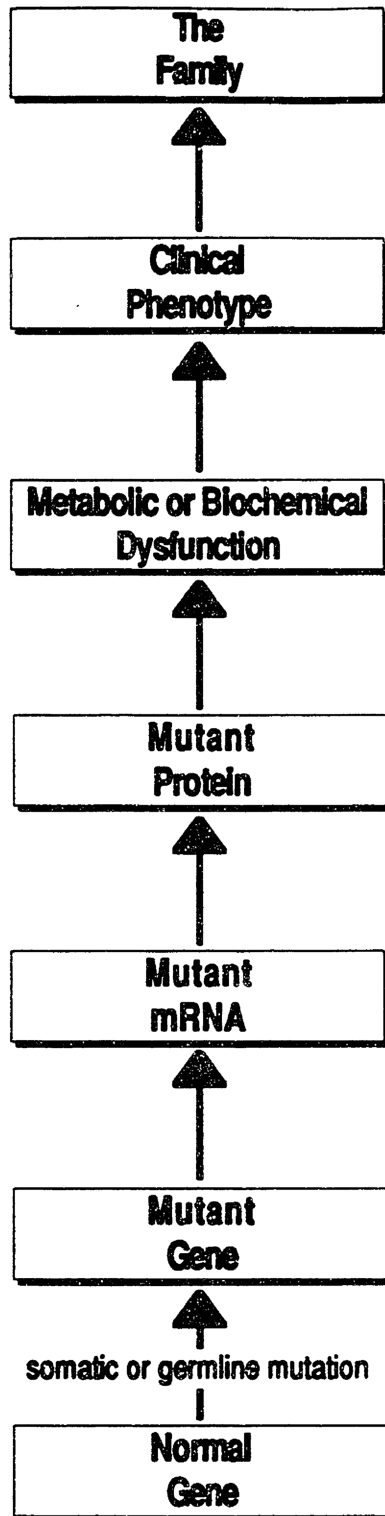
Occasionally the DNA sequence is changed or mutated, either by the action of a reactive chemical or radioactive mutagen, by the insertion of a DNA virus, or by an error in copying during cell division. As shown in **Figure 2.1**, a mutation in a gene causes a corresponding cascade of changes in the resulting mRNA and protein. While some mutations can have little or no effect, others can be devastating. For instance, cystic fibrosis, a lethal disease afflicting one child in 2000, is caused by a mutation, a deletion of just three DNA bases, which results in the loss of one amino acid in the membrane protein responsible for chloride transport. The treatment strategies outlined in **Figure 2.1** are discussed in detail in the next chapter.

The linkage between changes in gene, mRNA, protein and resulting body function represents a key insight into the cause of many diseases. However, a one-to-one relationship between genetic makeup (referred to as "genotype") and observable trait or symptom (referred to as "phenotype") is not always the case. Environmental factors can also play a major role in disease expression. **Figure 2.2** is a schematic diagram showing some representative human diseases arrayed as a continuum form purely genetic to purely environmental causes. The phenotype, what the patient experiences in terms of health or disease, is the result of the interaction of the genotype, what the patient inherited from his or her parents, and the environment, which the patient has cumulatively experienced.

Sometimes it is possible to compensate a genetically caused disease with an environmental modification, such as with diet or with drugs. For example, phenylketonuria, a form of mental retardation caused by a genetically inherited mutation in the gene coding for the enzyme, phenylalanine hydroxylase, can be prevented by the early strict restriction of phenylalanine in the diet.

In certain environmental circumstances, carrying one copy of a "defective" gene

Level of Intervention	Treatment Strategy



Treatment Strategy

- genetic counseling
- carrier screening
- presymptomatic diagnosis

- medical or surgical intervention
- patient education

- disease-specific compensation (dietary or pharmacologic)

- protein replacement
- enhancement of residual protein function

- antisense or ribozyme oligonucleotide therapy

- pharmacologic modulation of gene expression

- modification of somatic genotype by cell/tissue transplantation or by gene therapy

- partial prevention by reduced exposure to mutagenic or carcinogenic factors

Figure 2.1 The Central Dogma of Molecular Biology applied to the origin and treatment of genetic disease (from Thompson and others 1991).

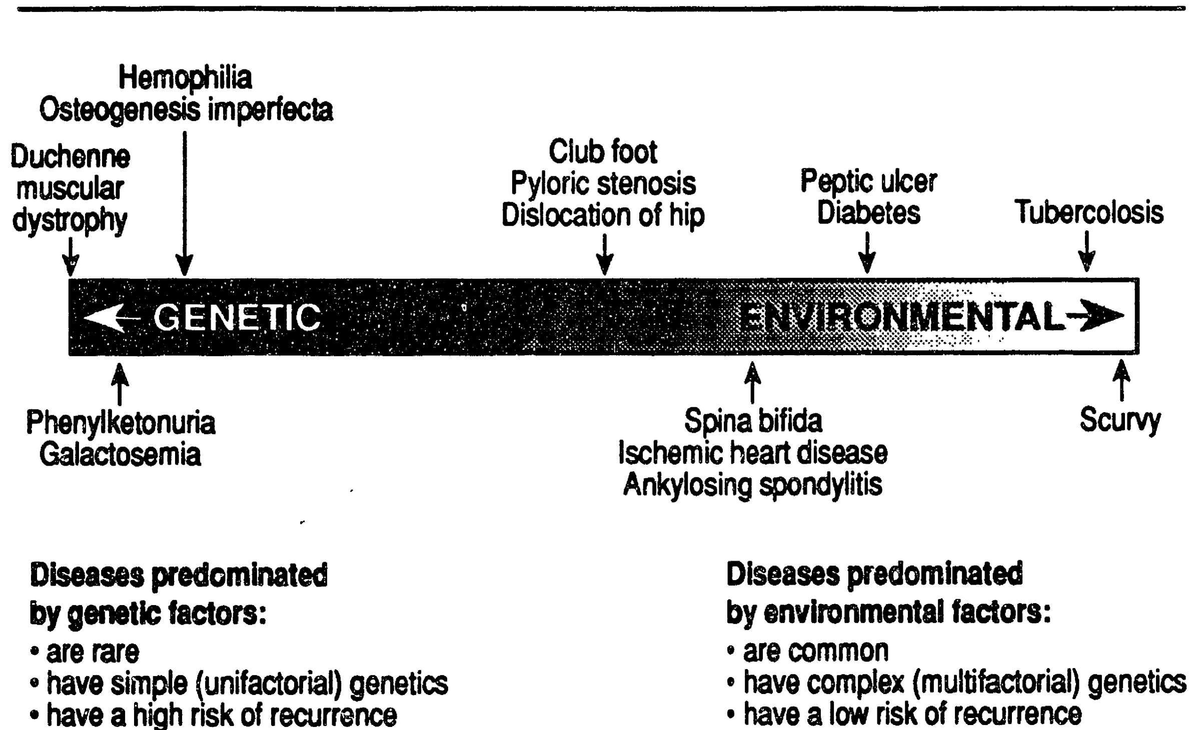


Figure 2.2 Human diseases arrayed on a continuum from purely genetic to purely environmental causes (redrawn from Emery and Mueller 1992).

can confer a selective advantage to an individual. For instance, sickle cell anemia is a severe debilitating disease caused by the inheritance of two defective copies of the gene for the beta chain of hemoglobin. If an individual (termed a "heterozygote") inherits only one defective copy (the other gene copy is normal), that individual will be resistant to malarial infection, and will not only have a better chance for survival but will also have a higher reproductive fitness. He or she will be more fertile, and will produce even more children carrying the sickle gene. In certain parts of Africa where malaria is endemic, as many as one person in 3 is heterozygous for the sickle gene, and is healthier than either of one's siblings who have two "disease" genes or two "normal" genes (Emery and Mueller 1992; Thompson and others 1991). Americans of African descent, because they are no longer in an environment where the malarial parasite lives, no longer have a selective advantage in carrying one copy of the sickle gene, Consequently, the heterozygote frequency has dropped from 1 in 3 to 1 in 10.

2.2 Genetic Variation and Biologic Wastage

Each individual in a population is an unique expression of an unique combination of genes. Selective pressures, such as weather, famine and pestilence, constantly challenge the members of a population with life threatening circumstances. Fortunately, there is usually enough variation within the individuals of a population that some will survive a catastrophe, the bubonic plaque for instance, live to reproduce, and pass on whatever genetic make-up they themselves inherited which allowed them to endure events which killed others.

Evolution works in an incremental fashion. If a slight genetic change confers a slight selective advantage, then that slight genetic change gets selectively passed on to the next generation, and then the next, in a geometric, exponentially expanding fashion.

Evolution is a game of very small and very large numbers. As long as only a few individuals can survive and reproduce, life goes on. If millions die along the way, it does not matter in evolutionary terms. Some organisms such as pollinating plants and spawning fish, which produce thousands, even millions,

of offspring in a single mating, base their reproductive stategy on this fact.

Life does not work to an absolute perfect standard. If a biological process is successful only part of the time, maybe only once in a million, that may be sufficient. For instance, it is estimated that 90-98% of all male human gametes are defective and that 75% of all human conceptions result in spontaneous self-abortion (Roberts and Lowe 1975). It is thought that this high degree of embryonic lethality is due to genetic errors during gamete formation, such as improper sorting of chromosomes and mutations in essential genes. Each union of sperm and egg is a test of biologic complementarity- an essential gene contributed by the father, should it be defective, must be complemented by the corresponding one from the mother, or the embryo will die.

Figure 2.3 is a flow chart indicating morbidity and mortality statistics at different stages of life. Biological wastage is greatest in the gametes and early embryos, but continues through birth and childhood. The study of genetic disorders is primarily the domain of pediatrics. Some genetically inherited diseases such as Huntingtons, however, do not manifest themselves until late in adult life. This constant selective pressure can be conceptualized as Nature's "genetic quality control process". In the words of two researchers, "The notion of controlling the problem of severe malformation at birth by early recognition and abortion is so good and simple that one wonders why Nature did not think of it first. We believe she did. There is now good evidence that product rejection by way of implantation failure and spontaneous abortion is her principal method of quality control. Evidence is accumulating that prenatal elimination may perhaps be the rule rather than the exception, with the implication that, in the world of early embryos, malformation may be the norm rather than the exception." (Roberts and Lowe 1975).

And so, genetic disease can be seen as an unfortunate but predictable byproduct of imperfect biological processes. These same imperfect mechanisms which generate error can also, on rare occasion, generate variation which has *positive* advantage. The ability of life to generate variation is an essential means of assuring survival of the species in the face of an ever-changing environment. It is our decision as an ethical society that those individuals who received an unlucky throw of the dice should be entitled to as normal a life as is possible. We are

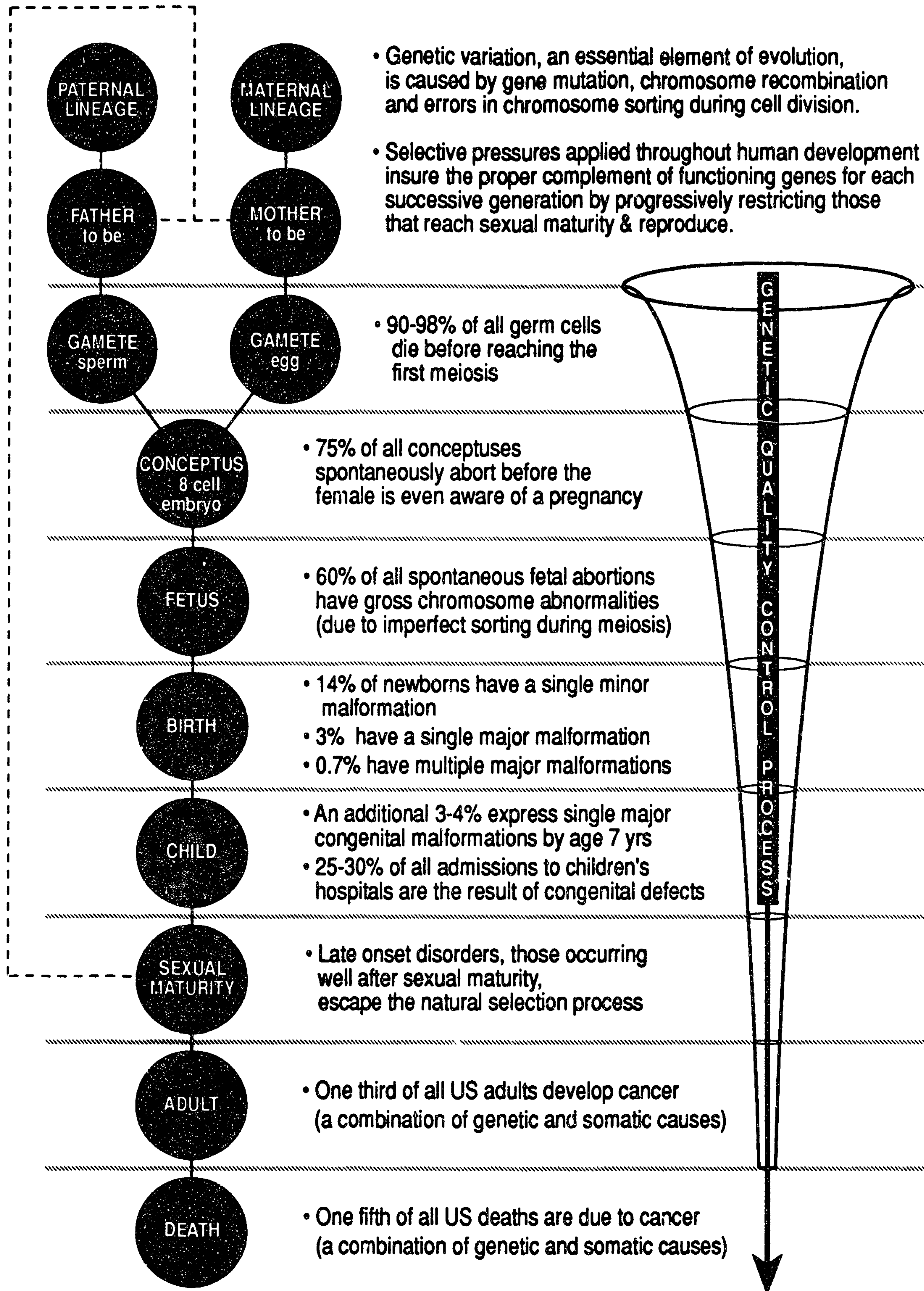


Figure 2.3 Prenatal and postnatal wastage is a major feature of life (Connor and Ferguson-Smith 1991; Milunsky 1992b; Roberts and Lowe 1975; Schimke and others 1986).

willing to invest our social efforts and resources to find ways of diagnosing and treating the less fortunate. We are also willing to encourage medical innovation by offering incentives and intellectual property protection to risk-taking commercial enterprises.

2.3 Cancer, Inherited and Acquired Forms

Cancer represents a special type of genetic disease. Not only are cancer genes transmitted in the normal fashion during sexual reproduction through the germline, but they can also occur in a differentiated somatic cell. Cancers which occur in childhood are typically inherited. Those that occur later in life may have an inherited component, but almost certainly have a postnatal somatic genetic event as well.

There are many varieties of cancer. The phenotype can be characterized in general as a loss of normal cell cycle controls. There is a vast array of genes that regulate when and under what stimuli (hormones, contact with other cells, etc.) a cell will divide, In cancer, some critical gene product becomes mutated and loses its normal regulative function. There are two basic categories of cancer genes- oncogenes and tumor suppressor genes. Oncogenes are variants of normal genes which, when mutated either in a germ cell or in a somatic cell, cause uncontrolled cancer growth. The presence of one copy of an oncogene is usually all that is needed for cancer transformation to occur. The normal copy of the gene (the "proto-oncogene") is present as well, but the oncogene is dominant over it.

Tumor suppressor genes do what their name implies- as along as they function properly, there is no cancerous growth. In tumor suppressor genes, the loss of one functional copy usually does not result in cancer. Only when *both copies* are mutated does cancer result. An individual who inherits one defective and one normal tumor suppressor gene will be at increased risk for cancer compared to an otherwise identical individual who inherits two normal copies. Both individuals have an equal probability of sustaining a somatic mutation in the other gene copy, but the individual who inherited a defective tumor suppressor gene is already "one down", having lost his or her back-up. This so-called

"double-hit" hypothesis explains many recessive mutations in tumor suppressor genes, but there can also be mutations in one copy which dominantly inhibit the function of the normal copy.

Like evolution, cancer is also a numbers game. There are about ten million million (10^{13}) somatic cells in an adult human body. There are perhaps several hundred oncogenes and tumor suppressor genes. The chance that one somatic cell in 10^{13} over the course of a 70 year lifespan will sustain one hit in an oncogene or two hits in a tumor suppressor gene is very high, perhaps even unity. The body's immune system has developed a constant patrol mechanism for such neoplastic cells. Evidence for this assertion comes from the observation that individuals immuno-compromised by chemo- or radio-therapy or by infection with HIV have a higher incidence of cancer.

As shown in **Figure 2.3**, one third of all adults in the USA develop some form of cancer and one fifth eventually die from it. Cancer is primarily an old person's disease, though, as previously discussed, there are tragic pediatric versions as well. Biological processes do not work to an absolute perfect standard. As long as the biological process, be it proper chromosome segregation between dividing cells or effective cancer cell detection by the immune system, works sufficiently to allow some individuals in the population to reach sexual maturity and successfully reproduce, life goes on. The propensity for cancer, because it occurs primarily in individuals who have already reproduced, is not likely to have been a critical trait in evolution. In fact, cancer can be viewed as having escaped the natural selection process. Only in recent history has the average lifespan been so high. Cancer is, in a sense, an unwelcomed byproduct of our improved quality of life, not because of our carcinogenic lifestyles, but because of our food abundance, sanitary engineering and medical system which allow us to live long enough for unwanted stochastic events to occur. As they say on the street, "Shit happens!". Our primordial ancestors were more concerned with getting enough to eat, escaping the jaws of saber-toothed tigers, and living to the ripe old age of twenty-five years than they were with worrying about the strength of their UV sun blocker or the number of cigarettes they smoked!

With the exception of viral infection, cancer represents the single most challenging disease to be understood and mastered. This is because a cancer cell

is virtually identical to an otherwise normal cell, except in only a few genes out of the hundred thousand total. If we only had a magic bullet! Anti-microbial drugs such as penicillin have shaped our expectation that a miracle agent against any disease is possible- we need only find or innovate it. The reason penicillin worked, however, is that a bacterial cell is very different than our cell- it has an extra outer wall, while ours does not. Penicillin inhibits the synthesis of this bacterial cell wall. Cancer cells, on the other hand, do not possess some gross biochemical or morphological difference from normal cells which can be easily exploited. They just grow out of control. What is needed in cancer diagnosis and therapy is a way to identify the cells with the subtle difference, exaggerate or amplify the difference, then exploit the difference to repair or preferably kill the cancerous cells. Gene-based diagnosis and therapy are currently the most promising shots we have at finding *the* magic bullet, should one exist.

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CHAPTER 3
COMPETITORS OF GENE THERAPY

3.1 Overview

This chapter is a review of the many approaches which compete with gene therapy. They include:

- genetic diagnostics and counseling
- special diets and treatments
- pharmacologic therapies
- protein replacement therapies
- cell or tissue transplants
- vaccines

When the competing therapies are pre-existing, they set the benchmarks for safety and efficacy which must be matched or preferably exceeded. Because doctors are conservative in their approach, a new therapy typically must offer substantial additional benefit to overcome the inherent inertia of medical practice. Existing therapies also set ceilings for the prices that can be charged for new therapies.

When there are no pre-existing alternative therapies, the benchmark becomes the standard medical practice for the passive management of the disease symptoms. Depending on the severity of the disease and the prevalence in the population, it is possible that all of the therapeutic approaches competing with gene therapy that are listed above will be researched simultaneously. Such is currently the case for cancer and AIDS. A technology lottery is developed where, financial capital, intellectual capital (brainpower), passion and luck become the critical ingredients of success.

It is clear in this era of healthcare cost containment that all new therapies, whether or not they are substitutions, must be able to justify their price. Cost-

benefit analysis should include savings in hospitalization and other medical costs, increased productivity of the patient (and their immediate family), and should address, if only qualitatively, the improvement in the patient's quality of life.

The most powerful competitor of gene therapy is that which is derived from the same technological base, namely DNA-based diagnostics. It is possible today to ascertain whether a prospective parent, an 8-cell embryo, a later stage fetus or a newborn child carries a gene causing disease. Using DNA analysis methods, diagnosis of an increasing number of genetic disorders can be made long before any symptoms appear. In combination with genetic counseling, this information gives parents the options of preventing the birth of an affected child through selective abortion or avoiding altogether (without abortion) the development of an affected embryo through *in vitro* fertilization and preimplantation testing procedures. It is also possible to substitute gametes from unaffected donors during the *in vitro* fertilization process (**Figure 3.1**).

All of the above methods require interventions into the normal reproductive process which, for personal or religious reasons, some individuals may totally reject. For them the options are: 1) pregnancy prevention through abstinence, contraception or sterilization, 2) adoption, or 3) take their chances that they may give birth to an affected child requiring a lifetime of treatment.

Genetic disorders vary greatly in severity. Some such as phenylketonuria, galactosemia and hypercholesterolemia can be controlled by special diet. Others can be moderated with drugs, by administering either synthetic manmade compounds or by supplementing deficient or missing naturally occurring substances. Some genetic disorders can be treated by cell or tissue transplants.

Gene therapy must compete with existing approaches in terms of safety, efficacy, cost and social acceptability. This chapter explores those approaches which compete with gene therapy.

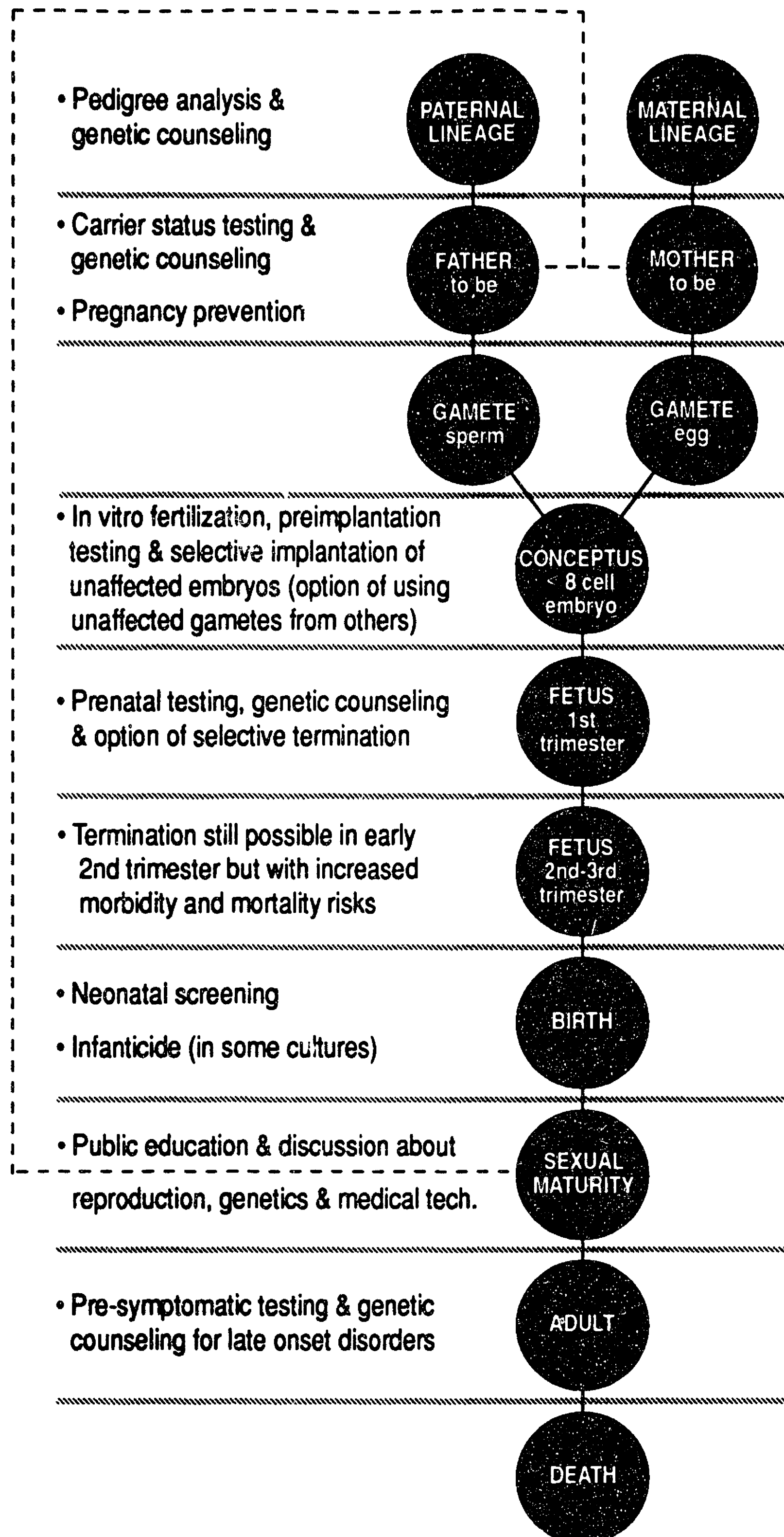

Figure 3.1 Possible diagnostic, reproductive and educational approaches to the prevention of inherited disease.

3.2 Disorder Prevention: Genetic Diagnostics and Counseling

The prevention of genetic disorders by early identification and selective termination is a formidible competitor to gene therapy. As the folk adage goes, an ounce of prevention is worth a pound of cure. The basic philosophy of prenatal genetic diagnosis is to reassure couples at risk that they may selectively give birth to unaffected children even if their reproductive risk for having affected children is high. Fetal defects serious enough to warrant elective abortion by parents occurs in less than 5% of all cases studied. Clinical experience shows that couples with as low as 10-25% risk of having an affected child often refrain from further pregnancies altogether unless prenatal diagnosis is available to them. The development of prenatal diagnostic technology has made it possible for these high risk parents to have children they would not have otherwise conceived. It has been noted that many more children have been born as a result of prenatal diagnosis than the small number of pregnancies that were terminated due to a grave fetal defect (Milunsky 1992a).

• **Population genetics.** Genetic diagnosis began as a simple empiric science. By observing and recording the frequency of occurrence of disorders in a defined population, it was possible to establish a newborn child's probable risk of inheriting those disorders. Analyses of the family histories yielded insights into the modes of inheritance: monogenic or multifactorial, dominant or recessive, autosomal or sex-linked. Families or ethnic groups with a higher than normal incidence could be alerted of increased risk.

For example, in Caucasians, approximately one child in 2000 is born homozygous for cystic fibrosis (CF), an autosomal recessive disorder. Through the study of population genetics, the Hardy-Weinberg Equilibrium in particular, it is possible to compute the carrier (heterozygous) frequency that must be present in the population to account for this frequency of affected births. If **m** is the mutant gene and **N** is the normal gene and **p** is the frequency of gametes with gene **m** and **q** the frequency of those with gene **N** so that **p + q = 1**, then the genotype frequencies in the progeny will be:

frequency:	p^2	2pq	q^2
genotype:	mm	mN	NN
phenotype:	affected	carrier	normal

Since we know for CF that $p^2 = 1/2000$ and that $p + q = 1$, we can calculate using simple algebra that the carrier (heterozygous) frequency $2pq$ must equal 1/23 (4.4%) or approximately 1/25 (4%).[6]

Table 3.1 gives the risks that a couple will birth a child with CF (Anderson and others 1990; U.S. Congress and Office of Technology Assessment 1992). Note that, if there is no history of CF in one's family, the risk that one is a carrier is the same as that of one's particular ethnic group as a whole. If, however, there is a history of CF, then the risk goes up depending on the relational proximity to the affected family member.

• **DNA based genetic tests.** Except for a few genetic diseases where carriers exhibit detectable biochemical abnormalities, there was until recently no way to conclusively advise prospective parents whether they were in fact carriers of a defective gene. For instance, parents learned of their carrier status for CF only by giving birth to an affected child (Shulman and others 1992). This situation has changed radically in recent years with advances in two areas:

- DNA based diagnostics
- Safe prenatal sampling techniques.

It is now possible to accurately assess carrier status for many single gene diseases by analyzing the DNA directly for specific mutations in the gene itself or indirectly for genetic marker sequences inherited along with the gene. In the direct genetic test, knowledge of the DNA sequences which flank the mutation are used in an elegant enzymatic amplification procedure known as Polymerase Chain Reaction (PCR) to produce large amounts of the mutated region of the gene, which can then be analyzed (Allman 1990). In the indirect genetic test, also known as linkage analysis, DNA from the blood of several family members is digested with restriction enzymes which cut the DNA at specific known sequences. These fragments are then analyzed for size by a technique known as gel electrophoresis. The restriction fragments, as they are called, are identified

[6] The Hardy-Weinberg Eqilibrium assumes 1) random mating, meaning no selection on the basis of phenotype and no consanguinity 2) no migration out of the population, so that CF homozygous stay in the breeding population and 3) the forward rate for new mutations is equal to the backward or reversion rate for old mutations. Since CF impairs reproductive potential, assumptions 1 and 2 are technically incorrect. However, since the affected phenotype is rare (1 in 2000), the error is minimal.

Individual	Probability of Being a Carrier as a fraction	as a percent
Negative family history		
• Caucasian	~ 1/25	4%
• African American	1/60 to 1/65	1.5 to 1.7%
• Asian American	1/150	0.7%
• Hispanic American	1/40 to 1/50	2 to 2.5%
Positive family history		
• Parent of individual with CF	1/1	100%
• Sibling of individual with CF	2/3	67%
• Niece or nephew with CF	1/2	50%
• Aunt or uncle with CF	1/3	33%
• First cousin with CF	1/4	25%

To calculate the possibility that a child will be affected with CF, multiply the carrier probability for each parent together and by 1/4 (the chance that both will contribute the CF gene). For example, if a parent has a sibling with CF and the other parent is Caucasian with no family history of CF, the chance of having an affected child would be 2/3 x 1/25 x 1/4 = 1/150.

Table 3.1 Probable risk of being a cystic fibrosis carrier as a function of family history (Anderson and others 1992; U.S. Congress and Office of Technology Assessment 1992).

by hybridization probes specific to the gene being tested. By comparing the sizes of the DNA fragments from the various family members it is often possible to trace the lineage of the disease gene from a specific generation member to the next (e.g., from grandad on mom's side to mom to daughter).

Table 3.2 is a partial list of single gene disorders amenable to prenatal or presymptomatic diagnosis using DNA tests (Gelehrter and Collins 1990; Larrick and Burck 1991). As will be discussed later, many of the DNA diagnostics currently available are directed at disorders that are also candidates for future gene therapy. This is no mere coincidence, since both procedures have common prerequisites, namely cloning of the normal gene and identification of the nucleotide mutation(s) causing the disorder. Because the complexity and regulatory requirements for a DNA diagnostic are much less than those for the corresponding gene therapy, the former will always reach the marketplace before the latter.

These molecular genetic tests, when combined with safe prenatal sampling techniques, have made it possible for parents to know if their pregnancy is affected or not for a specific disease. The parents then have the option of terminating the pregnancy provided abortion is legal in their jurisdiction. For an autosomal recessive disorder, there is a 25% chance the two carriers will conceive an affected fetus and a 75% chance the fetus will be unaffected (i.e, 25% homozygous normal plus 50% heterozygous carrier). Prenatal diagnosis must be done early enough for it to be useful in the decision to continue or safely/legally terminate a pregnancy, yet late enough for it to be safe to the fetus (Gelehrter and Collins 1990).

• **Amniocentesis** is the most common and safest prenatal sampling technique (Elias and Simpson 1992). During the 15th-18th week of gestation, a needle is inserted with the aid of ultrasonograph imaging into the mother's abdominal wall to withdraw a small amount of amniotic fluid, which bathes the fetus. Cells of fetal origin are isolated from the amniotic fluid and either analyzed directly or expanded in tissue culture (Hoehn 1992). The added risk of fetal loss due to amniocentesis is quoted at 0.5% or less and the risk to the mother is very minimal (Elias and Simpson 1992).

Autosomal Dominant

- Adult polycystic kidney disease
- Familial hypercholesterolemia
- Huntington's Disease
- Neurofibromatosis-1
- Retinoblastoma

Autosomal Recessive

- Adenosine deaminase deficiency
- alpha-Thalassemia
- beta-Thalassemia
- alpha1-Antitrypsin deficiency
- Cystic fibrosis
- Phenylketonuria
- Sickle cell anemia
- Tay-Sachs disease

X-linked Recessive

- Becker muscular dystrophy
- Duchenne muscular dystrophy
- Hemophilia A
- Hemophilia B
- Lesch-Nyhan syndrome
- Ornithine transcarbamylase deficiency

Table 3.2 A partial list of diseases amenable to prenatal and presymptomatic diagnosis by DNA analysis (Gelehrter and Collins 1990; Larrick and Burck 1991).

• **Chorionic villus sampling.** The problem with amniocentesis is that 15-18 weeks is rather late, necessitating performance of second and sometimes third trimester abortions to terminate a defective fetus (Shulman and others 1992). There is a need for a sampling procedure that will enable earlier detection so that abortions will be safer and less emotionally traumatic. While amniocentesis has been used as early as 9 weeks gestation, there appear to be problems and increased risks (Elias and Simpson 1992). An alternate early sampling technique is chorionic villus sampling (CVS). The chorion frondosum is a tissue of fetal origin that will ultimately develop into the placenta (Gelehrter and Collins 1990). As early as 8-10 weeks pregnancy it is possible to sample the chorionic villus using ultrasound imaging to direct a flexible poyethylene catheter through the vagina and cervix into the uterus. Alternatively, the tissue can be approached with a fine needle inserted into the abdomen. The overall rate of pregnancy loss after CVS depends on the operator's experience level, the gestation period and the maternal age (Brambati 1992). Controlled studies performed in Canada, the US and Denmark which compare the risks of CVS to amniocentesis are not fully conclusive. Brambati, in his review article cited above, was of the opinion that "when the highest technical standards are available, no obvious differences between [CVS and amniocentesis] techniques, in terms of sampling success and risks, should be expected". As a practical matter, CVS should only be performed by highly experienced staff in centers that perform the procedure routinely.

• **Fetal cells in maternal blood.** The problem with both amniocentesis and CVS is that they are invasive and carry with them low but nevertheless real risks for the fetus and the mother. A noninvasive technique would have the potential to revolutionize prenatal medicine. It is known that certain fetal cells such as erythroblasts, platelets, lymphocytes and trophoblasts enter the maternal blood system in very small numbers as early as 15 weeks gestation (Bianchi and Klinger 1992). Research is underway to develop enrichment techniques to concentrate these fetal cells from maternal blood so that their DNA can be analyzed. Genzyme and its subsidiary Integrated Genetics are currently working on cell separation methods based on physical and biological properties of fetal cells. In 1990 Genzyme granted exclusive commercialization rights for its fetal cell separation technology to Neozyme I, a research and development limited partnership, which Genzyme formed. Provided that adequate numbers of cells can be collected, there still remains the issue of how long the fetal cells remain in

the mother's blood. There are reports that they can last five years, making it very difficult to identify which pregnancy they represent (Lewis Holmes and Ellen Simpson, personal communication).

• **Preimplantation genetic diagnosis.** The problem with prenatal diagnosis is that the fetus is well developed before any suspected genetic disorders can be determined. Moreover, many couples are strongly opposed to abortion. Until recently, the only options for such couples were to 1) forego procreation 2) adopt or 3) take their chances that they may give birth to a child requiring a lifetime of treatment. Another approach is now possible, and that is *in vitro* (meaning "in glass") fertilization in combination with preimplantation diagnosis. Developed originally for couples having difficulty conceiving, *in vitro* fertilization is a process where eggs are removed from the female, placed in culture medium in a petri dish and fertilized with sperm collected from the male. After the zygote has undergone several cycles of cell division, it is placed back in the uterus of the woman donating the egg or a surrogate, where it develops as if it were normally conceived. It has been shown that one or two cells can be removed from an eight cell embryo without impairing subsequent development. This discovery opened up the possibility of preimplantation genetic diagnosis (Verlinsky and Kuliev 1991; Verlinsky and Kuliev 1992). In 1992 a team of researchers in London reported the successful birth to parents who were both cystic fibrosis carriers of a normal girl after *in vitro* fertilization and preimplantation diagnosis (Handyside and others 1992). Thus, *in vitro* fertilization combined with preimplantation genetic diagnosis is a way to allow only unaffected embryos to be selected for transfer to the uterus, thereby avoiding the need to terminate a pregnancy. The embryos that were shown to be affected were not implanted, but were cryopreserved in liquid nitrogen. While this approach is technically possible, it is very costly and not without risk. It is unlikely that such a procedure would be widely used and it is unclear that such invasive procedures would even be acceptable to individuals opposed to abortion in the first place.

• **Genetic counseling.** The advent of new genetic testing technologies has made the role of genetic counseling even more critical. The aim of counseling is to communicate as completely as possible an understanding of all the implications of the genetic disorder as well as a clear perception of the available options. In

our society, it is an inviolable right to found a family. All couples have a right to know if they are at increased risk of having children with a genetic disorder. **Table 3.3** is a list of the objectives of genetic counseling (Milunsky 1992a). It is important that professionals who provide counseling, namely physicians, board-certified genetic counselors and nurses, be nondirective, nonjudgemental and make every effort to preserve the patient's autonomy in decision-making. Counseling does not constitute telling families what they *should* do, but rather what they *can* do.

Genetic diagnostics and counseling have been discussed here as a "competitor" of gene therapy. That is, the fewer pregnancies with defects that reach term, the fewer potential candidates there are for gene therapy. While this is true in an abstract theoretical sense, the practical reality is that genetic diagnostics and counseling are absolutely necessary prerequisites for any effective and socially acceptable gene therapy program. The advent of gene therapy and other types of therapies will increase the need for genetic diagnostics and counseling. Fewer parents will elect selective abortion as more effective postnatal therapies become available to them.

• **Genetic screening.** Up to this point, genetic diagnosis has been discussed only for those individuals believed to be at increased risk on account of their family history. Another approach is to screen entire populations. Screening is most beneficial when there is an effective treatment for the disease.

Genetic screening using biochemical markers is routinely used as a mandatory procedure for the early recognition of affected individuals. For instance, newborn infants are routinely screened in most if not all states for phenylketonuria, an amino acid disorder which causes profound mental retardation if left undetected, but can be effectively treated by early restriction of dietary phenyalanine. Other diseases for which newborns are routinely screened include maple syrup urine disease, homocystinuria, galactosemia, congenital hypothyroidism, biotinidase deficiency and sickle cell anemia (Gelehrter and Collins 1990; Nichols 1988). As the cost of diagnostics goes down and concern for preventive pediatric medicine goes up, it is likely that more genetic diseases

Directed at the affected individual

- Decrease the pain and suffering due to the disease
- Advise as to whether treatment is possible
- Quote risk figures for offspring and other relatives
- Reduce anxiety and guilt
- Help patient to cope with the affliction

Directed at the parents

- Help couples make rational decisions about their reproduction
- Give family-planning options to at-risk matings
- Reduce anxiety and guilt
- Provide education about the disease in question
- Encourage couples to make their own decisions

Societal goals

- Eliminate genetic disease
- Prevent genetic disease
- Reduce the incidence of genetic disease
- Reduce the burden of genetic disease
- Decrease the frequency of deleterious genes
- Upgrade awareness of genetics in the public
- Influence mate selection

Table 3.3 The objectives of genetic counseling (Milunsky 1992a).

will be screened in newborns. Another use of genetic screening is to identify in the general population those individuals in their child bearing years who are carriers even though there is no family history of the disease. Classic examples of this type of screening include Tay-Sachs disease, sickle cell anemia and the thalassemias. There are no mandatory genetic screening programs of adult populations in the United States (U.S. Congress and Office of Technology Assessment 1992).

There are technical problems inherent in genetic screening, not because the tests are ambiguous but because diseases are often polymorphic. Take cystic fibrosis, for example. The disease is caused predominately by one very prevalent mutation, the deletion of a phenylalanine residue in the 508th position of the CFTR protein, referred to as the ΔF508 mutation, which accounts for 70% of the mutant CF genes in the population (Collins 1992). The problem is that the other 30% of the CF genes are represented by more than 170 different mutations (U.S. Congress and Office of Technology Assessment 1992). Current DNA tests assay for ΔF508 plus the 12 other most common CF mutations. The "ΔF508 plus 12" assay can identify about 85% of CF carriers in Caucasians (and 95% in Ashkenazic Jews). This means that, using the ΔF508 plus 12 assay, about 15% of the actual carriers go undetected. Since testing for all 170+ mutations is impractical, a *negative* test result does *not* guarantee that a person is *not* a carrier.

Using the ΔF508 plus 12 test means that some couples get test results that indicate one partner is a carrier and one is not, when in fact the negative (-) partner carries one of the rarer CF genes that is not assayed. Thus, while most couples whose test results are +/- are at zero risk of having an affected child, some couples with a +/- result are actually couples whose genetic status is +/+ (but is undetected) and who are really at 1 in 4 risk of bearing a child with CF. They are what is referred to as "false negative" couples.

