SELECTION AND LOCALIZATION OF CLONED DNA SEQUENCES FROM HUMAN CHROMOSOME 11

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SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF

DOCTOR OF PHILOSOPHY

at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May 1980

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ABSTRACT

Recombinant DNA techniques have been combined with somatic cell genetic methods to identify, isolate, and amplify fragments of human DNA localized at specific regions of human chromosome 11 selected as a model system. A library of genomic DNA segments has been constructed in Charon 4A from the DNA of a somatic cell hybrid carrying a portion of human chromosome 11 on a Chinese hamster ovary cell background. Using a nucleic acid hybridization technique which distinguishes human and Chinese hamster repetitive DNA, it has been possible to distinguish phages carrying DNA segments of human origin from those carrying DNA segments of hamster origin. 40 human segments have been isolated from chromosome 11 thus far, and 12 have been characterized in detail. For each DNA segment characterized, a sub-segment has been identified which carries no repetitive human DNA. These subsegments have been used as probes against a panel of hybrids containing various terminal deletions of chromosome 11. In each case an unequivocal chromosomal localization has been obtained. The same panel of cell hybrids has also been used in conjunction with cloned DNA probes to precisely localize the β-globin gene complex on chromosome 11. Both the strategies used for cloning chromosome specific DNA segments and for mapping them on a chromosome appear to be general. They can be extended to produce a fine structure map of chromosome 11 and other human chromosomes. This approach has promising implications for human genetics generally, for the human genetic diseases, and possibly for understanding of gene regulation in normal and abnormal differentiation.

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Title: Associate Professor of Biology

ACKNOWLEDGMENTS

I would like to extend my thanks to the many individuals who contributed to the successful completion of this work. First and foremost, I am indebted to David Housman, an inspired biologist, and an inspiring thesis supervisor who was a never-ending source of ideas and suggestions as well as support and encouragement. Second, I would like to thank Aviva Varsanyi-Breiner for her collaboration at the inception of these experiments, for correctly predicting the path to take, and for convincing me to follow it. I am also grateful to Sherry Keys for her excellent technical assistance and to Drs C. Jones, F.T. Kao, T.T. Puck, P. Gerald, G. Bruns, T. Maniatis and A. Bothwell for supplying the hybrid cell lines and some of the libraries used in these investigations. Finally I want to express my special appreciation to my wife, Maria, whose unfailing faith managed not only to bolster my confidence when experiments failed but also to spur me on at a more rapid pace when they did not.

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CHAPTER ONE

INTRODUCTION

THE HUMAN GENETIC MAP

In 1911 E.B. Wilson (1) was the first to assign a human gene (color blindness) to a particular chromosome (the X chromosome). Since that time the number of markers placed on the human chromosome map has expanded to over 300 (2,3). Three major methods have been used to assign chromosomal locations to these genes (2). First, genes have been mapped using the classical method of demonstrating linked inheritance in a family of an expressed polymorphism with a previously existing marker or karyotypic abnormality. Second, certain genes have been chromosomally localized by in situ nucleic acid hybridization of appropriate probes to human chromosome spreads. The successful application of this technique has thus far been limited to certain repetitive DNA sequences such as ribosomal DNA (4-7), and the histone genes (3). Finally a large number of markers have been localized by using somatic cell hybrids (usually human-Chinese hamster or human-mouse), which segregate human chromosomes, to demonstrate a correlation between the presence or absence of a particular phenotype (eg. production of a particular human protein) and the presence or absence of a particular human chromosome or chromosomal region in the hybrid cells (2).

Of the markers which have been assigned to human chromosomes,

approximately one third have been placed on the X chromosome which contains only 6% of the genomic DNA (2). The other 200 or so markers are spread over the 22 autosomes in a decidedly non-random fashion. Those loci which for which a specific chromosomal region has been determined tend to occur in clusters (as a result of the linkage method of mapping) and therefore large regions of the genome remain completely unrepresented. Any new methodology which could identify large numbers of new, potentially polymorphic markers would be of great interest for several reasons. First, new markers could be used to map by linkage analysis loci causing genetic disease. The markers currently available have proved insufficient in number to map most disease loci. Methods of antenatal diagnosis for various genetic diseases could potentially be based upon such linkage information. Second, increased numbers of markers could provide insight into chromosome organization and structure as well as the evolution of syntenic relationships in man. Third, the existence of new loci should aid in investigation of genetic mechanisms such as recombination and mutagenesis. Finally as McKusick and Ruddle (2) have so eloquently stated: " Mapping the human genome, like any uncharted terrain, is a challenge to the human intellect. Inevitably, information on the detailed gene anatomy of the chromosomes will also have its usefulness."

RECOMBINANT DNA TECHNOLOGY

In recent years, a new technology has emerged which has allowed the fine structure mapping and even the sequencing of specific genes. This technology is based on the ability to clone eukaryotic DNA segments by

physically attaching them to the DNA of plasmid (8) or bacteriophage vectors (9-11) and by replicating them in an appropriate bacterial host. Methods have been elaborated which permit the cloning of cDNA copies of mRNA molecules (12,13) and the screening of recombinants to identify molecules of interest (14). Similarly, bacteriophage vectors have been used to construct recombinant "libraries" containing all of the sequences of a particular eukaryotic genome (15) and specific gene sequences have been isolated from these libraries (16,17). In theory, any cDNA or genomic DNA segment could be cloned and isolated for study. In practice, a number of specific gene sequences have been isolated, mapped using site-specific restriction endonucleases and sequenced (18-21).

The advent of recombinant DNA techniques should have a profound impact on human gene mapping and provide a new impetus to all three of the methods of chromosomal localization discussed above. First, cloned DNA segments should permit the identification of large numbers of new polymorphic markers for linkage studies. Classical linkage analysis has usually been dependent upon differences in electrophoretic mobility, enzyme activity or antigenic determinants of proteins to provide markers (2). Cloned DNA sequences can be used as hybridization probes to identify individual differences in the location of restriction endonuclease cleavage sites (22). Investigators now can use the primary sequence of the genetic material itself as a source of polymorphism rather than depending on the expression of gene products and therefore can presumably map any region of the genome which contains single-copy DNA whether it be coding or non-coding.

Recombinant DNA techniques will also allow the expansion of

attempts to localize genes by in situ hybridization. Cloned DNA segments can not only provide pure isolates of repetitive DNA sequences (23) but can also provide the necessary probes for the refinement of in situ hybridization techniques to allow the mapping of single copy sequences (24).

Finally, the ability to isolate cloned human DNA segments will also facilitate the mapping of both coding and non-coding DNA sequences in somatic cell hybrids. Investigators will be able to detect these sequences by DNA-DNA hybridization and will no longer be dependent upon expression of human genes in hybrid cells.

FOCUS OF THE THESIS

The focus of this thesis will be to merge the techniques of somatic cell genetics and recombinant DNA to provide new approaches to the problems of gene mapping and gene organization in man. In chapter 2, the power of using cloned DNA as a probe for the presence or absence of particular gene in hybrid cells will be demonstrated by mapping the human β-globin gene complex on chromosome 11. Chapter 3 will use our current knowledge of DNA sequence organization in the mammalian genome to develop a methodology for cloning species specific DNA segments from hybrid cells. This methodology will be applied in chapter 4 to the isolation of DNA segments from the short arm of human chromosome 11. Finally, in chapter 5, an example of one practical benefit of this procedure will be presented as the cloned DNA sequences are used for some preliminary studies of deletions in chromosome 11 which are associated with the disease aniridia.

ORGANIZATION OF THE MAMMALIAN GENOME

Although recombinant DNA techniques have already been used to fine structure map small areas of mammalian genomes these studies have been confined to regions coding for well characterized proteins such as the hemoglobins or immunoglobulins (25,26). Our knowledge of the general organization of mammalian genomes however predates the development of recombinant DNA methods but results rather from experimental determinations of the rate of reannealing of denatured genomic DNA (27,28). Since the rate of reannealing of particular DNA sequence depends on the frequency of its occurrence in the genome, DNA segments can be classified by copy number per haploid genome.

The bulk of a mammalian genome generally consists of single copy DNA (one copy/haploid genome) interspersed with middle (intermediate) repetitive DNA (1000 to 100,000 copies/haploid genome). Generally, the genome also contains some highly repetitive (satellite) DNA (1,000,000 copies/haploid genome) which is found in long tandem arrays.

The mouse genome is consistent with this overall pattern of organization as demonstrated by Ginelli et al.(29). It consists of 76% non-repetitive DNA, 15% middle repetitive DNA (average repetition frequency= 20,000 copies/haploid genome), 8% highly repetitive DNA and 1% fold-back DNA (which reanneals instantaneously due to intramolecular inverted repeats).

The human genome presents a slightly different organization (30-32). Four kinetic components are detectable: highly repetitive or satellite DNA (2-6% of the genome), fast intermediate repetitive DNA (

6% of the genome), slow intermediate repetitive DNA (30% of the genome) and single copy DNA (60% of the genome). Both classes of intermediate repetitive DNA occur interspersed with single copy DNA but the fast repetitive DNA also has a component which occurs in satellite-like tandem arrays (31). The fast repetitive DNA appears to be largely dominated by a single family of repeated sequences (approximately 300 nucleotides in length) which are repeated 250,000 times per haploid genome (33).

The human and mouse genomes also differ somewhat in their pattern of interspersion of intermediate repetitive DNA. In the mouse, one third of the single-copy sequences are longer than 4500 nucleotides although less than 6% are greater than 11000 nucleotides in length (29). In the human genome, on the other hand almost all single-copy DNA sequences are less than 4500 nucleotides in length (31). Despite these differences, the overall pattern of fine interspersion of intermediate repetitive DNA and single copy DNA is present in all mammalian genomes studied thus far and, in fact, is found in almost all other eukaryotes except Drosophila (28). It is precisely the ubiquity of this pattern, along with the significant rate of evolution observed in intermediate repetitive DNA (34) which will be used in chapter 3 to devise a method for the isolation of DNA sequences from recombinant libraries based on their species of origin.

HUMAN CHROMOSOME 11

The ability to distinguish cloned DNA sequences by their species of origin along with the observed tendency of inter-species somatic cell

hybrids to segregate chromosomes of one species (35,36) allow the application of recombinant DNA technology to the selection of chromosomally localized DNA sequences as will be outlined in chapter 4 of this thesis. Chromosome 11 was chosen as a model system to explore this new technology in large part because of the pioneering work of Drs C. Jones, F.T. Kao and T.T. Puck (37-39). These investigators have isolated a stable human-hamster hybrid cell which contains chromosome 11 as its only human component thereby separating this chromosome from the rest of the human genome (37). They have furthermore identified three human cell surface antigens SA11-1, -2, and -3 which are expressed in the hybrid cells and have raised specific antisera against each of them. The antisera have been used following treatment with X-rays to select for hybrid cells which have lost the appropriate antigen (38). These generally result from a terminal deletion of the arm of chromosome 11 encoding the antigen. Because the genes for SA11-1 and SA11-3 are located on the distal portion of the short arm, these investigators have successfully selected a panel of hybrid subclones, each of which has retained a different proportion of the short arm (38). This panel has been used to allow deletion mapping of two enzyme markers (38,39), Acid Phosphatase-2 (ACP-2) and Lactate Dehydrogenase A (LDH-A), on the short arm of chromosome 11, but its further usefulness has been limited by the lack of markers assigned to this chromosome.

Chromosome 11 constitutes approximately 4.6% of the total human genome (40) but only 8 genetic markers have been assigned to it (3). In addition to the short arm markers, SA11-1 and -3, LDH-A and ACP-2 referred to above, the chromosome also contains the loci for SA11-2 and

Esterase A-4, ESA-4 on the long arm (3). Recently, Deisseroth et al. (41) have assigned the β globin gene complex to chromosome 11 and this assignment has been confirmed in this thesis. The final confirmed marker present on chromosome 11 is the WAGR locus involved in the aniridia-genital abnormality-renal tumour complex (3).

It has been observed that cytologically detectable deletions in the short arm of human chromosome 11 are sometimes associated with aniridia (a congenital eye malformation), genital abnormality, mental retardation, and a predisposition to Wilms tumor (a childhood Kidney tumor) in the heterozygous state (42,43). It is thought that each of these disorders is caused by deletion of one of a closely linked set of genes on the short arm (43). A chromosomal deletion was implicated in the disorders as early has fifteen years ago when Miller (44) first noted the high incidence of aniridia in Wilms tumor patients. Detection of a deletion did not occur until improved chromosome banding procedures made it possible in 1978 (44). Since that time a number of patients with similar deletions have been identified and the region of 11p13 has been identified as the most probable site of the WAGR complex (42-44).

It is clear that although chromosome 11 has relatively few conventional genetic markers, two of those which are present, the β-globin complex and the WAGR locus, are of great medical interest. The globin family of genes and their surroundings are also in the forefront of molecular biological studies of gene structure and control (25,45-47). These facts, combined with the excellent somatic cell genetic groundwork laid by Jones, Kao, and Puck (37-39) make chromosome 11 an ideal starting point for applying recombinant DNA techniques to

gene mapping.

CHAPTER TWO

CHROMOSOMAL LOCALIZATION OF THE β-GLOBIN COMPLEX

INTRODUCTION

The ability to map human genes at precise chromosomal locations, while of inherent interest, is likely to have important implications at both the practical and theoretical level. The accumulation of precise mapping information may prove relevant to investigations of linkage and position effects, to study of developmental processes in which particular genes are sequentially activated, and to diagnosis and understanding of diseases with abnormal patterns of gene activation.

Significant progress has been achieved in the assignment of human genes to specific chromosomes (2,3). Somatic cell hybrids have played an important role in such identifications (35). Although occasional regional assignment of such genes to sections of an arm has been reported, systematic mapping of the human genes with a precision comparable to that in Drosophila or the mouse has not yet been possible.

The development of techniques with the high degree of resolution of restriction endonuclease digestion, nucleic acid hybridization (22), and sequence determination (48,49) as well as the ability to purify and amplify specific human DNA sequences by using recombinant DNA methods opens the prospect of mapping the DNA of a human chromosome with a much greater precision than has previously been possible. These molecular

approaches have here been combined with somatic cell genetics to achieve precise chromosomal mapping of human genes.

Most mammalian genes which have thus far been localized on specific chromosomes are those present in cells capable of synthesizing the corresponding mRNA or protein. Recently, the human genes coding for the α and β chains of the hemoglobin molecule have been identified by molecular hybridization in solution and have been assigned to human chromosome 16 and 11, respectively, by using complementary DNA probes for human α-globin and β-globin gene sequences in human-mouse cell hybrids containing various combinations of human chromosomes (50,41). The recent development of molecular hybridization of nucleic acids transferred from agarose gels to nitrocellulose filters (22) offers another powerful method for detecting specific gene sequences. This method is highly sensitive and requires much less DNA than does molecular hybridization of nucleic acid in solution.

