Characterization of the Effects of Decreased Expression of Ribosomal Proteins on Cell Transformation and Cell Cycle Regulation

by

Yan Wang

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Signature of Author _____
Division of Toxicology
February, 1996

Certified by _____
Helmut Zarbl, Ph.D.
Thesis Supervisor

Accepted by _____
Associate Professor Peter Dedon
Chairman, Departmental Committee on Graduate Studies

This doctoral thesis has been examined by a Committee approved by the Division of Toxicology as follows:

Professor Peter Dedon _____

Professor David Schauer _____
Chairperson

Professor William Thilly _____

Helmut Zarbl, Ph.D. _____
Thesis Supervisor

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Abstract

A non-transformed revertant R2.2 was previously isolated from FBJ murine Sarcoma virus (v-fos) transformed Rat-1 fibroblasts. Previous genetic analysis indicated that one allele of ribosomal protein RPS3a gene is disrupted by plasmid integration in R2.2 revertant. Decreased expression of ribosomal protein RPS3a was shown to be responsible for reversion of v-fos transformation in R2.2 cells. This thesis is focused on investigating the molecular pathways by which decreased expression of ribosomal protein RPS3a mediates v-fos transformation reversion. In order to test the hypothesis that decreased expression of ribosomal protein RPS3a inhibits v-fos transformation by decreasing ribosomal accumulation, the expression of ribosomal proteins RPL5 and RPS6 was decreased in v-fos transformed cells by treatment with antisense oligonucleotides. Treated cells exhibit revertant phenotypes and decreased rates of protein synthesis, indicating that decreased ribosomal accumulation inhibits v-fos transformation. Compared to v-fos transformed cells, R2.2 revertant had increased doubling time, suggesting that decreased ribosomal accumulation resulted in altered cell cycle regulation. Cell cycle analysis indicated that most of the increase in doubling time in R2.2 revertant can be accounted by prolonged S phase. Consistent with this finding, the R2.2 revertant showed decreased cyclin A associated kinase activity and increased protein level of cyclin dependent kinase inhibitor, $p21^{Waf1}$, compared to parental v-fos transformed cells. Increased $p21^{Waf1}$ protein level was also detected in v-fos transformed cells treated with antisense oligonucleotides designed against several ribosomal proteins. These results indicated that inhibition of v-fos transformation by decreased expression of ribosomal proteins is associated with increased level of $p21^{Waf1}$ protein. The role of $p21^{Waf1}$ in v-fos transformation was further investigated by transfecting v-fos transformed cells with expression vectors encoding the full length $p21^{Waf1}$ protein, its C-terminus and its N-terminus.

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A	Adenine
bp	base pair
Ci	Curie
CAPS	3-[Cyclohexylamino]-2hydroxyl-1-propanesulfonic acid
EMS	Ethyl Methane Sulfonate
EDTA	Ethylene Diamine Tetra-acetic acid
FBJ	Finke-Biskis-Jinkins
G	Guanine
HEPES	4-[2-HydroxyEthyl]-1-PiperazineEthaneSulfonic acid
kb	1000 base pairs
LTR	Long Terminal Repeat
ug	microgram
uM	micromolar
mg	milligram
ml	milliliter
MEM	Minimum Essential Medium
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethanesulfonylfluoride
SDS	sodium dodecyl sulfate
Tris	Tris[hydroxymethyl]aminomethane
TCA	Trichloroacetic acid
TPA	12-tetradecanoyl-phorbol-13-acetate
T	Thymine
V	volts

Chapter 1
Literature Survey and Background

1.1 Cell transformation, oncogenes and tumor suppressors

1.1.1 Cell transformation

Cancer is one of the leading causes of death in the industrialized countries, second only to heart disease. Of a total of 11 million people who die annually in the industrialized countries, 2.3 million are killed by cancer (Lopez, 1991). In the United States, of 2,169,518 deaths in 1991, 514,657 cases, with a percentage of 23.7, were due to cancer (National Center for Health Statistics). Understanding the underlying molecular mechanisms of cancer and finding its cures are no doubt among the most important tasks of biomedical research.

Clinically, cancer is a malignant tumor characterized by the unchecked growth of a group of cells which invade into surrounding tissues and relocate to ectopic sites (metastases). Due to the difficulty in growing cancer cells under laboratory conditions and the advantage of reproducibility, in vitro transformed cell lines have been used as experimental systems to study tumorigenesis. Cell transformation is a process in which cultured cells are experimentally converted from "normal" states to neoplastic phenotypes. The phenomena was first discovered in early 1930s by Earle and his coworker (Earle and Voegtlin, 1938). In 1960s, the method of using cell transformation to investigate tumorigenesis was further developed by studying transformation in cells infected with tumorigenic DNA viruses and in cultures exposured to chemical carcinogens (Todaro and Green, 1966; Berwald and Sachs, 1963; Berwald and Sachs, 1965). Transformed cells usually acquired the properties of cancerous cells isolated from malignant tumors. Morphologically, transformed cells are usually more refractile and rounded compared to their normal counterparts. Transformed cells exhibit characteristics of loss of growth control, including loss of contact inhibition, loss of anchorage-dependent growth, and reduced dependence on growth factors. These characteristics are reflected in the abilities of transformed cells to overgrow a confluent

monolayer, to form microscopic colonies when suspended in semisolid media, and to grow in low-serum or serum-free media (Hanafusa et al., 1977; Tooze, 1980; Feramisco et al., 1985). Cell transformation is also linked to changes in cell metabolism, cytoskeleton and the expression of a variety of antigenic and biochemical markers, e.g., alkaline phosphatases. To meet the most stringent criterion for neoplastic transformation, cells must be able to form malignant tumors when introduced into susceptible animals.

It is now clear that cancer is a genetic disease resulting from accumulation of mutations in cellular genes. Studies of cell transformation in vitro have contributed significantly to the discovery of oncogenes and tumor suppressors, and to the establishment of the "multi-step" theory of carcinogenesis.

1.1.2 Oncogenes and tumor suppressors

Oncogenes are defined as viral or cellular genes that can induce one or more characteristics of neoplastic transformation when introduced, either alone or in combination with another gene, into appropriate cell types (Weinberg, 1989). Oncogenes have been found in RNA tumor viruses, DNA tumor viruses and cells isolated from tumor, or by screening for mutated genes in cell transformation assay.

About 85 years ago, the first oncogenic virus was identified by its ability to cause leukemia and sarcoma in chicken (Rous, 1911). This virus was named Rous Sarcoma Virus (RSV) and was later found to be a RNA tumor virus, also know as retrovirus. An intensive study of the oncogenic factors in RSV came in 1970s. The studies were greatly facilitated by the development of cell transformation assay, the isolation of transformation-defective mutants and the advent of recombinant DNA technology. Biochemical and genetic analysis of wild type and mutant viral genome led to the discovery of the transforming oncogene in RSV, v-src (Wang et al., 1975). v-Src specific sequences was found to hybridize to DNA from virtually all tested animals (Stehelin et al., 1976). Subsequent

studies indicated that the homology came from a large cellular gene which was named c-src. C-src is widely expressed in many kinds of tissues and is highly conserved during revolution (Spector et al., 1978; Parker et al., 1981; Shalloway et al., 1981; Shilo and Weinberg, 1981; Simon et al., 1983; Takeya and Hanafusa, 1983). v-src and c-src share similar structure but differ in their functions. These findings supported the hypothesis that v-src originated from normal cellular gene c-src and acquired transforming activity once captured by a retrovirus. This hypothesis was further substantiated by the discovery of other retrovirus-encoded oncogenes and their cellular counterparts--proto-oncogenes.

Cellular oncogenes which are not carried by retroviruses were discovered by studying tumors and transformed cells (Varmus, 1984). Proviral insertion, chromosomal translocation, gene amplification and point mutation result in changes in the structure or expression level of proto-oncogenes and thus convert them to oncogenes. These oncogenes were identified by gene transfer assays. In gene transfer assay, genomic DNA or cDNA libraries made from tumor cells are transfected into "normal" cells, such as the immortalized mouse NIH3T3 cells. Transformed cells isolated from transfected population are tested for the presence of foreign DNA and its ability to induce cell transformation is further tested in secondary transformation assay. This method has resulted in the discovery of a dozen of cellular oncogenes, including c-Ha-ras, c-Ki-ras, N-ras, c-trk, c-raf, and etc. (reviewed in Varmus 1984; Varmus 1987).

Proto-oncogenes assume a wide range of functions in cells. These functions include protein kinases, such as c-src (protein tyrosine kinase); growth factors, such as c-sis (B chain of the platelet-derived growth factor); signal transducers, such as c-ras (GTP/GDP binding protein); and transcription factors, such as c-fos, c-jun, and c-myc.

Oncogenes which have no apparent cellular origin have been discovered in DNA tumor viruses. Among them, two small DNA viruses, polyoma virus and simian virus 40 (SV 40) were studied the earliest (Reviewed in Tooze, 1980; Botchan et al., 1986; Salzman, 1986;

salzman and Howkey, 1987). Polyoma virus, isolated from leukemic mice, can induce neoplasms in a wide variety of cell types in mouse. The primary transforming oncogene product of polyoma virus was identified to be middle tumor antigen (mT). In SV 40, large tumor antigen (LT) mainly accounts for the transforming activity of the virus. Other DNA viruses that can induce cell transformation include the adenoviruses, the papillomaviruses, and the herpesviruses. Although the transforming mechanisms of DNA tumor viruses are not fully understood, the generally accepted hypothesis is that cell transformation results from interaction between the products of viral oncogenes and cellular proteins. These interactions lead to the activation of proto-oncogenes or inactivation of tumor suppressors. For example, the transforming activity of polyoma mT resides in its ability to form complex with proto-oncogene c-src and alter its regulation (Markland and Smith, 1987). SV40 LT was found to interact with tumor suppressors p53 and Rb (Lane and Crawford, 1979; Green, 1989). The interaction may sequester or inactivate the tumor suppressors and thus lead to cell transformation.

Besides oncogenes which can convert normal cells to neoplasm stage in a dominant fashion, there are another group of genes which contribute to tumorigenesis when inactivated. These genes are tumor suppressors. The importance of tumor suppressors in carcinogenesis is evidenced by the high incidence of loss-of-function mutation in naturally occurring tumors. Tumor suppressor genes have gained more and more attention in the past ten years. New tumor suppressors have been found and the functions of tumor suppressors, which were once mysteries, are now coming to light. The early indication of the existence of tumor suppressors came from somatic cells hybridization experiments. In most cases, when malignant human carcinoma cells were fused to normal human fibroblasts, the hybrid cells were not tumorigenic (Stanbridge, 1976; Stanbridge et al., 1982). Genetic analysis of families with high incidence of specific types of cancer, such as retinoblastoma and colon cancer, identified chromosomal deletions that segregate with the

diseases. The appearance of tumor often coincided with the deletion of the other allele of the same loci (Cavenee et al., 1983; Hansen and Cavenee, 1987). These findings led to the cloning of Rb tumor suppressor gene (Friend et al., 1986; Fung et al., 1087; Lee et al., 1987). Rb appears to function by inhibiting the activity of the E2F family of transcription factors. E2F binding sites are present in a variety of genes involved in initiating DNA synthesis. Deletion of the second allele leads to loss of heterozygosity of the locus. Studying loss of heterozygosity associated with tumor has been used as an approach to identify new tumor suppressor genes.

Tumor suppressor p53 was discovered through its ability to form complex with SV40 LT in virus-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Its function as a tumor suppressor was discovered by the finding that its locus was frequently deleted in a variety of tumors (Finlay et al., 1989; Baker et al., 1989; Baker et al., 1990). Patients with Li-Fraumeni syndrome have inherited mutations in p53 gene which predispose them to tumor development (Malkin et al., 1990). To date, p53 is documented as the most frequent mutated gene in human cancer (reviewed in Greenblatt et al., 1994). Wild type p53 has growth-inhibitory and transformation-suppression activity (Eliyahu et la., 1989; Finlay et al., 1989). It is a key factor in cell cycle check-point controls (see below). The most important function of p53 appears to regulate gene expression during cell growth, differentiation and in response to extra cellular signals. p53-responsible elements have been found in promoters of several genes, including the muscle creatine kinase, GADD45, mdm2, p53, and $p21^{Waf1}$.

A number of other tumor suppressor genes have been cloned as a result of studying gene inactivation in tumors. They include APC (Adenomatous Polyposis Coli) (Kinzler et al., 1991), DCC (Deleted in Colon Carcinoma) (Fearon et al., 1990), and NF 1 (Neurofibromatosis Type 1) (Cawthon et al., 1990).

1.1.3 The "multi-step" theory of tumorigenesis

Almost half a century ago, studies of mouse skin carcinoma formation pointed out that skin carcinogenesis was a multi-step process which included at least three stages: initiation, promotion and progression. In these experiments, mouse skin was first treated with chemicals called initiators, such as 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). These agents created invisible changes in mouse skin. After repeated treatment with promoters, usually the phorbol ester TPA (12-O-tetradecanoyl phorbol-13-acetate), papillomas appeared in initiated area and finally evolved into carcinoma. However, in non-initiated skin, application of promoters did not cause visible abnormalities. It was found that most initiators are strong mutagens. In contrast, promoters are only weakly or non mutagenic. Instead, they have strong growth promoting activity. These observations were combined with the notion that cancer results from mutations in cellular genes to form the "two-hit" model of skin tumor development. First mutation is created by initiator and is not sufficient for the development of tumor. However, it does equip the cell with growth advantage which allow it to expand clonally after treatment with promoters. During clonal expansion, a second mutation occurs in the population and the cell with both mutations become malignant (Hennings et al., 1983). A similar process was found in chemically induced rat liver tumors (Potter, 1984).

Although the mouse skin carcinogenesis is a simplified model of tumor development, the "multi-step" theory of tumorigenesis has been supported by results obtained from studies of epidemiology, transformation of primary cells in culture and tumor development in human. In human, the incidence of cancer as a function of age indicates that four to six mutations may be required for the appearance of clinically significant cancer (Armitage and Doll, 1957). Full transformation of primary cells usually happens in two stages--immortalization and transformation. These two steps require the cooperation of two oncogenes. In some cases, the oncogenes responsible for immortalization and

transformation can be separated. Clinically isolated premalignant lesions usually have mutations in a single proto-oncogene or tumor suppressor gene. Whereas, advanced carcinoma often exhibit a combination of activation of oncogenes or inactivation of tumor suppressor genes.

1.1.4 Studying revertants as an approach to dissect the transforming pathways of oncogenes

Full understanding of tumorigenesis and cell transformation requires knowledge about how the biochemical events initiated by mutations in proto-oncogenes and tumor suppressors confer neoplastic phenotypes. Specifically, it requires understanding of the molecular mechanism underlying the characteristics of transformed cells and tumor, such as loss of anchorage-dependent growth, loss of contact inhibition, decreased requirements for growth factors, changes in cell membrane and cyctoskeleton, angiogenesis, and invasiveness. These studies could be carried out by comparing the patterns of gene expression between normal cells and transformed cells using subtractive cloning or differential display of mRNAs. The potential problem of this method is the possibility that not all differentially expressed genes are related to tumorigenesis.

Molecular and genetic analysis of non-tumorigenic variants (revertants) isolated from transformed cells provides a potentially powerful tool to identify the genes that are important for cell transformation and dissect the biochemical pathways which mediate the transformation process (Bassin and Noda, 1987). Reversion can be achieved in one of several ways--inactivation of the original oncogenes, activation of tumor suppressor genes which suppress transformation in a dominant fashion, or inactivation of transformation effectors genes which are essential for the maintenance of transformed phenotypes. The later two categories of revertants have intact and functional transforming oncogene, however, they harbor genetic changes in cellular genes that are vital for transformation by

specific oncogene. Practically, since they are able to inhibit tumorigenesis, tumor effector/suppressor genes are potential targets for designing cancer therapeutic methods. A number of revertant cell lines arising from mutation in cellular effector genes or suppressor genes have been isolated from rodent fibroblasts transformed by either v-fos, v-ras, v-abl, v-fes, v-mos, or SV-40 LT (reviewed in Boylan and Zarbl, 1991; Haynes and Downing, 1988; Zarbl et al., 1987; Kho and Zarbl, 1993). Analysis of revertants includes molecular cloning of the putative transformation effector/suppressor genes, elucidating their cellular functions and identifying the downstream events which lead to the loss of tumorigenicity.

This thesis is focused on studying the mechanism of transformation reversion in a revertant cell line, R2.2, isolated from v-fos transformed Rat-1 fibroblasts.

1.2 v-fos oncogenes and the AP-1 transcription factors

The v-fos oncogenes were isolated from two murine retroviruses (FBJ-MSV and FBR-MSV) which can rapidly induce osteosarcomas in mice (Curran et al., 1982; Van Beveren et al., 1984). The gene products of v-fos and its cellular proto-oncogene, c-fos, are almost identical in size, with 381 and 380 amino acids, respectively. However, the C-terminal of the proteins, after amino acid 332, is different due to a deletion of 104 nucleotides near 3' terminus in v-fos gene (Van Beveren et al., 1983). The changes in carboxyl terminal appear to eliminate the ability of fos protein to down regulate transcription of c-fos gene (Wilson and Treisman, 1988). Compared to c-fos gene, v-fos genes also has a number of point mutations. Most of these mutations are not absolutely necessary for the transforming activity of v-fos. Substitution of glutamic acid for valine at residue 138 was shown to be crucial for the ability of FBR v-fos to immortalize cells. c-fos has the ability to transform fibroblasts if the c-fos protein level in cells is dramatically increased by (1) placing the gene under the control of a transcription enhancer leading to constitutive expression and (2)

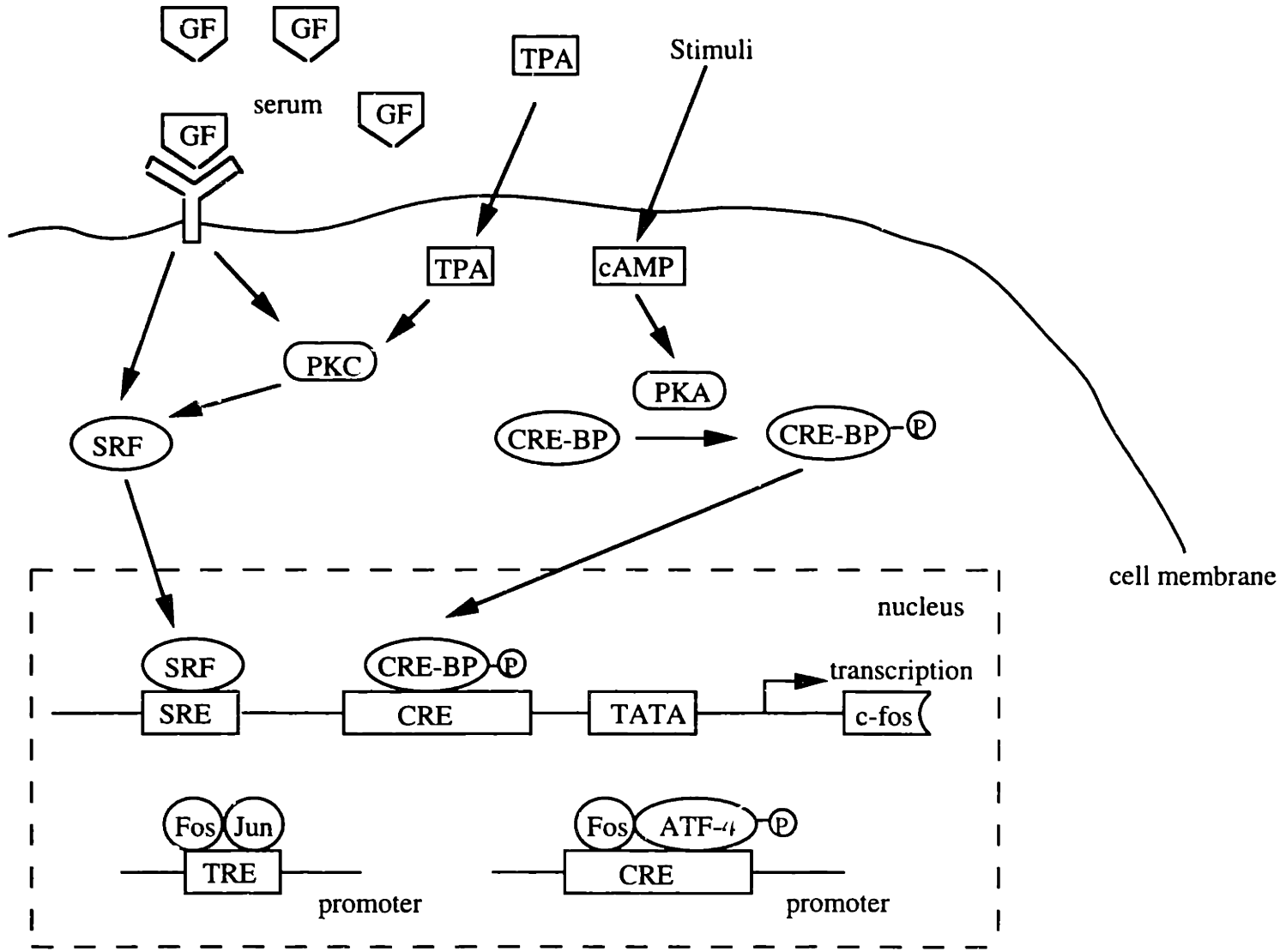
removal of a 67 bp AT-rich region from the 3' noncoding sequence (Miller et al., 1984; Lee et al., 1988). The 3' region of c-fos transcript was shown to promote mRNA degradation; its removal results in substantial increase of the half-life of c-fos mRNA (reviewed in Verma, 1986). When c-fos gene with these alterations was expressed in transgenic mice, the mice developed hyperplasia of bone and later osteosarcoma (Ruther et al., 1987). v-fos and c-fos proteins are both phosphorylated proteins, albeit with different phosphorylation patterns (Barber and Verma, 1987).

c-Fos protein is generally expressed at low level in proliferating cells. Its expression can be regulated by a wide variety of extra cellular signals, including mitogens, hormones, ionophores, stress, and drugs. These signals have been implicated in cell growth (Holt et al., 1983; Nishikura and Murray, 1987), and differentiation (Muller and Wagner, 1984; Diste et al., 1987). For example, c-fos is induced in PC12 phenochromocytoma cells in response to NGF (Morgan and Curran, 1991), in spinal cord neurons in response to sensory stimulation (Hunt et al., 1987), in lymphoid and myeloid cell lines in response to IL-2 and colony-stimulating factors, in mouse epidermal cells in response to active oxygen (Cerutli et al., 1989), and in HL60 cells upon differentiation (Muller et al., 1984). The expression of c-fos is also regulated during development (Ruther et al., 1987). During the development of mouse embryo, c-fos is expressed at higher level in undifferentiated tissue compared to differentiated tissues (Muller et al., 1983). The level of c-fos mRNA is 100-fold greater in human term fetal membranes than in other normal human tissues and cells (Muller et al., 1983). c-fos is one of the "immediate early response genes"--its expression can be rapidly and transient stimulated in quiescent cells following addition of serum or growth factors. The induction of c-fos mRNA occurs even in the presence of protein synthesis inhibitors, indicating that the factors responsible for c-fos transcription activation are already present in cells (Greenberg et al., 1986). c-fos expression was shown to be required for cell cycle progression (Kovary and Bravo, 1991).

The regulation of c-fos expression occurs at multiple levels. The identified regulatory elements in the promoter of c-fos gene include: (1) The TATA box (-25/-32) is required for the binding of RNA polymerase II, (2) two upstream elements control basal transcription. (3) serum response element (SRE) was identified at approximately -300 bp upstream. The binding between SRE and serum response factor (SRF) is essential for the induction of transcription in response to serum and growth factors (Treisman, 1985; Treisman, 1986; Gilman et al., 1986; Treisman, 1987). (4) Sequences at -60 bind to cAMP-binding-protein and mediate response to signals which increases the intracellular level of cAMP (Sheng et al., 1988). Since overexpression of Fos protein will result in cell transformation, it is important for cells to have negative regulatory mechanisms to control fos expression. c-fos mRNA has a short half-life. The 3' untranslated region of c-fos mRNA is responsible for its rapid degradation. Upstream sequences in c-fos promoter were implicated in the repression of transcription through interaction with the carboxyl terminus of c-fos protein (Wilson and Treisman, 1988). It is also reported that Fos protein decreases the elongation of fos mRNA.

v-fos and c-fos encode proteins that are nuclear transcription factors. The signal transduction pathways associated with Fos proteins are summarized in Figure 1. Fos proteins serve as transcription activators as well as repressors (et al., 1988; Wilson and treisman, 1988). The ability of Fos proteins to regulate transcription is essential for their transforming activity. Fos proteins themselves possesses weak DNA-binding activity and can not form dimers (Halazonetis et al., 1988; Nakabeppu et al., 1988). They can dimerize with Jun family proteins through leucine zipper regions to form AP-1 transcription complexes (Distel et al., 1987; Rauscher et al., 1988; Chiu et al., 1988). AP-1 complexes bind to TPA-responsive element (TRE) consensus sequence and regulate transcription in response to activated protein kinase C (PKC) (Ryder et al., 1987; Angel et al., 1987; Chiu et al., 1987). TPA activates proteins kinase C via diacylglycerol-mediated

Figure 1. Signal transduction pathways associated with Fos. Growth factors (GF) or TPA activate serum responsible factor (SRF) by protein kinase C (PKC) dependent or independent pathways. Activated SRF binds to serum responsible element (SRE) in c-fos promoter and increases c-fos transcription. Activated protein kinase A (PKA) phosphorylates cAMP responsible element (CRE)-binding proteins (CRE-BP) which subsequently bind to CRE in c-fos promoter and activate transcription. Fos protein does not bind to DNA by itself, nor does it form. Fos protein can form heterodimer with Jun family proteins or ATF-4 which is a member of CRE-BP family. The complexes regulate transcription by binding to TPA responsible element (TRE) or cAMP responsible element (CRE), respectively.


GF
GF
serum
GF
GF
TPA
Stimuli
TPA
cAMP
PKC
PKA
SRF
CRE-BP
CRE-BP
P
cell membrane
nucleus
SRF
CRE-BP
P
transcription
SRE
CRE
TATA
c-fos
Fos
Jun
TRE
promoter
Fos
ATF-4
P
CRE
promoter

pathways. Fos proteins can also regulate the activity of cAMP responsive element (CRE) in promoter. The CRE site is recognized by a family of proteins referred to as ATF or CRE binding proteins (CRE-BP). Fos proteins can form dimers with certain members of ATF/CRE-BP family and bind to CRE sites. The DNA-binding activity of Fos-Jun dimers was shown to be regulated by redox mechanism as well as phorphorylation (Abate et al., 1990).

c-Fos belongs to a group of factors which share homology. Other member of the group include Fra-1, Fra-2, and FosB (Zerial et al., 1989; Suzuki et al., 1991; Kerppola and Curran, 1991). They form dimers with Jun family proteins or ATF/CRE-BP family proteins with different affinities and DNA binding specificities.

The mechanism of v-fos transformation has not been completely understood. Existing evidence indicates that the transformation pathways of v-fos are shared by other oncogenes. Functional c-fos gene was shown to mediate the activity of other oncogenes. AP-1 activity is essential for cell transformation by ras (Lloyd et al., 1991). Cells that are resistant to transformation by v-fos are also resistant to H-ras and c-jun (Wisdom and Verma, 1990). Studying of non-tumorigenic revertants isolated from v-fos transformed cells has contributed significantly to the understanding of v-fos transformation. Revertants isolated from v-fos transformed cells could not be retransformed by v-Ha-ras, v-abl, and v-mos (Zarbl et al., 1987). Disruption of PKA signal transduction pathways has been implicated in the reversion process of revertant EMS-1-19 (Van Amsterdam et al., 1994). In revertant R2.2, decreased expression of ribosomal protein RPS3a was shown to be responsible for the revertant phenotypes (Kho and Zarbl, 1993). Studies in this thesis were designed to investigate the correlation between ribosomal accumulation, cell growth regulation and v-fos transformation.