Another important limitation of screening and indeed all DNA diagnostics is that newly occurring mutations go unnoticed. The average rate of mutation in a gene passed from parent to child is about one in a million, though it can range as high as one in ten thousand to as low as one in ten million (Gelehrter and Collins 1990; Thompson and others 1991). New mutations which have not been detected before will escape DNA diagnostic tests.

While there is no argument about making the test available on a voluntary basis to people who have a family history of CF, the controversy swells around the suggestion that the test should be applied to the population as a whole. Concerns about the widespread routine practice of screening for carrier status include loss of privacy, misuse or results by insurers, employers and others, the relative costs and benefits, inadequacy of quality assurance for testing facilities, staff training (including that for physicians) and finally the tests themselves. Without an adequate assessment of the potentials for discrimination and stigmatization of individuals on the basis of their genotype, there is the possibility of creating a "genetic underclass" that is uninsurable and unemployable (Greely 1992; Nelkin 1992; Nelkin and Tancredi 1989; Weinberg 1991). The Office of Technology Assessment has documented situations where individuals have been threatened with termination of health insurance coverage based on testing positive for genetic disorders even though they were asymptomatic. In one case a health maintenance organization (HMO) tried to bully a pregnant woman whose fetus tested positive for CF by threatening to withhold coverage for the baby should it be brought to term (Bluestone 1992).

Many ethicists have identified the social consequences of genetic testing and the new reproductive technologies to be one of the most challenging issues facing our society. It is important to note that 3-5% of the $3 billion federally funded effort to map and sequence the human genome is designated for research into the ethical, social and legal implications of such knowledge (Foreman 1991; U.S. Dept. of Health and Human Services and U.S Dept. of Energy 1990; Watson 1990). The ultimate social acceptance of gene therapy is intimately tied to these discussions.

3.3 Special Diets and Treatments

Certain genetic disorders can be treated by alterations in diet. Most inherited metabolic diseases can be characterized in two ways: 1) accumulation of toxic substances in the body or 2) absence of essential metabolic products. Examples of diseases amenable to dietary restriction are given in **Table 3.4** (Nichols 1988).

Disease	Substance Restricted
• Familial lipoprotein lipase deficiency	• Neutral fats
• Fructose intolerance	• Fructose
• Galactosemia and galactokinase deficiency	• Galactose
• Lactase deficiency	• Lactose
• Methylmalonic acidemia and propionic acidemia	• Protein
• Phenylketonuria	• Phenylalanine
• Refsum syndrome	• Phytanic acid
• Urea cycle disorders	• Protein

Table 3.4 A partial list of diseases treatable by dietary restriction (Nichols 1988).

• **Importance of early detection and therapy.** None of these diets are capable of reversing damage that has already occurred *in utero.* For instance, children with galactosemia often have learning disabilities and, in girls, ovarian abnormalities even though the disease is detected by screening at birth and dietary changes made shortly thereafter. This highlights two important issues during pregnancy: 1) the value of early prenatal detection and 2) influence of environmental and maternal factors such as diet and lifestyle.

• **Fetal therapy.** It is always better to know about a fetal genetic disorder earlier rather than later. Therapeutic options and efficacy are usually greater in early development (**Figure 3.2**). Fetal therapy or medical intervention inside the womb, including surgery, blood transfusion, bladder shunting and pharmacologic treatment, has been successfully performed and is an active field of research (Evans and others 1992). Fetal therapy is becoming an issue of *when* and *whom* to treat, either indirectly through the mother/placenta or directly into the fetus using a transabdominal approach. Prerequisite to these procedures are advanced diagnostic and imaging techniques. For those individuals who reject genetic diagnosis because of its affiliation with the sensitive subject of abortion, fetal therapy, which is also dependent on genetic diagnosis, offers an alternative justification for the technology.

• **Maternal factors** are critical to fetal development. For example, a mother with phenylketonuria (PKU) who is not on a restricted phenylalanine diet can have a baby who is born mentally retarded, even though the baby does not have the PKU genotype. In such cases, if dietary changes are made prior to conception or early in pregnancy, fetal development is normal.

• **Dietary restriction** is nontrivial. It is not only expensive and often unappetizing, but also requires strict compliance. Managing the diets of affected children and educating them to monitor their intake while away from home is a tremendous burden on a family. Secondary behavioral effects can also be a problem for the siblings who are unaffected and often feel neglected on account of their normality and the decreased attention they receive.

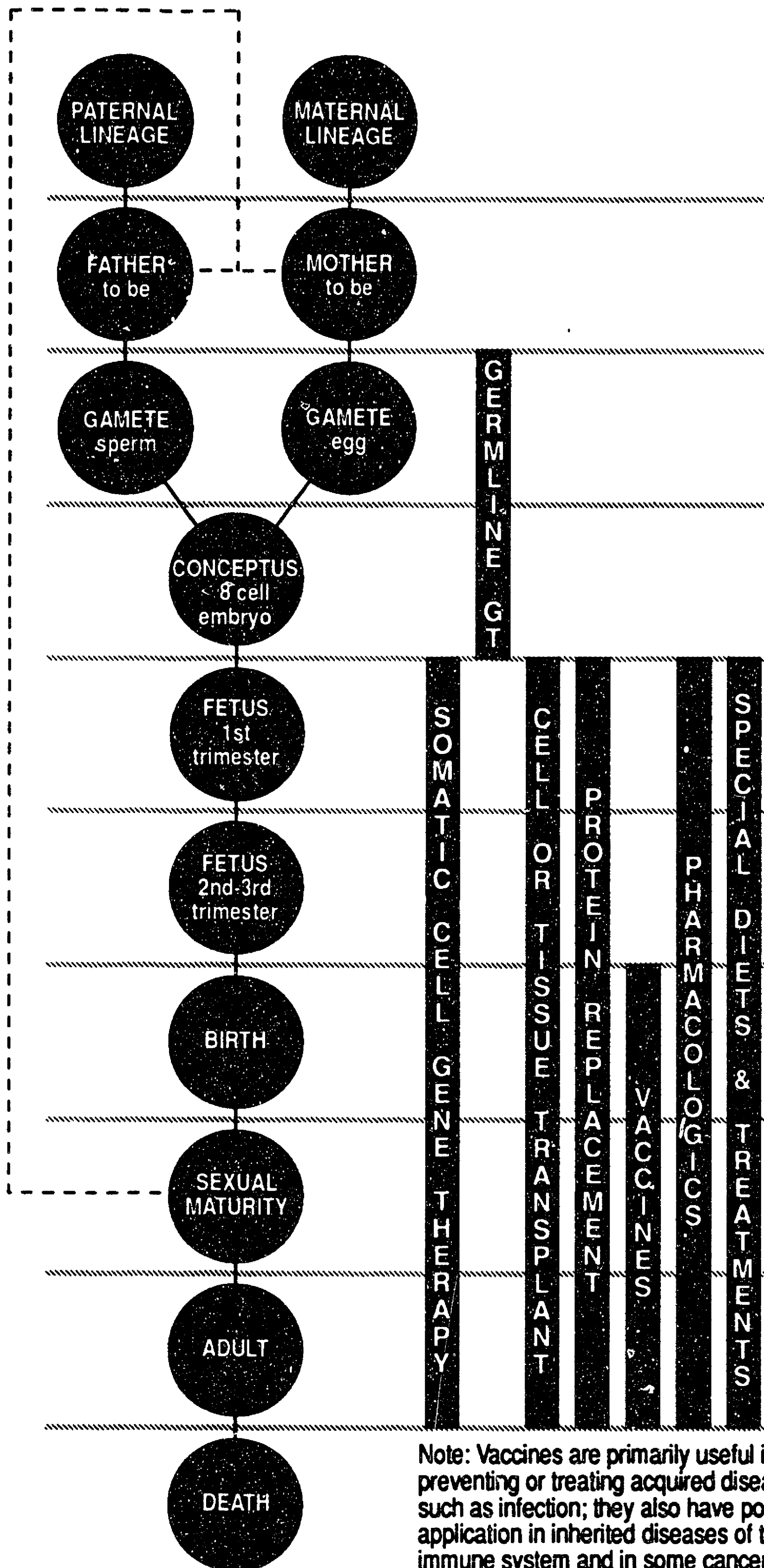

Figure 3.2 Possible therapeutic approaches to the treatment of inherited and acquired diseases.

• **Dietary supplementation.** Some genetic disorders are treatable by supplementing deficiencies in vitamins, coenzymes or other natural products:

- vitamin B6 (cystathioninuria)
- vitamin B12 (methylmalonic acidemia)
- biotin (proprionic acidemia)
- vitamin D (vitamin D resistant rickets)
- fat soluble vitamins A, D, E, K (cystic fibrosis)
- cortisone (adrenogenital syndrome)
- cysteine (cystinuria)
- thyroxine (congenital cretinism or hypothyroidism)
- uridine (oroticaciduria) (Emery and Mueller 1992).

• **Ecogenetics.** Genetic variations in susceptibility to environmental agents such as sunlight, foods, drugs, inhalants and infections are sometimes referred to as "ecogenetic" variations (Emery and Mueller 1992). Examples of environmental factors to be avoided by individuals with certain genetic disorders include:

- sunlight (albinism and xeroderma pigmentosum)
- smoking and air pollutants (alpha1-antitrypsin deficiency)
- smoking and fatty foods (familial hypercholesterolemia)
- certain muscle relaxants (pseudocholinesterase deficiency)
- barbiturates (porphyria) (Milunsky 1992b).

Research in this area is likely to increase as more and more attention is focused on the prevention of common diseases. Many social and ethical issues will arise if and when this new knowledge of genetic variation in susceptibility to environmental factors is factored into healthcare policy.

• **Miscellaneous treatments.** Lastly, there are many special treatments that are employed to manage the symptoms of genetic disorders. These range from a complete fix to lifelong chronic therapy. Examples are:

- surgical removal (colectomy for polyposis coli)
- surgical modification (congenital heart disease)
- cosmetic surgery (cleft palate)
- blood letting (idiopathic hemochromatosis)
- plasmapheresis (familial hypercholesterolemia)

3.4 Pharmacologic Therapies

• **Molecular basis of action.** Drugs are molecules which intervene in the biochemical workings of the cell. Drugs often act to interfer with the function of cellular proteins such as receptors, or enzymes. If they inhibit a reaction, they are referred to as "antagonists"; likewise, if they stimulate a reaction, they are referred to as "agonists". Drugs can intervene in cellular processes at any level: they can induce or suppress gene transcription, alter messenger RNA processing and translation or inhibit/activate protein function. They can alter membrane permeability and they can disrupt structural proteins responsible for cellular architecture.

Figure 3.3 is an example of the many ways that one net result, in this case the up-regulation of a hormone, can be achieved. A key concept is that molecules in the body are constantly being synthesized and degraded. Speeding up synthesis has the same net effect as slowing down degradation. One pharmaceutical compound may work by stimulating synthesis of a critical biochemical while another may achieve the same end by slowing down degradation. The relative therapeutic value of the two approaches may depend on undesireable side-effects or unfavorable interactions with other drugs administered at the same time. Other compounds may achieve the desired endpoint by affecting transport across cell membranes. For naturally occurring substances, it is often possible to supplement them directly.

• **Delivery aspects.** Drugs are commonly used in the maintenance and management of genetic disorders. Stepping up one level of abstraction, gene therapy can be thought of as a mechanism for the continuous delivery of a therapeutic chemical. Similarly, there are a multitude of drug delivery techniques and devices that accomplish similar tasks: drug impregnated polymers that can be implanted subcutaneously or intraperitoneally, mechano-electrical devices controllable by bio-feedback signals such as heartbeat, bone marrow ports, transdermal patches, radio-controlled pills and even "smart gels" which change their permeability to stimuli such as pH or voltage (Nanavati and Fernandez 1993; Travis 1993b).

- turn-down rate of absorption by other cells
- inhibit degrading enzymes in blood
- supplement blood hormone level by injection

- turn-up rate of synthesis
- turn-down rate of degradation
- turn-up rate of secretion

Sending Cell
(produces & secretes hormone into the blood)

Blood
(shaded area represents the concentration gradient of hormone emanating from sending cell)

- turn-up rate of absorption
- turn-down rate of degradation
- change the shape of the hormone molecule to increase specific activity
- change the shape of the hormone receptor to increase sensitivity

Receiving Cell
(absorbs hormone from the blood & becomes activated)

Figure 3.3 Possible pharmacologic approaches to up-regulate the biological action of a protein hormone.

Protein therapeutics until recently have not been deliverable by ingestion. They do not survive the digestive enzymes and extremes in pH. Research is underway to encapsulate proteins so that they can be administered orally, survive the gastrointestinal passage and be released intact at a proper locus for absorption across the intestinal membrane into the blood (Carrell 1993). Two encapsulation approaches are under commercial development: 1) thermally condensed amino acid microcapsules known as protenoids (Emisphere Technologies, Inc., Hawthorne, NY) and 2) microemulsions in which the internal phase is water and the external phase is lipid (Affinity Biotech, Inc., Boothwyn, PA).

Exogenously administered drugs have the benefit that they are amenable to quick changes in dosage should there be an abrupt change in the patient's medical status. Gene therapy, in contrast, is not as flexible. Cells which constitutively produce the therapeutic transgene product cannot be up- or down-regulated. As will be discussed later, some gene therapy protocols include insertion of a "suicide gene" which allows transformed cells to be killed in the event of an emergency by administering a drug such as ganciclovir. A better knowledge of the intracellular regulation of gene expression is necessary before inserted transgenes can be regulated *in vivo*. Without such detailed knowledge, externally administered proteins have the comparative advantage because of their ease in varying dosage.

• **Pharmacogenetics.** As mentioned previously, there is a genetic basis for the variability that is found in the response by different individuals to drugs. For instance, when soldiers in World War II were given the antimalarial drug, primaquine, about 10% of the Afro-americans and a smaller number of Caucasians (usually of Mediterranean origin) developed acute hemolytic anemia. Analysis of the blood of these individuals revealed that they had a mutant polymorphic form of the enzyme, glucose-6-phosphate dehydrogenase (G6PD), which was less stable than the normal form. The mutant form had a half-life of only 13 days compared to 62 for the normal form, resulting in a drop in the level of this essential enzyme in the red blood cells of the anemic soldiers (Gelehrter and Collins 1990).

Polymorphisms in drug metabolizing enzymes are thought to account for many of the observed racial differences in the therapeutic efficiency of drugs. For

instance, Orientals and Caucasians exhibit variable sensitivity to beta-blockers. Orientals are more susceptible to alcohol-induced facial flushing and palpitations due to the altered catalytic activity of their alcohol metabolizing enzymes, alcohol dehydrogenase and acetaldehyde dehydrogenase (Emery and Mueller 1992).

• **Traditional approach to drug discovery.** The ideal therapeutic compound is a small organic molecule, orally deliverable, and of novel composition of matter so that it can be patented. The process of discovery of such chemicals, as practiced by the pharmaceutical industry over the last 100 years, has relied on the brute force screening of vast libraries of chemicals obtained from many sources. It is often necessary to examine 20,000 - 40,000 compounds and mixtures of compounds to identify a promising pharmaceutical candidate. For instance, antibiotics were originally discovered in extracts of soil where certain molds grew. After purification and characterization of the active agent, organic chemists synthesize variations of this "lead compound" to see if its efficacy and safety can be improved. Classical medicinal chemistry has relied heavily on trial and error to discover these initial lead compounds. The process is slowly changing through a better understanding of the molecular basis of disease.

• **Biotechnological approach to drug discovery.** Biotechnology offers more sophisticated tools to what is basically the same approach. Using advances in molecular biology and genetics, it is possible to reduce some but not all of the trial and error by developing a clearer understanding of the molecular processes underlying the disease mechanism. The genes for critical proteins are mapped, isolated, sequenced, cloned, expressed and tested for therapeutic activity. The end products of this method are large, naturally occurring proteins usually with short physiological half-life, limited shelf-life, inconvenient mode of delivery (i.e., non-oral), and difficult and expensive manufacturing procedures. Because they are naturally occurring products, patent protection is more difficult. Biotechnology has yielded few novel therapeutics. Most of the field's products are compounds that were identified long before the advent of recombinant DNA techniques. Protein replacement therapy is discussed later.

• **Structure-based rational drug design.** This approach uses advanced 3-dimensional imaging techniques such as protein nuclear magnetic resonance

(NMR), X-ray crystallography and supercomputers to model and predict the active sites of proteins which interact with each other, with nucleic acids, or with small organic compounds. By using recombinant DNA techniques such as site specific mutagenesis, genetic variations of receptor proteins, prepared in milligram quantities, can be analyzed to determine the structural conformations necessary for biological function. Rational drug design thus combines the traditional small organic molecule synthesis and screening techniques perfected by the pharmaceutical industry with the newer molecular genetic protein engineering techniques developed by biotechnology to discover novel small molecules with therapeutic capability. These small molecules are often referred to as "mimetic drugs" because of their ability to imitate or mimic the pharmacological action of larger, more complex pharmaceuticals. It may be possible, for instance, to compensate for the defective CF transmembrane protein by administering a small molecule which binds to the protein and changes its architecture such that proper biological function is restored.

• **Antisense and other nucleic acid drugs.** It has been shown that cells actively take up nucleic acids. If the sequence of bases of the added nucleic acid is complementary to that of a naturally occurring messenger RNA in the cell, the compound can form a complementary double helix which inhibits translation of that mRNA into protein. This is known as "antisense" inhibition, because the therapeutic nucleic acid has a sequence which is opposite or complementary to that of the target nucleic acid. Most administered DNA is destroyed by nucleases in the cell. However, if the DNA oligonucleotide (about 20 bases in length) is made with a non-naturally occuring synthetic backbone, for instance sulfur is substituted for phosphorus, the compound retains its binding specificity but is resistant to intracellular nucleases. The synthetic backbone gives the antisense compound a longer *in vivo* half-life and, incidentally, a stronger patent position than its naturally occurring analogue. Antisense compounds are, in a matter of speaking, informational drugs. That is, the information contained in their sequence is what determines their biological activity. The same basic chemistry can be used to make a vast number of formulations, each highly specific. A oligonucleotide only 20 bases long (most genes are in the thousands of bases) can exist in 4^{20} or about a *million-million* possible different combinations.

It has been suggested that antisense oligonucleotides offer a new methodology

for rational drug design (Robert D. Rosenberg, personal communication). Just as physical techniques such as X-ray crystallography can be used to probe the *spatial* interactions of molecules, antisense technology can be used to probe the *kinetic* interactions of molecules in metabolic pathways. The ability to exogenously control enzymes by down-regulating their synthesis from mRNA templates means that biochemical pathways can be rationally probed to elucidate rate limiting steps and alternate pathways.

What distinguishes antisense therapy from gene therapy, since both are nucleic acids? The difference is subtle. Antisense is more like a traditional drug since it is made by chemical means and acts directly, no replication, transcription or translation being required. Gene therapy requires at a minimum that the nucleic acid, which is made biologically, behave *in vivo* like a gene, by being transcribed into RNA which may act directly or be processed further by translation into protein. Gene therapy can embrace an antisense modality. For instance, a gene encoding an antisense RNA complementary to a sequence of the HIV-1 virus that is essential for replication, when introduced into cultured human cells, blocked the production of infectious HIV-1 by greater than 99% (Chatterjee and others 1992).

Exogenously administered nucleic acids can do more than just inhibit messenger RNA translation. Certain sequences are capable of binding double-stranded DNA to form triple-stranded helices, raising the possibility that gene replication and transcription into RNA could be regulated by nucleic acid drugs. Certain RNA sequences also have catalytic, enzyme-like properties. These RNA enzymes or "ribozymes" as they are called are capable of splicing themselves and other RNAs, making them potentially useful as selective blockers of RNA processing in the nucleus.

• **Drug design by directed molecular evolution.** The extraordinary diversity that exists in populations of randomly synthesized nucleic acids has led researchers to devise selection screens to isolate and amplify those particular molecules that can perform a specific useful task, such as binding to and inhibiting the action of thrombin (Bock and others 1992). Thrombin inhibiting drugs are used to prevent clotting during coronary-bypass surgery. Currently used protein drugs may also stimulate unwanted immune reactions. A nucleic

acid that could inhibit thrombin action without eliciting adverse side effects would be very useful. The problem, however, is *which* nucleic acid out of millions upon millions of possibilities works? No problem. Researchers have successfully used a technique referred to as "selective evolution of ligands by exponential enrichment" or SELEX for short. Using DNA synthesizer machines, they constructed a random combinatorial library of $>10^{13}$ individual sequences that were 60 nucleotides long. Recall that there are 4^{60} or 10^{36} possible sequences 60 nucleotides long, so their sample library represents only one in 10^{23} of the possible unique libraries of similar size. The 60-mer DNA molecules were linked on each end with PCR primer sequences so that any rare molecules which bound to thrombin, which was immobilized on a solid support, could be amplified and sequenced. Out of the 10^{13} possible candidates, they found 32 distinct sequences which bound to and inhibited thrombin.

Other researchers have taken this selection/amplification process a step further by adding mutation at each round or "generation". By repetitively using selection procedures which only allow molecules that meet a functional criterion (e.g., catalyze a specific reaction) to be amplified and by introducing mutations at each round of selection and amplification, researchers were able to direct the *in vitro* evolution of a ribozyme resulting in a 100 fold increase in activity (Beaudry and Joyce 1992; Culotta 1992).

The obvious power of these techniques, which have been jokingly referred to as "Darwin in a test tube", is that it is not necessary to rationally predict how a molecular entity is going to do a specified task. Instead one need only let it "do its thing", and then *select*, then *amplify*, then *mutate* to get it to do even *better*. Instead of "rational design", one has "irrational design coupled with rational selection" (Amato 1992; Brenner and Lerner 1992). A new way of looking at populations of molecules is developing. Statistical polling techniques are used to identify and isolate candidates from extremely large and diverse populations of molecules. Researchers now talk about a "fitness landscape" or "catalytic task space" to refer to a domain defined by a functional subset of molecules within a "combinatorial repertoire" (Edgington 1993).

The potential diversity contained in nucleotide sequences is on a scale as big as the universe is large. This potential diversity is so extraordinarily vast that

Nature cannot have possibly expressed them all, even given all of geological time. "Nature has missed some opportunities" (Paul Schimmel, personal communication). Future advances in molecular evolutionary sciences are going to surprise us with amazing therapeutic capabilities we could never have possibly imagined. Some of these surprises will become integrated into gene therapies. Others will find expression in small molecule mimetic drugs.

3.5 Protein Replacement Therapies

If modern molecular diagnostics and genetics can identify a missing or defective enzyme as the cause of an inborn error of metabolism, it seems logical that pharmacological replacement might be beneficial (Larrick and Burck 1991) (**Table 3.5**). One of the oldest forms of protein replacement is insulin for diabetes mellitus. Hormone deficiencies lend themselves well to replacement therapy because hormones naturally circulate in the blood. Pharmacologic introduction of a hormone into the circulatory system is a relatively minor biochemical intervention. The trafficking of the exogenously introduced hormone follows the same route to the target cell's membrane receptors, where it triggers a normal cascade of intracellular signals.

The introduction of proteins which do not normally reside in the blood however present two major problems: 1) rapid inactivation or removal of the foreign protein, thus necessitating chronic therapy, and 2) immune reaction to the foreign protein, thus preventing an efficacious therapy.

• **Chemical modifications to lengthen halflife.** In some cases, proteins can be chemically modified to increase *in vivo* halflife by protection from proteases and antibodies. An example is adenosine deaminase (ADA). Lack of this enzyme causes loss of lymphocytes and resulting severe combined immune deficiency (SCID) in children. Attempts to administer exogenous ADA purified from cow (bovine) tissues were not successful because the enzyme was quickly inactivated in the blood. Researchers found, however, that if the bovine ADA was first chemically linked to polyethylene glycol (PEG), an inert waxy substance, it was protected from degradation (Hershfield and others 1987). The PEG also

Disease	Replaced Protein
• Diabetes mellitus	• Insulin
• Pituitary dwarfism	• Growth hormone
• Congenital hypothyroidism	• Thyroid hormone
• Hemophilia A	• Factor VIII
• Hemophilia B	• Factor IX
• Cystic fibrosis	• Pancreatic digestive enzymes
• Gaucher's disease	• Glucocerebrosidase
• Adenosine Deaminase Deficiency (SCID)	• Adenosine Deaminase-PEG
• Tay-Sachs	• Hexosaminidase A
• Congenital trypsinogen deficiency	• Trypsinogen
• alpha1-Antitrypsin deficiency	• alpha1-Antitrypsin
• Fabry's disease	• alpha1-Galactosidase

Table 3.5 A partial list of diseases treatable by protein replacement (Larrick and Burck 1991).

appeared to block the immune response to the protein. As a consequence of covalent attachment with PEG, the bovine ADA halflife was increased from a few minutes to days. The successful preliminary results using PEG-ADA therapy have raised the important question about the suitablity of gene therapy, given the relative risk/benefit ratios of the two approaches (Mulligan 1991).

• **Contamination from uncontrolled biological sources.** Replacement proteins are typically extracted from animal or human sources. ADA is obtained from cow tissues, glucocerebrosidase from human placenta and factor VIII from human blood. Contamination can occur by naturally occurring impurities which "co-purify" with the desired protein. Hormones for example, which are biologically active in trace amounts and not easily detectable by physical assays, can contaminate highly purified therapeutic protein preparations.

Contamination by viruses or proteinaceaous infectious particles (prions) (Pruishner 1991; Watson and others 1987) is an everpresent problem with biological sources. Between 60-80% of the hemophiliacs receiving factor VIII preparations before 1984 contracted HIV from contaminated sources (Nichols 1988). Prior to 1984, blood products were not screened for HIV. Hemophiliacs were the unfortunate victims of a therapy which treated one disease but caused another, namely AIDS. Once an infectious agent is identified and an assay is developed, quality control procedures can be implemented to essentially eliminate it as a risk. While protein purification and assay technology have advanced to a very high level of sophistication, the problem is, however, that one does not know conclusively everything that might be contaminating a therapeutic protein, which is frequently extracted from tissues pooled from hundreds, even thousands of individuals.

• **Advantages of production using recombinant methods.** The advent of recombinant DNA technology has eliminated the need to extract proteins from biological sources of questionable purity. By transfecting an established laboratory cell line with the gene for the therapeutic protein, it is possible to grow the cells in bioreactors under very stringent quality control procedures, harvest them and purify the protein. Examples of therapeutic proteins now made by recombinant DNA techniques and approved for commercial use include insulin, human growth hormone and factor VIII. Genzyme is currently building

a facility in Boston to produce recombinant glucocerebrosidase for Gaucher's disease.

Mention should also be made of the fact that many proteins require the post-translational addition of sugars (glycosylation) for biological activity. Bacterial cells do not glycosylate proteins, while mammalian cell lines such as Chinese Hamster Ovary (CHO) cells do. Mammalian cells, even though they are more costly to culture, are frequently the cell line of choice on account of their glycosylation capability.

• **Transgenic animals as bioreactors.** Another potential source of recombinant proteins is transgenic animals. Genes can be introduced into the embryonic cells of farm animals such as pigs, sheep or goats. By putting the gene for the desired therapeutic protein under the control of a tissue specific promoter, for example that for the milk protein casein, expression of the subject protein in an easily harvestable form, in this case milk, is possible. As a first step to making the cystic fibrosis transmembrane protein in farm animals, researchers have successfully made transgenic mice which express the protein in their mammary glands (DiTullio and others 1992).

Transgenic production of therapeutic proteins represents a potentially revolutionary improvement in reducing the cost of protein drugs. In the words of David Housman, one of the founders of Integrated Genetics, a pioneering firm in transgenics which was later acquired by Genzyme, "It's much cheaper to feed a goat some hay than it is to feed a tissue culture cell media and serum every day!". Genzyme has recently formed a separate company dedicated to transgenic "pharming" of protein drugs.

• **"Renting the cure vs owning it".** Protein replacement therapy requires a lifetime of continued pharmacologic treatment, mostly if not exclusively by injection. The costs can be very high. Factor VIII treatment for hemophilia can range from $50,000 to $300,000/year (Rosenberg 1992). One interviewee likened protein therapy to "renting the cure" while gene therapy offered the possiblity of "owning the cure". Possession of the gene within one's body is like owning the real estate instead of renting the use of it by the day.

Many biotech firms with proprietary interests in recombinant proteins are starting gene therapy development programs to insure they have a continuing participation in the field, irrespective of the modality which proves safer, more efficacious, more economic or more socially acceptable.

3.6 Cell or Tissue Transplants

Another way of vesting or empowering an individual to manufacture his or her own missing protein (or other gene product) is by cell or tissue transplantation. This is a form of gene therapy in that the beneficial gene is transfered to the patient not as an isolated entity, but within the genomic background of a cell from an acceptable donor. The recipient becomes in effect a chimera, not only at the specific genetic locus of therapeutic interest, but at many other loci as well.

• **Tissue rejection.** Tissue transplantation can be of several basic types (Connor and Ferguson-Smith 1991):

- Autograft: from self to self
- Isograft: between identical twins
- Allograft: between same species
- Xenograft: between different species

Autografts and isografts are genetically identical to the recipient; rejection by cell-mediated immunity is not a problem for these transplants. Xenografts, however, will always be rejected unless immunosuppressive treatments are administered simultaneously or unless the foreign antigens of the donor cells are stripped or masked. Allografts are intermediate in terms of rejection. Donors must be as antigenically similar to the recipient as possible. Compatibiliity is measured by comparing the ABO blood group and HLA tissue type of the recipient and prospective donor. Another technique is to mix immune-competant lymphocytes from both donor and host in culture. If the match is compatible, then rejection will not occur. If the match is poor, rejection will occur despite the use of immunosuppressants. This test is critical for bone marrow transplantation.

• **Graft-versus-host disease.** Immunocompetant cells, which originate in the

bone marrow of the donor, can mount an attack on the cells of the host if they are antigenically different. This phenomenon is known as "graft-versus-host" disease. The transplanted immune cells recognize the host cells as "foreign" and begin to attack them, causing serious damage to the skin, liver, intestines and other tissues. The probability that two siblings will have matching antigens, i.e., be histcompatible, is only 1 in 4. Only about 30% of the patients with diseases treatable by transplantation can find a histocompatible donor (Nichols 1988).

• **Bone marrow transplantation.** A partial list of genetic disorders amenable to tissue transplantation therapy is given in **Table 3.6** (Larrick and Burck 1991). Bone marrow transplantation (BMT) has been a successful therapy for many well-defined genetic disorders. The bone marrow is the main blood forming organ in adults. It contains a rare cell known as a *totipotent stem cell* which can not only give rise to more of itself but can also differentiate into 1) *lymphoid progenitor cells* and 2) *hematopoietic progenitor cells.* The lymphoid progenitor cells give rise in turn to the two basic elements of the immune system, the *T-lymphocytes*, which are responsible for tissue graft rejection and other aspects of cell-mediated immunity, and the *B-lymphocytes*, which are the antibody-producing cells. The hematopoietic progenitor cells give rise in turn to *red blood cells, platelets* and scavenger cells known as *mononuclear phagocytes*. Each branch of this differentiation from the totipotent stem cell is controlled by the micro-environment, the close proximity of one type of cell to another, and by growth factors known as cytokines.

In order to successfully transplant or engraft new stem cells into a patient's bone marrow, it is necessary to make room for them by destoying the patient's existing bone marrow with highly toxic chemotherapeutic drugs, alone or together with massive levels of radiation. This procedure creates the necessary "physiological space" for the new cells, which otherwise could not compete and outgrow the existing ones (Nichols 1988). During this period of engraftment and cellular repopulation, the patient is highly susceptible to infection. The longterm effects of chemo- and radio-therapy are not known, but most patients do become sterile. Bone marrow therapy has three major drawbacks: 1) lack of histocompatible donors 2) risk of graft-versus-host disease and 3) severity and side effects of chemo- and radio-therapy.

Bone Marrow Transplantation

- Adenosine deaminase deficiency
- Chediak-Higashi syndrome
- Chronic granulomatosis disease
- Fanconi's anemia
- Gaucher's disease
- Hunter's syndrome
- Hurler's syndrome
- Infantile agranulocytosis
- Lesch-Nyhan syndrome
- Nucelotide phosphorylase deficiency
- Osteopetrosis
- Sickle cell anemia
- Thalassemias
- X-linked agammaglobulinemia
- Wiskott-Aldrich syndrome

Liver Transplantation

- alpha1-Antitrypsin
- Congenital tyrosinemia
- Type IV glycogen storage disease
- Type II hyperlipoproteinemia
- Wilson's disease

Kidney Transplantation

- Polycystic kidney disease
- Cystinosis

Lung Transplantation

- Cystic fibrosis

Pancreas Transplantation

- Cystic fibrosis
- Diabetes mellitus (islet cells)

Table 3.6 A partial list of diseases treatable by tissue transplantation (Larrick and Burck 1991).

• **Solid organ transplants.** Also indicated in **Table 3.6** are solid organ transplants which have been used to treat genetic diseases. The risks associated with organ rejection and lifelong suppression of the immune system are very great. Consequently, these procedures are reserved for cases where organ failure is imminent.

3.7 Vaccines

There are two types of immune response which the body uses to fight infectious agents such as bacteria and viruses:

- Humoral immunity by the B-lymphocytes
- Cell-mediated immunity by the T-lymphocytes.

• **Humoral immunity** is an organism's first line of defense. Millions of B-lymphocytes, which originate in the bone marrow, circulate in the blood, constantly scanning for the presence of foreign substances. Each B-lymphocyte, through a process of gene shuffling, makes a unique antibody which is presented on its outer cell surface and which functions as an antigen receptor. Antibodies are four-chained proteins capable of binding with precise specificity to foreign proteins and other substances known collectively as antigens. When an antigen binds to an antibody on the surface of a specific B-lymphocyte, it triggers the clonal expansion of that particular B-lymphocyte and activates the resulting progeny cells to secrete those specific antibodies into the blood. The foreign substance, when complexed with this antibody, is then cleared from the blood by cells known as phagocytes. If the organism is challenged with the same foreign substance at a later date, the second response will be more rapid and produce more antibodies than the first. The first contact imparts "memory" so that the body is prepared for a second invasion should it occur. A state of immunity against blood-born invasion is thus established (Roitt 1991).

• **Cell-mediated immunity** is an organism's second line of defense. It is directed against cells that have already been infected or have taken up foreign proteins from the environment. Viruses and certain bacteria are capabable of hiding within cells, out of "view" of the B-lymphocytes. A totally separate acquired immunity system has evolved to address this problem. T-lymphocytes, which

also originate in the bone marrow but migrate to the thymus gland, are specialized to identify cells that harbor intracellular parasites. T-lymphocytes are similar to B-lymphocytes in that they have antigen receptors on their cell surfaces but differ in that they do not secrete antibodies. Instead, T-lymphocytes scan the surfaces of body cells "looking" for antigen signals, fragments of degraded foreign proteins, indicating that the cell is infected. These fragments are bound to molecules known as MHC-I glycoproteins and "presented" on the surface of the infected cell where they are recognized by a specific prekiller (unactivated) $CD8^+$ T-lymphocyte. In a manner which is entirely analogous to that of the B-lymphocyte, this specific $CD8^+$ T-lymphocyte undergoes clonal expansion into cytotoxic "killer" T-lymphocytes that then kill the infected cell.

If a cell has taken up previously synthesized foreign proteins from the surroundings by endocytosis, they too are digested into peptide fragments, complexed to a different glycoprotein known as MHC II, and similarly presented on the cell surface for the scrutiny of patrolling T-lymphocytes. This time a specific $CD4^+$ T-lymphocyte recognizes the cell "presenting" the fragment complexed with MHC II and undergoes clonal expansion into helper T-lymphocytes that assist B-lymphocytes in mounting an antibody response against the cell (von Boehmer and Kisielow 1991).

• **Non-live vaccines.** Vaccines can be made from killed viruses or from proteins extracted from them. Vaccines can also be made by recombinant DNA techniques using gene fragments that code for viral proteins. These killed or nonliving vaccines stimulate humoral immunity through a B-lymphocyte mediated antibody response. Humoral immunity is effective as a preventive vaccine. It is not, however, effective in destroying cells that are already infected.

• **Live attenuated vaccines.** Vaccines made from live attenuated viruses are capable of stimulating both humoral immunity and cell-mediated immunity. The latter is necessary to kill an infected cell. Live attenuated viruses are essentially gene transfer products. For example, the Sabin polio vaccine is a live virus whose disease causing ability has been neutralized, but whose ability to infect human cells has not. When a patient takes this vaccine, the virus infects the patient's cells, producing viral proteins which stimulate both a cytotoxic T-lymphocyte and an antibody response.

Current attenuation technology cannot insure that the disease causing ablility is permanently disabled. Attenuated viruses therefore can be a risk for the vaccinated patient as well as individuals in contact with vaccinated patients. Live attenuated vaccines for viruses such as HIV, hepatitus B, hepatitus C, herpes simplex and others have not yet been developed.

• **Gene transfer based immunotherapeutics.** Alternative vaccines are being developed which use non-replicating retroviral vectors to deliver genes which code for antigenic proteins from contagious viruses such as HIV (Jolly and Warner 1990; Jolly and Warner 1991; Warner and others 1991). The objective of these vaccines is to safely stimulate a cell-mediated immunity as well as an antibody response that would be effective in not only preventing infection but also in treating patients who are already infected. Such gene transfer based immunotherapeutic approaches must compete with the more traditional vaccine technologies.

3.8 Summary

Figure 3.4 is a summary of all the approaches to the prevention or the treatment of genetic diseases which have been discussed here. The use of vaccines and the resistance to bacterial and viral pathogens have also been included as there is a genetic component to the susceptibility to infection.

Several themes recur throughout this chapter. One is that prevention of genetic disorders by prenatal diagnosis and selective abortion, while it is the most effective procedure, is unacceptable to many couples. *In vitro* fertilization and preimplantation genetic diagnosis may offer an acceptable alternative.

Another theme is that there are usually more therapeutic options available the earlier a genetic disorder is detected. Fetal therapy, either directly through a transabdominal approach or indirectly through the mother's blood and the placenta, will become of increasing importance as a result of improved prenatal imaging and diagnostic techniques. The recent reversal of the twelve year ban on fetal research by the new presidential administration will accelerate investigation in this area.

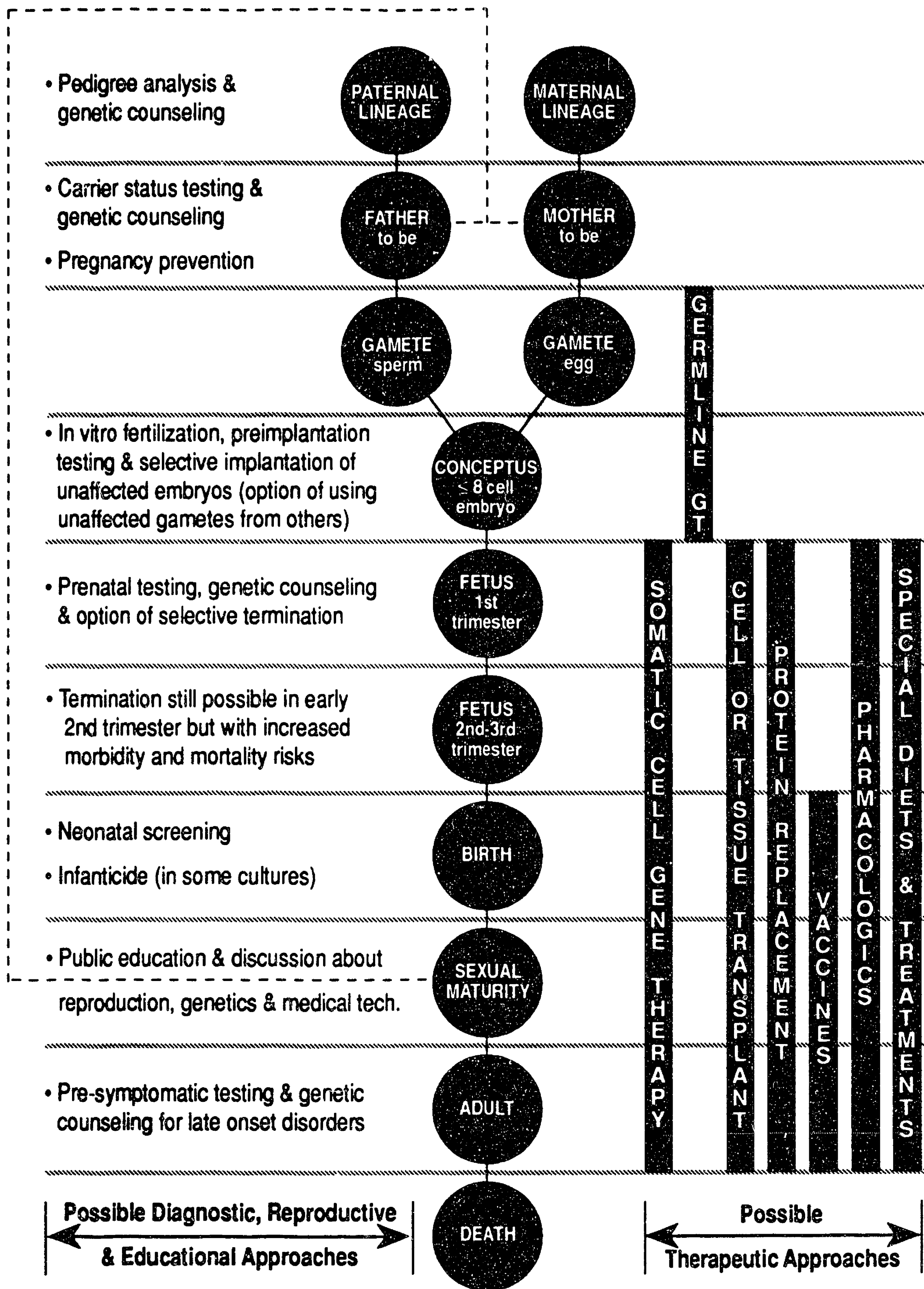

Figure 3.4 Possible approaches to the prevention or treatment of genetic diseases.

