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# Viral oncogenes in human fibroblasts

Resnick-Silverman, Lois A., Ph.D.

City University of New York, 1991

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# VIRAL ONCOGENES IN HUMAN FIBROBLASTS

by

**Lois Resnick-Silverman**

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

This manuscript has been read and accepted by the Graduate Faculty in Biology in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

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## Abstract

### VIRAL ONCOGENES IN HUMAN FIBROBLASTS

by

LOIS RESNICK-SILVERMAN

Adviser: Professor Harvey L. Ozer

To clarify the role of SV40 T antigen in the transformation of human fibroblasts this laboratory has generated immortalized transformants (HAL) using origin-defective mutants of SV40 encoding a temperature sensitive large T antigen (tsA58). At the permissive temperature (35 °C) HAL cells have properties resembling those of cell lines transformed by wild type SV40. However at the restrictive temperature (39 °C) HAL cells are unable to proliferate or form colonies. T antigen binding to the cellular proteins Rb and p53 is reduced upon temperature shift up.

Although PyLT antigen is capable of immortalizing rat embryo fibroblast (REF) cells, transfection of human fibroblasts with PyLT does not result in extension of their lifespan or immortalization. Stable introduction of PyLT into HAL cells by retrovirus vector does not result in the rescue of any colonies at the restrictive temperature.

Whereas E1A 12S protein has been shown to immortalize REF cells and to rescue tsA58-transformed REF cells at the restrictive temperature, this is not the case in HAL cells. Transfection of a plasmid encoding the E1A 12S protein and the human HPRT gene into HAL cells results in colonies which express the 12S protein but are still temperature-sensitive. A rare clone, Ad-1, showed restored growth at 39 °C. These cells are not revertants of the tsA58 genome but the reasons for the extended viability of Ad-1 cells at 39 °C remain unknown. Immunoblots have shown that polyoma LT antigen or E1A can be immunoprecipitated with antiserum to Rb protein in these cell lines, demonstrating that the binding of Rb alone is insufficient for the rescue of HAL fibroblasts. These studies and those of others indicate that multiple cellular and viral functions are involved in SV40-mediated transformation and immortalization of human fibroblasts.

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My husband, Gary, whose unique style of support and excellent word processing skills brought this manuscript to fruition.

This thesis is dedicated to my children, Jesse and David.

# ABBREVIATIONS

| | |
|---|---|
| Ad | Adenovirus |
| BRK | Baby rat kidney |
| CHO | Chinese hamster ovary |
| CFU | Colony forming unit |
| CKII | Casein kinase II |
| CR 1, 2, 3 | Conserved regions 1, 2, 3 |
| DAG | Diacylglycerol |
| DMBA | Dimethyl benzoic acid |
| E1A, 1B 2, 3, 4 | Adenovirus early region proteins 1A, 1B, 2, 3, and 4 |
| EGF | Epidermal growth factor |
| EOC | Efficiency of colony formation |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| HAT | Hypoxanthine, Aminopterin and Thymidine |
| HEL | Human embryonic lung cell line |
| HLA | Human leukocyte antigen |
| HMW | High molecular weight |
| HPRT | Hypoxanthine guanine phosphoribosyl transferase |
| HPV | Human papilloma virus |
| IF | Intermediate filaments |
| ITR | Inverted terminal repeats |
| L1-L5 | Late proteins 1 through 5 |

| | |
|---|---|
| LTR | Long terminal repeat |
| M-CSF | Macrophage colony stimulating factor |
| MEF | Mouse embryo fibroblast |
| MHC | Major histocompatibility complex |
| MMTV | Mouse mammary tumor virus |
| M-MuLV | Moloney murine leukemia virus |
| MNU | Methylnitrosourea |
| 4-NQO | 4-Nitroquinoline-1-oxide |
| PCNA | Proliferating cell nuclear antigen |
| PDGF | Platelet derived growth factor |
| PI | Phosphatidyl inositol |
| Py | Polyoma |
| PyLT | Polyoma large T |
| PyMT | Polyoma middle T |
| PyST | Polyoma small T |
| Rb | Retinoblastoma |
| REF | Rat embryo fibroblast |
| RFLP | Restriction fragment length polymorphism |
| SV40 | Simian virus 40 |
| SVLT | Simian virus 40 large T |
| SVST | Simian virus 40 small T |
| 6-TG | 6-Thioguanine |
| TPA | Tumor promoting agent |
| ts | Temperature sensitive |
| VP1, 2, 3 | Viral protein 1, 2, 3 |

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# Chapter 1

## INTRODUCTION

### 1.1 Senescence In Human Fibroblasts

A normal diploid cell is committed to a finite life span. Under tissue culture conditions, normal cells will initially demonstrate a high proliferative capacity (phase II). Yet, with subsequent population doublings, a steady decline in the growth rate of normal cells is observed until the non-replicative phase predominates (phase III) and death becomes inevitable. Phase III cells are characterized by an increased cell volume, a lengthening in the cell cycle time due to a prolonged G1 phase and an exponential decline in the number of the cells able to synthesize DNA. It is believed that after 50-60 population doublings human cells arrest in G1 or at the G1/S boundary. Unlike quiescent cells which are blocked in G0, aging cells demonstrate a level of thymidine kinase, DNA polymerase and histone H3 comparable to that of young actively dividing cells. The quinacrine dihydrochloride staining pattern and the ability to enlarge their thymidine triphosphate pool prior to S phase suggest that senescent cells are blocked in late G1 or G1/S. The induction of various cell cycle dependent genes by mitogens can also be used to monitor progression through the cell cycle. Cytoplasmic m-RNA levels of c-*myc* (early G1), p*53* (mid G1) and c-Ha-*ras* (mid-late G1) indicate that these genes are fully expressed in senescent cells (Rittling et al. 1986, Olashaw et al. 1983, Pendergrass et al. 1985). This is strong evidence that senescent cells arrest just prior to DNA synthesis.

This *in vitro* aging process has become a model for cellular senescence because it parallels the *in vivo* condition. Our understanding of growth and senescence is enhanced by the study of the mechanisms which are overcome by cells that no longer obey the signals coupled to normal growth.

## 1.2 Immortalization of Fibroblasts

Historically, fibroblasts were the first cells successfully propagated in tissue culture. These cells respond well to the mitogenic proteins such as PDGF, which is found in serum. Although it is known that other cell types can be induced to grow when provided with specific growth factors, fibroblasts still remain the most common cell type used for experimental studies. Notwithstanding the fact that fibroblasts are not the dominant cell type of any organ, they are found in all organs of the body. Furthermore, fibroblasts can become malignant tumors (fibrosarcomas account for 0.2% of all human tumors) or benign fibromas.

The ability to become immortalized differs greatly when comparing human fibroblasts to rodent fibroblasts. Although rodent cells will immortalize spontaneously at a rate of $2x10^{-6}$ per cell per generation (Kraemer 1986), human fibroblasts rarely, if ever, evolve into immortal cell lines spontaneously. There has been one reported case of spontaneous transformation of a fibroblast culture derived from a patient with melanoma (Mukherji 1984). It has been validated by HLA analysis. These cells appear morphologically changed, aneuploid, exhibit anchorage independence and form progressively growing sarcomas in athymic mice.

Whereas chemical carcinogens will readily transform rodent cells, no one has successfully produced malignant transformed human fibroblasts (which make invasive tumors in athymic mice) by carcinogenic treatment. Although numerous cell lines have been described which possess one or two features of a transformed cell (after chemical carcinogen treatment), few, if any, are bona fide transformed cells.

One of the best documented studies came from Kakunga (1977), who transformed cells originating from a lip biopsy using 4-nitroquinoline-1-oxide ("4-NQO"). These cells were reported to produce nodules (1/10 injected) in athymic mice which were removed and diagnosed as fibrosarcoma. However, when the normal cell line and its transformed derivative were subjected to isozyme analysis, chromosome studies, HLA and RFLP analysis, the results suggested that the fibrosarcoma cell line was not derived from the original normal cell line. Although this appears to be a bona fide fibrosarcoma, its pedigree is unknown.

Two immortalized human cell lines have been generated (1/260 attempts) after repeated treatments with 4-NQO (Namba 1978). When these cells were injected into nude mice, tumors were rarely produced and only then after a long latent period. These cell lines are chromosomally abnormal and display altered cell morphology. One has acquired diminished growth factor dependence.

Anchorage independent growth in human fibroblasts has been induced by carcinogens in a number of different laboratories (Friedman and Shin 1974; Milo and DiPaolo 1977, 1978, 1981, 1988; Sutherland 1980, 1981, 1988; Salinkas 1981; Maher 1982; Wang 1986; McCormick 1986, 1987). This criterion has been used by many as the endpoint of transformation. However, it is important to note that when injected into athymic mice, the tumors which sometimes grow (and have been characterized as poorly differentiated, undifferentiated mesenchymal, fibrosarcoma or nodular growths) are noninvasive, do not grow progressively and often regress. Cells derived from both these tumors and the anchorage independent cells do not grow indefinitely in culture. McCormick and Maher (1988) postulate that this property arises from a mutation-like event consistent with the pattern seen in classic mutational markers.

## 1.3 Transformation of Human Fibroblasts

In terminology recommended by the Tissue Culture Association (Schaeffer 1983) malignant transformation refers to cells which form true tumors and grow progressively in a susceptible host. These cells are characterized by the infinite ability to grow in tissue culture, formation of colonies in soft agar and growth factor independence. The conclusion that more than one or two events are required for malignant transformation is supported by two observations (a) only one unequivocal example of spontaneous transformation has been confirmed and (b) no cells displaying the full range of transformed traits have ever been induced by carcinogen treatment.

Why cultured human fibroblasts (and epithelial cells according to Chang 1986) are more refractory to malignant transformation than rodent fibroblasts is nonetheless intriguing. One possible explanation is that cultured rodent cells give rise spontaneously to karyotypically abnormal cells whereas cultured human fibroblasts remain karyotypically stable until senescence (Thompson and Holliday 1975). McCormick and Maher (1988) suggest that it is this stability which accounts for the rarity of spontaneous infinite life span variants. This hypothesis is supported by the fact that the karyotype of the bona fide human spontaneous immortal cell line is abnormal compared to the matched normal parent. Additionally, human immortal cell lines which emerge from SV40 transformed cells (i.e. cells which have acquired the properties of focus formation, low serum dependence and anchorage independence) are rare and chromosomally abnormal (Sack 1981). Fusion experiments between immortal SV40 and normal fibroblasts suggest that the infinite life span trait is recessive (Periera-Smith and Smith 1983). It has been calculated that if, in fact, two alleles need to be inactivated for expression of this trait, the frequency of this event occurring spontaneously would be $< 1x10^{-12}$ per cell per generation.

## 1.4 Multi-Step Hypothesis for Cell Transformation

That the process of tumorigenesis requires multiple steps has been supported by the following *in vitro* and *in vivo* observations:

1) Transformation of rat embryo fibroblasts ("REF") or mouse embryo fibroblasts ("MEF") with individual oncogenes is inefficient.

2) Transformation of established rodent cells is readily accomplished with individual oncogenes.

3) Certain combinations of oncogenes will transform REF or MEF (Land 1983, Ruley 1983).

4) Human immortal cells developed by Namba (1986) and McCormick (1989) using 4-NQO can be malignantly transformed by an 'activated *ras*'. Although normal fibroblasts and mesothelial cells that have been transfected with Ha-*ras* or v-*sis* are not tumorigenic and do not have infinite life spans (Fry 1988, Tubo 1987).

5) Human fibrosarcomas have been found to contain multiple activated oncogenes, e.g. human fibrosarcoma SHAC contains an 'activated' N-*ras* gene and a modification of the c-*myc* gene (Suarez et al. 1987).

6) A survey of 12 human solid tumors or tumor cell lines revealed that 40% (5/12) expressed two or more altered oncogenes. In three of these five, N-*ras*, c-*myc* and c-Ha-*ras* had undergone independent and distinct alterations. The most frequently altered oncogene accompanying a ras activation was c-*myc*, which displayed elevated expression due to gene amplification (Suarez et al. 1987).

7) Four genetic alterations were examined in 53 colorectal tumors and 53 adenomas (14 late stage, 12 intermediate and 27 early) (Fearson et al. 1990). Greater than 90% of the carcinomas had more than two alterations. Seven percent of the early adenomas contained more than one alteration. The number of alterations increased with the progressive stage of the adenoma. Two late stage adenomas contained all four genetic alterations supporting the hypothesis that additional alterations are required for malignancy to occur.

## 1.5 Oncogene Hypothesis

The oncogene hypothesis has become the unifying theory whereby the action of carcinogens and other genetic disturbances can be explained at a genetic level, i.e. that perturbations of normal genes can result in neoplastic changes. This theory links the normal cellular function of growth and differentiation with the aberrant condition of neoplastic transformation. The cataloguing of cellular oncogenes (more than 60 to date) continues in the hope that light will be shed on the intricate circuitry that controls normal growth.

In the past, many researchers have utilized NIH3T3 cells (an infinite life span, aneuploid mouse cell line) to assay for the transforming activity of various oncogenes and proto-oncogenes. In fact, by using the criteria of focus formation, growth in soft agar and growth in nude mice, *ras* oncogenes are highly efficient at transforming 3T3 cells (Shih et al. 1979, Cooper et al. 1980, Blair 1982). However, no phenotypic changes are produced when *myc* is used for transfection (Blair et al. 1982, Land et al. 1983). These cells already have acquired the necessary alterations for all but the final step in transformation. In order to examine the earlier steps involved in cell transformation, human fibroblasts and rat or mouse embryo fibroblasts must be employed.

The early experiments of Land et al. 1983 and Ruley 1983 using REF cells established a requirement for the activation of two proto-oncogenes in malignant transformation. A model was created for the existence of two classes of oncogenes. Class A oncogenes have a nuclear location and are involved in the immortalization of REF cells. This class includes the following genes: polyoma large T antigen, adenovirus E1A, c-*fos*, c-*myb*, c-*ski*, papilloma virus E6 and the c-*myc* family (comprised of v-*myc*, c-*myc*, N-*myc*, L-*myc* and R-*myc*). Class B oncogenes are responsible for morphologic transformations and include *ras* (comprised of N-*ras*, H-*ras*, K-*ras*), v-*erb*, v-*fes/fps*, v-*src*, v-*fms*, polyoma middle T antigen (MT) and adenovirus E1B. These gene products are associated with a membrane bound or cytoplasmic designation. SV40 T antigen, which can singly transform REF cells, traverses both A and B classes.

This model includes cellular genes (proto-oncogenes) whose transduction by retroviruses (oncogenes) makes them potentially capable of inducing transformation. Without exception viral oncogenes contain deletions, mutations, insertions or amplification relative to their corresponding proto-oncogenes. By supplementing the *ras* gene, a class B oncogene, with a class A oncogene, a fully transformed phenotype was achieved (Land et al. 1983, Ruley 1983). By substituting other class B oncogenes to serve as surrogates for *ras*, the same effect was achieved. See Table 1. It would be convenient, yet simplistic, to be able to sort oncogenes accordingly. However, it is now known that vigorous expression of single oncogenes may be sufficient for transformation. It has been shown that wild type c-*ras* can sustain indefinite growth of otherwise normal cells. It has been established by Spandidos et al. 1984, Small et al. 1988, and Keath et al. 1984 that rodent cells can become tumorigenic due to c-*myc.* Vennstrom 1984 demonstrated that embryonic rodent cells can be transformed by c-*myc* .

## 1.6 Class B Oncogenes

The products of class B oncogenes include protein kinases, GTP binding proteins, growth factors and growth factor receptors (See Table 2). The normal counterparts of these proteins are involved in the generation of a cascade of events which ultimately cause the DNA in the nucleus to start replicating. Signal transduction through a growth factor receptor is viewed in the following way. A growth factor binds to its receptor, the receptor becomes transiently activated. This in turn activates other proteins involved in the growth-stimulatory pathway. It also activates the production of a variety of small regulatory molecules called second messengers. These signals are ultimately transmitted to the nucleus, where the expression of specific genes is induced, resulting in cell division (See Figure 1).

Oncoproteins have been identified which play a role in every part of this scenario. (1) Growth factor proteins include v-*sis* which encodes an altered version of one of the chains of PDGF and int-2 whose product is related to FGF. (2) Transmembrane receptor proteins include *fms* (M-CSF receptor), *erb*B (EGF receptor), *neu* and *ros*. (3) Membrane associated oncogenes include the *src* family which has tyrosine kinase activity and the *ras* family which binds guanine nucleotides. (4) Cytoplasmic oncoproteins include *raf* and *mos*. These oncoproteins have serine-threonine kinase activity and may be similar to protein kinase C.

There are two well known mechanisms of signal transduction: through G proteins and through kinases and phosphatases.

Transduction Through G Proteins. G proteins are receptor activated proteins which mediate signal transduction through the exchange of bound GDP for GTP. This activates the protein which in turn promotes the activation of effectors such as ion channels and phospholipase C. Phospholipase C can generate two second messengers, diacylglycerol (DAG) and inositol triphosphate. DAG activates protein kinase C which appears to be involved in a host of stimulus response pathways (i.e. mitogenesis in response to growth factor stimulation, release of serotinin from platelets, release of acetylcholine from neuronal cells). Activated protein kinase C associates with the cell membranes and provides access to membrane substrates like pp60 *src*. This kinase has also been found to be the intracellular receptor for phorbol esters. Phorbol esters compete with DAG for its protein kinase binding site, causing prolonged activation of the enzyme and thus providing an explanation for their tumor promoting properties.

Scolnick (1979) recognized a similarity between the *ras* proteins and those of the G protein family. He demonstrated that normal p21 (the protein encoded by the *ras* gene) was a guanine nucleotide binding protein capable of binding GTP and GDP with a high affinity. He also speculated that those nucleotides control the p21 activity. Biochemical analysis of oncogenic *ras* mutants has suggested that alterations in GTP hydrolysis or GTP/GDP exchange may, in fact, be responsible for their transforming activity (Bos 1988, Barbacid 1987). More recent evidence has supported the hypothesis that *ras* proteins are controlled by GTP and GDP. GTP-bound forms are active and GDP forms are inactive (Trahey and McCormick 1987, Satoh et al. 1987). GAP (GTPase activating protein), a likely candidate for a *ras* effector protein, is putatively responsible for the conversion of p21-GTP to p21-GDP (Trahey and McCormick 1987, Gibbs et al. 1988, McCormick 1989). Oncogenic p21 proteins with mutations at positions 12, 59, or 61 are unaffected by GAP and remain in their active GTP-bound state.

Activated *ras* has been found in a significant proportion of all tumors. However, its incidence varies considerably with tumor type. It is associated with 20-40% of all colorectal tumors and acute myeloid leukemias, but is virtually absent in breast tumors and stomach cancer (Bos 1988). The presence of activated *ras* only in tumor DNA and not normal DNA from the same individual suggests that *ras* indeed plays some role in transformation.