1.3 Protein synthesis and ribosomes

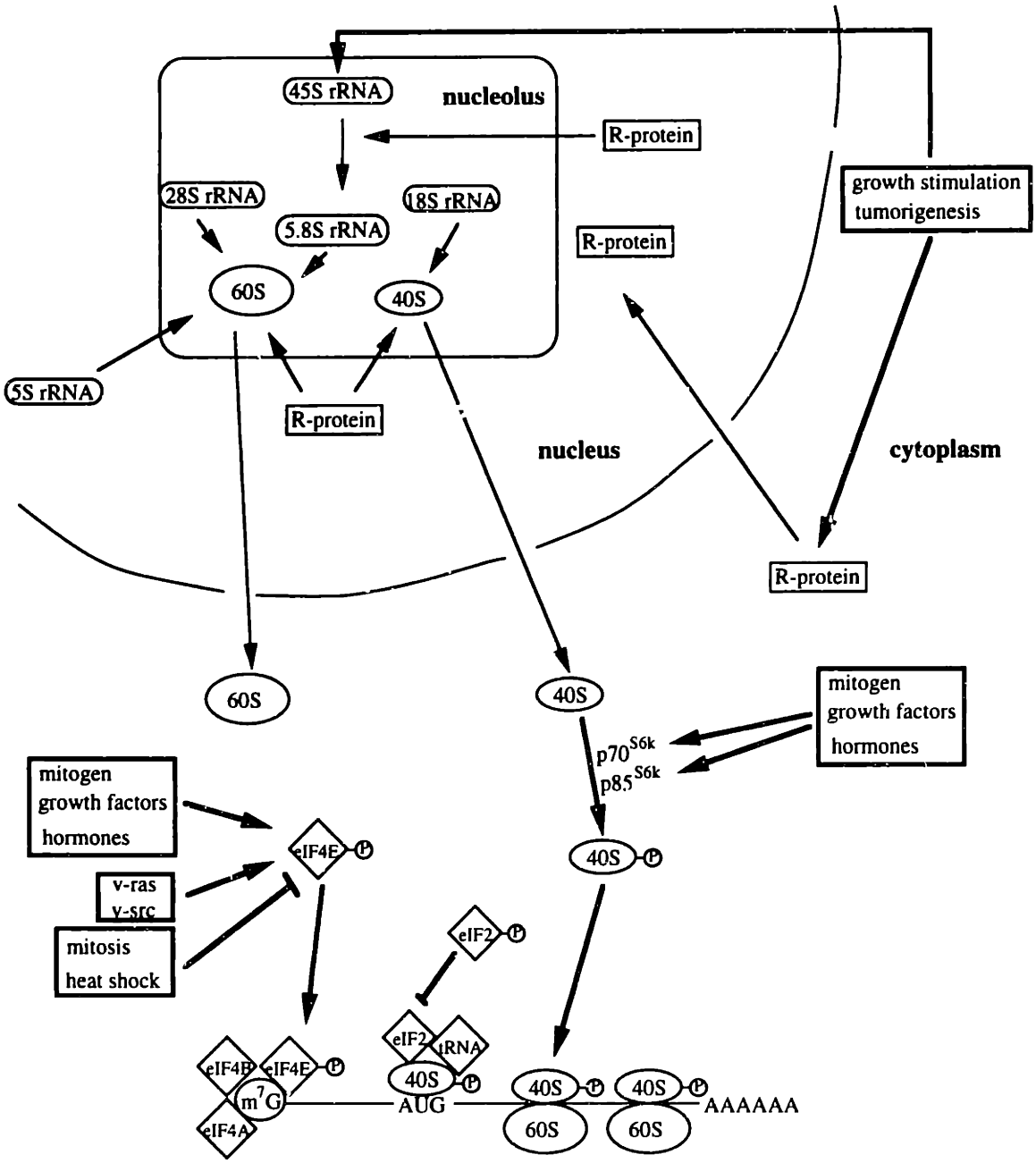
1.3.1 Eukaryotic ribosomes and translation machinery

Eukaryotic ribosomes consist of 40S and 60S subunits which are ribonucleoproteins (Wool et al., 1990a). The 40S subunit is composed of one molecule of 18S rRNA and ~30 proteins; The 60S subunit is composed of one molecule of each 5S, 5.8S, and 28S rRNA, and ~50 proteins. The function of the ribosomes is to synthesize proteins. In addition to ribosomes, protein synthesis requires the participation of initiation factors, elongation factors and termination factors at different stages of translation.

The rate of protein synthesis in cells can be regulated at multiple levels, including ribosome content, levels of auxiliary factors, phosphorylation of ribosomal proteins and auxiliary factors (Figure 2). Most of the mechanisms that regulate protein synthesis were found to function at translation initiation. The steps involved in translation initiation include: formation of a ternary complex (eIF-2·GTP/Met-tRNA) and its subsequent binding to the 40S subunit, activation of the mRNA by eIF-4A, eIF-4B, and eIF-4F which in part results in unwinding of mRNA secondary structure near the 5' m^7G cap structure, and the formation of 48S initiation complex, in which mRNA joins the 40S ribosomal subunit. eIF-2 is a complex of eIF-2α, eIF-2β and eIF-2γ. eIF-4F is composed of eIF-4A, eIF-4E and eIF-4γ. The last step is the rate limiting step in translation initiation. Four individual, but interacting polypeptides, eIF4A, eIF-4B, eIF-4E and eIF-4γ are required for the formation of the complex.

Existing evidence indicates that translation initiation factors eIF-2α and eIF-4E are important regulators of translation initiation. eIF-4E is an evolutionally conserved protein with a molecular weigh of 25 Kd (reviewed in Rhoads et al., 1991). It is the only factor that can bind to the mRNA 5' cap structure alone. It is also the least abundant initiation factor, with 0.01-0.2 molecules per ribosome, compared to 0.5-3 molecules per ribosome for other initiation factors (Hiremath et al., 1985; Duncan et al., 1987). The low abundance

Figure 2. Schematic illustration of the regulation of ribosomal biogenesis and protein synthesis. Regulatory pathways are indicated by thick lines. Ribosomal assembly takes place in nucleolus and parallels rRNA processing. During growth stimulation and tumorigenesis, the expression of ribosomal proteins and ribosomal RNAs increases. Initiation factors eIF-4A, eIF-4B and eIF-4E bind to "cap" structure and unwind mRNA. A complex composed of 40S ribosomal subunit, Met-$tRNA_f$ and eIF-2 binds to activated mRNA and forms 48S initiation complex. This step is rate limiting in translation initiation process. Phosphorylated eIF-4E binds to mRNA with higher affinity and thus increases the rate of protein synthesis. Phosphorylation of eIF-2 prevents it recycling and inhibits protein synthesis. $p70^{S6K}$ and $p85^{S6K}$ can be activated by growth promoting signals. Phosphorylation of ribosomal protein S6 in 40S subunit by $p70^{S6K}$ or $p85^{S6K}$ increases the rate of protein synthesis. R-protein = ribosomal protein.



TRANSLATION

of eIF-4E suggests that it is the rate-limiting factor of translation initiation. Expression level and phosphorylation of eIF-4E were shown to correlate positively with increased rate of translation. Increased expression of eIF-4E correlates with an increase in translation rate and cell growth in a wide variety of cell types (reviewed in Rhoads, 1993). Decreased expression of eIF-4E in Hela cells has been shown to cause a slow down of growth rate, decreased translation rate and disaggragation of polysomes (De Benedetti et al., 1991). Much evidence indicates that eIF-4E phosphorylation is necessary for its function in translation initiation. Phosphorylated eIF-4E has a 3-4 fold greater affinity for cap analogs and mRNA than non-phosphorylated eIF-4E (Minich et al., 1994). Only phosphorylated eIF-4E is present in 48S initiation complex (Joshi-Barve et al., 1990). Increased eIF-4e phosphorylation occurs in response to growth factors, mitogens, hormones and cell transformation. During mitosis or following heat shock, translation is reduced concomitantly with a reduction in eIF-4E phosphorylation (Duncan et al, 1987; Duncan and Hershey, 1989; Lamphear and Panniers, 1990). eIF-2α is also a phosphoprotein (Kimball et al., 1991). However, phosphorylation of eIF-2a prevents its recycling and inhibits protein synthesis.

Another important regulator of protein synthesis is ribosomal protein S6. It is rapidly phosphorylated when cell are stimulated to grow or divide (Stewart and Thomas, 1994 and the reference therein). The enzymes responsible for the phosphorylation of S6 are the $p70^{S6k}$ and $p85^{S6k}$, which are produced from the same transcript by differential splicing. $p70^{S6k}$ and $p85^{S6k}$ can be activated by a variety of mitogenic stimuli. Of the 40S ribosomal proteins, only S6 is known to become phosphorylated in response to mitogens. Phosphorylation of S6 is strongly correlated with increased rate of protein synthesis, suggesting that S6 phosphorylation is involved in up-regulating translation. Studies have shown that inactive ribosomes move into actively translating polysomes in parallel with increased levels of S6 phosphorylation. Phosphorylated 40S subunits were preferentially

recruited into polysomes after mitogenic stimulation as compared to non-phosphorylated subunits. It is suggested that S6 phosphorylation increases the affinity of 40S subunits for stored mRNP particles (mRNA protein particles) in translation initiation. In vitro, it has been demonstrated that phosphorylated 40S subunits translate mRNA more efficiently than the non-phosphorylated form (Palen and Traugh, 1987). Through the use of chemical cross-linking and protection studies, S6 was shown to localize to interface between 40S and 60S subunits. It is a site where the association between ribosome 40S subunit with mRNA, tRNA, initiation factors and elongation factors (Nygard and Nilsson, 1990; Morley and Thomas, 1991) takes place. Ribosomal protein S6 has the potential to directly interact with mRNA and its phosphorylation was implicated in the selective translation of messages with the 5' polypyrimidine tract (Jefferies et al., 1994).

Protein synthesis plays a major role in the regulation of gene expression with approximately 100-fold range of translational efficiencies among cellular mRNAs (Koch et al., 1980). Under similar conditions, the translation efficiency of certain mRNA is largely determined by its 5' end untranslated region (UTR). The features that could affect translation efficiency include the presence of m^7G cap, sequence surrounding the AUG codon, secondary structure and the length of UTR (reviewed in Kozak, 1991a). The poor translation of many mRNAs that encode oncoproteins, growth factors, transcription factors and other critical regulatory proteins can probably be explained by the highly structured 5' end sequence in these mRNAs (Kozak, 1991b).

Several studies have suggested that some ribosomal proteins may have more than one function. A chromatin-associated pool of S6 was reported recently (Franco and Rosenfeld, 1990; Reinhard et al., 1994). The function of chromatin-associated S6 is unknown. It was speculated that it may play a role in the up-regulation of ribosome biogenesis. In Drosophila, maintaining certain level of ribosomal protein S6 was shown to have an effect on tumor suppression in hemoepoietic system (Stewart and Denell, 1993; Watson et al.,

1993). Two cDNAs which encode the Drosophila homologues of rat ribosomal protein S3 and the acidic ribosomal protein PO have been identified and were reported to encode proteins which are also associated with the nuclear matrix of chromatin (Grabowski et al., 1991; Wilson et al., 1993). Both these two Drosophila proteins and the rat ribosomal protein S3 have been claimed to have AP (apurinic/apyrimidinic) endonuclease activity which is involved in DNA repair (Wool, 1993).

1.3.2 Protein synthesis and cell growth

Numerous lines of evidence indicates that changes in ribosomal accumulation and the rate of protein synthesis are associated with cell growth, proliferation and differentiation (Nomura et al., 1984; Mager and Planta, 1991) (figure 2). Cell cycle progression requires continued protein synthesis. There is a period in mid G1 phase when cells are extremely sensitive to inhibitors of protein synthesis. An increase in the rate of protein synthesis is required both for entry into cell cycle and progression of S phase (Brooks, 1977). When cell growth is stimulated by growth factors (Mauck and Green, 1973), hormones (DePhilip et al., 1980; Davies et al., 1986), or trauma (Dabeva and Dudov, 1982), there is corresponding increase in ribosome production. In contrast, synthesis of ribosomes can be reduced or arrested in serum-starved cells (Emerson, 1971; Cooper, 1973), quiescent cells (Becker et al., 1971; Tushinski and Warner, 1982), and cells undergoing terminal differentiation (Jacobs et al., 1985; Bowman, 1987). The rate of protein synthesis increases in response to treatment of cells with growth factors, cytokines, hormones and mitogens (reviewed in Rhoads, 1991; Sonenberg, 1993,). Regulation of translation rate involves phosphorylation of ribosomal protein S6 and initiation factors eIF-2α, eIF-4E, and eIF-4B. Initiation factors, such as eIF-2α, eIF-4E and eIF-4B are phosphorylated in response to a wide variety of extra cellular stimuli (reviewed in Hershey, 1991). Phosphorylation of eIF-4B, eIF-4E and ribosomal protein S6 positively correlates with

increased translation rate and cell proliferation, whereas phosphorylation of eIF-2α results in inhibition of translation and is associated with suppression of cell growth (Jagus et al., 1981; Hershey, 1989).

Deficiencies in ribosomal proteins have been shown to have adverse effects on growth and development. The dominant minute phenotypes in Drosophila, which includes short thin bristles, slow development, reduced viability and small body size, are the results of mutations in 50-60 different minute genes (Lindsley et al., 1987). The minute loci have been suggested to encode ribosomal protein (Kongsuwan et al., 1985). Ribosomal protein S4 can rescue a temperature-sensitive Syrian hamster fibroblast mutant which demonstrates G1 arrest and defective in DNA, RNA and protein synthesis under non permissive temperature (Watanabe et al., 1991). Deficiency of ribosomal protein S4 was shown to be responsible for Turner syndrome in human (Fisher et al., 1990).

1.3.3 Protein synthesis and tumorigenesis

Tumorigenesis is the result of unchecked cell growth, proliferation and differentiation. Protein synthesis which is responsible for the accumulation of one of most important macromolecules has direct effects on cell growth and plays important roles in tumorigenesis.

Increased expression of a number of ribosomal proteins has been found in a variety of tumors and transformed cells (Table 1).

Cap-binding protein eIF-4E, which participates in the unwinding of mRNA secondary structure, was shown to be mitogenic. Micro-injection of eIF-4E into quiescent 3T3 fibroblasts induces DNA synthesis (Smith et al., 1991). The level of eIF-4E mRNA can be induced by c-myc activation (Rosenwald et al., 1993). Overexpression of eIF-4E is associated with cell transformation and tumor development. Transcriptional activation of eIF-4E by chromosomal translocation or gene amplification was detected in several tumors

(Marcu et al., 1992). Over expression of eIF-4E in NIH 3T3 and Rat-2 cells results in malignant transformation (Lazaris-Karatzas et al., 1990; De Benedetti and Rhoads et al., 1991). eIF-4E can cooperate with either v-myc or E1A to transform primary rat embryo fibroblasts (Lazaris-Karatzas and Sonenberg, 1992). Transformation by eIF-4E results in Ras activation (Lazaris-Karatzas et al., 1992). eIF-4E may also play an important role in the transformation process by other oncogenes. Rinker-Shaeffer et al. (1993) demonstrated that expression of antisense RNA to eIF-4E in ras transformed rat embryo fibroblasts partially reversed the transformed phenotypes of these cells.

Table 1. Overexpression of ribosomal proteins in tumors and transformed cell lines

Source	Ribosomal proteins[a]	References
colon adenocarcinoma and carcinoma tissues	S2,S3,S6,S8,S12,P0,L5, L31,P2,S27a,S19	Chester et al., 1989 Sharp et al., 1990 Mafune et al., 1991 Pogur-Geile et al., 1991 Chiao et al., 1992 Barnard et al., 1992
colon adenocarcinoma cell line SW48	S3a	Kho, 1992
Wilms' tumor cell line G401	L9	McMaster et al., 1992
ovarian carcinoma cell line PA-1	S2,S3a	Chiao et al., 1992 Kho, 1992
hepatocellular carcinoma tissue and cell line	P0	Ou et al., 1987 Barnard et al., 1992
esophageal carcinoma	S15	Shiga et al., 1990
bladder carcinoma cell line T24	S3a	Kho, 1992
Rat-1 cells transformed by v-fos	S3a, S16, L26	Kho, 1992
Rat-1 cells transformed by v-fes, v-fms, v-sis, v-mos	S3a	Kho, 1992

[a] The expression was compared to either adjacent normal tissue or normal diploid fibroblasts.

Phosphorylation is important for the transforming activity of eIF-4E. Phosphorylation of serine 53 is essential for eIF-4E to transform NIH 3T3 cells and CHO cells (Lazaris-Karatzas et al., 1990). The correlation suggests that its transforming activity is associated with its ability to increase translation initiation.

Overexpression of eIF-4E was reported to correlate with increased translation of mRNA with extensive 5' secondary structures (Loromilas et al., 1992a). These structures have been found in mRNAs of growth factors and growth factor receptors (Sato et al., 1990), for example, c-sis (Rao et al., 1986), human FGF (Bates et al., 1991), tyrosine kinase lck (Marth et al., 1988), and c-myc (Darveau et al, 1985). mRNAs which are translated more efficiency in response to increased level of eIF-4E include those encoding ornithine aminotransferase (Fagan et al, 1991), cyclin D1 (Rosenwald et al., 1993), and ornithine decarboxylase (ODC) (Shantz and Pegg, 1994). These proteins were shown to participate in cell growth control. Cyclin D1 is required for G1 progression (Xiong et al., 1991). ODC is the rate-limiting enzyme in polyamine synthesis, which plays an essential role in cell growth and differentiation (Pegg, 1988).

Expression or modification of other translation auxiliary factors have also been implicated in tumorigenesis. Overexpression of elongation factor-1α (EF-1a) increases the susceptibility of mouse fibroblasts to transformation by chemical carcinogens and ultraviolet radiation (Tatsuka et al., 1992). The phosphorylation of eIF-2α correlates with inhibition of initiation of protein synthesis (Hershey, 1989). Overexpression of a dominant negative mutant form of eIF-2α kinase that is unable to phosphorylate eIF-2α can transform NIH 3T3 cells (Koromilas et al., 1992b).

1.4 The regulation of cell cycle

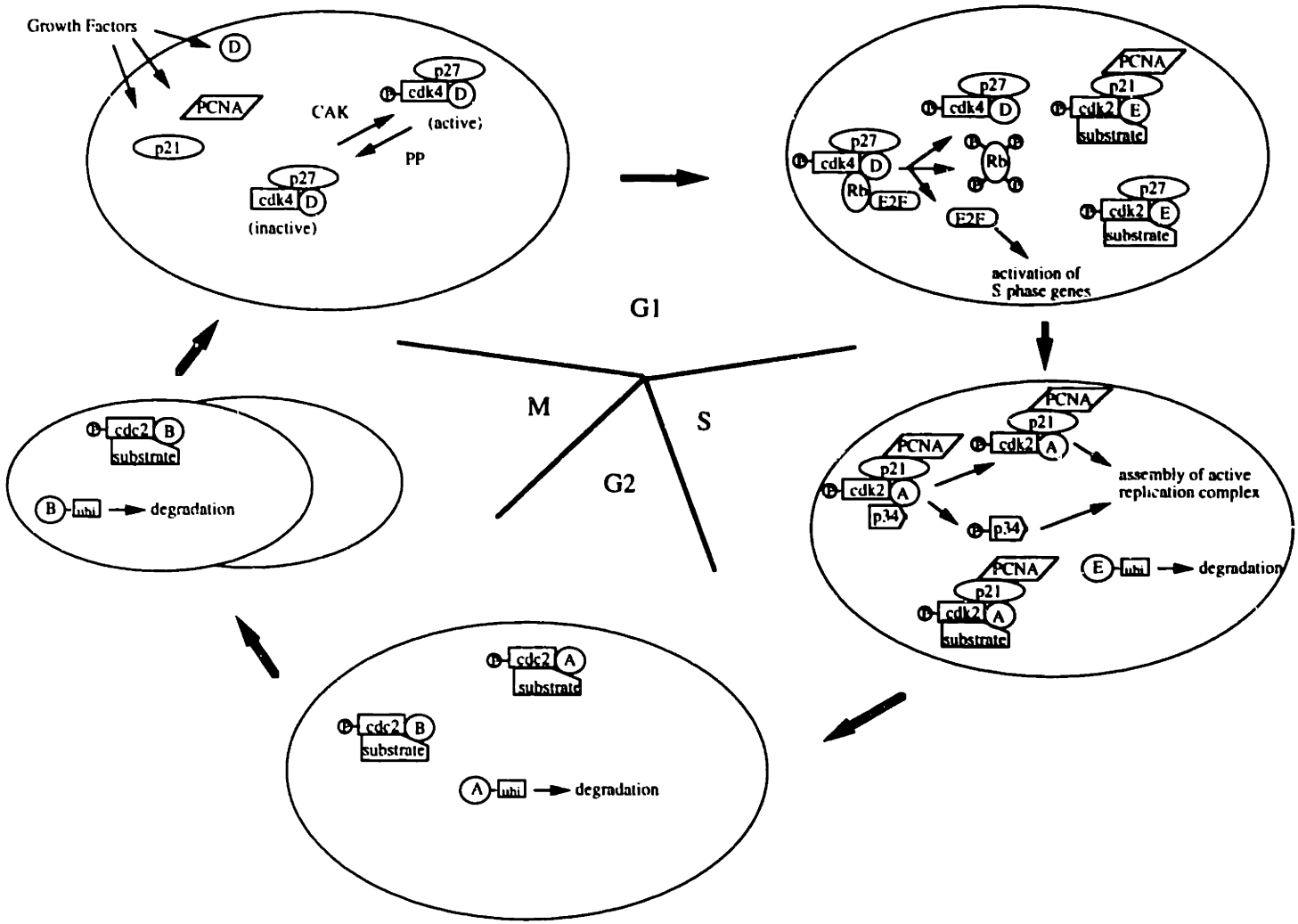
1.4.1 The regulation of cell cycle in mammalian cells

The cells division cycle of eukaryotic cells is a highly ordered process which consists of G1 (the first gap) phase, S phase, G2 (the second gap) and M (mitosis) phase. Chromosomes are replicated in S phase and partitioned into daughters cells during M phase. Non dividing cells suspend the cycle after mitosis and just before DNA synthesis. After removal of mitogens in many differentiated mammalian cells, they go into a state called "G0" . Some cell types may arrest only transiently and then undergo apoptosis, because growth factors required for their proliferation are also needed for their continued survival.

In eukaryotic cells, this process is tightly controlled by the sequential formation, activation, and subsequent inactivation of different cyclin-cyclin dependent kinase (Cdk) complexes (Reed, 1992; Pines, 1993; Sherr, 1993) (Figure 3). Cyclins were first discovered in marine invertebrates as proteins that accumulated to high levels in interphase and were abruptly destroyed in mitosis during the synchronous cell division of the early embryo (Evans et al., 1983; Swenson et al., 1986). The activation of p34 kinase by binding of cyclins is required for G2/M phase transition (Swenson et al., 1986; Minshull et al., 1989; Murray and Kirschner, 1989; Solomon et al., 1990). Molecules that regulate cell cycle are highly conserved during evolution (reviewed in Nurse, 1990; Cross, 1990). A number of mammalian cyclins have been identified by their ability to rescue yeast mutants which are defect in cell cycle progression. The mammalian homologues of p34 kinase are identified as different cyclin-dependent kinases (Cdks).

The activity of cyclin-Cdk complexes is regulated by phosphorylation and binding to inhibitors (Gould et al., 1991; Krek and Nigg, 1991; Solomon et al., 1992, 1993; Gould and Nurse, 1989; Polyak et al., 1994). In cdc2, one of the Cdks, phosphorylation of threonine 161 by cdc2-activating kinase (CAK) and dephosphorylation of threonine 14 and

Figure 3. Regulation of cell cycle progression by cyclins, Cdks and Cdk inhibitors. The expression of cyclin D can be activated by growth factors. Cyclin E is expressed later than cyclin D in G1 phase. The activity of both cyclin D-Cdk4 and cyclin E-Cdk2 complexes is rate limiting for G1 progression. A putative physiological substrate of cyclin D-Cdk4 complex is Rb. Phosphorylation of Rb releases E2F which can subsequently activates genes involved in S phase. Cyclin A and Cdk2 are both required for the onset of S phase and S phase progression. Cyclin A and Cdk2 localize to the site of DNA replication during S phase. p34, which is a subunit of replication protein A (RP-A), is a putative substrate of cyclin A-Cdk2 complex. Phosphorylation of p34 is thought to be important for the assembly of active replication complexes. Cyclin A forms complex with Cdc2 during late S phase and G2 phase. The function of cyclin A-Cdc 2 is unknown. Cyclin B is expressed in G2 and M phases. Its catalytic partner is Cdc2. Degradation of cyclin B is required for mitosis exit. The activity of cyclin-Cdk complexes can be regulated though phosphorylation by Cdk activating kinase (CAK). Binding of Cdk inhibitors, such as $p21^{Waf1}$ and $p27^{Kip1}$, to cyclin-Cdk complexes could inhibit their activity. The inhibition depends on the stoichiometry of the inhibitors in the complexes. Once finishing their functions, cyclins are degraded by ubiquitin mediated proteolysis. (Circles with a letter in the middle indicate cyclins. ubi = ubiquitin)


Growth Factors
D
PCNA
p21
CAK
p27
cdk4
D
(active)
PP
p27
cdk4
D
(inactive)
G1
S
G2
M
p27
cdk4
D
Rb
E2F
PCNA
p21
cdk2
E
substrate
activation of
S phase genes
PCNA
p21
cdk2
A
p34
assembly of active
replication complex
E
ubi
degradation
cdc2
B
substrate
B
ubi
degradation
cdc2
A
substrate
A
ubi
degradation

tyrosine 15 by protein phosphotase is required for activation (Solomon, 1993). CAK is composed of catalytic subunit Cdk7 and regulatory subunit, cyclin H (Fisher and Morgan, 1994; Makela et al., 1994). Interestingly, cdk7 and cyclin H are homologous to other Cdks and cyclins, respectively.

In G1 phase, cells respond to various signals, such as growth factors and differentiation, and make the decision whether to commit to proliferation or to go into G0 state (Pardee, 1989). Once Chromosomal replication is initiated, cells become relatively refractory to extra cellular signals. The cyclins which control G1 progression were identified as cyclin D and cyclin E (reviewed in Sherr, 1993). Increased expression of cyclin D1 and cyclin E was shown to shorten G1 phase, reduce cell size and decrease growth factor requirements (Quelle et al., 1993; Ohtsubo and Roberts, 1993), indicating that cyclin D1 and cyclin E are rate-limiting factors for G1 progression.

Three D-type cyclins (D1, D2, D3) have been identified as "delayed early response" genes to growth factor stimulation (Matsushime et al., 1991). They are expressed combinationally and differentially in different cell lineages (Kiyokawa et al., 1992; Matsushime et al., 1991a; Matsushime et al., 1991b). The major D type cyclin expressed in fibroblasts is D1 (Won et at., 1992). D type cyclins form complexes with Cdk2, -4, -5 and -6 (Bates et al., 1994; Mutsushime et al., 1992; Meyerson et al., 1994; Xiong et al., 1992). In fibroblasts and macrophages, Cdk4 appears to be the most prominent partner. In proliferating macrophages and fibroblasts, oscillations of the cyclin D1 and Cdk4 proteins are not readily apparent, although both components are somewhat more abundant during late G1 (Baldin et al., 1993; Matsushime et al., 1992; Matsushime et al., 1991). However, cyclin D1 is preferentially localized in nucleus during G1 phase followed by its marked decay in S phase (Baldin et al., 1993). D type cyclins are synthesized as long as growth factor stimulation persists and exhibit only moderate oscillations during the cell cycle with peak levels achieved near G1/S. Upon growth factor withdrawal, the level of cyclin D

decreases rapidly. It is speculated that D type cyclins actually serve as sensors of growth factors.

Much evidence indicates that the physiological substrates of cyclin D-Cdk complexes include tumor suppressor Rb (Resnitzky and Reed, 1995). Part of the evidence came from the physical interaction between cyclin D1 and Rb protein. The levels of Rb is stable during cell cycle, however, its activity is regulated by phosphorylation. Quiescent cells contain a hypophosphorylated form of pRb which are phosphorylated sequentially as cells progress through G1 and becomes a hyperphosphorylated form at the G1/S transition. It remains in this hyperphosphorylated form through out the S and G2 phases and is dephosphorylated as cells exit from mitosis (Buchkovich et al, 1989). Phosphorylation of pRb was shown to be required for cells to progress through mid-G1 and into S phase. The function of pRb in controlling cell proliferation is though to be mediated by its interaction with a number of cellular proteins, including c-myc, N-myc, cyclins, E2F, etc. (reviewed in Sherr, 1994; Weinberg, 1991). In human tumors, mutation and deletion of Rb were frequently detected in the region of protein-protein interaction. Phosphorylated Rb releases the inhibitory constraint on E2F which can subsequently activate transcription of genes important for S phase, including DNA polymerase α and dihydrofolate reductase (Dynalacht et al., 1994; La Thangue, 1994; Nevins, 1992; Nevins, 1994). Oncoproteins such as SV40 T antigen, adenovirus E1A, and human papillomavirus E7 facilitate the G1-S transition, in part by binding to pRb and releasing E2F.

Cyclin E is synthesized in late G1 and associated with Cdk2 to form complexes that can phosphorylate histone H1 in vitro. The activity of cyclin E-Cdk2 complex reaches maximum during G1-to-S phase transition (Dulic et al., 1992; Koff et al., 1993). In cells arrested in G1 by cell-cell contact or the treatment of transforming growth factor β, the activity of cyclin E-Cdk2 complex is decreased by binding to Cdk inhibitor, $p27^{Kip1}$, suggesting that the activity of cyclin E is important for G1 progression (Polyak et al.,

1994). Cyclin E is thought to be essential for initiating DNA replication (reviewed in Heichman and Roberts, 1994). Microinjection of antibodies against cyclin E into human fibroblasts prevents the initiation of S phase (Ohtsubo and Roberts, 1993).

The cyclin associated with S phase is primarily cyclin A which is expressed during G1-to-S transition. Microinjection of anti-cyclin A antibodies or plasmids encoding antisense cyclin A cDNA into fibroblasts during G1 phase resulted in cyclin A depletion and inhibition of the initiation of DNA synthesis, indicating that cyclin A is required for the onset of S phase (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992). Other evidence suggests that cyclin A may act to ensure the temporal relationship between S phase and mitosis (Walker and Maller, 1991). Cyclin A forms complex with Cdk2 during G1-to-S transition and in S phase; with Cdc2 in late S phase and in G2 phase. Both cyclin A-Cdk2 complex and cyclin A-Cdc2 complex are able to phosphorylate histone H1 in vitro. The importance of Cdk2 in S phase progression is indicated by the evidence that microinjection of Cdk2-specific antibodies into cultured human fibroblasts blocks S phase entry (Pagano et., 1993; Tsai et al., 1993). The function of cyclin A-Cdc2 has not been determined.