This technique has here been applied to a panel of deletion clones derived from a hybrid containing chromosome 11 as its only human component (37,36) in order to precisely localize the β-globin gene complex within the chromosome. The initial hybrid designated J1 contains the entire genome of the Chinese hamster ovary cell (CHO-K1) and the single human chromosome 11. The subclones of J1 used in this study were prepared by treatment with chromosome-breaking agents followed by selection in antisera specific to particular human cell surface antigens plus complement. The use of these specific antisera is possible because of the large immunologic differences between human and Chinese hamster cells. These antisera made possible the selection of clones that had lost various combinations of the antigen markers

SA11-1, -2, and -3. Biochemical analysis for lactate dehydrogenase A (EC 1.1.1.27) (LDH-A) and acid phosphatase 2 (EC 3.1.3.2) (ACP-2) and cytogenetic analysis for banding patterns were used to characterize each deletion subclone.

Ten subclones displaying different terminal deletions of chromosome 11 were selected for mapping the β-globin gene complex. Direct assay by DNA-DNA hybridization was required because the globin genes are not expressed in these hybrid cells. in the present study, cloned DNA fragments of the β and δ globin genes isolated by recombinant DNA techniques from genomic DNA were used as probes in the Southern hybridization procedure (22). This assay procedure provided an unequivocal test for the presence or absence of the DNA sequences of the β- and δ-globin genes with a sample of DNA of 20 micrograms or less. By using this combination of somatic cell genetics and cloned DNA probes it was possible to precisely localize the β-globin gene complex on chromosome 11.

MATERIALS AND METHODS

Cells. The Hela cell and human fibroblasts were used as sources of human DNA. The Chinese hamster ovary cell (CHO-K1), hybrid clone J1 and a panel of regional hybrid clones J1-7, J1-8, J1-9, J1-10, J1-11, J1-21, J1-23, J1-27, and J1-37 were used as clones containing varying amounts of human chromosome 11. All cells were maintained in alpha medium (without nucleosides) supplemented with 13% fetal calf serum.

Preparation of DNA. Cells were grown in Corning T-150 flasks to an

approximate cell density of 2×10^8 cells per flask. The cells were washed several times with phosphate-buffered saline and harvested by trypsinization. The cells were collected by centrifugation and washed again with phosphate-buffered saline. The cell pellet was resuspended in 10 ml of TNE buffer (10mM trisHCl pH 8.0, 100mM NaCl, 1mM EDTA). An equal volume of TNE buffer containing 1% sodium dodecyl sulfate was added slowly, and the solution was mixed gently. Proteinase K (EM Laboratories, Elmsford, NY) was then added to a final concentration of 200 µg/ml. This solution was incubated for 19 hr at 37°C and then extracted once with an equal volume of buffer-saturated phenol and three times with chloroform:isoamyl alcohol, 24:1 (vol/vol). The aqueous phase was removed with a wide-mouth pipette after the final extraction, and 2.5 vol of isopropyl alcohol was added and gently mixed inversion. The DNA precipitate was removed with a curved tip glass rod without spooling. The excess isopropyl alcohol was drained and the DNA was transferred to a tube containing 2 ml of TE buffer (10mM TrisHCl pH 8.0, 1mM EDTA), incubated for 12 hours at 25°C without shaking, and stored at 4°C.

Digestion of DNA with Retriction Enzyme. Aliquots (20 µg of DNA were digested with restriction enzymes in 0.1 ml final volume as follows:

(a) Digestion with Xba 1. To each DNA aliquot was added 15 units of Xba I (New England Biolabs) in the presence of 0.15 M NaCl,6 mM TrisHCl pH7.9, 6mM MgCl , 6mM 2-mercaptoethanol.

(b) Digestion with EcoRI. To each DNA aliquot was added 60 units of EcoRI (Boehringer Mannheim) in the presence of 100 mM TrisHCl pH7.5, 50mM NaCl, 10mM MgCl , 2mM 2-mercaptoethanol.

Digestion with either enzyme was carried out for 2 hr at 37°C. The restriction enzyme was inactivated by incubation of the sample for 5 minutes at 65°C.

Gel Electrophoresis and Hybridization of DNA. The sample was made 8% in Ficoll (MW 400,000) (Sigma) and loaded on a 1% agarose gel in a Blaircraft gel apparatus. Electrophoresis was effected at 40 V for 15 hr. The gel was stained with ethidium bromide (0.5 µg/ml) for 30 min, and the DNA was visualized with ultraviolet light. The gel was then incubated for 45 min in 1M NaOH with gentle shaking and the for two successive 20 min periods in 1M TrisHCl pH7.6, 1.5M NaCl. The DNA was then transferred to nitrocellulose as described by Southern (22) except that 20XSSC was used instead of 6XSSC. Filters were baked after transfer for 2 hr under reduced pressure at 80°C. The filter was prehybridized in a sealed plastic bag (Seal'n Save, Sears) at 67°C for 3 hr in a solution containing 10ml of 6XSSC, 10XDenhardt's Solution (0.2% bovine serum albumin, 0.2% polyvinylpyrollidone, 0.2% Ficoll), 100 µg/ml denatured salmon sperm DNA or calf thymus DNA, and 0.1% SDS. The filter was then rinsed in 3XSSC and air dried.

For hybridization the filter was placed in a plastic bag with one end open. Hybridization solution (17 µl/cm^2) was added (6XSSC, 10XDenhardt's, 100 µg/ml carrier DNA, 0.1% SDS and $3X10^6$ cpm of ^{32}P-labeled hybridization probe per ml). Air bubbles and excess hybridization solution were removed by rolling a test tube over the bag. The area of the bag containing the filter was then sealed. Hybridization was carried out at 67°C for 24 hr. The filter was then washed at 67°C in 500 ml portions of the following: 1 hr with 3XSSC,

10XDenhardt's,0.1%SDS; three times for 1 hr with 2XSSC, 0.1% SDS; and 1 hr with 1XSSC, 0.1% SDS. The filter was blotted dry and subjected to autoradiography with XR-5 x-ray film (Kodak), with a Dupont Cronex intensifying screen, at -70°C for 2-7 days.

Preparation of Hybridization Probes. Two cloned DNA fragments were used as hybridization probes. Both were derived from the human β-globin genomic clone HβG-1 isolated by Maniatis and coworkers (16). A probe specific for the β-globin coding region (Hβ1) and a probe specific for the δ-coding region (Hδ1) were obtained by digestion of the HβG-1 clone with the restriction enzyme Pst I and subcloning of the appropriate fragments in the plasmid vector pBR322. These clones were kindly provided by T. Maniatis. DNA was prepared from Hβ1 and Hδ1 as described by Wilson et al. (51). The plasmid DNA was labeled with ^{32}P to a specific activity of approximately $2X10^8$ cpm/μg by the procedure of Rigby et al. (52).

RESULTS

Characterization of deletion subclones

Ten deletion subclones were used in this study. Each has been previously been assayed for the enzyme markers LDH-A and ACP-2, and for the antigen markers SA11-1,-2, and-3 which are encoded on chromosome 11 (Table I). The locations of these markers on chromosome 11 is shown in Figure 1. Because several of the subclones have the same phenotype

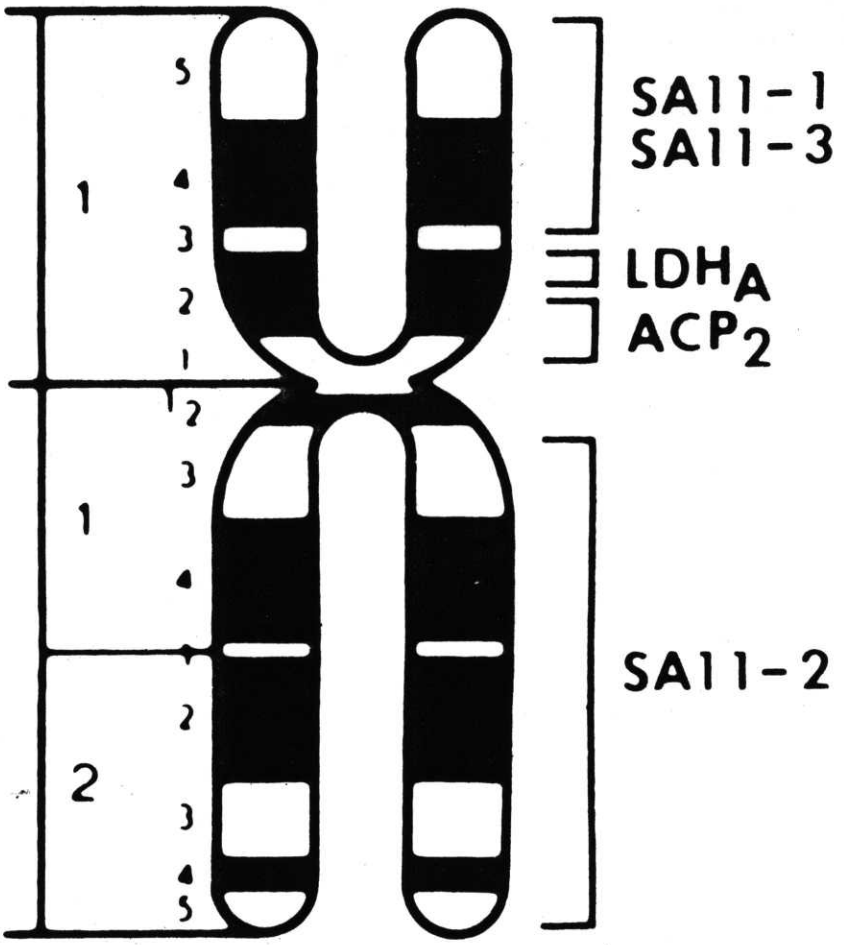

5
4
3
2
1
1
1
2
3
4
2
2
3
4
5
SA11-1
SA11-3
LDH$_A$
ACP$_2$
SA11-2

FIGURE 1: Diagram Showing the Locations on Chromosome 11 of Antigen and Enzyme Markers.

The locations of the loci SA11-1, SA11-2, SA11-3, LDH-A, and ACP-2 are shown along with the giemsa-banding pattern of chromosome 11. The designation of major regions and band numbers displayed in the figure is explained in the text.

TABLE I - CHARACTERIZATION OF CHROMOSOME 11 DELETION CLONE PANEL

Cell Hybrid	Terminal Deletion	Presence of Specific Human Markers					Assay for β-Globin Gene
		SA11-1	SA11-3	LDH_A	ACP_2	SA11-2	
J1	None	+	+	+	+	+	+
J1-7	p11-pter	-	-	-	-	+	-
J1-8	p1205-pter	-	-	-	+	+	+
J1-9	p1205-pter(?)	-	-	-	+	+	-
J1-10	p1208-pter	-	-	-	+	+	+
J1-27	Not Tested	-	-	-	+	+	+
J1-37	Not Tested	-	-	-	+	+	+
J1-23	p13-pter	-	-	+	+	+	+
J1-21	Not Tested	-	-	+	+	+	+
J1-24	Not Tested	-	-	+	+	+	+
J1-11	q13-qter	+	+	+	+	-	+

? See Chapter 4

with respect to these markers, they have also been characterized by chromosome banding techniques. In some cases, subclones with identical complements of markers differed in the extent of their respective deletions by cytogenetic analysis. Figure 1 displays the numbering system used for bands visualized by Giemsa staining of chromosome 11. The long arm consists of two major regions designated q1 and q2. Bands in each region are numbered sequentially beginning proximally and proceeding distally. Since the long arm of any chromosome is denoted by "q", the various bands of the long arm are q11, q12, q13, q14 (in region q1) and q21, q22, q23, q24, q25 (in region q2). the short arm consists of only one major region which contains five bands, p11, p12, p13, p14, p15. The termini of the long arm and of the short arm are designated qter and pter respectively. Locations within visible bands can be described by following the recommendation of the Paris Conference Supplement (53) for designation of breakpoints within a chromosomal band. The band is subdivided into 10 equal parts (numbered 01,02,...,10), the 01 region being closest to the centromere. Thus, in Figure 1 the location of LDH-A would be in the interval p1208-p1210 and the location of ACP-2 would be denoted as the interval p11-p1205. The subdivision of bands is determined subjectively and is therefore subject to considerable error. Furthermore the visualization of banding patterns of human chromosomes in hamster-human hybrid cells is more difficult than in human lymphoblasts (in which the banding pattern of chromosome 11 was originally described). Thus, the breakpoints estimated for the clones used in this study (Table I and Figure 10) are likely to be accurate at the level of the visible bands but the intraband designations listed in Table I should only be used to imply

relative locations of the breakpoints within the same band.

Demonstration of the Absence of the Human β-Globin Gene in CHO-K1 and Its Presence in the J1 Hybrid

The initial experiments in this study were designed to determine whether the Southern hybridization procedure could be used to specifically detect the human β-globin gene in a human-hamster hybrid cell. DNA was isolated from the J1 cell line and from the parental hamster line CHO-K1 and was digested with restriction endonuclease Xba I, fractionated by agarose gel electrophoresis, transferred to a nitrocellulose filter, and hybridized to a cloned DNA probe for the human β-globin gene. A typical experiment is shown in Figure 2. An intense hybridization was evident in the 12-13 kb region for J-1 (lane 2) but not for CHO-K1 (lane 1). Hybridization in this region was also observed with a sample of human DNA treated in a similar manner. These results indicate the presence of the human β-globin gene in J1 and its absence in CHO-K1, thus confirming the original assignment of the gene to human chromosome 11 by Deisseroth et al. (41).