Advances in gene therapy will depend on the development of the same core technology and base knowledge that supports competing approaches such as recombinant protein replacement therapy and small molecule mimetic drugs.

The ethical, legal and social implications of genetic and reproductive technologies will continue to occupy a key position in policy discussions. The ever increasing rate of scientific and technical advances and options will push society to make decisions faster than it has in the past. The social acceptance of gene therapy will depend in part on the acceptance of other biotechnology based diagnostics and therapies.

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CHAPTER 4
BASICS OF GENE THERAPY

4.1 Overview

This chapter will provide an introduction to the basic issues of gene therapy. It will begin with a discussion of the social, scientific and technical distinctions between gene repair vs gene enhancement and between somatic cells vs the germline. These two pairs, when "crossed" with each other, form a 2x2 matrix, and each of the four possible combinations will be discussed.

Next, a brief summarization of the major discoveries of modern molecular biology will be presented as a conceptual framework which is useful for organizing intervention strategies that are possible with gene therapy.

Finally, the practical concerns involved in implementing and enabling gene therapy will be discussed.[7] The field is evolving very rapidly. Any attempt to be encyclopedic would be doomed to certain failure; the specifics described in this thesis will no doubt be out-of-date before this weighty tome hits the library shelf. It is the hope of this author that he will have provided a "snapshot" of the fleeting details that will be sufficient to gain a glimpse of the more enduring fundamental issues which will persist into the future.

Specifically, there are at least eight basic practical issues which must be addressed in the design of any gene therapy protocol:

1. What is the gene (including regulatory sequences) being transferred?

2. What is the gene transaction- a new addition, a repair, or a knockout?

3. Is the gene integrated into the host genome or is it a free nuclear entity

[7] Manufacturing and regulatory concerns are discussed in Chapter 6.

and is it necessary to pass the gene on to clonal progeny?

4. What is the vector?

5. What is the delivery method?

6. What is the length of action and is multiple treatment necessary?

7. What is the target cell and what percentage must be successfully hit?

8. What are the therapeutic objectives at the molecular, cellular and organismic (clinical) levels?

The clinician will tend to approach these issues by moving upwards from the bottom of the list while the scientist and technologist will tend to approach these issues by moving downwards from the top of the list. The entrepreneur, on the other hand, will tend to oscillate between both approaches, as well as include social and market factors such as public acceptance, investor willingness, pricing, regulation, proprietary position, etc. All of these approaches working in concert are necessary to bring any socially sensitive technology to commercial fruition. Gene therapy is perhaps the ultimate socially sensitive technology. Unlike other technologies, gene therapy is not about changing things *around* us. Gene therapy is about changing things *within* us.

4.2 Scope of Gene Therapy

There are two classes of cells which are of upmost importance to the discussion of gene therapy: germline cells and somatic cells. Early in the development of all vertebrate embryos, certain cells are singled out to become progenitors of the gametes, the ovum (egg) in females and the sperm in males. These primordial germline cells, as they are called, migrate to the developing gonads, the ovaries in females and the testes in males, where they proliferate by a cell division process known as mitosis. Later in life they undergo a different type of cell division known as meiosis where they differentiate into gametes, losing one-half their chromosomes in the process. The remainder of the cells in the developing embryo proliferate by mitosis and differentiate into somatic (meaning "body")

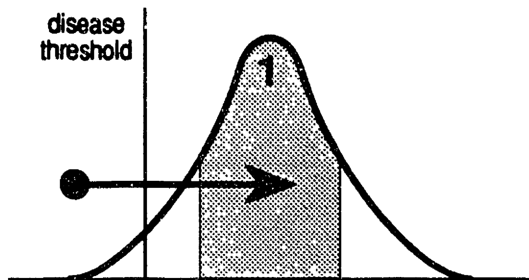
tissue such as muscle, skin and brain.

Only the germline cells have direct significance to the next generation. It is their genetic endowment that will be passed on to the next generation when the process of sexual reproduction, the fertililization of the ovum by the sperm, is repeated once again. The somatic cells of the parent also have significance to the next generation, but only indirectly, relating to the ability of the parent to reach the age of sexual maturity and successfully mate. For example, the muscle cells, one of many differentiated somatic cell types, are obviously essential to escape predators. In our early nomadic stages of evolution, a child with an inherited defect in his muscle cells would not survive the rigors of life in the wild and would not live long enough to reproduce; his germline cells would not be passed on in the population. The ensemble of somatic cells that make up an individual must pass a collective "fitness test" before the genes of that individual are passed on in the "gene pool" of the population.

The egg and the sperm contain all the information necessary to make more of themselves via the germline cells as well as all the other different types of somatic cells. Cells which can make more of themselves as well as, under certain circumstances, differentiate into specialized cells with unique functional capabilities, are known as "stem cells". The germline cells are the ultimate "totipotent" stem cell. There are also somatic stem cells such as the hematopoietic (blood-forming) stem cells which differentiate under the influence of growth factors into all the specialized cells circulating in the blood. They will be discussed later.

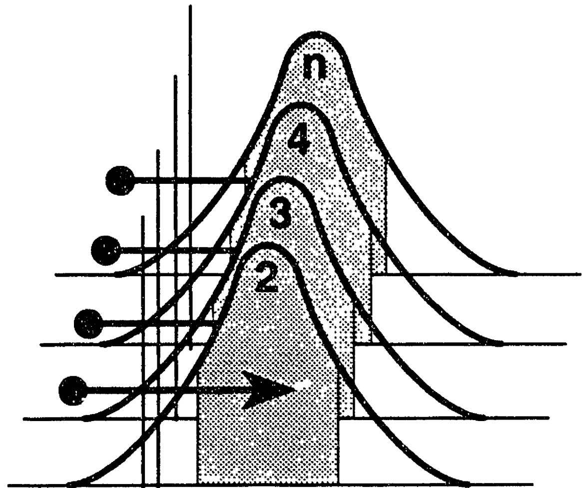
As shown in **Figure 4.1,** there are four basic categories of possible human genetic modification, depending on cell type and functional objective.

• **Somatic Cell Repair.** A central tenet born out of years of experimentation is that each somatic cell in an individual contains the same set of genes. What makes a muscle cell different than any other somatic cell type is that only a certain subset of the total endowment of genes is expressed. The genes that are not essential for muscle function are present, but silent. A muscle cell also has many "housekeeping genes" which are expressed in all cells, for example those involved in oxidative metabolism. When a deleterious mutation occurs in a



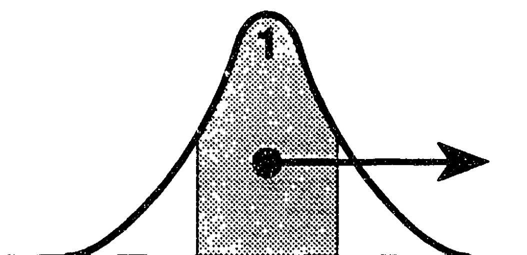
Somatic cell repair

add a gene to any cell in the body
(except a sex cell) to correct a deficiency
in an individual (generation #1)



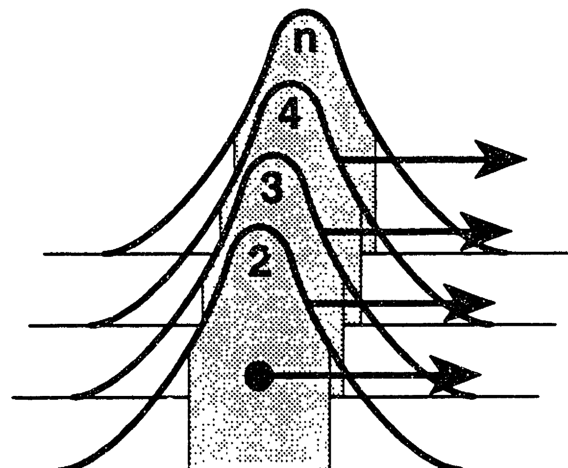
Germ line repair

add a gene to a cell destined to become
an egg or a sperm to correct a deficiency
in an individual's progeny (generations #2 to n)



Somatic cell enhancement

add a gene to any cell in the body
(except a sex cell) to confer a
new trait or augment an existing trait
in an individual (generation #1)



Germ line enhancement

add a gene to a cell destined to become
an egg or a sperm to confer a new trait
or augment an existing trait
in an individual's progeny (generations #2 to n)

Figure 4.1 The four basic categories of possible human genetic modification.

gene, only those cells where the gene is expressed are affected. For example, Duchenne Muscular Dystrophy (DMD) is a muscle degeneration disease in boys caused by the inheritance of a mutant dystrophin gene on the X chromosome inherited from the mother. The gene is primarily expressed in the muscle (Gelehrter and Collins 1990), though dystrophin mRNA has been found in the brain, which may explain mental retardation in some patients (McKusick 1992). There are major technical reasons (such as the size of the gene) and biological reasons (such as the morphology of muscle cells) why DMD would be very difficult to correct with gene therapy. But if it could, and it may, it would be an example of somatic cell repair. The benefit would accrue to the patient. As depicted in **Figure 4.1**, the individual's deficiency in the gene product, the protein dystrophin, would be brought into the normal (shaded) range. There would be no effect on the progeny because the germline cells would be unaffected.

• **Somatic Cell Enhancement**, as the name suggests, involves altering the normal, non-diseased somatic cells to exhibit an enhanced characteristic or an altogether new characteristic. An example of an enhanced characteristic might be the addition of a gene to "overproduce" human growth hormone in a child of normal stature in order to gain even greater height. An example of an altogether new characteristic might be the introduction of a gene that confers intracellular immunity to an infectious agent, in effect a vaccine with preventive (prophylactic) properties. A gene coding for an antisense RNA or a protein that could block replication of HIV would be such an application.

In contradistinction to somatic cell repair, where restoration of "normality", the absence of disease, is very clear, somatic cell enhancement enters a murky realm. The first question the NIH-RAC asks of any proposed somatic gene therapy protocol is "What is the disease to be treated?" (Walters 1991). Researchers are required to present a convincing argument that the disease is real and is serious enough to warrant use of an unproven approach such as gene therapy. A proposal to enhance an existing or to create a new human capability, rather than to cure a disease, would be most certainly excluded by this question.

A gene therapy protocol to treat hereditary dwarfism with the gene for human growth hormone might clear the RAC's hurdle, but enhancing a normal child's

height merely at the discretionary request of the child's parents would most certainly not. The benefit would not be commensurate with the risk. The use of gene therapy to enhance resistance to a pathogen, particularly a very serious one for which there is no effective therapy, such as HIV, would seem to be an appropriate risk. The use of killed or latent viruses as preventive vaccines for diseases such as polio would also serve as a precedent in this particular case.

Several issues underly somatic enhancement, be it by genes or any other means. The first is the potential for the abuse of technology for arbitrary or capricious reasons, without proper consideration of the risks inherent in the procedure. Certain cosmetic surgeries such as elective breast augmentation with silicone implants might fall into this category, though the situation would seem to be different in the context of restoration after a mastectomy due to cancer.

Another issue is whether we as a society condone the use of technology to modify one's body to gain some perceived or real competitive advantage. Assuming one has made an informed decision about the risks and the benefits, does one have the right to gain an advantage over others by chemical means? Olympic athletes, for example, are disqualified from competition if they are found to have taken body-building steroids. They are regarded as having gained an unfair and unsafe advantage and as having transgressed the spirit and intent of the game. There is both a medical and a moral decision here.

Underlying these issues is an even more fundamental one, and that is the lack of adequate public knowledge and understanding. The March of Dimes Birth Defects Foundation recently commissioned Louis Harris and Associates to conduct a public opinion poll on genetic testing and gene therapy (Louis Harris and Associates 1992). Among the findings were that, while the vast majority (89%) of Americans approve of using gene therapy to treat genetic diseases, they (86%) also admit to knowing little or nothing about it. Forty-three percent also said that they approve of using gene therapy for eugenic (non-therapeutic) purposes such as improving the physical characteristics or intelligence of children. Commenting on the results of the poll, Dr. Jennifer Howse, Foundation President, noted, "As with other rapidly changing technologies, advances in gene therapy seem to have outpaced the public's understanding of its possibilities" (Kling and Stein 1992).

This public knowledge gap represents perhaps the greatest vulnerablility for gene therapy as an emerging industry. If the primary source of public information about genetic engineering and biotechnology comes from sensational books and movies, for example, about greedy, unscrupulous scientists cloning dinosaur DNA, then the prospects for understanding are bleak. The numerous errors in the fictional premise of the book, Jurassic Park, have been discussed (Bains 1993). On the other hand, there are articles appearing in the popular press not about fictional harm but about real life benefits, such as the two girls who first received gene therapy for severe combined immunodeficiency due to ADA deficiency (Thompson 1993a). No beneficial technology is without risk. A balanced presentation to the public is of immense importance.

• **Germline Repair,** as shown in **Figure 4.1**, involves therapy which affects not just one individual, but all future progeny. The candidate target cells for germline gene therapy are 1) the gametes themselves 2) the embryonic stem cells or 3) the gamete producing cells in the mature gonads (the oogonia and spermatogonia). The genetic material in sperm is densely compacted making it a technically difficult target. The egg, unfertilized or newly fertilized, on account of its relatively large size and ease of handling, is the most likely target. Transgenic techniques developed for laboratory and farm animals are highly inefficient and not suitable in terms of acceptable safety and efficacy criteria in their current state for human use. The day will come, however, when the technical issues are no longer a barrier, leaving the ethical, legal and social issues to be resolved, if they have not already.

Germline repair is understandably a very sensitive subject. In 1986, the bioethicist Dr. LeRoy Walters wrote, "No aspect of gene therapy is more highly charged than that of germline or germ-cell therapy; it might seem, therefore, politically prudent to avoid the subject. But what is politically prudent may not be ethically responsible. In fact, timely ethical discussion of this issue, before germline gene therapy in humans is technically feasible, may assist future policymakers in their deliberations." (Walters 1986). Walters, who is currently Chair of the NIH-RAC, has proposed that the formal public discussion process should begin on germline gene therapy (Walters 1991).

There are several circumstances that have been discussed in the literature where

germline gene therapy might be warranted (Walters 1986). The first are situations where there is an argument for *efficiency*. Any successful candidate disease for somatic cell gene repair is also an eventual candidate for germline gene repair. If the somatic approach is proven to be successful for the cure of single gene recessive disorders like cystic fibrosis or Gauchers disease, then individuals treated with somatic cell gene therapy will grow to maturity and presumably be capable of reproduction. They will, by virtue of the medical intervention, become homozygous "carriers" capable of passing on the defective gene to their progeny at a frequency higher than that of heterozygote carriers.[8] While their children likewise could be phenotypically cured by somatic cell gene therapy, as they were, some parents may consider it more efficient to prevent the transmission of the defective gene to the next generation, if some method were available. Germline gene repair would be such a method. As was discussed in Chapter 3, prenatal diagnosis followed by selective abortion and preimplantation genetic diagnosis of *in vitro* fertilized embryos are two currently available techniques which can achieve the same objective. These approaches of course would not work for two homozygous parents, because all of their offspring would also be homozygous; germline gene repair would be their only option.

A second situation where germline gene repair might be warranted is where the disease can only be treated by germline gene therapy. In 1986, Dr. Walters suggested that the blood-brain barrier, for instance, might prevent the somatic treatment of brain cells involved in hereditary nervous disorders. Since that time, the herpes simplex virus, which normally infects neurons, has been engineered as a potential vector for brain cell gene therapy (Breakefield and DeLuca 1991). One could imagine that there are other circumstances where, for spatial or temporal reasons, somatic cell gene therapy is not possible, for instance an inherited disease which manifests itself early in embryonic development, necessitating genetic modification of the embryonic stem cells. This is, of course, highly esoteric and far removed from the mainstream of health care practice.

• **Germline Enhancement** is the most extreme and controversial form of gene therapy, evoking in some Orwellian nightmares of totalitarian control, Nazi

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[8] Two heterozygous parents have a 25% chance of birthing an affected homozygous child. In contrast, one heterozygous parent and one homozygous parent have a 50% chance, while two homozygous parents have a 100% chance.

attempts to create a "super race" and so forth. While political abuse of technology is an ever present possibility, there may be some situations, when evaluated in a relatively neutral and objective manner, where germline enhancement might be defensible in the future. Any justifiable enhancement of somatic cell function, given a sufficient history of success and given safe, effective methods of germline gene transfer, is a candidate for future germline enhancement. The usefulness of the new trait would have to be considerable to warrant a move with such deep evolutionary consequences. Conceivably, a gene that confers resistance to an intractable pathogen such as HIV, should one exist, might be such a candidate. As discussed previously, our species is in an evolutionary contest with HIV; the use of genetic technology may be our greatest, if not sole, competitive advantage in the fight.

Another possible appliciation of germline enhancement might be in the correction of evolutionary "mistakes", mistakes which are non-lethal in nature but which nevertheless reduce health and longevity (James Larrick, personal communication). For instance, the entire human species, unlike most other mammals, lacks the ability to synthesize ascorbic acid (also known as Vitamin C). This deficiency is due to an ancient mutation which inactivated our gene coding for L-gulonolactone oxidase (we actually have vestigial remnants of the gene in our chromosomes- a living "fossilized gene", as it were). As a result, we must obtain ascorbic acid entirely form dietary sources. While gross deficiency in ascorbic acid causes scurvy, the classic dietary disease which afflicted transoceanic mariners deprived of fresh fruit, mild deficiency, the sort associated with the contemporary diet, has been linked with diseases such as cancer and atherosclerosis (Rath and Pauling 1990). Linus Pauling, the famous chemist, has been advocating for years that we should be ingesting gram quantities of ascorbic acid on a daily basis (Pauling 1970). Hypoascorbemia, the disease of ascorbic acid deficiency, has been referred to as a "public" inborn error of metabolism in humans, because we all suffer from it, and as such, goes unnoticed as a disease masquerading as "normal" (McKusick 1992). One possible application of germline gene enhancement might be to restore a functional copy of the L-gulonolactone oxidase gene, thereby reversing that unfortunate mutational loss suffered by our earliest ancestors, for which we are the unlucky beneficiaries. Evolutionary loss of uricase, which renders humans vulnerable to gout, is a situation that is similar to the loss of L-gulonolactone oxidase (Victor

McKusick, personal communication).

Each of the four types of gene therapy, somatic cell repair, somatic cell enhancement, germline repair and germline enhancement, carries with it considerable ethical, legal and social issues which must be discussed and debated before any commercial application can procede. We are already well into this process for somatic cell repair. As the March of Dimes opinion poll showed, the public, while positive about the potential benefits, really does not understand what gene therapy is. It is clear that this is still a source of vulnerability for the nascent gene therapy industry. More effort needs to be expended in promoting public discussion of the relative risks and benefits of gene therapy.

In the past there has been a "taboo mentality" surrounding the discussion of topics such as somatic cell enhancement and germline intervention. It took ten years to debate somatic cell gene repair before the first human clinical trials were allowed to proceed. Now, while the technical means do not yet exist, is the time to begin the formal public process for the ethical assessment of somatic cell enhancement and germline intervention.

4.3 The Central Dogma as Molecular Roadmap

The gene is the most basic functional unit of life. Made of deoxyribonucleic acid arranged in two, anti-parallel and complementary strands, the gene is capable of encoding information in its linear array of nucleotides and is capable of being replicated in a semi-conservative manner (that is, one strand of the original double helix goes to each daughter cell). These two properties make it an ideal storage medium in the cell, which must direct operations and which must divide, passing on the encoded information to the daughter cells. The Central Dogma of Molecular Biology is given in **Figure 4.2**. The DNA (gene) resides in the nucleus, where it is copied into RNA, which is transported to the cytoplasm, where it is translated into protein. Proteins are the functional workhorses of the cell, performing a myriad of metabolic and structural roles. A change in the DNA (gene), accidental or intentional, will set off a cascade of changes, ultimately

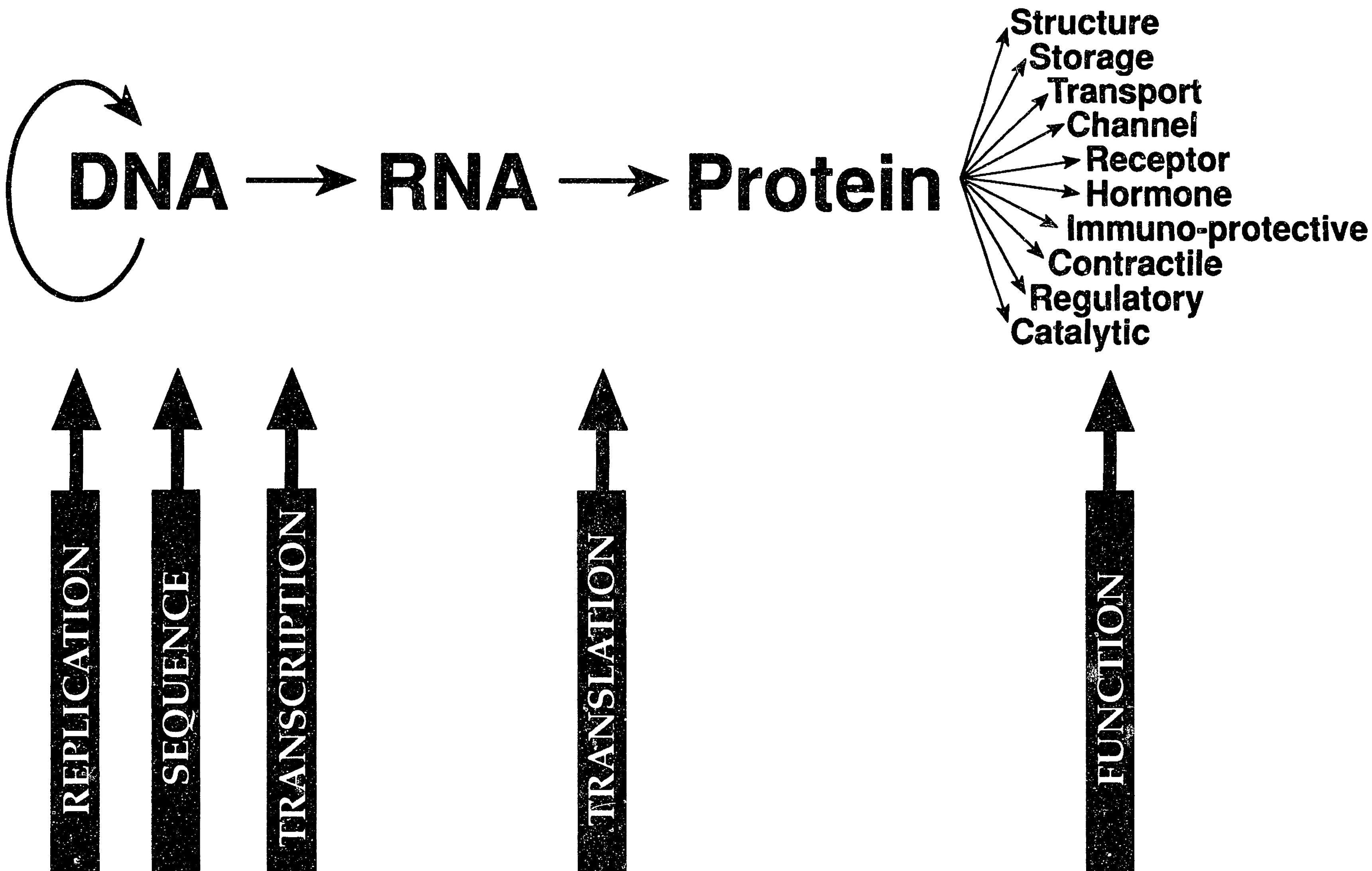

Figure 4.2 The Central Dogma of Molecular Biology indicating the sites of possible intervention using gene therapy.

resulting in a change of protein function.[9] The gene can be thought of as being in the "first position", capable of affecting all events downstream. This is the reason why gene therapy is so powerful as a platform technology. There is virtually no cellular process that cannot be altered by gene therapy.

In addition to serving as a template for protein synthesis, RNA has two other useful properties. The first is as an antisense molecule, capable of specifically binding to a complementary sequence in the messsenger RNA, thereby preventing translation into protein. In some cases, RNA can also bind to double-stranded DNA to form a triple helix, which may be useful in regulating events at the DNA level such as replication, transcription or recombination. The second useful property of RNA is that certain sequences known collectively as "ribozymes" are capable of binding to and catalytically cleaving RNA, thereby inactivating the target RNA. Both antisense RNA and ribozymes can be conceptually thought of as exogenously delivered drugs, and as with all pharmaceuticals, the main challenges are targeting and stability. As was discussed in Chapter 3, antisense therapy can be delivered as a DNA analogue with a synthetic backbone resistant to nuclease degradation. Gene therapy, because it acts on the "first position" in the flow of genetic information, can also incorporate antisense and ribozyme approaches along with recombinant proteins (and small peptide mimetics) using the same vector technology. In fact, it is theoretically possible to deliver all three modalities, each under separate control and all within a single DNA construct.

4.4 The Targeted Gene

The gene can be placed in its organismic context by conceptualizing it as the bull's-eye of a target as shown in **Figure 4.3**. The surrounding organizational levels can be viewed as concentric rings of the target, which increase in both complexity and physical scale as one proceeds radially outward from the center. On the outermost ring are the exogenous (outside the body) agents such as

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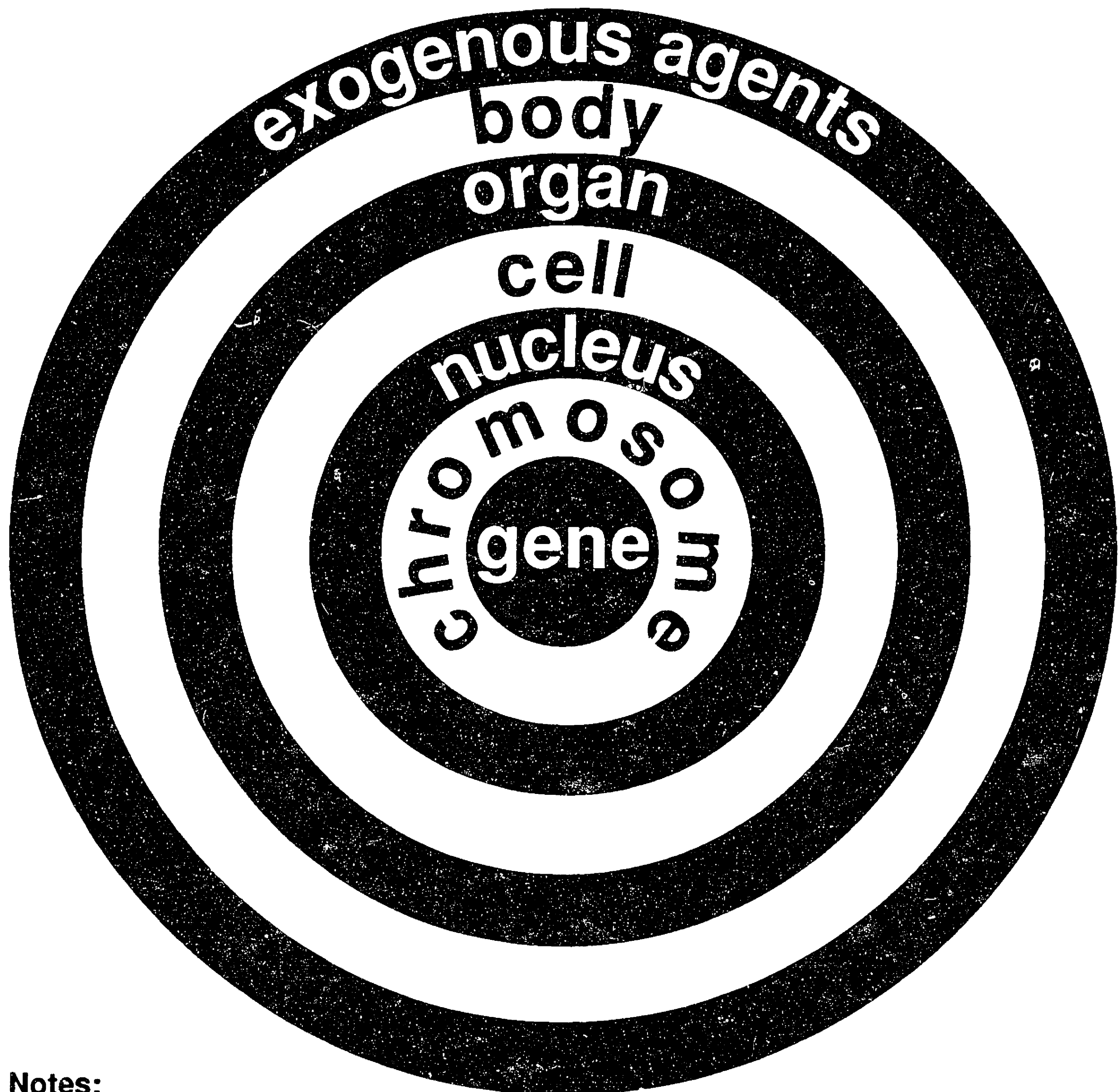
[9] To be precise, not all DNA changes or mutations result in a change in protein function. Some are neutral because 1) some amino acids are coded by multiple DNA triplet codons so the DNA change does not result in an amino acid change 2) some of the DNA does not code for proteins or is edited out during RNA processing (the intron sequences) or 3) parts of protein molecules are not critical to function and can be changed with no effect.



Notes:

• **exogenous agents:** includes gene transfer vectors, viral packaging cells (when inserted *in situ* as in certain tumor treatments), allogenic transplant cells, universal donor cells, drugs and other external factors such as ionizing radiation capable of turning genes on or off

• **body:** systemic introduction by intravenous injection or other means; risk to gamete producing cells; fetal therapy *in utero*

• **organ:** specific targeting of organs such as by intra-muscular injection, use of catheters, inhalation, dermal application, rectal route for colon cancer, etc.

• **cell:** tropic or "homing" agents such as viral vectors (live or dead) which may be engineered with cell-specific receptor ligands such as antibodies; *ex vivo* where cells are removed, cultured, treated and then re-introduced

• **nucleus:** agents such as adenovirus capsid proteins capable of intracellular trafficking through the cytoplasm and nuclear pores into the nucleus

• **chromosome:** random insertion such as by retrovirus, specific insertion such as by adeno-associated virus, episomal introduction such as by adeno-virus or bacterial plasmid, homologous recombination using flanking sequences and *ex vivo* selection/culturing techniques, introduction of a human artificial chromosome (HAC) capable of replication and segregation during mitosis (not achieved yet)

• **gene:** cDNA copy of the messenger RNA or full (un-edited) genomic gene, choice of regulatory DNA elements such as promoters and enhancers

Figure 4.3 The layers of the gene transfer target.

isolated genes, gene vectors, physical delivery devices and the like which can be manipulated by the practice of engineering. Perhaps the exogenous agents should be more appropriately modeled as the "arrow" because their sole purpose is to take aim at the gene in the bull's-eye position, negotiating around and through the domains of the body, organ, cell, nucleus and chromosome.

• **Constitutive vs Regulative Expression.** The gene is only part of the picture, however. While the gene codes the structural sequence that defines the gene product (the antisense RNA, ribozyme or protein, whichever the case may be) the gene must be also be regulated in terms of three basic parameters:

- temporal- when in development is the gene active?
- spatial- in what tissue(s) is the gene active?
- amplitude- how much of the gene product is made?

Regulation of these parameters is provided by DNA adjacent to the structural gene. These sequences, referred to collectively as the promoter/enhancer region, bind proteins known as transcription factors which affect the ability of the RNA polymerase to transcribe the structural gene. The promoter/enhancer serves as an "address" to the gene in much the same way that an area code serves as an address to a phone (Hood 1992). The human body has evolved an elaborate "molecular area code" system to turn on the 50,000 to 100,000 genes at just the right time, in just the right tissue, and in just the right amount. It is as if one digit controls the temporal, another the spatial, and another the amplitude.

Gene therapy, in order to be safe and effective, must address the issue of expression amplitude. How much product is needed to reverse the disease phenotype? Is the gene product toxic if it is made in high amounts? How fast is the product turned over, or secreted, or absorbed? All of these questions must be fit into what is known as a pharmacokinetic model, which is a system dynamics approach integrating all the relevant inputs, outputs and feedback loops.

The simplest form of gene regulation is termed "constitutive", meaning the gene is turned on all the time. The promoter/enhancers of certain viruses are constitutive. So are those of many genes coding the "housekeeping" enzymes, which are needed on a continuous basis to maintain cell function. The use of a constitutive promoter/enhancer in a gene therapy protocol would require

demonstration that the gene product is not overproduced, or is not toxic if it is overproduced. If the gene product is toxic when overproduced, a mechanism is needed to down-regulate it. For instance, the treated cells could always be removed if they were first physically encapsulated. Alternately, they could be killed by the insertion of a "suicide gene" which can convert a prodrug into a toxic substance, thereby killing the cell.

Another approach would be to use a promoter/enhancer which can be regulated, either by an endogenous naturally occurring chemical or by an exogenous one such as a drug. Gene therapy for insulin-dependent diabetes, an autoimmune disease which attacks the insulin-producing cells in the pancreas (Atkinson and Maclaren 1990), for instance, would require that the engineered insulin gene be inducible by the same internal metabolic stimuli that normally influence the expression of the gene. The expression of the transferred insulin gene would have to dynamically respond to fluctuations in body glucose and other metabolites.

Some promoter/enhancers are specific to tissues. They are very useful for insuring that expression only occurs in a certain tissue. If some of the gene vector were to unintentionally or non-specifically find its way to another tissue, there still would be no gene expression. For instance, malignant melanoma is a highly lethal cancer arising from the pigment-producing cells of the skin (Cooper 1993). The danger, of course, is that once the cancer cells spread throughout the body, surgical excision is no longer an option. A systemic therapy would be the only option remaining. Currently available treatments such as chemo- and radio-therapy kill all dividing cells in an unselective way. Here's how a tissue specific promoter/enhancer might be of use: Both normal pigment-producing skin cells and melanoma cells express tyrosinase, the enzyme responsible for melanin formation, but only the melanoma cells are rapidly dividing. One approach being considered is to make a retroviral vector carrying a "suicide" gene such as thymidine kinase (TK) that is under the control of the tyrosinase promoter/enhancer . When introduced into the patient, the vector can only integrate itself into the chromosomes of rapidly dividing cells and the TK gene can only be expressed in cells where tyrosinase is expressed (i.e., the skin cells, both normal and cancerous). The patient would then be given ganciclovir, a prodrug which would be metabolized by the TK in the melanoma cells to a toxic

DNA precursor, killing the melanoma cells as they divide. The vector will incorporate into other rapidly growing cells such as the hair follicles or the blood stem cells, but the TK will not be expressed because the tyrosinase promoter/enhancer is not activated in such cells.

The human erythrocyte-specific enhancer/promoter has recently been isolated, sequenced and patented by Irving M. London and group at the Harvard-MIT Division of Health Sciences and Technology (Tuan and others 1992). This regulatory element is extremely useful in gene therapy for red blood cell disorders such as sickle cell anemia and thallassemia. By placing one (or both) of the hemoglobin genes under the control of this erythrocyte-specific enhancer/ promoter, it will be possible to perform gene therapy at the blood stem cell level- only the derivative red blood cells will express the hemoglobin genes, while the derivative white blood cells, which also carry the transferred gene, will not. This strategy is identical to that followed by Nature during embryogenesis, where all cell types are endowed with an identical genetic heritage, but differentiate themselves by selectively expressing only some of their genes. The gene therapy operation is performed "upstream" in an undifferentiated but continuously replenishing stem cell, and then expressed later "downstream" in a derivative cell.

Another very promising approach is to use naturally occurring or newly engineered promoter/enhancer elements that respond to exogenous agents such as drugs. The ability to modulate the expression of any transferred gene at will by simply administering a drug, which itself clears the body with a short halflife, is enormously appealing. The physician is in the position of immediately modifying gene expression based on the patient's everchanging performance. Sending a gene into a patient's body is like sending a satellite into outer space. Mission Control directs the operation from afar, using radio-signals or chemo-signals to control events occurring over distances infinitely large or infinitesimally small.

One of the most well-known drug inducible promoter/enhancers is that for metallothionein, a cysteine rich protein that binds and transports heavy metals such as zinc and copper. The protein is thought to be involved in heavy metal detoxification (Richard Palmiter, personal communication). When mice are fed

zinc ions, the metallothionein promoter/enhancer is turned on in response, activating transcription of its adjacent gene and eventual synthesis of the metallothionein protein. In a famous experiment first demonstrating the principle of transgenics, Palmiter and Brinster put the rodent growth hormone gene under the control of the metallothionein promoter/enhancer and injected the DNA construct into fertilized mouse eggs. The fusion gene was incorporated into the genomes of some of the mice, resulting in a strain of mouse which grew to twice its normal adult size if fed zinc ions (Palmiter and others 1982). The zinc, acting as an exogenous "drug", induced the metallothionein promoter/ enhancer to turn on the artificially linked growth hormone gene, resulting in abnormally large growth.

GeneMedicine, a company newly founded by researchers from the Baylor College of Medicine in Houston, has developed a proprietary promoter/ enhancer which they refer to as a "gene switch". According to company literature, "... the functionality of the gene switch for controlling *in vivo* gene expression using an orally applied drug has been demonstrated in human cells *in vitro*, leading the Company to anticipate that it can develop advanced gene medicines that mimic the natural pulsatile delivery of numerous clinically important proteins."

The development of such drug-inducible promoter/enhancers will require extensive understanding of intracellular signal transduction pathways, that is, how the drugs actually get the transcription factors, the DNA sequence-specific proteins which bind to the enhancer, to activate the RNA polymerase. One could imagine a selection of co-developed pairs of gene switches and corresponding activating drugs which could be linked in a modular manner to tissue-specific switches. A "mix and match" procedure using various gene switches arranged in series would, in theory, allow exogenous control of any gene in any tissue.

Wallace H. Steinberg, the prophetic venture capitalist who founded the leading gene therapy company, Genetic Therapy, Inc. (Gaithersburg, MD), as well as the leading gene discovery company, Human Genome Sciences (Gaithersburg, MD), has predicted that, by the year 2000, one out of every two drugs will work by turning genes on and off and that "all drug companies in the world will use genomic data as their Rosetta stone for the development of new drugs and

diagnostic procedures. No science will be more important to the future of medicine than genomic research." (Sugawara 1992). The future wide-scale use of drugs to regulate the expression of genes, either naturally occurring or introduced as an externally controllable element, seems highly likely.

• **Gene Addition vs Gene Replacement vs Gene Inactivation** An important distinction must be made between adding a new copy of a gene versus replacing all or a portion of an existing one. All autosomal genes, those not located on the X or the Y chromosomes which control the inheritance of gender, are represented in each somatic cell by two copies, one inherited from the mother and the other from the father. The two copies can be identical or they can be slightly different, that is, polymorphic. There are, for instance, over 170 known mutations in the CFTR membrane protein that cause cystic fibrosis (U.S. Congress and Office of Technology Assessment 1992).[10] For many metabolic functions, the presence of at least one functional copy of the gene is sufficient- a heterozygous carrier, possessing one normal gene and one gene with a deleterious mutation, does not experience the symptoms because the normal gene can make enough enzyme to satisfy the cell's needs. The disease phenotype is said to be *recessive*, that is, not expressed in the heterozygous condition. If two carriers mate, however, there is a 25% chance that their child will be homozygous, that is, will inherit both mutated copies, will have *no* functional gene and will therefore exhibit the disease phenotype. Just as the presence of one normal gene copy is sufficient for the heterozygous carrier to escape disease symptoms, therapeutic addition of one normal gene copy to the cells of the homozygous patient should, in theory, be sufficient to reverse the disease symptoms. The patient's treated cells after gene therapy will each have a total of three copies of the gene, that is, two dysfunctional copies inherited from the parents and one medically transferred normal copy, referred to as a *transgene*.