Together, cyclin A and Cdk2 form higher-order quaternary complexes with a protein (p107) related to the retinoblastoma gene product (pRb) and with the transcription factor E2F, suggesting that the cyclin may indirectly govern gene expression during S phase (Cao et al., 1992; Devoto et al., 1992; Ewen et al., 1992; Faha et al., 1992; Pagano et al., 1992a; Shirodkar et al., 1992). In human cells, cyclin A and Cdk2 also exist in another quanternary complex with proliferating nuclear antigen (PCNA) and a Cdk-inhibitor, $p21^{Waf1}$ (Xiong et al, 1992). PCNA is the processivity factor of DNA polymerase δ, which is required both for leading-strand DNA replication and gap-filling synthesis in excision repair of DNA damage (Prelich et al., 1987a, 1987b; Prelich and Stillman, 1988; Toschi and Bravo, 1988; Shivji et al., 1992). DNA replication in mammalian cells occurs

in discrete nuclear foci. Cyclin A and Cdk2 , along with PCNA, colocalized to DNA replication foci throughout S phase in myotube, suggesting direct roles of these proteins in the control of DNA replication (Cardoso et al., 1993). In vitro studies of SV40 replication indicated that Cdks are necessary for assembling initiation complexes containing unwound DNA at replication origins (Roberts, 1993). Recent experiments provided evidence that Cdks promote unwinding of origin DNA within initiation complexes on cellular chromosomes (reviewed in Heichman, 1994). Cdk inhibitor $p21^{Waf1}$ does not prevent the assembly of RPA complexes containing double-stranded DNA, but does prevent the unwinding of DNA within these preinitiation complexes. One of the putative substrate of cyclin A-Cdk2 complex is RPA34, a single-stranded DNA-binding protein. RPS34 is one of the three subunits of replication protein A (RP-A) which is required for DNA replication. Phosphorylation of RPA 34 was proposed as a key regulatory event controlling the onset of DNA replication (Din et al., 1990). RPA34 can be phosphorylated by Cdc2 and Cdk2 immunoreactive complexes in vitro (Elledge et al., 1992).

Mitosis was shown to be controlled by cyclin B-Cdc2 complex. Cyclin B is synthesized in G2 and is rapidly degraded in mitosis. Degradation of cyclin B is required for mitotic exit (Murray et al., 1989). Formation of prereplicative complexes on double-stranded DNA is inhibited by the mitotic cyclin B-Cdc2 complex (Adachi and Laemmli, 1994), providing a mechanism for preventing S phase entry before completion of mitosis.

The protein levels of Cdks usually remain constant during cell cycle while the levels of cyclins oscillate. The degradation of cyclins is mediated by ubiquitin-dependent proteolysis pathways. A region of ~40 amino acids from the N-terminus is required for the destruction of cyclin A and cyclin B1 (Hunt, 1991; Pines, 1993). A sequence, Pro-Glu-Ser-Thr, within the C terminal region of the G1 cyclins is correlated with their rapid turnover. In some cases (cyclin A and cyclin B2), destruction of the protein requires binding and,

probably, phosphorylation by Cdks prior to proteolysis (Stewart et al, 1994). This requirement adds another mechanism to the control of cyclin-Cdk complex formation.

1.4.2 Cdk inhibitors

Accumulating evidence indicates that Cdk inhibitors are important mediators of the effects of extra cellular stimuli on cell cycle regulation (figure 4). The Cdk inhibitors can be grouped into two classes based on sequence homology: p15, $p16^{Ink4}$ and p18, which are specific for Cdk4 and Cdk6; and $p21^{Waf1}$ and $p27^{Kip1}$, which have broad specificity.

$p21^{Waf1}$ is capable of inhibiting a broad spectrum of cyclin-Cdk activities, including cyclin A-Cdk2, cyclin E-Cdk2, cyclin D-Cdk4, and, to a lesser extent, cyclin B-Cdc2 (Nasmyth and Hunt, 1993). Its inhibitory activity depends on the amount of $p21^{Waf1}$ present in cyclin-Cdk complex. While cyclin-Cdk complexes containing a single $p21^{Waf1}$ molecule are catalyticaly active, those containing multiple $p21^{Waf1}$ subunits are not. Changes in the stoichiometry of $p21^{Waf1}$ were sufficient to account for the conversion (Zhang et al., 1994). It has been suggested that different cyclin-Cdk complexes have different capacity for $p21^{Waf1}$ binding. For example, Cyclin D1 complexes become saturated at lower levels of $p21^{Waf1}$ accumulation than cyclin E complexes (Dulic et al., 1994). $p21^{Waf1}$ also inhibits the activity of PCNA in DNA replication. However, PCNA-dependent gap-filling repair synthesis is not blocked by $p21^{Waf1}$ (Li et al., 1994), suggesting that PCNA plays different roles in replication and DNA repair.

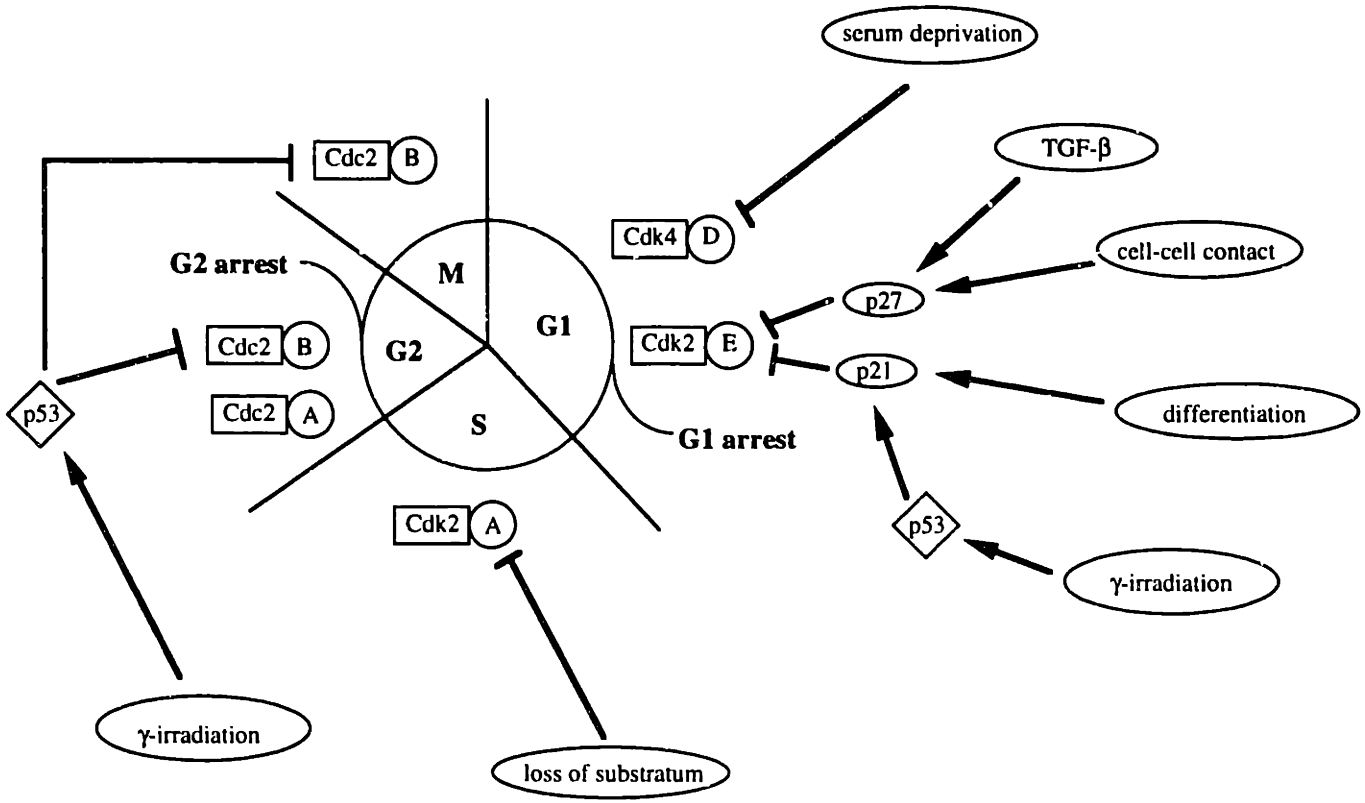
$p21^{Waf1}$ was also cloned as a gene which can be induced by wild type but not mutant p53 (El-Deiry et al., 1993). Its transcription is under the direct control of p53 tumor suppressor. $p21^{Waf1}$ synthesis is increased when p53 is induced after DNA damage by ionizing irradiation (Dulic et al., 1994). In fibroblast, $p21^{Waf1}$ is necessary for G1 arrest induced by γ-irradiation. In p53-negative cells from Li-Fraumeni patients, $p21^{Waf1}$ is absent from cyclin-Cdk complexes (Xiong et al., 1993b). The significance of $p21^{Waf1}$ in

the regulation of cell cycle progression is indicated by its presence in cells arrested under various conditions. $p21^{Waf1}$ was induced by MyoD upon myocyte terminal differentiation; increased $p21^{Waf1}$ correlates with increased binding to Cdks and inhibition of cyclin-Cdk activity (Guo et al., 1995; Jiang et al., 1994; Steinman et al., 1994). The expression of $p21^{Waf1}$ is increased in senescent cells and quiescent cells (Noda et al., 1994). Overexpression of $p21^{Waf1}$ usually causes complete block of cell cycle and results in cell death (El-deiry et al., 1993). $p21^{Waf1}$ is also up-regulated by many environmental stimuli, including cytotoxic and cytostatic agents (Johnson et al., 1994), and serum stimulation (Michieli et al., 1994). The implication of $p21^{Waf1}$ induction in these situations has not been determined.

$p27^{Kip1}$ is a heat-stable factor identified in epithelial cells that were arrested in late G1 by cell-cell contact or treatment with TGF-beta (Polyak et al., 1994). These cells have hypophosphorylated Rb protein (Laiho et al., 1990). They express both cyclin E and Cdk2 but do not contain catalytically active cyclin E-Cdk2 complexes (Koff et al., 1993). $P27^{Kip1}$ inactivates active Cyclin E-Cdk2 complexes as well as prevents Cdk phosphorylation by CAK. Overexpression of $p27^{Kip1}$ arrests cells in G1 phase (Toyoshima and Hunter, 1994). Like $p21^{Waf1}$, the inhibition of cyclin E-Cdk2 activity by $p27^{Kip1}$ depends on the stoichiometry $p27^{Kip1}$ in the complexes. In proliferating cells, $p27^{Kip1}$ is expressed but sequestered, probably by cyclin D-Cdk4 complexes, and is thus unavailable to interact with cyclin E-Cdk2 complexes. In contact inhibited cells and cells treated with TGF-B, the expression of cyclin D and Cdk4 is decreased. Although the expression of $p27^{Kip1}$ is not induced under these circumstances, intracellular level of free $p27^{Kip1}$ increases due to its release from cyclin D1-Cdk4 complex.

$p27^{Kip1}$ is also capable of inhibiting cyclin D1-Cdk4, cyclin A-Cdk2 and cyclin B1-Cdc2 protein kinase activity with different efficiency in vitro (Toyoshima and Hunter, 1994). $p21^{Waf1}$ and $p27^{Kip1}$ share similar N-terminal domains which interact with Cdks.

Figure 4. Inhibition of cell cycle progression by extra cellular growth inhibitory signals. Serum deprivation decreases the expression of cyclin D. In cells arrest in G1 phase by cell-cell contact or treatment with TGF-β, the intracellular concentration of Cdk inhibitor, $p27^{Kip1}$, is increased. The expression of another Cdk inhibitor, $p21^{Waf1}$, is increased in G1 arrest following γ-irradiation and terminal differentiation. Increased level of $p27^{Kip1}$ and $p21^{Waf1}$ correlates with decreased activity of cyclin E-Cdk2 complex. γ-irradiation also causes G2 arrest by inhibiting cyclin B-Cdc2. Repression of cyclin A expression is partially responsible for growth arrest when non-transformed cells are put into suspension.


serum deprivation
TGF-β
cell-cell contact
Cdc2 B
Cdk4 D
G2 arrest
M
G1
p27
Cdk2 E
G2
Cdc2 B
p21
differentiation
p53
Cdc2 A
S
G1 arrest
Cdk2 A
p53
γ-irradiation
γ-irradiation
loss of substratum

The first discovered member of the family of Cdk4-specific inhibitor is $p16^{Ink4}$. In tumor cells which have inactivated Rb, $p16^{Ink4}$, which is not normally detected in Cdk complexes, was found associated with Cdk4 (Bates et al., 1994; Xiong et al., 1993b). $p16^{Ink4}$ binds to Cdk4 and displaces cyclin D (Serrano et al., 1993). In the absence of Rb, these cells may no longer require the activity of Cdk4 or cyclin D1 for G1 progression, and thus escape the control of growth factors (Lukas et al., 1994; Tam et al., 1994). The implication of $p16^{Ink4}$ in tumorigenesis is suggested by the discovery that the multiple tumor suppressor 1 (MTS-1) locus may code for $p16^{Ink4}$ (Kamb et al., 1994).

1.4.3 Cell cycle and tumorigenesis

The formation of tumor is the result of abnormalities in cell growth, proliferation and differentiation. The common characteristic of all transformed cells is their ability to proliferate under conditions which would arrest growth in normal cells. Therefore, it is not surprising to find a close connection between tumorigenesis and cell cycle regulation.

Cyclin D1 was identified as the putative proto-oncogene, PRAD1, located on band q13 of human chromosome 11. This is the site of the BCL1 rearrangement in certain lymphomas and leukemia (Motokura et al., 1991; Withers et al., 1991). Overexpression of cyclin D1 resulted from chromosomal translocation (Withers et al., 1991; Rosenberg et al., 1991; Seto et al., 1992; Williams et al., 1992), retroviral insertion (Lammie et al, 1992), and gene amplification (Lammie et al, 1991; Jiang et al, 1992; Schuuring et al., 1992) of the same locus have been found in lymphomas, squamous cell tumors, and breast carcinoma. Overexpression of cyclin D1 cooperates with Ha-ras to induce transformation of primary rat embryo fibroblasts (Lovec et al, 1994). In ras transformed cells, there is increased expression of cyclin D1 and accelerated G1 progression (Liu et al., 1995).

Cyclin E was also found to be overexpressed in many types of human tumors. The cyclin A gene was disrupted by the insertion of hepatitis B virus in one clonal hepatoma (Wang et

al., 1990), resulting in the production of a chimeric protein which lacks the cyclin destruction domain.

Anchorage-independent growth is one of the characteristics of transformed cells. Continued expression of cyclin A is important for the initiation of S phase during anchorage-independent growth (Guadagno et al., 1993). In suspension culture, synchronized normal rat kidney (NRK) cells arrest cell cycle in late G1 and express cyclin E but not cyclin A. Ectopic expression of cyclin A in NRK cells will allow cells to replicate several rounds in suspension. However, continued expression of cyclin A does not abolish anchorage-dependence, suggesting other factors are involved in the regulation of anchorage-dependent growth.

Inhibitors of cyclin-Cdk complexes were found to be potential tumor suppressors. $p16^{Ink4}$ inhibits Cdk4 by binding in competition with cyclin D. The $p16^{Ink4}$ locus is frequently rearranged, deleted, or mutated in tumor cell lines (Kamb et al., 1994; Nobori et al., 1994) and in tumors isolated from human (Mori et al., 1994; Caldas et al., 1994). Ectopic expression of p16 was shown to inhibit transformation of primary cells induced by ras and myc oncogenes (Serrano et al, 1995). In normal cells most of the cyclin A-Cdk2 complexes were found associated with $p21^{Waf1}$, a Cdk inhibitor which can be induced by p53. This association is absent in most transformed cells (Xiong et al., 1993). Decreased expression and abnormal distribution of $p21^{Waf1}$ were observed during colon carcinogenesis (El-Deiry et al., 1995). The expression of $p21^{Waf1}$ was found decreased in several transformed human cell lines compared to normal fibroblasts. The decrease in both $p21^{Waf1}$ expression and its association with cyclin-Cdk complexes precedes neoplastic transformation, suggesting a possible causal relationship between the two events.

Tumor suppressors p53 and pRb were shown to be key regulators of cell cycle progression. Phosphorylation of Rb in late G1 is essential for cells to enter S phase (reviewed by Hinds and Weinberg, 1994). Hypophosphorylated Rb binds and inactivates

transcription factor E2F. Cells lacking Rb protein may escape signals which control G1 progression and the on set of S phase.

In response to γ-irradiation, most eukaryotic cells exhibit transient delays in both G1 and G2 phases, to allow for repair prior to DNA replication or mitosis (Hartwell and Weinert, 1989). This mechanism of cell cycle control is called checkpoint control (reviewed in Murray, 1994). It ensures the accurate transmission of genetic information and maintains genomic stability. p53 tumor suppressor was shown to be the key factor in both G1 and G2/M checkpoints in mammalian cells (Kastan et al., 1992; Kuerbitz et al., 1992). Loss of p53 function will result in replication or segregation of damaged DNA, and thus leads to genomic instability. This pathway has been suggested as one of the mechanisms by which mutations in p53 contribute to tumorigenesis. Recent evidence indicate that checkpoint control is at least partially mediated by the induction of $p21^{Waf1}$ by p53. Deletion of $p21^{Waf1}$ in transgenic mice did not affect the migration-associated differentiation of intestinal epithelial cell lineage or the p53-dependent apoptosis following γ-irradiation. However, $p21^{-/-}$ mouse embryo fibroblasts are impaired in their ability to undergo G1 arrest following DNA damage (Brugaroias et al., 1995).

1.5 Isolation and characterization of R2.2 revertant from v-fos transformed Rat-1 fibroblasts

The isolation of non-tumorigenic revertants from FBJ v-fos transformed rat fibroblasts was greatly facilitated by the availability of the rapid selection method developed by Zarbl et al. (Zarbl et al., 1987). This method is based on prolonged retention of fluorescent molecule Rhodamine 123 in the mitochondria of v-fos transformed cells compared to normal cells and revertants. v-fos transformed cells are stained with Rhodamine 123 followed by overnight destaining in media. Revertants which retain less Rhodamine 123

can be isolated by fluorescent activated cell sorting (FACS). This selection protocol is more efficient than traditional methods which include the use of toxic selection agents.

On the basis of this method, a number of revertants have been successfully isolated from v-fos transformed Rat-1 fibroblasts following DNA transfection or treatment with mutagen ethyl methanesulfonate (EMS) (Zarbl et al., 1987; Kho and Zarbl, 1993). These revertants retain intact and functional v-fos oncogenes, indicating that they have sustained mutations in cellular genes which are important for v-fos transformation. Two of them, EMS-1-19 and R2.2, have been characterized in detail. In EMS-1-19 cells, the mutation results in decreased expression of transcription factor JunB. Decreased JunB expression is partially responsible for the revertant phenotypes (van Amsterdam et al., 1994). Although the nature of the mutation has not been determined, evidence suggests a defect in the PKA signal transduction pathways. This thesis is focused on the study of R2.2 revertant. Following paragraphs serve as a brief description of the isolation and initial characterization of R2.2 revertants performed by Dr. Choon-Joo Kho.

In an attempt to isolate suppressors of v-fos transformation from normal cells, v-fos transformed Rat-1 fibroblasts (1302-4-1 cells) were co-transfected with a cDNA expression library prepared from normal human foreskin fibroblasts (from Hs68 cell line constructed using the pCDM8 vector) and a selectable marker, the pMex-neo plasmid. After G418 selection, drug resistant cells were stained with Rodamine 123 and analyzed by FACS. Cells which demonstrated the least fluorescence were collected and re-examined for Rhodamine 123 retention and morphology by epifluorescence and phase-contrast microscopy, respectively. R2.2 was one of the putative revertants identified. After 30 minutes staining with Rhodamine 123 followed by overnight destaining, the median fluorescent intensity in R2.2 cells is ~20 fold lower than that in v-fos transformed cells, yet still 2-3 fold higher than that in Rat-1 cells. R2.2 cells exhibit reduced cloning efficiency (0.04%) in semi-solid medium compared to v-fos transformed cells (~13%). The

dramatically decreased tumorigenicity of R2.2 revertant was confirmed by it inability to induce tumor in syngenic Fisher 344 rats after subcutaneous injection of 10^6-10^7 cells. No tumor was observed over a 20 week period in animals injected with R2.2 cells, whereas large tumors (10-30 g) were detected in animals injected with v-fos transformed cells. When injected into nude mice, R2.2 cells did give rise to small tumors, but only after a relatively long period (60 to 80 days) compared to 10 days with v-fos transformed cells. The nodules induced by R2.2 revertant did not exceed 0.1 g, even at 18 weeks after injection.

The existence of an intact and functional v-fos oncogene in R2.2 revertant was confirmed by Southern blot analysis and by retrovirus rescue experiments (Kho, 1992). The expression level of the 3.8 kb v-fos mRNA in R2.2 cells was comparable to that in v-fos transformed cells.

Hybrids between R2.2 revertant and v-fos transformed cells were tumorigenic, indicating that the revertant phenotypes in R2.2 cells are recessive to transformed phenotypes. Therefore, it is likely that R2.2 revertant sustained mutation in transformation effector gene which is necessary for the maintenance of transformed phenotypes.

Southern blotting with probes from the pCDM8 cDNA vector did not detect any signal from R2.2 cells, indicating that R2.2 cells do not harbor any human cDNA. Instead, a single copy of the selectable marker plasmid, pMex-neo, was found to integrate into the genome of R2.2 cells. These results raised the possibility that integration of pMex-neo plasmid disrupted an effector gene essential for v-fos transformation.

The sequence flanking the plasmid integration site was cloned from the genomic library of R2.2 revertant by using the neo gene sequence as probe. Comparison of the sequence flanking the integration site in Rat-1 cells, v-fos transformed cells and R2.2 revertant ruled out any complex genomic rearrangements or gross deletion. In order to determine if integration of pMex-neo plasmid disrupts a transcribed region, non-repetitive sequences

from the integration site were used probes to analyze RNA from Rat-1 cells, v-fos transformed cells and R2.2 revertant by Northern blotting. One of the probes detected a unique 1.2 kb transcript in all the cell lines. Integration of pMex-neo plasmid in R2.2 revertant does not produce novel mRNA species. Screening of a oligo(dT)-primed lambda-gt10 cDNA library prepared from v-fos transformed cells resulted in the cioning of a cDNA of 883 nucleotides with a single open reading frame (orf) of 792 nucleotides. This orf is capable of encoding a protein of 264 amino acids with a predicted moiecular weigh of 29,945 Dalton. The predicted amino acid sequence indicates that it is a highly basic protein with a potential nuclear localization signal and nine potential phosphorylation sites.

The identified mRNA was expressed ubiquitously in all the organs and cell lines examined. Southern blot analysis indicated that this gene is highly conserved during evolution. The gene was latter found to encode rat ribosomal protein RPS3a by direct comparison of the predicted amino acid sequence with the sequence of the amino-terminal 48 amino acids of purified rat RPS3a. The ribosomal localization of RPS3a was confirmed by cell fractionation and indirect immunofluorescence using an antibody raised against RPS3a.

The expression RPS3a mRNA is induced ~5 fold in v-fos transformed cells relative to parental Rat-1 cells. In R2.2 cells, the mRNA level of RPS3a is decreased 50% compared to 1302-4-1 cells, presumably as a result of mono-allelic disruption of the gene by the integration of pMex-neo plasmid. The differences at mRNA level are reflected in similar changes at the protein levels. The rate of protein synthesis is also decreased 50% in R2.2 cells compared to v-fos transformed cells. Decreased expression of RPS3a protein in v-fos transformed cells by expressing antisense RNA produced similar non-tumorigenic phenotypes as in R2.2 revertant. Increased RPS3a level in R2.2 revertant by ectopic expression restored cell transformation. These results indicated that decreased level of RPS3a protein in R2.2 revertant is responsible for the inhibition of v-fos transformation.

Increased expression of RPS3a is a general feature of transformation in Rat-1 fibroblasts. Compared to parental Rat-1 cells, increased RPS3a mRNA levels were observed in cells transformed by v-fes, v-fms, v-sis and v-mos oncogenes. Unusually high expression of RPS3a mRNA was also detected in human tumor cell lines, including T24 (bladder carcinoma), PA-1 (ovarian teratocarcinoma) and SW 48 (colon adenocarcinoma).

Human RPS3a cDNA was cloned from human foreskin fibroblast cell line Hs68 using rat RPS3a cDNA as probe. The human RPS3a cDNA is 89% identical to that of rat with only two amino acid substitutions at the protein level. Rat RPS3a protein shares 63% homology with the yeast MFT1 (Mitochondrial Fusion Targeting) protein which was shown to participate, in an unknown manner, in the import of protein into mitochondria (Garrett et al., 1991). The MFT1 protein was later found to encode yeast ribosomal protein rp10 (Takakura et al., 1992; Metspalu et al., 1992). Rat RPS3a gene is 66% identical to the plant gene cyc07 which is preferentially expressed in the S phase of cell cycle (Kodama et al., 1991) and may play a role in S phase progression (Ito et al, 1991). Interestingly, the expression of RPS3a mRNA is induced during S phase in synchronized human fibroblasts. In mouse embryo fibroblasts, RPS3a homologue was identified as a gene which can be rapidly induced by tumor necrosis factor-alpha (TNF-a) (Gordon et al., 1992). In normal Rat-1 cells, the expression of RPS3a mRNA can be stimulated by serum. Whereas in v-fos transformed cells, the expression of RPS3a is constitutive. These results suggest that RPS3a play important roles in the regulation of cell growth.

Although there is still a lack of direct evidence, RPS3a is likely to be essential for the function of ribosomal. Disruption of RPS3a homologues in yeast resulted in non-viable spores. Expression of antisense RNA of RPS3a in Rat-1 cells resulted in a dramatic decrease in the number of colonies obtained after transfection. Similar to ribosomal protein RPS6, RPS3a was shown to localize on the interface of 40S and 60S subunits (Figure 5). It can be crosslinked to eIF-2, Met-tRNA$_f$ and mRNA (Westermann and Nyagrd, 1983;

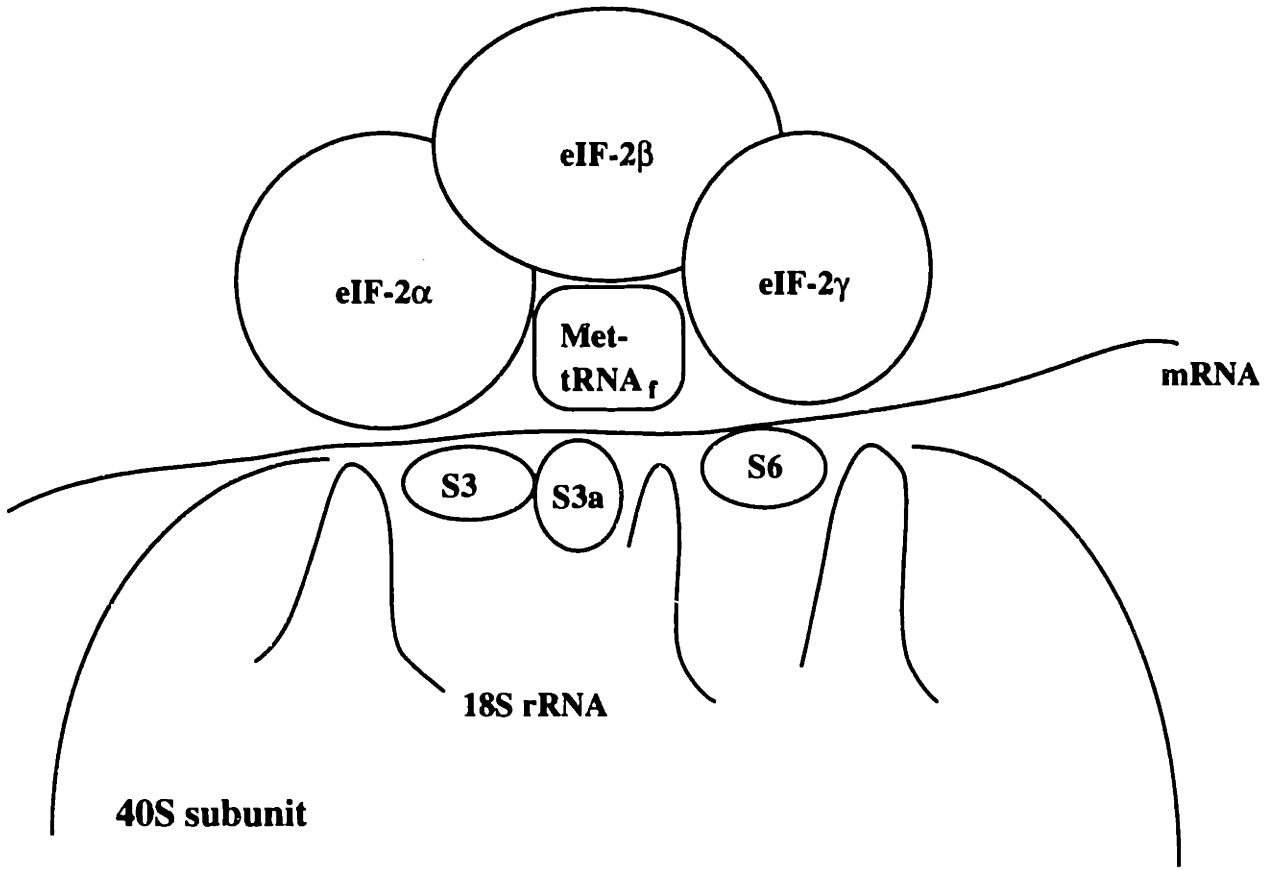


Figure 5. The position of RPS3a in 40S ribosomal subunit.