Demonstration that the β-Globin Gene Complex Lies in the Interval between the Respective Deletions of Clones J1-7 and J1-10

As an initial experiment to localize the β-globin gene complex on chromosome 11, deletion subclones J1-7, J1-9, J1-10, J1-11 and J1-23 were assayed for the presence of the β-globin gene. All three Xba I-digested DNA samples from J1-10, J1-11, and J1-23 showed hybridization in the 12-13 kb region (Figure 2, lanes 5-7). These

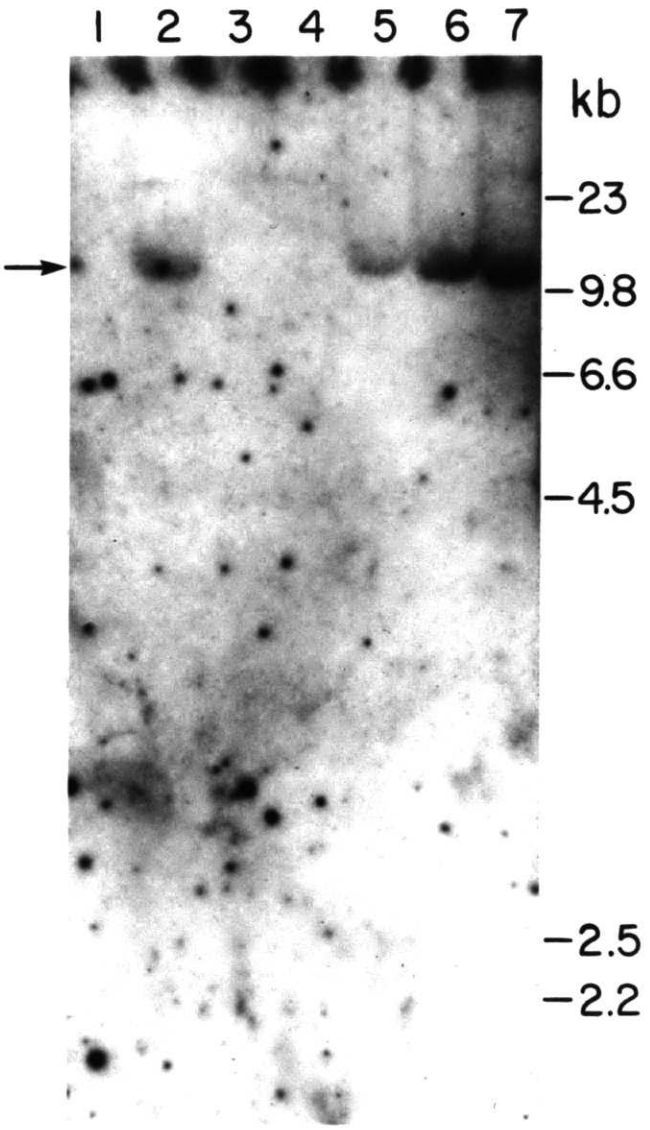

1 2 3 4 5 6 7
kb
23
9.8
6.6
4.5
2.5
2.2

FIGURE 2: Hybridization of Hβ1 Probe to Xba I-digested Cell DNAs. DNA extracted from the indicated cell line was digested with Xba I, fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labeled Hβ1 plasmid DNA. DNA derived from λcI857Sam7 DNA digested with HindIII was run in an adjacent lane; these fragments ran at the positions indicated on the right and serve as approximate size markers. A sample of human DNA was run in another lane and the position of the band of hybridization, corresponding to the fragment described by Lawn et al. (16) is indicated by the arrow. Lanes: 1, CHO-K1; 2, J1; 3, J1-7; 4, J1-9; 5, J1-10; 6, J1-11; 7, J1-23.

results imply that the β-globin gene is not present on the portion of the short arm deleted in J1-10 (p12ɔ6-pter). With DNA from J1-7 and J1-9, no significant hybridization was observed in the 12-13 kb region.

To confirm this preliminary result, similar experiment was performed using DNA preparations digested with the restriction endonuclease EcoRI, which cuts the β-globin gene within the coding region to yield 6.5 and 4.0 kb fragments (54,55). The 6.5kb band contains the portion of the β-globin gene which codes for the 5' end of the mRNA; the 4.0 kb segment includes the portion of the gene encoding the 3' end of the mRNA. As expected, a sample of CHO-K1 DNA (Figure 3, lane 1) failed to show significant hybridization at either 6.5kb or 4.0kb. Hybrid clones carrying partially deleted chromosome 11 exhibited the hybridization pattern consistent with the pattern seen in Figure 2 for Xba I-digested DNA. Clones J1-10, J1-11, and J1-23 displayed intense hybridization in the 6.5kb and 4.0kb regions while clones J1-7 and J1-9 did not.

Another experiment designed to confirm these results was performed using a probe corresponding to a different portion of the 12kb Xba I fragment. This probe included the section of the fragment corresponding to the coding sequences of the human δ-globin gene. Basic details of this experiment were similar to the experiment shown in Figure 2. Exactly the same set of results was obtained (Figure 4). Intense hybridization was observed in the 12-13 kb region of the Xba I-digested DNA of clones J1, J1-10, J1-11, and J1-23, but no significant hybridization was observed for clones J1-7 and J1-9 or for CHO-K1. Because the β- and δ-globin genes lie about 7kb apart (16), these results confirm the localization of these genes to the short arm

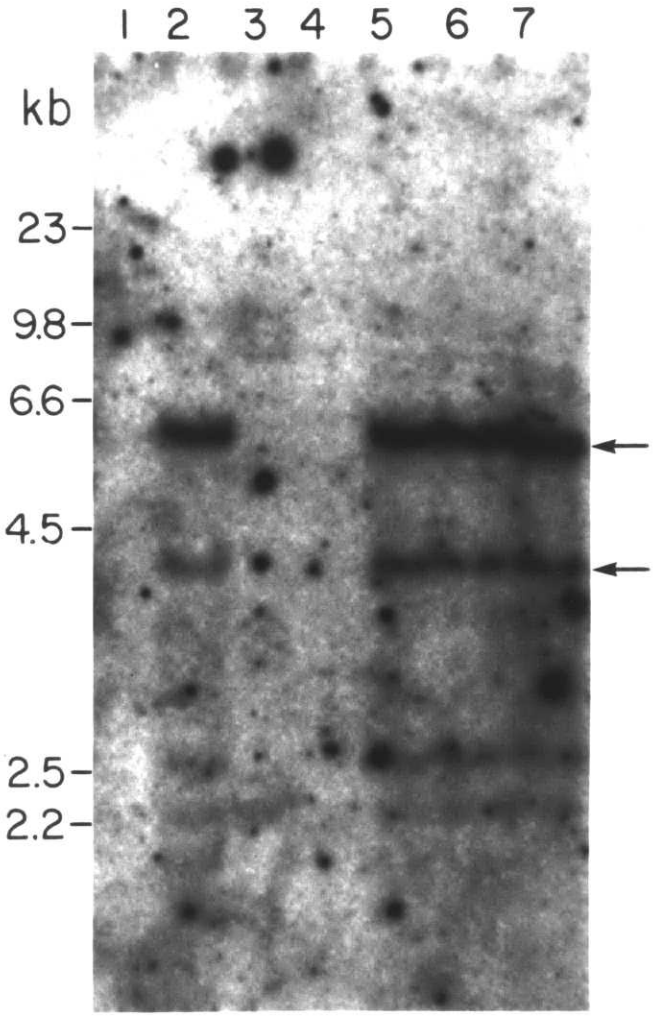

1 2 3 4 5 6 7
kb
23
9.8
6.6
4.5
2.5
2.2

FIGURE 3: Hybridization of Hβ1 Probe to EcoRI-digested Cell DNAs. DNA extracted from the indicated cell line was digested with EcoRI, fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labeled Hβ1 plasmid DNA. DNA derived from λ cI857Sam7 DNA digested with HindIII was run in an adjacent lane; these fragments ran at the positions indicated on the left and serve as approximate size markers. A sample of human DNA was run in another lane to locate the positions of the normal human 6.5kb and 4.0kb EcoRI fragments (indicated by the arrows on the right) on this gel. Lanes: 1, CHO-K1; 2, J1; 3, J1-7; 4, J1-9; 5, J1-10; 6, J1-11; 7, J1-23.

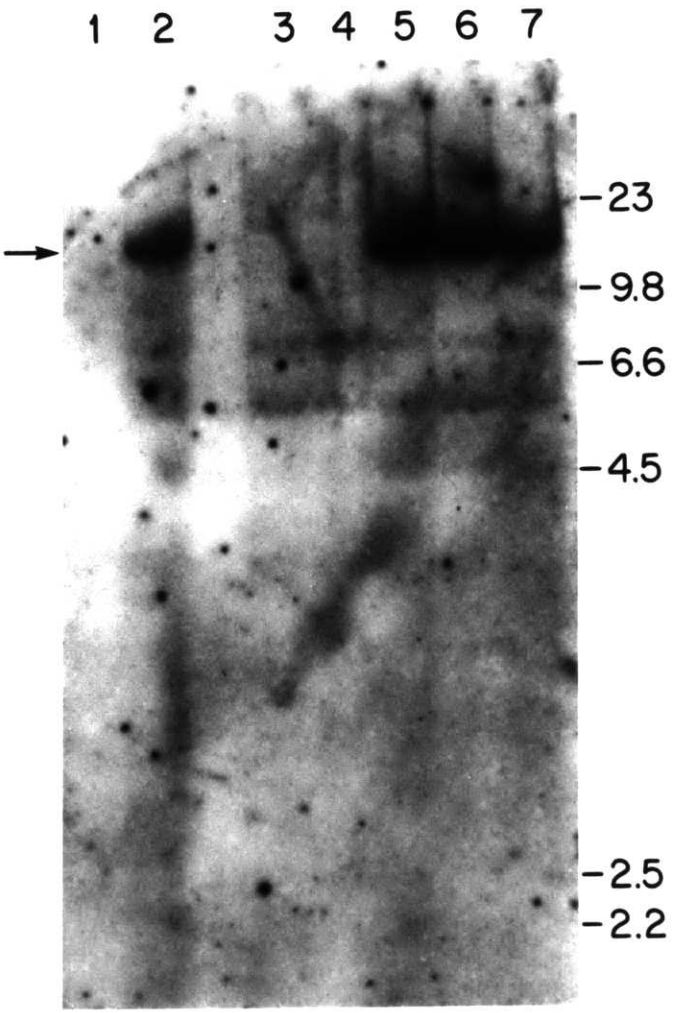

1 2 3 4 5 6 7
23
9.8
6.6
4.5
2.5
2.2

FIGURE 4: Hybridization of Hδ1 Probe to Xba I-digested Cell DNAs. DNA extracted from the indicated cell line was digested with Xba I, fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labeled Hδ1 plasmid DNA. DNA derived from λcI857Sam7 DNA digested with HindIII was run in an adjacent lane; these fragments ran at the positions indicated on the right and serve as approximate size markers. A sample of human DNA was run in another lane and the position of the band of hybridization, corresponding to the fragment described by Lawn et al. (16) is indicated by the arrow. Lanes: 1, CHO-K1; 2, J1; 3, J1-7; 4, J1-9; 5, J1-10; 6, J1-11; 7, J1-23.

of chromosome 11.

Precise Localization of the β-Globin Genes

The results outlined above imply that the β-globin gene complex is located in the intraband region p1205-p1208 on the short arm of chromosome 11. The precision of this assignment depends on the assumption that each of the deletion subclones resulted from a single terminal deletion of the short arm with no secondary internal deletion. Such a secondary deletion would be difficult to detect cytologically and could lead to an incorrect assignment. In subsequent experiments, to be described in Chapter 4, it was discovered that clone J1-9 appears to contain an internal deletion and could therefore yield misleading results. The results presented above therefore only allow the assignment of the β-globin gene complex to the region p11-p1208.

In order to further localize these globin genes and to minimize the effect of any undetected deletions, the number of deletion subclones under examination was expanded. DNA from each cell line was digested with enzyme EcoRI, fractionated on an agarose gel and transferred to nitrocellulose. The cloned β-globin probe described previously was used to assay for the presence of the 6.5kb and 4.0kb fragments in each DNA (Figure 4). Both fragments were present in the DNA of clones J1-10, J1-11 and J1-23 as expected, and were also present in the DNA of clones J1-8, J1-21, J1-24, J1-27, and J1-37. Neither fragment was present in the DNA of CHO-K1 or clone J1-7.

These results are consistent with the assignment of the β-globin gene complex to the region p11-p1205. The globin genes are clearly

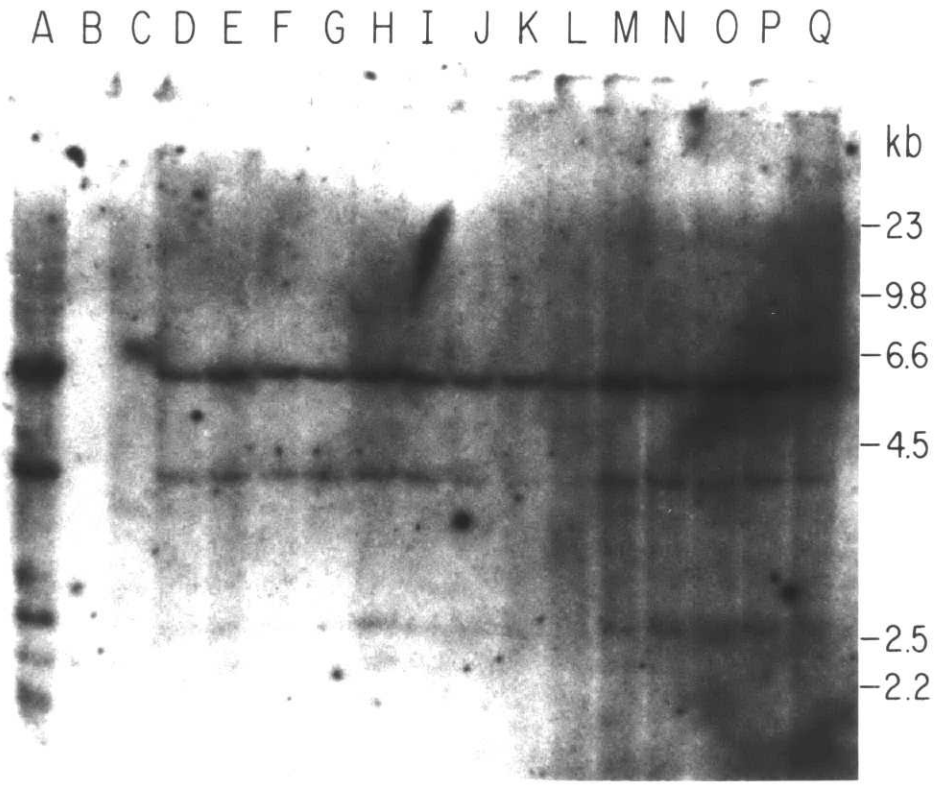

A B C D E F G H I J K L M N O P Q
kb
23
9.8
6.6
4.5
2.5
2.2

FIGURE 5: Hybridization of Hβ1 Probe to EcoRI-digested DNAs from an expanded Panel of Hybrid Clones. DNA extracted from the indicated cell line was digested with EcoRI, fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labeled Hβ1 plasmid DNA. DNA derived from λcI857Sam7 DNA digested with HindIII was run in an adjacent lane; these fragments ran at the positions indicated on the left and serve as approximate size markers. Lanes: A, J1-11; B, CHO-K1; C, J1-7; D, J1-8; E, J1-10; F, J1-27; G, J1-37; H, J1-23; I, J1-21; J, J1-24; K, 157A2; L, 157A6; M, 157A9; N, 156E5; O, 156E10; P, 156F3; Q, 156F8. The source of the DNAs from lanes K-Q will be discussed in Chapter 5.

closer to the centromere than the LDH-A locus but their position with respect to the ACP-2 locus remains uncertain. Testing for ACP-2 and β-globin in more subclones in which the breakpoint of the deletion has occured centromeric to the LDH-A locus should eventually resolve this issue.