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[10] As discussed in Chapter 3, one single mutation accounts for about 70% of the CF carriers while the rarer 170+ mutations account for the other 30%. The 13 most common mutations account for about 85% of the CF carriers in Caucasian and 95% in Ashkenazic Jew populations. The remaining 157+ mutations are very rare, perhaps some even newly arising in single individuals. Since each CF patient inherits two copies of the gene, there are, in theory, $>170^2$ or >28,900 possible variations of CF. Patients which inherit different mutations of the disease causing gene are referred to as *compound heterozygotes*. Much of the variation found in the symptoms of CF is thought to be the result of compound heterozygosity (David Housman, personal communication). Since there is no evidence that inheriting the gene from Mom is different than inheriting it from Dad (a phenomenon known as genomic imprinting; see Moore and Haig 1991), we should halve our number of theoretically possible CF variations from >28,900 to >14,450.

What is important in recessive genetic disorders is that the defective gene either makes no gene product at all, or makes a defective product which does *not* interfere with the function of the normal gene's product. The defective gene(s) is(are) passive or recessive, making the restoration of function possible by the normal gene contributed by the other parent or by the medical addition of a normal transgene. It is not necessary to remove or otherwise inactivate the mutant gene, only to insure that at least one gene copy per cell works.

In contradistinction to autosomal recessive disorders, there are *autosomal dominant disorders* where the disease *is* expressed in the heterozygous condition-only one copy, not two, of the mutant gene is sufficient to cause the disease. Dominant disorders represent an unwanted gain of function. Unlike the recessive inheritance pattern, which implies that no gene product or a defective gene product is made, the dominant inheritance pattern says nothing about the molecular basis of the disease. The thesis author knows of at least two molecular mechanisms for dominant disorders, and there are probabley more.

The first is a regulatory mutation where the mutant gene simply makes too much of an otherwise normal gene product, which is toxic at high concentrations (Herskowitz 1987).

The second is a situation where the product of the mutant gene acts to overcome, dominate or inactivate the product of the normal gene. While most recessive disorders result in defective enzymes, dominant disorders often result in defective structural, carrier or receptor proteins that are frequently composed of multiple subunits (Connor and Ferguson-Smith 1991). An example of a dominant disorder is osteogenesis imperfecta, a disease resulting from mutations in Type I collagen, the major structural protein of bone and other fibrous tissues. Type I collagen is a multimeric triple helical protein, composed of two identical protein chains encoded by a gene on chromosome 17, and one similar but distinct protein chain encoded by a gene on chromosome 7 (Thompson and others 1991). A mutation in either one of the two genes will result in the synthesis of defective protein chains which complex with normal protein chains, resulting in dys-functional triple helices. In this manner, the mutant gene impairs the function of the normal gene, and is accordingly said to be a *dominant negative gene.*

Because the dominant negative gene impairs the function of the normal gene, simply adding another normal gene (as in a recessive disorder) will not solve the problem. The dominant negative gene must be *replaced or inactivated*. Much as some word processing software programs are capable of editing text with a "search and replace" function, an equivalent genetic mechanism is needed to find the mutant gene, remove it and replace it with a normal copy. This is the domain of the newly emerging field known as "gene targeting" (Sedivy and Joyner 1992).

In a process that is not well understood, two DNA double helices, when sharing a region of similar sequence and when complexed with certain nuclear proteins known as recombinases, will break strands in the region of sequence similarity and anneal by base pairing to the complementary strand of the other double helix, resulting in the exchange of DNA sequences adjacent to the site of strand crossover. This phenomenon, known as homologous recombination, also occurs in normal cellular DNA repair and in genetic recombination, a process in gametogenesis whereby similar pieces of chromosomes of maternal and paternal origin are exchanged.

One possible approach to correct a dominant negative gene on a chromosome would be to replace it with a normal gene using homologous recombination. In this procedure, one would use a gene vector to introduce into the cell nucleus a normal version of the gene flanked on either end by sequences homologous to those flanking the target gene on the chromosome. These sequences would pair with those flanking the target gene, two recombination events would occur, and the net result would be an *excision* of the dominant negative gene and an *insertion* of the normal transgene into the same chromosomal site. Such an approach would also work for a portion of the gene, for instance, by using the sequences that flank either side of a point mutation or a deletion mutation. Alternatively, since there already is one functional gene in the cell, the dominant negative gene could be cut out altogether by using a transgene construct that has no gene in the middle but just the sequences flanking the target chromosomal gene. This method is called a gene "knockout". All of these approaches have been shown to work, for instance, in the generation of transgenic amimals by injection of DNA into newly fertilized eggs. The problem, however, is that the frequency is very low (1 success in 100 to 1000 tries). The success rate can be increased dramatically (1 success in 10 tries) by using flanking DNA sequences cloned from

isogenic strains of mice (Kirby Smith, personal communication), suggesting that gene replacement or removal by homologous recombination, for it to be a possibility in gene therapy, might require sequencing (or otherwise determining) the patient's specific mutation as well as the flanking sequences of sufficient length to effect a high frequency of recombination. Better basic knowledge of the recombination process, particularly the enzymology, is needed before there can be any gene replacement therapy in humans. Another issue discussed later, but which is relevant here, is that one must have knowledge about the fraction of targeted cells that must be successfully "hit" to reverse the symptoms of the disease.

As already mentioned, a mutant dominant negative gene or its product could also be inactivated. There are at least five possible mechanisms to suppress the expression of such a gene:

1) knockout gene with homologous recombination (already discussed)

2) block translation of mRNA with a specific antisense RNA sequence (which does not inhibit mRNA of normal gene)

3) destroy mRNA with a specific antisense RNA coupled with ribozyme activity (which does not destroy mRNA of normal gene)

4) block post-transcriptional processing or translation of mRNA by expressing subregions of mRNA which act as decoys, competitively inhibiting the action of essential regulatory factors or processing enzymes

5) inhibit protein product of dominant negative gene with another protein or polypeptide fragment possessing truncated domains involved in specific functional interactions (which likewise does not inhibit protein product of normal gene)- a protein decoy to inactivate the unwanted dominant negative gene product.

These are all approaches which interfer with the flow of genetic information as generalized in **Figure 4.2**, and every one is amenable to delivery by gene therapy. The problem is finding which RNA sequences or which protein sequences are capable of effecting such highly specific tasks. Fortunately, a method has been developed by Igor Roninson and group at the University of Illinois at Chicago (Holzmayer and others 1992). The technique involves creating libraries of randomly digested DNA fragments (700 base pairs or less) inserted into plasmid

expression vectors and screening them for their ability to change a selectable target phenotype, such as conferring resistance to viral or phage infection, or causing suppression of tumor cell growth. Cells expressing the desired phenotype are cloned, the plasmid expression vectors isolated, and the inserted DNA sequenced. Active DNA fragments, termed "genetic suppresssor elements" or GSEs, are confirmed by retransfection. Protein GSEs are distinguished from antisense RNA GSEs by inactivation following insertion of a frame shift mutation, which negates biological activity of proteins but not antisense RNA. This technology has been recently patented (Roninson and others 1993) and licensed to Ingenex, Inc. (South San Francisco, CA), a newly formed company financed by the Castle Group Ltd. (New York, NY), which is developing gene therapy delivery of GSEs against cancer and AIDs (Igor Roninson and Louis Bucalo, personal communication).

• **Chromosomal Integration vs Epigenetic Element** An important element in designing a gene therapy protocol is whether it is necessary to achieve integration into the chromosomes or not. Basically, any gene therapy that targets stem cells or the progenitor of a clonal line such as skin fibroblasts, where the intention is to achieve gene expression after one or many rounds of cell division, must have the transgene incorporated into the host genome. This will insure that the transgene will be replicated in a heritable fashion along with the host chromosomes during mitosis and that one copy will therefore go to each daughter cell.

If the target cell is a terminally differentiated cell that will not undergo any further cell division before the gene-of-interest is expressed, then an epigenetic (meaning outside the genome) element is satisfactory, and perhaps desirable. There is always the risk of insertional mutation when transgenes are incorporated into chromosomes, and this risk can be avoided by using an epigenetic element. For instance, a foreign gene which integrates into the genome can disrupt normal gene function, resulting in cell death. This is not a serious concern because it is a rare event and the body can easily afford to lose one of its 10^{13} cells.

What the body cannot afford, however, is accidental activation of an oncogene or inactivation of a tumor suppressor gene, both of which can lead to cancer. It is

thought that it takes the occurrence of such an event in only one cell in the body to cause cancer. The progeny of this one cell then spread uncontrollably throughout the body. If the disease being treated is highly lethal, malignant melanoma, for example, a low level risk of a secondary cancer due to the unintended action of a viral vector five years from now is acceptable, given that the patient may only have five months to live if nothing is done. Malcom Brenner, who has conducted numerous gene marking protocols using retroviral (RV) vectors, commented at a recent gene therapy conference that the risk his terminal cancer patients incurred during the retroviral marking of their bone marrow cells was no greater than that which they incurred by driving on the expressway to and from the hospital. Alternative treatments such as chemo- and radio-therapy are also very mutagenic. As of May 1992, there was a cumulative experience with RV mediated gene transfer and therapy equivalent to 106 monkey-years and 23 patient-years and as of that date, there was no observable pathology as a result of gene transfer (Anderson 1992). Three other monkeys, however, did develop lymphomas after bone marrow transplantation and gene transfer using a helper virus contaminated RV preparation (Anderson 1992). Both the RAC and the FDA require all clinical protocols to be strictly free of helper virus.

In addition to RV, adeno-associated virus (AAV) also integrates into the genome. DNA will integrate by itself via the cell's normal DNA repair mechanisms; the frequency is very low (< 1 in a million cells), but this is sufficient if *in vitro* selection and cell expansion methods are used (see description of Transkaryotic Therapies in Chapter 5).

Adenovirus and Herpes Simplex Virus, which do not integrate, are also being developed as gene vectors. Plasmids, circular DNA grown in bacteria, are perhaps the simplest and safest gene vectors. While all viral gene vectors are engineered to be replication incompetant, there is, however, a small chance that they can recombine with latent viruses in the patient and thereby gain virulence. Plasmids, on the other hand, can be engineered so that they do not share this risk. They contain no mammalian origin of replication, and therefore cannot multiply in the cell. Being composed of 100% DNA, plasmids can be purified to an extremely high degree whereas viruses are always contaminated with unknown cellular material coming from the producing ("packaging") cells in

which they are made. As discussed later, plasmids must be used in connection with a physical or chemical method to achieve a high level of gene transfer whereas viruses have evolved their own cell entry methods.

• **Random vs Specific Locus Integration** As mentioned above, gene therapy of stem cells and clonally expanded cells both require that the gene-of-interest be integrated into the host genome. RV is commonly referred to in the literature as exhibiting "random" insertion. The thesis author has not yet done a detailed review of the scientific literature to locate work that proves this statement conclusively. There may be patterns to insertion that have important consequences to the use of RV as gene vectors.

A virus that is reported to possess site specific integration is AAV, which integrates at a locus on chromosome 19. As reported by Barrie Carter of Targeted Genetics at a recent conference, there are certain sequences which must be preserved when the AAV is engineered for gene therapy, or the insertion specificity will be lost. The hope for AAV as a potential gene vector is that the specificity will reduce the risk of insertional mutation. Richard Mulligan has recently pointed out that AAV inserts in a region of chromosome 19 that has been implicated with chromosomal rearrangements giving rise to B cell leukemias, and that this puts a cloud over any safety advantages that AAV might have over random integration by RV (Mulligan 1993). When asked about this point, French Anderson reiterated the fact echoed by others that AAV to date has not been directly associated with any pathology or tumorigenicity (French Anderson, personal communication). Another useful feature of AAV in certain applications is that, unlike RV, it can infect cells which are not dividing.

Insertion of "naked" non-virally introduced DNA into the host genome by normal cellular DNA repair mechanisms can be random, but it can also be specific via a homologous recombination event (see above). As discussed in Chapter 5, Transkaryotic Therapies is developing both modes of non-viral DNA integration for their *ex vivo* gene therapy approach. This company believes that non-viral techniques are much safer than viral ones and that treated cells should be characterized before re-entry into the patient. A list of the company's safety and efficacy criteria is given in **Table 4.1**.

1. The genetically engineered cells should be characterized fully before they are implanted into the patient. Characterization should include demonstration that the cells are healthy (in that they grow normally, produce no toxic substances, and contain no infectious agents) and function properly (in that they produce the desired amount of therapeutic protein).

2. The genetic change to the patient should be minimized. A single cell derived from the patient should be engineered to possess the desired therapeutic properties. This cell should then be propagated, resulting in a uniform population of identical cells for implantation.

3. The function of the genetically engineered cells must be predictable based on the function of the cells prior to implantation. The engineered cells must function identically both inside and outside the patient - accurate dose/response relationships must be developed. In this way, it should be possible to accurately determine the optimal number of cells to be implanted based upon the age, size, and clinical condition of the patient.

4. The physician should be able to detect, monitor, and modulate the genetically engineered cells following implantation. The cell's production of the therapeutic protein should be capable of responding to pharmacologic agents administered or prescribed by the physician.

5. The genetically engineered cells should produce the therapeutic protein in response to physiologic demand. For example, for the treatment of diabetes, insulin-producing cells must secrete insulin in response to normal physiologic stimuli such as carbohydrates and proteins, re-creating the regulatory system that had been destroyed in the patient.

6. The system should be flexible. It should be possible to use cells isolated from different tissues and to reimplant them into different anatomical sites as dictated by clinical considerations.

7. The system must have clear therapeutic benefits and must not subject the patient to undue risk or the population to any risk. The use of infectious agents should be avoided.

Table 4.1 One company's safety and efficacy criteria for an "ideal" ex vivo gene therapy system (source: Transkaryotic Therapies Inc.).

• **Chromosomal Gene vs cDNA** Eukaryotes, evolutionarily advanced organisms such as ourselves which possess a cellular nucleus and which reproduce by specialized sex cells, exhibit a very unusual gene structure. In contrast to the prokaryotes, which do not possess a nuclear membrane and which divide vegetatively, the eukaryotes have pieces of DNA called "introns" interspersed within the coding sequences ("exons") of their genes. When the gene is transcribed into RNA, the intron sequences are edited out, and the adjoining exon sequences spliced to form the messenger RNA (mRNA), which is then transported out of the nucleus to the cytoplasm where it is translated into protein. The degree of intron interspersion is highly variable and can be quite extensive. **Table 4.2a** is a list of some representative genes showing their size and the corresponding size of their mRNA; **Table 4.2b** is a plot of the same data suggesting that bigger genes have their mRNAs edited more extensively than smaller ones. For instance, the mRNA transcribed from the alpha hemoglobin gene (800 bases) undergoes a 1.6 fold edit, while that of the parathyroid hormone gene (4,200 bases) undergoes a 4.2 fold edit and the dystrophin gene (>2 million bases) a >125 fold edit.

No one knows with certainty the significance of this peculiar discontinuity in eukaryotic genes, which is absent from the genes of prokaryotes such as bacteria. One theory is that the introns are merely vestiges of viruses which randomly inserted themselves into the coding regions of genes, and that eukaryotic cells evolved defensive editing mechanisms "to get rid of the trash" before translation into protein. As evidenced by **Table 4.2a**, these additional sequences are quite a burden to be born for every gene in every cell; it would seem to be a waste of resources and a major handicap given the ruthless competitive process of natural selection that is placed on the organism, *unless*, the presence of such intron sequences conferred some evolutionary advantage (Phil Sharp, personal communication). Wally Gilbert and colleagues at Harvard have speculated that exons may represent recurring structural motifs in proteins and that introns may facilitate the recombination of exons to combinatorially generate proteins with novel function and possible evolutionary advantage (Dorit and others 1990; Gilbert 1985). Based on comparisons of known protein sequences, they estimate that only 1000 to 7000 exons are needed to construct all proteins. Introns would serve as facilitators of exon shuffling and would, in effect, "speed up" evolution; if this were so, the cost to maintain introns would be more than offset by the

	Genomic size (kb)	cDNA (mRNA) (kb)	mRNA as a % of Gene size	Gene size divided by mRNA size	Number of introns edited
Small genes					
• alpha globin	0.8	0.5	62.5%	1.6	2
• beta globin	1.5	0.6	40.0%	2.5	2
• Insulin	1.7	0.4	23.5%	4.3	2
• Apolipoprotein E	3.6	1.2	33.3%	3.0	3
• Parathyroid hormone	4.2	1	23.8%	4.2	2
Medium genes					
• Protein C	11	1.4	12.7%	7.9	7
• Collagen I pro-alpha-1	18	5	27.8%	3.6	50
• Collagen I pro-alpha-2	38	5	13.2%	7.6	50
• Albumin	25	2.1	8.4%	11.9	14
• HMG CoA reductase	25	4.2	16.8%	6.0	19
• Adenosine deaminase	32	1.5	4.7%	21.3	11
• Factor IX	34	2.8	8.2%	12.1	7
• Catalase	34	1.6	4.7%	21.3	12
• LDL receptor	45	5.5	12.2%	8.2	17
Large genes					
• Phenylalane hydroxylase	90	2.4	2.7%	37.5	12
Giant genes					
• Factor VIII	186	9	4.8%	20.7	26
• Thyroglobulin	>300	8.7	<2.9%	>34.5	>36
• Cystic fibrosis transmembrane regulator	≈250	6.5	≈2.6%	≈38.5	26
Mammoth genes					
• Duchenne muscular dystrophy dystrophin	>2000	≈16.0	<0.8%	>125	>60

Table 4.2a Post-transcriptional editing of messenger RNA is extensive (data from McKusick 1992).

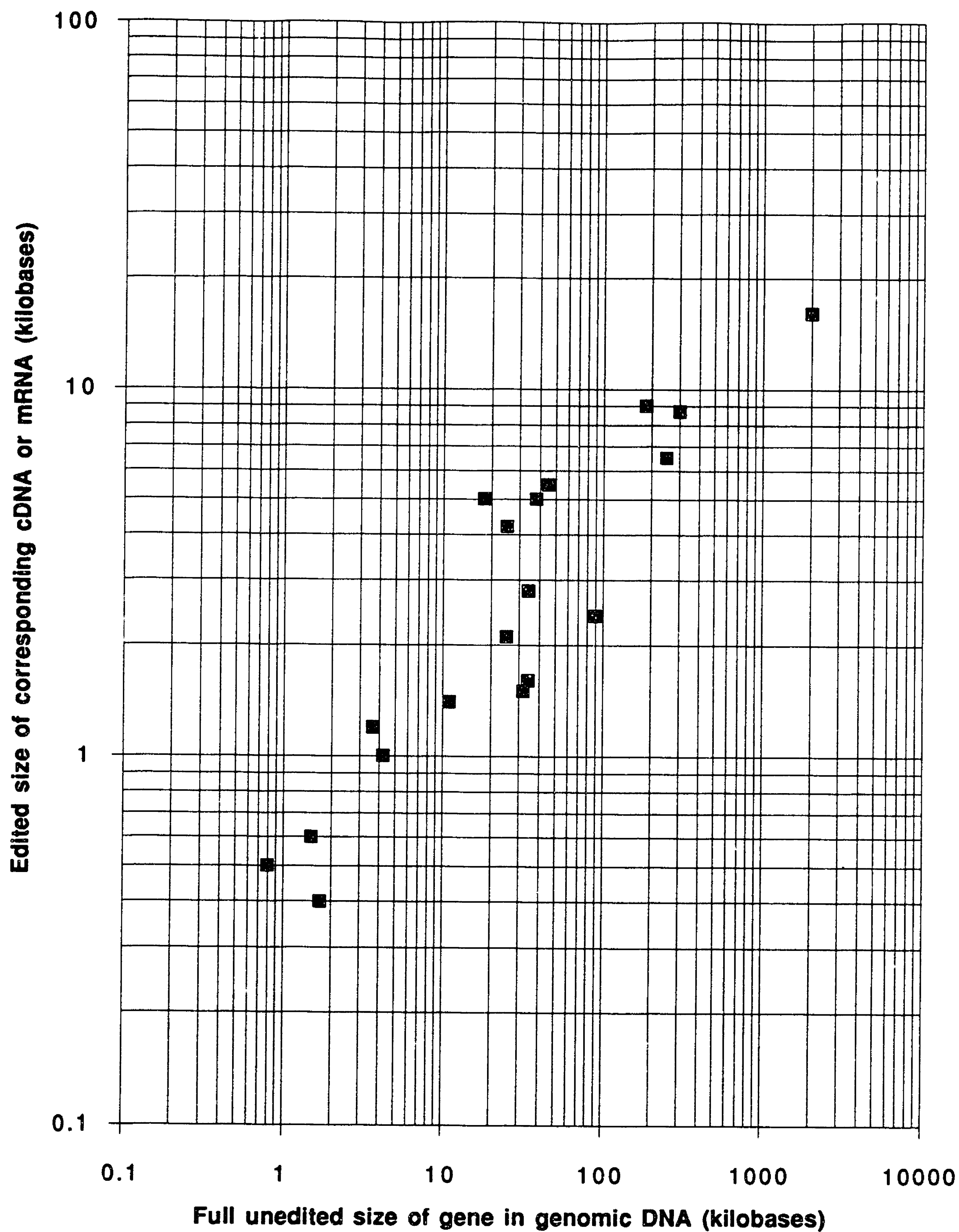


Table 4.2b Graph showing extent of post-transcriptional editing of messenger RNA (note: selection of particular genes is arbitrary and may not be representative of all genes).

benefits of enhanced competitive advantage.

What is the significance of exons and introns to gene therapy? All gene therapy to date uses a synthetic intron-free version of the real chromosomal gene, which is made by enzymatically copying the mRNA to make a complementary DNA or cDNA . The use of the intron-free cDNA has the practical advantage that the gene is much smaller and can usually be fit into viral vectors, which have insert size constraints (discussed later).[11] The mRNA that is made in the cell nucleus from the intron-free cDNA, while it will code for a fully functional protein, will not be processed the same way that a high molecular weight RNA transcript off the normal chromosomal gene will. While RNA transcripts of intron-free cDNA will probably be adequate for constitutively expressed genes, they may not be for those genes requiring more complex intra-nuclear regulation. The biological functions of RNA, particularly in the nucleus, are not well understood. Compared to DNA and protein, RNA is the next great frontier of molecular biology (David Housman, personal communication). As research progresses, we may find that certain gene therapy applications need the normal chromosomal version complete with exons. As discussed later, there are some vector systems under development that do not have an intrinsic upper size limit the way viruses do.

4.5 The Targeted Cell

• **Stem Cell vs Differentiated Cell** Stem cells renew themselves as well as serving as the progenitors of the specialized or differentiated cells. Germ cells are the ultimate stem cell, and understandably receive the most attention in any discussion about gene therapy. But the somatic stem cells represent a related set of concerns, and are of much greater immediate clinical significance. When evaluating an unproven therapy, margins of safety become a very important concern. Just as genetic modification of germline stem cells evokes concern for the well-being of derivative generations, so does genetic modification of somatic

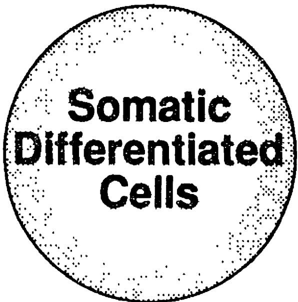
[11] At a recent conference, Barrie Carter reported that he had to chop off part of the cystic fibrosis transmembrane protein cDNA in order to get it to fit in the AAV vector, thus creating for himself a new set of experiments to show that the safety and efficacy of the truncated version is the same as the untruncated one.

stem cells evoke concern for the well-being of all cells in a patient that are derived from them. **Figure 4.4** is a graphic that attempts to map concerns about genetic intervention that are common to somatic differentiated cells, somatic stem cells, and germline stem cells. The bold arrows represent the big discontinuous jumps in the magnitude of concern when considering the germline. They are obvious. What may not be so obvious, however, are that there are also jumps (albeit much smaller) in the magnitude of concerns when moving from genetic intervention of somatic differentiated cells to that of somatic stem cells.

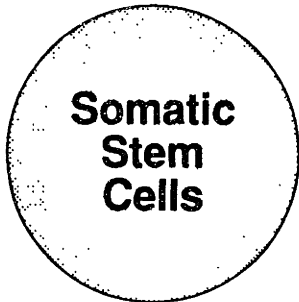
It is better for an unforeseen adverse reaction to occur in a terminally differentiated somatic cell, which will die within a matter of weeks or months, than a somatic stem cell, which will last the patient's lifetime. Gene therapy performed on terminally differentiated cells is like using a drug which will clear the body within a predictable period of time. The side-effects, if any, will usually last for only a small, manageable period of time. If initial clinical trials show that there are no adverse reactions when genes are added to differentiated cells, *then and only then* is it prudent to consider a more powerful, more permanent level of intervention. For instance, the initial gene therapy clinical trial performed on September 14, 1990 to correct adenosine deaminase (ADA) deficiency was performed on T cells, a differentiated cell derived from the hematopoietic (blood-forming) stem cells . It was not until May 4, 1993 that researchers and regulators ventured to perform the procedure on the actual stem cells, which were taken from a newborn's umbilical cord.

The hematopoietic system is the most well understood somatic lineage in the body. Knowledge of stem cells in other organs is not so thorough. Researchers, for instance, are still searching for stem cells in the liver (Travis 1993a). The liver possesses a remarkable ability to regenerate itself. A rat with two/thirds of its liver surgically removed can regenerate the organ to original size in just two weeks. During this regeneration process, mature hepatocytes break out of their normal quiescent state and begin to grow rapidly, convincing some researchers that there is no stem cell. Still others present gene marking studies indicating that a certain "oval" cell may give rise to the two major liver cell types, the hepatocyte and the bile epithelial cell.

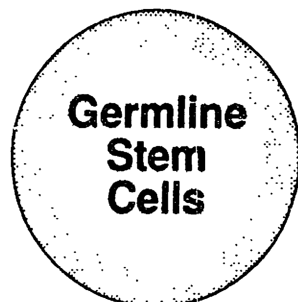
Multiple dosages are given to patient over time as cells age and die



One dosage treats patient for a very long time period (possibly for life)



One dosage would in theory treat patient as well as all subsequent progeny



Increasing Therapeutic Value

• Duration of effective therapy • Efficiency (amount of protein produced per gene dosage) • Side effects may be reduced with fewer dosages

Increasing Patient Risk

• Activation of proto-oncogenes • Inactivation of tumor suppressor genes • Gene becomes un-regulatable • Loss of ability to cancel treatment

Increasing Environmental Risk

• Unwanted exposure of healthcare professionals, patient's family (including future progeny) and public-at-large to gene transfer vectors

Increasing Ethical Concern

• Unforeseen effects on future generations

Figure 4.4 Factors associated with the choice of a target cell for gene repair (enhancement is not considered).

The existence of stem cells in the lung is also unclear. This has immediate importance to developers of gene therapy for cystic fibrosis. At a recent conference, Barrie Carter presented evidence using AAV vector, which integrates into the chromosomes of epithelial cells lining the lung airway, that certain cells continue to e› ʳess the CFTR transgene as long as six months after introduction, suggesting that some "stem-like" cells may exist in the lung.

The field of somatic gene therapy is likely to provide a major impetus to cell lineage and differentiation research. Not only are stem cells the ultimate somatic target for gene therapy, but knowledge about the control of their differentiation by protein growth factors (cytokines) is critical to understanding many diseases as well as embryogenesis.

• **Dividing vs Non-dividing Cells** Most cells in the adult are quiescent. At any point in time a small fraction are dividing to replace those that die. Cancer represents a major departure form this normal situation. In cancer, normal regulation of cell growth is lost. The rate of cell division far outstrips the rate of cell death, resulting in a bulking of cell mass known as a tumor. If the tumor cells adhere to one another, staying in a discrete physical locus in the body, the tumor is referred to as benign and can be removed by surgery if detected early. If, however, the tumor is not detected early and is allowed to progress, the tumor cells may become malignant, that is, lose their self-adhesion and spread (metastasize) throughout the body, thus precluding removal by surgery. This gross difference in the rate of growth between normal and malignant cells forms the basis of most current chemo- and radio-therapy, both of which are intended to kill rapidly growing cells by interfering with DNA synthesis.

Retrovirus (RV), because it is a RNA virus which integrates into the DNA of its host, must first make a DNA copy of itself. This provirus, as it is called, can only integrate when the cell is dividing, a feature of the RV life cycle which makes this virus very useful in transferring therapeutic genes into rapidly dividing cancer cells. RV, for example, is an ideal gene vector for a tumor imbedded in the brain, because the surrounding cells are quiescent. Even if the RV enters the non-dividing cells, it will not be able to incorporate and express the transgene.

For some applications, RV will not work. For non-dividing cells, other viruses

such as AV, AAV and Herpes Simplex Virus are used, as well as non-viral methods such as liposomes.

• **Physical or Biological Access to Cells** For some cell types, *ex vivo* gene therapy is not an option. While the ultimate goal of all gene therapy is to develop targetable, injectable vectors which can by systemically administered, it will be necessary in the early stages of the field to exploit features that are unique to the targeted cell or organ which facilitate localized *in situ* treatment. These may employ techniques such as isolating the blood circulation to an organ followed by localized perfusion of gene vectors or exploiting tissue geometries such as cavities which can be temporarilly isolated. Certain target cells will present some very difficult gene delivery challenges. Metastasized cancer cells, which have already been mentioned, will be very difficult on account of their geographic dispersion; receptor-mediated delivery seems to be the only option for reaching these cells, provided the defensive mechanisms in the blood can be stealthily evaded. Another class of cells which will be very difficult to treat with gene therapy will be those which exist in delicate architectural configurations, such as neurons.

Viruses have co-evolved with the human species. There is probably not a single human cell type over the course of evolutionary history that some virus has not figured out a way to invade and commandeer for its own self-interested purposes. Neurons, the most sophisticated expression of cellular architecture, are no exception. Herpes Simplex Virus type 1 (HSV-1) is capable of entering postmitotic (meaning permanently non-dividing) neurons at nerve terminals, migrating along axons and dendrites, crossing synapses, and entering the nucleus (Breakefield and DeLuca 1991; Breakefield and others 1992). HSV's unique capabilities make it ideal as a gene vector for brain cells. While it it true of all technologically manipulatable viruses, the ability to re-engineer these pathogens into agents of health by exploiting their parasitic niche biology for our own purposes is *particularly* gratifying in the case of the brain. HSV, unlike most drugs, can effectively cross the blood-brain barrier to deliver antisense, ribozyme or peptide drugs that would otherwise never get across. HSV can enter the brain from the peripheral nervous system, making it theoretically possible to selectively treat specific neural circuits. Research is also underway to develop HSV gene vector delivery to specific regions of the brain by direct stereotactic

injection.

Certain brain tumors are infectable by retrovirus. In a protocol recently approved by the RAC, Ken Culver in collaboration with Genetic Therapy, Inc. is preparing to inject mouse RV producing cells directly into the center of a tumor in a patient's brain . The RV carries the gene TK which, as has been discussed, converts the nucleoside analog, ganciclovir, to a toxic phosphorylated derivative. Why inject cells producing virus, why not the virus alone? There are two reasons. The first is that it is not possible to introduce enough virus otherwise. RV currently cannot be concentrated from the cell culture supernatant from which it is harvested; a titer yield of 10^6 pfu (plaque forming units) per milliliter is typical. Since a tumor is typically 10^8 cells, it would take an injection equivalent to 100 milliliters to deliver sufficient virus to infect every cell. This is clearly not feasible, though there are experiments being performed with a specialized catheter to infuse the tumor with fluid containing RV over a period of time. The other reason for injecting the RV producing cells is that only a fraction of the tumor cells are dividing at any one time; in order to get a high infection rate, the virus must be present over a period of time for the RV to enter at the right time in the cell cycle. Since RV is cleared by antibodies made by the immune system, the RV needs to be constantly replenished. Using a mouse model, Culver has found that not all the tumor cells need be infected in order to kill the tumor. As little as 10% infection can kill the rest of the tumor. This phenomenon has been termed the "bystander effect"; the mechanism is not well understood, perhaps it is cell-to-cell cytoplasmic communication. The mouse cells (which would be rejected by the host immune system in a matter of weeks if no ganciclovir were given) are killed along with the tumor upon treatment with the drug. Histological sections through tumors in mouse brains treated by this technique showed a very clean contraction of the tumor mass. Culver referred to the method in a recent conference as "molecular surgery".

Certain organs form natural vesicles which facilitate topical delivery of gene vectors. Cancers that line the inside of the bladder, for example, could be treated by temporarily isolating the tissue from normal urine flow, injecting liquid containing the vector, and then washing excess vector away. Gary Nabel at the University of Michigan has developed a specialized catheter for the delivery of gene vectors to tubular tissues such as arteries, the colon or the prostate. The

device, which is licensed to Vical, consists of two inflatable balloons "skewered" on a linear element through which fluids can be introduced and removed. The catheter is snaked into the tissue. When the balloons are inflated, they create two septa at either end of the resulting coelom or cavity, which can then be flushed and treated with vector.

Aerosol delivery to the pulmonary system is another very promising method being actively pursued. Adenovirus, which causes the common cold, and which has a natural affinity for lung epithelial cells, is being developed as a vector for delivery of the cystic fibrosis gene (see Chapter 5). Initial clinical trials will target the mucosal lining of the nose. These trials are not intended to provide any therapeutic benefit, but will serve to address certain safety issues.

Other gene therapy delivery techniques include

- topical delivery to the skin to treat certain cancers or hair loss
- treatment of other mucosal linings such in the eye, mouth, genitals, etc.

• **Cell Homing Approaches** Cells in the body identify themselves with "signature" receptors presented on their outer membranes. Blood-born cells of the immune system constantly patrol the body for cell surface messages that indicate the presence of "non-self", be it viral infection or very early stage cancer. Protein growth factors released into the blood or the extracellular matrix (which surrounds and holds tissues together) interact with membrane receptors, which in turn trigger cascades of intracellular events critical to body function. Viruses infect cells by evolving coat proteins which can serve as "keys" to these receptor "locks" on the cell surface. A detailed knowledge of receptor-mediated events such as these is necessary to develop a gene vector capable of homing to a specific target. Viral vectors offer "ready made" cell homing devices, but they are not specific enough. After all, viruses have developed their receptor specificities for their own biological purposes. HSV enters neurons very well, bit it also enters any other cell in the body as well; it's not picky. We can't expect to piggy-back our targeting expectations on re-engineered viruses forever. Advanced stage synthetic vectors will have to synthesize the lessons learned from the study of viruses, growth factors and other receptor-mediated events into instuments capable of targeting any cell type, including the most difficult and most evasive cell of all, the cancer cell.

• **Cells as Generic Platforms** For certain puposes, it may not be necessary to express a protein in the cell in which it is normally expressed. There may be more easily accessible cells which can be used as "platforms" to produce a deficient gene product. Factor VIII, for instance, the clotting factor which hemophilia A patients lack, is produced in the leukocytes, where it is then secreted into the blood (Zacharski and others 1968). Instead of targeting the blood stem cells, which give rise to the leukocytes, skin fibroblasts could be used instead. Removing a quarter inch diameter plug of skin from a patient is alot less invasive and costly than removing bone marrow. Use of platform cells requires, however, that the cells are capable of regulating gene expression and processing (usually by cleaving peptide bonds and by adding sugar residues) and secreting the protein properly.

Platform cells need not be removed from the patient. Muscle, for example, is an ideal *in vivo* platform cell that is easily accessible by intramuscular injection. It is an ideal target for the introduction of naked DNA coding for antigenic peptides which stimulate the body's intracellular immune response (see section on Vical in Chapter 5). There is a limit on how much transgenic protein can be produced by this process, but fortunately not much is need to elicit the immune response.

A logical extension of using cells as a generic platform is to develop universal donor cells. The mouse RV producing cells which are injected into brain tumors have already been discussed. Cell Genesys is developing a universal donor cell to serve as a transplantation platform for the production of therapeutic proteins (see Chapter 5). Key to this approach is the requirement to knockout the donor cell's HLA (tissue type) genes so that the cells are not "seen" as foreign and rejected by the recipient's immune system . Using the Factor VIII example from above, instead of making a new "batch" for each hemophilia patient, one universal cell transplant product would theoretically meet the needs of all. Freedom from adventitious agents such as HIV and hepatitus, which tragically contaminated early hemophiliac blood products, including those agents for which we are currently unaware, would be an everpresent concern. A universal product, as opposed to many individual patient batches, is more easily regulated by the FDA, allowing higher standards of quality assurance and quality control to be implemented.

4.6 Gene Transfer Technology

A vector is the key enabling technology that makes gene therapy possible. Since specific vectors are discussed later within the context of commercial ventures in Chapter 5, some summary remarks will be made here. There are three basic categories of vectors:

- Non-viral vectors
- Viral vectors
- Advanced synthetic vectors

• **Non-viral vectors** Mammalian cells naturally take up "naked" DNA from the surrounding environment. Passage across the cell membrane can be facilitated by physical methods, which include direct injection into the nucleus with a fine glass needle, electroporation where an electric current is used to temporarily disrupt the cell membrane, ballistics where DNA is adsorbed onto microscopic particles which are fired at cells with a "gene gun", precipitation with calcium phosphate or by complexing the DNA with cationic liposomes consisting of a bifunctional molecule possessing a positively charged moiety which binds the DNA by ionic attraction and a lipophilic moiety which can fuse with the cell membrane. Lastly, DNA can be attached by an ionic salt-bridge to a cell specific ligand which will bind to its corresponding receptor on the cell surface.

Entry of DNA into the cell usually occurs by a process known as endocytosis, where portions of the outer membrane bud inward, taking with them any macromolecules which have bound to the membrane. Most endosomal particles and their contents are transported to the lysosomal membrane complex within the cell where they are degraded. Occasionally, the DNA may escape from the endosome into the cytoplasm, where, by a process that is not well understood, it finds its way to nucleus as an epigenetic element. If the cell is terminally differentiated, the DNA fragment will be destroyed when the cell dies, while if the cell divides, it will end up in one of the daughter cells, which will eventually die. In either event, the DNA's existence is transient. On rare occurrence, the DNA may be incorporated into the chromosomal DNA, by homologous recombination or by normal DNA repair mechanisms. *Ex vivo* techniques can be employed to select and clonally expand the cells where these rare integration events have occurred. *In vivo*, however, the fate of foreign DNA, including any

production of gene product, is likely to be short-lived. A summary of the key properties of non-viral vectors is given in **Table 4.3**.

• **Viral vectors** Viruses over the course of evolution have taken the path that foreign DNA takes when it enters the cell and optimized it, vastly improving the chances of getting to the nucleus and becoming biologically active. AV, for instance, not only has antennae-like ligands which efficiently bind to cell receptors that trigger endocytosis, but also has evolved proteins which facilitate disruption of the endosome, release into the cytoplasm, as well as homing to the nucleus. RV, whose genome is made of RNA, first makes a DNA copy of itself, which then actively integrates into the chromosomal DNA.

The main objectives of viral vector engineering are 1) to gut as many genes as possible out of the virus to make "room" for insertion of the transgene-of-interest while retaining its ability to infect the target cell and 2) to insure that the virus is not capable of replicating once inside the target cell. Both of these goals are cleverly accomplished by removing the genes coding for proteins essential to the life cycle of the virus and engineering them into the genome of a "packaging" cell line, where they are constitutively expressed. The recombinant virus now has room for splicing in the transgene. It also is allowed to keep sequences which trigger encapsidation or virus formation and which are necessary to infect the target cell. Some replication incompetant viruses require "helper viruses" to help them make the proteins they need. These helper viruses must not be allowed to contaminate the final preparation, however. Viruses, being complex heterogeneous biological products, are easily contaminated during their manufacture. They present many challenges to the commercial producer and the governmental regulator (see Chapter 6).

The flip side of re-engineering viruses which have co-evolved with us is that they have wild-type cousins in important places, like inside our bodies. All of us harbor some latent viruses. This creates some critical concerns, the first being the risk that the engineered viral vector may genetically recombine with one of these endogenous viruses and become virulent by gaining the genes it needs to replicate. Another concern which relates to efficacy, and perhaps safety as well, is that many patients will, prior to gene therapy, have already developed antibodies to the wild-type strains. This should not affect an *ex vivo* protocol

	Naked DNA (by injection or electroporation)	CaPO4 Precipitate	Liposomes (± Cationic)	ASGP-Polylysine	Killed AV-Polylysine-±Transferrin
Safety					
• Integration into target host genome?	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)
• Select for "normal" transfected cell?	Yes, if done ex vivo	Yes, if done ex vivo	Yes, if done ex vivo	Yes, if done ex vivo	Yes, if done ex vivo
• Select for homologous recombination or or site specific integration?	Yes, if done ex vivo	Yes, if done ex vivo	Yes, if done ex vivo (no known data, however)	Yes, if done ex vivo (no known data, however)	Yes, if done ex vivo (no known data, however)
Efficacy					
• Gene expression when injected in vivo?	Transient, but depends on promoter	Transient, but depends on promoter	Transient, but depends on promoter	Transient, but depends on promoter	Transient, but depends on promoter
• Gene expression if cells first cloned ex vivo?	Stable, but depends on promoter	Stable, but depends on promoter	Stable, but depends on promoter	Stable, but depends on promoter	Stable, but depends on promoter
• Variety of target cell types?	Many cell types	Many cell types	Many cell types	Dividing liver cells (or use nafenopin)	Any cell type, dividing or not
• Vector itself elicits an immune response?	No	No	?	Possibly	Yes
Technical Feasibility					
• Vector can be injected directly in vivo?	Yes	Yes	Yes	Yes	Yes
• Maximum size of DNA insert?	Unlimited size	Unlimited size	Unlimited size	Unlimited size	Unlimited size
• Vector stability at room temperature?	Very Stable	Very Stable	Very Stable	Stable	Stable

References for Tables 4.3 and 4.4:
(a) John Monahan, personal communication
(b) Xandra Breakefield, personal communication
(c) Doug Treco, personal communication
(d) Curiel 1993
(e) Collins 1992
(f) Recombinant AV can be engineered to carry a 30k bp gene (Xandra Breakefield, personal communication)
(g) W. French Anderson, personal communication

Table 4.3 Key properties of dead viruses or non-viral vectors currently used as gene transfer vectors.

because the target cells can be removed from the immune system, but may seriously interfere with an *in vivo* protocol. For instance, most of the adult population have been infected at some period in their life with wild-type AV and are already sero-positive. *In vivo* use of an AV vector, such as that planned to deliver the CFTR gene to the lungs of cystic fibrosis patients, may require additional approaches to overcome or sidestep the immune response. Possibilities include giving additional vector to compensate for the inactivation, giving immunosuppressive drugs, giving "decoy" antigens or removing AV-antibodies from the patient's blood. Multiple *in vivo* therapy with viral vectors, as is anticipated for cystic fibrosis, will only aggravate the immune response problem over time. A summary of the key properties of replication-incompetant viral vectors is given in **Table 4.4**.