Although one can not exclude the possibility that *ras* mutations are epiphenomena, arising as the result of the higher mutation rates observed on transformed cells, it is generally accepted that *ras* bestows a selective advantage to tumor cells. In fact, there is some suggestion that v-Ha-*ras* may contribute to tumor progression by augmenting gene instability, thereby encouraging further critical mutations. This evidence comes from work by Harris et al. 19 , who introduced v-H-*ras* into primary normal human bronchial epithelial cells. A small number of colonies grew out in soft agar which appeared to be phenotypically transformed and gave rise to tumors in nude mice. These cell lines produced the v-H-*ras* protein when passaged in nude mice and resulted in progressive chromosomal rearrangements characterized by hyperploidy and marker chromosomes. Wong and co-workers (1986) have observed that *ras* transformed cells show 100-fold greater rates of cell : cell fusion. These spontaneously fused cells were hyperdiploid and contained increased numbers of chromosomes.

Mutant *ras* may be associated with the earliest phases of transformation, although conflicting evidence has been reported. In 1987, Bos et al. examined several adenoma-carcinoma sections from patients with colorectal cancer. Pre-malignant adenomas frequently abut carcinoma tissue affording the unusual opportunity of comparison. Oligonucleotide hybridization to exclusively carcinoma or adenoma tissue showed that of 6 cases in which activated *ras* was present in the carcinoma, 5 of the companion adenomas also expressed activated *ras*. This suggests that activation may be an early step. Reynolds et al. 1986 has reported that 3 of 10 spontaneous hepatic adenomas of mice contain activated c-H-*ras*.

MNU, known to induce G-A transitions (Zarbl 1983), was injected as a single dose into rats. Three months later mammary tumors expressing H-*ras* were recovered. Since MNU only has a half life of 30 minutes, it was concluded that activation of *ras* was an early event, predisposing a cell to becoming a tumor (Barbacid et al. 1987). Gullino et al. 1985 demonstrated that ovariectomy reduces tumor formation, suggesting that hormones may serve as tumor promoters for initiated cells. Using a mouse skin carcinoma model, Balmain et al. 1983, induced tumors with DMBA followed by TPA treatment. All tumors and pre-malignant papillomas were found to contain H-*ras*. When v-H-*ras* was substituted for the initiation step (DMBA treatment), the results was the same. Dotto et al. 1985 has verified the observation that REF cells containing activated *ras* are strongly stimulated by phorbol esters. It was found that foci were formed with 100% efficiency. Initiated cells remain contact-inhibited and will continue to proliferate only in the presence of tumor promoters.

<u>Transduction involving protein kinases and phosphatases.</u> Protein kinases and phosphatases regulate enzyme function through the phosphorylation or dephosphorylation of either serine-threonine residues or tyrosine residues. Cellular processes as diverse as carbohydrate metabolism or control of cellular growth are profoundly affected by protein phosphorylation.

Serine-threonine residues are by far the most common sites of protein phosphorylation within a cell. Protein kinase C, the best known serine-threonine kinase, plays a pivotal role in multiple signalling pathways. It is activated by the second messengers $Ca^{++}$ and DAG which are produced after the stimulation of many types of receptors. An overexpressed version of cloned protein kinase C can induce partial transformation of NIH3T3 and rat fibroblasts (Persons et al. 1988, Housey et al. 1988). Although not yet considered a proto-oncogene, it is related to a group of oncogenes with serine-theonine kinase activity (c-*raf*).

Not nearly as well understood as G proteins and second messengers which activate kinases like protein C are the protein kinases which phosphorylate tyrosine residues. Many growth factor receptors including those for epidermal growth factor, platelet derived growth factor, insulin and macrophage colony stimulating factor are tyrosine kinases. Several altered forms of these receptors (v-*erb*B, v-*sis*, v-*fms*) are oncogenes. It is likely that they also play a major role in growth regulation. A vigorous search for the substrates of these kinases has resulted in the identification of two proteins that may play an important role in the signal transduction pathway (Druker et al. 1989). These investigators propose that one is a novel lipid kinase (involved in the early proliferative response to certain growth factors) and the second is a cytoplasmic serine-theonine kinase which is the product of the c-*raf* proto-oncogene whose activation is associated with stimulation or transformation by certain growth factors or oncogenes.

Using the stimulation of quiescent cells with PDGF as an example of signal transduction, Druker et al. have proposed the activation of two pathways (See Figure 2). Pathway I involves three steps. First PDGF binds to its receptor, thereby increasing the tyrosine kinase activity of its receptor. Second, the activated receptor phosphorylates several proteins on tyrosine residues, including the 85kD phosphatidyl-inositol ("PI") kinase and possibly phospholipase C. Third, these membrane proteins can then activate other members of the signal transduction cascade and generate small second messengers such as DAG and inositol triphosphate.

Pathway II includes two steps. First, when PDGF is bound, cytoplasmic c-*raf* protein is brought to the membrane, phosphorylated on tyrosine and activated. Activation results in the auto-phosphorylation of its serine-theonine residues. Second, either the activated c-*raf* then transmits a growth stimulatory signal to the nucleus or it may activate other kinases in the cascade.

## 1.7 Class A Oncogenes

The nuclear retroviral oncogenes (i.e. *myc*, *fos*, *myb*) have short half lives, which make them well suited for involvement in the control of cell cycle and differentiation functions. Expression of *myc* is low during G0 and increases with mitogen stimulation. It appears to be temporally related to the increase of other nuclear oncogenes. The c-*myc* product is considered critical for allowing cells to pass from G1 to S phase (Campisi et al. 1984, Gonda and Metcalf 1984, Coppola and Cole 1986, Lachmann et al. 1986). If c-*myc* is reduced by the use of anti-sense oligodeoxyribonucleotides, cells will move to G1 following stimulation but cannot enter S phase. It is possible that deregulated c-*myc* expression may modulate expression of other proliferation genes in order to move through S phase (Cleveland et al. 1980) or it may affect DNA synthesis directly (Studzinski et al. 1986).

It is through the c-*fos* protein that transmembrane signals exert their influence in the nucleus. C-*fos* may mediate gene regulatory events induced by such signals which lead to cell proliferation or differentiation. Pledger et al. 1977 and Singh et al. 1983 have proposed that competence factors like PDGF induce c-*fos* and AP-1 to make cells responsive to a second group of factors, 'progression factors' such as EGF which in turn are required for induction of DNA synthesis. Antisense RNA experiments suggest that expression of fos is necessary for serum induction of DNA synthesis in quiescent fibroblasts (Holt et al. 1986, Nishikura and Murray 1987).

Thompson et al. 1986 has demonstrated that c-*myb* expression is associated with cell proliferation in non-fibroblasts. Like c-*myc* and c-*fos*, c-*myb* mRNAs have a short half life. During periods of exponential growth, c-*myb* RNA levels are 10 fold higher than that of non-dividing cells.

## 1.8 DNA Tumor Viruses - SV40 and Polyoma

Viral DNA oncogenes (papovaviruses and adenoviruses) are not associated with naturally occurring tumors *in vivo*. Their gene products can, however, induce transformation of cultured cells *in vitro*, and can cause tumors in immunologically compromised or neonatal animals. Unlike retroviral oncogenes, DNA tumor virus oncogenes are of viral, not cellular, origin and are intimately involved in the life cycles of these viruses. They are structurally dissimilar to retroviral oncogenes, yet they may serve similar functions based on their ability to 'complement' each other in tumorigenic assays.

In permissive mouse, monkey and human cells, polyoma, SV40 and adenoviruses respectively, have productive life cycles that lead to the production of progeny virions and the ultimate death of the host cell. The entire viral genome is expressed during infection including the late functions which are responsible for packaging the viral DNA into capsids. In cells which are non-permissive for viral replication, only a subset of viral genes encompassing a region shown to be essential for cell transformation is expressed. A small percentage of non-permissive cells become transformed when portions of the viral DNA become stably integrated into the host genome. Transformation does not appear to be the primary function of polyoma, SV40 or adenovirus oncogenes in their natural hosts. These viruses are dependent on cellular encoded proteins for the replication of their DNA genomes. Both SV40 and polyoma will induce cellular division and synthesis in a resting population. This is achieved by the action of a virally encoded protein which causes the cell to ignore its own regulatory signals and enter into its cell cycle.

It has recently been shown that the ability of SV40 large T antigen to initiate viral DNA synthesis is controlled by phosphorylation (Graessner et al. 1987, McVey et al. 1989). In a resting cell, many of the serine residues of the T antigen become phosphorylated; Thr 124 remains unphosphorylated. In this state, T antigen is unable to initiate viral DNA synthesis. The enzyme(s) required to phosphorylate Thr 124 (possibly cdc2 kinase) and remove the repressing serines (possibly PP2A), are cell cycle dependent and active only in cycling cells (S phase). The binding of T antigen to p*53* and retinoblastoma may act to release the infected cells from their G1 block and allow progression through the cycle. This will then activate cdc2 kinase and PP2A which will act to convert the T antigen into a form able to initiate DNA replication. A quiescent cell is pushed into cycle, so that the viral DNA can be replicated to a high copy number. This will occur in S phase in concert with the cellular DNA. In a transformed cell, where cell lysis is not the outcome, the viral DNA (although itself unable to replicate) continues to generate this signal giving rise to a cell which can divide under conditions where normal cell division is inhibited.

The papovaviruses SV40 and polyoma are ideal subjects to study as (1) they grow easily in tissue culture; (2) they are easily purified and manipulated; and (3) their genomes are less than 5 kb. Although initial interest in SV40 and polyoma focused on their oncogenic potential, these simple model systems have provided information on transcription, RNA processing, DNA replication, gene regulation, DNA structure and DNA-protein interactions.

SV40 and polyoma virus genomes are small double stranded covalently closed circular DNA molecules, 5243 base pairs and 5292 base pairs respectively (See Figures 3 & 4). The two viruses have identical structures and they are similar in both their chemical compositions and the molecular biology of their interactions with permissive and non-permissive host cells. Both DNA molecules have a single origin of replication which proceeds bi-directionally from this point and terminates 180 degrees away. Both genomes specify three late proteins, VP1, VP2 and VP3 which are made after the onset of replication and destined to become incorporated into progeny virions. Their dissimilarity lies in the strategies chosen for early gene expression. SV40 encodes one protein (large T antigen) for both viral replication and virally induced transformation whereas polyoma encodes a large T antigen as the DNA replication protein and a middle T antigen for transformation. Both SV40 and Py encode highly a homologous small t antigen whose functions, up until recently, have not been well defined. Pallas et al. 1988 and Walter et al. 1988 demonstrated that the small t antigens of SV40 and polyoma and polyoma middle T associate with two cellular polypeptides (p61-63kD and p36-37kD). The 61-63kD polypeptide has been identified as the regulatory A subunit of PP2A (Walter et al. 1990) and the 36-37kD polypeptide as the catalytic subunit of PP2A (Pallas et al. 1990). $PP2A_c$ has been shown to remove serine phosphates from large T antigen and may be cell cycled regulated. Thus the association of small t with these cellular polypeptides may help to ensure the activation of large T antigen.

During the early phase of infection, viral proteins required for the induction of host-cell functions and initiation of DNA replication are synthesized. The SV40 early transcription unit is transcribed into two mRNAs with identical 5' and 3' termini but different splice junctions. The smaller 2.3 kb (16S) mRNA encodes large T antigen, a protein of 94,000 daltons apparent molecular weight which is found predominantly in the nuclei of infected cells and is essential for replication and transformation. The larger 2.6 kb (19S) mRNA encodes small t antigen, a protein of 17,000 daltons apparent molecular weight. SV40 Large T antigen is a nuclear multifunctional DNA binding phosphoprotein whose role is well established in viral DNA replication. It recognizes a series of pentanucleotides with a consensus sequence of 5'G(A>G)GGC3' and is the only viral protein required for SV40 DNA replication in permissive monkey cells. The protein binds to the viral origin, catalyzes an origin dependent duplex opening reaction followed by bi-directional unwinding of the DNA strands by use of its helicase activity.[1] The

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[1] For a review of *in vitro* replication of DNA containing the SV40 origin, see Hurwitz et al. 1990.

origin binding domain overlaps with the helicase-substrate domain which encompasses both helicase substrate binding and ATPase domains (Wun-Kim and Simmons 1990). Autoregulation, cell immortalization, helicase activity transformation, host range for replication, helping adenovirus grow in monkey cells and activation of viral and cellular genes are other features of this protein (Tooze 1980). It has the ability to bind adenylated nucleotides (Bradley et al. 1984), host p*53* (McCormick et al. 1981) and the retinoblastoma protein (Harlow 1986). Figure 5 illustrates the phosphorylation sites and functional domains of the T antigen.

The 2.9kb polyoma early region utilizes all three potential reading frames to yield three different mature mRNAs. This economical overlapping is resolved by differential splicing to produce three functional mRNAs which are translated to large, middle and small T antigens (Berk and Sharp 1978, Kamen et al. 1980). The mRNAs share common 5' and 3' regions, differing only in size and position of the intron to be excised (See Figure 6). The large, middle and small T antigens have molecular weights of 100,000, 48,000 and 22,000 daltons respectively. In 1981 Kamen et al., using separate plasmids with the coding sequences of each of these RNAs, was able to establish individual roles for these proteins (Zhu et al. 1984).

SV40 T antigen is capable of immortalizing primary cells and transforming primary and established cells. The nuclear location of large T is not required for efficient transformation. It is believed that either only a small amount of nuclear large T is required for transformation or that transformation is mediated by a non-nuclear form of large T (Fischer-Fantuzzi et al. 1987). Transformation may be accomplished without the DNA binding, DNA replication ATPase, or helper function activities of T antigen.

The binding and stabilization of p*53* by SV40 T has captured the interest of many because of its association with the transforming function of large T (See 1.10 Anti-Oncogenes). This property is one which is not shared by PyLT and might provide some insight into the different mechanisms used by these viruses to transform cells.

Although PyLT is less well characterized than SV40, it too is a nuclear DNA binding phosphoprotein whose consensus sequence is the pentanucleotide 5'GA/G GGC3' (Cowie and Kamen 1986). It has two discrete nuclear localization sequence elements, one of which is homologous to the SV40. It has known ATPase (Gaudray et al. 1980) and nucleotide activities (Clertent et al. 1984). In the permissive mouse cell, large T alone is sufficient for replication of viral DNA and the early events of the lytic cycle. In non-permissive primary rat cells, large T is responsible for initiating and maintaining indefinite growth[2] (Land et al. 1983, Rassoulzadagen et al. 1983) and diminishing a

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[2] It is necessary to clarify the application of the term immortal which is now commonly used to describe a phenotype of both rodent and human cells. It is my belief that a distinction must be made.

In rodent cells, it is very likely that immortal colonies are variants that are generated spontaneously and the phenotype which is actually being scored following the introduction of selected oncogenes including polyoma large T is colony forming ability.

I do not believe that an equivalent situation exists in human cells, where there are no reported findings of spontaneous 'immortals'. In such a situation immortalization will be judged, not by colony forming ability, but by an acquired capability for indefinite growth. Hence, I will use the terms colony forming ability in rodent cells and immortalization in human cells to reflect these differences.

cell's need for growth factors from serum. It is required in conjunction with middle T to transform primary cells and its need is constitutive for sustaining the transformed phenotype. (Rassoulzadagen et al. 1983). The colony forming function has been localized to the N-terminal domain of the large T protein.

Benjamin and his colleagues demonstrated that PyLT and SV40 large T assume different roles in transformation. By analyzing host range transformation defective (hrt) mutants of polyomavirus it became clear that the mutations responsible for the hrt phenotype were not in the A gene (which encodes large T antigen), but mapped to a sequence in the large T antigen intron which was unique to the polyoma middle T and small T coding regions (Eckhart 1977, Fluck et al. 1977, Feunteun et al. 1976, Soeda and Griffin 1978, Carmichael and Benjamin 1980, Lania et al. 1980a). Using just the cDNA encoding middle T antigen, Treisman et al. 1981 demonstrated that established rodent cell lines required only middle T antigen for transformation. Cell lines transformed by PyMT become anchorage independent and tumorigenic (Cherington et al. 1986, Morgan et al. 1988, Rassoulzadegan et al. 1982, Treisman 1981).

How this oncogene transforms is not yet fully clear. What is known is that middle T can induce cellular DNA synthesis and cell division in resting cells (Stoker and Dulbecco 1969, Fried 1970, Graessman et al. 1981). It is associated with a tyrosyl-specific protein kinase activity (Eckhart et al. 1979, Schaffhausen and Benjamin 1979, Smith et al. 1979) which is derived from a small amount of c-*src* which is complexed to a small proportion of middle T antigen molecules (Courtneidge and Smith 1983,1984). There has been much speculation that polyoma transformation is the result of the change in phosphorylation and subsequent activation of c-*src* complexes (Courtneidge 1985, Yonemoto et al. 1985, Cartwright et al. 1986). PyMT has also been associated with a novel phosphorylation event, catalyzed by the complex formation of MT antigen, pp60$^{c\text{-}src}$ and PI kinase (Kaplan et al. 1986, Whitman et al. 1985, Druker et al. 1989). More recently, PyMT has been associated with the catalytic and regulatory subunits of PP2A (Pallas et al. 1990). Although genetic analysis suggests that each of these functions is necessary for transformation, none of these activities have been found to be singularly sufficient.

Integration of viral (either SV40 or Py) DNA containing a functional early region has been associated with the transformed phenotype (Sambrook et al. 1968, Botchan et al. 1976, Lania et al. 1980b). The sites appear to be random in nature since no common sequence or structural feature of either the cellular or viral DNA sequences can be detected. Integration results from a recombination event involving 2-5 base pairs of homology at the recombinant joint. Gross rearrangements of cellular DNA occur at the site of integration (Hayday et al. 1982, Ruley et al. 1982, Stringer 1982). Transformation results from the contribution of a specific viral oncogene(s) to the cell which may exert its influence in a multistep process.

## 1.9 Adenoviruses

Adenoviruses, like polyoma, share the requirement for expression of at least two viral functions in order to transform primary cells. Interest in this virus became fired in the 1960's when the oncogenic potential of Ad12 and Ad18 was revealed. These were the first human viruses shown to be oncogenic in newborn hamsters. Adenoviruses are categorized into five groups, housing 37 serotypes which are distinguished by the differing abilities to cause tumors in rodents. Nearly all of the current knowledge about genes and proteins involved in adenovirus transformation has come from *in vitro* transformation studies using the non-oncogenic serotypes 2 and 5. Despite intensive investigation, no adenovirus sequences have been detected in human cancers representing approximately 90% of the cancer in the United States.