DISCUSSION

The experiments presented here make it possible to assign the interval, consisting of 34 kb, containing the cluster of globin genes Gγ-Aγ- δ- β (56) to the approximate region p1205-p11 of the short arm of human chromosome 11. The sensitivity of the method and the relatively small number of cells required commend this approach for mapping other genes that are not expressed directly in cell hybrids. It should be noted that a number of other investigators have also combined somatic cell genetics and recombinant DNA techniques to map the β-globin gene complex on chromosome 11. Scott et al. (57) were able to confirm the presence of the β-globin gene on chromosome 11 while excluding it from the region 11q23-qter using a human-mouse hybrid which contained a single chromosome of human origin. This chromosome resulted from a translocation of most of chromosome 11 to the q25-26-qter region of the X chromosome. Lebo et al. (58) used combination of Southern hybridization and chromosome sorting to test a number of cells containing translocations involving chromosome 11 for the location of the β-globin gene complex. They initially assigned these genes to the distal portion of the short arm, including the regions 11p13,11p14 and

11p15 in disagreement with the study presented here. Upon reanalysis of the critical karyotypes, however, the assignment was changed to include the regions of 11p12, 11p13, 11p14, and 11p15. Similarly, both Sanders-Haigh et al. (59) and Jeffreys et al (60) have used a series of somatic cell hybrids containing translocated pieces of chromosome 11 in conjunction with the Southern blotting technique to localize the β-globin gene to the region of 11p11-11p15. None of these investigators were able to map the globin gene with the precision that was made possible here by the use of a panel of deletion subclones.

While the present study utilized hybrids containing the single human chromosome 11 and its deletion subclones as a model system to explore the possibilities of this approach, similar methods could be applied to the mapping of other human genes. Two cellular requirements are necessary to perform the type of fine structure genetic analysis described here. One is the availability of hybrids retaining one or a small number of specific human chromosomes. There are currently numerous cell lines which meet this requirement (61-63) and which if taken together represent all of the human chromosomes (63). The second requirement involves methods for securing a series of subclones with varying terminal deletions in a given chromosome. It has been demonstrated that low doses of x-rays in combination with a negative selection against a distal marker is extremely effective in producing such deletions (38,39). Selection against the presence of a cell surface antigen was used in this study, but negative selection schemes based on the loss of a particular enzyme activity should be equally effective.

The resolving power of the method presented here presumably can be

increased by isolating large numbers of deletion clones of chromosome 11 that have lost LDH-A. Each of these subclones will presumably have lost a different amount of the short arm and they should eventually yield an exact localization of the β-globin genes. They should also prove useful for the fine structure mapping of the genetic region which includes ACP-2, the β-globin gene complex and LDH-A as further DNA and protein markers become available.

CHAPTER THREE

SPECIES-SPECIFIC REPETITIVE DNA

INTRODUCTION

The application of recombinant DNA methodology has provided a broad range of new approaches to the problem of gene organization in higher eukaryotes. The ability to construct recombinant libraries from nuclear DNA (15) permits the application of this methodology to the analysis of the entire genome of any eukaryotic organism. This chapter describes hybridization probes and conditions which will identify cloned DNA sequences in such a library based on their species of origin. The approaches described here provide the basis for a method to isolate chromosomally localized DNA segments from man or from the mouse. They also have implications with respect to the organization and evolution of repeated DNA sequences in mammals.

MATERIALS AND METHODS

Cells. The G149A5 hybrid cell line containing a single mouse chromosome 16 on a hamster background and its parent lines AdeC$^-$ DTR and C3H/HeJ fibroblasts were supplied by Dr P. Gerald and were maintained as in Chapter 1.

Recombinant Libraries. The human library used in this study was supplied by Dr T. Maniatis (15). The hamster library and G149A5 library were constructed by inserting 15-20 kb fragments from a partial EcoRI digest of CHO-K1 DNA into Charon 4A as described in chapter 4. The mouse library, constructed in essentially the same fashion from the DNA of BALB/c mouse was provided by Dr A. Bothwell.

Hybridization Conditions. Benton-Davis Filters were soaked for 3 hours at 67°C in 500 ml of 4XSET (1XSET= 30 mM TrisHCl pH 8.0, 150mM NaCl, 2mM EDTA), 10XDenhardt's solution, 0.1% $Na_4P_2O_7$, 0.1% SDS. Each set of filters was then prehybridized in a sealed plastic bag in 10 ml of the same solution with the addition of 100 µg/ml denatured calf thymus DNA. After 1 hour at 67°C, the bag was opened and $5X10^6$ cpm of the appropriate probe was added. ^{32}P-labeled probes (S.A. $2X10^8$ cpm/µg) prepared by nick translation of human, hamster or mouse DNA (52). Hybridization was for 24 hr at 67°C. The filters were then washed in 500 ml portions of the following solutions at 67°C: 1 hour in 3XSET, 0.1% $Na_4P_2O_7$, 0.1% SDS; 2X 0.5 hours in 2XSET, 0.1% SDS; 2X 0.5 hours in 1XSET, 0.1% SDS; and 1 hour in 0.3XSET. The filters were blotted dry and subjected to autoradiography using XR5 X-ray film (Kodak) at -70°C with a Dupont Cronex intensifying screen.

RESULTS AND DISCUSSION

The goal of the experiments described here was to identify hybridization probes and conditions which distinguish cloned DNA

segments from three mammalian species, the mouse, the Chinese hamster, and man. Previous studies on the distribution of repeated DNA sequences in the DNA of mammals including the mouse and man indicate that repeated DNA sequences are interspersed among unique or single copy sequences in a highly complex pattern (28). In particular, it has been shown that almost all unique DNA sequences are within 4000 base pairs of such a repeated DNA sequence (30,31).

These results imply that if a recombinant DNA library were constructed from genomic DNA 15kb to 20kb in length then almost all of the recombinant clones in such a library should include at least one reiterated DNA sequence. In order to test this hypothesis, a hybridization probe consisting of repeated DNA sequences was prepared and hybridized to phages from such a library by the plaque-hybridization procedure originally described by Benton and Davis (64). Preliminary experiments led to the conclusion that a hybridization probe of this type could be obtained simply by incorporating high specific activity ^{32}P deoxynucleotide triphosphates into the genomic DNA of the appropriate species by the nick translation procedure (52). Individual phages from libraries constructed from 15-20kb DNA segments isolated from human, Chinese hamster and mouse cells were spotted in 10 by 10 grids on a fresh lawn of bacteria and after incubation overnite at 37 °C, DNA from the phage spots was transferred to nitrocellulose filters. Filters were made in triplicate from each grid and one filter from each group was hybridized to a high specific activity probe obtained by nick translation of the genomic DNA from one of the three species. Hybridization to DNA on the filter was detected by autoradiography. The outcome of this procedure is shown in

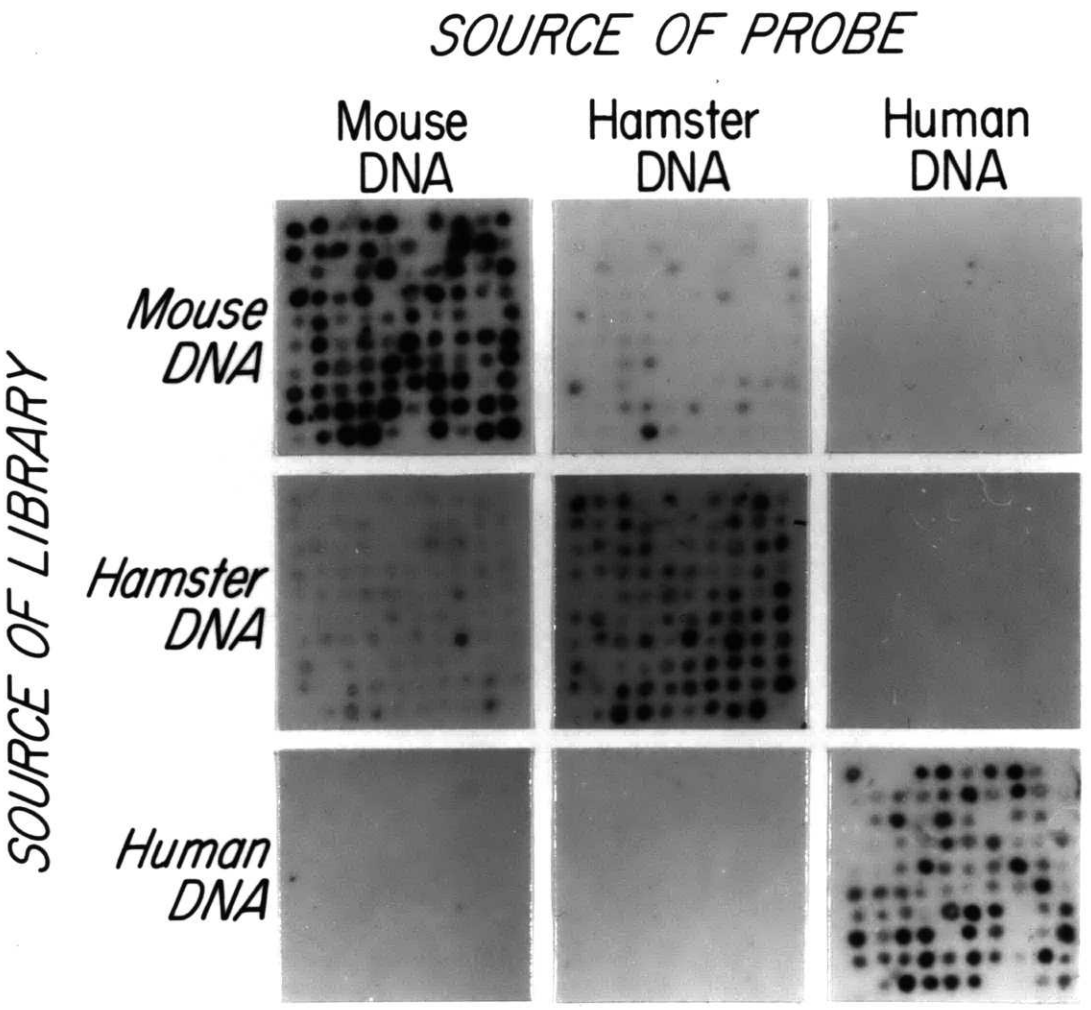

SOURCE OF PROBE
Mouse DNA
Hamster DNA
Human DNA
SOURCE OF LIBRARY
Mouse DNA
Hamster DNA
Human DNA

FIGURE 6: The Detection of Species-Specific Reiterated DNA in Recombinant DNA Libraries.

Individual phages from the indicated libraries were spotted in 10X10 grids on bacterial dishes and triplicate filters were made from each plate. These were hybridized to nick translated mouse, hamster or human DNA as indicated.

Figure 6. It is readily apparent that a very high proportion of the clones hybridize to the DNA probe from the homologous species. Results from an extensive series of experiments of this type are summarized in Table II. In all three cases over 90% of the clones in the library are detected by the homologous DNA probe.

It is also apparent in Figure 6 that none of the clones in the human DNA library exhibit significant hybridization to the mouse or hamster DNA probes. Similarly, none of the clones in the mouse or hamster libraries exhibit significant hybridization to the probe obtained from human DNA. These results are significant for a number of reasons. The ability to distinguish cloned DNA segments on a species-specific basis can be used in conjunction with appropriately segregated somatic cell hybrids between human and hamster cells or between human and mouse cells to isolate cloned human DNA segments from specific regions of individual human chromosomes. This methodology has already proved useful for the isolation of such cloned DNA segments from human chromosome 11 as will be described in chapter 4 and from the human X chromosome.

The observation that over 90% of the clones in the libraries give a positive hybridization signal in response to a homologous hybridization probe provides further confirmation of the original observations of Britten and Davidson and subsequent workers (28) on the degree of interspersion of repeated DNA sequences in mammals. The failure of a human DNA probe to hybridize significantly to hamster or mouse DNA clones and the failure of a mouse or hamster probe to hybridize to human DNA clones is a further indication that significant evolutionary divergence has occurred among the interspersed repeated DNA sequences

TABLE II - HYBRIDIZATION OF RECOMBINANT PHAGES TO HOMOLOGOUS AND HETEROLOGOUS SPECIES PROBES

	Proportion of Clones Showing Significant Hybridization (Out of 1000 Tested)		
	MOUSE LIBRARY	HAMSTER LIBRARY	HUMAN LIBRARY
Mouse Probe	96%	48%	<1%
Hamster Probe	37%	98%	<1%
Human Probe	<1%	<1%	96%

of human and rodent DNA (34).

The pattern of hybridization of the mouse and hamster DNA probes to clones from the mouse and hamster DNA libraries is also of interest for a number of reasons. The fact approximately 60% of the mouse clones which do hybridize to a mouse DNA probe fail to hybridize to a hamster DNA probe indicates that chromosomally localized mouse DNA segments can be isolated from hamster-mouse somatic cell hybrids which have been segregated to contain specific mouse chromosomes.

Preliminary experiments involving a somatic cell hybrid segregated to contain mouse chromosome 16 on a Chinese hamster chromosomal background have led to the successful isolation of eight cloned DNA segments localized to mouse chromosome 16. A recombinant library consiting of 15-20kb EcoRI fragments from the hybrid cell G149A5 was constructed in the recombinant vector Charon 4A. Individual phages from this library were spotted into grids as above and duplicate filters were made from each grid. One filter of each pair was hybridized to nick-translated mouse DNA while the other was hybridized to nick translated hamster DNA. Phages displaying more intense hybridization with mouse DNA probe than with hamster DNA probe were selected as clones of putative mouse origin. Two of these phages were further analyzed. Nitrocellulose filters were prepared containing EcoRI-digested DNA from the hybrid cell line (G149A5) containing mouse chromosome 16, from the CHO parent line (Ade-C$^-$ DTR) and from C3H fibroblasts (from which the chromosome 16 was derived). Subfragments of the two putative mouse clones were selected (in order to avoid repetitive DNA), labeled by nick translation and used as probe against these filters. Figure 7 shows the results of this experiment. The

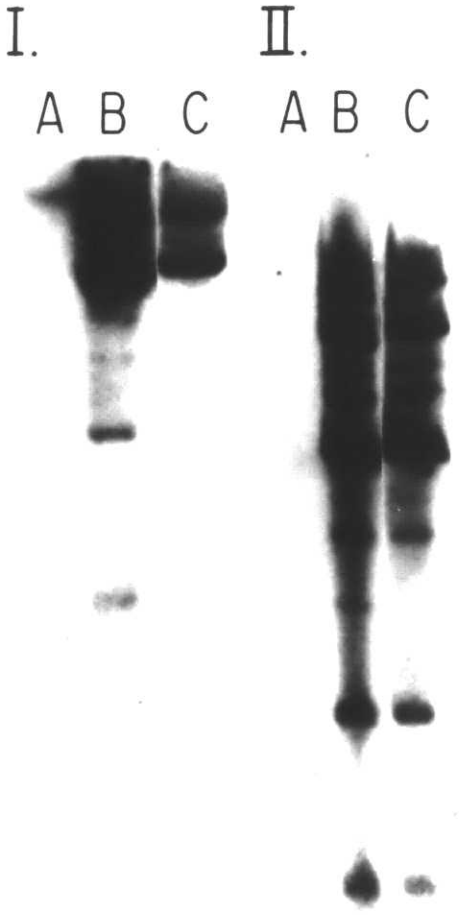

I.
II.
A B C
A B C

FIGURE 7: Hybridization of clones M16-3 and M16-4 to CHO, C3H mouse and G149A5 hybrid cell DNA

Panel I- A 1.5kb EcoRI band from phage M16-3 was selected for use as probe (because it failed to hybridize to ^{32}P-labeled mouse DNA in an assay similar to that used in Chapter 4 for human DNA clones) against EcorI digested DNA from CHO (cell line AdeC^{-} DTR), C3H mouse fibroblasts and G149A5 hybrid cells (which contain a mouse chromosome 16 on a hamster background). The DNAs had been resolved on a 1.44% agarose gel. The phage M16-3 possessed a total of 5 EcoRI fragments (5.5, 3.0,2.9,1.7, and 1.6 kb)

Panel II- A similar experiment to panel I was performed except that the gel used was 1% agarose. In this case, the probe was a 1.9kb HindIII fragment from phage M16-4 which contained a single EcoRI fragment of 13kb.