• **Advanced synthetic vectors** The basic technical problems with viral vectors that are cited above come from the fact that we are too close to viruses and they are too close to us. Throughout our reciprocal co-evolution, we have been their prey and they have been our predators. When they have evolved ways to hijack our cells, we in turn have evolved ways to thwart them. Trying to jerry-rig a virus into an agent of health, a therapeutic gene vector, is inherently fraught with problems. We are willing to live with the risks that come with this approach in order to make a first stage tool that is desperately needed to fight diseases for which there are no effective cures, such as cancer and AIDS. For other diseases such as Gauchers where there are effective (though costly) alternative cures, the risks associated with viral vectors may exceed the benefits.

Another approach is to take the lessons learned from the study of viral mechanisms and re-employ the basic principles to develop synthetic vectors which achieve therapeutic objectives with a minimum of side problems. This is the method of attack taken by David Curiel and Boehinger Ingelheim (see Chapter 5 for a detailed discussion). Using AV which has been killed by ultraviolet light, they have shown in an elegant set of experiments that the endosome-lysing and nuclear-homing capabilities of the dead virus can be used to efficiently transfer a fully functional gene attached to the *outside* of the virus with a simple salt bridge. Moreover, if the AV does not naturally infect a cell, it can be made to do so by addition of a specific ligand which binds to a receptor on the target cell. The transgene may be of unlimited size and need not be under the

	Retrovirus (RV) single stranded RNA recombinant virus	Adenovirus (AV) double stranded DNA recombinant virus	Herpes Simplex (HSV-1) double stranded DNA		Adeno-Associated (AAV) single stranded DNA recombinant virus
			amplicon (plasmid) plus helper virus	recombinant virus	
Safety					
• **Any harmful effects from parent virus?**	Tumorigenic (a)	Pathogenic (a) Tumorigenic (a)	Pathogenic (a)	Pathogenic (a)	No Currently Known Diseases (a)(g)
• **Replication-incompetent vector possible?**	Yes	Yes	Yes	Yes	Yes
• **Recomb. of vector w/ endogenous viruses?**	Possible	Possible	Possible	Possible	Possible
• **Virus specific proteins expressed in host?**	No (b)	Yes (b)	Yes (b)	Yes (b)	No (a)
• **Integration into target host genome?**	Yes & randomly	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)	Rare (<1 cell/million) & random (DNA repair)	Yes & at a single site on Chromosome 19 (a)
Efficacy					
• **Stability of gene insertion in host's genome?**	Variable (a)	Maintained as an episome in nucleus (b)	Maintained as an episome in nucleus (b)	Maintained as an episome in nucleus (b)	Very stable (a)
• **Stability of gene expression in host cell?**	Depends on promoter	Depends on promoter	Depends on promoter	Depends on promoter	Depends on promoter
• **Frequency of infectivity of host cell?**	Variable	High for epithelial cells & variable for others (b)	High	High	High
• **Variety of target cell types?**	Must be dividing cells	Epithelial cells are best but it enters any cell (b)	Nerve cells are best but it enters any cell (b)	Nerve cells are best but it enters any cell (b)	Can be any cell type, dividing or not (a)
• **Vector itself elicits an immune response?**	Yes	Yes	Yes	Yes	Yes
Technical Feasibility					
• **Maximum size of DNA insert?**	6k-7k bases (c)	6k-8k base pairs (d,f)	10k-15k base pairs (b)	35k base pairs (b)	4.2k-4.6k bases (a,b)
• **Vector stability at room temperature?**	Unstable	Stable	Stable	Stable	Very Stable
• **Vector titer or yield (per milliliter)?**	Low (10^6) (e)	Very High (>10^10)(e)	Very High (10^10)(b)	Very High (10^10)(b)	High (10^8/ml) (e)
• **Vector can be injected directly in vivo?**	No, unstable in blood	Yes	Yes	Yes	Yes
• **In situ packaging cell injection is possible?**	Yes (b)	?	Yes (b)	Yes (b)	?

Table 4.4 Key properties of replication-incompetent live viruses currently used as gene transfer vectors.

control of viral promoter agents. These experiments suggest that a synthetic polypeptide vector could be specified and engineered to deliver a gene of any size to the nucleus of any cell for which a specific receptor ligand can be identified. Immunogenicity is still a likely problem, however. More research will be needed to further disect the functional properties of viruses into component parts which can be re-assembled and re-embodied in synthetic viral vectors.

4.7 Gene Therapy Programming

The future development of gene therapy will require a systematic ordering of the gene constructs, targets, delivery techniques and medical objectives. As vectors and delivery methods are improved and expanded, a taxonomy of possible approaches will develop. **Table 4.5** is a first pass at such an ordering system. There are eight basic questions that need to be asked when designing a gene therapy protocol:

1. What is the gene (including regulatory sequences) being transferred?
2. What is the gene transaction- a new addition, a repair, or a knockout?
3. Is the gene integrated into the host genome or is it a free nuclear entity and is it necessary to pass the gene on to clonal progeny?
4. What is the vector?
5. What is the delivery method?
6. What is the length of action and is multiple treatment necessary?
7. What is the target cell and what percentage must be successfully hit?
8. What are the therapeutic objectives at the molecular, cellular and organismic (clinical) levels?

For illustrative purposes, the answers to these questions for one of the gene therapy protocols under development for cystic fibrosis are provided in the

Gene Construct	Gene Transaction	Final Nuclear Status of Gene	Vector	Delivery & Frequency	Target Cell	Objectives: Molecular	Objectives: Cellular	Objectives: Clinical
cDNA	Add new gene (+)	Random c'some insertion	Naked Plasmid	Ex vivo culture	Tumor Infiltrating Lymphocytes	Add functioning gene to fix recess. disorder	Reverse effects of protein deficiency	Reverse symptoms of genetic disorder
full unedited genomic DNA	Repair existing gene by homol. recomb. (Δ)	Specific c'some insertion	CaPO4 Pcpt	In vivo methods:	Peripheral Blood Lymphocytes	Add gene to confer drug resistance	Essential cell survives during chemotherapy	Protect vital cells (eg, blood stem cells) during chemotherapy
+ Promoter DNA	Delete(knockout) existing gene by homol. recomb. (-)	Episome (replicative)	Liposomes (± Cationic)	Intra-muscular	Bone Marrow	Add "suicide" gene to confer drug sensitivity	Unwanted cell is killed by chemotherapy	Cause tumor to recede
+ Enhancer DNA		Episome (non-replicative)	ASGP-Polylys	Intra-venous	Tumor Cells	Homolog. repair of gene to fix domin. disorder	Reverse unwanted domin. gain of function	Block replication of infectious agent such as HIV
± Homologous flanking DNA (if recombination event req'd)		Human Artificial C'some (HAC) (not yet possible)	Dead AV-Poly-lysine-Ligand	Intra-peritoneal	Skin Fibroblasts	Add gene coding for antigen to elicit intracellular immune response	Present antigen fragments as MHC1 complx to cytotoxic T cells	Slow down aging process
			Retrovirus	Sub-cutaneous	Hepatocytes	Add growth factor gene	Stimulate cell expansion in vivo	
			Adenovirus	Trans-dermal	Lung Epithelium			
			Adeno-Assoc. V.	Inhalation	Neurons			
			Herpes Smplx V.	Mucosal				
			Univ. Donor Cell	Catheter				
			Packaging Cell	Gel				
				Cell capsule				

Illustrative Example: Cystic Fibrosis

Gene Construct	Gene Transaction	Final Nuclear Status of Gene	Vector	Delivery & Frequency	Target Cell	Objectives: Molecular	Objectives: Cellular	Objectives: Clinical
CFTR cDNA + AV Promoter	Add new gene (+)	Episome (non-replicative)	Adenovirus	In vivo method: Inhalation once every 2-3 months	Lung Epithelium	Add functioning Chloride Channel Protein	Restore proper ion balance in lung epithelial cells	Reduce mucous buildup & opportunistic infection

Table 4.5 Combinatorial "pull-down" menu showing possible options for the construction of gene therapy protocols.

lower panel of **Table 4.5**.

As the field of gene therapy develops and the successes and failures are recorded, a body of knowledge will emerge which will inform clinicians of their options and "most-likely-to-be-successful" approaches for newly targeted diseases.

CHAPTER 5
COMMERCIALIZATION OF GENE THERAPY

5.1 Critical Success Factors in the Business of Gene Therapy

The business of gene therapy requires the integration of many critical factors over a long span of time. **Figure 5.1** is a list of the major factors essential for success. Above all, there is a constant need for large amounts of capital. Under-funded companies are simply not going to make it through the clinical trials, even if they have worked out their science and technology, licensed their genes and negotiated their distribution channels. Consequently, many gene therapy companies have struck strategic alliances with larger pharmaceutical firms, who supply capital usually in exchange for equity and/or distribution rights.

Another element which is essential is the diagnostic capability to assess and monitor the benefit of the therapy. Gene therapy companies must develop alliances with commercial diagnostic companies. If no such companies exist, the gene therapy developers may find themselves in the diagnostics business by default, a situation they may not have anticipated in their original business plan.

Companies developing an *in vivo* product can follow the distribution structures developed by the pharmaceutical industry. *Ex vivo* cell service companies have more complicated distribution issues to address. Will the service be centralized? How will the patient interface be handled? Who in the field will collect the cells, ship them, introduce them into the patient and monitor therapeutic success? If the cell service is decentralized, who will perform it? How will off-site personnel be trained? How will quality assurance controls be implemented?

Lastly, the manufacturing facility and processes will need to be scaled up to make vectors for use in the clinical trials. The company will have to demonstrate to the FDA that current Good Manufacturing Practices are being followed.

Factor	Requirements
Financial Staying Power	✓ Access to adequate sources of capital to cover the high costs of R&D, clinical testing, regulatory approval, manufacturing & distribution
Disease Criteria	✓ Severity of disease must be great ✓ Alternate therapies must be costly and/or ineffective or non-existent ✓ Gene therapy must present a favorable risk-benefit ratio ✓ Patient population must be of sufficient size ✓ Patient population must be of sufficient affluency to afford new therapies
Scientific Knowledge	✓ Mapping and sequencing of the gene(s) responsible for the disease trait ✓ Sufficient understanding of the molecular basis of the disease ✓ If a protein deficiency disease, clinical data showing that protein replacement is an effective therapy ✓ Analytical techniques sufficient to develop commercial diagnostic tests for the gene and the gene product ✓ Identification of an appropriate target cell with sufficiently defined biology ✓ An animal model with disease symptoms comparable to those in humans
Gene Estate	✓ Ownership of proprietary rights to the gene either by 1) being the first to discover and patent the gene or 2) acquisition of a license (preferably exclusive) from the gene owner
Gene Transfer Technology	✓ Access to an appropriate gene delivery technique obtained by license or by internal R&D effort secured by patents ✓ Expertise in vector construction, testing and manufacture
Manufacturing Technology	✓ Must engineer facility and process for increased scale of operation & solve related manufacturing concerns
Relations with Suppliers	✓ Strong contractual relations with suppliers of critical goods and services ✓ Establishment of multiple (FDA approved) suppliers where possible
Regulatory Approval	✓ Must present convincing evidence that product is safe and efficacious ✓ Must demonstrate that production facility & processes follow current Good Manufacting Practices ✓ Must determine product lability or shelf life
Alliance with Diagnostic Firms	✓ Must develop strategic alliances with firms offering diagnostic tests for the gene & the gene product in order to assess & monitor the benefit of the therapy ✓ If no such firm exists, diagnostics must be included in gene therapy business plan
Clinical Base	✓ Identification of potential patients by using commercially available DNA diagnostics ✓ Establishment of newborn and carrier status screening programs ✓ Education of physicians & patients about gene therapy as an alternative ✓ Adequate longterm tracking of safety and efficacy parameters in patients
Distribution	✓ Training of a sales force with specialized knowledge ✓ Establishment of contractual relations with clinics if decentralized ex vivo service ✓ Quality assurance procedures for shipping cells if centralized ex vivo service
Public Acceptance	✓ Society must be willing to accept risks associated with gene therapy ✓ Payers of healthcare must be willing to reimburse costs of gene therapy

Figure 5.1 Critical success factors in the business of gene therapy.

Investment in manufacturing plant and equipment will need to be made up front to manufacture the vectors for clinical trials, without any assurance that an approved-for-market product will result.

5.2 Companies Developing Gene Therapy

Research presented here (**Table 5.1**) has identified four major companies which have entered the gene therapy commercialization field since a similar survey in the Spring of 1992 (Chow 1992). They are Avigen, GenVec, SyStemix and Theragen. The field is expanding in several regards. In addition to the companies who are developing the gene transfer technologies, there are also the companies or institutions who hold proprietary positions in the genes themselves. With the rate of human gene discovery being approximately one per day (Waldholz 1993) and with less than 3000 of the estimated 50,000-100,000 genes identified to date (McKusick 1992), the institutions holding the patents on the genes are becoming a major constituency in the development of gene therapy. For instance, Genetics Institute (Cambridge, MA), which produces a recombinant form of Factor VIII, has licensed its interest in the gene to Genetic Therapy for the purpose of developing a gene therapy for hemophilia A. Other than licensing the gene, Genetics Institute is not active in this development (Garen Bohlin, personal communication). This is an indication that companies heavily invested in the production of recombinant proteins are recognizing the potential competitive threat of gene therapy and are diversifying their positions to insure their future participation in the marketplace.

The gene for cystic fibrosis is another case where licensing will play an essential role in the development of the gene therapy. The discovering institutions, the University of Michigan and the Hospital for Sick Children in Toronto, have filed patent applications and have indicated that, if the patents issue, they intend to license the technology to others on a non-exclusive basis. At present there are five separate groups pursuing gene therapy for cystic fibrosis. Genzyme, one of the five, is already well along the way in negotiating a licensing agreement with the probable gene patent holders (Gregory Phelps, personal communication).

Lastly, there are firms developing adjunct technologies such as cell separation

Year Founded	Company Name	Location	Ownership/Funding
1981 1992	Genzyme Corp. • Neozyme II (spinout)	Cambridge, MA	Public/$700MM Mkt Capit. Public SWORD/$75 MM
1986	Genetic Therapy, Inc.	Gaithersburg, MD	Public/$140MM Mkt Capit.
1986	Somatix Therapy Corp.	Alameda, CA	Public/
1987	Vical Inc.	San Diego, CA	IPO on 2/93
1987	Viagene, Inc.	San Diego, CA	IPO on 1/93
1988	Cell Genesys, Inc.	Foster City, CA	IPO on 1/93
1988	SyStemix, Inc.	Palo Alto, CA	Public/$160MM Mkt Capit. 60% ownership by Sandoz
1988	Transkaryotic Therapies Inc.	Cambridge, MA	Private
1989	TargeTech, Inc.	Meriden, CT	Public (acquired 1992 by Immune Response Corp.)/ $200MM Mkt Capit. Parent
1990	Targeted Genetics Corp.	Seattle, WA	Private spinout of Immunex Corp. which retains 40% ownership
1991	Theragen	Ann Arbor, MI	Private
1992	Avigen, Inc.	Alameda, CA	$10MM priv. placement by The Castle Group, NYC
1993	GenVec	Montgomery County. MD	$8.5MM priv. placement by Hillman Medical Ventures & upto $17MM invested by Genentech
1993	Boehinger Ingelheim/ Curiel	not determined	negotiation in progress

Table 5.1 Companies developing gene therapies as of the Spring of 1993.

methods which are essential elements of *ex vivo* gene therapy. Companies such as Applied Immune Sciences (Santa Clara, CA), CellPro (Bothell, WA) and SyStemix (Palo Alto, CA) are developing cell sorting methods for bone marrow cells, which are useful in conventional bone marrow transplants as well as in *ex vivo* gene therapies.

• **Avigen** is a newly formed company which is developing the adeno-associated virus (AAV) as a vector for gene therapy. Avigen's goal is to develop gene therapy products which can be distributed as traditional pharmaceuticals (John Monahan, personal communication). The company does not want to be in the *ex vivo* cell service business, which it sees as high risk, due to the labor intensive aspects and quality assurance challenges and due in part to the liabilities of processing materials with contagions such as HIV.

The adeno-associated virus offers several advantages over existing retroviral vectors:

- non-random insertion
- non-tumorigenic
- no need for dividing cells.

AAV is a common human virus and is not associated with any known diseases. Approximately 80% of the population is seropositive for AAV. The wild type always integrates within human chromosome 19 at the same locus (±5 base pairs), thus reducing significantly the risk of oncogene activation or cancer suppressor gene inactivation. Once inserted, it does not disengage or "pop out" of the chromosome; unlike retroviruses which show variable stability, AAV integration is stable. Lastly, AAV does not require cells to be actively dividing, while retroviruses do. For instance, liver gene therapy with retroviral vectors in lab animal models requires removal of up to 75% of the organ in order to stimulate cell division. An hepatectomy of this magnitude would be unacceptable as a therapeutic protocol.

Avigen has targeted three initial target areas:

- sickle cell anemia
- AIDS
- multiple drug resistance in normal cells of cancer patients

Research has shown that as little as 5% normal beta-hemoglobin expression will correct sickling in red blood cells. Avigen is developing a sickle cell gene therapy that can be accomplished *in vivo* by insertion of a needle into the bone marrow. Their DNA construct consists of the beta-globin gene, which is public domain, in combination with their erythroblast specific promoter and their AAV strain, both of which have patents pending. Avigen's AAV does not insert at the same chromosomal site as the wild type; the company would not divulge the locus of insertion.

Avigen is developing a gene therapy for the treatment for AIDS using the AAV vector to transfect bone marrow cells with a fusion gene coding for a calcium ion dependent RNAase which is linked to the HIV capsid protein subunit. The strategy is to get this fusion protein to incorporate into the virus particle as it forms in the cytoplasm. The RNAase is inactive inside the cell where the calcium ion concentration is *micro*molar. When the virus particle buds off the cell and enters the blood, where the calcium is *mili*molar (one thousand times greater concentration), the nuclease will be activated and will cut up and destroy the HIV RNA genome.

The multiple drug resistance program involves the transferral to bone marrow cells of cancer patients of a gene that codes for a membrane transport protein that pumps out cancer drugs such as taxol and other chemotherapeutics.

• **Boehinger Ingelheim/Curiel** is a collaboration between the european pharmaceutical firm Boehinger Ingelheim, which owns the private Research Institute of Molecular Pathology in Vienna, Austria and David Curiel of the University of North Carolina at Chapel Hill. While no venture has been formed (David Curiel, personal communication), negotiations are underway to begin commercialization of technology which, in the opinion of this thesis author, offers the most promising direction in the search for what has been termed the "Holy Grail" of gene therapy, namely *a targetable, injectable vector*.

Boehinger Ingelheim and Curiel are developing vectors using dead adenovirus (AV) which can transport DNA of unlimited size to the nucleus of any cell which possesses a unique membrane receptor and isolatable corresponding ligand. In order to describe their approach, it is necessary to take a brief detour into the

mechanism of AV infection. Certain cells, such as epithelial cells in the lung, have receptors on their outer membrane surface which specifically bind to antennae-like fiber proteins projecting from the capsid shell of the AV. All eukaryotic cells have a complex system of internal membranes known as the Golgi Apparatus which are constantly fusing with the outer membrane, sending macromolecules outward to the surroundings (exocytosis) and inward from the surroundings (endocytosis). When the AV ligand binds to its receptor on the membrane, it triggers a process known as receptor-mediated endocytosis, that is, a piece of the membrane binding the AV buds inward (invaginates) to form a vesicle floating free in the cytoplasm with the AV inside. Once formed, this vesicle, known as an endosome, undergoes acidification, which is thought to induce a change in the shape of the AV capsid proteins, allowing them to interact with and disrupt the membrane, thus releasing the AV into the cytoplasm (Curiel 1993; Zatloukal and others 1992). Once in the cytoplasm, the AV capsid proteins also have the ability to bind to cytoplasmic elements which lead to the pores of the nuclear membrane (Darnell and others 1990). In an energy dependent process not well understood, the AV is transported into the nucleus where its DNA is released and its genes expressed by RNA polymerase and associated transcription factors present in the nucleus.

Researchers developing replication-incompetant AV as vectors for gene therapy are effectively exploiting this endosomolytic and nuclear homing capability of AV to get their gene-of-interest into the nucleus. They have three nagging problems, however. First, there is the safety issue that their virus can gain virulence by genetic recombination with endogenous AV, which is widespread, having infected almost all of the adult population. Second, expression of their gene is obligated to be under the control of viral promoter/enhancer elements. This complicates tissue-specific expression and regulation. Third, they are limited to genetic inserts under 8,000 base pairs (8kb), the maximum amount of foreign DNA that can be stuffed inside the capsid.

Boehinger Ingelheim and Curiel propose the following solution to these three problems: First, solve the safety problem by inactivating the AV with ultraviolet radiation. Killed AV cannot replicate and, more critically, cannot recombine with endogenous virus. It can, however, trigger the receptor-mediated endocytosis, lyse the endosome, and home to the nucleus just like the live virus. Second,

instead of splicing the therapeutic gene into the viral genome where it is under control of the virus' regulatory elements, put it outside the virus by attaching it to the surface of the AV with a weak electrostatic salt bridge using the polycation, polylysine. Third, since the gene is outside the capsid, it can be any size. Boehinger Ingelheim and Curiel can transfer 48kb cosmid DNA with the same efficiency achieved with 6kb plasmid DNA and are currently investigating the transfer of yeast artificial chromosome (YAC) DNA of a size in the millions of base pairs.

Gene size is likely to be a critical hurdle in the future of gene therapy. Most approaches today are using cDNA, which is a DNA version of the messengerRNA, which is itself a highly edited version of the original gene. For instance, while the Factor IX gene is 34kb, its corresponding mRNA is only 2.8kb, a twelvefold reduction in size due to post-transcriptional editing (McKusick 1992). The use of cDNA excludes or bypasses the biological intricacies of nuclear processing of the pre-mRNA, a stage that is most likely critical in the poorly understood nuances of gene regulation. Evidence for this assertion is the fact that some thalassemias are the result of splicing disorders of the beta-globin chain (Gelehrter and Collins 1990).

Not all cells have AV receptors on their surfaces. Those that do not are not infectable and are not candidates for gene therapy using live or dead AV. Boehinger Ingelheim and Curiel however have found a way to apply their killed AV vector technology to such cells by linking a cell specific ligand. They call it a "ternary molecular conjugate vector" on account of the three components: 1) DNA/polylysine, 2) dead AV, and 3) cell specific ligand. Using human transferrin as the ligand, they were able to transfer reporter genes (luciferase) into HeLa cells, a human cell line which has transferrin receptors but no AV receptors. Neither transferrin/DNA nor AV/DNA by themselves worked, because the former lacked the endosomolytic and nuclear homing properties of AV and because the latter lacked the receptor properties of the transferrin.

This experiment suggests that, given the existence of a purified cell-specific ligand, one can target a gene of any size to any cell using a dead virus. Once the endosomolytic and nuclear homing properties of AV capsid proteins are better understood, it may be possible to do away with the dead virus altogether. It is

conceivable that a recombinant fusion protein could be engineered which possesses the three functional moieties, cell-specific ligand, capsid protein and polylysine for DNA binding all in one continuous polypeptide. Such a molecular conjugate vector would come very close indeed to the "ideal targetable, injectable vector" mentioned earlier. The possibility of an adverse immune reaction, particularly if repeated injections are required, would still remain as a hurdle to be overcome by such a product.

Within the year David Curiel will move from the University of North Carolina to head the newly formed Gene Therapy Institute at the University of Alabama. Discussions regarding the formation of a commercial venture to develop this technology are underway with Boehinger Ingelheim. Malignant melanoma is planned to be the first disease target.

• **Cell Genesys** is developing the technique of homologous recombination to replace, activate or inactivate specific genes in cells. In homologous recombination, a vector is prepared which contains sequences which are similar or homologous to sequences already present in the target gene. In the middle of this homologous sequence can be placed additional unrelated sequences or deletions. When this vector is introduced into cells, it will recombine with the cell's DNA. While the mechanism is not fully understood, it is thought to involve overlapping or "crossing over" of the complementary strands and pairing interactions between the "sticky ends" of the two DNA duplexes (Sedivy and Joyner 1992). This phenomenon occurs as frequently as one event in a thousand (Thomas and Capecchi 1986; Thomas and Capecchi 1987; Thomas and others 1986), which is suitable for selection by a growth or drug resistance method. The net effect is that mutation by deletion or by addition of DNA sequences can be achieved at specific genetic sites, allowing one to modulate specific gene activity. This emerging field is referred to simply as "gene targeting".

Cell Genesys is applying gene targeting in three areas:

- universal cell transplant products
- human therapeutic protein products
- human monoclonal antibody products.

Universal donor cells have the genes that code for unique protein sequences on

the cell surface inactivated by gene targeting. These proteins, known as human leukocyte antigens (HLA), confer a unique immunological "fingerprint" on all the cells of an individual, making tissue transplantation between individuals with differing HLA tissue types impossible without immunosuppressive drugs. Cell Genesys is developing universal donor cells for three tissues frequently used as transplants:

- keratinocytes (skin) for severe burns and impaired wound healing
- retinal epithelial cells (eye) for macular degeneration
- myoblasts for muscle wasting diseases.

In addition to making these donor cells, which are "invisible" to the immune system, Cell Genesys is also making universal immune cells, known as T cells, which can bypass the recipient's HLA identification system and function properly. Such cells would be useful in patients with diminished immune systems, such as those with AIDS.

Universal donor cells can also be used themselves as "gene transplant vectors". The cells become in effect a "genetic platform" which can be configured to deliver gene products of therapeutic value.

The other two areas where Cell Genesys is using gene targeting, human therapeutic protein products and human monoclonal antibody products, are concerned not with the delivery of genes to a patient but instead with the production of classically administered therapeutic proteins made by novel bioprocessing methods. In the first case, the company proposes to take established human cell lines and *activate* the desired gene to produce a given human therapeutic product. Normally, a human gene is added into an animal cell line. The recombinant cells then have to be passaged to select the particular cells which stably express the desired gene product. By gene targeting, the regulator genes that control expression of an already existing but dormant gene could simply be "switched on".

The human monoclonal antibody production program involves the use of gene targeting to insert human antibody genes into mouse embryo stem cells, which give rise to mice with humanized immune systems. Humanized monoclonal antibodies are less likely to evoke allergic responses when administered to

humans than those which the mouse normally produces.

• **Genetic Therapy (GTI)** is one of the oldest of the gene therapy companies. It is also has the greatest number of protocols approved by the National Instiitutes of Health Recombinant DNA Advisory Committee (NIH-RAC) (see Appendix B). The company is closely tied with the NIH, having signed numerous Cooperative Research and Development Agreements (CRADAs) which grant joint ownership or the exclusive license of proprietary technology developed by certain investigators at the NIH. One of the key investigators is W. French Anderson, formerly Chief of the Molecular Hematology Branch of the National Heart, Lung and Blood Institute of the NIH. Anderson began working on gene therapy at NIH in 1968. In 1992 he moved to the University of Southern California where he continues to be funded by GTI. Other key gene therapy researchers at NIH with which GTI has CRADAs include Michael Blaese, who along with Anderson did the ADA deficiency and Tumor Infiltrating Lymphoctye human trials, Robert Gallo, who is a leader in AIDS research and Arthur Nienhuis, a bone marrow and hemoglobinopathy expert.

GTI is pursuing many different gene vector constructs including retroviruses, adenoviruses and adeno-associated viruses as well as the packaging cell lines which are used to grow these replication incompetant viruses. GTI's commercial strategy is to license the therapeutic genes, construct the vectors and sell them to clinical institutions who would do *ex vivo* therapy on the cells of their patients. GTI is also developing direct *in vivo* methods but not as the initial target market.

The diseases that GTI is targeting are indicated in **Tables 5.2** to **5.5**. As can be seen, GTI is pursuing eight monogenic disorders, two AIDS programs, eleven different cancer programs as well as one therapy related to preventing clotting after cardiac surgery.

GTI has prepared many of the vectors used in cell marking protocols. While these procedures offer no direct therapeutic benefit to the patient, they are often used as a monitor for the progress of other therapies. Gene markers also serve as valuable controls for the vector technology itself. Some observers view the use of gene markers in humans as somewhat unethical since the patient incurs some (albeit small) risk but receives no therapeutic benefit (John Monahan, personal

	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
Monogenic Diseases														
• Cystic fibrosis				✔	✔	✔								
• Emphysemia (alpha1-antitrypsin def.)				✔										
• Fabry's disease (alpha-galactosidase)						✔								
• Gaucher's disease (glucocerebrosidase)				✔		✔					✔			
• Hemophilia A (Factor VIII deficiency)				✔			✔		✔			✔	✔	✔
• Hemophilia B (Factor IX deficiency)				✔					✔			✔		✔
• Hemo. B (gamma Carboxylase, a Fac. IX activator)												✔		
• Hypercholesterolemia (LDL receptor)				✔					✔			✔		
• Parkinson's disease (tyrosine hydoxylase)							✔				✔			
• SCID (ADA deficiency)				✔										
• Sickle Cell Anemia	✔													
• Short stature (human growth hormone)												✔		
• Thalassemias	✔													

Table 5.2 Monogenic diseases targeted by gene therapy companies as of Spring 1993 (source: company literature).

	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
AIDS														
• anti-HIV T cell expansion										✔				
• anti-HIV donor blood stem cells								✔						
• Fusion RNAase-HIV capsid protein	✔													
• HIV cell-mediated immunotherapeutic													✔	✔
• Soluble CD4 receptor				✔										
• HIV Replicating Protein Decoy				✔										
• Universal anti-HIV T cells			✔											
Other Viral Diseases														
• Cytomegalovirus (specific CD8+ CTL)										✔				
• Hepatitus B virus (antisense)									✔					
• Hepatitus B (immunotherapeutic)													✔	✔
• Herpes simplex (immunotherapeutic)													✔	✔
• Influenza A virus (immunotherapeutic)														✔
Other Infectious Diseases														
• Malaria (immunotherapeutic)													✔	

Table 5.3 Infectious diseases targeted by gene therapy companies as of Spring 1993 (source: company literature).

Cancers	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
• Brain (unspec. gene w/ herpes simplex vector)											✔			
• Brain (thymidine kinase-ganciclovir kill)				✔										
• Breast cancer				✔								✔	✔	✔
• Cervical cancer													✔	
• Childhood (AML) leukemia				✔										
• Chronic myeloid leukemia (CML)				✔										
• Colon cancer				✔										✔
• Lung cancer				✔										
• Malignant melanoma		✔		✔			✔					✔	✔	✔
• Multiple Drug Resistance	✔													
• Neuroblastoma				✔										
• Ovarian cancer							✔							
• Pancreatic cancer													✔	
• Renal cancer				✔			✔							
• Tumor Infiltr. Lymphocytes (IL-1,2,4, TNF,etc)				✔										
• Various non-therapeutic cell marker genes				✔										

Table 5.4 Cancers targeted by gene therapy companies as of Spring 1993 (source: company literature).

	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
Miscellaneous Diseases														
• Alzheimer's disease (NGF)							✔							
• Alzheimer's disease (unspec. gene)												✔		
• Alzheimer's disease (unspec. gene w/ HSV)											✔			
• Anemia (EPO)												✔		
• Anti-clot during cardiac stents (t-PA)				✔										
• Autoimmune disorders (prTGF-beta)														✔
• Blood vessel growth after angioplasty														✔
• Diabetes, adult-onset (insulin gene)												✔		
• Diabetes, juvenile-onset (insulin gene)												✔		
• Hematopoietic stimulating factors								✔						✔
• Immunomodulatory proteins (cytokines)														✔
• Ischemic heart disease (FGF-5)														✔
• Liver failure				✔										
• Macular degeneration in eye			✔											
• Osteoporosis (calcitonin gene)												✔		
• Rheumatoid arthritis (IL-1 in synovial cells)											✔			
• Universal donor & receptor cells			✔											
• Universal donor hematopoietic stem cells								✔						

Table 5.5 Miscellaneous diseases or techniques targeted by gene therapy companies as of Spring 1993 (source: company literature).

communication).

Of special note is the strategic alliance with Sandoz Pharma Ltd. In November 1991, GTI entered into a royalty-bearing license agreement with Sandoz covering areas of immunology/inflammation, cancer and cardiovascular disease. Sandoz will pay upto $13.5MM over three years for research in these three areas in exchange for exclusive world wide rights to use the technology to make and sell products. Sandoz also bought $10MM of GTI stock.

• **Genzyme** is a well-diversified biopharmaceutical company pursuing gene therapy as well as several of the competing technologies mentioned in this study. Of particular note is the company's activity in cystic fibrosis. In the diagnostics area, Genzyme's subsidiaries, Integrated Genetics and Vivigen, are offering DNA diagnostic services for the 16 most common cystic fibrosis mutations. The company's scientists in association with Tufts University have also been successful in developing transgenic mice which secrete the normal CFTR protein in the membranes associated with the fat globules of milk (DiTullio and others 1992). The availability of large amounts of the protein opens the possibility of a protein replacement therapy, perhaps by aerosol delivery of a liposome formulation. Most protein replacement procedures are via the blood and do not involve membrane proteins. Whether an exogenously administered membrane protein can properly interculate itself into a cell membrane and whether such a protein can survive an aerosol delivery without denaturation are unique challenges of this project.

The other approach Genzyme is taking on cystic fibrosis is gene therapy using an adenovirus to deliver the gene to the cells of the lung via an aerosol. In December 1992, Genzyme in collaboration with the University of Iowa received approval from the NIH-RAC to do human trials. These initial trials will involve swabbing the vector on the nasal passages of CF ΔF508 homozygotes with mild to moderate symptoms over age 18 who are already sero-positive for adenovirus. Samples of nasal epithelial cells will then be gently brushed off and tested for CFTR mRNA. Success of the transfection from a physiological standpoint will be assessed by changes in chloride transport as measured with a small electrode *in situ*. The prior immunity to adenovirus brings up an important point about the use of viral vectors derived from commonly occurring wild type strains.

Virtually everyone has been exposed to adenovirus and has developed antibodies which block any systemic dispersion in the body. Only techniques which deliver the vector directly to the target cell can escape the humoral immune system; examples are *ex vivo* treatment which removes the target cells from the antibodies in the serum or aerosol delivery to the exteriormost cells of the lung.

In March 1992, Genzyme raised $75MM for cystic fibrosis protein and gene therapy programs through the formation of Neozyme II, a Stock Warrant Off-Balance Sheet R&D (SWORD) Limited Partnership. Genzyme can buy back the technology from Neozyme II at any point in time per an established schedule. The funding is not split between the the protein and gene approaches in a fixed proportion. Rather the funding is approved based on the relative progress of the two approaches, which is assessed periodically (Gregory Phelps, personal communication).

There are five research groups pursuing cystic fibrosis gene therapy, and three of them have already announced commercial partners. The obvious question arises: is there a big enough market to support 3-5 firms? A "back of the envelope" calculation is as follows: There are approximately 30,000 patients in the US and also about that many in Europe. CF is primarily a Caucasian disease, occurring in Asian and other world populations at a much lower frequency. While prenatal diagnostics is expected to result in fewer CF births, the life expectancy of the existing affected population is going up due to improved medical maintenance. So as an approximate measure, assume that the world market which can 1) pay for gene therapy and 2) be economically reached by marketing and distribution channels is stable at about 60,000 worldwide. It is unlikely that CF gene therapy will result in a permanent "cure" for the individual; that would require transduction of lung progenitor stem cells, which are probably not going to be accessible by an aerosol-delivered vector, if indeed such cells even exist. Periodic treatment will probably be necessary. If the cost of periodic CF gene therapy is on the order of $20,000/year, the total world market would be 60,000 times that or $1.2 billion/year, a market size that could easily support five independent commercialization efforts. This approximation is obviously full of assumptions, such as third party healthcare payers are willing to pay $20,000/year/patient. Nevertheless it gives a "ballpark" estimate.

In addition to cystic fibrosis, Genzyme is also pursuing a gene therapy for Gaucher's Disease and, to a lesser extent, Fabry's Disease. Genzyme is a major producer of glucocerebrosidase, the protein replacement therapy for Gaucher's Disease. This enzyme is currently purified from pooled human placenta, but will be made by the company using recombinant DNA cell culture methods in the near future.

• **GenVec** is a single purpose gene therapy company whose birth was announced by a press release from Genentech (So. San Francisco, CA) on March 3, 1993. Ronald Crystal, former Chief of the Pulmonary Branch of the National Heart, Lung and Blood Institute of the NIH, is the founder of GenVec. In December 1993 Crystal, who at the time had a CRADA with Genetic Therapy, received approval from the NIH-RAC to do human trials for a cystic fibrosis gene therapy similar to Genzyme's. Between December and March there was a falling out between Crystal and GTI. On the same day as the GenVec announcement by Genentech, the NIH-RAC announced approval of a CF protocol proposed by GTI in collaboration with Jeffrey Whitsett of the Childrens Hospital at the University of Chicago (see Appendix B for a list of all RAC approved protocols).

Seed capital of $8.5MM is being provided by an investor group led by Hillman Medical Ventures (Horsham, PA) (Liebert 1993). Genentech will provide GenVec with up to $17MM in research support, milestone payments and future equity investments in exchange for worldwide marketing rights to any CF gene therapy product that is developed. Genentech's involvement in CF is not new. The company has recently developed a recombinant DNAase, named Pulmozyme, which relieves mucous congestion by reducing the viscosity caused by DNA released from lysed cells in the mucous.

• **Somatix**, like GTI, is one of the oldest gene therapy companies. The company is based on retroviral technology developed by Richard Mulligan of the Whitehead Institute at MIT. Other scientific luminaries associated with the company include Fred Gage and Ted Friedmann of UC San Diego and Inder Verma of the Salk Institute.

The company acquired Hana Biologics in 1991 and GeneSys Therapeutics in 1992. Hana Biologics focused on the transplantation of mammalian cells capable

of producing specific proteins while GeneSys focused on gene transfer techniques for neural disorders.

Somatix is concentrating its research programs on cancer, neural diseases, and blood disorders. In the cancer program, tumor cells are removed from the patient and transfected with retroviral vectors containing genes for lymphokines, which stimulate the immune system to recognize certain tumor associated antigens, resulting in the destruction of other tumor cells bearing the same antigens.

The neural research is concerned with Parkinson's disease and Alzheimer's disease. In both diseases, non-neural cells are taken from the patient and genetically modified *ex vivo*, then reimplanted in the appropriate position in the brain. For Parkinson's the gene would code for tyrosine hydroxylase, an enzyme essential in the biogenesis of the neurotransmitter dopamine, and for Alzheimer's the gene would code for nerve growth factor (NGF) to stimulate the growth of cells which normally degenerate in the disease.

Somatix is also working on gene therapies for both hemophilia A and B.

• **SyStemix** is a new entrant into the gene therapy area, having recently appointed James Mulé, former Senior Investigator at the National Cancer Institute, as Director of Gene Therapy. SyStemix is an example of a company with core technology that is closely related to *ex vivo* gene therapy, namely cell fractionation and tissue transplant therapy. The company has developed proprietary methods to isolate hematopoietic stem cells (HSCs), which are the most important cells in bone marrow transplants. HSCs not only can divide to make more of themselves, but they also can differentiate into any one of the specialized blood cells in the circulation. They are very rare, only one in 2000 bone marrow cells is an HSC. The ability to continuously replenish all types of blood cells such as red blood cells, platelets, T cells and many others makes HSCs ideal targets for permanently curative gene therapy.

SyStemix has also developed a proprietary mouse, SCID-hu, which has a human immune system. This mouse is of enormous utility in testing any therapy for any disease that involves the human immune system. Instead of doing preclinical

studies in primates, which is very expensive, subject to animal rights concerns, and often inconclusive because of differences between monkeys and humans, it is possible to test the human immune system directly in the form of a surgical transplant onto a mouse background or "platform" .