Adenoviruses are DNA viruses with genomes that average 35-40kb.[3] The genome has been shown to be a double stranded, non-permuted, non-redundant DNA molecule lacking terminal repeats, but containing inverted terminal repetitions ("ITRs") (Garon et al. 1972, Wolfson and Dressler 1972). Unlike papovaviruses, they code for their own histone-like proteins. They possess early and late genes, with the onset of replication being the dividing line. Despite the complication that sequences from both complementary strands were expressed early as well as late in infection (Landgraf-Leurs and Green 1973), a transcription map was available by 1974 (See Figure 7). Four blocks of early genes (E1-E4) are scattered along the genome, two being transcribed from one strand and two from the complementary strand. Individual promoters control each early region (Berk and Sharp 1977, Evans et al. 1977). Further analysis of the E1 region revealed that it contained three transcription units - E1A, E1B and polypeptide 1X (Wilson et al. 1976). Late proteins L1-L5 are mainly structural proteins. Early gene regulation is complex and it is clear that the genes are regulated at many different levels.

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[3] For a review of gene expression and replication, see Pettersson et al. 1986.

Region E1A is expressed first, followed by E1B, E3, E4 (Nevins et al. 1979, Lewis and Matthews 1980) and lastly E2. The enhancer elements preceding E1A are responsible for the immediate activation of the promoter (Hearing and Shenk 1983, Hen et al. 1983, Imperial et al. 1983, Osbourne et al. 1984). The other early promoters are transactivated by the E1A region.

E1A and E1B proteins are required for the fully transformed phenotype (Graham et al. 1974, 1978, Frost and Williams 1978, Jones and Shenk 1979, McKinnon et al. 1982, Chinnadurai 1983). Products of the E1B gene alter morphology, hasten growth rate, prevent contact inhibition and promote focus formation, anchorage independence and tumorigenicity. Two major proteins (19kD and 55kD) are encoded by E1B gene. Vectors encoding the 19kD or the 55kD protein have been co-transfected into primary rodent cells in conjunction with a plasmid encoding the E1A gene. Each of these proteins can act cooperatively with E1A to produce transformed foci (although at half the frequency of an intact E1B gene)(White and Cipriani 1990). The 55 kD protein, like SV40 large T antigen, will bind cellular p*53*, suggesting that it plays a role in abolishing the tumor suppressive effects of this gene (Sarnow et al. 1982). The 19kD protein is responsible for anchorage independent growth and the disruption of the vimentin IF network (White and Cipriani 1990).

E1A proteins are required for the efficient expression of early viral genes whose products are essential for viral DNA replication (Berk 1979, Jones and Shenk 1979). Other properties related to E1A expression include colony formation in primary rodent cells (Houweling 1980), induction of cellular DNA synthesis in quiescent cells (Quinlan and Grodzicker 1987), transcriptional activation of E1B and other early regions of the viral genome (Berk 1979, Jones and Shenk 1979), transactivation of viral and cellular genes (Liu et al. 1985), the repression of transfected genes linked to the SV40, polyoma or adenovirus 2 E1A enhancer (Borrelli et al. 1984, Velcich and Ziff 1985) and the repression of transcription of cellular genes (Hen et al. 1985).

E1A is processed by alternative in-frame RNA splicing into two overlapping mRNAs, 12S and 13S (Berk and Sharp 1978, Chow et al. 1979, Kitchingham and Westphal 1980) which share common 5' and 3' termini (Perricaudet et al. 1979, Baker and Ziff 1981). The two proteins, 243aa and 289aa, differ only by the 46aa unique to the 13S RNA (See Figure 8). Analysis has revealed the polypeptides to be very heterogeneic, due to extensive post-translational modification (Harlow et al. 1985). The 'immortalization' function of E1A for rodent cells is one which is shared by *myc* and PyLT (antigen) genes and like these products, it is located in the nucleus. Functional substitution of these genes can be seen in cooperativity experiments. E1A can cooperate with PyMT or activated H-*ras* to transform primary cells (Ruley 1983). PyLT and members of the *myc* family can substitute for E1A in these assays (Eliyahu et al. 1984, Jenkins et al. 1984, Land et al. 1983, Farada et al. 1984, Ruley 1983, Schwab et al. 1985, Yancopoulous et al. 1985).

The adenoviral transforming proteins can be used to transform or 'establish' a variety of cell types including fibroblasts, epithelial cells and retinoblasts (Houweling et al. 1980, Montell et al. 1984). E1A is capable of collaborating with polyoma MT, T24

Harvey *ras* or Ad5E1B genes to fully transform cultured BRK cells (Ruley 1983, Schneider et al. 1987).

Although either the 12S or the 13S protein will complement PyMT or T24 *ras* in the transformation of BRK cells (Zerler et al. 1986), a retroviral vector encoding the 13S protein (rv13s) has been shown to have greatly reduced potential to immortalize various epithelial primary rodent cell types that the rv 12S can readily induce to proliferate and establish (Cone et al. 1988). These findings, in addition to the observation that adenovirus deficient in 12S expression replicated less well than wild type in a growth arrested human fibroblast line and induced lower levels of cellular DNA synthesis in a G0 arrested restrictive rodent cell line (Spindler et al. 1985), support the hypothesis that 12S protein is required to activate cellular DNA replication in the quiescent cells in which adenoviruses replicate. Further support comes from experiments using E1A viruses containing 12S cDNA in place of the normal E1A region. They can infect BRK cells and stimulate cellular DNA synthesis as well as cellular proliferation (Quinlan and Grodzicker 1987). This particular study included a hybrid virus Ad5.SVR4 in which the E1 region of Ad5 has been replaced with the early region of SV40, making the kinetics of expression of the T antigens and the 12S polypeptide similar. Infection with Ad5.SVR4 also resulted in levels of induction of cellular synthesis and cell proliferation similar to that of the 12S virus. SV40 T antigen, however, provided the additional functions such as growth in soft agar, high plating efficiency, loss of epithelial cell characteristics and disarranged cytokeratins to fully transform the BRK cells.

Attempts to sort out individual E1A activities has resulted in an extensive array of E1A mutants. It is the unique region (CR3 in Figure 8) of 13S polypeptide which is responsible for activation of the adenovirus early genes. A single amino acid change in this 46aa region will severely impair transactivation. This region is not required for immortalization or transformation (Lillie et al. 1986, Moran et al. 1986). Exon 1 of E1A contains two highly conserved regions, CR1 and CR2 (or domains 1 and 2), which are required in combination for full transformation of BRK cells with *ras*. Introduction of these domains on separate plasmids will result in focus formation (Moran and Zerler 1988). However, deletions in either region (aa 1-85 or aa 121-127) will result in a loss of transforming ability (Whyte et al. 1988, Moran et al. 1986). Whereas mutant viruses containing in frame deletion-substitutions in CR1 (aa40-77) are non-essential to the growth of Hela cells, this region provides two distinct functions in infected BRK cells: induction of DNA synthesis and focus formation (Smith and Ziff 1988). This correlation has been shared by Moran and Zerler (1988) who also demonstrated that deletions in CR2 resulted in cells which accumulated a DNA content greater than in $G_2$/M, suggesting that domain 2 may be involved in controlling the normal cessation of DNA synthesis before progression through the rest of the cycle. Using deletion mutants to analyze the cell cycle effects of the three domains, Zerler et al. (1987) concluded the N-terminal (aa 1-85) is sufficient for induction of synthesis of proliferating cell nuclear antigen ("PCNA") and the stimulation of DNA synthesis in quiescent BRK cells (Induction of PCNA is also a requirement for SV40 replication *in vitro*, Prelich et al. 1987). CR2 was required for the induction of mitosis and the active proliferation of BRK cells.

The actual mechanisms which underlie these function have yet to be elucidated. One approach to this problem has been to identify cellular proteins that associate with the E1A polypeptide. Immunochemical studies have led to the identification of a number of cellular proteins which co-immunoprecipitate with E1A (Yee and Branton 1985, Harlow et al. 1986, Egan et al. 1988, Whyte et al. 1989). The three most prominent proteins have relative molecular weights of 300kD, 107kD and 105kD (107 and 105kD proteins have been described previously as a 110kD protein). Any mutation that destroys the binding of the E1A protein to any of these three host proteins will also inactivate the ability of E1A to cooperate with *ras* in the transformation of BRK cells (Whyte et al. 1988a,b). This suggests that E1A exerts its transforming and immortalizing capabilities through these interactions.

Deletion mutants have been used to map out the regions of E1A by analyzing the ability of mutant proteins to form complexes with cellular proteins. Any mutations to the aminoterminus (aa 1-76) were sufficient to eliminate co-precipitation of the 300kD protein. Mutations in non-contiguous sequences encoding aa 30-60 and aa 121-127 abolished the interaction with the 105kD protein and mutations to aa 122-129 eliminated the interaction with the 107kD protein (Whyte et al. 1989). With the exception of the amino terminal amino acids (aa 1-40) which play a role in the binding of the 300kD protein, many of the sequences involved in the binding of the 300, 107 and 105 kD proteins, lie within conserved regions 1 and 2 (Figure 8) which have been found to be essential for complementing activated *ras*.

The report that the 105kD protein is the product of the Rb gene (Whyte et al. 1988) has raised considerable speculation about the transforming mechanism of such complexes. Rb is a nuclear phosphoprotein with DNA binding activity (Lee et al. 1987) whose normal role may involve cell cycle regulation through its association with a dominant nuclear oncogene like E1A.

It has recently been shown that p105-Rb will also complex with SV40 large T in virus infected and transformed cells (DeCaprio et al. 1988, Ludlow et al. 1989). This binding also appears to be directly related to the transforming properties of the protein, since mutants which fail to bind p105-Rb do not transform (Kalderon and Smith 1984, Cherington et al. 1984, DeCaprio et al. 1988). The papovaviruses contain a conserved sequence that is similar to the conserved domain 2 (CR2) of E1A and that is required for Rb binding (Figge et al. 1988)(See Figure 9). Moreover, it has been shown that this region from SV40 large T can substitute functionally for the homologous sequence on E1A (Moran 1988). Dyson et al. 1990 has used *in vitro* assays to show that the large T of several polyoma type viruses can also bind to Rb. Phelps et al. 1988 have described similar sequences in the E7 proteins of several human papilloma viruses and Dyson (1989) has demonstrated the ability of the HPV-16 E7 transforming protein to bind Rb *in vitro*. The significance of this functional motif and its interaction with p105-Rb may certainly underlie the transforming functions of DNA tumor viruses.

## 1.10 Anti-Oncogenes

Another class of genes whose expression inhibits transformation has recently come into focus. The loss of such genes may have profound effects on neoplastic conversion.

The concept of tumor 'suppressor genes' dates back to the first somatic hybridization experiments. When normal cells are fused with malignant cells, the resultant hybrids were less tumorigenic. Concomitant with chromosome loss was the reappearance of the ability to induce tumors (Stanbridge 1985). Somatic cell fusions between Hela cells and normal human diploid fibroblasts have implicated chromosomes 11 and 14 in tumor suppression (Stanbridge 1981). In 1986 Stanbridge demonstrated the suppression of Hela cell tumorigenicity by introducing a single copy of a fibroblast chromosome (tX:11). Other evidence has come from the observation that there is an associated loss of hamster chromosome 15 in rare tumorigenic cell lines emerging from hamster embryo fibroblasts infected with v-H-*ras*.

The loss of these tumor suppressor genes (anti-oncogenes or recessive oncogenes) is now associated with a growing list of human malignancies, including retinoblastoma, Wilms' tumor, neuroblastoma, small cell lung carcinoma, acoustic neuromas, colon carcinoma, breast cancer, bladder cancer, neurofibromatosis and chronic myelogenous leukemia. Two well characterized examples are those of the Rb locus and Wilms' tumor locus where RFLP probes have shown homozygosity of alleles in the region of these loci in tumor tissue compared to heterozygosity in constitutional cells (Lee et al. 1987). The studies of Weissman et al. 1987 provide direct evidence that the genetic information present on the human chromosome 11 suppresses the malignant phenotype of Wilms' tumor cells. By using microcell hybridization to introduce the normal chromosome 11 into a Wilms' tumor cell line, variant cells (which expressed similar transformed traits in culture) were unable to form tumors in nude mice. Cytogenetic analyses have suggested that the familial form of this cancer is associated with deletions in chromosome p11.

Familial retinoblastoma is a disease in which young children develop multifocal tumors in the retina. Non-familial retinoblastoma develops in slightly older children and is characterized by unifocal, unilateral tumors. Knudson (1971) proposed in the 1970's that two 'hits' in a recessive gene were required for the development of human retinoblastoma. Comings (1973), concurring with this somatic mutation theory, suggested that in the familial form, one allele of a relevant autosomal gene was inherited in a defective form in all somatic cells. The neoplastic phenotype was acquired in retinal cells which somehow lost the function of the second allele. In non-familial (sporadic) retinoblastoma, the assumption is that both allelic mutations would have to be acquired somatically.

Cytogenetic and molecular analyses have supported the prediction that retinoblastoma cells often have two altered allelic copies of a single gene Rb-1 located on chromosome 13q14 (Cavanee et al. 1983, Dryja et al. 1986, Friend et al. 1986, Fung et al. 1987, Lee et al. 1987a, McGee et al. 1989, T'Ang et al. 1989). Should these affected individuals survive the hereditary tumor, they are at an increased risk to develop secondary tumors of mesenchymal origin (Hansen et al. 1985). These tumors are also defective in their Rb-1 locus. Tumor cell lines which have been reported to carry the Rb-1 defect include small cell lung, breast, bladder and sporadic osteogenic sarcomas. (Bookstein et al. 1989, Harbor et al. 1988, Lee et al. 1988, Varley et al. 1989, Yokota et al. 1988, Horowitz et al. 1989 and Toguchida et al. 1989).

The product of the Rb-1 gene is a differentially phosphorylated 105 or 110 kD nuclear protein (Rb) with DNA binding activity. The loss of function of this protein may be tied to loss of growth regulation. A finding which offers unexpected implications for the mechanism of action of this gene product is that Rb can form a specific complex with at least 3 major viral transforming products: SV40 large T, adenovirus E1A and human papilloma virus E7 (HPV-E7) (DeCaprio et al. 1988, Dyson et al. 1989a, Whyte et al. 1988). Genetic evidence suggests a link between the ability to complex with Rb and the ability to transform (DeCaprio et al. 1988, Egan et al. 1989). E1A, SV40 large T and HPVE7 share a small region of amino acid homology that is necessary for high affinity binding with Rb (See Figure 9). When mutations are introduced into this region, the frequency of transformation by E1A or SV40 T antigen is strongly reduced. It is therefore logical to argue that one aspect of the transforming activity of these viral oncogenes is the modulation of the Rb growth suppression function.

It was neither through its transduction by oncogenic retroviruses, nor through genetic analysis that *p53* came to light as a player in the cell transformation story. Its discovery came about through direct association with SV40 large T antigen (Lane and Crawford 1979, Linzer and Levine 1979) and the adenovirus type 5 E1B 55 kD protein (Sarnow et al. 1982). The most striking effect of these complexes was to stabilize the *p53* (normally found in low levels with half lives of 6 to 30 minutes) molecule by increasing its half life 100 fold. The observation that *p53* levels were elevated and stabilized in many chemically transformed and tumor derived cell lines (Dippold et al. 1981) led to the logical conclusion that transformation was the result of either the enhancement of function or increased levels of p*53*. This hypothesis was supported by several studies in which the 'immortalization' of rodent cells resulted from overexpression of p*53* (Jenkins et al. 1984, Rovinski and Benchimol 1988) and transformation of primary rodent cells resulted from the cooperation of an activated *ras* gene with p*53* (Eliyahu et al. 1984, Parada et al. 1984). Recent evidence (Finlay et al. 1988, Eliyahu et al. 1988, Hinds et al. 1989) has clearly demonstrated that the wild type sequence of p*53* does not encode an oncogenic protein. Wild type p*53* protein plus activated *ras* will not result in any REF transformants. The original genomic clone of p*53*, derived from a BALB/c mouse library and several p*53* cDNA clones all encode mutations at residue 135 resulting in the substitution of a valine for an alanine and the subsequent activation of the p*53* protein for cooperation with *ras*.

The activated p*53* proteins bind poorly (if at all) with SV40 T antigen; they form complexes with hsc 70 and do not express the conformation dependent epitope recognized by the murine-specific monoclonal antibody PAb 246. These properties suggest that transformation activation arises from a loss-of-function mutation. In the case of SV40 T antigen or E1B protein-wild type p*53* complexes, transformation may result from the sequestering and/or inactivation of the wild type p*53* (Zantema et al. 1985).

In this context, p*53* and the retinoblastoma protein are very similar. SV40 T antigen contains two separate domains, one located between amino acid 105-114 which binds to the retinoblastoma protein and another domain lying between amino acid 272-517 which binds the p*53* protein (Figure 5). Structural and functional studies have shown that these domains are involved with cell immortalization and transformation (Srinivasen et al. 1989). The adenoviruses have analogous domains which are located on the separate proteins E1A and E1B. The association of Rb and p*53* with DNA tumor oncogenes in conjunction with the studies showing the widespread occurrence of Rb and p*53* mutations in human tumors (retinoblastoma, osteosarcoma, small cell lung cancer, ductal breast cancer, stomach cancer and colorectal cancer) strengthens the view that these genes indeed may play a pivotal role in tumor suppression. In a recent model of colorectal tumorigenesis (Fearson 1990) the accumulation of mutationally activated oncogenes (*ras*) coupled with mutational activation of tumor suppressor genes becomes central to the appearance of colorectal tumors. More than 75% of colorectal carcinomas contain deletions in chromosome 17p (the region to which the p*53* gene has been mapped), whereas adenomas infrequently contain such a loss. The coupling of the point mutation of the p*53* gene with the loss of the remaining wild-type allele is a frequent occurrence in colorectal cancers, lung, breast and brain tumors.

Finlay et al. 1989 and Eliyahu et al. 1989 have demonstrated tumor suppressive effects of wild type p*53* by comparing the number of foci recovered from the transfection of REF with either *ras* + p*53*-mutant or *ras* + p*53*-mutant + p*53*-wild type. They also compared *ras* + E1A with *ras* + E1A + p*53*-mutant and *ras* + E1A + p*53* wild type. In each case, inclusion of p*53* wild type dramatically reduced the number of foci. Analysis of the foci which were recovered from the experiments which included p*53*-wt contained mutations similar to those observed in tumors *in vivo*. Such results are supportive of the hypothesis that p53-wt could act as a suppressor of transformation.

## 1.11 Objectives

Much of the research in this laboratory has focused on the role of SV40 genes in the immortalization and transformation of human diploid fibroblasts (Neufeld et al. 1987, Radna et al. 1989). It has been established that (1) the frequency of transformation of human cells with SV40 is significantly lower than that of rodent cells; (2) the majority of transformed clones isolated from human cells transfected with origin defective SV genomes (encoding either wild type or temperature sensitive T antigens) have extended yet limited lifespans; and (3) it is only a rare transformed cell which becomes immortal in contrast to rodent cells in which immortalization is common in most of the SV40 transformants. This latter finding has led to two hypotheses: (1) the A+B hypothesis insufficiently accounts for the phenomena associated with complete cell transformation in human cells and (2) these phenomena might be explained more accurately by a model that requires an additional event (designated C) for immortalization. We speculate that this event involves cellular factors (Figure 10).