In both panels the order of the lanes is : A, Chinese hamster; B, C3H mouse; C, G149A5 Hybrid.

probe from clone M16-3 (panel 1) showed intense hybridization to DNA from both the C3H mouse (lane B) and the hybrid cell (lane C). Light hybridization was also detected to a single fragment in hamster DNA (lane A). The probe from clone M16-4 also hybridized to a number of fragments present in both C3H DNA (lane B) and in G149A5 DNA (lane c) as well as one fragment present in C3H DNA but not present in the hybrid cell DNA. This fragment presumably derives from a mouse chromosome other than 16. Clone M16-4 showed no hybridization to hamster DNA (lane A). The intense hybridization of both of these clones to C3H DNA suggests that they are of mouse origin while the chromosome complement of the hybrid cells demands that, if this is the case, they must derive from mouse chromosome 16. Clearly the application of this approach to chromosomal localization of cloned mouse DNA segments can serve as a powerful adjunct to classical methods of gene mapping in the mouse.

A further point of interest is that many mouse clones do show some hybridization to a hamster DNA probe and many hamster clones hybridize to a mouse DNA probe. In both cases the hybridization to the homologous probe is generally more intense than to the heterologous probe. These results are consistent with the view that the degree of evolutionary divergence of the interspersed DNA sequences between mouse and hamster is less than the degree of divergence of these sequences between rodents and humans (34). While this is not unexpected, an interesting aspect of the result shown in Figure 6 is that the repeated DNA sequences represented in different clones in the hamster and mouse libraries appear to exhibit different degrees of evolutionary divergence from each other based on their relative intensity of

hybridization with each probe. Because of the essentially qualitative nature of the current series of experiments it cannot be determined whether the degree of evolutionary divergence represents a continuum or if two or more dicrete classes of such sequences exist, one class completely divergent (no cross-hybridization) and the other still largely homologous. A quantitative spot hybridization assay such as that described by Kafatos et al. (65) might decide this issue. Furthermore an analysis of the way in which these sequences have diverged in species in which some homology between interspersed repeated DNA can still be detected might shed some light on the mechanism by which these sequences remain related as a family in a given species. A systematic comparison of cloned DNA segments which contain either conserved or non-conserved repeated DNA segments might be of value in this context. The procedures described here would be appropriate for the identification of such segments.

CHAPTER FOUR

CLONED DNA SEGMENTS FROM HUMAN CHROMOSOME 11

INTRODUCTION

There are two major practical reasons for attempting to achieve the most complete possible fine structure analysis of the human genome. First, delineation of the DNA sequence of individual genes can be used to diagnose the presence of human disease. These procedures can be applied to in utero situations so that birth of tragically defective babies can be prevented. But perhaps of even greater ultimate importance to biomedical science is the fact that definition of DNA sequences over large chromosomal regions, including both sequences specifying protein structures as well as the non-coding intervals within and between these sequences, appears to promise greater understanding of physiological and biochemical mechanisms of human gene regulation. The genetic data so achieved should illuminate many aspects of medicine and developmental biology including situations not usually considered to lie within the narrow scope of the term "genetic disease".

The application of recombinant DNA techniques has already had a significant impact on human genetics. The isolation of cloned human globin genes (10,66) has been instrumental in the analysis of the fine structure of these genes (55,67,68) and in determining the physical basis of the genetic lesions responsible for the α and β thalassemia

syndromes (69,70). Cloned globin probes also have been used to exploit polymorphism in restriction enzyme recognition sites as a basis for antenatal diagnosis of sickle cell anemia and δβ-thalassemia (71,72). However, a general method for producing DNA probes from specific chromosomal regions has not been available. At present approximately 300 genetic markers have been assigned to specific chromosomal locations, but these are not sufficient in number to permit linkage studies in many cases where such information would be important to the intrauterine diagnosis of genetic disease. In addition these markers are too few in number to permit fine structure mapping of specific genetic regions.

In the present study, an approach is presented which uses recombinant DNA methods in combination with somatic cell genetics to isolate cloned DNA segments from defined regions of human chromosome 11 which has been selected as a model system and to map these on the chromosome. The approach is general and appears applicable to the isolation of cloned DNA segments from other human chromosomes. The construction of a fine structure map of a human chromosomal region at the DNA level appears feasible using this technology. The ability to achieve this goal should have a significant impact on human genetics at a variety of levels.

MATERIALS AND METHODS

Preparation of DNAs. DNA was prepared from Hela, CHO-K1, and hybrid cells as described in Chapter 2. Digestions were performed under the reaction conditions suggested by the supplier of the restriction

enzymes (New England Biolabs) except in the case of EcoRI (Boeringer-Mannheim) where the reaction conditions specified in Chapter 2 were used. After inactivation at 65°C, digested DNAs were extracted, once with phenol and twice with chloroform:isoamyl alcohol 24:1, and then precipitated with etnanol. DNAs were resuspended in a minimum volume of TE buffer (10mM TrisHCl pH8.0, 1mM EDTA).

Charon 4A bacteriophage was propagated in liquid culture and its DNA prepared as previously described (73). Charon 4A arms were separated from the internal fragments by sucrose gradient centrifugation of EcoRI-digested phage DNA (15). 15-20 kb DNA fragments from CHO-K1 and J1-11 were isolated after partial EcoRI digestion by the method of Maniatis et al. (15). These DNAs were ligated to Charon 4A arms as described by Varsanyi-Breiner et al. (73). An in vitro encapsidation procedure was used to obtain J1-11 and CHO-K1 libraries (73,74). The total human library was kindly provided by Dr T. Maniatis.

Screening of J1-11 Library. Grids of recombinant phages were obtained by transfer with sterile toothpicks of individual plaques from plates with 100-300 p.f.u. onto a fresh lawn on a 150mm bacterial dish. Duplicate filters were made from each grid as described by Benton and Davis (64). and were hybridized to total nick translated Hela DNA or CHO-K1 DNA respectively (10^6 cpm/filter, S.A. $2x10^8$ cpm/μg) as described by Varsanyi-Breiner et al. (73). Clones containing human DNA sequences were chosen by virtue of their hybridization to Hela DNA but not to CHO-K1 DNA. The corresponding phage were plaque-purified and DNA was prepared for them as for Charon 4A.

Preparation of Clone-Specific Hybridization Probes. Each putative human-Charon 4A recombinant DNA was digested with EcoRI and a number of other restriction enzymes (BamHI, SacI, HindIII, HaeII) and the resulting fragments were first resolved by agarose gel electrophoresis and then transferred to nitrocellulose. Filters were hybridized as above to nick translated Hela DNA to locate fragments containing repetitive human sequences. For each clone a fragment not hybridizing to the Hela DNA probe was chosen for use as probe against similar filters containing EcoRI digested DNAs from the panel of deletion hybrids. This fragment was prepared by the freeze-squeeze procedure (75) and nick translated to high specific activity by the method of Rigby et al. (52).

RESULTS

The following strategy was employed for the isolation of DNA segments from chromosome 11. First, a library of recombinant clones was constructed from the DNA of the hybrid cell J1-11 which contains the short arm and centromeric region of chromosome 11. The procedure used was similar to that described by Maniatis et al. (15). DNA fragments 12-20kb in length were isolated from the hybrid cell DNA, after partial digestion with EcoRI, by size fractionation on a sucrose gradient. These fragments were then cloned in the λ phage vector Charon 4A which can accept 8 to 22 kb of foreign DNA. Each recombinant phage therefore contained a single 12-20 kb fragment of human or Chinese hamster origin.

The second step was to use the species specific probes described in

the previous Chapter to identify those phages carrying DNA of human origin. An additional test of the ability of the of the methods described in Chapter 3 to distinguish DNA of human origin from that of hamster origin is shown in Figure 6. Individual phages from libraries of human DNA, hamster DNA or J1-11 DNA were spotted in a grid on a fresh lawn of bacteria. After growth of the phage, the DNA from each spot was transferred to a nitrocellulose filter. ^{32}P-labeled human DNA was then used as a hybridization probe against these phage DNAs. As expected, the hybridization probe is effective in discriminating cloned DNA segments of human origin. The first two rows of the grid consisted of Charon 4A clones carrying Chinese hamster DNA. None of these clones hybridized to the probe. The next two rows contained clones picked at random from a library of human DNA in Charon 4A. Almost all of these clones hybridized to the human DNA probe. Included in the array was the phage HβG1 carrying the coding regions for the human β- and δ-globin genes isolated by Lawn et al (16). The DNA of this phage hybridized intensely with the probe. In the remaining rows of the grid, the phage contain cloned DNA segments from the hybrid cell J1-11. Of the clones in this array, one out of 234 hybridized intensely with the human DNA probe. A systematic survey of over 20,000 clones from this library yielded approximately 40 recombinants of putative human origin.

Twelve clones were selected at random from the initial 40 for further characterization. The goal of these studies was to determine the distribution of reiterated DNA sequences within the cloned DNA segments in order to locate restriction fragments containing little or no repetitive DNA. The analysis of DNA from one of the clones, H11-3,

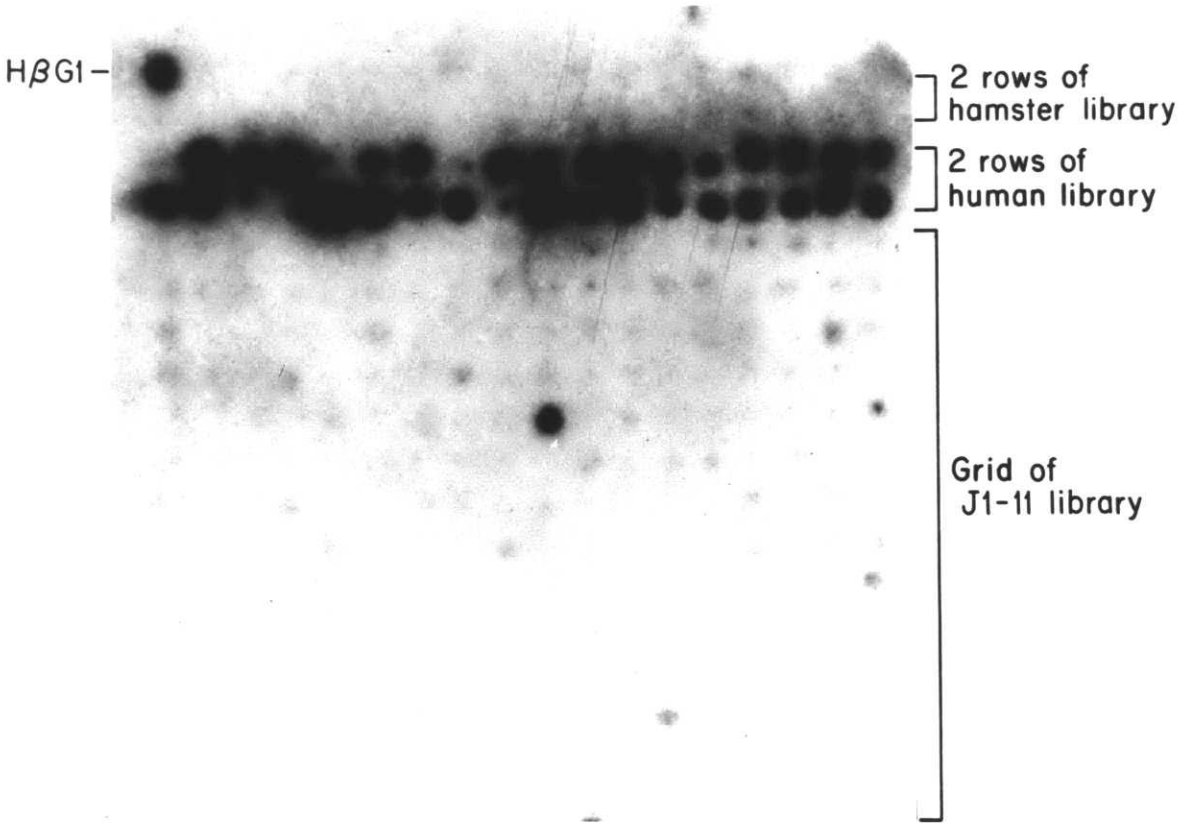

HβG1
2 rows of hamster library
2 rows of human library
Grid of J1-11 library

FIGURE 6: Detection of Cloned Human DNA Segments in The J1-11 Library.

Individual phage from the indicated recombinant libraries were spotted on a 150 mM bacterial Dish as described in Materials and Methods. A filter replica was made and hybridized to 10^6 cpm of ^{32}P-labeled human DNA. The human β-globin genomic clone HβG1 (16) was also spotted in this array.

is shown in Figure 9. The DNA from this clone was digested with the restriction enzymes EcoRI or BamHI and fractionated on a 1% agarose gel. The DNA fragments were then visualized by staining with ethidium bromide. The pattern obtained is shown in Figure 9, panel I. EcoRI digestion of clone H11-3 DNA (lane A) produced four fragments: the Charon 4A arms (x and z), a dimer of these arms annealed at the terminal cohesive ends (w) and a single inserted fragment (y) of 15 kb. BamHI digestion (lane B) produced 9 fragments: 4 consisting entirely of DNA from the Charon 4A arms (c,d,e,h) and 5 containing at least some of the human cloned sequence (a,b,f,g,i).