Westermann et al., 1979; Takahashi and Ogata, 1981; Westermann et al., 1981). RPS3a has been further mapped to the protuberance of the subunit, a location where binding to initiation factors occurs (Lutsch et al., 1990). The evidence suggests that RPS3a may play a role in translation initiation.

Since RPS3a is important for the function of ribosomes, it is possible that decreased expression of RPS3a inhibits v-fos transformation by decreasing ribosomal accumulation. However, the role of RPS3a as a v-fos transformation effector gene can be equally well explained by a second function of RPS3a which is independent of ribosomes. Part of the studies in this thesis were designed to distinguish these two hypotheses. This thesis also investigated the possible mechanism of v-fos transformation reversion in R2.2 revertant by studying cell cycle regulation and tested the effects of decreased expression of RPS3a on tumorigenicity in Hela cells.

Chapter 2
Materials and Methods

2.1 Cell lines

Rat-1 is an established embryonic fibroblast cell line from Fisher 344 rat. 1302-4-1 is a tumorigenic cell line obtained by transformation of Rat-1 cells with FBJ v-fos oncogene. R2.2 is a revertant cell line isolated from 1302-4-1 cells. One allele of ribosomal protein RPS3a gene is disrupted by integration of pMex-neo plasmid in R2.2 cells. C5 is a retransformed cell line isolated from R2.2 revertant after transfection with an expression vector of ribosomal protein RPS3a. The characteristics of the Rat-1, 1302-4-1, R2.2 and C5 are summarized in table 2.

Hela (ATCC CCL2) and Saos-2 (ATCC HTB 85) were obtained from American Type Culture Collection.

Table 2. Properties of Rat-1, 1302-4-1, R2.2 and C5.

Cell Lines	v-fos	Relative RPS3a Protein Level[a]	Rhodamine 123 Retention[a]	Tumorigenicity
Rat-1	-	1	1	-
1302-4-1	+	5	30	+
R2.2	+	2	2	-
C5	+	5	> 30	+

[a] The values were compared to those of Rat-1.

2.2 Plasmid

pcDNA3 (Invitrogen) is a mammalian expression vector with the expression of exogenous gene under the control of human cytomeglovirus (CMV) enhancer-promoter (Figure 6). It contains the neomycin phosphotransferase gene which confers resistance to neomycin (G418). pcDNA3-p21, pcDNA3-p21N and pcDNA3-p21C were constructed by

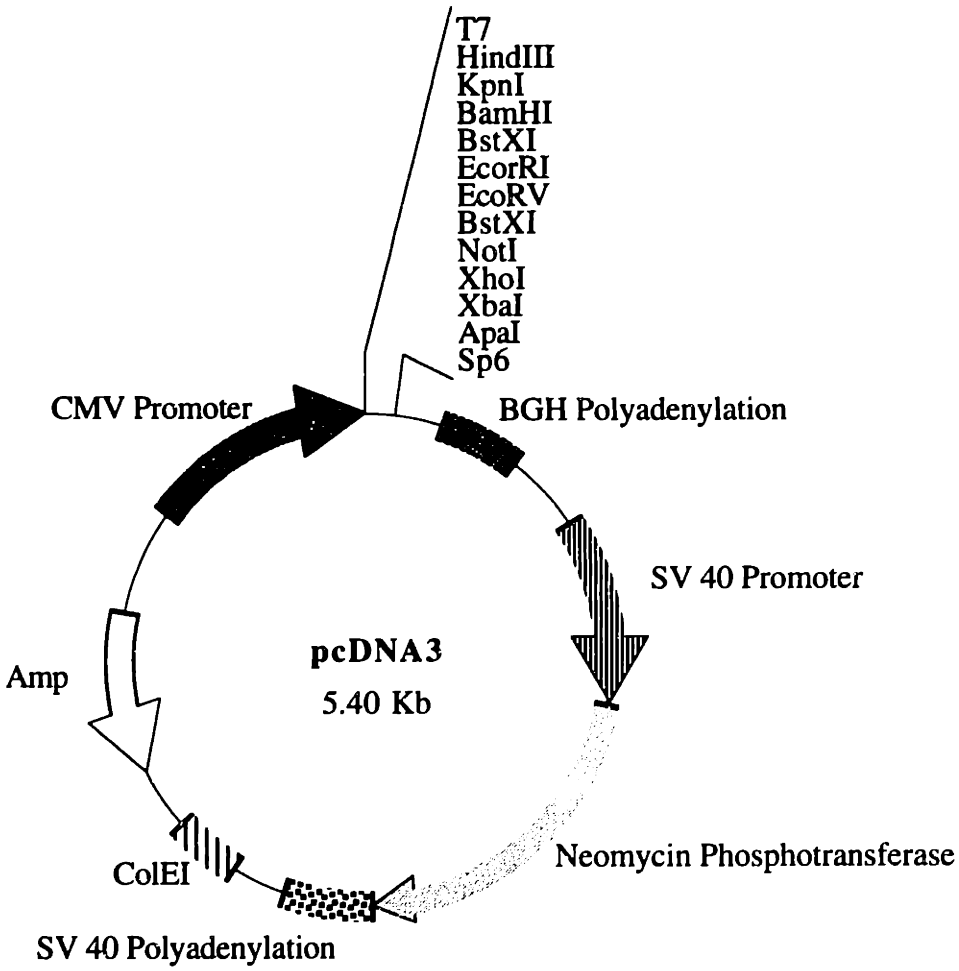


Figure 6. Map of pcDNA3.

cloning of the full-length coding region of human $p21^{Waf1}$ gene (amino acids 1-164), sequence encoding amino acids 1-90 of the gene or sequence encoding amino acids 87-164 of the gene into BamHI and XhoI sites of pcDNA3, respectively.

pMSG (Pharmacia) is an inducible mammalian expression vector. Genes are expressed from the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Figure 7). pMSG-S3a(-) was constructed by cloning of 5' 305 base pairs of human RPS3a cDNA in antisense direction into XhoI and SmaI sites of pMSG.

2.3 Cell culture

Hela (ATCC CCL2) and its derivatives were cultured in Eagle's minimum essential media (MEM) supplemented with 10% Nu serum (Collaborative Research) and non-essential amino acids (Gibco/BRL). Other cell lines were cultured in MEM supplemented wtih 10% fetal bovine serum and non-essential amino acids. Cells were maintained at 37°C in incubators with 5% CO_2. Mycoplasma contamination was examined periodically by Hoechst stain method using Mycoplasma Stain Kit (ICN) and was found to be negative in all cases.

2.4 Soft agar growth assay

Cells were suspended at indicated concentration in complete media (i.e.. MEM supplemented with 10% serum and non-essential amino acids) containing 0.33% noble agar (Difco) and overlaid on 2 ml solidified 0.55% agar medium in 35 mm wells. Plates were kept at 4°C for 20 minutes before incubation at 37°C. Plates were fed with 1 ml media containing 0.33% agar twice a week. At the end of 4 weeks, colonies were stained with 1 ml p-iodonitrotetrazolinium violet (Sigma, 0.5 ug/ml in PBS) in incubators for 2

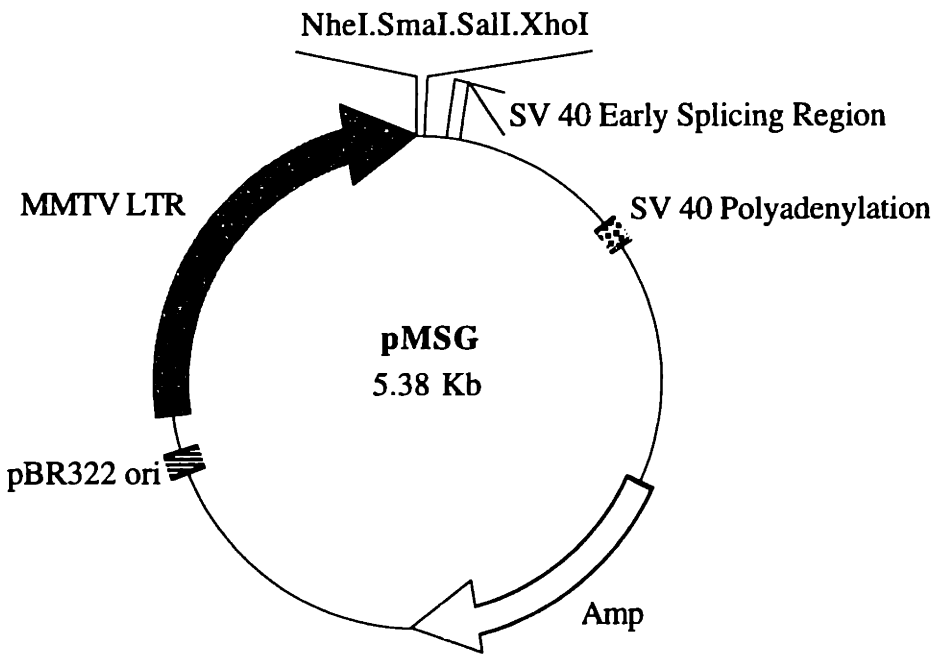


Figure 7. Map of pMSG.

days and dried on filter paper. Experiments were performed in triplicates and repeated at least twice.

2.5 Transfection and selection

Exponentially growing cells were trypsinized, pelleted and resuspended in complete media at 2 X 10^7 cells/ml. A 0.5 ml aliquot of and indicated amount of DNA were mixed in electroporation cuvette. Electroporation was carried out at 270 volts, 960 uF for Hela cells; 350 volts, 960 uF for rat cells; and 290 volts, 960 uF for Saos-2 cells. After electroporation, cells were plated on three or five 100 cm plates in fresh media. Transfected cells were selected with 200 ug/ml G418 (Gibco/BRL) in complete media 36 to 48 hours after transfection. Two weeks later, colonies started to appear. Four weeks later, individual colonies were transferred to 24-well plates by pasteur pipet. When counting of the number of clones was necessary, colonies were stained with xylene cyanole (0.4% xylene cyanole FF, 70% methanol, 10% acetic acid) and destained with water.

2.6 Construction of plasmids

Plasmid was purified from E.coli using the alkaline lysis method (Sambrook et al., 1989). DNA was quantitated by spectrophotometry. Restriction enzyme digestion and ligation were performed under conditions recommended by manufacturer (New England Biolabs or Gibco/BRL). DNA fragments were purified from agarose gel using Qiaex (Qiagen). Ligated DNAs were electroporated into E.coli strain DH5a using Gene Pulser (Biorad). The designed clone was identified by restriction mapping.

2.7 Flow cytometry analysis

Trypsinized cells were counted by Coulter counter (Coulter Electronics, Inc.) and pelleted at 800 X g for 10 min. The cell pellet was resuspend in stain solution (3% w/v

polyethylene glycol (PEG) 6,000, 50 ug/ml propidium iodide, 100 ug/ml RNAase A, 0.1% Triton X-100, 4 mM sodium citrate [pH 7.8]) at 2 X 10^6 cells/ml. After incubation at 37°C for 20 minutes, equal volume of salt solution (3% w/v PEG 6,000, 50 ug/ml propidium iodide, 0.1% Triton X-100, 0.4 M NaCl, pH 7.2) was added. The suspension was pipetted up an down to disrupt clumps. Stained nuclei were analyzed by flow cytometry immediately or stored in dark at 4°C over night before analysis.

Flow cytometry analysis of DNA content was performed using FACScan (Beckton Dickinson) according to manufacturer's instruction. The distribution of cells in cell cycle stages was analyzed using ModFit software (Verity Software House, Topsham, Maine).

2.8 Cell synchronization

Cells were grown to 50% - 70% confluent. Cells were washed three times with phosphate buffered saline (PBS) and incubated at 37°C for 55 hours in isoleucine free MEM (formulated with Selectamine kit from Gibco/BRL) containing 10% dialyzed fetal bovine serum and non-essential amino acids. Cells were then incubated in complete media containing 2 mM hydroxy urea (Sigma) for 14 hours. Cells were released from hydroxy urea block by washing with PBS three times and incubating in complete media. The viability of cells was monitored by trypan blue staining and was over 90% in all cases.

2.9 Treatment of cells with phosphorothioate oligonucleotides

Cells were cultured on 60 mm plates in 2 ml complete media containing phosphorothioate oligonucleotides at indicated concentration. Cells were changed to fresh media containing phosphorothioate oligonucleotides every three days. Other conditions were the same as untreated cells. Phosphorothioate oligonucleotides were dissolved in sterile PBS and added directly to the plates at indicated concentration. In soft agar growth assay, cells in agar were fed with medium containing phosphorothioate oligonucleotides at

indicated concentrations. Antisense phosphorothioate oligonucleotides were designed to be complementary to the 5' end of mRNA which contains the translation start site. The antisense oligonucleotide sequence for rat ribosomal protein RPS3a (RPS3a-AS) is 5' CCC GAC CGC CAT GGT GCC GC 3'; for rat ribosomal protein L5 (RPL5-As) is 5' TCA CGA ACC CCA TCT GCG GA 3'; for rat ribosomal protein S6 (RPS6-AS) is 5' GCT TCA TCT TGA CAC AGC CG 3'. Control oligonucleotides used in the experiments were a random 20-mer phosphorothioate oligonucleotide and a sense phosphorothioate oligonucleotide of ribosomal protein L5 (RPL5-S), 5' TCC GCA GAT GGG GTT CGT GA 3', which is complementary to RPL5-AS.

2.10 Measurement of the rate of protein synthesis

Approximately 5 X 10^4 cells were plated in 35 mM plates 2 days before labeling. Cells were changed to 1 ml fresh media containing 10 uCi L-[3,4,5-^{3}H(N)]-leucine (NEN, 140 Ci/mmol). At time points after labeling, media was taken out and plates were washed three times with cold PBS. 1 ml ice-cold 10% trichloroacetic acid (TCA) was added to each plate. After 10 minutes incubation on ice, TCA was taken out and discarded. The plates were washed twice with 0.5 ml 10% TCA with 10 minutes incubation at 4 ^{o}C for each wash, followed by washing with 1 ml methanol at room temperature. The plates were dried and TCA insoluble material was dissolved in 1 ml 0.3 N NaOH containing 1% sodium lauryl sulfate (SLS). A equal volume aliquot of the solution from each cell line was used for scintillation counting. A parallel set of unlabeled plates was trypsinized to determine cell number. The counts were normalized with cell number.

2.11 Rhodamine 123 retention

Exponentially growing cells were incubated at 37 ^{o}C with 10 ug/ml Rhodamine 123 (Sigma) in complete media for 30 minutes. Cells were then washed with three changes of

fresh media with incubation at 37°C for 30 minutes between each change. After the third change of media, cells were incubated at 37°C for 16 to 18 hours. Cells were trypsinized and suspended at ~5 X 10^5 cells/ml. Fluorescent intensity of the cells was analyzed by flow cytometry using FACScan (Becton Dickinson) using previously established parameters.

2.12 Western blotting

Cells were washed 3 times with cold PBS and lysed in ice-cold RIPA buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.5% SDS, 1 mM PMSF, 30 ug/ml aprotinin). Cell lysate was passed through 21 G needle several times to sheer chromosomal DNA and centrifuged at 12,000 X g in microcentrifuge for 30 minutes. Protein concentration in supernatant was determined by D_C protein assay kit (Biorad). Fifty ug of total protein was mixed with either 2X of 5X SDS sample buffer (1X: 2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-Cl [pH6.8], 0.001% Bromophenol blue) and resolved by SDS-PAGE. For the detection of RPS3a, protein was transferred to Immobilon (Millipore) in CAPS buffer(10 mM CAPS in 10% v/v methanol) using a Transblot (Biorad) apparatus following the recommendation of manufacturer. For the detection of other antigens, transfer was performed in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3).

After proteins were transferred, Immobilon membranes were either processed immediately for antigen detection or dried and stored at 4 °C for later use. Membranes were blocked in TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) containing 5% dried non-fat milk for 1 hour before incubation with antibody diluted in blocking solution on a rocking platform. Membranes were washed three times in TBST with five minutes incubation between each wash. Membranes were then incubated with either Horseradish peroxidase conjugated or biotinylated secondary antibody (Amersham) following manufacturer's recommendation. Antigen was detected by using enhanced

chemiluminescence (ECL) system (Amersham). The intensity of the signals was quantified by scanning autoradiograms with a Pharmacia-LKB laser densitometer.

2.13 Immunoprecipitation and protein kinase assay

Cells were washed three times with cold PBS and lysed in ice-cold H1 lysis buffer (10 mM Tris-Cl [pH7.4], 1% Triton X-100, 0.5% NP40, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM sodium othrovanadate, 10 mM PMSF, 30 ug/ml aprotinin). Cell lysate was passed through 21 G needle several times and centrifuged to remove cell debris. The protein concentration of supernatant was determined by D_C protein assay kit (Biorad) and adjusted to be the same for all cell lines with H1 lysis buffer. Proteins were incubated with a polyclonal anti-cyclin A antibody (Santa Cruz, sc-751, 1:100 dilution) for 1 hour on ice followed by incubation with 20 ul/ml protein A-agarose beads (Santa Cruz) in cold room on a rotating device over night. Immunocomplex captured on protein A-agarose beads was washed three times with 1 ml cold H1 lysis buffer and four times with 1 ml cold H1 kinase buffer (20 mM Tris [pH7.4], 7.5 mM $MgCl_2$, 1 mM DTT). In each wash, beads were suspended in buffer and collected by centrifugation. After the final wash, beads were resuspended in one tenth of the starting volume of cell lysate. The suspension was used in protein kinase assay or quick frozen in liquid nitrogen and stored at -80°C.

For protein kinase assay, 10 ul of immunocomplex suspension was mixed with 5 uCi γ-^{32}P-ATP, 50 uM ATP, 4 ug histone H1 in H1 lysis buffer with a volume of 30 ul. The reaction mixture was incubated at 37°C for 30 minutes. At the end of incubation, the reaction was stopped by adding equal volume of 2X SDS sample buffer and boiling. A fraction (10 - 20 ul) of the reaction was resolved on 10% SDS-PAGE. Phosphorylated histone H1 bands were visualized by autoradiograph of dried gels.

2.14 RNA purification

Total RNA was prepared from cells using the method of Chomczynski and Saachi (Chomczynski and Saachi, 1987). Briefly, cells were washed twice with PBS and lysed in plated with guanidium thiocyanate. Cell lysates were sonicated and extracted with water saturated phenol followed by extraction with chloroform. RNA was precipitated with isopropanol and dissolved in diethyl pyrocarbonate (DEPC) treated water.

2.15 RNAase protection assay

^{32}P-UTP labeled RNA probes was synthesized from linearized plasmid by T7 RNA polymerase. DNA templates were removed by DNAase digestion. RNAase protection assay was carried out as described (Sambrook et al., 1989). Briefly, 20 ug total RNA was co-precipitated with labeled probes by ethanol. The pellet was resuspended in hybridization buffer containing 80% formamide and incubated at 48°C over night. The hybridization mixture was then digested with RNAase A and RNAase T1. After RNAase inactivation and ethanol precipitation, protected fragments were resolved on 6% denaturing PAGE. The gels were dried and exposed to X-ray films. The gels were also exposed to phosphoimager screens. The radioactivity in each band was quantified using ImageQuant software (Molecular Dynamics).

2.16 Gel mobility shift assay

Exponentially growing cells were trypsinized, washed once with cold PBS and once with cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCL, 1.5 mM $MgCl_2$, 0.5 mM DTT). Cell pellets were incubated in at least 5 volume of buffer A for one hour. Cells were then disrupted by passing through 26 G needle 5 times. Nuclei were collected by centrifugation. Buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) was added drop-wise to nuclei pellets. The volume of buffer C was about one third of the volume of initial cell pellet. The

mixture was rocked at 4°C for 1 hour and centrifuged at top speed in microcentrifuge for 15 minutes. Supernatant was used for gel shift assay or aliquoted, frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined using D_C protein assay kit (Biorad).

Double stranded oligonucleotide probes used in the experiments were: AP-1 consensus oligonucleotide (Santa Cruz) 5' - CGC TTG ATG ACT CAG CCG GAA - 3' (AP-1 binding site is underlined.); AP-1 mutant oligonucleotide (Santa Cruz) 5' - CGG TTG ATG ACT TGG CCG GAA - 3' (Substituted bases are in bold face.); SP-1 consensus oligonucleotide (Santa Cruz) 5' - ATT CGA TCG GGG CGG GGC GAG C - 3'. Oligonucleotides were labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs). 3 ug of total protein from nuclear extract was incubated with labeled oligo-nucleotide probes and 1 ug of poly (dI·dC) in 10 ul of buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 2.5 mM DTT) at room temperature for 20 minutes. Reaction mixture was resolved by 4% native PAGE. The position of oligonucleotides was visualized by autoradiograph.

Chapter 3

Decreased Expression of Ribosomal Proteins Inhibits v-fos Transformation in Rat-1 Fibroblasts

3.1 Introduction

Both the v-fos oncogene and c-fos proto-oncogene encodes transcription factor which form heterodimers with Jun family proteins and regulate transcription of genes by binding to AP-1 site in promoter region (Distel et al., 1987; Rauscher et al., 1988; Chiu et al., 1988). c-fos proto-oncogene is involved in regulating the response of cells to extracellular signals during development and cell growth (Nishikura and Murray, 1987; Ruther et al., 1987; Lloyd et al., 1991). Expression of v-fos and constitutive expression of c-fos induce neoplastic transformation in many cell types. Their function as transcription factors is essential for their transforming activity. Therefore, it was hypothesized that v-fos and c-fos induce cell transform by altering the expression of genes. Altered gene expression will result in a spectra of biochemical changes in cellular components which comprise the transforming pathways. However, genes whose expression are regulated by Fos proteins may not necessarily contribute to cell transformation.

Non-tumorigenic revertants can arise spontaneously from transformed cells at low frequency. The reversion frequency can be increased by chemical mutagenesis, insertional mutagenesis or DNA mediated gene transfer. Transformation reversion may result from inactivation of the transforming oncogene or mutations in cellular genes which are essential for cell transformation. Studying of the second category of revertants provides a unique approach to identify biochemical pathways involved in the transformation process. Since the factors mutated in revertants are essential for the tumorigenicity of transformed cells, they are potential targets for designing cancer therapeutic methods. A rapid protocol for the selection of revertants from v-fos transformed cells was developed by Zarbl et al. This method is bases on the loss of prolonged retention of fluorescent molecular Rodamine 123 in the mitochondria of revertants compared to v-fos transformed cells.

A non-tumorigenic revertant, R2.2, was isolated from v-fos transformed Rat-1 fibroblasts after DNA transfection by its loss of prolonged Rhodamine retention. The revertant retains an intact v-fos oncogene and the retrovirus which carries v-fos oncogene can be rescued from R2.2 revertant by infecting the cells with a helper virus. The level of v-fos mRNA in R2.2 revertant was comparable to that in transformed cells. Somatic cell hybridization experiments indicated that the genetic change(s) in R2.2 cells is recessive. Thus, R2.2 revertant has sustained inactivation mutation in gene(s) which is essential for v-fos transformation. Detailed genetic analysis indicated that a single copy of pMex-neo plasmid integrated into the genome of R2.2 revertant. The integration disrupted one allele of ribosomal protein RPS3a gene (Kho and Zarbl, 1993). The expression of RPS3a protein is decreased 50% in R2.2 revertant compared to v-fos transformed cells, presumbly resulted from mono-allelic disruption of RPS3a gene.

Ribosomal protein RPS3a is highly conserved during evolution. Disruption of its homologues in yeast resulted in non-viable spores. Expression of RPS3a antisense RNA in Rat-1 cells greatly reduced the number of colony after transfection. Therefore, RPS3a is likely to be essential for the function of ribosomes. Consistent with this hypothesis, a 50% decrease in the rate of protein synthesis was observed in R2.2 revertant compared to v-fos transformed cells.

Ectopic expression of ribosomal protein RPS3a in R2.2 revertant increased the protein level of RPS3a and the rate of protein synthesis, and in the meanwhile restored transformed phenotypes. Decreasing the protein level of RPS3a by antisense RNA in v-fos transformed cells resulted in similar revertant phenotypes as in R2.2 cells. Therefore, decreased RPS3a protein level is responsible for the revertant phenotypes in R2.2 cells.

Since RPS3a is a ribosomal protein, it is possible that decreased RPS3a level inhibits v-fos transformation by decreasing the number of functional ribosomes. However, this hypothesis does not exclude the possibility that the transformation reversion is mediated by

a second function of RPS3a which is independent of its role in ribosomes. Several ribosomal proteins were suggested to have more than one function. For example, rat ribosomal protein RPS3 and its two homologues in Drosophila were reported to associated with nuclear matrix of chromatin and have AP (apurinic/apyrimidinic) endonuclease activity which is involved in DNA repair (Grabowski et al., 1991; Wilson et al., 1993; Wool, 1993). Distinguishing between the above two possibilities is pivotal to the subsequent study of the biochemical pathways involved in the reversion process.

If decreased RPS3a level inhibits v-fos transformation by decreasing ribosomal accumulation. Then, decreased expression of other ribosomal proteins which are essential for the function of ribosomes should also generate revertant phenotypes in v-fos transformed cells. Ribosomal proteins RPL5 is involve in the early stage of ribosomal assembly and rRNA processing. Phosphorylation of RPS6 can be stimulated by mitogenic signals and is strongly correlated with increased rate of protein synthesis (Stewart and Thomas, 1994 and the references therein). Therefore, ribosomal proteins RPL5 and RPS6 are likely to be essential components of ribosomes. Decreasing the expression of these two ribosomal proteins should decrease the number of functional ribosomes. In this study, v-fos transformed cells were treated with antisense oligonucleotides of ribosomal protein RPL5 and RPS6 to decrease their expression. Cells treated with antisense oligonucleotides exhibit revertant phenotypes as indicated by their inability to grow in semi-solid media. The results support the hypothesis that decreased ribosomal accumulation inhibits v-fos transformation.

3.2 Results

3.2.1 Treatment of cells with antisense oligonucleotides

In order to decrease the expression of ribosomal proteins RPL5 and RPS6, v-fos transformed cells were treated with antisense oligonucleotides. The following consideration was taken into account in designing oligonucleotides to maximize their effectiveness: (1) 12-22 nucleotides long; (2) high Tm of binding to complementary oligonucleotides; (3) low secondary structure; (4) close to the translation initiation site; (5) minimum self dimer formation. The oligonucleotides were synthesized as phosphorothioates which are resistant to nucleases. A random oligonucleotide was used as a negative control. Cells treated with antisense oligonucleotide of RPS3a served as positive control.

The effective concentration of oligonucleotide was reported to range from 0.5 uM to 20 uM. v-fos transformed cells were treated with 5 uM of each oligonucleotide initially. Extensive cell death was observed in cells treated with antisense oligonucleotides of ribosomal protein RPL5 and RPS3a. Since cell death was not observed in cells treated with control oligonucleotides and antisense oligonucleotide of RPS6, the cell death was likely to have resulted from inhibition of ribosomal accumulation. The toxicity of antisense oligonucleotides of RPL5 and RPS3a indicated that they functioned efficiently to decrease the level of the ribosomal proteins. The concentrations of these two oligonucleotides were decreased until no significant cell death observed. The sequence and working concentration of the oligonucleotides are summarized in Table 3.

After being cultured in media containing oligonucleotides for two weeks, cells treated with antisense oligonucleotides exhibit flat and non-refractile morphology, whereas, untreated cells and cells treated with control oligonucleotide remained rounded and refractile results not shown). The slow response is consistent with the slow turnover of ribosomal proteins.

Table 3. Sequence, working concentration and Tm of the antisense oligonucleotides.

Oligonucleotides	Sequence	Concentra-tion (uM)	Tm (°C)[a]
control	Randon	5	-
RPS3a-AS	5' CCC GAC CGC CAT GGT GCC GC 3'	1.5	72
RPL5-AS	5' TCA CGA ACC CCA TCT GCG GA 3'	3	64
RPS6-AS	5' AGC TTC ATC TTG ACA CAG CC 3'	5	60

[a] Tm = 2 X (A+T) + 4 X (G+C).

3.2.2 Soft agar growth assay of cells treated with antisense oligonucleotides

The ability of cells to grow in semi-solid medium is strongly correlated with tumorigenicity in fibroblasts. In order to test the soft agar growth ability of v-fos transformed cells treated with antisense oligonucleotides, cells were seeded at 5 X 10^4 cells/ml in agar in the presence of oligonucleotides at appropriate concentration. Cells were fed with media containing oligonucleotides twice a week. Three weeks later, colonies were stained and dried on filter paper. The colonies appear as black dots (Figure 8). Compared to untreated cells and cells treated with control oligonucleotides, cells treated with antisense oligonucleotides of ribosomal proteins gave rise to greatly reduced number of colonies, suggesting that these cells have lost this transformed phenotype.