Experiments involving the isolation of a series of pre-immortal and immortal cell lines from human cells transfected with an origin defective SV40 genome encoding a temperature sensitive T antigen may provide some insight into the "C" event (Figure 10). The phenotype of the immortalized and transformed cell is referred to as $A^+B^+C^+$ in contrast to the transformed pre-immortal phenotype which is $A^+B^+C^-$. When cells of either of these phenotypes are shifted up to 39°C, the labile T becomes inactive and the cells die. The resultant phenotype in the pre-immortal must be $A^-B^-C^-$ whereas in the immortal cell it may be either $A^-B^-C^-$ or $A^-B^-C^+$. Identification of the $A^-B^-C^+$ phenotype is impossible because without the ($A^+B^+$) function, the cells die. It is possible, however, to supply an "A" oncogene in an attempt to rescue these cells. In an $A^-B^-C^-$ scenario, replacement of the "A" function (becoming $A^+C^-$) will still not rescue the cells because they cannot form colonies. If, on the other hand, the alternative situation exists ($A^+B^-C^+$), colonies can be rescued when provided with an "A" oncogene because they are still immortal. This laboratory favors a model in which T antigen facilitates some stable alteration of a cellular gene which results in the release of cells from the G1 or G2 block.

Our model is not dissimilar to that of Wright, Pereira-Smith and Shay (1989), in which they propose a 2-stage immortalization scheme. M1, which is characterized by loss of mitogen responsiveness, the production of a protein inhibitor of DNA synthesis and arrest in G1 is overcome by the DNA synthesis-stimulating activity of SV40 T antigen. These cells exhibit an extended life span and continue to divide until they reach M2. Unless the cells inactivate M2 they enter 'crisis' and die. It is postulated that M2 involves an entirely independent mechanism limiting proliferation. Inactivation of M2 occurs through a rare mutational event which results in a cell being immortalized. Although it is not thought that SV40 T antigen plays a direct role in the inactivation of M2, the possibility is not excluded that the induction of chromosomal abnormalities may help to inactivate the gene responsible for M2. Immortalization requires persistent expression of both M1 and M2.

The phenomenon of multistep immortalization of human fibroblasts will be addressed in two ways: (1) by examining the effect of polyoma large T antigen on the growth of a normal human diploid fibroblast cell line (HS74), and (2) by infecting or transfecting polyoma large T antigen or E1A 12S protein into a cell line (HAL) previously transformed and immortalized by an origin defective SV40 gene encoding a heat-labile large T antigen (tsA58 SVori$^-$). The subsequent shifting up to the restrictive temperature for T function creates the opportunity for other nuclear oncogenes to rescue the human cell line. In this way, oncogenes with analogous means of immortalization may be identified. In addition, this kind of experiment would also shed light on the role of the cellular factors involved in immortalization and transformation.

Relevant proteins, such as the retinoblastoma (Rb) or p53 protein will be analyzed for their association to SV40LT, polyoma large T antigen or E1A 12S. This system provides a unique opportunity to examine the expression of oncogenes and their associated cellular factors in two situations, (1) at the permissive temperature where they can be expressed without the cell's dependence on them and (2) at the restrictive or non-permissive temperature, whereby expression of the oncogene becomes obligate for the survival of the cell.

# Chapter 2

# Materials and Methods

## Cell Culture Procedures

All human cell lines are grown in DF10 or DF12 medium supplemented with 10% Fetal Bovine Serum (Hyclone). The antibiotics penicillin "G" phosphate and streptomycin sulfate (Hazelton 10,000 U/100ml) are routinely added to the medium. Temperature sensitive cell lines are maintained in a humidified 35°C incubator with 7.5% $CO_2$ atmosphere whereas all other cell lines are maintained at 38.5°C.

If required for selection procedures, DF12 medium + 10% FBS will be supplemented with either HAT ($5x10^{-5}$M hypoxanthine, $5x10^{-6}$M aminopterin, $1x10^{-5}$M thymidine) or G418-Sulfate (GIBCO, #860-1811, total activity 250 $\mu$g/ml for human cells, 1mg/ml for mouse cells). Unless otherwise stated, cells are always retained under selection reagents.

Cells are passaged by brief treatment with trypsin-EDTA mixture (0.5gm/L trypsin and 0.2gm/L EDTA, Hazelton). Following addition of DF12 + 10% FBS, the cells are centrifuged for 5 minutes at 1000 rpm in an IEC clinical tabletop centrifuge.

Cell counts were obtained with Royco Tissue cell counter after dilution in phosphate buffered saline (PBS - 150mM NaCl, 3mM $NaH_2HPO_4$, 7mM $NaH_2HPO_4$, pH 7.4). Cell lines were frozen in Nunc Cryotubes at a minimum concentration of $1x10^6$ cells/vial. The cells are suspended in DF12 + 10% FBS + 10% DMSO and stored either in liquid nitrogen or in a -70°C freezer.

## Isolation of High Molecular Weight Mammalian Cellular DNA
(Small et al. 1982)

Confluent Petri dishes (100mm) are washed once with PBS and scraped with a teflon covered razor blade into 2ml PBS. The cells are centrifuged approximately 5 minutes at 1000 rpm and the supernatant is discarded. The pellet can either be frozen at -70°C or resuspended in 5ml of sterile lysis buffer (50mM Tris pH 7.5, 20mM $NA_2EDTA$, 100mM NaCl). Sodium dodecyl sulfate (SDS) (Bio Rad Electrophoresis Grade #161-0301) is added to a final concentration of 0.1% followed by immediate immersion in a 65°C water bath for 15 minutes. The tubes are cooled to room temperature and freshly prepared Proteinase K (Boehringer Mannheim, 10mg/ml stock) is added to a final concentration of 100 mg/ml, then inverted for thorough mixing and incubated in a 50°C water bath for 48 hours. The cell lysate is then extracted with an equal volume of buffer saturated redistilled phenol containing 0.1% hydroxyquinoline (Sigma #Q3126). The samples are rocked gently for 10 minutes and then centrifuged at 3000 rpm for 15 minutes at room temperature to separate the two phases. The aqueous phase is removed with a wide bore pipette and re-extracted with phenol choloroform:isoamyl alcohol (48:48:2) until there is no visible protein interphase. This is followed by an extraction with chloroform:isoamyl alcohol (24:1). The viscous aqueous phase is transferred to a new tube in which the salt concentration is adjusted to 100mM with 5.0M NaCl. 2.5 volumes of ice-cold 95% ethanol is added. The DNA precipitates as a white mass upon inversion of the tube. The DNA is hooked with an pasteur pipette, washed once in 70% ethanol and then transferred to a new tube containing 1ml to 2ml of sterile TE (10mM Tris pH 7.5, 1mM EDTA). After the DNA goes into solution (this may be hastened by incubation at 37°C), the concentration of DNA is determined by diluting the sample 1:100 in TE and reading the UV absorption at OD 260. (1 OD=50$\mu$g/ml double standard DNA).

### Hirt Extraction (Hirt 1967)
### (Isolation Of Low Molecular Weight DNA)

Confluent 100mm Petri dishes containing mammalian cells are washed 3 times with PBS at room temperature. The PBS is completely aspirated and 1ml of Hirt lysis buffer (0.6% SDS, 10mM EDTA, 10mM Tris pH 7.6) is added to each dish. After a few minutes each dish is swirled until lysis is complete (the dish should look clear). The lysate is added to an Oak Ridge polycarbonate centrifuge tube to which 5M sodium chloride is added to a final concentration of 1M. The tubes are inverted and stored overnight at 4°C. The lysates are centrifuged at 25,000 rpm for 30 minutes at 4°C in a Beckman Type 50 Ti rotor (or 45 minutes at 4°C in a microfuge when using eppendorf tubes) and then transferred to polypropylene centrifuge tubes. The supernatant is extracted once with buffer equilibrated phenol:chloroform:isoamyl alcohol (48:48:2) and once with chloroform:isoamyl alcohol (24:1). The pellet may be redissolved in TE and processed in a similar fashion. Sodium chloride is added to a final volume of 200mM and the DNA is precipitated with 2.0 volumes of ice cold 95% ethanol overnight at -20°C. The DNA is recovered by centrifugation in a Sorvall HB-4 rotor at 15,000 rpm for 45 minutes (or 15 minutes in a microfuge when using eppendorf tubes). The supernatant is discarded, the DNA pellet is briefly dried in a speed vac (Savant) and then resuspended in TE.

### Growth of Bacteria and Amplification of Plasmid
### Amplification in Rich Medium
### (Maniatis 1982, p.88)

In brief, 10ml cultures of *E. coli* HB101 are initiated from either single colonies or bacterial stocks with LB medium containing the appropriate antibiotic. These cultures are gradually scaled up to 500ml cultures to which 170$\mu$g/ml chloramphenicol is added at log phase for amplification of the plasmid.

### Harvesting of Cells and Isolation of Plasmid DNA Cleared Lysate Technique.

Amplified cultures are centrifuged at 40°C in a Sorvall GSA rotor for 10 minutes at 6000 rpm. Pellets (which may be frozen at this point at -20°C) are placed on ice and resuspended in 1ml cold Buffer A (50mM Tris, pH 8.0; 20% sucrose) per 100ml original culture. Transfer the suspension to 12ml Oak Ridge tubes (not more than 4ml to each tube) and add $Na_2$EDTA, pH 8.0 to 50mM while mixing gently. Add freshly made lysozyme stock (10mg/ml in Buffer A) to a concentration of 1mg/ml. Invert this mixture and incubate on ice for 15 minutes. Add an equal volume of Triton lysis buffer (0.2% Triton X-100, 50mM Tris, 50 mM EDTA, pH 8.0) to the suspension which should appear notably viscous. Invert and incubate again for 15 minutes on ice. This suspension is centrifuged in a 50 Ti rotor at 35,000 rpm for 20 minutes at 4°C.

The supernatant should be carefully removed and 25ml aliquots transferred to each 70 Ti polyallomer tube which will also receive 25g CsCl (BRL #5507UB) and 2.5ml of a 10mg/ml stock of ethidium bromide. The tubes are inverted and then centrifuged at 32,000 rpm for 40 to hours at 20°C.

Two bands in the center of the tube are easily visualized under UV light. The plasmid band (lower band) is removed by puncturing the side of the tube with a syringe. The ethidium bromide is removed by repeated (4 to 5 times) extractions with 1-butanol saturated with water until all the pinkish color disappears from the aqueous phase. Dialyze this phase against 2 to 3 changes of TE.

Deproteinize the aqueous phase by extracting twice with buffer saturated phenol:chloroform:isoamyl alcohol (48:48:2) followed by extraction with chloroform:isoamyl alcohol (24:1). Add NaCl to a final concentration of 250mM and 2.5 volumes of ice cold 95% ethanol. Precipitations may be carried out overnight at -20°C or for 20 minutes at -70°C. If using eppendorf tubes, centrifuge DNA at 4°C for 10 minutes in microfuge, decant the alcohol and dry for 10 minutes in a speed vac. Use a minimal amount of TE to resuspend the DNA. Analysis of plasmid DNA for concentration and quality the preparation should be carried out by electrophoresis on a 0.8% horizontal agarose gel using appropriate markers.

### Rapid Small Scale Isolation of Plasmid DNA (Boiling Method)
### (Method of Holmes and Quigley 1981 described in Molecular Cloning 1982, p.366)

In brief, 1.5ml of an inoculated overnight culture is centrifuged, lysed with lysozyme and placed immediately in a boiling bath. The cellular DNA and debris is pelleted in a microfuge. The plasmid is precipitated from the supernatant with 2.5M acetate and isopropanol. The plasmid DNA is centrifuged and dried in a speed vac and resuspended in TE. A portion of this may be analyzed by electrophoresis on a 0.8% horizontal agarose gel.

### $CaPO_4$ Transfection
### (Modification of Graham and van der Eb, 1973)

Recipient cells are plated at a concentration of 3-5x$10^5$ cells/100mm dish 16 to 24 hours previous to transfection.

The calcium-phosphate precipitates of DNA are prepared as follows:

1. Appropriate aliquots (I do not usually exceed 2.5ml) of 2x Hepes Buffered Saline, pH 7.0-7.1 (2x HBS:280mM NaCl, 50mM Hepes [Calbiochem Ultrol Grade #391338], 1.5mM $Na_2HPO_4 \bullet 12H_20$ [Baker #1-3822]) are added to sterile 13ml plastic Falcon tubes. The 2x HBS is prepared from 10X stocks which may be frozen at -20°C for future transfections.

2. A 2X DNA-$CaCl_2$ solution is prepared in the following manner. A 20x stock of $CaCl_2$ (2.5 M $CaCl_2 \bullet H_20$ Mallinkrodt #4160) is diluted 1:10 in TE to give a final concentration of 250mM $CaCl_2$. This solution should include and account for the DNA. Each 1ml precipitate should have an absolute DNA concentration of 10$\mu$g to 20$\mu$g. Generally I include 10$\mu$g of carrier (salmon sperm DNA) with varying amounts of plasmid vector. I add the 20x $CaCl_2$ to the TE followed by the DNA.

3. The 2x DNA-$CaCl_2$ solution is mixed with an equal amount of 2X HBS before 1ml aliquots of the precipitate are added to each 100 mm dish. Using 2 automatic pipettors, air is bubbled into the tube containing the 2x HBS while an equal amount of the 2x DNA-$CaCl_2$ solution is added in a dropwise fashion. (Continue to bubble in air

to insure an even and fine precipitate. Allow the tubes to stand for 30 minutes before adding the precipitates to the dishes.)

4. Gently resuspend the precipitates and add 1ml dropwise over the surface of the medium in the Petri dishes.

5. Reincubate the cultures for a minimum of 3 hours at the appropriate temperature in a $C0_2$ incubator. The amount of time will vary with the cell line; some cell lines will tolerate overnight exposure.

6. Aspirate the medium containing the precipitates and re-feed the cultures with fresh medium. Selection is generally begun 48 to 72 hours after transfection. (See tissue culture procedures).

### Retroviral Infection
(Jat 1986)

The producer cell lines are grown to 90% confluency in 100mm dishes. The medium is removed and replaced with 5ml (1/2 usual volume) of DF12 + 10% FBS. After no more than 12 hours, the medium is harvested by passage through a $.45\mu$ filter (S&S). The filtered medium is used immediately for infection, although freezing at -70°C is possible.

Two ml of medium containing retrovirus and $8\mu g/ml$ polybrene (Aldrich) are used to infect recipient cells which have been plated the night before at a density of $3 - 5x10^5$ cells per 100mm dish. The medium from each dish of recipient cells is aspirated, replaced with the 2ml of retrovirus and incubated at the appropriate temperature for two hours with occasional rocking. Eight ml of complete medium is then added to each dish. Forty-eight hours after infection, the dishes are split at 1:2 and 1:20 into selection medium containing 300$\mu$g/ml G418. Visible colonies are apparent 14-21 days after infection. Individual colonies are picked with cloning cylinders or multiple colonies are pooled and grown to mass culture.

## DNA Analysis

All manipulations of plasmids (see Table 1) were carried out by standard procedures or under the recommended conditions of the supplier.

Restriction digests of plasmid and high molecular weight DNA were monitored by horizontal gel electrophoresis (Maniatis 1982). Transfer of the DNA to nitrocellulose filters was performed according to the method of Southern (1975) as described in Current Protocols (1987). Subsequent hybridization and autoradiography of Southern filters was accomplished by standard procedures (Stahl et al. 1979). $^{32}$P-labeled probes were made according to the specifications included in the Random Primed DNA Labeling Kit (BMB #1004-760, Feinberg and Vogelstein 1983).

## Random Primed DNA Labeling
(Feinburg and Vogelstein 1983)

Briefly, 50ng of heat denatured DNA is mixed with dATP, dGTP, dTTP (0.5 mmol/l each), 10x reaction mix and 50$\mu$Ci [$\alpha^{32}$P] dCTP. Klenow enzyme (DNA polyermase I) is added before incubation at 37°C. After 30 minutes the reaction is stopped and loaded onto an STE equilibrated quick spin column (BMB #100408) of G-50 Sephadex. The activity of the recovered probe is determined by scintillation counting.

## Recovery of DNA from Agarose Gels
## (Elutip-d, S&S)

In brief, the DNA of interest is electrophoresed and then sliced out of a low melting agarose gel (LMP agarose BRL). While being incubated at 65°C in low salt buffer, the Elutip-d column is prewashed with high salt buffer followed by warm low salt buffer. The melted DNA sample is slowly pushed through the syringe followed by a wash with warm low salt solution. The DNA is eluted from the column with the high salt solution to which cold ethanol is added in order to precipitate the DNA. After incubation at -70°C, the DNA is pelleted, washed with 70% ethanol and recentrifuged. The pelleted DNA is dried and redissolved in TE buffer.

## Immunoassays

Mammalian cell extracts were prepared from 100mm dishes which had been seeded at 35°C. In a typical experiment, 1-2x$10^6$ cells were inoculated and either maintained at 35°C or shifted to 39°C for 3 to 4 days. Extracts were typically prepared directly on the dish at 4°C using 0.5 to 1ml of lysis solution containing 120mM NaCl, 0.5% NP40, 50mM Tris-HCl, pH 8.0 and a mixture of protease inhibitors including PMSF (0.15mM), leupeptin (0.3 mg/ml), $\alpha 2$ macroglobulin (0.1 mg/ml) and aprotinin (0.1 TIU). The disrupted cells were harvested by scraping with a rubber policeman and centrifuged in a microfuge for 15 minutes at 4°C. The supernatant was routinely used directly for further analysis.

Extracts containing approximately 1-2mg protein were immunoprecipitated by the addition of the appropriate monoclonal antibody (see Table 2) and sepharose conjugated

A protein (Radna et al. 1989). In the case of Rb-1, an additional incubation with rabbit anti-mouse IgG was included. The immunoprecipitate was washed 5 times in buffer containing 100mM NaCl, 0.5% NP40, 1mM EDTA, 20mM Tris-Hcl, pH 8.0 and the protease inhibitor mixture, and dissolved by heating in a boiling water bath (100°C, 1 minute) in 50$\mu$l of buffer containing 6% SDS, 250mM NaCl, 2.5M mercaptoethanol, 20% glycerol, and bromphenol blue. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as described in Current Protocols (Laemmli 1970, Towbin et al. 1979). Viral and cellular proteins were then detected by appropriate monoclonal antibodies and either affinity purified goat anti-mouse IgG conjugated with horseradish peroxidase or alkaline phosphatase obtained from Boehringer-Mannheim and used according to specifications provided by the supplier. Extracts were analyzed by use of the following mouse monoclonal antibodies: SV40 T antigen was assayed by PAb 419, PyLT by F4, adenovirus E1A by M73, Rb-1 by C36 and p53 by PAb 421; PAb 210E8 against the *E. coli* RNA polymerase $\beta$ subunit was used as a negative control.