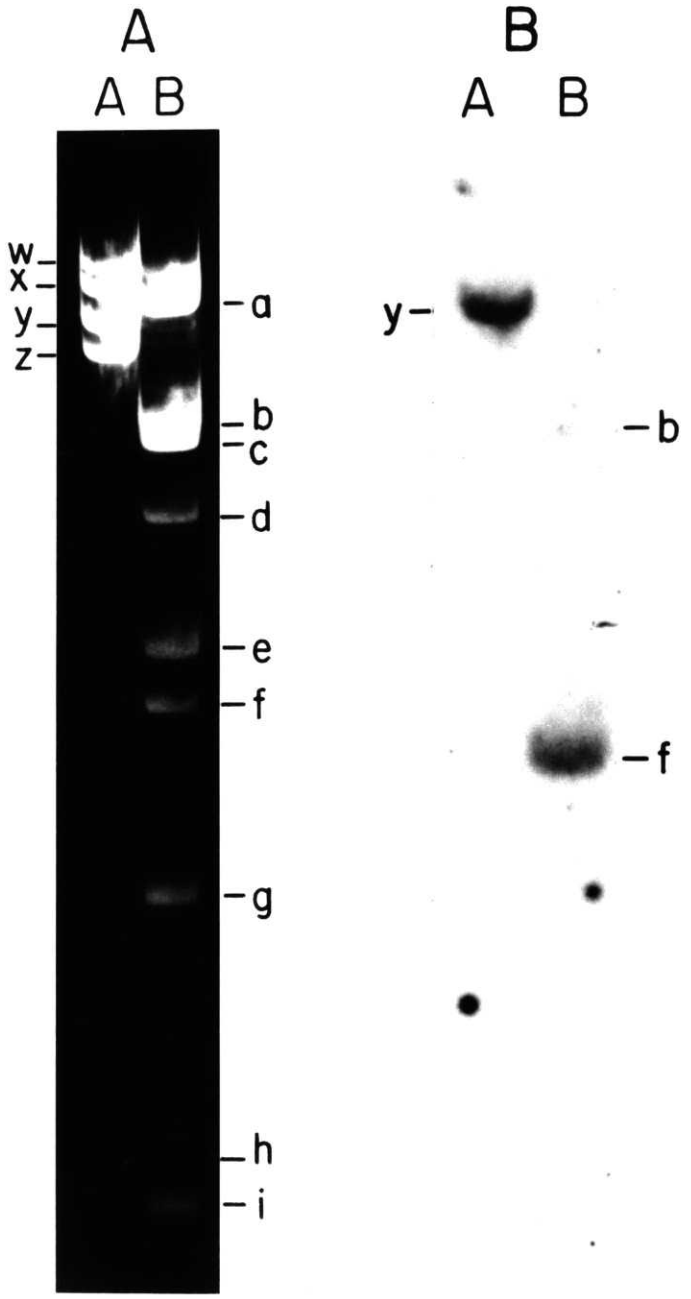
Following visualization of the DNA fragments, the DNA was transferred to a nitrocellulose filter and was hybridized to ^{32}P-labeled human DNA ($2\lambda10^8$ cpm/μg). The results are shown in Figure 9, panel 2. Although fragment y from the EcoRI digestion and two of the DNA fragments (b,f) from the BamHI digestion hybridized to the human DNA probe and therefore presumably contained some reiterated human DNA sequences, three fragments (a,g,i) produced by BamHI digestion of clone H11-3 did not hybridize to the probe under these conditions and therefore were presumed to contain only single-copy DNA. One of these fragments, g, was chosen as a probe for further hybridization experiments.

In order to localize this cloned DNA segment to a specific region of chromosome 11 an experiment similar to those used in Chapter 2 to map the β-globin gene was performed. DNA was isolated from the following cell lines: CHO-K1, the parent cell line of the human-hamster hybrids; J1 carrying the entire human chromosome 11 on a Chinese hamster background; and the four deletion subclones J1-11,

FIGURE 9: Characterization of Clone H11-3

Panel I: Ethidium bromide stained restriction fragments of clone H11-3. lanes: A, EcoRI; B, BamHI. Restriction fragments were resolved on a 1% agarose gel and visualized by staining with 0.5 μg/ml ethidium bromide. Fragment y is the single 15kb human EcoRI fragment inserted in this clone.

Panel II: Hybridization of restriction fragments of clone H11-3 to ^{32}P- Human DNA. the fragments seen in Panel I were transferred to nitrocellulose and hybridized to ^{32}P-Human DNA. Fragments b and f contained some human repetitive DNA. Fragments c, d, e, and h consisted entirely of Charon 4A vector DNA. Fragments a, g, and i showed no hybridization to the ^{32}P Human DNA and fragment g was chosen for use as a "single-copy" sequence probe.


A
A B
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A B
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b
f

J1-7, J1-10, and J1-23 whose breakpoints divide chromosome 11 into 5 convenient regions as represented in Figure 10. The DNAs were digested with EcoRI, fractionated on an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled fragment 'g' from DNA clone H11-3. Based on the pattern shown in Figure 9, this probe is expected to hybridize to a 15 kb DNA fragment in EcoRI-digested DNA. As expected, hybridization was observed in this region of the gel in DNA samples from hybrid lines J1 and J1-11(Figure 11). The pattern of hybridization to clones J1-7, J1-10 and J1-23 can be used to ascertain the chromosomal location of the DNA fragment isolated and cloned in H11-3. The presence of intense hybridization in DNA isolated from all three cell lines indicates that the H11-3 segment is located in the centromere-linked region delineated by the breakpoints of deletion subclones J1-7 and J1-11.

A number of DNA clones were mapped in a similar fashion to H11-3. In each case a probe fragment containing only single-copy DNA was prepared (with the single exception of clone H11-13 discussed below) and the criterion for presence of the cloned DNA segment in a hybrid was the detection of an EcoRI band similar in size to one of those cloned in the phage.

The characterization of the 12 recombinant phage chosen for analysis is shown in Table III and the results of the mapping experiments are summarized in Table IV. At least one clone was localized to each of the regions of the short arm of chromosome 11 delineated by the panel of cell hybrids (Figure 10). Two experiments displaying some interesting features of the mapping experiments are shown in Figure 12.

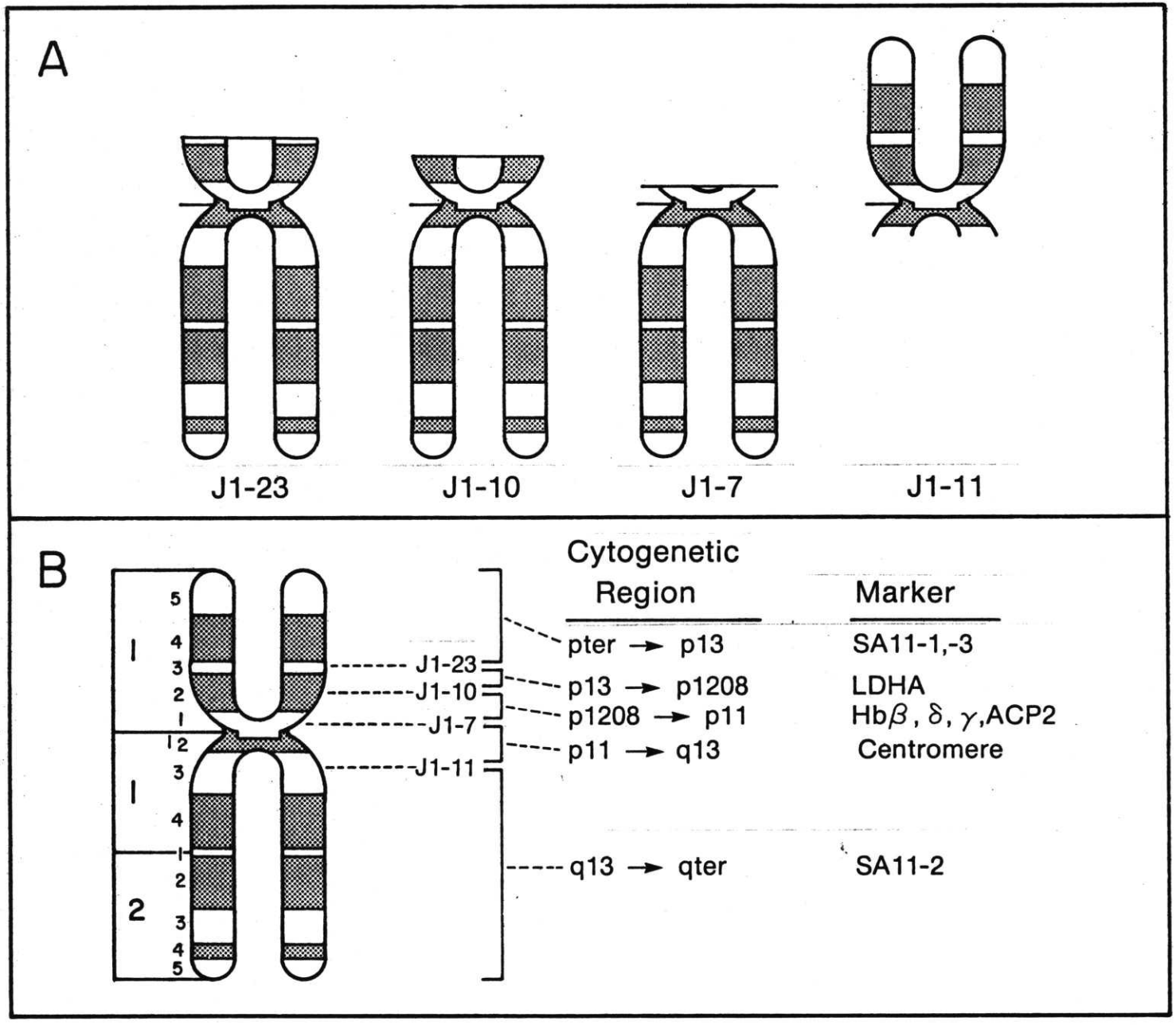
A
J1-23
J1-10
J1-7
J1-11
B
Cytogenetic Region
Marker
pter → p13
SA11-1,-3
p13 → p1208
LDHA
p1208 → p11
Hbβ, δ, γ,ACP2
p11 → q13
Centromere
q13 → qter
SA11-2
J1-23
J1-10
J1-7
J1-11
1
2
1
2
3
4
5

FIGURE 10: A) Diagram showing the various terminal deletions of human chromosome 11 in the four cell hybrids.

B) Schematic representation of human chromosome 11 with arrows indicating the breakpoints at which terminal deletions have occurred in the four clones. The four breakpoints divide chromosome 11 into five cytogenetic regions each of which is characterized by the various markers indicated.

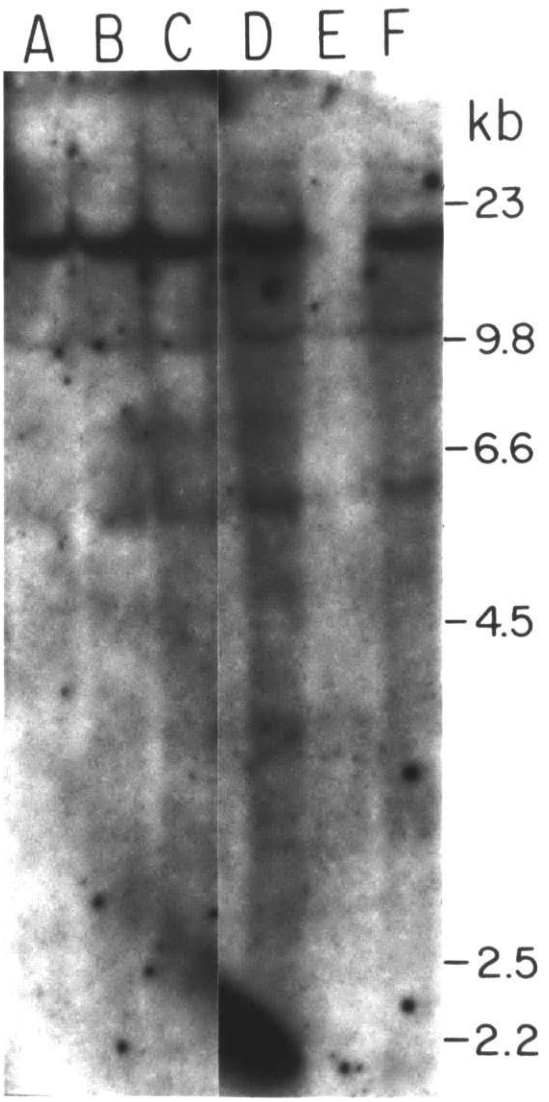
A B C D E F
kb
23
9.8
6.6
4.5
2.5
2.2

FIGURE 11: Localization of Clone H11-3 on Chromosome 11. EcoRI digested DNA from cell lines J1-11 (A), J1-23 (B), J1-10 (C), J1-7 (D), CHO-K1 (E) and J1 (F) was fractionated on a 1% agarose gel at 40 V for 15 hr and was transferred to nitrocellulose. The filter was hybridized to nick translated fragment "g" from a BamHI digest of clone H11-3 (see Figure 9). A HindIII digest of λ cI857Sam7 was run in a parallel lane to provide approximate size markers.

TABLE III - CHARACTERIZATION OF RECOMBINANT CLONES FROM J1-11 LIBRARY

Putative Human Clone	EcoRI Fragment(s) Inserted (Kb)	Characteristics of Probe Fragment Chosen	
		Size (Kb)	Enzyme Used
H11-3	15	3.0	BamHI
H11-6	9.5, 5.2	2.5	HaeII
H11-8	19	2.1	HindIII
H11-9	10, 4.9	1.6	HindIII
H11-11	12	2.0	SacI
H11-13	2.7, 2.4, 2.1, 2.0, 1.7	2.7	EcoRI
H11-17	15	3.2	SacI
H11-19	8.9, 7.8	1.6	SacI
H11-20	17	3.2	SacI
H11-21	10.5, 5.6	3.0	BamHI
H11-22	11.5	2.5	SacI
H11-39	16	1.4	SacI

TABLE III- Characterization of Recombinant Clones from J1-11 Library.

The indicated clones were analysed to determine the distribution of repetitive sequences in the human insert. DNA from each recombinant was digested with enzymes EcoRI, BamHI, HindIII, SacI or HaeII and the fragments were resolved on 1% agarose gels. The DNA was transferred to nitrocellulose and each filter was hybridized to 10^6 cpm nick translated Hela DNA as described in Materials and Methods. The sizes of the EcoRI fragments inserted in the phages was determined relative to a parallel lane of HindIII-digested λcI857Sam7 DNA. For each recombinant a fragment was located which failed to hybridize to the probe and which was therefore presumed to be derived from human 'single-copy' DNA. This fragment was utilized in subsequent experiments as a probe against appropriately digested human or human-hamster hybrid cell DNAs.

TABLE IV - MAPPING OF CLONED DNA SEGMENTS ON CHROMOSOME 11

Recombinant Clone	Presence in Deletion Hybrid										Localization
	J1-11	J1-7	J1-8	J1-9	J1-10	J1-27	J1-37	J1-23	J1-21	J1-24	
H11-3	+	+		-	+			+			q13-p11
H11-6	+	+		-	+			+			q13-p11
H11-8	+	-			-			+			p1208-p13
H11-9	+	-			-			+			p1208-p13
H11-11	+	-	-		-	-	-	-	+	+	p13-pter
H11-13	+	-		-	-			+			p1208-p13
H11-17	+	+			+			+			q13-p11
H11-19	+	+			+			+			q13-p11
H11-20	+	-			+			+			p11-p1208
H11-21	+	-			+			+			p11-p1208
H11-22	+	-			+			+			p11-p1208
H11-39	+	-			-			+			p1208-p13

Empty spaces indicate hybrids not tested.