SCID (an acronym for Severe Combined Immune Deficiency) mice have no immune system of their own; they are highly susceptible to infectious agents and they cannot reject tissue transplants such as from humans. SyStemix co-founders Irving Weissman and Mike McCune discovered that transplantation of three fetal human tissues (thymus, liver and lymph node) could restore the immune system of SCID mice. Such surgically constructed chimeric mice were able to resist infectious challenges that killed the controls. The human fetal liver supplies immature immune cells while the thymus supplies T cells. Together they migrate to the lymph node where they interact with other immune cells and are "trained" to recognize foreign antigens. An example of the power of SCID-hu mice in testing therapies is given by the fact that it took SyStemix only *four weeks* to successfully predict the most effective dosage of the AIDS drug AZT, compared with the *four years* that it took Wellcome to do the same with human clinical trials (S. G. Warburg Securities 1992).

SCID-hu provides SyStemix with a formidible experimental tool to develop a gene therapy for AIDS. The company is using HIV infected SCID-hu mice to model the following curative therapeutic strategy: 1) remove bone marrow from AIDS patients, 2) purify the subpopulation of HSCs which are not infected with HIV 3) insert a gene which prevents replication of HIV, 4) reintroduce the genetically engineered cells back into patients after their immune system has been eradicated by chemotherapy and 5) repopulate the immune system with the HIV-free and HIV-resistant cells.

The use of mice with human immune systems has generated some safety concern and some caution regarding possible experimental artefacts. It has been shown that HIV in mice with engrafted human cells is capable of genetically recombining with naturally occurring endogenous mouse retroviruses and creating strains with novel biological properties, including increased virulence (Marx 1990). When two viruses reproduce in the same cell, they can exchange genes coding for coat proteins, allowing them to infect new types of cells. Gallo

and others found that human immune cells could be infected with certain strains of mouse leukemia virus (Lusso and others 1990). When HIV was added to these cells that were pre-infected with the mouse retrovirus, the HIV acquired genes from the mouse virus that enabled it to replicate more rapidly. Two concerns were raised. First, from a biosafety perspective, strict quarantine of SCID-hu mice is necessary to prevent the release of new viral strains that could pose a health risk to either humans or mice or both. Second, mouse models of human AIDS may be subject to artifacts arising from the presence of naturally occurring mouse retroviruses. Both concerns are addressable, and presumably are being dealt with in the appropriate manner.

It is noteworthy that Sandoz has acquired 60% of SyStemix. In June 1992 the two companies also signed an agreement to jointly identify and isolate the gene that codes for the stem cell growth factor (SCGF) responsible for HSC self-renewal. Such a gene would have application in radiation and chemotherapy, bone marrow transplantation and immunosuppressive disorders. Recombinant SCGF would have utility in *ex vivo* cell culture expansion and the gene itself could be introduced into HSCs to regulate or maintain cell differentiation once they are reintroduced into the patient.

Sandoz also has significant holdings in another gene therapy company, Genetic Therapy. Sandoz, by virtue of its strategic alliances with US biotech companies and research institutions, is becoming a formidible player in gene and associated cell therapies. The Swiss-based multinational pharmaceutical firm also recently signed a $300MM agreement with the Scripps Research Insititute, which has raised some eybrows because the Institute's research is supported in part by federal funding, leading some critics to assert that the US government is indirectly subsidizing a foreign-based entity (Anderson 1993c). Commercial activity using federally funded university technology is undergoing scrutiny by legislators. Some are using public funding as a justification for price controls on new drugs and therapies.

One of the co-founders of SyStemix is Irving Weissman, a Howard Hughes Medical Institute Researcher on the staff of Stanford Medical School. As a result of Sandoz's large infusion of capital into SyStemix, Hughes officials determined last October 1992 that Weissman had a conflict-of-interest, which led to his

subsequent resignation as a Hughes investigator (Anderson 1993b). Stanford did not consider Weissman's holdings in SyStemix to be in conflict with University policy. This particular situation underscores a growing tension between academic science and industry. It is not surprising that the brightest and most successful academic researchers may also be entrepreneurially inclined. Managing conflicts in ways that preserve academic ideals, innovation and technology transfer is a critical component of maintaining global competitiveness.

• **TargeTech** is developing an *in vivo* gene delivery system that is specific for the liver. Not only is the liver an organ involved in many inherited metabolic disorders, but it also has a large mass making it suitable as a cellular "platform" for the production of gene therapy products not normally made in the liver. Liver cells (hepatocytes) possess unique receptors which bind and internalize a common serum protein known as asialoglycoprotein (ASGP). In 1987, the scientific co-founders of TargeTech, Drs. George and Catherine Wu of the University of Connecticut School of Medicine, found that plasmid DNA complexed to ASGP via a covalently linked cation such as polylysine could be efficiently (85%) targeted *in vivo* to hepatocytes (Wu and Wu 1987; Wu and Wu 1988). Moreover, experiments with reporter genes indicated that the plasmid DNA is capable of expression. The plasmid does not appear to integrate into the hepatocyte genome nor does it appear to replicate *in vivo*, as evidenced by the fact that it maintains the same methylation patterns and restriction enzyme sensitivity as it did when originally produced in *E. coli* bacterial fermentation. TargeTech has not performed experiments to prove conclusively that genomic integration does *not* occur at some low frequency (George Spitalny, personal communication). [12]

Plasmid gene expression is transient, lasting about a day (Wu and others 1991). If, however, hepatocyte replication is stimulated by two-thirds partial hepatectomy, then gene expression persists for at least two weeks. It is thought that replicating cells, which have morphologically different endosomal membranes, do not degrade the plasmids as rapidly as quiescent, non-replicating

[12] This is to be contrasted to work by Transkaryotic Therapies (discussed later) where, using a different method of plasmid DNA delivery, genomic integration is achieved regularly at a frequency of 10^{-6} and constitutes an essential element of the company's gene therapy approach.

cells. Surgical hepatectomy is traumatic and not a viable adjunct clinical procedure for use with gene therapy. It is possible, however, to stimulate hepatocyte replication by pharmacologic means using a hypolipidemic agent such as nafenopin, which has been shown to maintain plasmid expression in rats for as long as 9 weeks (Wu and others 1990) . Such a pharmacologic adjunct procedure may be required for the ASGP vector to work in humans as well.

TargeTech is developing the ASGP vector for the gene therapy of hyper-cholesterolemia. Using a strain of rabbit that is genetically hyperlipidemic, researchers have been able to reduce total serum cholesterol by 20-30% for at least 6 days following transfer of a gene coding for the low density lipoprotein (LDL) receptor (Wilson and others 1992). Expression was low, however, for the reason mentioned above, achieving a level that was only 2-3% of normal. If repeated injection of the ASGP/DNA complex is necessary to effect a treatment, there is also the potential unexplored problem of an adverse immune response.

TargeTech is currently focusing its effort on an ASGP-mediated delivery to the liver of antisense nucleotides for the treatment of hepatitus B. The company was recently acquired by The Immune Response Corporation. Working together with the clinical expertise in its parent firm, TargeTech hopes to enter clinical trials with its Hepatitus B product by early 1994.

• **Targeted Genetics** is developing an *ex vivo* procedure known as adoptive immunotherapy. Immunosuppressed individuals, such as those with leukemia who have undergone radiation treatment to kill cancerous bone marrow cells, are particularly susceptible to opportunistic infections such as cytomegalovirus (CMV), which can cause a fatal pneumonia. Targeted Genetics is working on a technique to bolster the treated patient's killer T cell population with cells that are "trained" *in vitro* to attack CMV in the body. Prior to radiation treatment, bone marrow cells are taken from the patient. From this population of cells are isolated just the T cells which recognize the CMV antigen. When they are cultured in the presence of the antigen and a growth factor known as interleukin-2 (IL-2), these T cells are "trained" to identify the surface antigens of any CMV-infected cell in the body and kill it (Hoffman 1992; Riddell and others 1992). It currently takes 6-12 weeks of cell culturing to produce a sufficient number of CMV specific T cells to restore immunity in a patient. One use of gene therapy

that Targeted Genetics is pursuing is to insert the gene for IL-2 into the T cells so that they can stimulate their own growth after being released in the patient's body, thus dramatically shortening the lengthy culture expansion period and reducing the number of times the cells must be administered.

The company has also developed a fusion gene, termed HyTK, which has two functions: 1) resistance to the antibiotic hygromycin, which is useful as a positive selection marker during cell culture and 2) sensitivity to ganciclovir, a drug which is useful as a safety measure, allowing a genetically modified cell released in the body to be specifically killed if an unforeseen problem should arise (Lupton and others 1991). This fusion gene, while having no therapeutic value *per se*, is a powerful adjunct when attached to the therapeutic gene. For instance, should the IL-2 gene in the T cells mentioned above over-produce and begin to expand the T cells circulating in the patient beyond the desired number, an injection of ganciclovir would trigger the "suicide" function of the HyTK fusion gene and specifically kill the genetically engineered cells.

Targeted Genetics' parent company, Immunex (Seattle), has decided to spin off Targeted Genetics while retaining a 40% equity position after completing a private institutional placement. Immunex is currently seeking a partner.

• **Theragen** is developing gene therapies for three target markets. 1) The immediate focus is Gaucher's disease, a lysosomal storage disorder caused by a deficiency in the enzyme, glucocerebrosidase. 2) In two to four years the company will develop vector mediated therapies for arthritis by inserting the gene for IL-2 in cells from joint or synovial tissues. 3) In four to eight years the company anticipates vector mediated treatments for nervous system disorders such as Alzheimer's, Parkinson's and brain cancer.

Theragen is not only using retroviruses but adeno-associated viruses, herpes simplex viruses and liposomal vectors as well. Theragen has licensed from the University of Florida and DNX Corporation (Princeton, NJ) the use of the AAV vector system for the treatment of arthritis and other disorders of joint and connective tissues. The company has developed its own proprietary herpes simplex virus (HSV) vector for the treatment of neurodegenerative disorders. HSV is capable of infecting mature neural cells and residing in a latent state

where foreign gene expression can occur (Holloway 1991). Insertion of the enzyme, tyrosine hydroxylase, into neural cells of Parkinson's disease patients may be an effective way to restore the brain's ability to make dopamine, an essential neurotransmitter.

Theragen scientists, John Barranger and Paul Robbins, have succeded in transfering the glucocerebrosidase gene to correct Gaucher's disease into the bone marrow of mice using a retroviral vector (Ohashi and others 1992; Stipp 1992). They achieved near 100% insertion of 1-2 copies/diploid genome and expression that persisted 4-7 months after transplantation. Questions remain about whether the technique would require destroying a human patient's existing bone marrow cells before introducing the new modified ones. If that were so, the procedure would probably be too risky.

Genzyme, which sells annually over $100MM of glucocerebrosidase as a protein replacement therapy, has come under criticism by policymakers and healthcare reformers who charge that patients must spend as high as $500,000 annually for the enzyme. Genzyme, however, maintains that the cost falls to under $60,000/year as doses are gradually lowered during the first two years of treatment. Theragen projects that it could earn revenues of $25 to 100MM annually from a gene therapy for Gaucher's disease.

• **Transkaryotic Therapies (TKT)** uses nonviral *ex vivo* methods to transfect cells with therapeutic genes. Doug Treco, Director of Research at TKT, cites the following drawbacks of retroviral vectors: 1) possible restoration of virulence by recombination of non-replicative vectors with endogenous viruses 2) possible activation of cancer oncogenes or inactivation of cancer suppressor genes by random insertion of vector 3) low titer or difficulty in getting high yields of vector 4) relatively small size of gene insert (7000 base pairs maximum) precludes therapies with larger genes 5) low levels of protein expression, cells are able to shut down or reduce expression of virally inserted genes and 6) only dividing cells are infected, thus precluding cells such as neurons and many other cells of adult tissues.

Richard Selden, TKT's founder, showed in 1987 that naked double stranded DNA (linear or circular) could be introduced into L cells, a mouse fibroblast tumor cell

line, by administration of DNA co-precipitated with calcium phosphate (Selden and others 1987a). Approximately one in a million cells stably inserted the DNA into its genome. These cells could be isolated by culture in a medium with a drug that killed any cells not transfected with the DNA, which contained not only the therapeutic gene but also a gene conferring resistence to the drug. Selden found that these cells could be expanded in culture and implanted in mice where the therapeutic gene product (in this case, human growth hormone) was successfully expressed and secreted.

This experiment set the following therapeutic paradigm for TKT:
1) remove a tissue biopsy from the patient 2) dissociate the cells in culture medium 3) transfect (using no virus) with therapeutic gene linked to a selectable drug marker 4) select individual transfected cells using the drug marker 5) clone the individual cells 6) characterize the clones for expression of therapeutic gene 7) characterize the clones for "normality" such as normal growth behavior in cell culture, no tumor formation when injected into animals, normal chromosomal karyotype, no contamination with infectious agents, etc 8) *ex vivo* expand the clone showing the best characteristics and 9) reimplant the transfected cells back into the patient.

The first test of TKT's approach in human clinical trials is expected to begin late 1993 for the treatment of hemophilias A and B. A "skin punch" consisting of a patch of skin about 1/4 inch in diameter will be removed and genes for Factor VIII or Factor IX or gamma carboxylase, an enzyme activator of Factor IX, will be introduced as described above. TKT is working out the details of the trials with the FDA. Because the company has deliberately stayed clear of using any NIH money, it is not necessary to come before the NIH-RAC, which TKT management views as politically "pro-viral" in make-up.

TKT admits that their method of DNA delivery shares with retroviral vectors the problem of random insertion, but that their methods of cell characterization prior to reimplantation reduce the risks a "billion-fold" over that for retroviruses. The company hopes to reduce the risks even more with gene targeting by homologous recombination, which is being developed, not as a way to repair a damaged micro-lesion in an existing gene, but instead as a way to insert a new gene in a region of a chromosome where 1) no cancer genes are currently known

to occur and 2) expression of the gene is high. As more and more cancer oncogenes and suppressor genes are mapped, TKT can continuously improve its targeting method. Using optimized versions of standard gene transfer methods like calcium phosphate co-precipitation, Polybreane cationic complexes, microinjection and electroporation, TKT has been able to improve the frequency of homologous recombination by a factor of 100. While early researchers such as Capecchi (Thomas and others 1986) achieved efficiencies of one in a thousand cells, TKT can get one in ten. TKT is investing heavily in homologous recombination research because the company views it as an essential enabler of more sophisticated gene therapies for diseases where careful gene regulation is required.

TKT's *Stage I* program is to focus on diseases with significant markets where protein replacement, using naturally occurring or recombinant sources, has a proven track record. The already mentioned protein factors for hemophilia have extensive therapeutic histories. Fine tuning the dosage is not necessary. By adjusting the number of engineered fibroblast cells reimplanted back into a patient, TKT expects to achieve adequate dosage control. Treatment is effective as long as expression in the serum is equal to at least 10% of normal; over-expression is not toxic. Other first-stage gene therapies for diseases with extensive protein treatment history include short stature due to human growth hormone deficiency and anemia due to erythropoietin deficiency. These diseases have windows of acceptable therapeutic protein concentrations that are relatively *simple, wide and static.*

The company's *Stage II* program is gene therapy for diseases where the windows of acceptable therapeutic protein concentration are in contrast relatively *complex, narrow and dynamic* . These diseases require therapies that necessitate detailed knowledge of intracellular signal transduction and the physiological regulation of gene expression (Selden and others 1987b). Such a disease is insulin-dependent diabetes where the blood levels of insulin vary dynamically in response to the blood levels of glucose. Insulin will come off patent protection in the mid-90s, making gene therapy for diabetes mellitus commercially attractive, provided satisfactory mechanisms for physiological gene regulation *in vivo* can be developed.

Also included in the company's *Stage I I* program are gene therapies for diseases where a protein has not yet been proven to be an effective therapy. Hypercholesterolemia is such a disease. TKT is interested in not only the rare familially inherited cases of this disease, but also in the more common form which afflicts a much larger patient population. In addition to the low-density-lipoprotein (LDL) receptor, TKT is investigating two other candidate proteins and their genes which are involved in hypercholesterolemia.

One way to exogenously (meaning outside the patient) regulate a transgene is with a drug inducible promoter; administration of a drug with no other function but to activate a promoter controlling a transgene could be used to regulate expression of the LDL receptor, for example. TKT is researching this technique as well as mechanical cell encapsulation as ways to regulate gene product dosage.

Lastly, TKT's *Stage III* program is the discovery of new disease genes and the development of novel proteins for the treatment of disease. As mentioned previously, most gene therapy companies can be considered as gene transfer contractors, achieving commercial equity by developing proprietary gene transfer or chromosomal construction technologies. The other major way to develop equity in the human gene estate industry is to *own* the gene by being the first to discover it, file and obtain the senior patent position. TKT ultimately wants to generate its own flow of proprietary disease genes. The company plans to develop gene estate for its own account, not that of another entity to which it must pay royalties or act as a fee-for-services contractor. To accomplish this long term goal, TKT is developing its own gene isolation technology using yeast artificial chromosomes (YACs), which are capable of cloning large human DNA fragments the size of millions of base pairs. YACs are a major research subject in the federally funded Human Genome Project. TKT's efforts are part of a growing trend to voluntarily "privatize" this government project. In 1993, private investment in genome related science and technology is expected to be as much as half of the $170MM being spent by the federal government (Anderson 1993a).

TKT is looking for strategic partners. The company does not need an alliance to enter clinical trials and is prepared to begin testing the end of 1993 without a partner. Funding is provided by an initial seed investment of $6MM by Warburg

Pincus Capital Company (New York) followed three years later by another round of $15.5MM by a group led by Warburg Pincus.

• **Viagene** is developing gene therapies for the treatment of severe viral infections such as AIDS, Hepatitus B and herpes simplex, cancers, and hemophilia A. With the exception the Factor VIII gene therapy for hemophilia, all of the company's research is focused on the development of gene-based immunotherapeutics, which stimulate the cell-mediated immune system to produce antigen specific cytotoxic T cells.

The AIDS program is representative of their strategy, which is to start with *ex vivo* tests to prove the principle, then switch to a direct *in vivo* product for clinical and commercial development. In the *ex vivo* AIDS program, which is just starting Phase 1 clinical trials, skin cells are removed from patients and transfected with retroviral vectors containing coding sequences for HIV envelope proteins that function as the antigens. After verification that the viral antigens are being produced by the skin cells, the cells are administered intramuscularly back into the patients, where it is anticipated that specific cytotoxic T cells will recognize the HIV antigen as a MHC-I complex on the cell surface and mount an immune response against the engineered cells as well as any other infected body cells. Unlike most gene therapies which require a substantial amount of transfected tissue to adequately replace a missing or defective protein, the cell-mediated immune response needs only a limited number of cells to be effective.

The direct vector HIV immunotherapeutic, which is in preclinical testing with mice and primates, is not patient specific and does not require the culturing of a patient's cells; quality control testing is restricted to the vector itself. If the direct *in vivo* vector research proceeds rapidly, the company expects it will replace the *ex vivo* product.

Both direct and *ex vivo* HIV immunotherapeutic work are being done in collaboration with the Green Cross Corporation of Osaka, Japan, which has agreed to fund upto $40MM over a four year period in exchange for equity and exclusive, worldwide rights to use and sell the HIV immunotherapeutic products.

The cancer program involves the *ex vivo* transfer of genes coding for lymphokines into a patient's tumor cells. Lymphokines enhance the expression of MHC proteins, the substances which must complex with intracellularly produced antigens before they can be presented on the cell surface. Cancers often evade the host's immune system by suppressing the expression of the MHC proteins. The use of gene transfer drugs in a small population of the tumor cells may reverse this condition sufficiently to evoke the body's immune system to attack all cancer cells in the body. This systemic approach is of particular importance in cancers which have spread throughout the body or are not amenable to surgical removal. Candidate cancers include cervical, breast, pancreatic and melanoma.

Viagene is also collaborating with Bayer on a retroviral vector for hemophilia A. Bayer is supplying the Factor VIII gene, which it has licensed fron Genentech and upto $9MM in support over a three year period in exchange for an exclusive worldwide license to market any hemophilia A product resulting from the development program.

• **Vical** is developing the direct transfer of therapeutic genes using plasmid DNA, which is grown by bacterial fermentation, purified and dispensed in a vial, just like conventional drugs. The key discovery, made by Vical scientists Philip Felgner and Robert Malone in collaboration with Jon Wolff of the University of Wisconsin, is that, under certain conditions, some muscle tissues are able to take up DNA and express the coded sequences as proteins for periods of weeks to several months (Wolff and others 1990). The finding, which actually was a control for a more elaborate experiment to engineer the muscles of mice to express foreign proteins, was a total surprise (Cohen 1993).

The naked DNA method has application in many diseases. Not only is it potentially useful for muscle diseases such as Duchenne's muscular dystrophy but also for more body-wide diseases where the muscle tissue can serve as a "protein factory platform". A major limitation is that one injection can only reach a limited number of muscle cells, so diseases such as Duchenne's, which require most if not all cells to be transfected, must await better targeting techniques, perhaps liposomes.

For immunotherapeutic applications or "gene vaccines", only a few cells need be transformed. Several techniques are possible in addition to intramuscular injection. One is DNA nose drops, which may provide mucosal immunity against respiratory viruses. Another being developed by Agracetus, Inc. (Middleton, WI) involves the coating of DNA on tiny gold beads which can be shot into the skin with a "gene gun". Such a device may be used in the future for population-wide single dose innoculations against a wide array of pathogens and cancers using a cocktail of genes, an "omnivax".

Vical has partnered with Merck to develop DNA-based vaccines for human and veterinary application. The success of their joint effort in eliciting a cell-mediated response against the human influenza A virus in mice was recently reported (Rhodes and others 1993; Ulmer and others 1993). Mice were injected in the quadriceps three times at 3-week intervals with plasmids coding for influenza A viral antigens. Controls received either no injection or injection of a blank vector (no viral antigen sequences). Thirty days after being challenged with a lethal dose of the virus, 90% of the mice receiving the experimental immunization were living, compared to only 20% of the injected controls and 0% of the blank vector controls. In addition to such *preventive* vaccine applications, the Vical/Merck team is also developing *therapeutic* vaccines for patients already infected with diseases such as AIDS, herpes and hepatitus B.

Traditional chemotherapy for cancer seeks to kill rapidly dividing tumor cells. The problem is that normal cells which are also rapidly dividing, such as cells of the intestinal epithelium and of the bone marrow, are also killed as a side-effect. The therapy is often more life-threatening than the original disease. Researchers believe that the body's immune system is continuously monitoring all cell surfaces to detect spontaneously occurring cancer cells, which are specifically killed. Some escape detection however. A possible therapeutic strategy would be to inject directly into the tumor some DNA sequences coding for an antigen that the body would recognize as foreign. Vical, in collaboration with Gary Nabel of the University of Michigan, is developing such a "cancer vaccine" for malignant melanoma. The rationale is to directly deliver DNA sequences coding for a human histocompatibility antigen (HLA) that is different than the patient's. Differing human HLA antigens are responsible for organ transplant rejection, which inevitably occurs unless immunosuppressive drugs are administered.

Instead of *avoiding* the rejection, doctors want to *evoke* it, as if the patient's own tumor were an organ transplanted from an unacceptable donor. As of the end of December 1992, six patients with malignant melanoma have been treated with this method (see RAC approved gene therapy protocol #13 in **Appendix B**). Blood and tissue samples indicate the expression of the administered "foreign" HLA DNA and the presence of specific cytotoxic T cells in the blood. No signs of toxicity have been observed so far (Vical IPO Prospectus 1993). Depending on the outcome of this initial trial, Vical and Nabel may ask the FDA for permission to conduct further trials with other melanoma patients.

Vical is also developing intravascular catheters to deliver genes to heart tissue as a way of treating atherosclerosis, the buildup of plaque on the inner surfaces of blood vessel walls. Fibroblast Growth Factor Type 5 genes, when injected directly into the heart muscle of the rat, have been shown to release the expressed growth factor protein into the extracellular spaces of heart muscle cells and stimulate the formation of new blood capillaries in the local area. By improving blood flow to the heart muscles, Vical scientists hope to counteract the atherosclerosis.

In addition to the therapies mentioned above, Vical is also developing other applications for their naked DNA plasmids where only small amounts of expressed proteins are required to produce the desired therapeutic results. They include immunomodulatory proteins such as cytokines, other growth factors and the clotting proteins Factors VIII and IX for the treatment of hemophilias A and B respectively.

As an adjunct to Vical's *in vivo* plasmid DNA technology, the company is also developing the use of lipid materials known as cytofectins to facilitate gene transfer into certain tissues. Cytofectins, which are cationic or positively charged, form complexes with DNA molecules, which are anionic or negatively charged. These cytofectin/DNA complexes are efficiently taken up by cells in a process known as endocytosis. Degradation of the DNA by intracellular nucleases is reduced, and expression into protein is increased.

Vical believes their technology to be superior to others on the basis of 1) convenience 2) universality 3) safety 4) flexibility of therapy 5) ease of

commercialization and 6) cost-effectiveness.

5.3 Company Differentiation by Technology and Mode of Delivery

A summary of the vector technologies and modes of delivery used by the surveyed gene therapy companies is given in **Table 5.6.** By far the strongest commercial issue emerging from the interviews and company literature is the mode of delivery. *In vivo* gene delivery is "product intensive" while *ex vivo* gene delivery is "service intensive".

All firms agreed that *in vivo* delivery is the ideal method from a production and distribution perspective. A "gene in a bottle" fits most closely with established pharmaceutical manufacturing, marketing and distribution procedures. General practice physicians could dispense the genes just like any routine vaccine. More complex procedures, such as inhalation of adenovirus where specialized equipment or facilities might be required, could be done in clinics on an outpatient basis. Avigen, TargeTech, Viagene and Vical are clear proponents of *in vivo* gene therapy, choosing vectors and diseases amenable to this mode of delivery.

In vivo gene therapy, however, does not have the built-in patient response controls that *ex vivo* gene therapy has. *Ex vivo* methods allow the cells to be characterized for their physiologic properties prior to re-implantation. All gene therapy protocols must go through a "proof of principal" phase with the Food and Drug Administration that involves exhaustive *ex vivo* testing, even if an *in vivo* product is the ultimate objective. Companies such as SyStemix, Targeted Genetics and Transkaryotic Therapies, who see themselves exclusively as *ex vivo* cell service firms, must make a basic decision either to centralize their operation, by bringing patients or patients' cells to their facility, or to decentralize, by licensing their technology to clinics and perhaps assigning some of their staff to remote locations. *Ex vivo* treatment is labor and facility intensive, which implies not only increased cost but also increased risk of a possible error in handling. While the liabilities associated with *in vivo* delivery pertain mostly to the product itself, *ex vivo* delivery has the additional liabilities associated with a service business as well.

	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
Naked Plasmid DNA													I	I
Chemical/Physical & MicroInjection												E		
Liposome/Plasmid DNA Complex											?			I
ASGP/Plasmid DNA Complex									I					
Dead Adenovirus/Plasmid DNA Complex		E/I												
Adenovirus				I	I	I	I				?			
Adeno-Associated Virus	I			I							?			
Retrovirus				E			E			E			E	
Herpes Simplex Virus											?			
Universal Donor Cells (made ex vivo with non-viral DNA & techniques that select for homologous recombination)			I											

(Key: "E" = Ex vivo, "I" = In vivo, "?" = mode not specified)

Table 5.6 Vector technologies and modes of delivery used by gene therapy companies as of Spring 1993 (source: company literature).

Regarding company differentiation by vector technology, the older firms such as Genetic Therapy and Somatix are the ones most committed to the retrovirus, the original gene therapy vector. Questions of safety and technical problems pertaining to titer yields and acceptable cell targets have caused some of the newer companies to develop methods that are free altogether of viruses or use viruses which are regarded as safer and more versatile on a number of technical grounds. One such virus is adeno-associated virus, which Avigen, Genetic Therapy and Theragen are developing. At least six of the thirteen companies surveyed, Cell Genesys, TargeTech, Theragen, Transkaryotic Therapies, Viagene and Vical, are developing gene therapies without use of any viruses.

5.4 "Outside Institutions" forming Strategic Alliances with Gene Therapy Companies

A summary of companies forming strategic alliances with gene therapy companies is given in **Table 5.7**. These companies, many of whom are large, established, muti-national pharmaceutical firms, possess complementary assets which the gene therapy companies need, namely capital, regulatory, marketing, manufacturing and distribution expertise. The gene therapy companies, on the other hand, possess complementary assets which the large pharmaceutical companies need, namely innovative therapies to feed their commercial pipelines.

Marketing and distribution rights are most commonly transferred to the pharmaceutical partner. Frequently there is R&D collaboration. Bayer, for example, is supplying Viagene with the Factor VIII gene, which it licensed from Genentech, and is responsible for clinical studies and regulatory approval for the hemophilia A gene therapy product. Bayer has the worldwide license to market the product while Viagene retains the worldwide right to manufacture, although Bayer has the option to purchase Viagene's manufacturing right in exchange for a manufacturing royalty based upon a previously agreed upon formula.

	Avigen	Boehinger Ingelheim	Cell Genesys	GTI	GenVec	Genzyme	Somatix	SyStemix	TargeTech	Targeted Genetics	Theragen	TKT	Viagene	Vical
Boehinger Ingelheim (deal in progress)		?												
DuPont Merck											✓			
Genentech					✓									
Green Cross Corp (Osaka, Japan)													✓	
JT Immunotech (Japan Tobacco)			✓											
Merck														✓
Miles (Bayer AG)													✓	
Parke-Davis											✓			
Sandoz				✓				✓						

Table 5.7 "Outside institutions" forming strategic alliances with gene therapy companies as of Spring 1993 (source: company literature).

5.5 Technology Transfer from Universities and Research Institutions to Gene Therapy Companies

Biotechnology was born in the 1970s in the academic laboratory. Since that time the university has continued to be a major source of basic knowledge and enabling technology for the industry.

In 1980, passage of Public Law 96-517 (the Bayh-Dole Act) by Congress gave universities title to their inventions arising from US government sponsorship. Subsequent guidelines and executive orders have given academic institutions the freedom to exploit their inventions as they wish, consistent with the public interest in the transfer of technology to the commercial sector (Nelsen 1989; Nelsen 1991).

Effective university interaction with industry requires management of the relation so that the commitment of the university to basic science and research is not unduly skewed by the lure of for-profit activity. John Preston, Director of the MIT Technology Licensing Office, points out that conflict-of-interest is an inherent component of the industry-university relation and that the only way to totally eliminate it would be to stop the relation altogether and stop the entrepreneurial activity of university researchers. This would be devastating to the U.S. economy, which benefits enormously from the commercialization of university generated invention. Instead, what MIT and other universities have done is to establish policies which guide university faculty and inventors in their relations with industry. The conflict-of-interest policies for MIT are given in **Table 5.8.**

Of particular note is that, while MIT will not invest any cash in a start-up, it will accept equity in lieu of royalty payments. This is a non-cash form of investment which is consistent with the proactive goals of the Institute to support entrepreneurial effort while, at the same time, not interfering unduly with the mechanisms of the open market to raise capital.

Table 5.9 provides a mapping of interactions between gene therapy companies and universities/research institutes. Every company has at least one relationship which they list in their literature. (In-licensing of pre-existing technology could

University Employee	• May not (in general) take a line position with any company • Must file annual statement of outside professional activities
University Faculty	• May found, sit on board and consult with any company • May not (in general) engage in outside activities more than one day per week (may take an unpaid leave of absence from University if necessary) • May use University equipment for outside activities subject to University's reimbursement policies
University Inventor (applies to employees, who may be students, staff, or faculty)	• Must disclose all outside company relationships annually with department head • If Inventor owns stock in Company Licensee, s/he must sign "Conflict Avoidance Statement" and agree: 1) not to use students at University for Company sponsored projects 2) not to restrict or delay public release of university research 3) not to take direct or indirect research support from the Company • Department head may elect to create oversight committee to review relationships with Company Licensee
University Technology Licensing Office (TLO)	• May not invest cash in Company Licensee in any financing round • May take equity position in Company in lieu of royalty payments, provided it is <20% (typically <10%) • May not sit on Company Licensee board at any time
University Corporation	• May not invest cash in any Company Licensee in the first round • May elect (but seldom does) to invest cash (University Endowment) in subsequent rounds as in any unaffiliated company
Company Licensee	• May not locate on campus • Is encouraged to locate "on the edge" of campus in buildings owned and rented by University as "incubator space"

Table 5.8 Current Conflict-of-Interest Policies of MIT (from MIT literature and interviews with technology transfer officers).

Gene Therapy Company • Collaborating University or Research Institute	Principal Investigator(s) at University or Research Institute	Subject
Avigen		
• Indiana University School of Medicine	A. Srivastava	AAV Vector
• The Johns Hopkins University School of Medicine	J. Boeke	anti-HIV fusion capsid protein/nuclease
Boehinger Ingelheim		
• University of North Carolina at Chapel Hill	D. Curiel	Dead AV-Polylysine-Transferrin Vector
• Research Institute of Molecular Pathology, Vienna, Austria	M. Cotten	Dead AV-Polylysine-Transferrin Vector
Cell Genesys		
• New England Deaconess Hospital (Harvard Medical School)	? (? = not specified)	anti-HIV T cell transplant products
• Scripps Research Institute	?	anti-HIV T cell transplant products
• National Institute of Allergy & Infectious Diseases (NIH)	?	anti-HIV T cell transplant products
• National Eye Institute of the NIH	?	Retinal cell surgical implantation
• Dana Farber Cancer Institute (Harvard Medical School)	?	anti-inflammatory monoclonal antibodies
GTI		
• University of Southern California	F. Anderson	gene therapy of blood disorders
• National Institute of Neurological Disorders & Stroke (NIH)	E. Oldfield & Z. Ram	brain cancer
• Harvard University	?	HIV therapy using gene "vpx"
• University of Cincinnati Children's Hospital Medical Center	J. Whitsett	cystic fibrosis
• NIH	M. Blaese, F. Anderson, S. Rosenberg	ADA deficiency
• NIH	S. Rosenberg, F. Anderson, M. Blaese	tumor infiltrating lymphocyte marker
• University of Pittsburgh	M. Lotze	malignant melanoma marker & IL-4 tumor vaccine
• University of California at Los Angeles	J. Economou	tumor infiltrating lymphocyte marker
• NIH	S. Rosenberg, F. Anderson, M. Blaese	cancer therapy using Tumor Necrosis Factor gene
• NIH	S. Rosenberg, F. Anderson, M. Blaese	cancer therapy using Interleukin-2 gene
• St. Jude Children's Hospital (Memphis, TN)	M. Brenner	marker genes for acute myelogenous leukemia & neuroblastoma

Table 5.9 In-licensing of technology from (or collaboration with) universities and research institutes by gene therapy companies as of Spring 1993 (from company literature).

Gene Therapy Company • Collaborating University or Research Institute	Principal Investigator(s) at University or Research Institute	Subject
GTI (continued)		
• M. D. Anderson Cancer Center (Houston, TX)	A. Deisseroth	marker gene for chronic myelogenous leukemia
• Indiana University	K. Cornetta	marker gene for acute leukemia
• NIH	A. Nienhuis	cancer therapy using multiple drug resistance gene
• Baylor College of Medicine	F. Ledley	marker gene for liver failure
• Albert Einstein College of Medicine (Yeshiva University)	L. Reid	gene therapy for liver diseases using liver stem cells
• Fred Hutchinson Cancer Research Center (Seattle, WA)	D. Miller	Factor VIII & IX gene therapy for hemophilias A & B
• NIH	A. Nienhuis	gene therapy for sickle cell anemia and thalassemia
• NIH	R. Gallo, M. Blaese, F. Anderson	HIV therapy using soluble CD4 receptor gene
GenVec		
• National Heart, Lung & Blood Inst. (NIH)	Ronald Crystal	cystic fibrosis
Genzyme		
• University of Iowa College of Medicine	M. Welsh	cystic fibrosis gene therapy
• University of Michican	F. Collins	non-exclusive license for cystic fibrosis gene (in negotiation)
• Toronto Hospital for Sick Children	L. C. Tsui	non-exclusive license for cystic fibrosis gene (in negotiation)
Somatix		
• Whitehead Institute for Biomedical Research (MIT)	R. Mulligan & O. Danos	retroviral vectors and packaging cells & endothelial taget cells
• University of California at San Diego	F. Gage & T. Friedmann	gene therapy for Parkinson's disease
• Salk Institute	I. Verma	skin fibroblasts as targets for hemophilia gene therapy
• California Parkinson's Foundation	W. Langston	gene therapy for Parkinson's disease
• The Johns Hopkins University School of Medicine	D. Pardoll	tumor therapy using lymphokine genes
SyStemix		
• Stanford University School of Medicine	I. Weissman	stem cell isolation
• Stanford University School of Medicine	M. McCune	human tissue in immunocompromised mammalian hosts

Table 5.9 (continued) In-licensing of technology from (or collaboration with) universities and research institutes by gene therapy companies as of Spring 1993 (from company literature).

Gene Therapy Company • Collaborating University or Research Institute	Principal Investigator(s) at University or Research Institute	Subject
TargeTech		
• University of Connecticut School of Medicine	G. Wu, C. Wu	ASGP mediated liver targeting & gene therapy
Targeted Genetics		
• Fred Hutchinson Cancer Research Center (Seattle, WA)	P. Greenberg, S. Riddell	marker gene and lymphokine genes in HIV specific T cells
Theragen		
• University of Pittsburgh	J. Barranger, P. Robbins	gene transfer technology for Gaucher's disease
• University of Florida (with DNX Corporation)	?	adeno-associated virus (AAV) vector for gene therapy
• Princeton University	?	AAV helper virus
TKT		
• Massachusetts General Hospital (Harvard Medical School)	R. Selden	autologous cell gene therapy
• University of North Carolina (Chapell Hill, NC)	D. Stafford	exclusive license for gamma carboxylase gene
• University of Texas Southwestern Medical Center at Dallas	?	non-exclusive license for low-density-lipoprotein receptor gene
Viagene		
• Walter Reed Army Institute of Research	R. Ballou	anti-malarial vaccines
• Duke University Medical Center	?	lymphokine immunotherapeutics for malignant melanoma
• Wisconsin Alumni Research Foundation	?	packaging cell lines used in retroviral vector production
Vical		
• University of Michigan & Howard Hughes Medical Institute	G. Nabel	foreign HLA antigen gene therapy for malignant melanoma
• University of Chicago	J. Leiden	Growth Factor FGF-5 gene therapy for atherosclerotic blockage
• University of Michigan	E. Nabel	Growth Factor FGF-5 gene therapy for atherosclerotic blockage
• University of California at San Diego	D. Carson	immunomodulatory therapy using lymphokine genes
• Wisconsin Alumni Research Foundation	?	intramuscular injection techniques for gene therapy

Table 5.9 (continued) In-licensing of technology from (or collaboration with) universities and research institutes by gene therapy companies as of Spring 1993 (from company literature).

not always be clearly separated in the literature from ongoing collaboration or sponsored research.) What is clear, however, is that gene therapy companies depend on universities not only for trained research staff but also for technology. There is every indication that good university relations will continue to be a source of competitive advantage for gene therapy companies as they compete with themselves and with foreign companies at home and in the international market.

5.6 Human Gene Estate Development as a Newly Emerging Industry

• **Speculation on industry evolution.** How will this new industry that transfers human genes evolve? Will it assume the structure of the drug industry or will it be different? The gene transfer industry has two basic ownership positions:

- ownership of the gene
- ownership of the gene transfer technology

Ownership of genes typically resides with the universities and research institutions who discovered them, though there is no reason high net worth individuals, pension funds or investment trusts couldn't also own the rights to genes, perhaps as an asset within their diversified portfolio. Ownership of the gene transfer technology, including manufacturing and distribution, will most probably reside with for-profit companies, because they are the only entities incented to take on the challenge of continuously developing the technology.

The two ownership interests must merge in the marketplace to create a commercially viable product. In addition, there must be the usual cadre of development professionals, the lawyers, accountants, bankers, and insurers to do the general business work. And finally there must be the medical profession to perform patient diagnosis, gene delivery and followup.

This gene development industry bears a resemblance in its activity and in its professional structure to the land development industry. Land development requires a similar ownership position, either by conveyance of a deed (which is like a patent) or a lease (which is like a license). In land development, most of the technology is public domain, though there are proprietary building construction

systems in use today. Gene transfer companies are like construction contractors with their own proprietary technology.

Figure 5.2a shows the existing structure of the commercial Real Estate Industry and **Figure 5.2b** shows a possible analogous structure of what the commercial Gene Estate Industry might look like if it evolved similarly. (This is a highly speculative and hypothetical proposition for discussion purposes only.) Were the field to evolve in this direction, one would expect the emergence of "gene developers", who put together deals between "gene owners" and "gene transfer contractors", gain the necessary regulatory approvals, secure debt with bankers and place equity with "gene syndicators", who might issue "Gene Estate Investment Trusts" or "GEITs" as investment instruments.