## Protein Extracts
(Ito et al. 1977)

Confluent 100mm Petri dishes are placed on a level metal tray which is sitting in a large pan containing ice. The medium is aspirated and the dishes are washed once with cold PBS. The cells of each dish are scraped using a teflon coated razor blade into 2ml of cold PBS. The cell suspension is centrifuged in a 15ml conical centrifuge tube at 1000 rpm for 5 minutes at 4°C. The supernatant is discarded and 150$\mu$l of cold extraction buffer (20mM Tris-Cl pH 8.0, 80mM NaCl, 20mM EDTA) containing freshly added PMSF and DTT (final concentrations of 2mM and 1mM, respectively) is added to a pellet of 5X$10^6$ cells. Generally two confluent dishes yield 2-3x$10^7$ cells or a pellet of approximately 0.1ml to which is added 1ml of extraction buffer. The extraction mixture is frozen and thawed 3 times. The mixture is transferred to an eppendorf tube and centrifuged in a microfuge at 12,000 rpm for 15 minutes at 4°C. The extract is decanted off and 50$\mu$l aliquots are either used immediately or kept frozen at -70°C.

# Chapter 3

# RESULTS

## The Effect of PyLT Antigen on the Growth of HS74 Cells

### 3.1 Construction of pPyLT-S

The early phase of this project focused on effecting a change in the growth of human fibroblasts by the introduction of polyoma large T coding sequences into HS74 cells. These human fibroblasts of bone marrow origin have a well documented limited lifespan (Neufeld et al. 1987). They have a modal number of 46 chromosomes including two X chromosomes which show no obvious abnormalities by karyotypic analysis. They are non-refractile to the standard Ca-$PO_4$ transfection assay thus making them a suitable candidate for these studies. Using an approach which proved to be successful for the introduction of origin-defective SV40 into HS74 (see Small et al. 1982 and Neufeld, Ph.D. Dissertation 1986) a series of experiments has been completed in which various polyoma constructs were co-transfected with pRSV-neo into HS74.

Three plasmids were examined: pPyLT1 which expresses polyoma large T coding sequences under its own regulatory control (Rassoulzadegan et al. 1982), pPSVE1-Bla which expresses all three polyoma T antigens under the control of SV40 regulatory sequences (Muller et al. 1984) and pPyLT-S which expresses polyoma large T antigen under the control of SV40 regulatory sequences. Based on evidence from de Villiers et al. 1983, replacement of the enhancer sequences of a polyoma plasmid recombinant with that of SV40 becomes 400% more efficient in the expression of polyoma large T in primate cells. In order to circumvent the species limitation of the polyoma enhancer, a plasmid (pPyLT-S) containing a hybrid transcription unit was constructed (see Figure 11). This unit is composed of the SV40 promoter fused to the early region of polyomavirus. The polyoma early region contains a precise deletion of the large T intron sequences, thus precluding expression of polyoma middle and small T antigens and allowing only the expression of large T antigen.

Three plasmids were used to achieve this construction. The first KP45 is a 2.6kb plasmid derived from pBR322 which contains a substantial deletion between base pairs 676 and 2365 and contains multiple cloning sites in the polylinker region (See Figure 12). The second plasmid pPSVE1-Bla (Figure 13) was also generated in pBR322. It contains the entire polyomavirus early region under the control of the SV40 early promoter. In order to maximize expression of the polyoma virus T antigens, the binding sites for polyoma large T (nucleotide positions 147 and 153) involved in repression of early transcription were omitted. The HindIII C fragment of SV40, containing all the viral regulatory elements including the origin for DNA replication, early and late promoters and early start sites for transcription has been fused to the polyoma early region at nucleotide position 154. This plasmid synthesizes all three polyoma T antigens. The third plasmid pPyLT1 (Rassoulzadegan et al. 1982)(Figure 14) is a recombinant plasmid which contains a modified polyoma virus genome under its own regulatory sequences cloned into the BamHI restriction site of pAT153. Restriction fragments encompassing the large T intron (410-794) have been removed from the full length genomic DNA and have been replaced with the corresponding shorter fragment from the cDNA clone. Its coding capability is restricted to only the large T antigen.

The strategy for achieving the construction of pPyLT-S is the following. First, to clone a large restriction fragment from pPSVE1-Bla which includes the polyoma large T intron into KP45, which will serve as an intermediate vector. Second, substitute for the correspondingly shorter fragment from pPyLT1. Third, this large restriction fragment, which now contains the appropriate deletion of the large T intron, will be isolated and cloned back into pPSVE1-Bla. This new plasmid is termed pPyLT-S.

Construction of the first intermediate vector. The first intermediate construction (pLars1, see Figure 15) is generated as follows. First, pPSVE1-Bla (9.4kb) is digested with EcoR1 and BamHI to generate two fragments which are gel purified by electroelution, A (6.5kb) and B (2.9kb). Second, KP45 (2.6kb) is digested with EcoR1 and BamHI to yield a 2.3kb and a 0.3kb fragment. Third, the 2.3kb fragment from KP45 (containing the origin of replication and ampicillin resistance gene) is ligated to the B fragment (2.9kb) of pPSVE1-Bla. The resulting plasmid, pLars1, contains two AccI sites, both in the polyoma sequences.

Substitution of shorter fragments. pPyLT-1 is cut by AccI six times to release five resolvable fragments (see Figure 16). The 748bp AccI fragment spans nucleotides 369 to 1500 in the polyoma DNA encompassing the large T intron deletion between nucleotide 410-795. Gel purification of this fragment was facilitated by including BglI in the AccI digest to remove the fragment of closest proximity to the 748bp fragment and then electroeluting this band of interest.

The corresponding fragment (1130bp) in pLars1, which includes the large T intron, is then excised by digestion with AccI. The remainder of the plasmid (4.1kb) is gel purified and ligated to the 748bp AccI fragment from pPyLT-1. Several transformant colonies are screened with four enzymes. EcoRI and BamHI are included as linearizers, AccI is used to confirm the presence of the 748bp fragment and BamHI and AvaI are used to determine the orientation of the 0.748kb fragment. The correct orientation yields two fragments whose sizes are 2.31kb and 2.56kb. The plasmid meeting these criteria (pLars24 - 4.87kb) is grown up and amplified in *E.coli* (see Figure 17).

Isolation and cloning of the EcoRI-BamHI Fragment. The purified DNA from pLars24 is cut with EcoRI and BamHI to release the modified insert from the KP45. The two fragments obtained are 2.55kb and 2.3kb respectively, the former being the modified insert of interest. An additional digestion with PvuI cuts only the 2.3kb vector fragment making the EcoRI-BamHI fragment more accessible for gel purification.

Fragment A, the 6.5kb EcoRI-BamHI fragment of pPSVE1-Bla is then ligated to the 2.55kb EcoRI-BamHI fragment from pLars24, generating pPyLT-S (Figure 11). Eight colonies are picked for enzyme analysis and comparison to pPSVE1-Bla. Linearization with EcoRI or BamHI yields a single band of approximately 9.05kb, which runs just below the linearized molecule of pPSVE1-Bla. AvaI cuts once in the plasmid sequences and once in the fragment derived from pPyLT1 to yield 6.0kb and 3.0kb fragments. Because the large T intron also contains an AvaI site, the pPSVE1-Bla plasmid has an additional fragment.

Digestion with AccI yields five fragments: 4.4kb, 1.59kb, 2.0kb, 748bp and

309bp. When pPSVE1-Bla is cut with AccI, it releases the same sized fragments with the exception of the 748bp fragment. This AccI fragment is 1.13kb, 385 base pairs larger (accounting for the large T intron) than its counterpart in pPyLT-S.

The last diagnostic enzyme used is PvuII which cuts once in the vector and three times in the insert to yield four fragments: 4.4kb, 2.88kb, 947bp and 888bp. Only the 947bp fragment differs in pPSVE1-Bla. This 1.3kb fragment is also 385 base pairs larger than its counterpart.

The plasmid meeting these criteria (pPyLT-S) is, therefore, similar to pPSVE1-Bla in all respects except that it contains a deletion of base pairs 410-795 in the early region rendering it incapable of making middle T or small T. To test for the ability of pPyLT-S to make a functional large T, a replication assay is performed which entails the co-transfection of permissive mouse 3T3 cells with pPyLT-S and pJLPYO, a 2.68kb plasmid containing the origin of replication for polyoma. As a positive control for large T expression, pPSVE1-Bla and pJLPYO are also tested. After 48 and 72 hours, low molecular weight plasmid DNA is isolated by the Hirt extraction procedure (see Materials and Methods). The DNAs are cut with DpnI and SalI. The input DNA is methylated at the adenine residues which lie within the sequence GATC. These sequences will be recognized and cleaved by DpnI. If a functional T is being made, pJLPYO will be able to replicate in the mouse cells. It will be resistant to DpnI digestion because it is no longer methylated. SalI is used to convert all replicating forms into a single unit length DNA. Following electrophoresis and Southern blot hybridization, the linear form of pJLPYO is evident when it is co-transfected with either pPyLT-S or pPSVE1-Bla (Figure 18). Separate transfection of these plasmids does not result in the replication of their respective DNA molecules. Taken in combination, the results of the restriction enzyme analysis and the replication assay indicate that pPyLT-S contains a deletion of the large T intron and that it is capable of synthesizing a functional large T antigen.

### 3.2 Comparison of Py and SV40 Promoters in HS74
### Transactivation of an Exogenous Promoter by Py.

In order to evaluate the effects of pPyLT-S expression in human cells, we exploited an assay system which was successful with SV40 large T antigen. These assays involved either the transfection of HS74 whereby foci formation is the outcome or the co-transfection of HS74 with a plasmid conferring drug resistance whereby growth in selective medium results in colony formation. Although we would not expect morphological transformation to result from the transfection of PyLT into HS74, focus production was nonetheless compared amongst pPyLT-S, pPyLT-1 and pPSVE1-Bla in HS74. No foci were observed in any of these transfections, a result which was expected for pPyLT-S and pPyLT-1 but somewhat surprising for pPSVE1-Bla since transformation might occur with polyoma large and middle T antigens.

Transfection of HS74 cells with pRSVneo has been demonstrated (Litzkas et al. 1984) to yield G418$^R$ colonies at a frequency of 1 in $10^6$ cells. In contrast, transfection of HS74 with pRNS-1 (pRSVneo containing SV40 origin defective (ori$^-$) DNA inserted into the BamHI site) will yield 10-30 times more G418$^R$ colonies. The observation of a transactivating effect of SV40 large T led to an attempt to demonstrate a similar effect of PyLT. If such an effect could be shown, it would be possible to determine whether there is a difference between polyoma expression under its natural promoter or under the promoter of SV40.

Table 5 summarizes the results from a series of experiments typified by the transfection of HS74 with 2μg/ml pRSVneo and the transfection of HS74 with increasing concentrations (2μg, 5μg, 10μg) of pPyLT-1 or pPyLT-S together with 2μg of pRSVneo. All transfections are followed by selection in medium containing 250μg/ml of G418 for three weeks. As previously observed, transfection with pRSVneo yields 1-2 G418$^R$ colonies/10$^6$ cells. Those dishes receiving from 2 to 10μg of pPyLT-S and pRSVneo resulted in G418$^R$ colonies, whereas no colonies were observed in dishes which were co-transfected with pRSVneo and pPyLT-1. These results confirmed the expectation that the polyoma promoter is less effective in human cells.

By co-transfecting 2μg/ml pRSVneo with 10μg pPyLT-S, the number of G418$^R$ colonies increases to 30/10$^6$ cells. An equivalent number of colonies can be achieved with pRSVneo alone when the concentration of this plasmid is increased to 10μg/ml. It was apparent from such an experiment that the PyLT protein was responsible for the transactivating effect on the expression of the exogenous neo promoter.

## 3.3 Fate of G418$^R$ Colonies

Since the presence of polyoma large T antigen greatly influences the number of G418$^R$ colonies, there is some assurance that after co-transfecting low concentrations of pRSVneo (1-2μg) with appropriate amounts of pPyLT-S, the surviving colonies contain, at least initially, a functional large T.

The morphology of the G-418$^R$ colonies resulting from co-transfection with pPyLT-S appeared flat and untransformed, similar to HS74 cells. Fifty colonies were picked by cloning cylinders. Although growth in medium and G418 appeared vigorous at the onset, all 50 colonies underwent crisis and died. They retained a healthy fibroblast appearance for a few weeks, but eventually resembled a typical senescent population, i.e. large, multi-nucleated and vacuolating cells. These colonies survived no longer than control colonies which had been transfected with RSVneo alone. Table 6 illustrates the lifespan of colonies picked from two typical experiments. Therefore, it appears that polyoma large T antigen does not extend the lifespan of normal human fibroblasts.

Co-transfection into HS74 poses some severe limitations in this analysis. It is necessary to determine whether or not the non-selectable plasmid (pPyLT-S) becomes integrated into the cell and continues to be expressed or if its presence is just transient. This task was not possible because death precludes all efforts to gather enough cells for DNA purification, protein extraction or frozen storage.

One of the limitations of the Ca-$PO_4$ technique is that few cells actually incorporate the transfected DNA. An optimal experiment yields the maximum 15 G418$^R$ colonies per $5x10^5$ cells. Additionally, each dish requires $10\mu g$ of pPyLT-S + $2\mu g$ pRSVneo making each experiment expensive in terms of the amount of DNA used. Retroviral mediated DNA transfer was chosen as an alternative for the following reasons: (1) it promised to be a highly efficient gene transfer system; (2) both dominant markers and non-selectable markers are expressed through strong retroviral transcriptional signals; (3) the selection of the dominant marker ensures retention of the non-selectable marker; (4) the integration of the vector takes place at the LTR's, thereby ensuring that there will not be disruption in the coding sequences for the genes of interest.

The next four sections offer a detailed description of the retroviral system: Section 3.4 describes the packaging cell line system; Section 3.5 describes the retroviral vector and Section 3.6 discusses generation of the producer cell line.

### 3.4 Packaging Cell Line

PA317 (American Type Culture Collection #CRL 9078) is an amphotropic packaging cell line generated by co-transfection of NIH3T3 $TK^-$ cells with the herpes simplex virus thymidine kinase gene (a BamHI fragment in pBR322) and the packaging construct, pPAM3, a derivative of pPAM[4]. The PAM packaging construct is the product of a systematic series of deletions made in the replication competent amphotropic retrovirus AM-MLV. This entails a deletion of the retroviral packaging signal, all of the 3' LTR, part of the 5' LTR and the site for second-strand DNA synthesis (See Figure 19). These deletions obviate packaging, reverse transcription or integration of any virus transcribed from pPAM3. PA317, the cell line to emerge from this co-transfection, contains a non-transmissible retrovirus which provides trans-acting factors that are required for the packaging and transmission of retroviral vectors. It can produce viral vector titers as high as $10^6$ to $10^7$ colony forming units ("CFU")/ml when transfected with recombinant retroviral vectors. These viruses are capable of infecting mouse, rat, cat, dog and human cells.

For the purpose of generating a producer cell line capable of making virus that

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[4] For a complete description, see Miller and Buttimore 1986.

expresses the polyoma large T antigen, the plasmid pZIPNeoSV (PyLT)1 (Jat et al. 1986) was chosen for transfection into PA317.

## 3.5 Retroviral Vector

pZIPNeoSV(x) (Cepko 1984)(See Figure 20) contains a unique Bam site into which the BglI-BamHI fragment (nucleotides 87 to 4633) of a recombinant polyoma plasmid (pPyLT) was cloned. pPyLT (Rassoulzadegan et al. 1982) contains a deletion of the large T intron (bp 410-794) rendering it incapable of expressing either small or middle T antigens and thereby enabling unique expression of large T. The pZIPNeoSV(x) vector consists of a Moloney murine leukemia virus ("M-MuLV") transcriptional unit derived from an integrated M-MuLV provirus (Hoffmann et al. 1982) and pBR322 sequences necessary for the propagation of the vector DNA in *E.coli*. The M-MuLV sequences retained in the vector include the long terminal repeats (LTR's), sequences necessary for the reverse transcription of the viral genome, sequences for the encapsidation of viral RNA (Mann et al. 1983) and 5' and 3' splicing signals involved in the generation of the subgenomic viral env RNA. The unique restriction sites (BamHI and XhoI) were inserted in place of the sequences encoding the gag-pol and env polypeptides. Sequences encoding G418$^R$ in mammalian cells and kanamycin resistance in *E.coli* as well as sequences encoding the SV40 and pBR322 origins of replication have been introduced into the XhoI site. The pZIPNeoSV(PyLT)1 vector makes use of the strong retrovirus transcriptional signals to promote the expression of both dominant selectable markers (neo) and non-selectable cDNA sequences (polyoma LT).

## 3.6 Generation of Producer Cell Line

Highly transmissible virus carrying the gene for polyoma large T antigen is generated by transfection of pZIPNeoSV(PyLT)1 into PA317 cells and selection in G-418. 10$\mu$g of vector are transfected according to the standard calcium phosphate procedure of Graham and van der Eb (1973) into recipient PA317 cells which are grown at 37°C and maintained in DF12 + 10% FBS + HAT medium. PA317 cells are seeded at a density of $5x10^5$ cells/100mm dish. Three hours later, the medium is aspirated and replaced with DF12 + 10% FBS + HAT medium. Forty-eight hours later, selection is begun with complete medium containing 1mg/ml G-418. Colonies are picked with cloning cylinders and grown up to mass culture. The medium of each producer cell line is screened for infectivity with the retrovirus infection protocol that appears in Materials and Methods. These lines are maintained in selective medium containing HAT and G-418.

## 3.7 Infectants for the Expression of Polyoma

Figure 21 illustrates the generation of HAL infectants. When PA317 cells were seeded at a density of $1x10^6$ cells/dish and transfected with pZIPNeoSV(PyLT) at a concentration of 10$\mu$g/ml, 30-100 $G418^R$ colonies/dish were generated. Ten colonies (C1-C10) and five pooled dishes (1-5) were chosen for analysis. When maintained in selective medium containing both G-418 and HAT, all of the producer cell lines expressed polyoma large T. Each pooled dish or picked clone was expanded and grown up for infection of HAL cells, a human cell line chosen to be the control recipient. (Although a description of these cells is provided in the following chapter, for purposes herein HAL cells are human fibroblasts which have been conditionally transformed by an origin defective SV40 genome encoding a temperature sensitive large T antigen). Infection with cell-free medium ("supernatants") from producer cell lines (C1-C10) generated from single picked clones resulted in G-418$^R$ HAL cells which also expressed polyoma large T. Infection using supernatants from pooled producer cells did not result in the expression of polyoma large T by any of the G-418$^R$ cell lines. The CFU's of six producer cells lines (C1, C3, C7, C8, C9 and C10) are shown in Table 7. They ranged from 0.5 CFU/ml to 1000 CFU/ml. The virus containing medium from C9 was used to simultaneously infect HS74 and HAL cells. Although 750 colonies were obtained with HAL cells as expected, none were obtained in HS74 cells. Since I was unable to improve the frequency of HS74 cells expressing polyoma large T, it therefore seemed logical to switch the focus to the effect of polyoma large T antigen in HAL cells.