Figure 12-I shows an experiment in which clone H11-9 was mapped to the region p1208-p13 of the short arm. The probe used was a 1.6kb HindIII fragment of the H11-9 insert which failed to hybridize to ^{32}P-human DNA and was therefore presumed to contain only 'single copy' DNA. In Figure 12-I, hybridization was observed at the expected 10kb fragment size (the size of one of the inserted EcoRI fragments in H11-9) in EcoRI-digested DNA from cell lines J1-11 and J1-23 but not in the DNA from cell lines CHO-K1, J1-7, or J1-10. A similar pattern of hybridization was observed at 4.5kb. Since no fragment of this size is found in clone H11-9, this band must result from a cross-hybridizing sequence also present on chromosome 11. Similarly when this fragment was used as probe against EcoRI-digested Hela DNA, a number of fragments were observed in addition to those which ran at 10kb and 4.5kb. These fragments may have been due to a different pattern of EcoRI sites in the two alleles present in these cells, or more likely, to the presence of cross-hybridizing sequences present on other chromosomes. If the latter is the case then some of the DNA which is here referred to a 'single copy' actually occurs several times per genome.

Figure 12-II shows an experiment in which clone H11-13 was localized to the p1208-p13 region of the short arm of chromosome 11. This experiment is noteworthy because it demonstrates that even when no fragment containing only 'single copy' DNA can be found in a cloned segment, the cloned DNA can often still be mapped by using as probe a fragment containing some repetitive sequence DNA. The probe used in this case was a 2.7kb EcoRI fragment which showed some hybridization to ^{32}P-human DNA. As can be seen in Figure 12-II a considerable

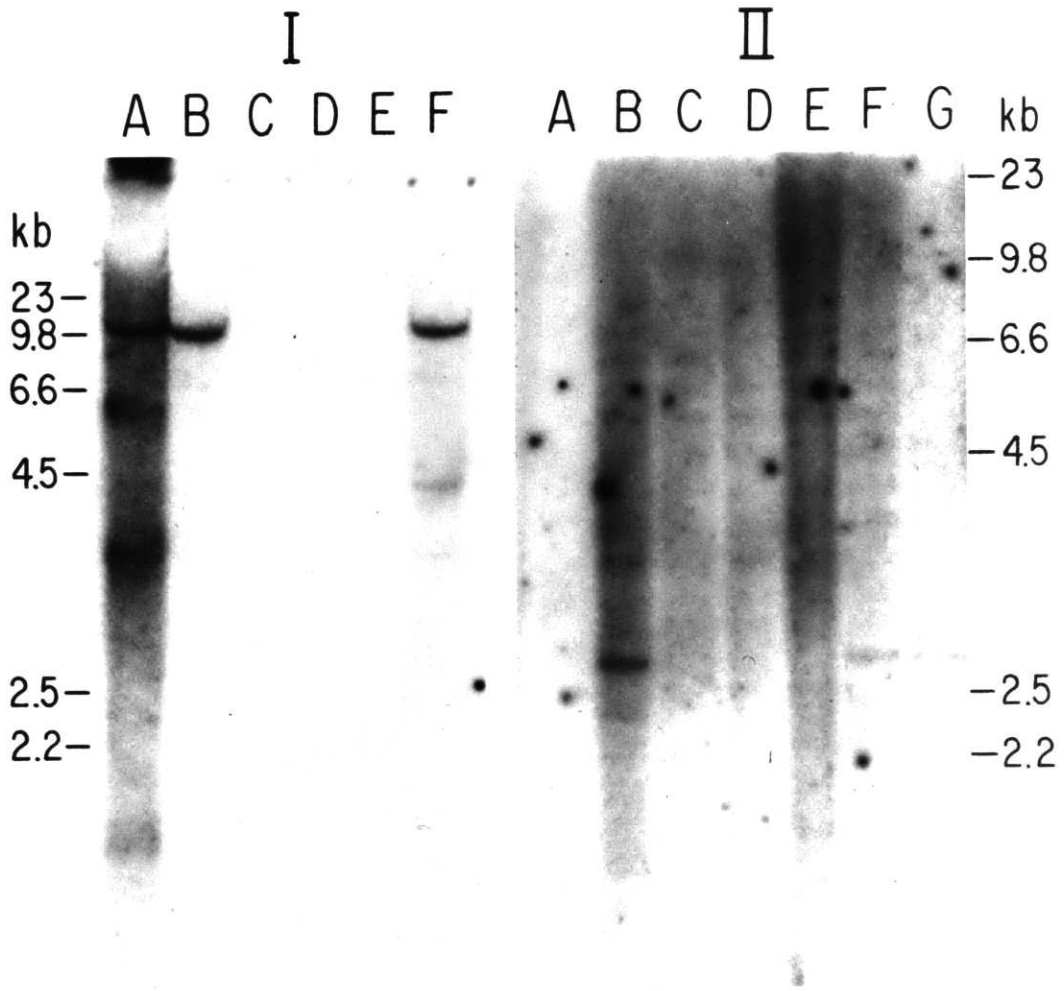
I
II
A B C D E F
A B C D E F G kb
kb
23
9.8
6.6
4.5
2.5
2.2
23
9.8
6.6
4.5
2.5
2.2

FIGURE 12: Panel I- Localization on chromosome 11 of the DNA segment from clone H11-9.

EcoRI-digested DNAs were transferred from a 1% agarose gel and hybridized to a ^{32}P-labeled restriction fragment derived from clone H11-9 as described in Materials and Methods. Lanes: A, Hela; B, J1-23; C, J1-10; D, J1-7; E, CHO-K1; F, J1-11.

Panel II-Localization on chromosome 11 of the DNA segment from clone H11-13.

This experiment was performed as in Panel I except that the probe, in this case derived from clone H11-13, was known to contain some repetitive sequence DNA. Lanes: A, CHO-K1; B, J1; C, J1-7; D, J1-9; E, J1-10; F, J1-23; G, J1-11.

The size markers in both Panels are derived from a HindIII digest of λcI857Sam7 DNA.

background haze of repetitive sequence hybridization was visible for any hybrid DNA but not for CHO-K1 DNA. Despite this repetitive DNA hybridization, it was possible to detect significant hybridization to the expected 2.7kb EcoRI fragment in the DNA of cell lines J1, J1-11, and J1-23. No such fragment was observed in DNA from hybrids J1-7, J1-9, or J1-10. While it is clear that fragments containing some repetitive DNA can be used to chromosomally localize cloned DNA sequences in hybrid cells containing only one human chromosome, they can not be used as probes against cells containing a full complement of human chromosomes. Therefore, in the present study, fragments containing only 'single copy' DNA were used in most cases.

A number of other experiments in which cloned DNA sequences were mapped on chromosome 11 are shown in Figures 14, 15, and 16. These results are summarized in Table IV and will not be discussed in detail here.

One final point of interest in these mapping experiments which was referred to in Chapter 2 is the apparently anomalous result that a DNA segment present in J1-7 is not present in J1-9. This appears to be the case for both clones H11-3 and H11-6. Given the lack of sensitivity of cytogenetic techniques to detect small deletions and the method by which the J1-9 cells were isolated (after UV irradiation) it is likely that J1-9 possesses not only a terminal deletion of the short arm of chromosome 11, but also an internal deletion proximal to the ACP-2 locus. This makes the deletion clone J1-9 useless for the current mapping experiments but with enough cloned DNA probes, it should eventually be possible to map the extent of this deletion. What this result does emphasize however is the advantage that the use of DNA

probes has over standard methods of characterizing the chromosome complements of somatic cell hybrids.

It should be noted that the clones isolated in this study yield a linear chromosomal map consistent with the assumption that the deletion hybrids were all (except J1-9) derived by terminal deletion of chromosome 11. If this is the case the structure of the termini of these deleted chromosomes would be of great interest since the presumably specialized telomeric sequences (76) would have been removed. It remains possible however that these deletions are internal (leaving telomeric sequences at the tip of the deleted chromosomes). If the deletions are indeed terminal, then it is also possible that the termini of the broken chromosomes have spontaneously "healed" or undergone rearrangement by the bridge-breakage-fusion cycle characteristic of chromosomes without telomeres (77,78). In any case, it would not be surprising if these type of rearrangements cause anomalies in the linear map as more DNA clones and cell hybrids are used. In fact, if the current methodology can be combined with classic linkage techniques of mapping (by using polymorphisms in restriction endonuclease recognition sites) then such rearrangements could be characterized in detail.

DISCUSSION

Chapter 2 of this thesis demonstrated how cloned radioactive DNA probes could be used with a panel of specific deletion mutants of a hybrid cell containing a single human chromosome to secure high-resolution, chromosomal mapping of gene sequences present in the

DNA probe. This chapter shows that a library can be constructed from the single human chromosome of such a hybrid and that the various DNA segments of this library can be mapped on the corresponding human chromosome. No RNA isolation is necessary. The method appears to be general and is based on the high species specificity of the reiterated mammalian chromosomal DNA demonstrated in Chapter 3.

Mammalian gene mapping has made significant progress in recent years, particularly by means of cytogenetic identification of relatively gross regions on each chromosome (79), and by development of powerful methods for determining DNA sequences (48,49). However, one of these techniques operates at the level of millions of base pairs while the other is limited to the handling of thousands. Therefore a large gap in resolving power exists which must be bridged before these techniques can be combined for systematic, high resolution mapping of the human genome. The methodology described in the present study illustrates how this gap can be filled. It would now appear possible to isolate from specific human chromosomes DNA segments of a size which permits complete sequence analysis, and to map these segments on the chromosome.

The methods outlined here extend recombinant DNA technology so that cloned DNA segments can be isolated and characterized based on their genetic map position alone, rather than on their ability to code for a specific mRNA. Human chromosome 11 was chosen for this study because of the opportunity to apply deletion mapping techniques to the localization of cloned DNA segments. The methods devised by Kao, Jones, and Puck (37-39) can be extended to isolate a large number of deletions in the short arm of chromosome 11. Previously the value of a

panel of such hybrid cells was limited by the availability of genetic markers on chromosome 11 and the ability to distinguish deletion end points cytologically. The present study provides an alternative approach to the problem of genetic fine structure which will permit detailed comparison of hybrid cell lines carrying cytologically indistinguishable mutations. A fine structure map at the DNA level of a specific region of chromosome 11 appears feasible in the immediate future by combining the recombinant DNA methodology described here with further deletion mapping.

These advances appear to make possible a number of new excursions in human genetics and genetic disease. These principles can be applied to any single human chromosome hybrid, of which a reasonable number have already been prepared (61,80,81). Indeed, it should also be possible to apply these methods to hybrids containing several human chromosomes.

The present approach appears capable of further development in the direction of materially increasing the resolving power of the mapping process by accumulation of more deletion mutants as well as more DNA probes. Moreover the probes themselves can furnish a new series of markers for genetic analysis. By use of X-irradiation and other chromosome breaking agents to increase the number of deletions in the chromosome under study, one can determine the frequency with which members of a pair of markers are lost or retained and relate these frequencies to their map distance apart, as has been shown by Goss and Harris (82-84) and Law and Kao (81,83). The availability of additional markers should aid the quantitation of mutagenic action which has been proposed for the screening of environmental mutagens and carcinogens

with the use of single human chromosome hybrids (86).

These probes also appear to extend powers of diagnosis of genetic disease in man. For the case of diseases due to homozygous recessive defects caused by gene deletion, the procedure is straightforward. For cases in which a dominant genetic defect can be identified with a significant deletion, rearrangement or polymorphism in DNA sequences it should be possible to prepare hybrids containing single copies of the specific chromosomes and test these with appropriate probes. Alternatively, one might be able to distinguish quantitatively by molecular hybridization with appropriate probes between situations involving single and double gene doses. Finally, it may be possible to detect different patterns resulting from treatment with particular restriction endonucleases and to use these polymorphisms as genetic markers. A similar procedure should be effective in identifying heterozygous carriers of recessive defects. Obviously these techniques would be applicable to cell samples obtained by amniocentesis and therefore could serve for prenatal diagnosis and screening.

CHAPTER FIVE

ANIRIDIA-ASSOCIATED DELETIONS OF CHROMOSOME 11

INTRODUCTION

There are many potential applications of the cloned DNA sequences described in the previous chapter. One of the most important possibilities of interest to clinicians and developmental biologists alike is the analysis of defects in chromosome 11 which in the heterozygous state can produce aniridia and predisposition to Wilm's tumor (3).

It has been estimated that the frequency of occurence of aniridia, a congenital malformation of the iris is one thousand times higher in Wilm's tumor patients than in the population at large (42,43,87). Since not all patients afflicted with aniridia develop Wilm's tumor and vice versa, it is not thought that they result from the lack of a single gene product. It has also become clear that patients who do have both aniridia and Wilm's tumor usually have cytologically detectable deletions in the short arm of chromosome 11 in the vicinity of p13 (42,43). Thus it is likely that the deletion of two or more distinct but linked genes results in these defects. The presumptive loci involved have been dubbed the WAGR complex (3). Also associated with the WAGR complex are other genetic defects including disproportionate growth of one side or one part of the body, overgrowth of the abdominal viscera, large red or brown birthmarks, genitourinary

abnormalities and mental retardation (43,88).

The availability of cloned DNA probes from the short arm of chromosome should allow a delineation of the specific region of the chromosome associated with each of these genetic defects. This study represents a preliminary attempt to identify sequences which have been deleted in two unrelated patients suffering from aniridia. Each of these patients has a detectable deletion constituting approximately 1/8 to 1/4 of the short arm of one chromosome 11 in the vicinity of p13. The ability to detect sequences from these regions could lead to a prenatal test for aniridia which would be useful in families with a history of Wilm's tumor. Perhaps of greater importance however is the possibility that this technology could result in a predictive test concerning predisposition to Wilm's tumor since this cancer is often curable if detected early.

MATERIALS AND METHODS

Cell lines. Cell lines 157A2, 157A6, and 157A9 were derived by fusing total nucleated blood cells cells from patient A with CHO-K1 cells and isolating independent clones which scored positive for LDH-A indicating the presence of a human chromosome 11. Since the hybrids have retained only a few human chromosomes (5-12) it is unlikely that any one cell line has retained both copies of chromosome 11.

Cell lines 156E5, 156E10, 156F3 and 156F8 were derived in a similar fashion using nucleated blood cells from patient B. Patients A and B were two unrelated children afflicted with aniridia. The hybrid cell lines were kindly provided by Dr P. Gerald.

DNA Preparation and digestion, Probe Preparation, Southern Blotting. All of these procedures were performed as described in Chapters 2 and 4.