The inability of cells treated with antisense oligonucleotides to grow in soft agar depends on continuous presence of the oligonucleotide. When cells seeded in soft agar were fed with media which did not containing antisense oligonucleotides, they gave rise to visible colonies (Figure 9). This result suggests that the antisense oligonucleotides are necessary for the revertant phenotypes, ruling out the possibility that the reversion resulted from selection, mutation or clonal expansion.

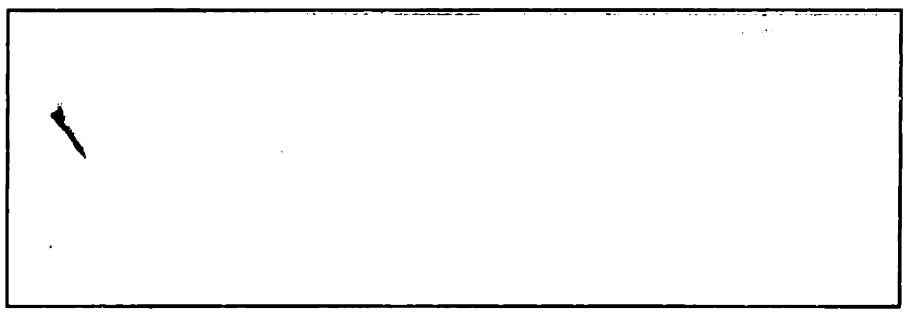
Figure 8. Soft agar growth of v-fos transformed cells treated with antisense oliogo-nucleotides of ribosomal proteins. v-fos transformed cells and v-fos transformed cells treated with appropriate concentration of indcated oligonucleotide were seeded at 5 X 10^4 cells/ml in 0.33% agar. Cells were fed with media with or without oligonucleotides. Colonies were stained 4 weeks after plating.

v-fos transformed cells

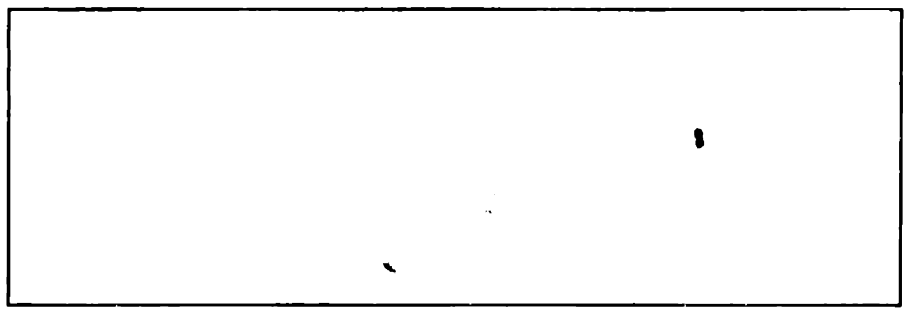
No oligo

Control oligo

Antisense oligo of ribosomal protein RPS3a



Antisense oligo of ribosomal protein RPL5



Antisense oligo of ribosomal protein RPS6

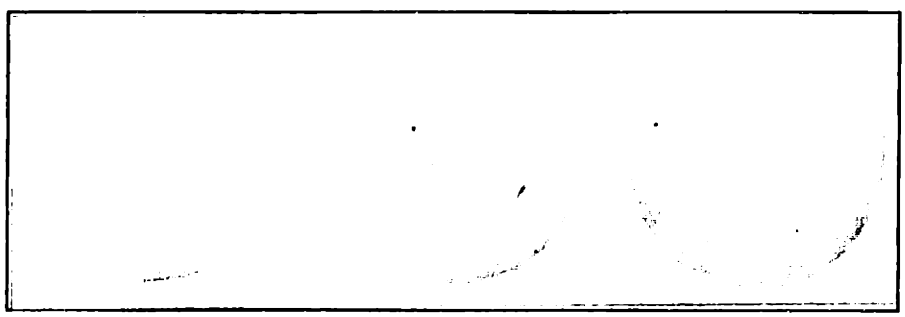
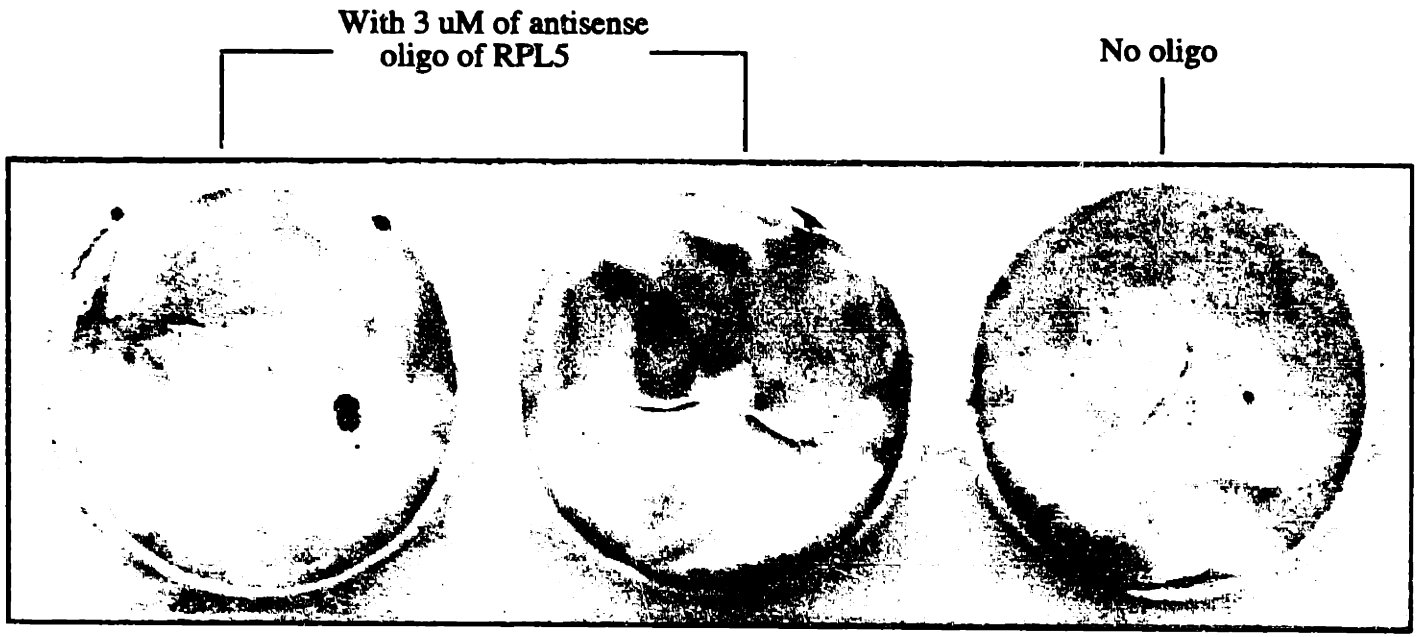


Figure 9. Soft agar growth of v-fos transformed cells treated with antisense oligonucleotide of ribosomal protein RPL5 in the presence and absence of antisense oligonucleotide. v-fos transformed cells treated with antisense oligonucleotide of ribosomal protein RPL5 were seeded at 5×10^4 cells/ml in 0.33% agar. Cells were fed with media with or without antisense oligonucleotide of RPL5 as indicated.


With 3 uM of antisense
oligo of RPL5
No oligo

The inability of cells to grow in soft agar is only one of the indications of transformation reversion. The tumorigenicity of v-fos transformed cells with decreased expression ofribosomal proteins RPL5 or RPS6 should be tested in syngenic rats. It is not feasible to treat the cells injected into animal with antisense oligonucleotides. Stable cell lines with decreased level of RPL5 or RPS6 can be created by expression antisense RNA in v-fos transformed cells and used for tumorigenicity study in animals.

3.2.3 Rate of protein synthesis in treated cells

The best way to test the efficacy of antisense oligonucleotides is to look for decreased expression of target proteins by immunoblotting. However, functional antibodies against ribosomal proteins RPL5 and RPS6 were not available when this study was conducted, and thus the effectiveness of antisense oligonucleotides had to be assessed by other methods.

Decreased expression of ribosomal protein RPS3a in v-fos transformed cells resulted in decreased rate of protein synthesis (Kho, 1992). This result suggested that the amount of functional ribosomes was rate-limiting for protein synthesis. Therefore, the rate of protein synthesis can be used as an indication of cellular level of functional ribosomes. If antisense oligonucleotides function effectively to decrease the level of ribosomal proteins, cellular concentration of functional ribosomes should be reduced provided that the ribosomal proteins are essential for the function of ribosomes. As a result, the rate of protein synthesis should decrease as well.

The rate of protein synthesis was measured by the rate of incorporation of ^{3}H-leucine into newly synthesized peptides. Exponentially growing cells were incubated with ^{3}H-leucine. At time points after labeling, cells were treated with 10% cold TCA to precipitate cellular protein and remove free ^{3}H-leucine. TCA precipitable radioactivity, expressed as counts per minute (cpm), was determined and normalized to cells number. Once the cellular

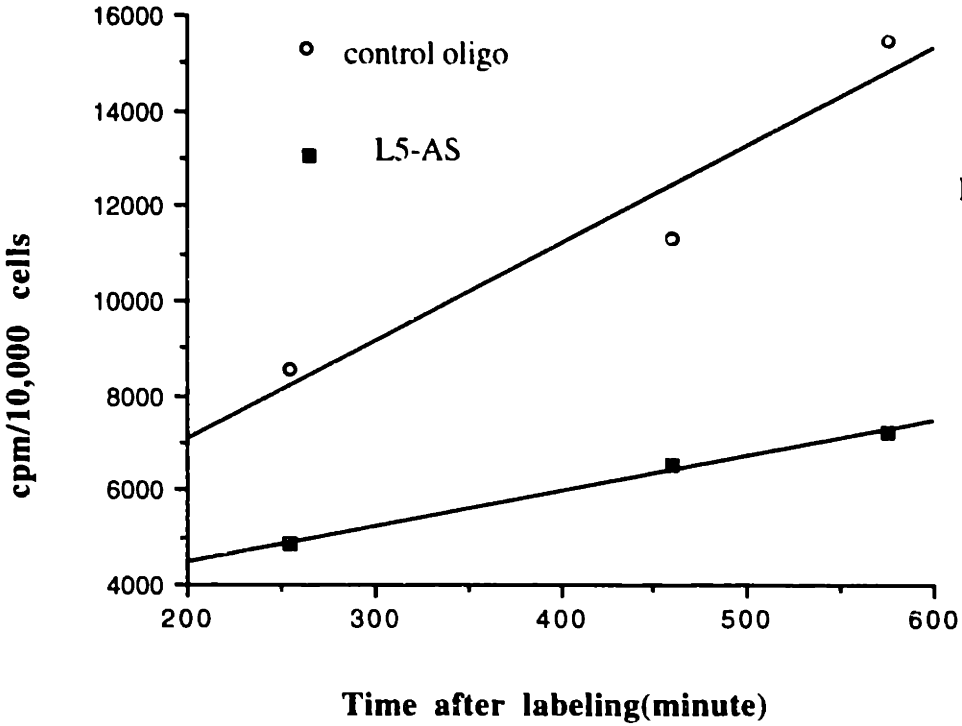
amino acid pool is saturated with ^{3}H-leucine, the incorporation of radioactivity into protein increases linearly as a function of time. The radioactivity per 10,000 cells was plotted against time (Figure 10). The slope of the line represents the rate of protein synthesis which is expressed as cpm per minute per 10,000 cells . The rate of protein synthesis in v-fos transformed cells treated with antisense oligonucleotide of ribosomal protein RPL5 was only about one third of the rate in cells treated with control oligonucleotide. This level is comparable to that was seen in R2.2 revertant.

3.3 Discussion

3.3.1 Antisense oligonucleotides as a means to decrease the expression of ribosomal proteins

Antisense oligonucleotide has been routinely used as an approach to decrease gene expression . It is proposed that binding of antisense oligonucleotides to target mRNA activates its degradation by ribonuclease H. The ability of oligonucleotides to activate RNase H is critical for their inhibitory activity (Wagner et al., 1993). Successful application of antisense oligonucleotides depends on the correct selection of oligonucleotide sequences to give strong inhibition of translation, high oligonucleotide stability and good uptake into cells. Effective antisense oligonucleotides are typically 15-22 nucleotides long. This length is in theory long enough to give a sequence that allows specific binding to target mRNA but short enough to be taken up into cells efficiently. Sequences containing the initiation codon and or surrounding sequence have been particularly successful as a target for antisense oligonucleotide. High concentration of oligonucleotides frequently leads to nonspecific inhibition of gene expression, cytotoxicity and induction of p53, which is capable of binding to the ends of single stranded nuclei acid. The effective concentrations of antisense oligonucleotides are reported to range from 0.5 uM to 20 uM, depending the

Figure 10. Rate of protein synthesis in cells treated with oligonucleotides. The rate of protein synthesis was measured in v-fos transformed cells treated with a random oligonucleotide (control) or antisense oligonucleotide of ribosomal protein RPL5 (L5-AS) according to the method described in Materials and Methods.



Rate(control) = 20.7 cpm/10,000 cells/min

Rate(L5-AS) = 7.6 cpm/10,000 cells/min

cell types and the genes to be inhibited. In this study, treatment of cells with 10 uM of oligonucleotides resulted in massive cell death in all cases. Modification of oligonucleotides and novel delivery methods allow low concentration of antisense oligonucleotides to be used in experiments. Most researchers now use antisense oligonucleotides that are phosphorothioate-modified because of their enhanced nuclease stability (Froehler et al., 1992). Modification with C-5 propyne pyrimidines greatly increased the affinity for complementary RNA and resulted in decreased effective concentrations (Wagner et al., 1993). Catonic lipids have been used to facilitate cellular uptake of antisense oligonucleotides (Bennett et al., 1992).

High Tm of binding to complementary oligonucleotides, low secondary structure, and lack of self complementary are also important factors that contribute to the effectiveness of antisense oligonucleotides. Interestingly, in this study we found that the effective concentrations of antisense oligonucleotides are inversly correlated their Tms (Table 3).

The half life of ribosomes have been estimated to be 5 to 10 days (Larson et al., 1991). The equilibrium of decreased levels of ribosomal proteins is likely to be achieved over a period of time. In this study, we found that morphological changes associated with the loss of transformed phenotype was only observed about two weeks after the initial treatment, which is not suprising given that the half-life of ribosomes is about 10 days.

3.3.2 Ribosomal proteins and protein synthesis in tumorigenesis

In order to test the hypothesis that decreased expression of ribosomal protein RPS3a inhibits v-fos transformation by decreasing ribosomal accumulation, v-fos transformed Rat-1 fibroblasts were treated with antisense oligonucleotides of ribosomal proteins RPL5 and RPS6 to decrease their expression. These two ribosomal proteins are likely to be essential for the function of ribosomes. Ribosomal protein RPL5 binds to and stabilize 5S rRNA in the early stage of ribosomal assembly (Steitz et al., 1988). Ribosomal protein

RPS6 is phosphorylated in response to mitogenic stimuli. Phosphorylation of RPS6 correlates with increased translation rate. Decreased protein levels of RPL5 and RPS6 are likely to result in reduced cellular level of functional ribosomes.

Compared to non-treated cells and cells treated with control oligonucleotides, v-fos transformed cells treated with antisense oligonucleotides of ribosomal proteins RPL5 or RPS6 exhibit dramatically decreased growth in semi-solid media (soft agar). The inability of cells to grow in soft agar was dependent on the continuous presence of antisense oligonucleotides. The results indicate that treatment with antisense oligonucleotides of ribosomal protein RPL5 or RPS6 inhibits v-fos oncogene induced cell transformation in Rat-1 fibroblasts. Although the levels of ribosomal proteins RPL5 and RPS6 were not studied due to the lack of specific antibodies, inhibition of v-fos transformation is likely to be mediated by decreased expression of ribosomal proteins RPL5 and RPS6. Consistent with this hypothesis, the rate of protein synthesis in cells treated with antisense oligonucleotide of ribosomal protein RPL5 was found to be decreased to about one third of the rate in cells treated with control oligonucleotide, same as seen in R2.2 revertant. These results support the hypothesis that decreased expression of ribosomal protein RPS3a mediates v-fos transformation reversion by decreasing the amount of functional ribosomes. Therefore, at least in v-fos transformed cells, maintenance of a thereshhold level of ribosomal accumulation is essential for cell transformation.

The importance of ribosomal proteins and protein synthesis in tumorigenesis has been indicated by a wealth of evidence. The expression of a number of ribosomal proteins was found increased in tumor tissues and transformed cells (Table 1). Although not likely to be the cause of malignancy, increased ribosomal accumulation may be important for the maintenance of tumorigenicity. Translation initiation factor eIF-4E is involved in the unwinding of mRNA secondary structure at or near the 5' m^7G cap site. Increased expression of eIF-4E induced transformation of primary cells in cooperation with v-myc or

E1A (Lazaris-Karatzas and Sonenberg, 1992). eIF-4E was shown to facilitate translation initiation of mRNAs with extensive secondary structures in their 5' untranslated regions. These secondary structures were found in mRNAs encoding growth factors, growth factor receptors and proto-oncogenes (Loromilas et al., 1992), suggesting that modulation of translation efficiency may change the balance between growth promoting factors and growth inhibitory factors.

Decreased level of ribosomal proteins results in decreased overall rate of protein synthesis. The decreased rate of protein synthesis seems to be offset by increased doubling time (see Chapter 4). The protein content of R2.2 revertant, expressed as ug of protein per one million cells, is similar to that of v-fos transformed cells (table 4). However, the pattern of gene expression could be changed by decreased ribosomal accumulation according to the following two hypotheses. This possibility is supported by the founding that the expression of Cdk inhibitor, $p21^{Waf1}$, is increased in R2.2 revertant compared to v-fos transformed cells (Chapter 4).

It is possible that decreased cellular level of ribosomes will affect the translation efficiency of mRNAs differentially. When the availability of ribosomes becomes the rate limiting factor, mRNA many compete with each other for binding to ribosomes. This possibility is supported by the observation that the synthesis of some proteins was decreased to a greater extent than the overall decrease in the rate of protein synthesis in R2.2 revertant (Kho, 1992). As a result, although the cellular protein content remains constant, decreased ribosomal accumulation results in altered gene expression pattern. Translation efficiency of mRNA is largely determined at the step of translation initiation. Under similar condition, the translation efficiency of mRNA is determined by its 5' end sequence elements, including the position and sequence context of translation initiation codon AUG and the secondary structure in 5' end (reviewed in Kozak, 1991). In the "scanning model" of translation initiation, a 48S complex formed by ribosomal protein 40S

subunit and translation initiation factors scans along unwound mRNA from the 5' end to the first AUG codon followed by binding of 60S subunit to yield the final 80S initiation complex (reviewed in Merrick, 1994). Decreased level of either subunit could affect translation initiation. However, it is not known if decreased level of 40S subunit or 60S subunit is equally effective in the suppression of v-fos transformation, because it is possible that decreased expression of proteins of one ribosomal subunit may affect the level of both subunits. In the early stages of ribosomal assembly, ribosomal proteins bind to 45S pre-rRNA and form a complex referred to as 80S preribosome. The pathways of ribosomal assembly has not been completely understood. The ribosomal proteins were suggested to facilitate the processing of 45S pre-rRNA which is spliced into 28S (60S subunit), 18S (40S subunit) and 5.8S (60S subunit) rRNAs (reviewed by Hadjiolov, 1980). Therefore, decreased expression of one ribosomal protein may affect the assembly of both subunits. This possibility can be tested by directly quantify the level of ribosomal subunits in R2.2 revertant or v-fos transformed cells treated with antisense oligonucleotides of RPL5 or RPS6.

The second hypothesis takes into consideration the fact that the cellular concentration of proteins also depends the volume of the cell, which was not determined in this study. With the same protein content, R2.2 revertant could have higher or lower protein concentration compared to v-fos transformed cells. In cases where the active enzyme is composed of several subunits, small changes in the concentration of individual factors could result in large changes in the concentration of the complex. As a result, the regulation of signal transduction and gene expression in cells could be drastically altered.

Therefore, decreased ribosomal accumulation could inhibit v-fos transformation by increasing the expression of transformation suppressor genes and/or decreasing the expression of transformation effector genes directly or indirectly. Identifying these transformation effector/suppressor genes is essential for understanding the molecular

mechanism of inhibition of v-fos transformation by decreased expression of ribosomal proteins.

Chapter 4

Decreased Expression of Ribosomal Proteins Altered Cell Cycle Regulation in v-fos Transformed Cells

4.1 Introduction

The study of oncogene induced cell transformation has contributed significantly to the understanding of tumorigenesis. The products of proto-oncogenes are usually important factors in the regulation of cells growth. They assume a wide range of functions including growth factors, growth factor receptors, protein kinases, transcription factors, signal transducers and cyclins. Thus, the studies of the properties of oncogenes/proto-oncogenes and the biochemical pathways of cell transformation also contributes to our knowledge about cell growth, proliferation and differentiation. The expression of a transforming oncogene induces a series of molecular and biochemical changes inside cells which altogether contribute to transformed phenotypes. Identifying these biochemical and molecular events is the key step in our understanding of cell transformation.

The study of non-tumorigenic revertants isolated from transformed cells has been used as an approach to identifying the biochemical pathways important for cell transformation. The revertants of most interest are those which retain intact and active oncogene, but harbor mutations in cellular genes which play important roles in the transformation process. By identifying the causal mutated genes in revertants and studying the biochemical pathways associated with these genes, we will be able to dissect the transformation process. Revertant cell line R2.2 was isolated from v-fos oncogene transformed Rat-1 fibroblasts (Kho, 1992). Genetic analysis indicated that one allele of ribosomal protein RPSa gene was disrupted by the integration of pMexneo plasmid. The protein level of RPS3a and the rate of protein synthesis were decreased in R2.2 revertant compared to v-fos transformed cells. Decreased level of ribosomal protein RPS3a was shown to be responsible for the revertant phenotypes. Further study indicated that decreased RPS3a protein level inhibits v-fos transformation by decreasing ribosomal accumulation, since treatment of v-fos transformed cells with antisense oligonucleotide designed against ribosomal proteins RPL5 and RPS6

also resulted in reversion of v-fos transformation and comparable decreases in the rates of protein synthesis. However, the mechanism by which decreased ribosomal protein level leads to transformation reversion has not been established.

The ultimate goal in our analysis of revertants is to elucidate the molecular events which are initiated by the mutations and finally lead to revertant phenotypes. Decreased ribosomal accumulation could have pleotropic effects on gene expression and our task is to identify genes which are important for the reversion of v-fos transformation. Much evidence has demonstrated a close connection between cell cycle regulation and tumorigenesis. This connection was suggested by the fact that the growth of tumor is the result of abnormalities in the control of cell growth, proliferation and differentiation. The major characteristic of transformed cells is their ability to proliferate under conditions which will arrest growth of normal cells. Recent evidence has demonstrated that abnormalities in cell cycle in cell cycle regulation are not only indicators of tumorigenicity but also contribute to tumorigenesis.

The eukaryotic cell cycle is divided into four stages, G1, S, G2 and M. The progression of cell cycle is controlled by the activity of different cyclin-cyclin dependent kinase (Cdk) complexes. Cdks which have protein serine/theronine kinase activity are the catalytic subunit of the complexes. Their kinase activity is activated by binding to cyclins which also provide substrate specificity. The activity of the cyclin-Cdk complexes is further regulated by phosphorylation and binding to inhibitors.

Increased expression of cyclins, which are positive regulators of cell cycle, have been detected in a variety of tumors. For example, overexpression of cyclin D1 resulted from chromosomal translocation, retroviral insertion, gene amplification has been detected in lymphomas, squamous cell tumors, and breast carcinoma (Withers et al., 1991; Lammie et al., 1992; Schuuring et al., 1992). Meanwhile, negative regulators of cell cycle, such as cyclin-dependent kinase (Cdk) inhibitors, were implicated in tumor suppression. Cdk4 inhibitor $p16^{Ink4}$ locus is frequently found inactivated by rearrangement, deletion or

mutation in tumor tissues and tumor cell lines (Kamb et al., 1994; Mori et al., 1994). Anchorage-independent growth which is a characteristic of cell transformation was reported to associated with continued synthesis of cyclin A (Guadagno et al., 1993). Tumor suppressors p53 and Rb were shown to play key roles in cell cycle regulation. Their roles in cell cycle are associated with their function as tumor suppressors. Phosphorylation of Rb in late G1, which results in the activation of transcription factor E2F, is necessary for cells to enter S phase (reviewed by Hinds and Weinberg, 1994). p53 is the key regulator of checkpoint control of cell cycle (Kastan et al., 1992; Kuerbitz et al., 1992). Abnormalities in checkpoint control is one of the mechanisms leading to tumor development. The function of p53 in checkpoint control was shown to be partially mediated by Cdk inhibitor $p21^{Waf1}$.

R2.2 revertant has an increased doubling time relative to v-fos transformed cells, suggesting that decreased ribosomal protein level may affect cell cycle progression. Changes in cell cycle regulation may contribute to the inhibition of v-fos transformation. A comparison of cell cycle regulation between R2.2 revertant and v-fos transformed cells could point out the biochemical pathways or a subset of pathways which mediate transformation reversion. The study presented here provides evidence to support of the hypothesis that altered cell cycle regulation contributes to the revertant phenotype of R2.2 revertant.

4.2 Results

4.2.1 The AP-1 binding activity in R2.2 revertant

The first step in analyzing revertants is to rule out the possibility that the non-transformed phenotypes resulted from inactivation of the transforming oncogene. Previous results indicate that the R2.2 revertant has functional v-fos oncogene. The level and size of

v-fos mRNA in R2.2 revertant is comparable to that in v-fos transformed cells. However, decreased expression of ribosomal protein could affect the expression of v-fos protein or its activity as a transcription factor. One mechanism by which decreased expression of ribosomal protein could inhibit v-fos transformation is to decrease the activity of v-Fos onco-protein. This possibility was suggested by the finding that the expression of $\alpha 1(I)$ procollagen mRNA, which is repressed in v-fos transformed cells, is partially restored in R2.2 revertant (Kho, 1992). The expression of Fos proteins was shown to be regulated at posttranscriptional level by the stability of mRNA. Fos proteins are phosphorylated at multiple sites. Although the function of phosphorylation has not been understood completely, phosphorylation of Fos proteins may represent a mechanism which regulates their activity. The ability of v-fos to form heterodimer with Jun family proteins and bind to AP-1 consensus sequence is necessary for its transforming activity. Changes in the expression or modification of Jun family proteins may affect the transforming activity of v-fos.

To test the above possibility, the expression of v-Fos protein was studied in exponentially growing cells by immunoblotting using antibody specific for Fos proteins (c-Fos and v-Fos) (Figure 11). The expression of Fos proteins was not detectable in growing Rat-1 cells. R2.2 revertant showed similar Fos expression level as v-fos transformed cells. Since the transforming activity of v-fos is mediated by AP-1 sites in promoters, the amount of factors bound to AP-1 sequence serves as a index to evaluate the function of v-fos. AP-1 binding activity was studied by gel mobility shift assay. ^{32}P labeled oligonucleotide containing the consensus AP-1 sequence was incubated with nuclear extract from equal number of cells. Oligonucleotides with protein bound migrate slower than free oligonucleotides in native PAGE and can be visualized by autoradiograph (Figure 12). The specificity of binding was demonstrated by competition experiments. The binding to labeled AP-1 oligonucleotides was titrated by 10 times excess of unlabeled AP-1

Figure 11. The expression of Fos protein in Rat-1, 1302-4-1, and R2.2 cells. 50 ug of total cellular protein from exponentially growing cells was resolved on SDS-PAGE. The expression of 55 Kd Fos protein was detect by a specific antibody.