# Chapter 4

## Results

### 4.1 Description of HAL Cells

HAL is a transformed and immortal human cell line which makes a temperature sensitive SV40 large T antigen. Its parent cell line SVtsA/HF-A (Radna et al. 1989) was isolated from a transformed focus resulting from the transfection of HS74 with an origin defective SV40 tsA58 genome (pSVtsA). These cells are temperature dependent for growth as confirmed by the absence of colony formation or repeated passage of mass cultures at 39°C. Immortal SVtsA/HF-A cells show a depletion of S phase cells and an accumulation of G2 arrested cells at the restricted temperature as demonstrated in cell cycle studies by flow cytometry (Ozer and Traganos, unpublished data). This is consistent with an absence of increase in cell number and an inhibition of DNA synthesis as measured by [$^3$H] thymidine incorporation. These cells express an immunoreactive large T (and small t) antigen at both 39°C and 35°C. However, pSVO (a plasmid containing a functional SV40 origin) is unable to replicate in SVtsA/HF-A (Radna et al. 1989) or HAL cells which have been shifted to the restrictive temperature (39°C), whereas, it replicates well in cells which remain at 35°C, thereby demonstrating that large T becomes non-functional at 39°C.

HAL cells can be rescued at the restrictive temperature by transfection with pRNS-1, a plasmid encoding wild type SV40 large T antigen and G-418 resistance. In such an experiment, G418$^R$ colonies are either picked or pooled at 35°C. 5x104 to 2x10$^5$ cells are generally seeded per 60mm dish at 35°C and subsequently shifted to 39°C or maintained at 35°C. The cell number is determined for duplicate dishes at both 35°C and 39°C at 24 hour intervals for one week. Figure 22 illustrates the temperature dependent growth of non-transfected HAL cells at 35°C and 39°C. When HAL cells were transfected with pRNS-1, cultures containing multiple colonies grew well at both temperatures. When individual colonies of transfected cells were tested, approximately 50% of the picked clones restored growth at 39°C. Figures 23 and 24 are growth curves of two different G418$^R$ clones picked at 35°C from a dish of HAL cells transfected with pRNS-1.

The use of HAL cells may provide practical as well as insightful advantages over HS74. Their continuous growth at 35°C obviates the problem of senescence occurring before analysis. These cells can be characterized at 35°C and then shifted to 39°C to assess the effect of the polyoma sequences. Cells which can be rescued at 39°C can be analyzed without the complication of a functional SV40 T antigen.

## 4.2 Producer Cell Lines

The HAL infectants (HC1, HC3, HC7, HC8, HC9, HC10) which resulted from the infection at 39°C by supernatants from the producer cell lines C1, C2, C7, C8, C9 and C10 (refer to Figure 21) were maintained as separate pooled cultures in 300$\mu$g/ml G-418. Three infectants, HC1, HC9 and HC10, were positive for the expression of polyoma large T antigen by western immunoblot analysis with pAbF4.[6] The left-hand side of Figure 25 is a composite immunoblot of producer cell lines and HAL infectants

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[6] The antibody (F4) used in this assay reacts with the NH3 terminus of the polyoma protein. It shows no cross-reactivity to the SV40 large T antigen.

which were screened for PyLT antigen expression. Lanes 4 and 5 contained protein extracts from two producer cell lines, C9 and C7 respectively. Protein extracts from their HAL infectant counterparts (HC9 and HC7) are shown in lanes 8 and 7 respectively. Unfortunately, infectants of the producer with the highest titered retrovirus (C7) did not make an immunoreactive large T antigen (lane 4). HC9 (lane 8) produced two species of polyoma large T, one which was full sized and one which was slightly truncated. Five single colonies were picked and grown up from low density cultures of HC9. Four of the five tested expressed the truncated polyoma large T protein. When protein extracts of HC1 (lane 2) and HC9-1 (lane 3) were compared to extracts prepared from PyT54 cells (a hamster cell line which contains an integrated copy of the polyoma early region)(lane 1) only HC1 produced solely a 100kD polyoma large T antigen which co-migrated with that of PyT54. HC10-7 also expressed a truncated polyoma large T protein. It is interesting to note that although all of the mouse producer cell lines made a full sized polyoma large T protein, corresponding HAL infectants did not necessarily make the same sized protein. The right-hand side of Figure 25 is an immunoblot for SV40 large T antigen in HAL cells (lane 12) and HAL infectants HC9 (lane 11), HC7 (lane 13) and HC8 (lane 14), verifying the quality of the extracts themselves.

## 4.3 Growth Characteristics

When HAL cells are seeded ($5 \times 10^4$ cells per 60mm dish or $2 \times 10^5$ cells per 100mm dish) at 35°C and subsequently shifted up to 39°C at day 3, no colonies are recovered. Without the presence of a functional large T antigen to maintain the transformed/immortalized phenotype, these cells 'senesce' and die. Therefore, to observe the effect of polyoma large T antigen under conditions in which the SV40 large T antigen is inactive, HC1-1, HC9 and HC9-1 were seeded at $2x10^5$ per 100mm dish at 35°C. After 48-72 hours, these dishes were shifted to the restrictive temperature for 14-21 days. No colonies have been rescued from such temperature shift experiments. No colonies have ever been observed involving direct selection of infectants at 39°C. Direct selection involved first infecting HAL cells at 35°C with the supernatant from the producer cell line (C9). Then, in conjunction with or following the addition of G418 (days 3, 7 and 21), these cultures were shifted to 39°C. The difference between direct and indirect selection is that in indirect selection stable $G418^R$ colonies are first isolated at 35°C, grown up and then tested for growth at the restrictive temperature. It should be noted that experiments involving direct selection at 39°C have not been uniformly successful even when transfecting a plasmid encoding wild type SV40 antigen (pRNS-1).

The efficiency of colony formation ("EOC") for HC9 and HC1-1 at 35°C is 0.4% and 0.2% respectively, i.e. 200 colonies per $5x10^4$ cells and 100 colonies per $5x10^4$ cells per 60mm dish. In parallel experiments HAL cells have an EOC of 0.8-1.0%, i.e. greater than 300 colonies per 60mm dish.

Figures 26, 27 and 28 illustrate the growth of HC9, HC9-1 and HC1-1 at 35°C and 39°C. Typically, each cell line is seeded at a density of $5x10^4$ cells per 60mm dish and is incubated at 35°C. Duplicate dishes are counted for each time point. Companion dishes are shifted up to 39°C at day 3 or when the cells reach a density of $1x10^5$ per dish. Thereafter, cell counts are determined every 48 hours in duplicate dishes at both temperatures. The saturation density is determined one week after the dishes reach confluency. The saturation density per 60mm dish for HAL, HC1-1 and HC9 was $7.25 \times 10^6$, $5 \times 10^6$ and $7.5 \times 10^6$ respectively. The doubling time at 35°C is 48 hours. A steady decline in the number of cells and an increase in the doubling time is evident at 39°C in all three cell lines. Despite the fact that an abundant amount of polyoma protein exists in these cells, the polyoma large T antigen does not restore growth at 39°C.

### 4.4 Analysis of HAL Infectants by Southern Blotting

High molecular weight ("HMW") DNA samples were prepared from the following: (1) producer cell lines which expressed polyoma large T antigen (P3) and their respective infectants which did not express polyoma large T (HP1, HP3); (2) producer cell lines which expressed polyoma large T antigen (C9) and their respective infectants which expressed inappropriately sized polyoma large T (HC9, HC9-1, HC9-2) and (3) the infectant expressing a full sized polyoma large T antigen (HC1-1).

The DNA samples (10$\mu$g) are either uncut, digested with BamHI which should release the polyoma large T insert (4.2 kb), or digested with XbaI which should result in a 6.6kb and a 2.8kb fragment containing polyoma sequences. XbaI cuts once in the LTRs and twice in the middle of the polyoma sequences. A restriction enzyme digest of the vector pZIPNeoSV(PyLT) (Figure 29) demonstrates that the input DNA contains the expected BamHI, XbaI and HindIII sites.

1) Figure 30 is a 3 hour exposure of BamHI and XbaI digested samples of P3, HP1, HP3 probed with a full length polyoma DNA. The producer line P3 shows the expected 4.2kb BamHI insert. Neither of the infectants HP3 or HP1 had this band. Instead they had bands of 6.0kb and 5.0kb, respectively. As expected, cutting with XbaI

generated the 6.6kb and 2.8kb fragments in the producer line but did not do so in HP3 or HP1. XbaI generated 6.0kb and 4.0kb fragments in the case of HP1 and 5.0kb and 3.5kb fragments in the case of HP3.

There are two conclusions to be drawn from this experiment. First, polyoma sequences are present in the infectants. However, the protein is not immunoreactive with the antibody (F4) used in the western blotting consistent with the absence of the expected restriction sites and a truncated protein missing the immunodeterminants. Second, the amount of DNA detected in the lanes of HP3 and HP1 far exceeded the quantity of the DNA seen in the lane of the P3, the producer line. This observation suggested that extrachromosomal sequences were being generated in the infectant lines.

This interpretation is demonstrated to be the case by the results shown in Figure 31, in which 2$\mu$g and 10$\mu$g uncut samples of P3, HP3, HP1, and HP4 DNA are electrophoresed, blotted and probed for polyoma. The producer line P3 (lanes 1 and 5) contains no free forms, whereas all the infectants contain at least one species of DNA, differing in its molecular weight for each of the three infectants.

2) Figure 32 compares BamHI digested and uncut DNA samples of the producer mouse cell line C9 with HC9, its matched HAL infectant and subclones of that line, HC9-1 and HC9-2. C9 contains no low weight molecular weight DNA in the uncut sample and releases the appropriate 4.2kb BamHI fragment. HC9 contains two predominant species of free polyoma DNA ($\approx$5.0kb and 6.0kb), one of which (5.0kb) is shared with HC9-1 and HC9-2. Cutting with BamHI produces two bands in HC9, $\approx$5.0kb and $\leq$1.5kb. There is some hint of a 4.2kb fragment. The pattern in HC9-1

and HC9-2 is the same in both BamHI digests, an approximately 5.0kb fragment and a slightly larger 5.5kb fragment. The faster mobility of the uncut DNA (lanes 2-4) indicates that the amplified sequences are likely closed circular structures (form I).

3) When the infectant HC1-1 is analyzed (see Figure 33), the 4.2kb fragment is seen in the BamHI digest (lane 12). Other bands are also seen (5.25kb, 3.0kb, 2.0kb) although in lesser quantities. The lane containing the uncut sample (lane 6) reveals several species of free DNA. When low molecular weight DNA samples (Hirt supernatants) are prepared (see Figure 34), the uncut and BamHI patterns of HC9, HC9-1, and HC1-1 (lanes 2, 8 and 5 respectively) are the same as was seen in their HMW preparations, the Hirt pellets (lanes 11, 10 and 12). This observation suggests that the bulk of the polyoma protein is coming from the extrachromosomal sequences and not the integrated vector.

The following observations are offered in summary: (1) The producer lines do not contain free polyoma DNA sequences, but do contain integrated copies of the vector which give a characteristic 4.2kb band upon cutting with BamHI. (2) The infectants have a considerable amount of polyoma DNA sequences, suggesting replication of the infectant DNA consistent with the presence of an SV40 origin in the retrovirus construct and SV40 large T antigen in the HAL cells. (3) Although there are several different sizes of such amplified DNA in these infectants, the 5.0kb species is the favored size. (4) Infectants which do not make an immunoreactive polyoma large T contain a variety of amplified DNAs which produce inappropriately sized polyoma fragments upon digestion with BamHI. (5) HC9, an infectant which makes two immunoreactive polyoma large T proteins, contains two species of amplified DNA, neither of which yield the 4.2kb polyoma DNA fragment when cut with BamHI. There is some evidence of a 4.2kb band in the BamHI digested Hirt pellet DNA, suggesting that the protein detected in the immunoblot is encoded from an integrated copy of the retroviral DNA. (6) HC9-1 and HC9-2 subclones of HC9, which make only the truncated version of polyoma large T antigen, have common 5.0kb amplified DNAs which do not yield a 4.2 BamHI fragment. There is no evidence of an integrated copy in the Hirt pellet DNA digested with BamHI. (7) HC1-1 is the only infectant which makes a full length immunoreactive polyoma large T and contains a characteristic 4.2kb BamHI fragment in its population of amplified DNA. It was, therefore, chosen for more detailed study.

### 4.5 Immunoprecipitation of PyLT & Retinoblastoma Protein

Based on reported findings of an *in vitro* association of polyoma large T antigen with the retinoblastoma protein (Dyson et al. 1990), immunoprecipitation experiments were performed. Determination of an *in vivo* association of these proteins was based on the immunoprecipitation of the Rb protein from protein extracts followed by the detection of bound polyoma large T antigen by western analysis.

Figure 35 is an immunoblot demonstrating Rb-PyLT complexes in HC-1 cells which had been cultivated for 4 days at 35°C (lanes 1,3 and 5) and 39°C (lanes 2,4, and 6). Protein extracts were immunoprecipitated with PAbC36 (a monoclonal antibody to the retinoblastoma protein). The immunoblot was then separated and reacted with either C36 (lanes 1 and 2) or monoclonal antibody F4 to PyLT (lanes 3 to 7). RB-1 protein is detected in cells at both temperatures with two bands at 35°C and only the faster migrating band at 39°C. A prominent band was observed at approximately 100Kd when the immunoblot was developed with F4. Comparison of lanes 3 and 4 shows that in HC1-1, PyLT antigen binds RB-1 irrespective of temperature. This band corresponded in position with immuno-affinity purified PyLT prepared from 293 cells (provided by K. Lawlor), which are active in *in vitro* viral DNA synthesis (Dermody et al. 1988), as shown in lane 7. Conversely, immunoprecipitates prepared from HC1-1 with control antibodies (e.g. the anti-E1A antibody M73) did not contain this band, as shown in lanes 5 and 6. The band of intermediate size in lanes 1-4 is a non-specific band unrelated to RB-1 or PyLT. The association of PyLT with Rb-1 is, therefore, not sufficient to rescue HC1-1 which remains temperature dependent.

## 4.6 Transfection of HAL cells with E1A 12S

The 12S protein encoded by the E1A early region of adenovirus was selected as the second oncogene to be included in this study. A summary of the features of the 12S protein which made it a desirable candidate include: (1) the induction of cellular DNA synthesis and cell cycle production in quiescent cells, (2) the ability to 'immortalize' primary epithelial cells, (3) the induction of DNA synthesis and proliferation of baby rat kidney (BRK) epithelial cells in the absence of serum and (4) the stimulation of growth factor production that induces quiescent primary BRK epithelial cells and primary rat hepatocytes to synthesize DNA and proliferate (Quinlan et al. 1988).

## 4.7 Construction of p12s-hprt

A construction was planned which combined the features of p12sE1a (a 4.1kb plasmid derived from pUC18 which contains the 12sE1A gene in the EcoRI-SstI sites of the polylinker region) and p4aA8 (a 4.5kb plasmid derived from pBR322 which contains the human *hprt* cDNA linked to the SV40 origin and the early promoter).[7] The hprt sequences would serve as a selectable marker in HAT medium in HAL cells since they are resistant to thioguanine, deficient in cellular *hprt*, and unable to form colonies in HAT medium. The plan for this construct included preserving the origin of replication from the pUC18 plasmid. Both p4aA8 and 12sE1A contain unique AatII and NdeI restriction sites which lie outside the areas of interest. In p4aA8 cutting with AatII followed by NdeI generated two fragments, 2.5kb and 2.0kb. In p12sE1A, the same enzymes generated fragments of 3.8kb and 0.26kb. The 2.5kb and 3.8 kb fragments were gel purified, ligated and used to transform DH-1 cells to ampicillin resistance. Seven colonies were picked for analysis with BamH1, EcoRI, SstI, NdeI and AatII. Four of the seven produced the appropriate sized fragments for a plasmid derived from the ligation of the 2.5kb fragment of p4aA8 and the 3.8kb fragment of p12sE1A. This construct, p12s-hprt (see Figure 36), is 6.3kb and can be linearized by EcoRI, SstI, AatII and NdeI. BamHI generates fragments of 4.0kb, 1.5kb and 0.8kb. It contains the 12sE1A gene between the EcoRI and SstI sites of the polylinker. The *hprt* gene and its

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[7] Co-transfection of these plasmids was not considered an option because of the results observed in a series of co-transfection experiments using p4aA8 and pRNS-1, p4aA8 and pRSVneo, pRSVgpt and pRNS-1, pRSVgpt and pRSVneo (See Appendix for a full description of the results of these experiments). The following conclusions were drawn from these experiments: (1) Retention of the second unselected plasmid will vary between different pairs of plasmids used in the co-transfection. (2) The transfection frequency of the unselected second plasmid in a co-transfection is always less than if that plasmid was selected for immediately following the co-transfection. (3) Undefined factors influence the 'incompatibility' of these coupled plasmids.

SV40 regulatory sequences lie between the ClaI and TthIII restriction sites.

## 4.8 Transfection

$3x10^5$ HAL (P6-P10) cells are transfected according to standard protocol with p12s-hprt (5μg, 10μg) or p4aA8 (1μg). The transfection was performed at 35°C with selection beginning three days after transfection. At 21 days the dishes transfected with 5μg p12s-hprt contained approximately 150 colonies. (Transfection with 10μg p12s-hprt had toxic effects on the cells). The dishes transfected with 1μg p4aA8 contained approximately 50 colonies each. Thirty colonies are picked with cloning cylinders and expanded into progressively larger wells until they are seeded into 100mm dishes. Only 11 survived the transfer into 24 well dishes. However, all of those colonies which grew successfully in 24 well dishes were also able to attain 100mm dishes. In addition, representative cultures containing multiple colonies are trypsinized and maintained in 100mm dishes (designated "pooled culture").

## 4.9 Screening For 12S Expression

Protein extracts prepared from 10 individual colonies and one pooled culture are immunoprecipitated overnight with M73 (a monoclonal antibody to the E1A 12S and 13S proteins). A western blot of the SDS-PAGE gel in which a panel of immunoprecipitates have been analyzed is then probed with M73 and horseradish peroxidase conjugated goat anti-mouse antibody. Extracts of HAL cells and 293 (a kidney cell line transformed by E1A and E1B) cells are included as controls.