RESULTS

The goal of this study was to evaluate the usefulness of cloned DNA probes for the investigation of deletions involving the WAGR complex of chromosome 11. Since each of the patients used in this study possessed one deleted and one normal chromosome 11, their DNA could not be tested directly for presence or absence of DNA sequences corresponding to any given cloned probe. Therefore, it was necessary to segregate the two chromosome 11s from each individual. To achieve this goal, somatic cell hybrids were constructed between cells from each patient and Chinese hamster ovary cells. These were allowed to segregate human chromosomes, and then cloned lines containing LDH-A, indicating the presence of a human chromosome 11 in the hybrid cell, were selected for further study. It is unlikely that any of the cell hybrids used here have retained both the deleted and the normal chromosome 11. Unfortunately, because of the difficulty of performing cytogenetic analysis on human-hamster hybrids, it was not possible to determine which hybrids contained a normal chromosome 11 and which had retained only the deleted chromosome. If the segregation of the two chromosomes occurred in an equal but random fashion then the probability is high that both the normal and deleted chromosomes are represented in the current panel of hybrid cell lines.

DNA was prepared from each cell line and the presence of sequences complementary to some of the cloned probes described in the previous chapter was assayed by the Southern blotting procedure. A typical assay is shown in Figure 13. In this experiment, the same 'single copy' probe derived from clone H11-3 which was prepared in the previous chapter was used to detect the presence or absence in these hybrid cell lines of the 15 kb EcoRI fragment inserted in this phage. Since clone H11-3 maps in the region p11-q13 on chromosome 11 and the aniridia-associated deletion is known to involve p13, it was expected that a 15 kb EcoRI fragment would be detected in all hybrid cell lines, regardless of whether they contained a normal or deleted chromosome 11. This in fact was the case, as shown in Figure 13. A second experiment with predictable results is shown in Figure 5. The deletions described thus far in aniridia patients are not thought to involve the β-globin locus. Thus if Hβ1 plasmid were used as a probe against EcoRI digested DNA from this panel of hybrid cell lines, one would expect to observe both the 6.5kb and 4.0kb fragments in all the hybrid lines. Once again, the predicted result was obtained (Figure 5).

A number of other cloned probes were tested against this panel of cell hybrids and the results of these analyses are shown in Table V. As can be seen, all but one of the sequences tested was present in all of the hybrid cells and are therefore unlikely to be included in the deletion of the short arm in these two patients. The testing of two of these clones, H11-17 and H11-20 are shown in Figures 14 and 15 respectively. These experiments act as controls for the one clone which was an exception to the rule. Clone H11-11 which maps to the region p13-pter of the short arm was not found in any of the aniridia

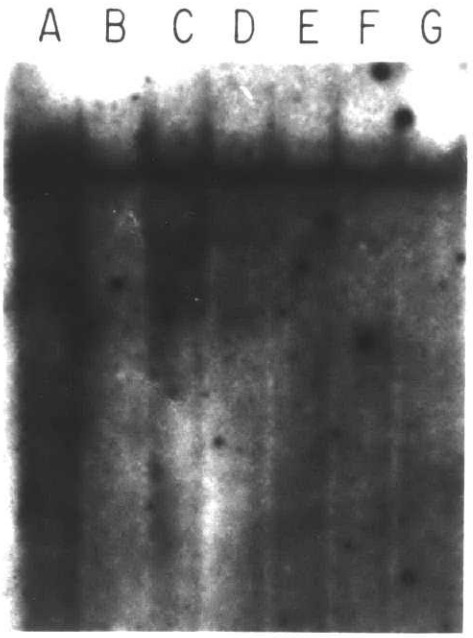
A B C D E F G

FIGURE 13: Presence in Aniridia hybrids of the DNA segment cloned in H11-3.

EcoRI digested DNAs were resolved on a 1% agarose gel, transferred to nitrocellulose and hybridized to nick translated fragment 'g' from clone H11-3 (see Figures 9 and 11). Lanes: A, 157A2; B, 157A6; C, 157A9; D, 156E5; E, 156E10; F, 156F3; G, 156F8.

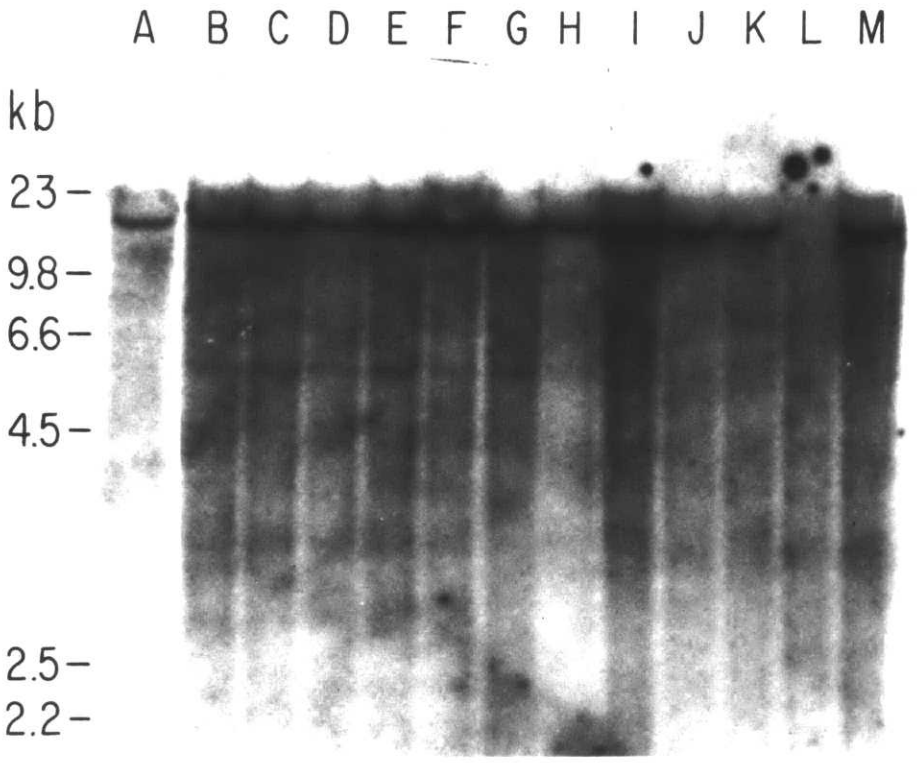
A B C D E F G H I J K L M
kb
23
9.8
6.6
4.5
2.5
2.2

FIGURE 14: Presence in deletion subclones and aniridia hybrids of the DNA segment cloned in H11-17.

EcoRI digested DNAs were transferred from a 1% agarose gel and hybridized to a nick translated 'single-copy' restriction fragment from clone H11-17. Lanes: A, Hela; B, 156F8;, C, 156F3; D, 156E10; E, 156E5; F, 157A9; G, 157A6; H, 157A2; I, J1-23; J, J1-10; K, J1-7; L, CHO-K1; M, J1-11.

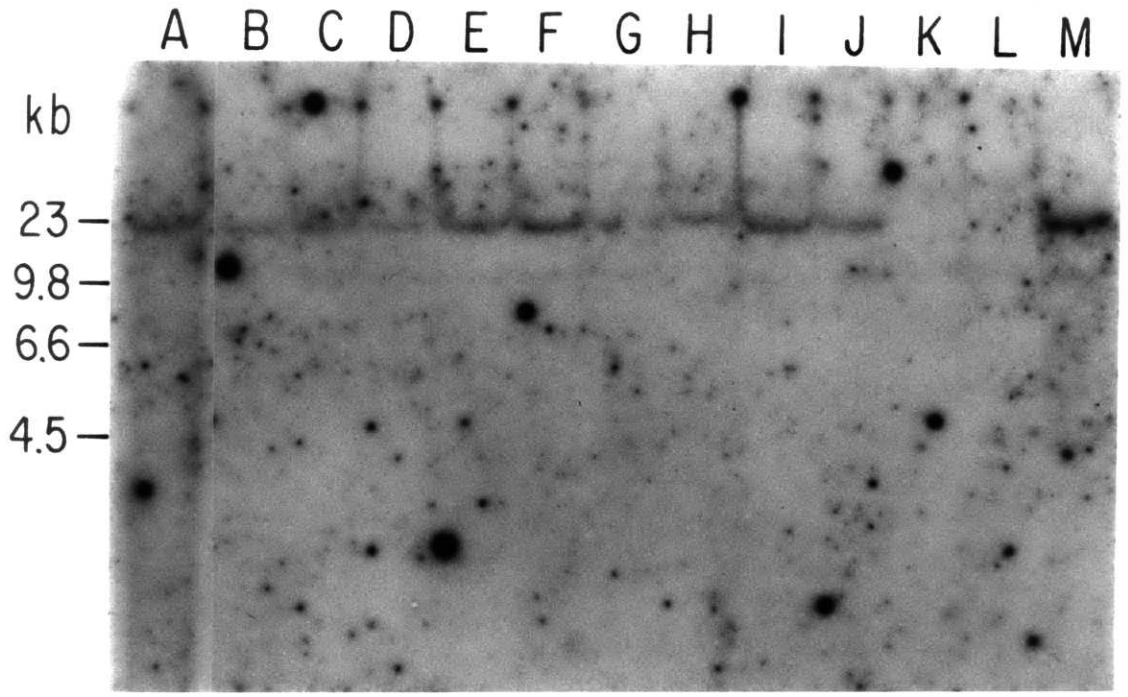
A B C D E F G H I J K L M
kb
23
9.8
6.6
4.5

FIGURE 15: Presence in deletion subclones and aniridia hybrids of the DNA segment cloned in H11-20.

EcoRI digested DNAs were transferred from a 1% agarose gel and hybridized to a nick translated 'single-copy' restriction fragment from clone H11-20. Lanes: A, Hela; B, 156F8;, C, 156F3; D, 156E10; E, 156E5; F, 157A9; G, 157A6; H, 157A2; I, J1-23; J, J1-10; K, J1-7; L, CHO-K1; M, J1-11.

TABLE V - PRESENCE OF CLONED DNA SEGMENTS IN ANIRIDIA HYBRID LINES

Recombinant Clone	Localization on Chromosome 11	Presence in Aniridia Hybrids						
		157 (Patient A)			156 (Patient B)			
		A2	A6	A9	E5	E10	F3	F8
H11-3	q13-p11	+	+	+	+	+	+	+
H11-8	p1208-p13	+	+	+	+	+	+	+
H11-9	p1208-p13	+	+	+	+	+	+	+
H11-11	p13-pter	-	-	-	-	-	-	-
H11-13	p1208-p13	+	+	+	+	+	+	+
H11-17	q13-p11	+	+	+	+	+	+	+
H11-19	q13-p11	+	+	+	+	+	+	+
H11-20	p11-p1208	+	+	+	+	+	+	+

hybrids tested. As can be seen in Figure 16, when a 2.0kb Sac I fragment from clone H11-11 was used as probe against EcoRI+Sac I-digested DNAs, a fragment of 2.0kb was clearly detectable in Hela DNA and J1-11 DNA but not in DNA from CHO-K1, J1-7, J1-10 of this clone to the region p13-pter on chromosome 11. Neither was a fragment of 2.0kb (or any other size) observed in any of the hybrid cells derived from the aniridia patients.

There are a number of possible explanations for this rather unexpected result. First, it is possible that clone H11-11 falls within the aniridia-associated deletion but that all of the hybrid cells contain the deleted chromosome. While this is unlikely if the segregation of the normal and deleted chromosomes was random but equal, it could occur if the presence of the deleted chromosome has a selective advantage due to the presence of a deleterious gene on the normal chromosome. If this were the case then DNA from the original patients should contain a band hybridizing to the probe. Unfortunately, these DNAs are not immediately available. A second possibility is that both patients possess a variant pattern of restriction enzyme recognition sites in this sequence which results in fragments which are too small to detect with the gel system used here (<0.8kb). This possibility could also best be tested using DNA from the patients themselves. This sequence may also have been absent due to deletion in the uncharacterized blood cells used for constructing the aniridia hybrids, even though it was present in the fibroblast used to construct the J1 hybrid and is also present in Hela cells. A fourth explanation for this result would be that the culture conditions used for maintaining the hybrid cells selected for cells containing


A B C D E F G H I J K L M
kb
23
9.8
6.6
4.5
2.5
2.0

FIGURE 16: Absence in deletion subclones and aniridia hybrids of the DNA segment cloned in h11-11.

The indicated DNAs were digested with EcoRI and then with Sac I and the resulting fragments were resolved on a 1.2% agarose gel. After transfer to nitrocellulose the DNAs were hybridized to a 2.0kb 'single-copy DNA' Sac 1 fragment which had been purified from the insert of clone h11-11. The hybridization was performed as described in Materials and Methods with the exception that 10% w/w dextran sulfate (Pharmacia) was included in the hybridization solution (89). Lanes: A, J1-11; B, CHO-K1; C, J1-7; D, J1-10; E, J1-23; F, 157A2; G, 157A6; H, 157A9; I, 156E5; J, 156E10; K, 156F3; L, 156F8; M, Hela.

chromosome 11s in which a terminal deletion had occurred distal to the LDH-A locus. This hypothesis could potentially be investigated using antisera to SA11-1 and SA11-3. Until these tests are performed, it remains unclear whether clone H11-11 falls within the aniridia-associated deletion or not.

DISCUSSION

The results presented here represent a first attempt to locate cloned DNA sequences which are deleted in patients suffering from aniridia. It is clear that many more DNA clones and many more aniridia patients will have to be examined before a precise definition of the sequences associated with the disease can be achieved. Since not all patients possess exactly the same size of deletion, testing many aniridia victims with a large number of DNA clones mapping to the vicinity of p13 should allow the delineation of the minimum region of overlap of the deletions and the identification of the critical gene(s) involved. A similar strategy could be employed in the case of Wilm's tumor, as well as in other deletion associated neoplasms such as retinoblastoma (90-92)

Cloned probes isolated in the manner presented in this thesis should also be useful for identifying disease associated chromosomal deletions which are too small to detect cytogenetically. This type of deletion may occur in the large number of Wilm's tumor patients in which no deletion has yet been found as well as in other inherited diseases. One such disease is familial aniridia which, unlike classic aniridia, is inherited as an autosomal dominant gene and is not

associated with a visible chromosomal deletion. In fact, it is not clear whether the familial aniridia locus maps to the p13 region of chromosome 11. DNA probes of the type described here used either directly or as linkage markers should provide an answer to this question.

While the application of the technology described in this thesis to the study of aniridia and Wilm's tumor is of fundamental interest, there are numerous other areas which could benefit from these methods. Many of these have been alluded to throughout this thesis including the potential use of cloned chromosomally localized DNA sequences in mouse and human genetic linkage and mapping studies as well as in basic investigations of chromosome structure, gene organization and expression, recombination, mutagenesis and evolution. It is clear that the pairing of somatic cell genetics and recombinant DNA will be a long and fruitful marriage.

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