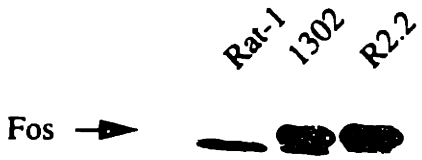
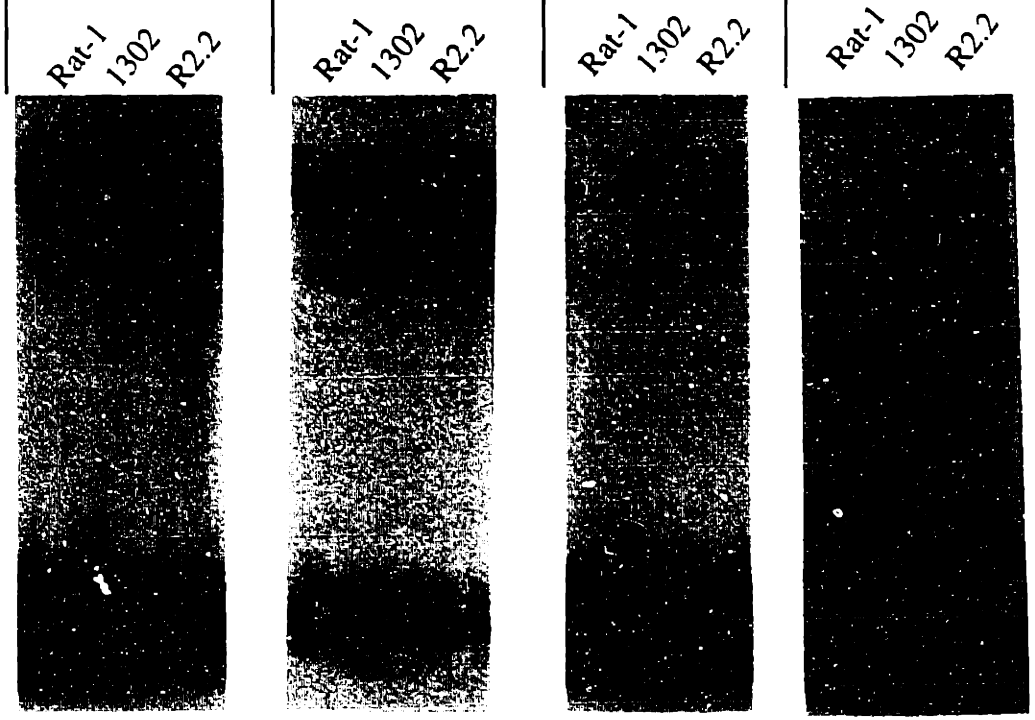

Rat-1
1302
R2.2
Fos

Figure 12. AP-1 binding activity in Rat-1, 1302-4-1, and R2.2 cells. 3 ug of nuclear protein from each cell line was incubated with ^{32}P-labeledoligonucleotide probes in the presence or absence of 10 fold excess of cold competitor. Oligonucleotide-protein complexes were detected by autoradiograph following electrophoresis in native PAGE.

labeled probe	AP-1	AP-1	AP-1	AP-1 (mutant)
cold competitor	none	AP-1 (mutant)	AP-1	none



oligonucleotides but not by unlabeled mutant AP-1 oligonucleotides. In normal Rat-1 cells, there was minimal AP-1 binding activity, consistent with the low abundance of Fos proteins and Jun proteins in proliferating cells. The AP-1 binding activity was similar in v-fos transformed cells and R2.2 revertant. These results indicate that decreased expression of ribosomal protein RPS3a did not affect the expression of v-Fos protein or its binding to AP-1 site. However, the ability of AP-1 bound v-Fos protein to regulate transcription can not be determined from the above experiments. The possibility that decreased expression of ribosomal protein RPS3a affects the ability of v-Fos protein to regulate transcription with out affecting its binding to AP-1 site still exists.

4.2.2 Distribution of cells in cell cycle stages

The doubling time of cell lines were calculated as the increase in cell number in exponentially growing cultures over a period of time (Table 4). Transformation of Rat-1 fibroblasts with v-fos oncogene resulted in increased doubling time, 17.7 hours in Rat-1 cells vs. 29.5 hours in v-fos transformed cells. Slower G1-to-S transition induced by v-fos was reported to be responsible for the increase (Balsalobre and Jolicoeur, 1995). Compared to v-fos transformed cells, R2.2 revertant has further increased doubling time, 38.6 hours in R2.2 revertant vs. 29.5 hours in v-fos transformed cells. An increase in doubling time could be due to increased duration of every cell cycle stage or a particular cell cycle stage. A direct comparison of the duration of each cell cycle stage between R2.2 revertant and v-fos transformed cells was performed to address this question.

In the exponentially growing cell population, a fraction of the cells at age t in the life cycle is proportional to $2e^{-at}$ where a = ln2/(generation time). The age t is zero at the beginning of the life cycle and equal to generation time at the end of the life cycle. Thus, the logarithm of the fraction is linearly related to age t. On the basis of this principle, a graphic method was developed to calculate the duration of each cell cycle from doubling time and

Table 4. Cell cycle distribution and the duration of cell cycle stages.

	Protein Content [a]	Doubling Time (hours)	G0/G1 phase		S phase		G2/M phase	
			percentage (%)	duration (hours)	percentage (%)	duration (hours)	percentage (%)	duration (hours)
Rat-1	-	17.7	51.24 ± 2.40	7.5	29.82 ± 1.04	5.7	18.94 ± 1.41	4.5
1302	114	29.5	55.62 ± 2.53	13.8	20.33 ± 0.36	6.2	24.05 ± 2.22	9.5
R2.2	139	38.6	48.88 ± 1.45	15.0	30.66 ± 1.31	13.0	20.46 ± 3.59	10.6

[a] The protein content of cells is indicated as ug of protein per one million of cells.

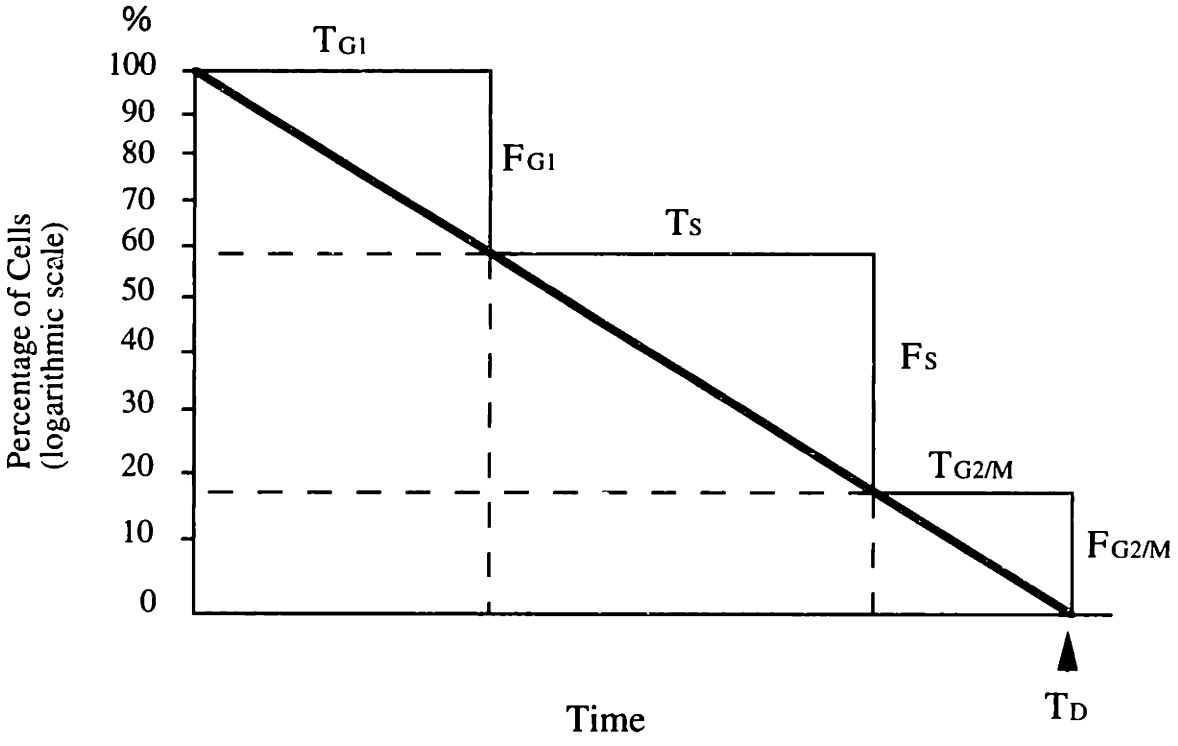


Figure 13. Graphic method of estimation of duration of cell cycle phases. T_D is the doubling time. F_{G1}, F_S, and and $F_{G2/M}$ are the distribution of cells in cell cycle phases. T_{G1}, T_S, and $T_{G2/M}$ are the duration of cell cycle phases obtained from the chart.

the distribution of cells in each cell cycle stage (Okada, 1967) (Figure 13). The distribution of cells in cell cycle stages was obtained from flowcytometry analysis of propidium iodide-stained nuclei. As shown in Table 4, the duration of S phase in R2.2 revertant was twice as long as in v-fos transformed cells, 13 hours vs. 6.2 hours. Whereas, there was not much change in the duration of G1 and G2/M phases, 15 and 10.6 hours in R2.2 cells vs. 13.8 and 9.5 hours in 1302-4-1 cells, respectively. The results suggested that decreased expression of ribosomal protein RPS3a the affected the progression of S phase.

4.2.3 Cyclin A associated protein kinase activity

The activity of cyclin-Cdk complexes were shown to regulate the duration of cell cycle stages. Increased expression of cyclin D1 or cyclin E resulted in accelerated G1 progression (Quelle et al., 1993). The cyclin identified in S phase is primarily cyclin A. Cyclin A is synthesized in late G1 phase, and its expression reaches maximum in G1-to-S transition and in S phase. During S phase, the major partner of cyclin A is Cdk2. The expression of both cyclin A and Cdk2 is required for the onset of DNA replication and S phase progression. Cyclin A and Cdk2 have been detected at DNA replication foci. It has been suggested that cyclin A-Cdk2 complex may phosphorylate factors involved in DNA replication. cyclin A associated kinase activity could potentially regulate the kinetics of S phase progression. Therefore, the activity of cyclin A was compared between transformed cells and revertants.

Since the expression of cyclin A oscillates during cell cycle, the activity of cyclin A was studied in synchronized cells to allow quantitative assessment. Cells were first synchronized in mid G1 by isoleucine deprivation which blocks protein synthesis. Cells were then released from isoleucine deprivation and cultured in complete media containing hydroxy urea. Hydroxy urea inhibits DNA polymerase and thus arrests cells at G1-to-S transition. Cells progressed through G1 phase and were arrested at G1-to-S transition,

since hydroxy urea inhibits DNA polymerase. Greater than 90% of cells were viable at the end of treatment, as monitored by trypan blue staining. The extent of synchronization was analyzed by flowcytometry. After 14 hours treatment with 2 mM hydroxy urea, approximately 80% of cells were in G1 phase (Figure 14(a)). Cells synchronized at G1-to-S transition were released from hydroxy urea block and allowed to progress into S phase for three hours. At this point, around 80% of 1302-4-1, R2.2 and C5 cells were in S phase, with only 49% of Rat-1 cells in S phase (Figure 14(b)). This effect may due to slow recovery of Rat-1 cells from hydroxy urea block. Cyclin A and associated factors were immunoprecipitated from cell lysate under non denaturing condition using a cyclin A specific polyclonal antibody. Cyclin A associated kinase activity was assayed by its ability to phosphorylate its canical substrate, histone H1. Phosphorylated histone H1 was resolved by SDS-PAGE and visualized by autoradiograph.

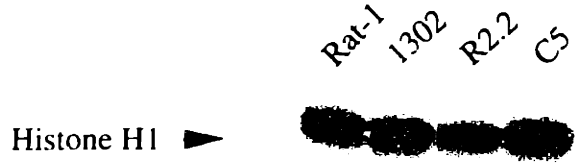
In cells synchronized at G1-to-S transition and in S phase, R2.2 revertant showed decreased cyclin A associated kinase activity compared to v-fos transformed cells. C5 is a retransformed cell line created from R2.2 revertant by increased expression of ribosomal protein RPS3a. The cyclin A activity in C5 cells was restored to a level that resembled in v-fos transformed cells. These results indicated that decreased expression of ribosomal protein RPS3a was responsible for decreased cyclin A activity in R2.2 revertant. Decreased cyclin A activity in R2.2 revertant was consistent with the observed decrease in the rate of S phase progression.

4.2.4 Expression of S phase associated factors

In an effort to identify the factors which affect S phase progression in R2.2 revertant, the expression levels of cyclin A, cyclin E, Cdk2, PCNA, $p27^{Kip1}$ and $p21^{Waf1}$ were compared among cell lines in synchronized and nonsynchronized cells. The expression of both cyclin A and Cdk2 could affect the activity of cyclin A-Cdk2 complex. Cyclin E is

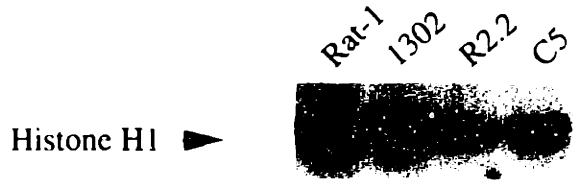
Figure 14. Cyclin A associated kinase activity. The degree of cell synchronization was indicated in the table.

(a)



	G1	S	G2/M
Rat-1	87.39%	5.15%	7.46%
1302	81.42%	4.19%	14.39%
R2.2	82.68%	8.35%	8.79%
C5	76.38%	13.44%	10.18%

(b)



	G1	S	G2/M
Rat-1	46.74%	49.89%	3.37%
1302	14.83%	80.53%	4.64%
R2.2	16.03%	78.42%	5.55%
C5	10.15%	89.36%	1.74%

expressed in late G1 and is classified as a G1 cyclin. It shares a common catalytic partner, Cdk2, with cyclin A. The expression level of cyclin E could potentially affect the cyclin A-Cdk2 binding by competing for the same partner. PCNA (proliferating cell nuclear antigen) is processivity factor of DNA polymerase δ which is responsible for leading strand synthesis in DNA replication. The level of PCNA could modulate the activity of DNA polymerase δ and thus affect S phase progression. p^{27Kip1} and $p21^{Waf1}$ are Cdk inhibitors capable of inhibiting the activity of Cdk2. In addition to being a Cdk inhibitor, $p21^{Waf1}$ can also bind to PCNA and inhibits PCNA-dependent DNA replication (Figure 15). Therefore, increased expression of $p21^{Waf1}$ or $p27^{Kip1}$ could inhibit S phase progression.

Cell lysates were prepared under denaturing condition from exponentially growing, nonsynchronized cells or cells synchronized at the G1-to-S transition by hydroxy urea. Equal amounts of total cellular protein were resolved on SDS-PAGE and transferred to nylon membrane. The proteins were detected by Western blotting using antigen-specific antibodies (Figure 16).

The expression of cyclin A, cyclin E, PCNA and $p27^{Kip1}$, was not significantly different between v-fos transformed cells and R2.2 revertant. The protein levels of Cdk2 were increased in R2.2 revertant compared to v-fos transformed cells. However, the protein levels in retransformed C5 cells are similar to that in R2.2 revertants instead of v-fos transformed cells. This result suggested that increased expression of Cdk2 in R2.2 revertant is not a result of decreased expression of ribosomal protein RPS3a. The protein levels of $p21^{Waf1}$ were increased dramatically in R2.2 revertants compared to v-fos transformed cells. The expression levels of $p21^{Waf1}$ in C5 retransformed cells were similar to that in v-fos transformed cells. Thus, increased level of $p21^{Waf1}$ in R2.2 revertant is a result of decreased expression of ribosomal protein RPS3a. Increased $p21^{Waf1}$ expression was detected in synchronized cells as well as in nonsynchronized cells. Since $p21^{Waf1}$ can inhibit the activity of both Cdk2 and PCNA, increased level of $p21^{Waf1}$ could be

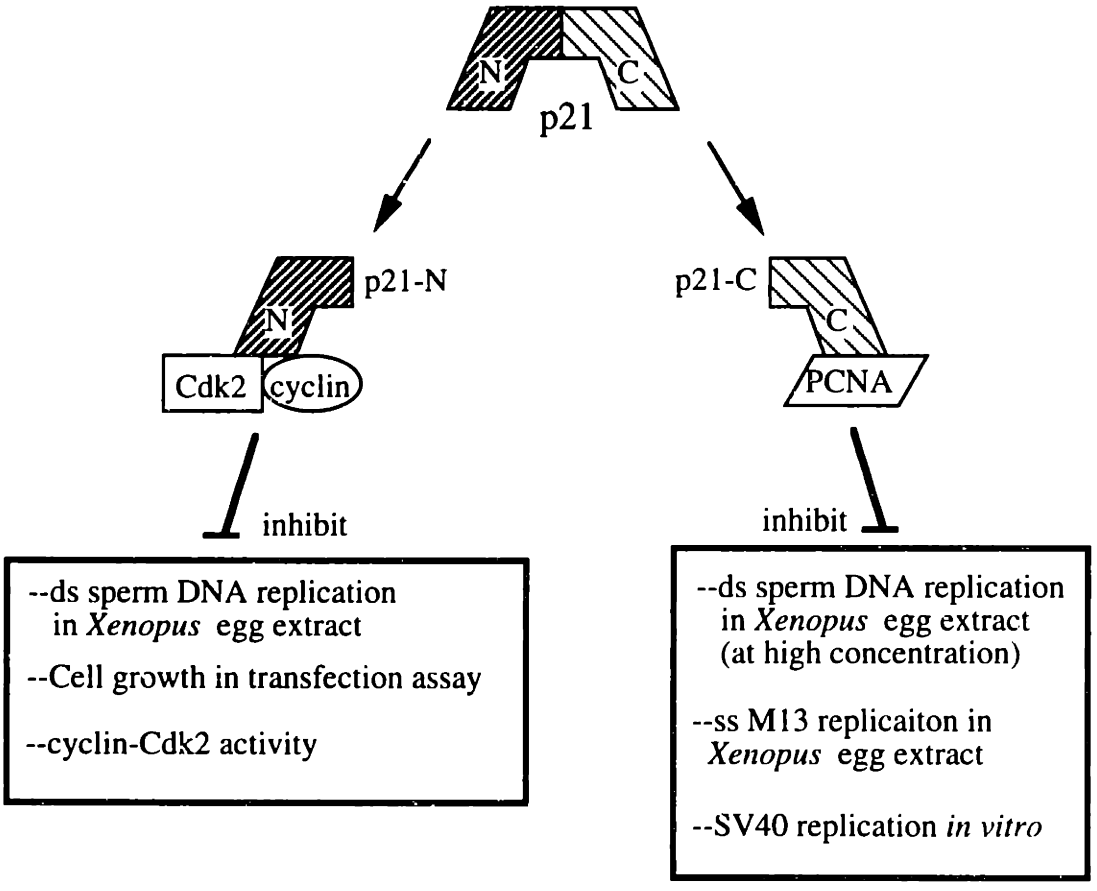
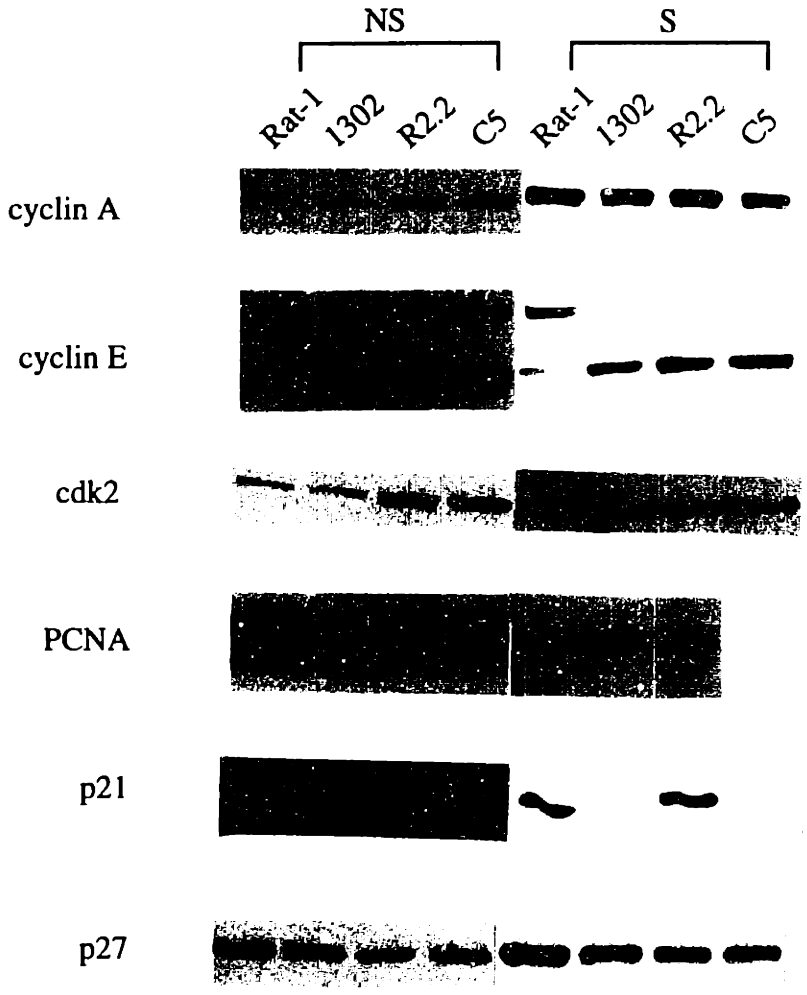


Figure 15. Different functions of the N-terminus and C-terminus of $p21^{Waf1}$.

Figure 16. The protein levels of factors that regulate S phase progression. Cell lysates were extracted from non-synchronized (NS) cells or cells synchronized at G1-to-S transition by hydroxy urea (S). The expression of the proteins was detected by Western blotting.


NS
S
Rat-1
1302
R2.2
C5
Rat-1
1302
R2.2
C5
cyclin A
cyclin E
cdk2
PCNA
p21
p27

responsible for decreased cyclin A activity and slower S phase progression in R2.2 revertant. Normal Rat-1 cells have similar $p21^{Waf1}$ protein levels comparable to those in R2.2 revertant, suggesting that high level of $p21^{Waf1}$ expression correlates with non-tumorigenic phenotypes. A comparison of $p21^{Waf1}$ expression between Rat-1 cells and v-fos transformed cells suggested that v-fos transformation represses $p21^{Waf1}$ expression. Consistent with this hypothesis, decreased expression of $p21^{Waf1}$ was reported in several transformed human cell lines compared to normal cells (Zhang et al., 1995).

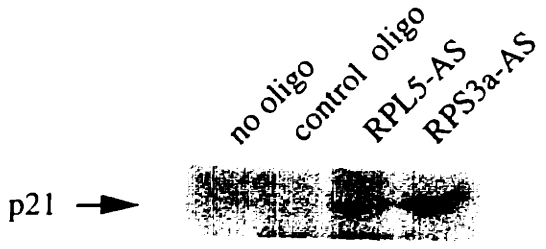
4.2.5. Expression of ^{p21Waf1} in v-fos transformed cells treated with antisense oligonucleotides of ribosomal proteins

We next tested the hypothesis that increased protein level of $p21^{Waf1}$ in R2.2 revertant was due to decreased ribosomal accumulation resulting from decreased expression of ribosomal protein RPS3a. The level of ^{p21Waf1} was studied in v-fos transformed cells treated antisense oligonucleotides of ribosomal proteins. Cells were maintained in media containing oligonucleotides at concentrations that gave a revertant morphology and decreased growth in soft agar. Equal amounts of total cellular protein from each cell line were analyzed by Western blotting. Increased expression of ^{p21Waf1} was observed in cells treated with antisense oligonucleotides of ribosomal proteins RPS3a and RPL5 compared to untreated cells and cells treated with control oligonucleotide (figure 17(a)).

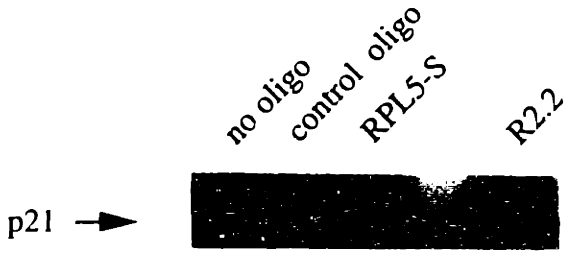
Previous studies argued that treatment of cells with oligonucleotides induces p53 expression by introducing the termini of single stranded DNA into the cell. Since the transcription of ^{p21Waf1} gene is under the direct control of p53, p53 induction could result in increased expression of ^{p21Waf1}. This possibility was ruled out by showing that ^{p21Waf1} protein level was not increased in v-fos transformed cells treated with a random oligonucleotide and a sense oligonucleotide of ribosomal protein RPL5 (Figure 17(a) and

Figure 17. The expression of $p21^{Waf1}$ in v-fos transformed cells treated with oligonucleotides as indicated. In (b), R2.2 revertant was used as a positive control.

(a)


no oligo
control oligo
RPL5-AS
RPS3a-AS
p21

(b)


no oligo
control oligo
RPL5-S
R2.2
p21

17(b)). These results support the hypothesis that decreased ribosomal accumulation induces $p21^{Waf1}$ expression in v-fos transformed cells.

4.2.6 The effects of increased expression of $p21^{Waf1}$ proteins in v-fos transformed cells

Whether increased $p21^{Waf1}$ protein level contributes to v-fos transformation reversion can be tested by ectopic expression of $p21^{Waf1}$ in v-fos transformed cells. $p21^{Waf1}$ protein inhibits the activity of Cdk2 and PCNA by its N-terminal domain and C-terminal domain, respectively. N-terminal domain and C-terminal domain demonstrated different functions in vitro assays (Chen et al., 1995) (Figure 15). The two domains were also shown to have different effects on cell growth. Overexpression of the full-length $p21^{Waf1}$ protein and its N-terminal domain inhibited cell growth in a number of human tumor cell lines, as indicated by a large decrease in the number of colonies obtained after transfection(El-Deiry et al., 1993; Chen et al., 1995). The C-terminus of $p21^{Waf1}$ did not appear to interfere with cell growth. $p21^{Waf1}$ also binds to cyclins, however, the binding domain has not been identified.

Extremely high level of $p21^{Waf1}$ may inhibit cell growth in v-fos transformed cells. However, intermediate level of $p21^{Waf1}$ may suppress v-fos transformation without causing cell death. $p21^{Waf1}$ could contribute to v-fos transformation reversion by inhibiting the activity of the factors it interacts with. In order to test this hypothesis, expression vectors containing the full-length cDNA of human $p21^{Waf1}$, the cDNA of N-terminal domain (amino acids 1-89) or C-terminal domain (amino acids 87-164) under the control of cytomeglovirus (CMV) promoter were transfected into v-fos transformed Rat-1 fibroblasts. The vector alone was transfected as a negative control. The vector used also expresses neomycin phosphotransferase gene which confers resistance to G418. The results indicated that overexpression of $p21^{Waf1}$ in v-fos transformed cells could inhibit cell growth. However, if we consider the fact that R2.2 revertant and Rat-1 cells have considerably

higher level of $p21^{Waf1}$ than v-fos transformed cells, it should also be possible to isolate transfectants which express the exogenous $p21^{Waf1}$ at intermediate levels.

After G418 selection, no colonies were observed on plates transfected with $p21^{Waf1}$ C-terminus (plasmid pcDNA3-p21C). This result was probably due to the inactivation of neomycin phosphotransferase gene in the plasmid, transfection of the same plasmid into Saos-2 cell line which was shown to be unaffected by C-terminus of $p21^{Waf1}$ also failed to give rise to any colonies.

Compared to plates transfected with the vector alone, fewer colonies were obtained from plates transfected with full length $p21^{Waf1}$ cDNA (plasmid pcDNA3-p21) and its N-terminus (plasmid pcDNA-p21N) (figure 18). These results confirmed the growth inhibitory effect of full length p21 protein and its N-terminus in v-fos transformed cells. The growth inhibitory effect of the plasmids also indicates that exogenous $p21^{Waf1}$ proteins were functioning properly in transfected cells.

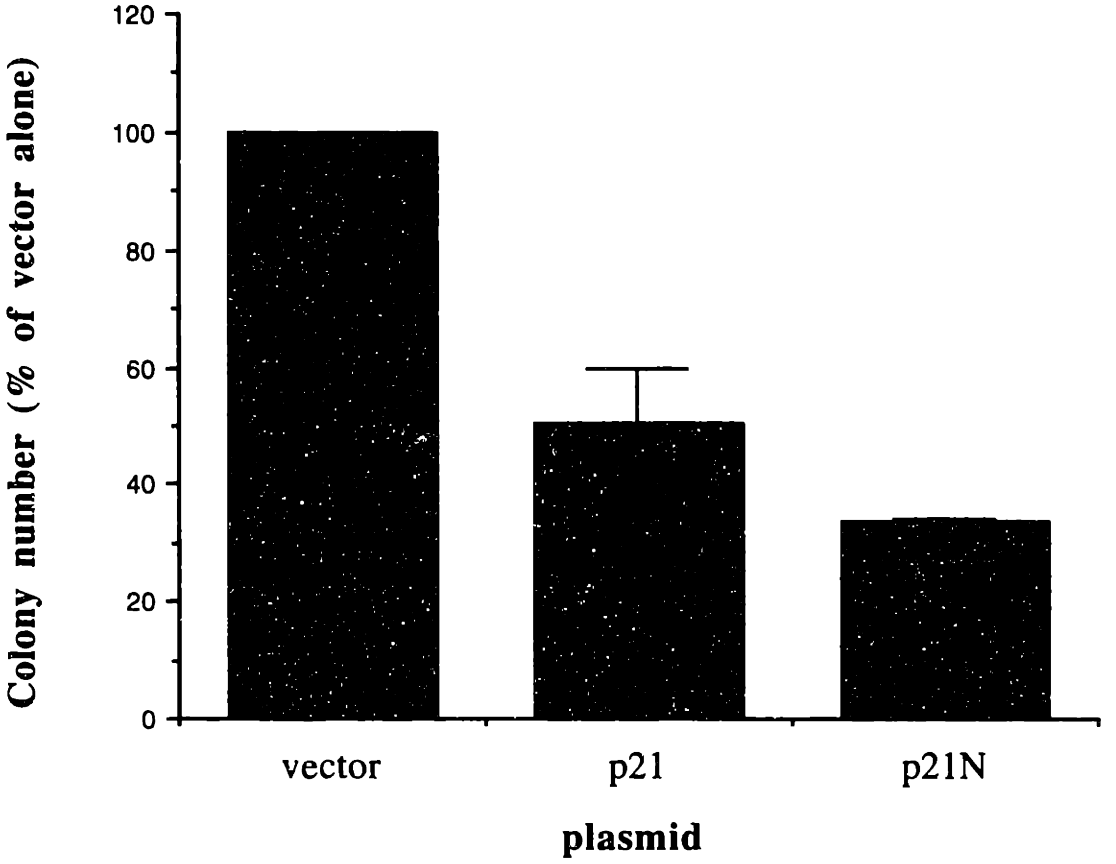
Colonies were isolated from transfected plates and the expression of exogenous $p21^{Waf1}$ will be analyzed by Western blotting. The phenotypes of the clones are still being studied.