• **Reverse architecting and reverse engineering of genes.** Another professional activity implied in this hypothetical scenario that has particular interest to the thesis author is that of the "gene architect". How would this role be distinguished from the "gene engineer"? Using the building construction industry analogy, the engineer is responsible for knowing *how to build*, while the architect is responsible for knowing *what to build*, based on the client's program requirements. It is difficult to conceive a role for an architect in the genetic repair of a simple monogenic recessive disorder since the issue of *what to repair* is already clear. There is a role, however, for an architect in designing therapies where the issue of *what to repair* is not self-evident, such as is the case in monogenic dominant disorders, polygenic disorders, cancer, AIDS (and, in the future, genetic enhancement). These situations may warrant a more ingenious or creative approach to gene therapy which is not simply an "overlay" of the underlying disorder. An unobvious, different and totally unrelated strategy may be necessary. For instance, gene therapy to kill a cancer cell may have nothing to do with the underlying cause of the cancer. In fact, a therapy which is capable of killing any cancer cell regardless of cause would have much greater utility than one directed at a particular oncogene or tumor suppressor gene, of which there are many. Designing a strategy for *what to build* in the way of a gene-based intervention or modification is clearly within an architect's professional capability and domain, given proper mastery of the specifics. Just as a bridge architect must be sufficiently familiar with the principles of engineering (e.g., statics, strength of materials, earthquake design) to engage in an effective

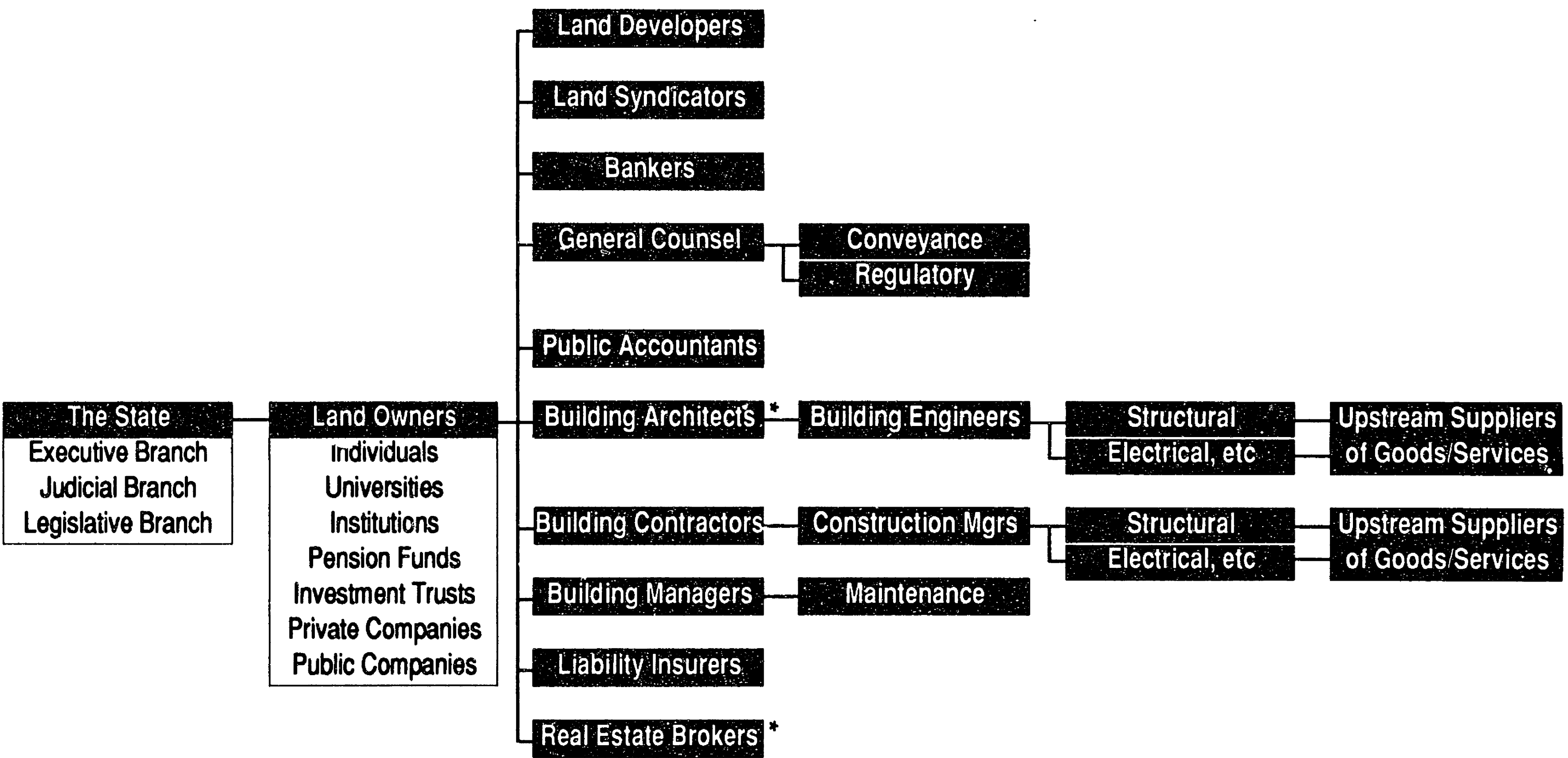


Figure 5.2a Existing structure of commercial Real Estate Industry.

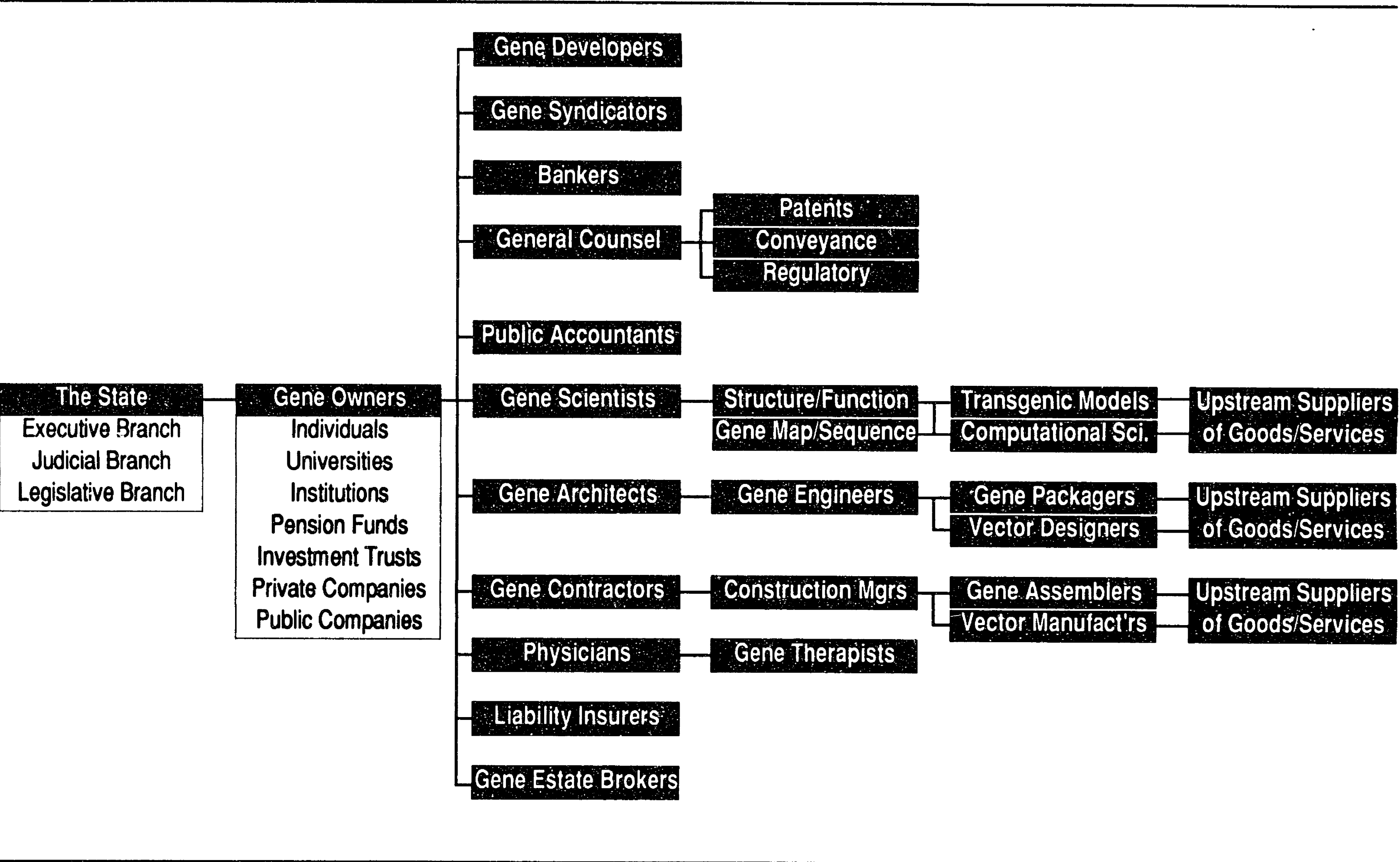


Figure 5.2b Possible analogous structure of commercial Gene Estate Industry in its mature stage.

collaboration with a structural engineer, so must a gene architect be sufficiently familiar with the principles of engineering and medicine (e.g., molecular genetics, methods of DNA fabrication, toxicology, pharmacology) to engage in an effective collaboration with a genetic engineer and a clinician.

The possibilities for the design of genes with novel function are extremely vast and virtually untapped. Using techniques such as structure-based rational design and directed molecular evolution, it is possible to design gene products (e.g., antisense RNA or proteins) with novel functional capabilities. An entire metabolic pathway consisting of a cascade of enzymes could be designed and constructed in this manner. The net effect would be to design novel function by "reversing" the flow of information that normallly occurs in Nature. Instead of *Gene → RNA → Protein → Resulting Function,* the design team, consisting of architect, clinician and engineer, would implement the following: *Desired Function → Protein → Gene.* [13]

The gene itself would be obtained by a process of "reverse architecting" and "reverse engineering", that is, working backward from the desired function of the gene using protein engineering and recombinant DNA technology.

The design of novel gene function represents an interesting departure from the real estate analogy. While land is a geographically fixed entity, the genetic diversity that can be expressed in DNA sequences is essentially unlimited. This can be demonstrated with the following ludicrous but nevertheless illustrative example:

> *Question:* If we could convert the entire mass of the earth into DNA nucleotides, how long a DNA molecule could we construct and still have one copy of every possible sequence combination present?
>
> *Approach:* Imagine a strand of DNA n nucleotides long. There are four different nucleotides that could occupy each of the n positions. A

[13] The RNA step is only needed in the forward direction; it could be bypassed when converting the protein sequence into the DNA sequence. Due to degeneracy in the Genetic Code, a given protein sequence could be converted into several different DNA sequences, all of which would translate into the same protein when transferred into a cell.

combinatorial library containing one strand for each possible sequence combination would consist of 4^n strands. The total number of nucleotides used to make the combinatorial library would be $n(4^n)$.[14]

Answer: As shown in **Figure 5.3**, the largest DNA molecule we could make using all the mass of the earth as nucleotides and still have one copy of every possible sequence combination is only 78 nucleotides long! This is a phenomenally small number given the fact that we have DNA that is six billion nucleotides long in each of our cells.

A typical gene is 1000 nucleotides long. Complete expression of the potential diversity present in less than one-tenth of a typical gene would exceed the mass of earth. This is an astounding result! For real estate to be even remotely analogous in scale to gene estate, we would probably have to include in our "land inventory" all the heavenly bodies in the universe.

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[14] Given constants: the average molecular weight of a nucleotide is 325 daltons, Avogadro's number is 6.023×10^{23} molecules/mole, and the mass of the earth is 6.0×10^{27} grams (Weast 1977).

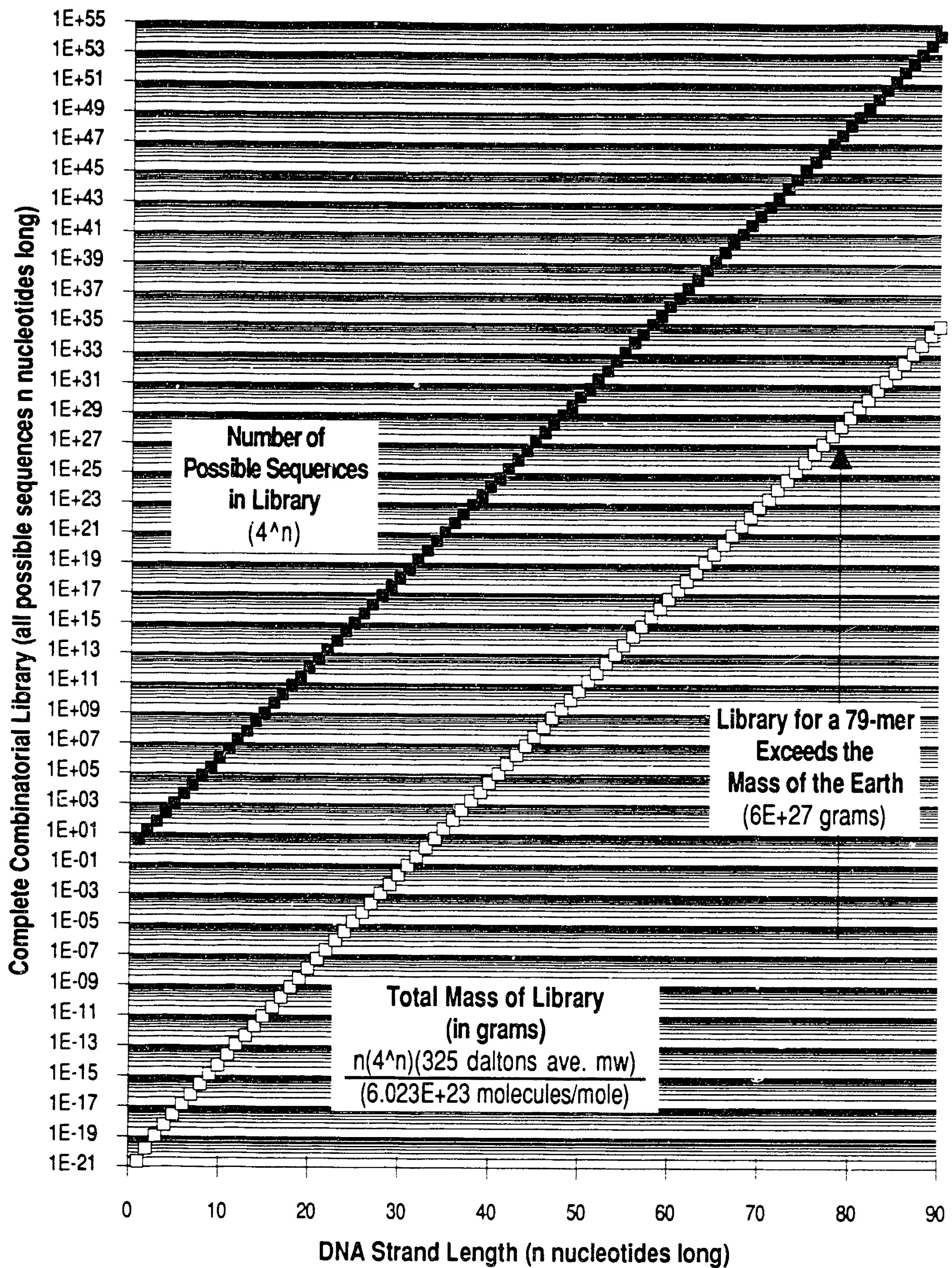

Figure 5.3 Relationship between DNA strand length, number of possible sequences and total mass of a Complete Combinatorial Library.

CHAPTER 6
REGULATION OF GENE THERAPY

6.1 Overview

Governmental regulation of the testing, manufacturing and marketing of pharmaceutical products has always represented a major barrier to commercialization. From the public point of view, regulation serves the function of protecting the public welfare by establishing and enforcing objective criteria for the safety and efficacy of products before and after introduction into the marketplace. The Food and Drug Administration (FDA), created by Congress in 1938 by the passage of the Federal Food, Drug, and Cosmetic Act, is responsible for regulating pharmaceuticals (as well as other products) intended for sale, barter, or exchange between US states and possessions as well as between the US as a nation and foreign countries.[15]

From the private point of view, regulation serves as an effective barrier to keep new entrants out of the marketplace. Only a well-capitalized firm with substantial research, development, clinical and manufacturing expertise can successfuly bring a drug to market. It is estimated that it takes on average 12 years and a capitalized cost of $231MM (in 1987 dollars) to bring a new chemical entity or drug to market (DiMasi and others 1991). Approximately two-thirds of the time and money are spent in laboratory tests and clinical trials designed to address the FDA's criteria. Small, under-capitalized companies, which include all but a few of the biotech companies, will not be able to finance this process, regardless of the ultimate value of their innovation. That is why many of the small gene therapy developers have struck strategic alliances with large pharmaceutical houses to gain access to capital as well as clinical and manufacturing expertise, all of which are essential to pass regulatory approval

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[15] The regulation of products made abroad by US or by non-US companies and the impact of US regulations on non-US companies operating in the US are important topics which have not been addressed in this thesis. They are key items for future investigation.

(please see Chapter 5 for further discussion of this issue).[16]

Regulation also is necessary to keep the industry moving forward. The establishment of regulatory guidelines and standards for products and manufacturing establishments assures the diffusion of good practices throughout the industry. Manufacturers who comply are allowed to operate and place product on the market while those that do not comply have their license revoked and are shut down. In both situations, the public welfare is protected.

6.2 Regulation by the Food and Drug Administration (FDA)

There are two departments within the FDA which are responsible for regulating chemical products intended for use in humans:

- Center for Biologics Evaluation and Research (CBER)
- Center for Drug Evaluation and Research (CDER).

The names would seem to indicate a rational division in jurisdiction based on living and non-living sources or methods of production. Inspection of the actual product classes regulated by each Center (**Table 6.1**) indicates, however, that historical and perhaps political factors are involved as well. All hormone products such as insulin and human growth hormone, for instance, are regulated as a "drug" by CDER. This is irrespective of the method of manufacturing, be it extraction from animal organs or production in cultured cells using recombinant DNA technology. In contrast, DNA is a "drug" regulated by CDER if it is synthesized by chemical means, but is a "biological product" regulated by CBER if made in living cells. Since the filing procedures are different, it is important to ascertain which department has jurisdiction for any given product.

CBER has been designated by the FDA to review gene therapy protocols (Epstein 1991). Therefore, while human growth hormone manufactured in recombinant cells is regulated as a "drug", a therapy involving introduction of the gene for human growth hormone into a patient's cells would be regulated as a "biological

[16] Access to channels of distribution, especially overseas, is also a major factor in the formation of strategic alliances.

A. Center for Drug Evaluation and Research (CDER) Product Regulation Responsibility

1. Naturally-occurring substances purified from mineral or plant source materials (excluding vaccines or allergenics)
2. Products produced from non-human animal or solid human tissue sources (excluding animal-derived procoagulant products or antisera, venoms, red cell replacement products, vaccines, allergenic products, products composed of living cells, & certain other products under CBER)
3. Antibiotics as defined by Section 507(a) of the FD&C Act, regardless of the method of manufacture.
4. Certain agreed-upon classes of substances constitutively produced by fungi or bacteria including disaccharidase inhibitors & HMG-CoA inhibitors.
5. Chemically-synthesized molecules (excluding vaccines and allergenics) including:
   a. Products produced by chemical synthesis that are intended to be analogues of cytokines, thrombolytics, or other biologics, or that function by binding to the receptors for biological products
   b. Chemically-synthesized mononucleotide or polynucleotide products, including products complementary to RNA or DNA sequences
6. Hormone products, regardless of method of manufacturing, e.g., insulin, human growth hormone, pituitary hormones.

B. Center for Biologics Evaluation and Research (CBER) Product Regulation Responsibility

1. Biological products subject to licensure:
   a. Vaccines, regardless of method of manufacture (for the purpose of this agreement, a vaccine is defined as an agent administered for the purpose of eliciting an antigen-specific cellular or humoral immune response)
   b. In vivo diagnostic allergenic products, in vivo diagnostic tests for DTH, & allergens regardless of the method of manufacture intended for therapeutic use as "hyposensitization" agents
   c. Human blood or human blood-derived products including placental blood-derived products, animal-derived procoagulant products & animal or cell culture-derived hemoglobin-based products intended to act as red blood cell substitutes
   d. Immunoglobulin products, whether monoclonal or polyclonal, produced in humans, animals bacteria, fungi, viruses or virus pseudotypes, or in cell culture
   e. Products composed of or intended to contain intact cells or intact microorganisms including bacteria, fungi, viruses or virus pseudotypes, or viral vectors
   f. Protein, peptide or carbohydrate products produced by cell culture, excepting antibiotics, hormones, products listed in A.3. above, & products previously derived from human or animal tissue & regulated as approved drugs
   g. Protein products produced in animal body fluids by genetic alteration of the animal, i.e., transgenic animals
   h. Animal venoms or constituents of venoms

Table 6.1 Product regulation responsibility assignments within the FDA (Kessler and others 1991).

B. Center for Biologics Evaluation and Research (CBER) Product Regulation Responsibility (continued)

2. Other product classes:
   a. Synthetically-produced allergenic products that are intended to specifically alter the immune response to a specific antigen or allergen
   b. Certain drugs used in conjunction with blood banking and/or transfusion

C. Exceptions

1. All products that are subject to approved or pending NDAs or PLAs as of the effective date of this agreement will be left under that regulatory mechanism & under the jurisdiction of the center that currently administers the NDA or PLA.
2. New products that use the same active ingredient(s) as the above approved products will be assigned to the same lead Center & regulated by the same mechanism (PLA or NDA) as the approved products. Questions about the similarity of active ingredients will settled by the CBER-CDER jurisdiction committee.

D. Combination Products

Products that are combinations of one or more drug and one or more biologic products will be assigned based on the product's primary mode of action. This mechanism assigns administration & product quality responsibility. The medical, pharmacological & other reviews will be [assigned by a different mechanism described in another section].

1. Assigned to CBER:
   a. Combination products that consist of a biological product from a product class subject to licensure (including biological products that have been chemically modified) combined with a radioactive component.
   b. Combination products that consist of a biological product component used as a mode of localization and a toxin component that is not itself a drug product (e.g., ricin A toxin) used as an effector
   c. Combination products that consist of a drug component and a biological component where the drug product enhances the efficacy or ameliorates the toxicity of the biological product
2. Assigned to CDER:
   a. Combination products that consist of a biological product component used as a mode of localization or used to affect the distribution of the product, combined with a nonradioactive drug component used as an effector
   b. Combination products that consist of a biological component and a drug component where the biological component enhances the efficacy or ameliorates the toxicity of the drug product.

Table 6.1 (continued) Product regulation responsibility assignments within the FDA (Kessler and others 1991).

product".

Since somatic cell gene therapy encompasses such a wide array of procedures, some *ex vivo*, some *in vivo*, some involving viruses, some not, and since the field is evolving so rapidly, it is not possible to adequately address regulatory issues in any manner except on a case-by-case basis. Any attempt to establish a set of standards would probably be out of date before it could be announced. What the CBER staff has done instead is to issue "Points to Consider" papers, which are not regulations or guidelines, but instead present the current thinking by the staff about important issues in product development and testing (Epstein 1991; Quinnan 1991). Gene therapy developers are encouraged to meet with CBER staff in the early stages before formal filing to present initial data and to establish a dialogue. CBER does not require any prior approval by any other regulatory body before consideration of a protocol. All correspondence with the FDA is kept confidential.

Figure 6.1 is a schematic flow chart outlining the CBER review and approval process for a traditional biological product. The first formal step is the filing of an Investigational New Drug (IND) Application, which presents preclinical animal testing and laboratory data and describes in detail the proposed human trial. CBER then reviews the Application, possibly requesting modifications. Assuming that all criteria are satisfactorily met, CBER grants approval for human clinical trials to begin. The sponsor must also gain all necessary approvals at the local level. This typically involves review and approval by the Local Institutional Review Board (IRB) of ethical issues and other matters pertaining to the protection of human subjects and by the Local Institutional Biosafety Committee (IBC) for conformance with recombinant DNA safety guidelines.

There are typically three phases to clinical trials. In Phase 1, safety issues of the proposed therapy are addressed. There is usually not a test of efficacy against disease of the proposed product in this phase. Depending on the specifics of the clinical trial, Phase 1 may be performed with normal human subjects or with disease patients.[17] In Phase 2, a pilot trial involving a small patient sample size is conducted to address efficacy. Phase 3 is a scaled-up version of Phase 2, typically

[17] All human subjects volunteer for participation in clinical trials. They must go through a lengthy informed consent process before acceptance in a program.

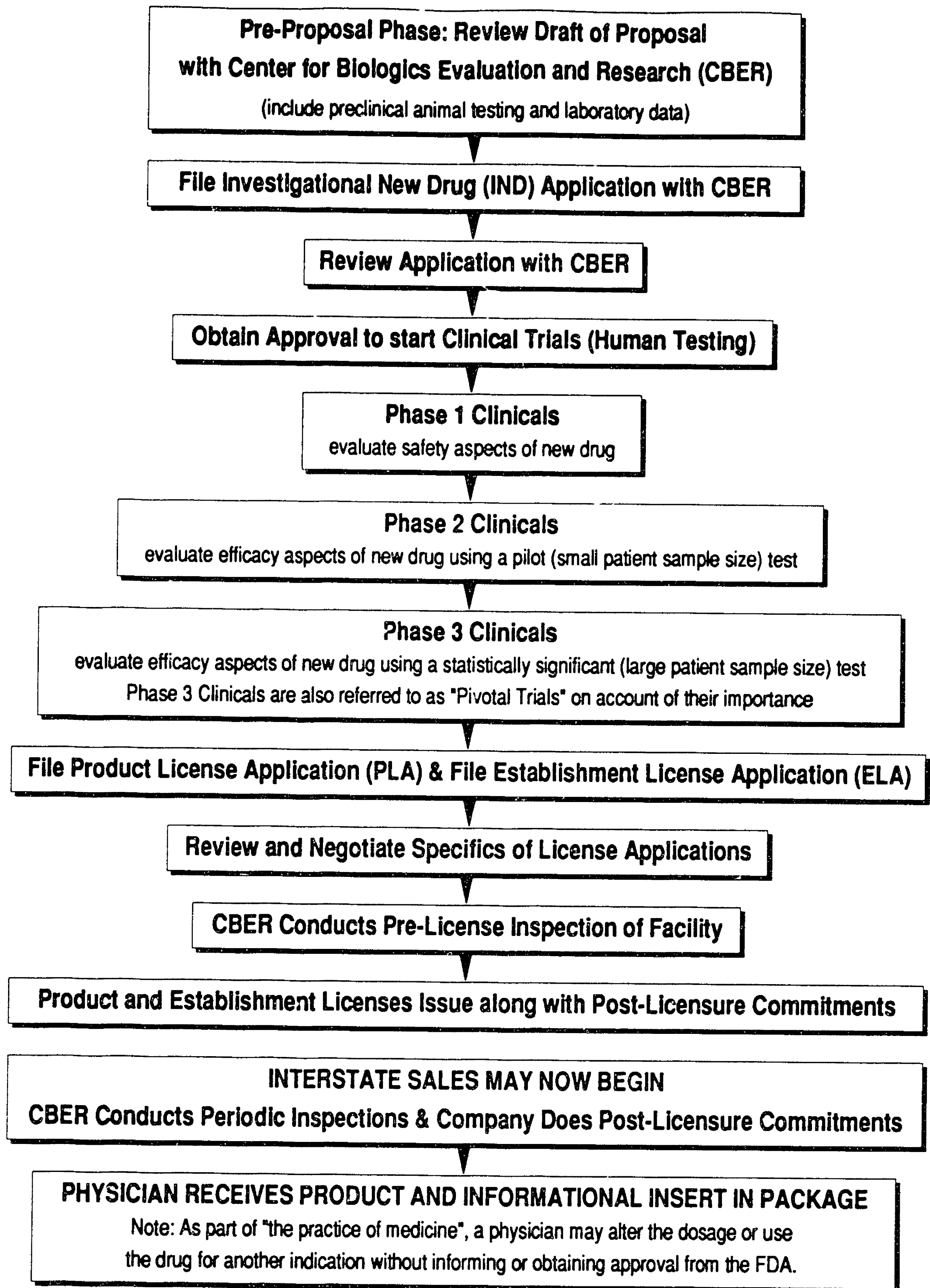

Figure 6.1 The review and approval process for a traditional biological product in the United States (data from FDA literature and interviews).

involving a much larger patient sample size, possibly tens of thousands of patients, and occurring over a period of years. Many gene therapies are targeting rare life-threatening diseases with very small patient sizes. Since no gene therapy protocols have advanced beyond Phase 1 as of the writing of this thesis, it is unclear how Phase 3 will be handled in these cases. It may, for instance, be modified in scope and combined with Phase 2 to accommodate the small patient population and urgency of therapy. Assuming the medical review of the clinical trials is positive, the sponsor may then file a Product License Application (PLA) and an Establishment License Application (ELA). Not only must the standards for the product be negotiated and approved, but the establishment itself, including the actual physical facility, the equipment, input materials, processing procedures and staff expertise must all be shown to comply with standards known as current Good Manufacturing Practices (cGMP).

After the successful negotiation of product standards and inspection of the facility, the Product and Establishment Licenses issue along with any Post-Licensure Commitments which the sponsor has agreed to perform. Interstate sales may now begin. It is important to note that physicians have considerable autonomy in the use of pharmaceutical products. Under a principle known as "the practice of medicine", a physician may alter the dosage or use the product for another indication not recommended by the sponsor or the FDA without informing or obtaining approval from the FDA. In such a situation, the local applicable tort law regarding malpractice would apply should a problem arise.

The right of physicians to use drugs as they see fit for "off-label" indications is often used by pharmaceutical companies as part of their strategy to get to market as quickly as possible. The objective is to gain approval for *an* indication, not necessarily the one(s) with the greatest therapeutic benefit or market size, but the one that is the easiest and fastest to achieve. By picking an indication with the clearest, cleanest and quickest clinical endpoint, drug companies can expedite their path to market. Meanwhile, they preserve their proprietary position with patents on the other indications and publish papers in the medical and scientific press. Experimental research on off-label use is picked up quickly by the leaders in the medical profession, and their resulting experiences diffuse quickly throughout practice. However, healthcare payers usually refuse to reimburse off-label uses. This puts the cost on the patient, who typically cannot afford the

drug treatment. In order for the drug companies to get paid for their products, they need FDA approval of the indications.

After the biological product is released in the market, CBER continues to monitor the safety, efficacy, and compliance with the terms of the Product and Establishment Licenses. Also monitored is the effectivity of the drug, that is how it is actually used in the field by practicing physicians; major variations from the approved use may necessitate modifications in product labeling. Any changes in manufacturing, formulation or labeling proposed by the sponsor must be pre-approved by CBER. Failure to do so may result in revocation of licenses, product recall, fines and imprisonment.

Another point to mention is that the FDA has no legal authority concerning the costs of an approved drug product or the reimbursement policies of healthcare payers. Cost-effectiveness, while not currently within the FDA's regulatory domain, may be included in the future. Indeed, two jurisdictions, Australia and Ontario (Canada), have recently drafted guidelines for cost-effectiveness studies to be submitted prior to securing government reimbursement status for certain products (Drummond 1993).

6.3 Regulation by the National Institutes of Health Recombinant DNA Advisory Committee (NIH-RAC)

The other federal body besides the FDA-CBER which regulates human gene therapy is the NIH-RAC. **Table 6.2** is a comparison between the two groups. Unlike the FDA-CBER, which is confidential and mandatory, the NIH-RAC is open to the public and only necessary if the sponsor receives funding from NIH.[18] Both CBER and RAC are concerned with basic sciences, clinical medicine and preclinical testing. CBER, however, focuses on manufacturing and product related issues while RAC provides a forum for social issues such as law, ethics and theology. RAC functions to prevent federal grant monies administered by

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[18] Some of the gene therapy companies interviewed for this thesis meticulously avoid any involvement of NIH monies, including in-licensing of technology funded by NIH, on account of the added regulation and the loss of confidentiality. Other companies, however, are of the opinion that RAC review, which includes public comment, serves to strengthen their position by public consensus that they are following state-of-the-art best practices.

National Institutes of Health Recombinant DNA Advisory Committee (NIH-RAC)	Food and Drug Administration Center for Biologics Evaluation and Research (FDA-CBER)
Jurisdiction	**Jurisdiction**
• Any entity receiving NIH funds for any project (whether for gene therapy or not) must submit proposed gene therapy protocols for review/approval or risk losing future funding	• Any entity intending to manufacture gene therapy products and market them across state borders must submit proposed gene therapy protocols for review/approval or face eventual legal action and possible fine and/or imprisonment
Accessibility	**Accessibility**
• Open to public (some meetings closed) • Six meetings per year	• Confidential (some meetings open) • Staff available for interaction and consultation on a day-to-day basis
Areas of Expertise, and of Information Scrutinized	**Areas of Expertise, and of Information Scrutinized**
• Basic sciences • Clinical medicine • Law • Ethics • Theology • Preclinical testing	• Basic sciences • Clinical medicine • Regulatory issues • Manufacturing processes • Quality Control • Preclinical testing

Table 6.2 The regulation of gene therapy in the United States (Jurisdiction data from interviews and Public Health Service Act (Section 351 of Part F of Title III); all else from Epstein 1991 reproduced with permission of publisher).

NIH from being used for unreviewed and unapproved experimentation in humans, as originally happened with Dr. Cline in 1980 (see Chapter 1).

The RAC review process is schematically diagrammed in **Figure 6.2**. In contrast to CBER, review of proposed protocols at the local level must be performed first before RAC will place them on the agenda. The Local Institutional Biosafety Committee (IBC) must sign-off on compliance with recombinant DNA safety guidelines and the Local Institiutional Review Board (IRB) must be satisfied that ethical issues and regulations regarding the protection of human subjects are properly addressed. RAC then announces the application in the Federal Register along with the date and place of the hearing, which is open to the public. Based on the presentation by the sponsor and subsequent discussions, the 23 members vote to make a recommendation to the NIH Director to accept or to reject the protocol. If the RAC approves the protocol and the NIH Director agrees, and if approval by CBER has been obtained, then clinical testing in humans may begin. Reporting procedures are summarized at the bottom of **Figure 6.2**. A list of protocols approved by RAC as of March 4, 1993 is given in **Appendix B** of this thesis.

In theory, approval of experimental gene therapy protocols are supposed to be based only on scientific and medical merits. As recent events have shown, political pressure can also be a major issue. On October 8, 1992, Senator Tom Harkin, who chairs the appropriations committee that oversees NIH's budget, wrote NIH Director Bernadine Healy requesting "compassionate use" of gene therapy for a politically well-connected patient dying of brain cancer (Thompson 1992). The requested therapy had previously been turned down by the RAC because there was too little preclinical data to warrant experimentation in humans. Healy initially wrote Harkin back saying that "there have not...been enough studies on this proposed treatment to even begin consideration on a compassionate plea basis." Since almost every patient treated with gene therapy to date is dying and would qualify on the same basis, she wrote, "it is not possible to make decisions as to which case is more worthy than others...Attempting such kinds of decisions would compromise the review process and not be in the best interests of such patients." (Thompson 1993b). On December 28, Healy did a surprising about-face. She over-ruled the RAC's prior decision, approved the therapy for the Senator's constituent, and called an

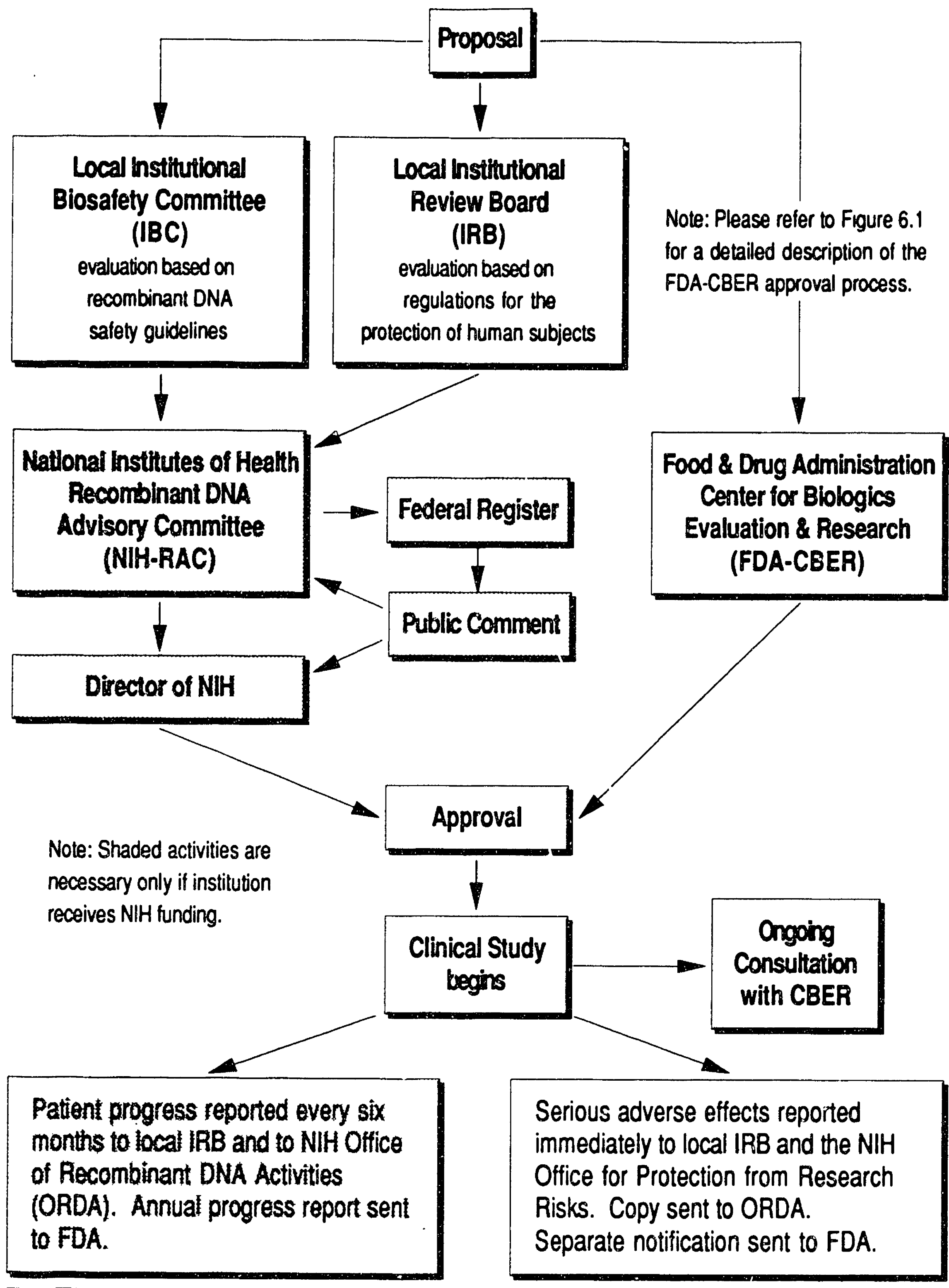


Figure 6.2 The review and approval process for human gene therapy trials in the United States (redrawn from Nichols 1988 and revised with assistance from the NIH-ORDA and FDA-CBER staffs).

emergency meeting of the RAC on January 14 to draft guidelines for the expedited consideration of single patient protocols. Healy's actions enraged many members of RAC. After a stormy meeting on January 14, the RAC voted to recommend to the NIH the following 10 points (National Institutes of Health 1993b):

1. NIH will strongly emphasize that the standard method of protocol submission is highly preferred.
2. The RAC will consider single patient protocols.
3. There will be no attempt to distinguish between research and treatment in the consideration of protocols.
4. Regardless of the method of review, the criteria must be the same for all protocols.
5. When time-sensitive circumstances prevail, the NIH will do an internal review.
6. To the extent that it is legally and practically possible, the Director of NIH will ask NIH experts, RAC members, and other experts to participate in protocol review.
7. Among other factors to be considered by the Director of NIH, is the consanguinity of the new protocol to existing protocols.
8. The NIH will report to the RAC following its internal review.
9. Protocols that are deferred or not approved by the RAC in its normal review process, are not eligible for expedited review.
10. In the development of any documents that are a part of this policy statement, the terms, compassionate use and compassionate treatment, will be deliberately avoided.

Approval of these recommendations is still pending as of the writing of this thesis. Also uncertain is the tenure of Bernadine Healy as Director of NIH in the new administration.

The FDA, in comparison, does have a policy in effect for single patient use and emergency use of experimental therapies. In her statement at the January 14

RAC meeting, Dr. Janet Woodcock, Director of the Office of Therapeutics of CBER, stated that the FDA has come under considerable pressure from patients, patient advocacy groups, and the general public to increase access to experimental therapies, particularly for AIDS and cancer (National Institutes of Health 1993a). The FDA provides these therapies with the disclaimer that patients may be exposing themselves to increased risk of toxicity and may be deferring themselves from other more effective therapies that might be available. The FDA discourages access before the dosage and toxicity of the drug have been determined in the Phase 1 trials. If all other therapies have been exhausted, the FDA will work with the patient's doctor and the investigator to provide access. Single patient use cases are usually time-limited situations due to the life-threatening condition of the patient. Emergency use pertains to situations of a medical emergency where there is not sufficient time to perform the required paperwork. Physicians can phone the FDA to request clearance for emergency use.