Although the "pooled culture" tested negative for the 12S polypeptide, 4 of 10 colonies proved to be positive for its expression. Figure 37 is a typical western blot of three negative, H/12s#4, H/12s#5, H/12s#7 (lanes 1,2, and 7) and one positive, H?12s#1 transfectant (lane 6). In lane 5, which contains the 293 extract, the 12S and 13S polypeptides migrate in the vicinity of the 43kD molecular weight marker, below the heavy chain immunoglobulin. Three bands (two major and one minor) were immunoprecipitated from the extracts of the positive HAL transfectant (compare lanes 5 and 6). The uppermost band co-migrated with the lower band of the 293 extract (the 12S polypeptide). No bands, other than the heavy chain immunoglobulin, are seen in the control lane (4).

In order to determine if the 12S protein was capable of binding cellular Rb protein, a similar panel of extracts was immunoprecipitated with C36 (a monoclonal antibody to Rb protein). If the 12S protein were associated with the Rb protein, it should be co-precipitated in this assay. The immunoprecipitates are electrophoresed, blotted and probed with M73. To monitor non-specific sticking of the 12S protein to the beads, duplicate extracts were immunoprecipitated with a control antibody (directed against *E. coli* RNA polymerase). Figure 38 shows that the 12S protein of the E1A12s positive transfectants (lanes 1 and 4) were capable of binding Rb (lanes 9 and 8). The two uppermost bands of the three identified in the anti-E1A immunoprecipitation were also seen in the anti-Rb immunoprecipitation (compare lanes 4 and 8).

In Figure 39 it is seen that the 12S polypeptide of H?12s#1 binds to Rb irrespective of the temperature. Compare lanes 2 (35°C) and 3 (39°C).

HAL cell extracts were also immunoprecipitated with C36 and then probed with pAb419 (a monoclonal antibody to SV40 T antigens) to confirm the binding of SV40 large T antigen with cellular Rb (see Figure 40). This experiment also showed that the SV40 T antigen in the H/12s#1 cells continued to bind Rb protein (lane 6) in the presence of the E1A 12S polypeptide which also bound the Rb protein.

### 4.10 Temperature Shift Experiments

Cultures from the initial transfection with 12s-hprt containing multiple macroscopic colonies at 35°C were shifted to 39°C. None of the colonies continued to grow vigorously, suggesting that E1A-12S did not result in efficient temperature correction.

Three of the four HAL transfectants which tested positive for the 12S protein were tested individually for their abilities to rescue the growth at 39°C. As a negative control, HAL cells which were transfected with p4aA8 were also shifted up to the non-permissive temperature. In the case of the first colony (H/12s#1), three 100mm dishes were seeded with $2x10^5$, six 100mm dishes were seeded with $1x10^5$ cells and eight 100mm dishes were seeded with $5x10^4$ cells. One 100mm dish ($1x10^5$ cells) was maintained at 35°C and the other dishes were shifted to 39°C after overnight attachment at 35°C. After four weeks, the dishes were examined for viable colonies. A total of seven colonies were observed at 39°C and picked with cloning cylinders. With one exception, they all resembled the morphology of the HAL parent, triangular and refractile. Expansion beyond a 12 well dish did not occur for five of the seven colonies. These colonies never grew vigorously and died within weeks after picking. The sixth colony (H/12#1-5), grew well at first and was expanded into 100mm dishes. However, after a few passages, the cells assumed an aberrant appearance and were frozen down.

The last colony was unique in its appearance. The cells were very flat and had a cuboid morphology. Only one small patch of slow growing cells was noted in the well of the 24 well plate. Four weeks later the cells were expanded into a 12 well plate. It took three weeks to reach confluence, at which time it was split into two wells and then finally into 60mm dishes. The cells appeared healthy and grew in islands which resembled those of epithelial cells. Figure 41 is a photograph of Ad-1, the clone which was rescued at 39°C, and H/12s#1, the HAL transfectant from which it was rescued.

The HAL/4aA8 transfectants were seeded at identical densities to that of the H/12s-hprt transfectants for the temperature shift experiments. No growth resulted at 39°C.

### 4.11 Growth Characteristics of Ad-1 and H/12s#1

The population of Ad-1 cells maintained at 39°C in HAT medium has retained a homogeneous flat epithelial-like morphology. It has remained slow growing (Figure 43) with a doubling time that exceeds 85 hours. Ad-1 cells grow to confluence ($\approx 6.8 \times 10^6$/100mm) and have a saturation density of $8.4 \times 10^6$ cells/100mm. The cells become tightly packed rather than "piling up". The EOC increases from 0.1% when seeding $1 \times 10^3$ cells per 60mm dish to 1.0% when seeding $1 \times 10^4$ cells per 60mm dish. Like HAL and H/12s#1, it cannot tolerate substantial decreases in the serum content of its medium. No cells remain after two weeks in dishes which have been fed medium with 3.0% or 0.5% fetal bovine serum.

The morphology of H/12s#1 closely resembles that of the HAL cells, triangular and dendritic until confluency. The doubling time of these cells when maintained at

35°C is 48 hours. In 100mm dishes, H/12s#1, like Ad-1 cells, reach a saturation density of $8.0\text{x}10^6$ cells, which is slightly less than the saturation density for HAL cells ($1.15\text{x}10^7$ cells). HS74, the normal human fibroblast cell line, has a saturation density of $1.5\text{x}10^6$ cells per 60mm dish. As can be seen in Figure 42, when $5\text{x}10^4$ H/12s#1 cells are seeded into 60mm dishes at 35°C, their numbers will steadily increase until confluency ($4.7\text{x}10^6$ cells) is reached. If companion dishes are shifted to 39°C on day 3, they will complete one population doubling, but then lose viability and eventually fall off the dish. The EOC for H/12s#1 at 35°C is 0.5% for $10^3$ cells per 60mm dish and 1.0% for $10^4$ cells per 60mm dish. No colonies except Ad-1 have grown when cultures of H/12s#1 have been shifted to 39°C.

One of 250,000 HAL cells seeded into a 100mm dish will form a colony at 39°C. The possibility that Ad-1 cells were not being rescued by 12S and were actually a revertant of HAL cells was addressed in the following ways: western analysis to determine the proteins and their bound cellular factors at 35°C and 39°C for HAL, H/12s#1 and Ad-1 (Section 4.12); southern analysis to obtain an indication of the comparative amounts of freely replicating DNA in H/12s#1 and Ad-1 (Section 4.13); and back selection of Ad-1 with thioguanine to select for cells which have eliminated their 12s-hprt sequences, and would be expected to have regained the temperature sensitivity of HAL cells (Section 4.13).

## 4.12 Characterization of Ad-1 by Western Analysis

4.12.1 E1A 12S Protein: Protein extracts of Ad-1 (39°C), Ad-1 (35°C), H/12s#1 (late passage), H/12s#1 (freeze down of early passage) and H/12s#11 were prepared (see Figure 44). The extracts were either immunoprecipitated with M73 (lanes 5-8) or C36 (lanes 1-4), separated by SDS-PAGE, electroblotted onto a filter and then probed with M73. The right hand side of Figure 44, lanes 5-8, illustrates that Ad-1 (39°C) cells (lane 5) make a 12S polypeptide that co-migrates with its counterpart in H/12s#1 (lanes 6 and 7). H/12s#1 cells were consistently maintained in HAT medium to ensure the retention of its plasmid (p12s-hprt). Yet, with increasing passage number, H/12s#1 made decreasing amounts of 12S protein (Compare lane 6 with lane 7). Ad-1 cells have continued to express the 12S protein for the four months that they have been in culture.

4.12.2 - Rb Protein: Lanes 1-4 of Figure show that both Ad-1 (39°C) and Ad-1 (35°C) make a 12S polypeptide which binds to Rb. In addition, the amount of 12S protein which binds Rb appears to be nearly equal to the amount of 12S expressed (compare lanes 5 and 4). As can be seen in lane 2, later passages of H/12s#1, make less 12S protein and consequently bind less Rb protein. Despite the observed binding of 12S to the Rb protein in H/12s#1, H/12s#3, H/12s#11 and H/12s#10, the cells die at the restrictive temperature. This suggests that provision of an Rb-E1A 12S complex is insufficient to complement the ts defect in HAL cells.

4.12.3 - *p53*. The heat labile SV40 T antigen of HAL cells is capable of binding p53 at the permissive temperature. When these cells are shifted up to a restrictive temperature, the large T antigen loses its ability to bind p53. If Ad-1 cells were revertants, the SV40 large T antigen should continue to bind p53 at the restrictive temperature. The following experiment included the cell line H/RNS-1P, which was isolated from the transfection of HAL cells with pRNS-1, a plasmid encoding the gene for wild type SV40 large T antigen and neomycin resistance. The cell line was generated from a culture of pooled colonies which were grown at 35°C in G418 medium. When shifted to the restrictive temperature, they continue to grow as a mass culture. This cell line serves as the positive control for the binding of SV40 T antigen to p53 at 39°C.

HAL, H/12s#1, and H/RNS-1P cells were seeded at 35°C at a density of $1x10^6$ cells per 100mm dish. Ad-1 cells were maintained and seeded at 39°C. The following day, companion cells were shifted to 39°C. On day three after shift up, protein extracts were prepared which were immunoprecipitated with PAb421 (a monoclonal antibody to p53) and subsequently probed on a blot for large T antigen with PAb419.

As seen in Figure 45, at 35°C HAL, H/12s#1 and H/RNS-1P make an SV40 large T antigen which binds p*53* (lanes 4,5, and 8). When shifted to 39°C, the SV40 large T antigen of HAL and H/12s#1 no longer binds p*53* (compare lanes 4 with 3 and lanes 5 and 6). When HAL cells are provided with a wild type large T, as in the case of H/RNS-1P, they will bind p*53* at both temperatures (compare lanes 8 and 9); however, Ad-1 cells do not make an SV40 large T which associates with p53 at 39°C, as seen in lane 2. The conclusion from this experiment is that Ad-1 cells are probably not revertants of the ts phenotype so it is possible that E1A 12s protein plays a role in the rescue of Ad-1 cells.

<u>4.12.4 - SV40 Large T</u>: The amount of SV40 large T antigen from HAL, H/12s#1, Ad-1(39°) and Ad-1(35°) extracts have been compared. Ad-1(35°) cells are Ad-1(39°) cells which have been maintained in HAT medium and switched back to 35°C

for approximately four weeks. In Figure 46 comparable amounts of protein for Ad-1(39°) and Ad-1(35°) were analyzed by immunoblot with PAb419. It was apparent that Ad-1(39°) cells (lane 5) contained less immunoreactive SV40 large T than its counterpart Ad-1(35°) (lane 6) and its parent H/12s#1 (lane 4) or HAL cells (lanes 1 and 2). Scanning densitometry, using the 1-D Autostepover/Videophoresis XV program from Bio-Med was performed. The optical density was measured for each band and the area of the peak was computed (See Figure 47). The extracts of HAL and H/12s#1 had equivalent amounts of protein. The areas of the peak representing the band for large T were 15098 and 17093 respectively. Extracts of both Ad-1(39°) and Ad-1(35°) had one half the amount of protein than the extracts of HAL or H/12s#1. Their large T peaks were 1162 and 8029 respectively. If normalized to the same protein content, the area of the peaks for HAL, H/12s#1 and Ad-1(35°) is 8-fold that of Ad-1(39°). HAL cells which have been grown at 35°C and HAL cells which have been shifted to 39°C for 3 days show equivalent amounts of SV40 large T antigen by immunoblot (data not shown). This is an indication that the heat labile T of Ad-1(39°) has lost its immunoreactivity at the non-permissive temperature. Ad-1(39°) can regain its levels of immunoreactive large T when switched back to the permissive temperature. The conclusion from this series of immunoblots is that the growth of Ad-1 cells at 39°C is not due to a reversion or overexpression of the temperature sensitive SV40 large T antigen and can only be explained as yet by undefined cellular events.

Table 8 is a summary of the proteins and protein complexes detected by western blotting in HAL(35°), H/12s#1(35°/39°), Ad-1(39°/35°) and H/RNS-1P(39°/35°). The profile of H/12s#1 and Ad-1 cells do not look substantially different, yet Ad-1 cells grow at 39°C and H/12s#1 cells do not. The survival of this rare variant of H/12s#1 cannot be due solely to the 12S-Rb complexes or one would expect many more colonies to have been rescued from H/12s#1 cells which have been shifted to the restrictive temperature.

### 4.13 Southern Analysis For Free Plasmid

p12s-hprt, the plasmid used to transfect HAL cells, contains the *hprt* gene linked to the SV40 origin and promoter. Although it is presumed that at least one copy has integrated into the genome of HAL cells, free copies of this plasmid can be released and/or replicate autonomously. Free copies of p12s-hprt can be detected as low molecular weight species in a Hirt supernatant. If Ad-1 cells were rescued by E1A function, the replicative function of large T should continue to be compromised, rendering it unable to generate additional free copies of the plasmid at 39°C. Whatever copies were present at 35°C when the cells were shifted to 39°C would be diluted with subsequent divisions of the cells.

Hirt lysates were prepared from Ad-1(39°), Ad-1(35°) and H/12s#1. A high molecular weight DNA sample of H/12s#1 was included in this panel. The Hirt supernatants were digested with AatII and NdeI which cuts within the plasmid sequences to release the 12S sequences (3.8kb) from the remainder of the 2.5kb plasmid. Uncut samples, along with the AatII and NdeI digested samples, were electrophoresed. A 1.7kb EcoRI-PstI fragment containing the E1A sequences from pE1A-WT (a gift of E. Moran) was used for making the radiolabeled probe. Figure 48 is the autoradiograph of this southern blot.

H/12s#1 contains one predominant species of unit length p12s-hprt DNA (lane 7). Since it is seen without enzyme digestion, it is freely replicating plasmid. The Ad-1(35°) cells also contain a substantial amount of free plasmid. The uncut pattern is the same as H/12s#1 (lane 4). The AatII-NdeI digested sample (lane 3) contains a major band which migrates at approximately 6.0kb and three lesser bands at ≈3.8kb, 5.5kb and 2.5kb. The 3.8kb band shows the greatest intensity. Linearization of p12s-hprt produces a 6.3kb fragment (seen as the major band in lane 3). Complete digestion with AatII-NdeI yields 3.8kb and 2.5kb fragments. The 3.8kb fragment which contains the 12S sequences is the second most prominent band in lane 3. On the other hand, the Ad-1(39°) cells contain no copies of free plasmid in the undigested sample (lane 6). There are two visible bands in the lane containing the AatII-NdeI digested sample (lane 5). The more intense of the two is approximately 10pg gene equivalents and migrates parallel to the linear (6.3kb) form in the Ad-1(35°), an estimated ≈75pg fragment. Without any form I in the undigested lane, it is uncertain whether the bands seen in the digested lane came from low molecular weight DNA in the Hirt supernatant.

Even if these bands are of low molecular weight origin, the amount present in the Ad-1(39°) cells is substantially less than the amount present in Ad-1(35°). This is in support of the conclusion that Ad-1 cells do not make an SV40 large T antigen capable of supporting replication and that these cells are not revertants.

### 4.14 Back Selection

A feature of HAL cells generated for the purpose of back selection is its resistance to 6-thioguanine (6-TG). In Ad-1 cells, the *hprt* gene is linked to the 12S gene. A loss of the integrated plasmid would render these cells negative for 12S and *hprt* expression. Such cells would grow in 6-TG at 35°C and should die when shifted to 39°C. If, however, Ad-1 cells were revertants, they would still be able to grow at the non-permissive temperature because their growth was not dependent on the presence of the 12S protein.

To this end, Ad-1 cells were shifted down to 35°C for one week. The HAT medium was removed and replaced with complete medium supplemented with hypoxanthine and thymidine. One week later, the cells were seeded into 100mm dishes at a density of $1\text{x}10^5$ and $2\text{x}10^5$ cells per dish. These cultures were fed with $5\text{x}10^{-5}$M 6-TG. Within one week, many of the cells died and those which remained appeared to undergo a morphologic change. Instead of having an epithelial shape, they began to branch out in a way that resembled the HAL cell line. The surviving cells grew into colonies which were picked four weeks after addition of the 6-TG. Thirty colonies were picked from dishes containing ≈80-100 colonies each. Although the growth of some colonies appeared to be vigorous, within four weeks they all died. When this experiment was repeated, no colonies grew out of 6-TG selection.

An explanation for the inability of to recover any $6TG^R$ colonies is that the cells were still $hprt^+$. The Ad-1 cells were maintained in HAT medium for seven days at 35°C before selection was removed. Then complete medium (without HAT) was added for an additional seven days prior to addition of 6-TG. This period of time would be sufficient to build up a reservoir of freely replicating plasmid. Unless a cell is free of its integrated and free copies of plasmid, it will be $hprt^+$ and it will die in 6-TG. This might require shifting Ad-1 from 39°C to 35°C with immediate removal of selection and prolonged growth in complete medium before the addition of 6-TG.

### 4.15 Immunofluorescence/Intermediate Filaments

The preliminary characterization of the intermediate filaments of Ad-1 cells was initiated because of two findings. First, Cone et al. (1988) demonstrated that the majority of rat primary cells which become 'immortalized' by rv12s (retrovirus encoding 12S sequences) were simple epithelial cells. Cultures from a wide range of organs (heart, pancreas, thyroid, adrenal, brain) contained a variety of cell types. Immunofluorescent studies prior to infection have shown that primary cultures contain vimentin-positive, cytokeratin-positive and desmin-positive cells. Yet after infection, only one or two types of foci appeared, and in most cases, these foci had epithelial morphologies. These cells were positive for cytokeratin 8 by immunofluorescent staining with LE41. The interpretation offered was that replication in epithelial cells was favored over other cell types. It is interesting to note that adenoviruses replicate in epithelial cells *in vivo*.

Second, it has also been shown (Roberts et al. 1985) that NIH3T3 cells infected with retroviruses containing the E1A 12S, 13S or 7S protein exhibited distinct alterations of their morphology. Cells containing the retroviral vector lacking the E1A sequences displayed a typical fibroblastic appearance like its parent. Specific proteins that influence cell structure and morphology were compared in these cell lines. The $\alpha$-tubulin mRNA levels were unaffected by the expression of any E1A product. However, the levels of fibronectin and collagen type I mRNAs were substantially reduced in the cells which expressed 12S or 13S protein. Cytokeratin expression was not examined in these cells.