4.3 Discussion

4.3.1 The significance of increased $p21^{Waf1}$ level in cell cycle regulation

Results obtained in this study indicated that inhibition of v-fos transformation by decreased expression of ribosomal proteins was accompanied by increased $p21^{Waf1}$ protein level. $p21^{Waf1}$ was cloned as an inhibitor of cyclin-Cdk2 complexes (Nasmyth and Hunt, 1993) and at the same time as a factor whose expression can be induced by wild-type but not mutant p53 (El-Deiry et al., 1993). $p21^{Waf1}$ protein is capable of inhibiting the kinase activity of Cdk2 by it N-terminal domain and inhibiting the function of PCNA in DNA replication by its C-terminal domain (Chen et al., 1995). The expression of $p21^{Waf1}$ was

Figure 18. Growth inhibitory effects of full length ^{p21Waf1} protein and ^{p21Waf1} N-terminus (amino acids 1-90). Equal amont of DNA of each plasmid was used for transfection.


Colony number (% of vector alone)
120
100
80
60
40
20
0
vector
p21
p21N
plasmid

found increased under various conditions which arrest cell cycle, including senescence (Noda et al., 1994), terminal differentiation (Guo et al., 1995; Jiang et al., 1994) and γ-irradiation (Dulic et al., 1994). $p21^{Waf1}$ deficient mouse fibroblasts have impaired G1 checkpoint control after γ-irradiation. All available evidence indicates that $p21^{Waf1}$ is an important regulator of cell cycle progression in response to growth-inhibitory signals.

In normal cells, cyclin A-Cdk2 exists predominantly in a quaternary complex with $p21^{Waf1}$ and PCNA (Zhang et al., 1993). In many transformed cells, these complexes are replaced by quinary complexes containing cyclin A, Cdk2, and a 45 Kd factor, p45 (Zhang et al., 1995). This switch is probably due to decreased expression of $p21^{Waf1}$ and increased expression of p45 in transformed cells. $p21^{Waf1}$ and p45 were shown to compete for binding to the cyclin A-Cdk2 complex. Cyclin A and Cdk2 are essential for the on-set of S phase and S phase progression. They localize to replication foci and may be responsible for the phosphorylation of RPA34, which is a subunit of replication protein A. It was shown that p45 is indispensable for the activity of cyclin A-Cdk2 in S phase both in normal cells and transformed cells. Therefore, the loss of $p21^{Waf1}$ in transformed cells may be partially responsible for the deregulation of entry into S phase under inappropriate conditions.

Many transformed cells grow more rapidly than their normal parental counterpart. However, v-fos transformation results in slower growth rate than normal cells. Increased or decreased doubling times are not likely to be the reason of gain or loss of tumorigenicity. Instead, the inability of transformed cells to respond to growth inhibitory signals and arrest cell cycle directly correlates with their tumorigenicity. Therefore, increased expression of $p21^{Waf1}$ may contribute to the reversion of v-fos transformation by restoring the response of cells to growth inhibitory signals, such as cell-cell contact and loss of anchorage. It will be interesting to investigate the role of $p21^{Waf1}$ under these conditions. For example,

anchorage-independent growth was shown to partially depend on the expression of cyclin A and, probably, the activity of cyclin A-Cdk2 complex. Increased level of $p21^{Waf1}$ could inhibit cyclin A-Cdk2 activity and thus abolish anchorage independent growth. In R2.2 cells, increased expression of $p21^{Waf1}$ protein could contribute to decreased cyclin A associated kinase activity and prolonged S phase progression. It is also possible that other unidentified factors are involved as well. Elucidating the underlying molecular mechanism of transformed phenotypes will assist us in understanding the connection between cell cycle regulation and tumorigenesis.

Ectopic expression of full length $p21^{Waf1}$ protein, its N-terminus or C-terminus in v-fos transformed cells will allow us to test if increased $p21^{Waf1}$ level is sufficient for v-fos transformation reversion and by what pathway. However, the absence of revertant phenotypes in transfectants with $p21^{Waf1}$ expression does not eliminate the role of $p21^{Waf1}$ in transformation reversion. It is believed that tumorigenicity results from a combination of many biochemical pathways and so does cell transformation. Most of these biochemical pathways have not been understood. In vivo, tumorigenic cells usually acquire a variety of biochemical features, including changes in cell metabolism, invasiveness and angiogenesis. It is still not clear how the transformed phenotypes studied in vitro correlate with the characteristics of cancer cells in vivo, although tumorigenic cells are usually "transformed" as tested by their ability to grow in soft agar, such a correlation does not exist in all cases. On the basis of these considerations, the role of $p21^{Waf1}$ in tumor suppression may not be unveiled by the methods used in this study. Decreased ribosomal accumulation could potentially affect the expression of many factors. It remains possible that v-fos transformation reversion is the result of several pathways working in concert.

4.3.2 The role of other cell cycle regulators

The level of cyclin E does not change among cell lines; however, two different forms of cyclin E with the estimated molecular weigh of 55 kDa and 40 KDa were expressed in the presence and absence of v-fos oncogene. The expression pattern does not seem to correlate with transformation reversion, since the expression of cyclin E in R2.2 revertant is the same as in v-fos transformed cells. The shift of cyclin E from 55 Kda to 40 Kda in tumor cells has been previously reported. Although the significance of the shift is unknown, it is speculated that the two forms of cyclin E arise from differential splicing.

The expression of Cdk2 was increased in R2.2 revertant compared to v-fos transformed cells. However, its level remained high in C5 cells, which are R2.2 cells retransformed by increased expression of ribosome RPS3a. Therefore, increased Cdk2 level in R2.2 revertant does not result from decreased ribosomal accumulation.

No correlation has been observed between decreased ribosomal accumulation and the expression levels of cyclin E, cyclin A, Cdk2, p27 and PCNA. However, decreased ribosomal accumulation may change their posttranslational modification, and thereby alter their their activity. For example, phosphorylation of Cdk2 by Cdk activating kinase (CAK) and its dephosphorylation at other sites are both necessary for the its activation. The pool of unknown cell cycle regulators is far from being exhausted. The existence of unidentified factors which are crucial for the inhibition of v-fos transformation in R2.2 revertant can not be excluded.

Chapter 5

The Effects of Decreased Expression of Ribosomal Protein RPS3a in Hela Cells

5.1 Introduction

Non-tumorigenic revertants can be isolated from cells transformed by oncogenes. Inhibition of cell transformation can be achieved by one of several ways--inactivation of the original oncogenes, activation of transformation suppressor genes which suppress transformation in dominant fashion or inactivation of transformation effectors genes which function recessively. The existence of the later two types of revertants, which have active transforming oncogenes, suggested that cancer cells could lose their tumorigenicity despite the presence of oncogenic mutations. Since mutations in transformation suppressor or effector genes could inhibit tumorigenesis, these genes are potential targets for anticancer drugs. On the basis of this concept, chemotherapeutic agents can be designed to increase the activity of transformation suppressors or decrease the activity of transformation effectors. Tumorigenic cells share many common features, suggesting that they have overlapping transformation pathways. Thus, transformation suppressors or effectors identified in one cell line may also play a similar role in the cell transformation in other cells.

R2.2 revertant was isolated from v-fos transformed Rat-1 fibroblasts. It has functional v-fos oncogene. However, one allele of ribosomal protein RPS3a gene was disrupted by insertion of pMexneo plasmid in R2.2 cells. The level of ribosomal protein RPS3a was decreased more than 50% in R2.2 revertant compared to v-fos transformed cells. Molecular analysis demonstrated that decreased protein level of RPS3a was responsible for v-fos transformation reversion. Further study indicated that decreased expression of other ribosomal proteins, namely RPL5 and RPS6, in v-fos transformed cells resulted in revertant phenotypes similar to that in R2.2 cells. Taken together, the evidence suggested that decreasing ribosomal accumulation inhibits v-fos transformation in Rat-1 fibroblasts.

It is well established that modulation of ribosomal accumulation and protein synthesis plays important roles in tumorigenesis. Increased expression of ribosomal proteins was observed in a variety of tumor tissues and transformed cell line compared to their normal counterparts. Although increased ribosomal accumulation is not likely to be the cause of malignancy, it may, however, be essential for the maintenance of tumorigenicity. Therefore, decreasing ribosomal accumulation could be used as a method for cancer treatment. In addition to causing decreased overall rate of protein synthesis, decreased cellular content of ribosomes may generate an imbalance among factors that regulate cell growth by changing the translation efficiency of their mRNAs differentially. The later machanism has been implicated in cell transformation by translation initiation factor eIF-4E. Overexpression of cap binding factor eIF-4E is capable of transforming NIH 3T3 cells and Rat-2 cells (Lazaris-Karatzas et al., 1990; De Benedetti and Rhoads et al., 1991). Overexpression of eIF-4E results in ras activation and increased translation of mRNA with extensive 5' secondary structures (Loromilas et al., 1992a).

Since increased expression of ribosomal proteins is a common characteristic in many transformed cells, suppression of cell transformation by decreased ribosomal accumulation may not be specific for v-fos transformed cells. Instead, decreased ribosomal accumulation may inhibit cell transformation in other transformed cell lines. As the first step to test this hypothesis, we tested the effects of decreased expression of ribosomal protein RPS3a on the tumorigenicity of Hela cells.

5.2 Results

5.2.1 Subcloning of Hela cells:

Hela (ATCC, CCL2) is a cell line derived from human cervical adenocarcinoma. The phenotypes of Hela cells are heterogeneous. The heterogeneity is demonstrated by non-

uniform morphology, aneuploidity with the number of chromosome ranging from 70 to over 100, and the existence of derivative cell lines with distinct properties. To avoid clonal variation which could potentially complicate the study, Hela cells were subcloned by the dilution method. Seven subclones (Hela 1.1 to Hela 1.7) were isolated and tested for their ability to grow in soft agar. One of the clones, Hela 1.1, which had the highest cloning efficiency in soft agar was selected for this study. However, as shown in later study, subcloning does not eliminate clonal variation completely. The possible reason is that inactivation of p53 by the E6 oncoprotein of human papilloma virus in Hela cells results in an extremely unstable genome.

5.2.2 Expression of antisense RNA of ribosomal protein RPS3a in Hela 1.1 cells

The expression of antisense RNA was used to as an approach to decrease the level of ribosomal protein RPS3a in Hela 1.1 cells. It was reported that antisense sequences containing the AUG start codon are most effective in decreasing the expression of target genes. A 320 bp fragment from the 5' end of human RPS3a cDNA was cloned into mammalian expression vector pMSG in antisense orientation to form pMSG-RPS3a(-). The expression of antisense sequence in pMSG is under the control of mouse mammary tumor virus (MMTV) long terminal repeat (LTR). MMTV-LTR has moderate basal expression level. The expression can be induced by over 10 fold by glucocorticoid hormone or its analog, dexamethasone. The induction is dependent on cellular expression of glucocorticoid hormone receptor. This vector has been used successfully for the inducible expression of exogenous genes in Hela cells (Klessig et al, 1984).

pMSG-S3a(-) was transfected into Hela1.1 cells along with 10 times excess of a selectable maker, pMexneo, which confers resistance to antibiotics G418. After G418 selection, colonies were isolated and propagated. The inducibility of antisense RNA expression was studied in one of the clones 1S12, cultured in media containing different

concentrations of dexamethasone. The expression of antisense RNA was detected by RNAase protection assay. Total cellular RNA was hybridized to ^{32}P-labeled single-stranded RPS3a sense RNA probe. After RNase digestion, protected double stand RNA fragments were resolved by denaturing PAGE. The expression of antisense RNA of RPS3a corresponded to the appearance of a 260 base pair fragment. The level of antisense RNA was quantified by exposing the gels to phosphoimager screen and normalized to the expression level of β-actin. As shown in Figure 19, the expression of antisense RNA in 1S12 cells can be induced by dexamethasone. Induction showed dose response to increased concentration of dexamethasone. Maximum induction occurred when the concentration of dexamethasone was reached 1uM . This concentration was used in subsequent experiment.

Transfectants isolated after G418 selection were cultured in media containing 3 uM of dexamethasone. The expression of antisense RNA of RPS3a was analyzed by RNAase protection assay and normalized to the level of β-actin.

5.2.3 Rhodamine 123 retention in clones with antisense RNA expression

A variety of transformed cells have the ability to retain the fluorescent molecule, Rhodamine 123, in their mitochondria over a period of time. Loss of prolonged Rhodamine 123 retention was used as a marker to isolate revertants from v-fos transformed fibroblasts (Zarbl et al., 1987). This selection method can be applied to other transformed cells by optimizing the detaining time. Two revertant, HA and HF, were successfully isolated from Hela cells based on their loss of prolonged Rhodamine 123 retention (Boylan et al., submitted).

The Rhodamine 123 retention was studied in Hela clones which expressed antisense RNA of RPS3a. Cells cultured in media containing 3 uM of dexamethasone were stained with 10 ug/ml of Rhodamine 123 for 30 minutes and washed three times with fresh media,

Figure 19. Dose response of RPS3a antisense RNA induction by dexamethasone. Clone 1S12 was cultured in media containing different concentrations of dexamethasone. The expression of RPS3a antisense RNA was detected by RNAase protection assay and normalized to the expression level of β-actin.

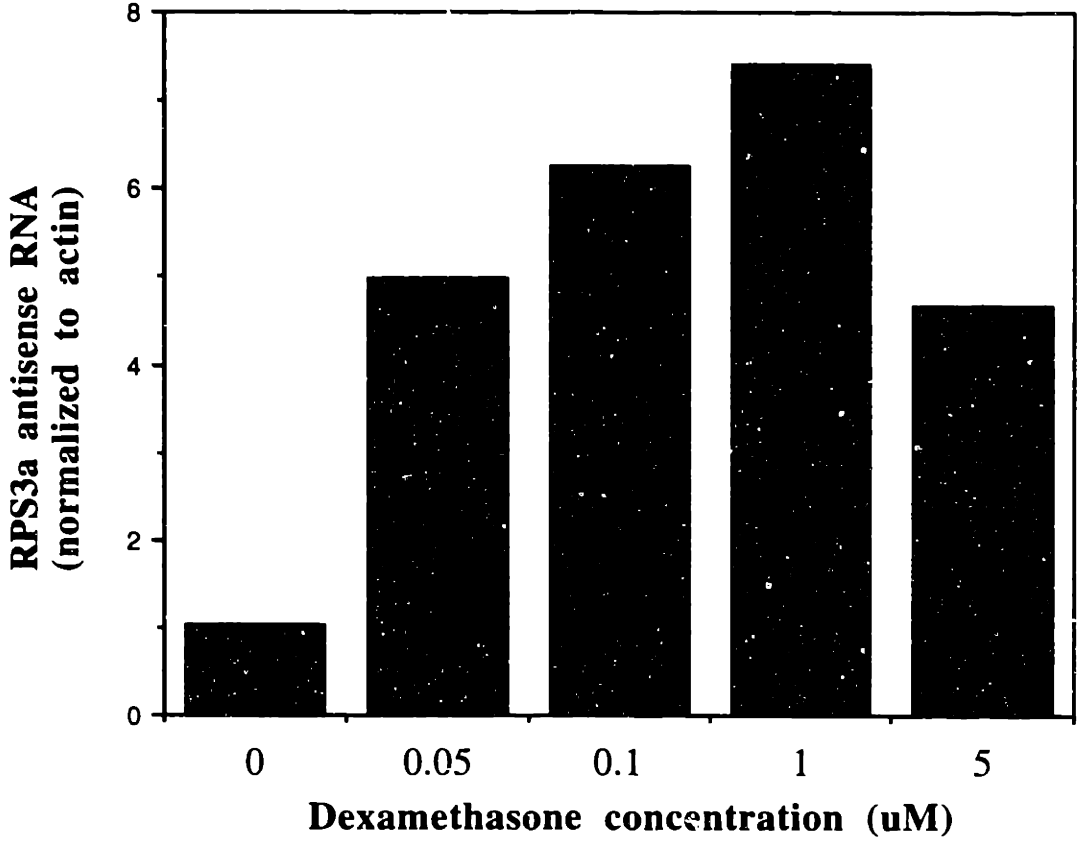

RPS3a antisense RNA
(normalized to actin)
8
6
4
2
0
0
0.05
0.1
1
5
Dexamethasone concentration (uM)

Figure 20. Rhodamine 123 retention of clones isolated after transfection of Hela 1.1 with pMSG-S3a(-). After staining cells with Rhodamine 123, the fluorescent intensity was quantified by flow cytometry analysis. The values were compared to that of Hela, which was given an arbitray unit of 1.

Fluorescent intensity
(relative to Hela)
0
1
2
Cell line
HF
IS2.24
IS12
IS2.1
IS2.18
IS2.7
IS2.26
IS2.31
IS2.21
IS2.10
Hela
IS2.6
IS2.13
IS2.15
IS2.22
IS2.5
IS2.33
IS2.28
IS2.17

followed by 18 hours of destaining in fresh media. The fluorescent profiles of stained cells were analyzed by flow cytometry. The median fluorescent intensity of the clones was compared to that of Hela 1.1, which was given an arbitrary unit of 1. As shown in Figure 20, clones 1S2.1, 1S2.7, 1S2.18, 1S2.24, 1S2.26, 1S2.31, and 1S12 exhibit around 50% decrease in their retention of Rhodamine 123. This was comparable to the level in Hela revertant HF, suggesting that they may have decreased tumorigenicity.

5.2.4 Expression level of RPS3a protein

The RPS3a protein level of was studied in clones 1S2.1, 1S2.7, 1S2.18, 1S2.24, 1S2.26, 1S2.31 and 1S12. Cells were cultured in the presence or absence of 3 uM of dexamethasone. 50 ug of total protein form each cell line was analyzed by Western blotting using a an antibody specific for RPS3a. Representative results are shown in Figure 21. The expression of ribosomal protein RPS3a was reproducibly decreased in clones 1S2.7 and 1S2.24 upon induction of antisense RNA expression by 3 uM dexamethasone (Figure 21 and Table 5). In Figure 21, clone 1S12 also showed decreased expression of RPS3a upon induction by dexamethasone. However, the decreased expression of RPS3a in 1S12 was not seen not reproducible. As in parental Hela 1.1 cells, the level of RPS3a remained more or less the same before and after dexamethasone induction in clones 1S2.1, 1S2.18, 1S2.26, and 1S2.31. Northern blotting analysis indicated that the expression of RPS3a mRNA is not decreased in 1S2.7 and 1S2.24 compared to parental Hela 1.1 cells (Table 5). These results suggested that the decreased expression of RPS3a protein in the listed clones is due to the expression of antisense RNA. This possibility was further supported by the finding that 1S2.7 and 1S2.24 had the highest expression of RPS3a antisense RNA after induction (Figure 22). In other clones, it is possible that the expression of antisense RNA was not high enough to cause a decrease in the protein level of RPS3a.

Table 5. Expression of RPS3a protein and RNA in clones 1S2.7 and 1S2.24.

	RPS3a protein level [a]		RPS3a RNA level [b]
	no dexamethasone	3 uM dexamethasone	
Hela 1.1	1.00 ± 0.00	1.00 ± 0.00	1.00
1S2.7	1.18 ± 0.12	0.46 ± 0.18	1.54
1S2.24	1.07 ± 0.15	0.45 ± 0.47	1.79

[a] The expression levels of RPS3a protein in clones were normalized to that of Hela.

[b] The level of RPS3a mRNA was assayed in cells treated with 3 uM dexamethasone. RPS3a mRNA levels were normalized to that of Hela.

5.2.5 Soft agar growth assay of clones

The ability of cells to grow in semi-solid media (soft agar) is often an indication of tumorigenicity. Cells cultured in the presence or absence of 3 uM of dexamethasone were seeded in 0.33% noble agar at indicated density. Cells seeded in soft agar were fed with media with or without 3 uM dexamethasone. At the end of the experiment, colonies were stained and dried on filter paper.

Clone 1S2.24 did not produce any visible colony even in the absence of dexamethasone (figure 23). This phenotype of 1S2.24 is similar to what was observed in HA which is a well characterized revertant previously isolated from Hela, suggesting that 1S2.24 may also be a revertant. However, the expression of ribosomal protein RPS3a was not decreased in clone 1S2.24 in the absence of dexamethasone. Therefore, the inability of clone 1S2.24 to grow in soft agar may not have resulted from decreased expression of RPS3a. Clone 1S2.24 will not be informative in assessing the effect of decreased expression of RPS3a on tumorigenicity in Hela cells.

Figure 21. The expression level of RPS3a protein in clones. Exponentially growing cells were cultured in media with or without 3 uM dexamethasone as indicated. 50 ug of total cellular protein from each cell line was resolved by SDS-PAGE. RPS3a protein was detected by Western blotting.

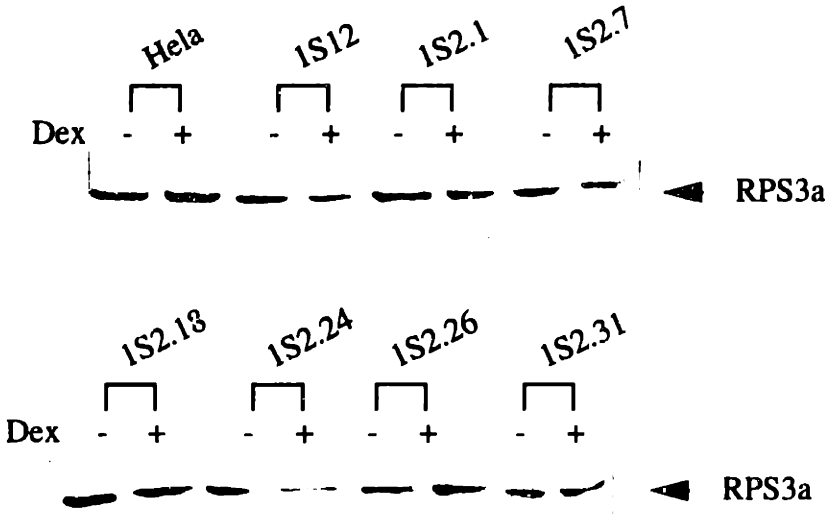
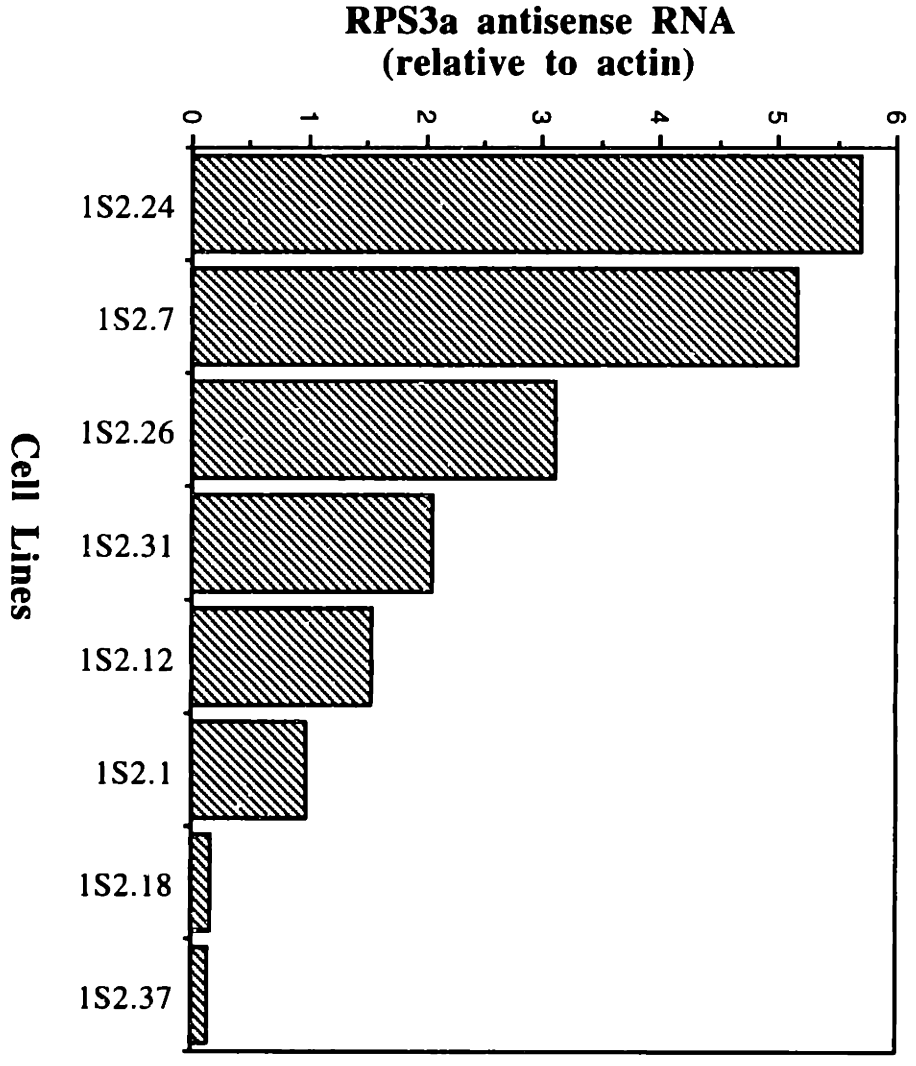

Hela
1S12
1S2.1
1S2.7
Dex - + - + - + - +
RPS3a
1S2.13
1S2.24
1S2.26
1S2.31
Dex - + - + - + - +
RPS3a

Figure 22. The expession of RPS3a antisense RNA in clones upon induction by 3 uM dexamethasone. Exponentially growing cells were cultured in media containing 3 uM of dexamethasone. The expression of RPS3a antisense RNA was detected in total RNA by RNAase protection assay and normalized to the level of β-actin.


RPS3a antisense RNA
(relative to actin)
0
1
2
3
4
5
6
Cell Lines
1S2.24
1S2.7
1S2.26
1S2.31
1S2.12
1S2.1
1S2.18
1S2.37

Clone 1S2.7 exhibit similar growth as parental Hela 1.1 cells in the absence of dexamethasone (Figure 23 and Figure 24). When cultured in 3 uM of dexamethasone, clone 1S2.7 gave rise to fewer colonies than parental Hela 1.1 but still more than revertant HA. This result indicated that clone 1S2.7 did not completely lose transformed phenotype but may have decreased tumorigenicity compared to Hela cells. The expression of ribosomal protein RPS3a was decreased 50% in 1S2.7 in the presence of 3 uM dexamthasone (Table 5). Thus, decreased proteins level of RPS3a may be responsible for the decreased soft agar growth of clone 1S2.7. However, it is also possible that decreased soft agar growth of clone 1S2.7 resulted from clonal variation, for example, different response to dexamethasone. In order to test this possibility, soft agar growth assay was performed on clones 1S12, 1S2.1, 1S2.18, 1S2.26, and 1S2.31, which did not show decreased expression of RPSa in 3 uM dexamethasone. These clones had comparable or lower growth in soft agar relative to clone 1S2.7 in the absence of 3 uM dexamethasone (Figure 25). When cultured in 3 uM of dexamethasone, clones 1S12, 1S2.1, 1S2.18, 1S2.26, and 1S2.31 formed more colonies than 1S2.7. This result indicated that the decreased soft agar growth in clone 1S2.7 in 3 uM of dexamethasone was due to decreased expression of RPS3a.

5.3 Discussion

Increased expression of ribosomal proteins is a characteristic of a variety of tumors and transformed cell lines. In previous study, it was shown that decreased expression of ribosomal proteins inhibits v-fos transformation, presumably by decreasing ribosomal accumulation. Decreased expression of ribosomal protein may suppress tumorigenic phenotypes in other transformed cells as well. If this hypothesis is proved to be true, decreasing the expression of ribosomes or ribosomal accumulation could be a potentially

Figure 23. Soft agar growth assay of Hela 1.1 and clones transfected with pMSG-S3a(-). Cells were seeded at 2 x 10^4 cells/ml (with dexamethasone) or 2 x 10^3 (without dexamethasone).