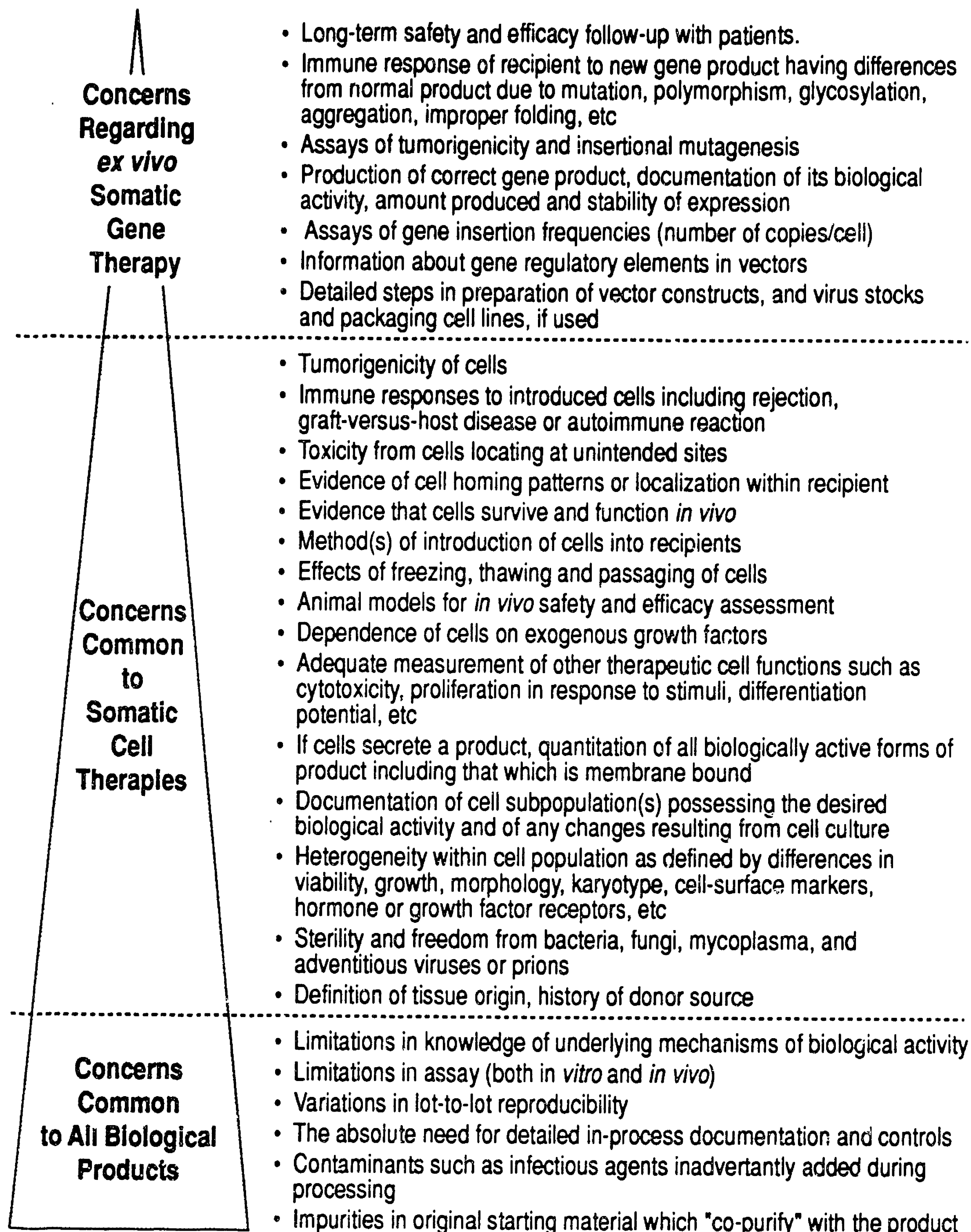
6.4 Regulatory Concerns in Human Gene Therapy

Gene therapy presents a challenge to regulation. There are many different diseases, target cells, vectors, and delivery methods. The field is very dynamic, making it impossible and not necessarily desirable to establish hard and fast regulations. The approach taken by CBER is to review each protocol on a case-by-case basis using a common set of principles.

Regulatory concerns can be grouped into three nested categories:

- Concerns Common to All Biological Products
- Concerns Common to Somatic Cell Therapies
- Concerns Regarding Somatic Gene Therapy.

Figure 6.3 presents a sampling of regulatory concerns for the first two categories as well as some in the third category which are specific for *ex vivo* somatic gene therapy (the regulatory concerns for *in vivo* somatic gene therapy would be different). The figure should not be viewed as a generalized check-list, but is provided merely to indicate the types of regulatory concerns that CBER addresses. The particular concerns that apply to any specific protocol are



Note: The above concerns are presented merely to provide the reader with a "flavor" of the types of issues involved in establishing safety and efficacy. They should not be interpreted as a generalized check-list. In practice, the CBER staff works with the sponsoring company to establish the specific scientific criteria appropriate for each protocol on a case-by-case basis. Please read upwards from the bottom to the top.

Figure 6.3 A sampling of regulatory concerns for *ex vivo* somatic gene therapy protocols (data summarized from Epstein 1991 and Quinnan 1991).

evaluated on a case-by-case basis. Working with the sponsoring company, the CBER staff establishes the specific scientific criteria needed to evaluate the protocol in the various phases of approval.

6.5 Some Reflections on the Future of Regulation

Society places its regulatory agencies in a nearly impossible situation. On the one hand, they are expected to exhaustively scrutinize all newly proposed products for safety and efficacy. And on the other hand, they are open to the everpresent criticism that they are unnecessarily delaying the entry of new beneficial products into the market. Obviously, there is an inherent tension between the need to evaluate and the need to release products. From a public relations perspective, the FDA has considerable downside for missing a safety problem, but, until recently, has had little upside for accelerating product approval. The tendency of the agency in the past has been to err on the conservative side, that is, to require more tests than might otherwise be considered necessary by those who stand to receive medical benefit from them. But at what point does the incremental increase in assurance of safety/efficacy fail to justify the associated incremental cost necessary to achieve it? The cost can be direct, such as additional clinical trials, which are born initially by the sponsoring company but ultimately passed on to the consumer as increased prices, and the cost can be indirect, such as delay of a product with a positive benefit/cost profile and possible loss of life in patients denied access to a drug hung-up in approvals. Simply put, in the time necessary to adequately study the side-effects of a drug, one life may be saved from an adverse reaction that would have otherwise gone unnoticed, but a thousand may die because the drug was mired in approvals and not released in the market. There are philosophical and statistical biases that hold the one life in higher regard than the thousand. Super-defensive drug approval may cost more lives on a net basis, but there is no liability, legal or political, for those who die as a result of the absence or unavailablility of a drug still in clinical trials- no liability until recently, however, when AIDS activists successfully pressured the FDA to release the experimental compound, AZT, to patients and to accelerate the approval process. The need for an evaluation of statistical biases in the approval of drugs was recently emphasized in a Biotech CEO Roundtable discussion chaired by MIT Sloan

School Dean, Lester Thurow. The implications for future global trade practices warrant continued study.

Biotech product life cycles are becoming shorter as the pace of innovation quickens. The typical biotech product of the 1990s will probably have an effective life of about five years before being made obsolete by the next one. When the time to approve drugs approximately equals their useful economic, technologic and therapeutic life, that means that the nth product will be introduced into the market about the same time its successor, the $(n+1)$th product will be entering clinical trials. Accelerating obsolescence will force society and its agents, the regulators, to find ways to achieve informative clinical endpoints faster.

Economist Lester Thurow has predicted that the country which can discover, develop, approve, manufacture, and distribute drugs the *fastest* will own the global market. Comparative advantage will go to the country which can find ways to bring drugs to market *faster* and *more efficiently*. Harmonization of drug approval procedures and cross-acceptance of clinical data between trading partners will become an important element in trade policy negotiations. In a world where economic competition replaces militaristic competition as the determinant of global power, the industry and government of each country will have to work very closely to make their nation's biotechnology effort as "lean and mean" as it possibly can be.

CHAPTER 7
CONCLUSIONS AND FUTURE ISSUES

1. This thesis has identified and explored the factors which are critical to the success of a commercial venture in the business of gene therapy.

2. Gene therapy is based on the flow of information in living systems at the molecular level and is designed to strategically intervene and modify this flow of information at the most basic level possible, the gene.

3. As a result, gene therapy offers the broadest technological platform for medical treatment known, incorporating antisense oligonucleotide, ribozyme, protein and small peptide mimetic drug modalities all within one single pharmacological construct.

4. Gene therapy is enabling a paradigm shift in the practice of medicine away from the treatment of the *phenotype* (the symptoms) and towards the treatment of the *genotype* (the underlying causes) of many diseases including AIDS, cancer, cardiovascular disease and genetic disease.

5. Advances in genomic sciences are laying the groundwork for revolutionary changes in the pharmaceutical industry of the next millennium. The new drugs of the future will be genes, or will be regulators of genes.

6. The social acceptance of gene therapy (in all its forms) will be critically dependent upon an informed public that is aware of the risks and benefits offered by this new technology.

7. The global economic race to commercialize biotechnology in general and gene therapy in particular may strain cultural differences in attitude regarding the appropriate use of such socially sensitive technology, particularly with regard to genetic enhancement and genetic modification of the germline.

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APPENDIX A: LOG OF EXPERTS INTERVIEWED

Date	Name, Title & Institution	Expertise
11/11/92	**Michael Rosenblatt, M.D.** Professor of Molecular Medicine Co-Director, Harvard-MIT Division of Health Sciences Boston, MA	• Pharmaceutical R&D
11/13/92	**Ghaleb H. Daouk, M.D.** Clinical Fellow Massachusetts General Hospital Charlestown, MA	• Renal physiology & disease • Biotech start-up issues
12/22/92	**Suzanne L. Epstein, Ph.D.** Director Laboratory of Molecular Immunology Division of Cellular & Gene Therapies Center for Biologics Eval. & Research Bethesda, MD	• FDA review of gene therapy
12/24/92	**Brigid G. Leventhal, M.D.** Professor of Pediatric Oncology The John Hopkins Hospital Baltimore, MD & Voting Member, National Institutes of Health Recombinant DNA Advisory Committee (NIH RAC)	• Clinical oncology • Review & approval of all gene transfer/therapy protocols by institutions receiving NIH funding
12/24/92	**Kirby D. Smith, Ph.D.** Professor of Molecular Biology The Johns Hopkins Sch. of Medicine & Kennedy Krieger Institute Baltimore, MD	• Human molecular genetics
1/11/93	**Stephen D. Lupton, Ph.D.** Dir., Dept. of Gene Expression Targeted Genetics Corporation Seattle, WA	• Retroviral vectors • Selectable marker/suicide fusion genes
1/11/93	**Richard D. Palmiter, Ph.D.** Professor of Biochemistry Howard Hughes Medical Institute University of Washington Seattle, WA	• Transgenic biology • Co-Founder, Targeted Genetics Corp.

1/12/93	**G. Stanley McKnight, Ph.D.** Professor of Pharmacology School of Medicine University of Washington Seattle, WA	• Cell signal transduction • Pharmacology
1/14/93	**Robert T. Schimke, M.D.** Professor of Biology Stanford University Stanford, CA	• Cell biology • Cancer biology • Gene amplification • Pharmacology
1/15/93	**James W. Larrick, M.D., Ph.D.** Scientific Director & Founder Palo Alto Institute for Molecular Medicine Mountain View, CA	• Gene therapy • Drug discovery
1/18/93	**John Monahan, Ph.D.** President & CEO Avigen Alameda, CA	• Commercialization of gene therapy using adeno-associated virus vectors
1/19/93	**Craig Muir** Manager, Drug Assay Group Tularik, Inc. South San Francisco, CA	• Molecular biology • Father of son with hemophilia A
1/19/93	**Karl Handelsman** Manager, Operations Tularik, Inc. South San Francisco, CA	• Molecular genetics • Commercialization of biotechnology
1/20/93	**John J. Serbin, Ph.D.** Director, Business Development Viagene, Inc. San Diego, CA	• Commercialization of gene therapy
1/20/93	**Geoffrey Wahl, Ph.D.** The Salk Institute for Biol. Sci. La Jolla, CA	• Cancer biology
1/21/93	**Inder M. Verma, Ph.D.** The Salk Institute for Biol. Sci. La Jolla, CA	• Gene therapy using retroviruses • Member, Board of Directors Somatix Therapy Corp.
1/21/93	**Ellie Ehrenfeld, Ph.D.** Dean of Biological Sciences University of California Irvine, CA	• Virology
1/21/93	**Donald Summers, M.D.** Associate Dean School of Medicine University of California Irvine, CA	• Virology

1/22/93	**Ellen Simpson, Ph.D., M.S.** Genetic Counselor Integrated Genetics Long Beach Memorial Medical Ctr Long Beach, CA	• Genetic counselling of expecting families
2/11/93 3/23/93	**Gregory D. Phelps** Senior VP of Corporate Development Genzyme, Inc. Cambridge, MA	• Biotech bus. development • Former President/CEO of Viagene
2/11/93	**Gail J. Maderis** Director, Corporate Development Genzyme, Inc. Cambridge, MA	• Biotech bus. development
2/18/93	**Garen Bohlin** Executive VP and CFO Genetics Institute, Inc. Cambridge, MA	• Biotech bus. development • In partnership with Genetic Therapy on hemophilia A&B gene therapy
2/18/93	**Lawrence S. Daniels** VP Marketing and Bus. Devel. Biogen, Inc Cambridge, MA	• Biotech bus. development
3/2/93	**Mark A. Findeis, Ph.D.** Senior Research Scientist TargeTech, Inc. Meriden, CT	• Chemical formulation of asialoglycoprotein - plasmid DNA complexes
3/2/93	**Timothy J. Gels** Manager of Business Development TargeTech, Inc. Meriden, CT	• Pharmaceutical business development
3/2/93	**George L. Spitalny, Ph.D.** VP Research & Development TargeTech, Inc. Meriden, CT	• Molecular biology of asialoglycoprotein - plasmid DNA complexes
3/2/93	**Alan G. Walton, Ph.D.** Principal Oxford Bioscience Partners Stamford, CT	• Venture capital in biotech • Round two investment in Genetic Therapy Inc. • Investor in Human Genome Sciences
3/22/93	**Lester C. Thurow, Ph.D.** Dean MIT Sloan School of Management Cambridge, MA	• Macroeconomics • Industrial economic policies

3/29/93	**Richard Horan** President SEQ, Limited Partnership Princeton, NJ	• Development of high speed, ow cost DNA sequencing technologies
4/1/93	**Linda Burch** VP, Business Development SyStemix, Inc. Palo Alto, CA	• Develop. of gene therapy for AIDS, immune diseases and cancers using hematopoietic stem cells
4/2/93	**Douglas A. Treco, Ph.D.** Director of Research Transkaryotic Therapies Inc. Cambridge, MA	• Gene therapy using physical/chemical methods & homologous recombination
4/7/93	**David T. Curiel, M.D.** Professor of Medicine University of North Carolina Chapel Hill, NC	• Gene therapy using UV-inactivated adenovirus-polylysine/plasmid DNA complexes
4/8/93	**John T. Preston** Director MIT Technology Licensing Office Cambridge, MA	• University technology transfer policy and practice
4/13/93	**Lita L. Nelsen** Officer MIT Technology Licensing Office Cambridge, MA	• University technology transfer policy and practice
4/15/93	**Becky Ann Lawson** Committe Management Officer Office of Recombinant DNA Activities National Institutes of Health Bethesda, MD	• NIH DNA biosafety programs
4/16/93	**Joyce Brinton** Director Office for Technology & Trademark Licensing Harvard University Cambridge, MA	• University technology transfer policy and practice
4/22/93	**Joseph F. Lovett** General Partner Medical Science Partners Brookline, MA	• Venture capital investment in start-up companies using technology developed at Harvard Medical School
4/28/93	**Barbara Handelin, Ph.D.** Director, DNA Diagnostics Laboratory Integrated Genetics/Genzyme Framingham, MA	• DNA diagnostics for cystic fibrosis, Huntingtons and other genetic diseases

4/30/93	**Irving M. London, M.D.** Professor Emeritus Harvard Medical School MIT Biology Department Harvard-MIT Health Sciences & Tech.	• Molecular biology of blood disorders • Founding scientist of Innogene Pharmaceuticals
5/6/93	**Henri Termeer** Chairman of the Board, President & CEO Genzyme Corporation Cambridge, MA	• Biotech bus. development • Development of gene therapy for cystic fibrosis
5/19/93	**Mark Dibner, Ph.D.** Institute Director Institute for Biotechnology Information North Carolina Biotechnology Center Research Triangle Park, NC	• Evolution of the biopharmaceutical industry
5/20/93	**Paul Gelep** Associate Director, Biotherapeutics Genzyme Corporation Cambridge, MA	• Development of recombinant thyroid stimulating hormone
5/21/93	**Xandra O. Breakefield, Ph.D.** Associate Geneticist NeuroScience Center Massachusetts General Hospital Charlestown, MA	• Treatment of neurodegenerative diseases & brain tumors with retroviral and herpes simplex gene transfer vectors
5/24/93	**Alison Taunton-Rigby, Ph.D.** Senior VP Biotherapeutics Genzyme Corporation Cambridge, MA	• Treatment of Gaucher's disease with protein replacement therapy • Cystic fibrosis gene therapy • Biotech bus. development
5/25/93	**Phillip A. Sharp, Ph.D.** Professor of Biology & Dept. Head MIT Biology Department Cambridge, MA	• Genome sciences • Post-transcriptional processing of pre-mRNA • Founding partner of Biogen
6/4/93	**Don Kirksey** Corporate Development Glaxo Pharmaceuticals Research Triangle Park, NC	• Evaluation of emerging technology including gene therapy
6/7/93	**W. French Anderson, M.D.** Professor of Biochemistry & Pediatrics University of Southern California Los Angeles, CA	• Hematological disorders • GTI collaborator • Did first human gene therapy protocol for ADA deficiency SCID
6/8/93	**Louis R. Bucalo, M.D.** President and CEO Ingenex Inc. South San Francisco, CA	• Cancer gene therapy business development

Date	Name	Topics
6/8/93	**Albert B. Deisseroth, M.D.** Prof. for Cancer Treatment & Research Chairman, Dept. Hematology MD Anderson Cancer Center, Univ. of Texas Houston, TX	• Cancer gene therapy
6/21/93	**Robert H. Zaugg, Ph.D.** Senior Director Business Devel. Vical Inc. San Diego, CA	• Gene therapy business development
6/21/93	**Philip L. Felgner, Ph.D.** Founder and Chief Scientist Vical Inc. San Diego, CA	• Liposome gene vectors • Naked DNA immunity
6/22/93	**Mimi Hancock, Ph.D.** Vice President Avigen Alameda, CA	• Commercialization of gene therapy using adeno-associated virus vectors
6/22/93	**Franklin M. Berger** Vice President, Research Josepthal Lyon & Ross Inc. New York, NY	• Biotech stock analysis
6/22/93	**Stephen M. Dalton** Vice President CoreStates Investment Advisors	• Biotech stock analysis
6/22/93	**Larry A. Couture, Ph.D.** Research Scientist Genzyme Corporation Framingham, MA	• Gene therapy of cystic fibrosis using adeno-virus vector
6/22/93	**Henry L. Nordhoff** President and CEO TargeTech Inc. Meriden, CT	• Pharmaceutical business development
6/23/93	**Victor A. McKusick, M.D.** Univ. Professor of Medical Genetics The Johns Hopkins School of Medicine Baltimore, MD	• Human genetics • Author of <u>Mendelian Inheritance in Man</u> • Key participant in Human Genome Project
6/25/93	**Gerard J. McGarrity, Ph.D.** Vice President & Director of Development Genetic Therapy, Inc Gaithersburg, MD	• Former Chairman, RAC • Gene therapy business development
6/25/93	**M. James Barrett, Ph.D.** President & CEO Genetic Therapy, Inc	• Gene therapy business development

	Gaithersburg, MD	
6/27/93	**Fred D. Ledley, M.D.** VP Clinical Research Devel. GeneMedicine, Inc. Houston, TX	• Principal Investigator on early RAC approved gene marking liver protocol • GeneMedicine Scientific Founder
6/27/93	**Douglas Jolly, Ph.D.** Department of Research Viagene, Inc. San Diego, CA	• Cancer immunotherapy using retroviral vectors
6/27/93	**Eric T. Juengst, Ph.D.** ELSI Branch Nat'l Center for Human Genome Research National Institutes of Health Bethesda, MD	• Ethical, legal and social implications of genetic research and practice
6/28/93	**Paul Boni** Research Assistant Mehta and Isaly Worldwide Healthcare Investments New York, NY	• Biotech stock analysis
6/29/93	**Johanna A. Griffin, Ph.D.** Director, Molecular Biology Boehinger Ingelheim Ridgefield, CT	• Assessment of emerging technology
6/29/93	**Gary J. Nabel, M.D., Ph.D.** Dept. Internal Medicine and Biological Chemistry Howard Hughes Medical Institute University of Michigan Ann Arbor, MI	• Cancer gene therapy • Specialized catheter for delivery of vectors
6/30/93	**Stephen M. Edgington** Senior Editor Bio/Technology Magazine New York, NY	• Development of biotechnology
7/21/93	**Igor B. Roninson, Ph.D.** Department of Genetics University of Illinois at Chicago Chicago, IL	• Inventor of method for isolating Genetic Suppressor Elements • First cloned Multi-Drug Resistance 1 gene
7/23/93	**David E. Housman, Ph.D.** Center for Cancer Research MIT Biology Department Cambridge, MA	• Founding scientist of Integrated Genetics • Helped clone many disease genes including myotonic dystropy and Huntingtons disease

8/8/93	**Ronald Crystal, M.D.** Chief, Division of Pulmonary and Critical Care Medicine Cornell University Medical College Ithaca, NY	• Founding scientist of GenVec • Adenovirus gene therapy of cystic fibrosis
8/9/93	**Jean-Michel H. Vos, Ph.D.** Depts. of Biochemistry & Biophysics University of North Carolina Chapel Hill, NC	• Development of Epstein-Barr viruses as Human Artificial Episomal Chromosomes for mapping and gene therapy
8/9/93	**Savio L. C. Woo, Ph.D.** Baylor College of Medicine Houston, TX	• Founding scientist of GeneMedicine • Gene therapy for hemophilia
8/20/93	**Eli Gilboa, Ph.D.** Duke University Medical Center Durham, NC	• First introduced French Anderson to retroviruses as gene vectors • Use of irradiated and genetically altered autologous tumor cells as a cancer vaccine

APPENDIX B: RAC APPROVED HUMAN GENE TRANSFER AND GENE THERAPY PROTOCOLS AS OF JUNE 8, 1993

001 (TR) Rosenberg, Steven A., National Cancer Institute;
The Treatment of Patients with Advanced Cancer Using Cyclophosphamide, Interleukin-2 and Tumor Infiltrating Lymphocytes.
Date of RAC Approval: 10-03-88
Date of NIH Approval: 03-02-89

002** (TH) Blaese, R. Michael, National Cancer Institute;
Treatment of Severe Combined Immune Deficiency (SCID) due to Adenosine Deaminase (ADA) Deficiency with CD34(+) Selected Autologous Hematopoietic Stem Cells.
Date of RAC Approval: 07-31-90
Date of NIH Approval: 09-06-90
Major Amendment-Date of RAC Approval: 02-10-92 to 02-11-92
Major Amendment-Date of NIH Approval: 04-22-92
Minor Modification: 06-07-93
Genetic Therapy, Inc.

003 (TH) Rosenberg, Steven A., National Cancer Institute;
Gene Therapy of Patients with Advanced Cancer Using Tumor Infiltrating Lymphocytes Transduced with the Gene Coding for Tumor Necrosis Factor.
Date of RAC Approval: 07-31-90
Date of NIH Approval: 09-06-90
Genetic Therapy, Inc.

004* (TR) Brenner, Malcolm K.; Mirro, Joseph; Hurwitz, Craig; Santana, Victor; and Ihle, James, St. Jude Children's Research Hospital;
Autologous Bone Marrow Transplant for Children with Acute Myelogenous Leukemia in First Complete Remission: Use of Marker Genes to Investigate the Biology of Marrow Reconstitution and the Mechanism of Relapse.
Date of RAC Approval: 02-04-91
Date of NIH Approval: 07-12-91
Minor Modification: 11-91
Genetic Therapy, Inc.

005* (TR) Brenner, Malcolm K.; Mirro, Joseph; Santana, Victor; and Ihle, James, St. Jude Children's Research Hospital;
A Phase I/II Trial of High Dose Carboplatin and Etoposide with Autologous Marrow Support for Treatment of Stage D Neuroblastoma in First Remission: Use of Marker Genes to Investigate the Biology of Marrow Reconstitution and the Mechanism of Relapse.
Date of RAC Approval: 05-31-91
Date of NIH Approval: 07-12-91
Minor Modification: 11-91
Genetic Therapy, Inc.

006* (TR) Brenner, Malcolm K.; Mirro, Joseph; Santana, Victor; and Ihle, James, St. Jude Children's Research Hospital;
A Phase II Trial of High-Dose Carboplatin and Etoposide with Autologous Marrow Support for Treatment of Relapse/Refractory Neuroblastoma Without Apparent Bone Marrow Involvement.
Date of RAC Approval: 05-31-91
Date of NIH Approval: 07-12-91
Minor Modification: 11-91
Genetic Therapy, Inc.

007* (TR) Deisseroth, Albert B., M.D. Anderson Cancer Center;
Autologous Bone Marrow Transplantation for Chronic Myelogenous Leukemia in which Retroviral Markers are Used to Discriminate between Relapse which Arises from Systemic Disease Remaining after Preparative Therapy Versus Relapse due to Residual Leukemic Cells in Autologous Marrow: A Pilot Trial.
Date of RAC Approval: 05-31-91
Date of NIH Approval: 07-12-91
Minor Modification: 04-19-93
Genetic Therapy, Inc.

008 (TR) Ledley, Fred D.; Woo, Savio; Ferry, George; and Hartwell, Whigennand, Baylor College of Medicine;
Hepatocellular Transplantation in Acute Hepatic Failure and Targeting Genetic Markers to Hepatic Cells.
Date of RAC Approval: 05-30-91
Date of NIH Approval: 07-12-91
Genetic Therapy, Inc.

009* (TR Lotze, Michael T., University of Pittsburgh School of Medicine;
The Administration of Interleukin-2, Interleukin-4, and Tumor Infiltrating Lymphocytes to Patients with Melanoma.
Date of RAC Approval: 05-30-91
Date of NIH Approval: 01-17-92
Minor Modification: 11-30-92
Genetic Therapy, Inc.

010* (TH) Rosenberg, Steven A., National Cancer Institute;
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Tumor Necrosis Factor (TNF).
Date of RAC Approval: 10-07-91
Date of NIH Approval: 10-15-91
Minor Modification: 7-9-92
Genetic Therapy, Inc.

011 (TH) Rosenberg, Steven A., National Cancer Institute;
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Interleukin-2 (IL-2).
Date of RAC Approval: 10-07-91
Date of NIH Approval: 10-15-91
Genetic Therapy, Inc.

012* (TH) Wilson, James M., University of Michigan;
***Ex vivo*" Gene Therapy of Familial Hypercholesterolemia.**
Date of RAC Approval: 10-08-91
Date of NIH Approval: 11-14-91
Minor Modification: 12-03-92

013* (TH) Nabel, Gary J., University of Michigan;
Immunotherapy of Malignancy by *In vivo* Gene Transfer into Tumors.
Date of RAC Approval: 02-10-92
Date of NIH Approval: 04-17-92
Minor Modification: 01-22-93
Minor Modification: 01-93
Vical

014 (TR) Cornetta, Kenneth, Indiana University;
Retroviral-Mediated Gene Transfer of Bone Marrow Cells during Autologous Bone Marrow Transplantation for Acute Leukemia.
Date of RAC Approval: 02-11-92
Date of NIH Approval: 04-17-92
Genetic Therapy, Inc.

015 (TR) Economou, James S. and Belldegrun, Arie, University of California at Los Angeles;
The Treatment of Patients with Metastatic Melanoma and Renal Cell Cancer Using *In Vitro* Expanded and Genetically-Engineered (Neomycin Phosphotransferase) Bulk, CD8 (+) and/or CD4(+) Tumor Infiltrating Lymphocytes and Bulk, CD8(+) and/or CD4(+) Peripheral Blood Leukocytes in Combination with Recombinant Interleukin-2 Alone, or with Recombinant Interleukin-2 and Recombinant Alpha Interferon.
Date of RAC Approval: 02-11-92

Date of NIH Approval: 04-17-92
Genetic Therapy, Inc.

016 (TH) Freeman, Scott M., University of Rochester School of Medicine, Rochester, New York;
Gene Transfer for the Treatment of Cancer.
Date of RAC Approval: 02-10-92
Date of NIH Approval: 02-05-93

017 (TR) Greenberg, Philip D. and Riddell, Stanley, University of Washington, Seattle;
Phase I Study of Cellular Adoptive Immunotherapy Using Genetically Modified CD8+ HIV-Specific T Cells for HIV-Seropositive Patients Undergoing Allogeneic Bone Marrow Transplant.
Date of RAC Approval: 02-11-92
Date of NIH Approval: 04-17-92
Targeted Genetics

018 (TH) Brenner, Malcolm K.; Furman, Wayne; Santana, Victor; Bowman, Laura; and Meyer, William, St. Jude Children's Research Hospital;
Phase I Study of Cytokine-Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed/Refractory Neuroblastoma.
Date of RAC Approval: 06-01-92
Date of NIH Approval: 08-14-92
Genetic Therapy, Inc.

019 (TH) Oldfield, Edward, National Institutes of Health;
Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir.
Date of RAC Approval: 06-01-92
Date of NIH Approval: 08-14-92
Genetic Therapy, Inc.

020 (TR) Deisseroth, Albert B., MD Anderson Cancer Center;
Use of Two Retroviral Markers to Test Relative Contribution of Marrow and Peripheral Blood Autologous Cells to Recovery After Preparative Therapy.
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92
Minor Modification: 04-19-93
Genetic Therapy, Inc.

021 **(TH)** Gansbacher, Bernd; Houghton, Alan; and Livingston, Philip, Memorial Sloan Kettering Cancer Center;
Immunization with HLA-A2 matched Allogeneic Melanoma Cells that Secrete Interleukin-2 in Patients with Metastatic Melanoma,
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92

022* **(TH)** Gansbacher, Bernd; Motzer, Robert; Houghton, Alan; and Bander, Neil, Memorial Sloan Kettering Cancer Center;
Immunization with Interleukin-2 Secreting Allogeneic HLA-A2 Matched Renal Cell Carcinoma Cells in Patients with Advanced Renal Cell Carcinoma.
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92
Minor Modification: 03-31-93

023 **(TR)** Dunbar, Cynthia, National Institutes of Health;
Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Multiple Myeloma.
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92

024 **(TR)** Dunbar, Cynthia, National Institutes of Health;
Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Metastatic Breast Cancer.
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92

025 **(TR)** Dunbar, Cynthia, National Institutes of Health;
Retroviral-Mediated Gene Transfer of Bone Marrow and Peripheral Blood Stem Cells During Autologous Bone Marrow Transplantation for Chronic Myelogenous Leukemia.
Date of RAC Approval: 06-02-92
Date of NIH Approval: 08-14-92

026 **(TR)** Walker, Robert E. , National Institutes of Health;
A Study of the Safety and Survival of the Adoptive Transfer of Genetically Marked Syngeneic Lymphocytes in HIV Infected Identical Twins.
Date of RAC Approval: 09-14-92

027 (TR) Schuening, Friedrich G., Fred Hutchinson Cancer Research Center
Phase I/II Study of the Use of Recombinant Human Interleukin 3 (rhIL3) Stimulated Peripheral Blood Progenitor Cell Supplementation in Autologous Bone Marrow Transplantation in Patients with Breast Carcinoma or Hodgkin's Disease.
Date of RAC Approval: 09-14-92
Date of NIH Approval: 02-05-93

028 (TR) Schuening, Friedrich G., Fred Hutchinson Cancer Research Center
Evaluation of the Use of Recombinant Human G-CSF Stimulated Peripheral Blood Progenitor Cell Supplementation in Autologous Bone Marrow Transplantation in Patients with Lymphoid Malignancies.
Date of RAC Approval: 09-14-92
Date of NIH Approval: 02-05-93

029 (TR) Schuening, Friedrich G., Fred Hutchinson Cancer Research Center
A Trial of G-CSF Stimulated Peripheral Blood Stem Cells for Engraftment in Identical Twins.
Date of RAC Approval: 09-14-92
Date of NIH Approval: 02-05-93

030 (TR) Deisseroth, Albert B., University of Texas MD Anderson Cancer Center;
Use of Retroviral Markers to Evaluate the Efficacy of Purging and to Discriminate Between Relapse which Arises from Systemic Disease Remaining after Preparative Therapy Versus Relapse due to Residual Neoplastic Cells in Autologous Marrow Following Purging in Patients with Chronic Lymphocytic Leukemia (CLL).
Date of RAC Approval: 09-14-92

031 (TH) Roth, Jack A., The University of Texas MD Anderson Cancer Center;
Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer.
Date of RAC Approval: 09-15-92

032 (TR) Brenner, Malcom K., St. Jude Children's Research Hospital;
A Phase II Trial of the Baxter Neuroblastoma Bone Marrow Purging System Using Gene Marking to Assess Efficacy.
Date of RAC Approval: 09-15-92
Date of NIH Approval: 02-05-93
Baxter Healthcare Corporation

033 (TH) Lotze, Michael T. and Rubin, Joshua T., University of Pittsburgh;
Gene Therapy of Cancer: A Pilot Study of IL-4 Gene Modified Antitumor Vaccines.
Date of RAC Approval: 09-15-92
Date of NIH Approval: 02-05-93
Genetic Therapy, Inc.

034* (TH) Crystal, Ronald G., National Heart, Lung, and Blood Institute;
A Phase 1 Study, in Cystic Fibrosis Patients, of the Safety, Toxicity, and Biological Efficacy of a Single Administration of a Replication Deficient, Recombinant Adenovirus Carrying the cDNA of the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator Gene in the Lung.
Date of RAC Approval: 12-03-92
Date of NIH Approval: 04-16-93
Minor Modification: 05-17-93
GenVec/Genentech

035 (TH) Wilson, James M., University of Michigan;
Gene Therapy of Cystic Fibrosis Lung Diseases Using E1 Deleted Adenoviruses: A Phase I Trial.
Date of RAC Approval: 12-03-92

036 (TR) Welsh, Michael J., Howard Hughes Medical Institute and Smith, Alan E., Genzyme Corporation;
Cystic Fibrosis Gene Therapy Using an Adenovirus Vector: *In vivo* Safety and Efficacy in Nasal Epithelium.
Date of RAC Approval: 12-04-92
Date of NIH Approval: 04-16-93
Genzyme

037 (TH) Culver, Kenneth, Iowa Methodist Medical Center, Des Moines, Iowa and Van Gilder, John C., University of Iowa, Iowa;
Gene Therapy for the Treatment of Malignant Brain Tumors with In Vivo Tumor Transduction with the Herpes Simplex Thymidine Kinase Gene/Ganciclovir System.
Date of RAC Approval: 03-01-93
Date of NIH Approval: 04-16-93
Genetic Therapy, Inc.

038 (TR) Heslop, Helen E.; Brenner, Malcom K.; and Rooney, Cliona, St. Jude Children's Research Hospital, Memphis, Tennessee;
Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T Lymphocytes to Recipients of Mismatched-Related or Phenotypically Similar Unrelated Donor Marrow Grafts.
Date of RAC Approval: 03-02-93
Date of NIH Approval: 04-16-93

039 (TR) Brenner, Malcom K.; Krance, Robert; Heslop, Helen E.; Santana, Victor; and Ihle, James, St. Jude Children's Research Hospital, Memphis, Tennessee;
Assessment of the Efficacy of Purging by Using Gene-Marked Autologous Marrow Transplantation for Children with Acute Myelogenous Leukemia in First Complete Remission.
Date of RAC Approval: 03-02-93
Date of NIH Approval: 04-16-93

040 (TH) Simons, Jonathon, Johns Hopkins Oncology Center, Baltimore, Maryland;
Phase I Study of Non-Replicating Autologous Tumor Cell Injections Using Cells Prepared With or Without Granulocyte-Macrophage Colony Stimulating Factor Gene Transduction in Patients with Metastatic Renal Cell Carcinoma.
Date of RAC Approval: 03-01-93
Somatix Therapy

041 (TH) Wilmott, Robert W., Whitsett, Jeffrey, Children's Hospital Methodist Center, Cincinnati, Ohio, and Trapnell, Bruce, Genetic Therapy, Inc., Gaithersburg, Maryland; **"A Phase I Study of Gene Therapy of Cystic Fibrosis Utilizing a Replication Deficient Recombinant Adenovirus Vector to Deliver the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airways.**
Date of RAC Approval: 03-02-93
Date of NIH Approval: 04-16-93
Genetic Therapy, Inc.

042 (TH) Boucher, Richard C., and Knowles, Michael R., University of North Carolina, Chapel Hill, North Carolina; **"Gene Therapy for Cystic Fibrosis Using E1 Deleted Adenovirus: A Phase I Trial in the Nasal Cavity.**
Date of RAC Approval: 03-02-93

043 (TH) Seigler, Hillard F., Duke University Medical Center, Durham, North Carolina;
A Phase 1 Trial of Human Gamma Interferon-Transduced Autologous Tumor Cells in Patients With Disseminated Malignant Melanoma.
Date of RAC Approval: 06-07-93

044 (TH) Deisseroth, Albert B.; Kavanagh, John; and Champlin, Richard, University of Texas MD Anderson Cancer Center, Houston, Texas
Use of Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Ovarian Cancer: A Pilot Trial.
Date of RAC Approval: 06-07-93
Genetic Therapy, Inc.

045 (TH) Nabel, Gary J., University of Michigan Medical Center, An Arbor, Michigan;
Immunotherapy for Cancer by Direct Gene Transfer into Tumors.
Date of RAC Approval: 06-07-93
Vical

046 (TH) Barranger, John A., University of Pittsburgh, Pennsylvania;
Gene Therapy for Gaucher Disease: Ex Vivo Gene Transfer and Autologous Transplantation of CD34(+) Cells.
Date of RAC Approval: 06-07-93
Theragen

047 (TH) Karlsson, Stefan and Dunbar, Cynthia, NIH, Bethesda, Maryland, and Kohn, Donald B., Childrens Hospital Los Angeles, Los Angeles, California;
Retroviral Mediated Transfer of the cDNA for Human Glucocerebrosidase into Hematopoietic Stem Cells of Patients with Gaucher Disease.
Date of RAC Approval: 06-07-93
Genetic Therapy, Inc.

048 (TH) Galpin, Jeffrey E., University of Southern Califiornia, and Casciato, Dennis A., University of California, Los Angeles, California;
A Preliminary Study to Evaluate the Safety and Biologic Effects of Murine Retroviral Vector Encoding HIV-1 Genes [HIV-IT(V)] in Asymptomatic Subjects Infected with HIV-1.
Date of RAC Approval: 06-07-93
Viagene

049 (TH) Nabel, Gary J., University of Michigan Medical Center, Ann Arbor, Michigan;
A Molecular Genetic Intervention for AIDS - Effects of a Transdominant Negative Form of Rev.
Date of RAC Approval: 06-07-93
Vical

050 (TH) Raffel, Corey, Childrens Hospital Los Angeles, Los Angeles, California, and Culver, Kenneth, Iowa Methodist Medical Center, Des Moines, Iowa;
Gene Therapy for the Treatment of Recurrent Pediatric Malignant Astrocytomas with In Vivo Tumor Transduction with the Herpes Simplex Thymidine Kinase Gene.
Date of RAC Approval: 06-08-93

051 (TH) Hesdorffer, Charles and Antman, Karen, Columbia University College of Physicians and Surgeons, New York, New York;
Human MDR Gene Transfer in Patients with Advanced Cancer.
Date of RAC Approval: 06-08-93

052 (TH) Ilan, Joseph, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio;
Gene Therapy for Human Brain Tumors Using Episome-Based Antisense cDNA Transcription of Insulin-Like Growth Factor 1.
Date of RAC Approval: 06-08-93

TR	=	Human Gene Transfer Protocol
TH	=	Human Gene Therapy Protocol
*	=	Minor Modification
**	=	Major Modification
Underline	=	RAC Approved/NIH Approval Pending (as of 6/8/93)

RAC Approved: 29 TH + 23 TR = 52 Total Protocols
NIH Director Approved: 15 TH + 21 TR = 36 Total Protocols

Commercial Affiliation as determined from the public literature (partial list)

(END OF THESIS)