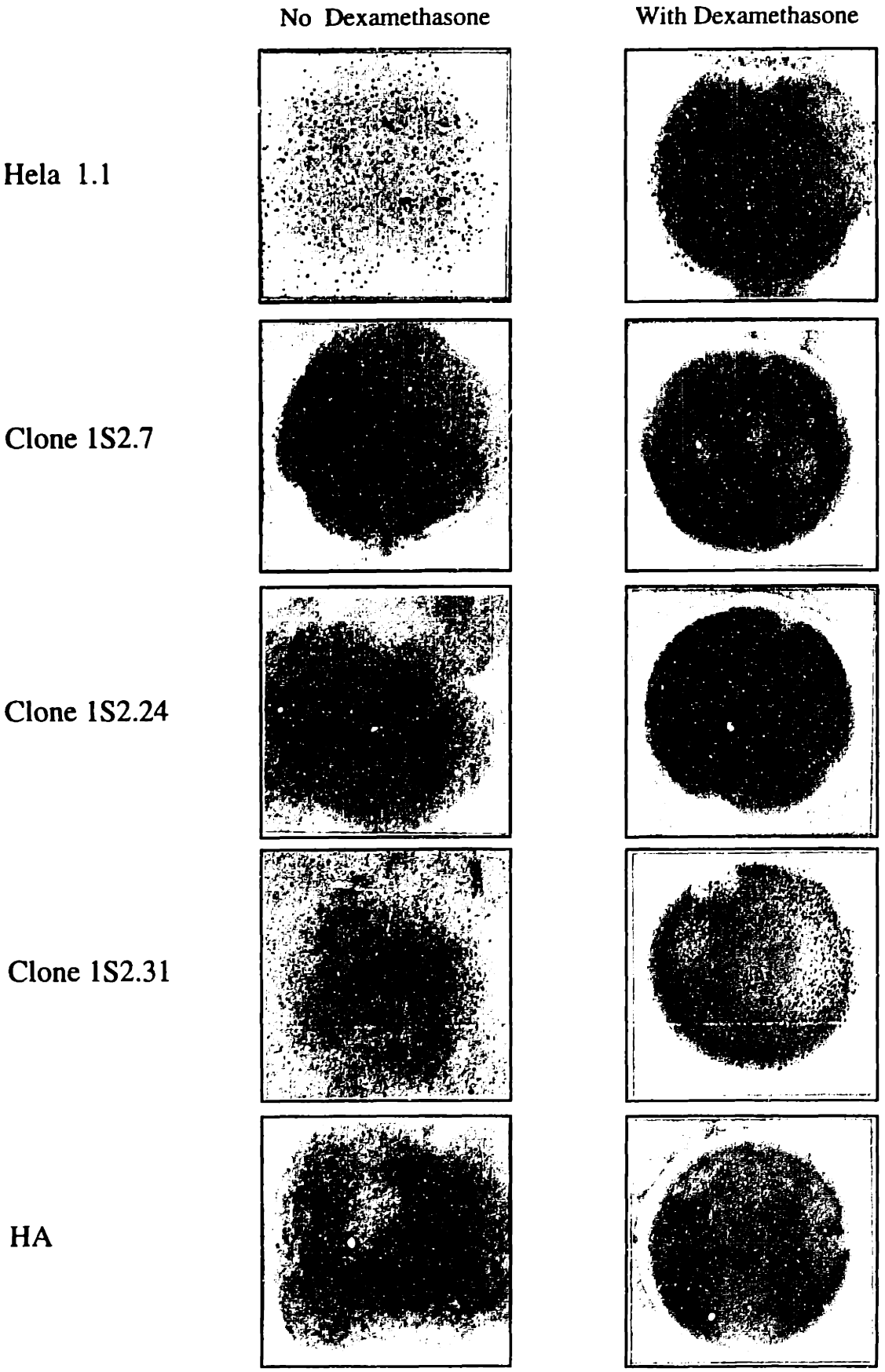

No Dexamethasone
With Dexamethasone
Hela 1.1
Clone 1S2.7
Clone 1S2.24
Clone 1S2.31
HA

Figure 24. Soft agar growth assay of Hela 1.1 and clone 1S2.7. Cells were seeded at 1 x 10^4 cells/ml with or without dexamethasone.

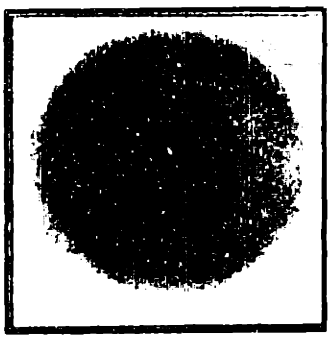
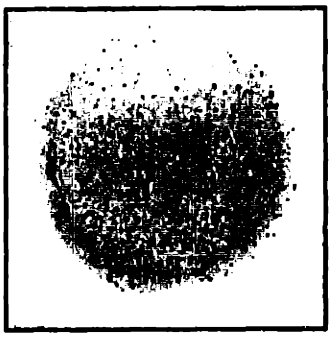
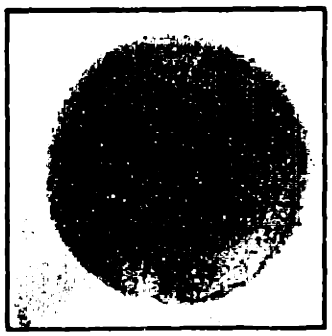
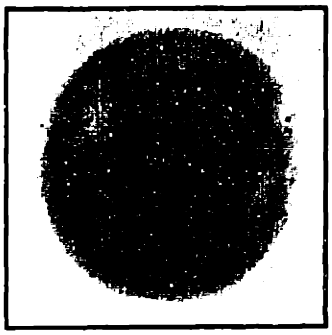
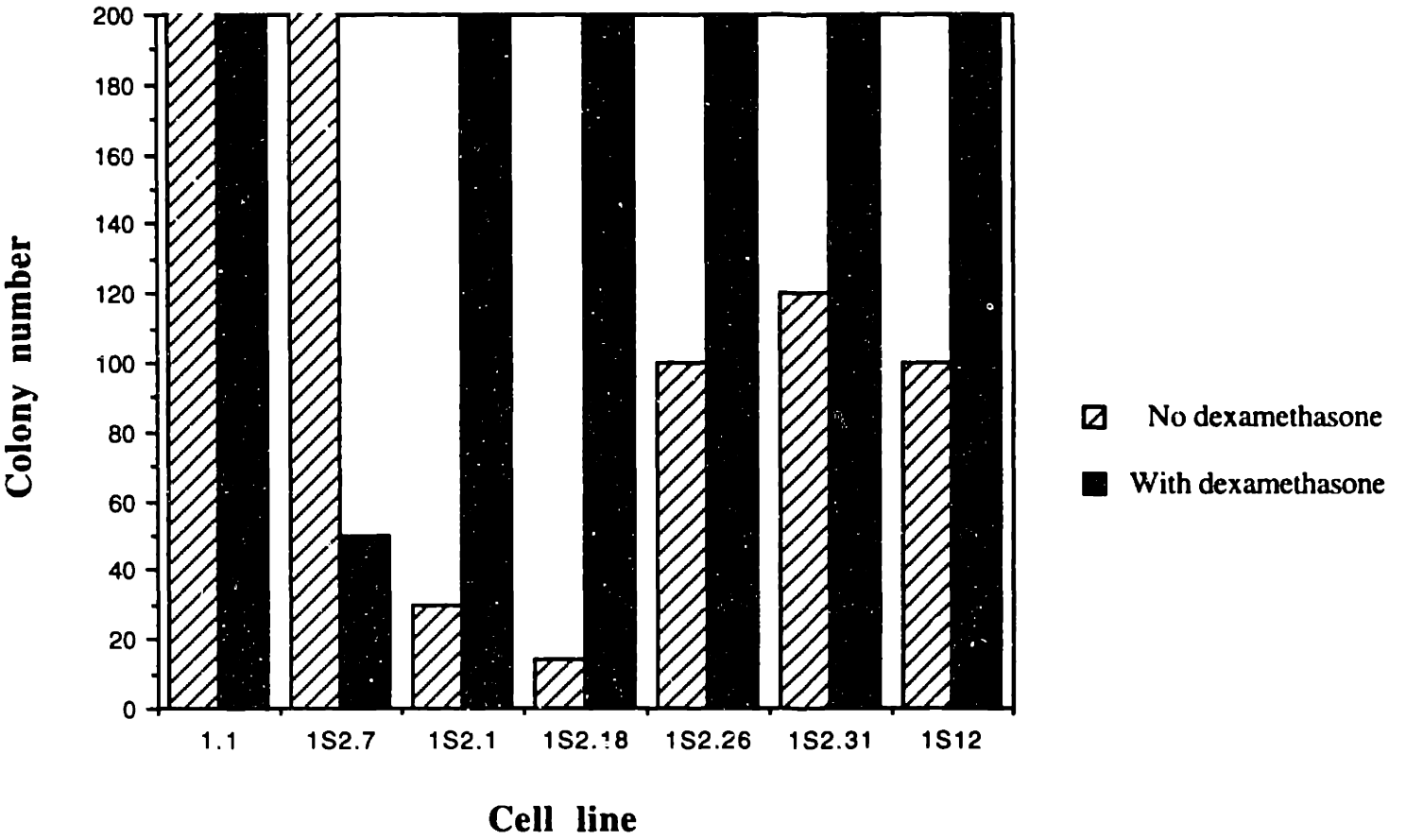
	No Dexamethasone	With Dexamethasone
Hela 1.1		
Clone 1S2.7		

Figure 25. Comparison of the number of colonies obtained in soft agar growth assay of Hela 1.1 and clones transfected with pMSG-S3a(-). Cells were seeded at 2 x 10^4 cells/ml (with dexamethasone) or 2 x 10^3 (without dexamethasone). At the end of the experiment, the colonies were stained with tetrazolium violet and counted.


Colony number
200
180
160
140
120
100
80
60
40
20
0
1.1
1S2.7
1S2.1
1S2.18
1S2.26
1S2.31
1S12
Cell line
No dexamethasone
With dexamethasone

useful anticancer method. Chemotherapeutic drugs could be designed to decrease ribosomal accumulation by (1) decrease the level of ribosomal proteins; (2) decrease the synthesis and processing of ribosomal RNA; (3) inhibit the assembly and transport of ribosomes; the ideal drugs would have the ability to be activated by high level of ribosomes or ribosomal proteins. The advantage of these drugs over traditional genotoxic agents used in cancer therapy is that they need not necessarily be genotoxic, and thus would not induce mutation, thereby reducing the risk of developing new tumors as a result of therapy. As a preliminary step to test the above hypothesis, the effects of decreased level of ribosomal protein RPS3a were analyzed in Hela cells.

The reasons for using Hela in this study include the following: (1) Hela cells were derived from human cervical carcinoma. Studies done in Hela cells have more practical meaning than in experimentally transformed cell lines which are much simplified models of tumor. The use of human cell line was important because the biochemical pathways of signal transduction may not be exactly the same in rodent cells and human cells. (2) Hela cells originated from epithelial cells from which most types of malignant tumors develop. More than 80% of the tumors occurring in human are carcinomas with an epithelial origin. (3) Hela cells are one of the best characterized tumor cell lines. The availability of information will facilitate the analysis of the results obtained. (4) The ability to isolate revertants from Hela cells suggested that tumorigenicity can be inhibited in Hela cells. The revertant cell lines can also serve as negative controls in the assay for tumorigenicity. (5) In Hela cells, E6 and E7 oncoproteins of human papillomavirus inactivate tumor suppressors p53 and Rb, respectively. The inactivation of p53 and Rb was suggested to contribute to the tumorigenicity of Hela cells. p53 has been documented as the most frequently mutated gene in human cancer. Thus, testing the effect of decreased expression of RPS3a in Hela cells will have broad significance.

In order to decrease the expression of ribosomal protein RPS3a, a subclone of Hela (ATCC CCL2), Hela 1.1, was transfected with an inducible antisense vector, pMSG-S3a(-). Among the cell lines isolated after G418 selection, 1S2.7 and 1S2.24 showed 50% decrease in the protein level of RPS3a upon induction with 3 uM of dexamethasone. Since the expression of RPS3a was not decreased in parental Hela 1.1 cells by dexamethasone treatment, decreased expression of RPS3a in clones 1S2.7 and 1S2.24 was likely to have resulted from induction of antisense RNA expression by dexamethasone. The decreased ability of clone 1S2.7 to growth in soft agar compared to parental Hela 1.1 and other clones indicated that the clone has partially lost the transformed phenotypes due to decreased expression of RPS3a. However, unlike in v-fos transformed cells, a 50% decrease in the level of RPS3a does not result in complete reversion in Hela cells. It is possible that the level of RPS3a has to be further decreased in Hela cells to inhibit cell transformation.

Isolated transfectants also showed decreased soft agar cloning efficiency with out decreased expression of RPS3a, such as clone 1S2.24. This phenomena may rise from disruption of other genes important from cell transformation and, again, clonal variation.

Questions may also be raised regarding the correlation between the loss of prolonged Rhodamine retention and tumorigenicity. Among seven clones which exhibit decreased Rhodamine 123 retention, only two (1S2.7 and 1S2.24) showed decreased soft agar growth. This is comparable to the previously observed frequency of revertants among Rhodamine negative cells (Boylan and Zarbl, unpublished results).The mechanism of loss of prolonged Rhodamine retention in revertants has not been understood. The correlation between Rhodamine retention and transformation reversion is only experimental, and may not happen in all cases. For example, decreased Rhodamine retention may result from clonal variation or inactivation of the genes related to the uptake or release of Rhodamine 123.

In summary, the evidence provided by this study supports the possibility that decreased expression of ribosomal protein RPS3a will result in partial loss of transformed phenotypes in Hela cells. The effects of decreased expression of RPS3a on the tumorigenicity of Hela cells can be further studied by generating clones with markedly decreased level of RPS3a and testing tumor formation in animal.

Chapter 6
Conclusions and Suggestions for Future Experiments

6.1 Summary and conclusions

A non-tumorigenic revertant, R2.2, was previously isolated from v-fos transformed Rat-1 fibroblasts following DNA transfection (Kho and Zarbl, 1993). One allele of ribosomal protein RPSa gene was disrupted by integration of pMexneo plasmid in R2.2 cells. Previous studies indicated that decreased expression of ribosomal protein RPS3a was responsible for the loss of tumorigenicity in R2.2 revertant. Thus, RPS3a is v-fos transformation effector gene which is necessary for the maintenance of transformed phenotypes. This thesis research was focused on investigating the pathways by which decreased expression of RPS3a mediates reversion from v-fos transformation. Since transformed cells usually exhibit similar phenotypes, it is likely that cell transformation by different oncogenic events may have overlapping transformation pathways and share transformation effector genes. Therefore, decreased expression of RPS3a may inhibit cell transformation in other transformed cells. This hypothesis was tested in Hela cells.

Since RPS3a is a ribosomal protein, decreased expression of level of RPS3a may inhibit v-fos transformation by decreasing ribosomal accumulation. However, this hypothesis does not rule out the possibility that decreased level of RPS3a mediates v-fos transformation reversion by a second function which is independent of ribosomal accumulation. To distingush between these two hypotheses, we studied the effects of decreased expression of ribosomal proteins RPL5 and RPS6 on v-fos transformation. v-fos transformed cells treated with antisense oligonucleotides of ribosomal protein RPL5 and RPS6 exhibit non-transformed morphology and greatly decreased abilities to grow in semi-solid media compared to untreated cells. Cells treated with a random control oligonucleotide had similar phenotypes as untreated cell. The antisense RNA induced phenotypic changes depended on the continuous presence of antisense oligonucleotides in culture media. The evidence indicated that treatment of v-fos transformed Rat-1 fibroblasts

with antisense oligonucleotides of ribosomal protein RPL5 and RPS6 resulted in loss of transformed phenotypes. The results support the hypothesis that decreased ribosomal accumulation inhibits v-fos transformation.

To further investigate the biochemical pathways involved in the inhibition of v-fos transformation by decreased ribosomal accumulation, we studied cell cycle regulation in v-fos transformed cells and the revertants. The fundamental characteristic of all transformed cells is their ability to proliferate under conditions that usually arrest cell cycle in normal cells. Abnormalities in cell cycle regulation has been suggested to contribute to cell transformation and tumorigenesis. In this study, it was found that the S phase was twice as long in R2.2 revertant compared to v-fos transformed cells, while the durations of G1 and G2/M phases were unaffected. Prolonged S phase in R2.2 revertant is concomitant with decreased cyclin A associated kinase activity. By analyzing the expression of factors which could regulation S phase progression, we found that the protein level of Cdk inhibitor $p21^{Waf1}$ was dramatically increased in R2.2 revertant compared to v-fos transformed cells. Increased level of $p21^{Waf1}$ was also observed in v-fos transformed cells treated with antisense oligonucleotides of ribosomal proteins. Thus, inhibition of v-fos transformation by decreased ribosomal accumulation is accompanied by increased $p21^{Waf1}$ protein level. p21Waf1 inhibits the activity of Cdk2 and PCNA by its N-terminus and C-terminus, respectively. Both Cdk2 and PCNA were shown to be necessary for S phase progression. Therefore, increased expression of $p21^{Waf1}$ in R2.2 revertant may be responsible for the prolonged S phase. $p21^{Waf1}$ was shown to mediate cell cycle arrest in response to extra cellular signals. Increased p21Waf1 level in v-fos transformed cells may restore their response to extracellular growth inhibitory signals and thus inhibit cell transformation. This hypothesis was tested in v-fos transformed cells by ectopic expression of full length $p21^{Waf1}$ protein, its N-terminus and its C-terminus. Consistent with observations in other cell lines, overexpression of full length $p21^{Waf1}$ protein and its N-

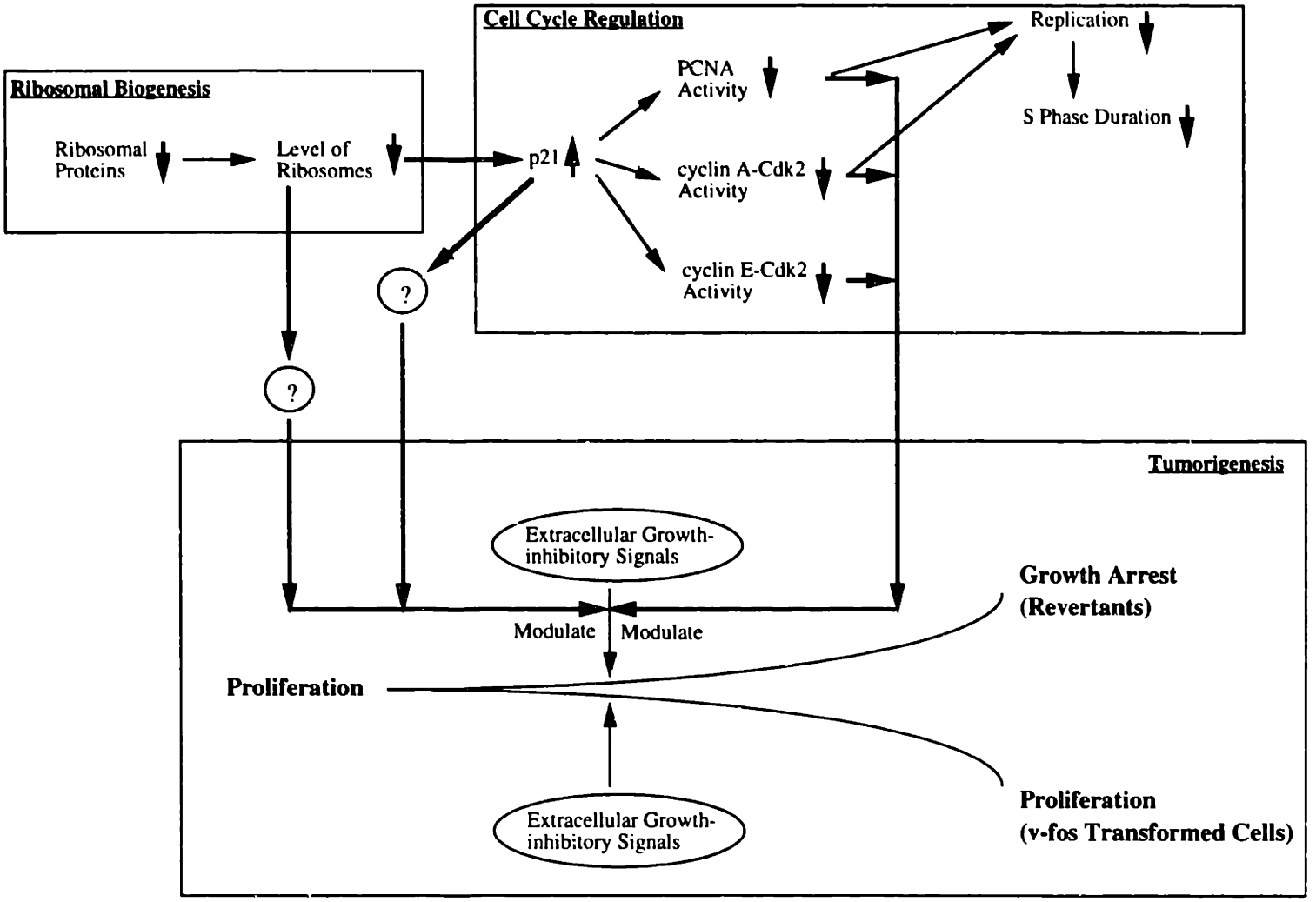
terminus demonstrated growth inhibitory effects in v-fos transformed cells, as indicated by a reduction in the number of colonies obtained after transfection compared with transfection with vector alone. The phenotypes of the colonies with exogenous $p21^{Waf1}$ expression are still being studied.

In order to test if decreased expression of ribosomal protein RPS3a will inhibit cell transformation in Hela cells, a vector which is capable of expressing antisense RNA of RPS3a from a dexamethasone-inducible promoter was transfected into Hela cells. Loss of prolonged Rhodamine 123 retention was used as an indicator to screen possible revertants from clones isolated after transfection. Clones which demonstrated decreased retention of Rhodamine 123 were analyzed for their expression of RPS3a protein. Clone 1S2.7 showed 50% decrease of the expression of RPS3a and decreased growth in soft agar compared to parental Hela cells upon dexamethasone induction. The results support the possibility that decreased expression of RPS3a results in a partial loss of transformed phenotypes in Hela cells. However, a 50% decrease of RPS3a protein level does not inhibit cell transformation completely in Hela cells.

6.2 Suggestions for future experiments:

On the basis of present knowledge, a model which depicts the biochemical pathways involved in the reversion process is shown in Figure 26. It is reasonable to assume that decreased expression of ribosomal proteins would affect the expression of many genes; increased $p21^{Waf1}$ protein level is one among them. $p21^{Waf1}$ was shown to be a putative tumor suppressor; increased $p21^{Waf1}$ level may be one of the pathways by which decreased expression of ribosomal proteins suppresses v-fos transformation. $p21^{Waf1}$ has been shown to bind Cdk2 and PCNA by different domains and inhibit their activity. Thus, increased level of $p21^{Waf1}$ may be contribute to decreased cyclin A-Cdk2 activity and

Figure 26. The roles of ribosomal accumulation and $p21^{Waf1}$ expression in v-fos transforamtion.


Ribosomal Biogenesis
Ribosomal Proteins
Level of Ribosomes
Cell Cycle Regulation
p21
PCNA Activity
cyclin A-Cdk2 Activity
cyclin E-Cdk2 Activity
Replication
S Phase Duration
?
?
Tumorigenesis
Extracellular Growth-inhibitory Signals
Modulate
Modulate
Growth Arrest (Revertants)
Proliferation
Proliferation (v-fos Transformed Cells)
Extracellular Growth-inhibitory Signals

prolonged S phase progression observed in R2.2 revertant. Increased $p21^{Waf1}$ protein level may change the response of cells to extracellular growth-inhibitory signals and thus bring cell cycle under control. The activity of Cdk2 and PCNA could potentially affect cell cycle progression. However, Cdk2 and PCNA may not be the only targets of $p21^{Waf1}$. Other unidentified partners of $p21^{Waf1}$ may also mediate the function of $p21^{Waf1}$. The following experiments are designed to further investigate the roles of $p21^{Waf1}$ and cell cycle regulation in v-fos transformation.

(1) Is increased expression of $p21^{Waf1}$ necessary for the revertant phenotypes in R2.2 cells?

Increased level of $p21^{Waf1}$ may be only one of pathways mediating the inhibition of v-fos transformation by decreased expression of ribosomal proteins. While increased $p21^{Waf1}$ level may not be sufficient for the suppression of v-fos transformation, it is still possible that high level of $p21^{Waf1}$ protein is necessary for the revertant phenotypes. If this hypothesis is true, decreased expression of $p21^{Waf1}$ in R2.2 revertant will result in retransformation. The expression of $p21^{Waf1}$ could be decreased by treatment with antisense oligonucleotides or expression of antisense RNA. Cells with decreased expression of $p21^{Waf1}$ after antisense oligonucleotide treatment or transfection of antisense vector can be analyzed for their phenotypes. The first method was tested in this thesis. The phosphorothioate oligonucleotide used was 5' CAG GAT CGG ACA TGG TGC CT 3'. However, the expression of $p21^{Waf1}$ was not decreased as indicated by Western blotting(data not shown). It was found that R2.2 revertant is more sensitive to oligonucleotide treatment that v-fos transformed cells. Treatment with 5uM of a random oligonucleotide caused significant cell death. Therefore, increasing the concentration of oligonucleotide was not feasible. It is possible that the concentration used is not sufficient

for the inhibition of $p21^{Waf1}$ expression. Oligonucleotides with different sequence, which may have higher efficiency, can be tested in future experiments.

The second method is to express antisense RNA of $p21^{Waf1}$ by stable transfection of an antisense vector. R2.2 revertant has the pMexneo plasmid integrated into its genome and expresses neomycin transferase which confers resistance to G418. The vector used for expression of antisense RNA should be transfected with another selectable marker, e.g.. hygromycin resistant gene.

(2) Does increased $p21^{Waf1}$ expression correlate with decreased activity of cyclin A?

In R2.2 revertant, which has decreased expression of ribosomal proteins RPS3a, increased v protein level is accompanied by decreased cyclin activity and prolonged S phase progression. Since $p21^{Waf1}$ is able to inhibit the activity of Cdk2, increased $p21^{Waf1}$ level may decreased the activity of cyclin A-Cdk2 complex and, as a result, increase the duration of S phase. In order to establish the correlation between increased $p21^{Waf1}$ level and decreased cyclin A activity, cyclin A-Cdk2 activity can be tested in v-fos transformed cells with decreased expression of other ribosomal proteins. Another approach is to decrease the expression of $p21^{Waf1}$ in R2.2 revertant by antisense RNA or antisense oligonucleotides and then test the activity of cyclin A-Cdk2 complex. Although this correlation does not prove that increased $p21^{Waf1}$ level contributes to v-fos transformation reversion, it provides a link between decreased expression of ribosomal proteins and altered cell cycle regulation.

(3) Does increased $p21^{Waf1}$ level change the response of cells to extracellular signals?

One possible way by which $p21^{Waf1}$ could contribute to suppression of v-fos transformation is to inhibit cell cycle progression in response to extra cellular growth inhibitory signals. $p21^{Waf1}$ could inhibit the activity of PCNA, which is required for chromosomal replication, and Cdk2, which associate with cyclin E in G1-to-S transition

and with cyclin A in S phase. Therefore, it is likely that increased $p21^{Waf1}$ level would have an impact on G1-to-S transition or S phase progression. Inhibition of cyclin E activity has been implicated in cell cycle arrest in response to cell-cell contact. Using a preparative suspension culture system, Guadagno et al. (1993) demonstrated that the inability of untransformed cells to grow in semi-solid media is accompanied by the absence of cyclin A expression at G1-to-S transition and increased cyclin A level could allow cells to go through several round of cell cycle in suspension. These results suggest that in untransformed cells the expression of cyclin A is anchorage-dependent. This system can be used to study the effects of increased $p21^{Waf1}$ expression on the response of v-fos transformed cells to extracellular growth-inhibitory signals. Cells can be synchronized at mid-G1 by protein synthesis inhibitor, mimisine, and seeded in preparative suspension culture. Cells will be recovered at time points after being released from mimisine block. The progression of cell cycle will be analyzed by flow cytometry. Cyclin-Cdk complex could then be immunoprecipitated from cell lysate. The presence of $p21^{Waf1}$ in immunocomplex can be analyzed by Western blotting. The response of cells to cell-cell contact can be studied in similar manner.

(3) Is decreased cyclin A activity essential for transformation reversion?

Decreased cyclin A activity could result in slower S phase progression. Although, increased S phase is not like to be responsible for transformation reversion, decreased cyclin A activity may be necessary to confer revertant phenotypes. In order to test this possibility, the effects of increased cyclin A activity on reversion can be studied in R2.2 cells. Increased cyclin A activity can be achieved by ectopic overexpression of cyclin A, Cdk2 or both. However, if decreased cyclin A activity is due to changes in the modification of the subunits, the above method will not be very useful.

(4) How is the expression of p21 regulated by ribosomal accumulation?

Increased level of $p21^{Waf1}$ protein in response to decreased expression of ribosomal proteins suggests that the expression of $p21^{Waf1}$ may be regulated at translational level.

It was suggested that the translation efficiency of mRNA with high GC content in its 5í untranslated region (UTR) is regulated by translation initiation. Overexpression of translation initiation factor eIF-4E facilitate the translation of cyclin D1. The GC content in 5í UTR of cyclin D1 mRNA is 71%.

2121 bp of $p21^{Waf1}$ cDNA cloned from human has a very long 3' untranslated region (1551 bp) and a fairly long 5' untranslated region. The GC content in its 5í UTR is 69%, with 52 GCs out of 75 bases. In rat $p21^{Waf1}$ mRNA, the GC content in 5í UTR is 61%, with 52 GCs out of 85 bases. Therefore, the translation of $p21^{Waf1}$ mRNA could be regulated by translation initiation. Comparing $p21^{Waf1}$ mRNA level between R2.2 revertant and v-fos transformed cells will provide evidence to address this possibility.

The above discuss invokes the hypothesis that decreased ribosomal accumulation facilitate the translation initiation of $p21^{Waf1}$ mRNA. To test this hypothesis, the 5' UTR of $p21^{Waf1}$ can be fused to a reporter gene, such as b-galatosidase. The construct can be transfected into R2.2 revertant and v-fos transformed cells. The protein level of the reporter gene can be normalized to its mRNA level. The differences in reporter protein levels between R2.2 revertant and v-fos transformed cells should provide the answer.

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