To determine if the morphology of Ad-1 cells was in any way reflective of the modulation of specific cellular genes, immunofluorescent staining of Ad-1 and HAL cells was performed with an antibody to vimentin and a polyclonal antibody to cytokeratins. Fluorescent micrographs revealed that both Ad-1 and HAL cells were vimentin positive. HAL cells displayed no cells which stained positive to the polyclonal antibody to cytokeratins. In contrast, approximately 10% of the Ad-1 cells became brightly fluorescent when stained with this antibody (Figure 49). The pattern seen was typical for intermediate filaments. The fibers encircled the nucleus, forming a "basket" which extended out to the periphery of the cell. The possibility cannot be excluded that induction of these cytoskeletal genes occurred originally in the parental HAL cell line. Knapp and Franke (1989) have shown that rare cells can emerge spontaneously in non-epithelial transformed culture lines which synthesize vimentin intermediate filaments and cytokeratins 8 and 18. In SV40 transformed fibroblasts, 0.1 to 0.5 percent of cells were positive for both cytokeratin 8 and 18. No cytokeratin positive cells were seen in HAL cells. It would be tempting to speculate that in Ad-1 cells, the alteration in the expression of these cellular genes occurred in response to the action of the 12s protein. An analysis of the induction of such cellular genes is beyond the scope of this thesis but would be interesting to pursue based on this intriquing finding.

### 4.16 Fate of Ad-1(39°)

After 14 weeks of continual culture at 39°C, a change in the morphology and growth capacity of Ad-1 cells became apparent. The cells looked less epithelial (cuboidal) and they no longer grew in tightly packed islands. The peripheral cells looked more triangular with spindly extensions. This became the predominant phenotype until 3-4 weeks later when no viable cells remained. Cell extracts prepared at the outset of this change revealed that these cells continued to make the 12S protein (Figure 50, lane 2).

Ad-1 cells which had been shifted and maintained at 35°C suffered a similar change, although its onset was delayed by a few weeks. Both cultures in the presence and absence of HAT medium underwent substantial losses of cells. The cells in the dishes maintained in HAT medium died. Colonies grew out of the dishes from which HAT was removed. The fate of these colonies has yet to be determined.

As of yet the reasons for this crisis remain undetermined. Extracts were prepared when a change in the growth of these cells was observed. These will be tested for immunoprecipitable 12S and SV40 large T antigens. The binding to Rb by these proteins will also be determined.

# Chapter 5

## Discussion

The immortalization potentials of two nuclear oncogenes, polyoma large T antigen and adenovirus E1A 12S protein were examined in a human fibroblast background. The effects of polyoma large T antigen were addressed in two ways. The first involved the introduction of PyLT into normal human fibroblasts in the hope of effecting a change in the lifespan of these normal cells. The second approach involved infecting a human fibroblast cell line which has been immortalized and transformed by a temperature sensitive SV40 T antigen with PyLT antigen and Ad12S protein. The effects of these proteins were monitored at the permissive and restrictive temperatures.

Transactivation of the promoter in pRSVneo was the only evident effect that the PyLT had in HS74. No G418$^R$ colonies were capable of continuous growth expansion. Although use of the SV promoter in PyLT-S clearly influenced the expression of PyLT (as determined by the transactivation of RSVneo), further characterization was not possible due to an insufficient number of cells, all of which succumbed to senescence before analysis. Therefore, it could not be determined whether polyoma continued to be expressed, was overexpressed or was even lost.

When this oncogene, together with the gene for resistance to G418, was introduced into immortal HAL cells at 35°C, those cell lines were stable for G418$^R$ and made copious amounts of PyLT. However they were not able to be rescued at 39°C. PyLT was thus unable to substitute for a wild type SV40 LT in this system. Similar results were observed when E1A 12S protein was transfected into HAL cells. With the exception of one clone (Ad-1) which has shown an extended ability to grow at the restrictive temperature, all attempts to generate rescued clones with the E1A 12S protein have failed. The results of these experiments suggest that these oncogenes may employ different 'immortalization' pathways in human cells which are inadequate in complementing a cell line which has already been immortalized by an 'establishment' gene.

The results of the experiments in which the human fibroblasts (HS74) were transfected with PyLT seem somewhat surprising in light of the similarities shared by SV40 LT and PyLT antigens and the effects that these antigens have in promoting the establishment of primary rat embryo fibroblasts. Both SV40 LT and PyLT are involved in the stimulation of host DNA synthesis, initiation of viral DNA replication, repression of early gene expression, the switch from early to late transcription and the events that lead to integration, excision and amplification of the virus DNA within the host chromosome. Whereas SV40 LT alone can initiate and maintain transformation of both primary cells and continuous cell lines, PyLT does not cause tumorigenic transformation. Rassoulzadegan et al. (1983) demonstrated that permanent cell lines could be established from REF cells which were transfected with PyLT. Such cell lines retained the growth properties of non-transformed fibroblasts, showed a diminished need for serum and were dependent on a continuous expression of PyLT for their growth. Asselin and Bastin (1985) have reported that the intrinsic frequency of establishment for REF cells as determined by use of a plasmid coding for $G418^R$ was 1 of 452 colonies or 0.2%. In this respect, REF cells resemble human fibroblasts more closely than other rodent cells which show a high rate of spontaneous establishment.

As a counterpoint to the results observed in this study, Strauss et al. (1989) has described successful results in human cells using a construct, pMTV-LT2, in which the coding sequences of polyoma large T antigen are linked to the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter. After transfection into human embryonic lung (HEL) fibroblasts, induced and non-induced colonies were isolated and assessed for immortalization properties. All of the non-induced and 10% of the induced colonies were characterized as immortal. Two facts made this interpretation questionable. The control cells, which were transfected with the background plasmid, were not treated with dexamethasone. Therefore the reversal of immortalization by removal of dexamethasone cannot be linked clearly to polyoma large T expression. Additionally, in the case of one immortal clone whose colony formation was not significantly influenced by dexamethasone, a sub-clone was isolated which no longer expressed polyoma large T antigen and yet was still immortal. This cell line was characterized as expressing >1000 polyoma mRNA molecules per cell. Strauss and co-workers (1989) have reported that high levels of PyLT antigen can be mutagenic; thus it would be plausible that this clone was immortalized due to the mutagenic activity of polyoma and not the oncogene product itself.

The retroviral vector pZIPNeoSV (PyLT) was used in an attempt to improve upon all of the limitations of co-transfections. The vector simultaneously transduced the polyoma large T antigen and resistance to G418-sulfate. Somewhat disappointing was the fact that the producer lines with the highest titers (1000 CFU/ml) did not result in infectants which expressed detectable polyoma LT in a western blot. Only 2 of 10 infectants made a full-sized PyLT. This result could be explained by an examination of the vector, pZIPNeoSV (PyLT), which was used (Figure 20) to generate the producer cell line. It contains an SV40 origin upstream of the polyoma and neomycin resistance gene and is separated from the 3' LTR by the pBR origin. The HAL cell line[8] makes a thermolabile SV40 T antigen which is capable of binding to an origin of replication at the permissive temperature. This endogenous SV40 T was able to facilitate replication and excision of heterogeneous sized proviral sequences. In fact, the infectants which were G418$^R$ but polyoma large T negative by western analysis did have polyoma sequences which were detected in the Southern blot analysis. Indeed, uncut samples of HMW DNA contained free DNA which included portions of the polyoma genome. If replication proceeded bilaterally from the origin but did not continue out to both LTRs, molecules would be excised and amplified which were not the full length of the provirus. One predominant species was linearized by BamHI and XbaI, suggesting that only one BamHI site of the polyoma insert and only one of the two LTRs (one XbaI site) was present. The polyoma LT being expressed by such a DNA would be deficient in its 5' sequences and would not be detectable by the antibody used in the western assay. (F4

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[8] Because human cells are semi-permissive for SV40 replication, the plasmid used to transfect the temperature sensitive T antigen into the human fibroblasts did not contain a competent origin of replication for SV40 T.

is a monoclonal antibody to the amino terminus of polyoma large T antigen.) This model would be consistent with results of Botchan et al. (1980) who demonstrated the heterogeneity of excision products in cells carrying integrated copies of SV40. However, in an analysis of the Hirt DNA derived from producer cells fused to COS cells, the predominant recombination event that freed the viral sequences from the flanking cellular DNA occurred within the direct repeat sequences of the LTRs and produced unit length DNA sequences. (Only occasionally were viral sequences isolated which contained one LTR, a deletion at either the 5' or 3' end of the viral genome and contained cellular sequences). It is, therefore, easy to account for the presence of free sequences, but it remains somewhat surprising that only 2 of 10 HAL infectants (HC1-1 and HC-10) generated unit length DNA which released a full sized polyoma large T from its unique BamHI site. By western analysis, HC1-1 made a level of PyLT comparable to PyT54, the hamster transformant which synthesizes polyoma large T antigen. To date, this has not been reported in a human cell line. Numerous unsuccessful attempts were made to obtain colonies which appeared to be rescued at 39°C.

The generation of free DNA ceases at the restrictive temperature. As a result, the role of the integrated copy of the retrovirus assumes greater importance because it becomes the sole contributor of polyoma large T antigen.[9] Initially, this should have

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[9] Although HC1-1 has been shown to contain an abundance of free DNA containing PyLT, there has been no verification of the integrated copy. The Hirt pellet can be probed for PyLT sequences but one cannot rule out the possibility of contamination by the Hirt supernatant. In the case of the infectants which did not make an immunoreactive PyLT, one could make a probe to the 5' end of the polyoma gene to obviate the masking effect of the free plasmid which reacts to a probe made to the whole molecule.

little impact because of the existing free DNA which expresses PyLT. However, as this population becomes diluted, the role of the integrated copy becomes essential for survival. No clones were observed after selection for 2 to 3 weeks at the high temperature, consistent with reduced polyoma LT due to loss of free DNA. However, this would not explain the lack of growth at 39°C as in the growth curve experiment. The free polyoma DNA would not be expected to be lost in this short period of time. Therefore, a sufficient amount of PyLT should have been present to elicit an effect at the restrictive temperature. The inability of these cells to grow at the restrictive temperature suggests that in human cells, polyoma large T antigen does not supply the replicative cell function(s) necessary to overcome the temperature sensitive block characteristic of the SV40 tsA gene.

With respect to the emerging view that the binding of cellular Rb by several families of DNA tumor viruses plays an important step in the transforming capabilities of these viruses, HC1-1 was analyzed for its Rb binding capacity. Dyson et al. (1990) have found that in an *in vitro* assay, the mixing of RB polypeptide with detergent lysates of tissue culture cells containing polyoma viral proteins will result in the formation of stable protein complexes. The relevance and existence of these complexes have not been addressed in an *in vivo* human system. When protein extracts of HC1-1 are immunoprecipitated with C36 (antibody to Rb) and then probed for polyoma large T in a western assay, a band co-migrates with authentic PyLT. This demonstrates that, indeed, there is an interaction between these molecules but the biologic effect of this interaction is insufficient to rescue the HAL cells at 39°C. The role of Rb will be further addressed in the discussion of the adenovirus E1A 12S protein.

Jat et al. (1986) has made use of the same vector, but has employed an ecotropic packaging cell line to create the infective retroviral virion used to transduce the PyLT into REF cells. He has reported that 50% of the G418$^R$ colonies went on to become established cell lines without passing through a 'crisis' period. Eight of these cell lines were shown to have immunoprecipitable PyLT which co-migrated with authentic PyLT antigen. Interestingly enough, when the same retroviral recombinant ($\psi 2LT_4$) was used to infect REF cells which were transformed by temperature sensitive SV40 T antigen (tsA58)[analogous to HAL cells], no complementation of the defect was observed at the restrictive temperature. The results of Jat's experiments suggest that the steps involved in 'establishment' may very well differ from those involved in the rescue of a temperature sensitive (SV40) transformed cell line.

Although raising a temperature sensitive SV40 transformed cell line to the restrictive temperature seems to revert the phenotype back to a normal cell which senesces due to its finite lifespan, biochemically the cell is no longer naive. A range of cellular activities may change or adapt to the synthesis of the SV40 T antigen. Even the provision of the wild type SV40 oncogene may not efficiently fully complement the defect in an altered cellular milieu. In the present set of experiments pRNS-1 was transfected into HAL cells at the permissive temperature. Single clones were picked in addition to dishes which were pooled and grown up. Although the pooled dishes ($\approx$50 colonies per dish) grew well at 39°C, only 50% of picked clones were able to be rescued at the restrictive temperature. This suggests that either the level of T expression differed from clone to clone or that the temperature sensitive clones were intrinsically different. Jat has demonstrated that the transfection of wild type SV40 (pZIPSV40) into four different temperature sensitive SV40 transformed REF cell lines resulted in rescue at 39°C in the case of only two of the cell lines. The four tsA cell lines sorted out into two categories: highly temperature sensitive and less temperature sensitive. The highly temperature sensitive group included cell lines which made higher levels of SV40 T antigen. Constructs which made the wild type SV40 large T antigen or the E1A 12S protein were unable to rescue the growth of the cells lines which were highly temperature sensitive. Yet the same constructs were able to rescue the less temperature sensitive cell line. The HAL cell line is considered moderately temperature sensitive and may resemble Jat's temperature sensitive line which proved difficult to rescue. Therefore, the testing of several temperature sensitive SV40 transformed human cell lines would confirm the inability of PyLT or E1A 12S to rescue the human cells.

That SV40 T antigen and adenovirus E1A share common transforming mechanisms has been brought to light by several observations. Amino acid residues 101-118 of SV40 T antigen bear some similarities to the E1A domain 2 (Stabel et al. 1985). The C-terminal half of domain 2 contains a motif which is shared by SV40 and several other nuclear oncogenes and is recognized by casein kinase II (CKII), a protein kinase which may be involved with cell cycle control. The N-terminal half of domain 2 contains an adenovirus conserved sequence (DLXCXE) which is also shared by papovaviruses.

The transforming activity of the SV40 T antigen is dependent upon the integrity of residues 105-114 (Kalder and Smith 1984, Cherington et al. 1988). (See Figure 5). A chimeric adenovirus which contains SV40 T antigen residues 101-118 in place of the E1A domain 2 is able to induce proliferation of primary BRK cells and cooperate with the ras oncogene to fully transform these cells. Two cellular proteins, p105K and p107K, whose co-precipitation depends on the presence of domain 2 of E1A, are also co-precipitated by SV40 LT and by the chimeric protein in infected HeLa cells.

The immunoprecipitation of protein extracts containing SV40 T antigen with monoclonal antibody to SV40 T antigen (PAb 419) or monoclonal antibody to 105-Rb (C36) co-precipitated T antigen and 105 Rb in each case. Deletion mutants containing structural alterations in the transformation domain failed to bind 105-Rb (DeCaprio et al. 1988). These mutants have been shown to be defective in the induction of focus formation and growth in soft agar in established rat and mouse cells (Kalderon and Smith 1984, Cherington et al. 1988), yet they show no defect in origin-specific DNA binding or nuclear localization to T antigen. As might be suspected since SV40 T antigen is capable of binding the 105kD protein, it is also able to bind the 107kD protein which requires aa 121-127 of domain 2 in the E1A region of adenovirus (Dyson et al. 1989). It too is a nuclear phosphoprotein whose under-phosphorylated form is the species to which SV40 T antigen binds. The 105 and 107 kD proteins associate with the E1A protein independently. The association of the 107kD protein with E1A is essential but not sufficient for transformation.

Analysis of mutations to the amino terminus (the region necessary for binding to the 300kD protein) indicates that this region also makes an essential contribution to the transforming potential of E1A (Lillie et al. 1987, Schneider et al. 1987, Stephens and Harlow 1987, Zerler et al. 1987, Moran and Zerler 1988, Smith and Ziff 1988, Whyte et al. 1988, Whyte et al. 1989).

The results of the present study suggest that the adenovirus 12S cannot rescue the growth of the SV40 transformed temperature sensitive human cell line (HAL) at the restrictive temperature. Yet, examination of the HAL transfectants which expressed the E1A 12S protein, (H/12S #3, #10 and #1) revealed that indeed the 12S gene product was associated with a p105-Rb (as was the SV40 large T antigen). Despite this, all but one clone (Ad-1 39°) died at the restrictive temperature. These results have reinforced the concept that Rb binding, in and of itself, is not sufficient to rescue these cells.

What then would explain the growth of this one variant clone at 39°C? A comparison of the cellular proteins bound by the E1A protein of Ad-1 and H/12s#1 might provide some insight. In one experiment (see Figure 51), H/12s#1 and Ad-1 39° cell lines were labeled with methionine free medium containing 100$\mu$Ci/ml of $^{35}$S methionine for two hours. Protein extracts were prepared and immunoprecipitated with monoclonal antibodies to E1A, Rb and p53. The autoradiograph of the gel revealed that the 12S protein ($\approx$45kD) of Ad-1 cells bound both a 105-107 kD protein and a >200kD protein. Whereas the 12S protein of H/12s#1 cells only bound a 105-107 kD protein. A longer exposure of this autoradiograph reveals a faint band at the >200kD position and a weak band at 43kD. This experiment might suggest that the cells of H/12s#1 make a mutant 12S protein which is immunoreactive to M73 but cannot bind or poorly binds the >200K protein. It retains the ability to bind p105-107 proteins. The one variant clone which grew at 39°C bound the 300kD protein in addition to the p105-107 (Rb) proteins. Obviously, such results must be repeated for confirmation of this finding. Riley et al. 1990 has shown similar results using various mutants of adenovirus E1A. E1A proteins bearing mutations in CR1 (the region containing the sequences which bind the 300kD protein and some of the non-contiguous sequences which bind the 110-Rb protein)(see Figure 8) did not rescue temperature sensitive SV40 T transformed REF cells. These cells retained a fibroblastic morphology like the temperature sensitive parent, rather than the epithelial morphology of adenovirus infected cells. E1A proteins containing mutations to CR2 (the region which binds the 105-Rb protein and 107K protein) rescued the temperature sensitive cells at a slightly lower level than wild type E1A. These proteins failed to bind 105 Rb yet retained their ability to bind the 300K protein.

In terms of the present study, the 12S protein in H/12s#1 should be re-evaluated for its ability to bind cellular proteins. If it is truly deficient in its ability to bind the 300K protein, it would be most interesting to transfect H/12s#1 with the construct containing CR1 and ascertain the effect of this independently functional domain.

More colonies should be picked and analyzed for the expression of the 12S protein. If other 12S expressing clones can be rescued at the restrictive temperature, analysis of the E1A mutants would become valuable for defining the regions of E1A which play a role in the rescue of tsa mutants. If no clones can be rescued from cell lines expressing a 12S protein which binds the 300kD and 110kD proteins, then the obvious conclusion is that E1A does not rescue human tsA cells.

In established rodent cell lines which have been transformed by temperature sensitive SV40 T antigen, shifting up to the restrictive temperature causes the cells to revert (lose their transformed phenotype) to the normal established phenotype. This suggests that the temperature sensitive SV40 T antigen denatures upon shift-up and results in the loss of its transforming ability. Could CR2 (which binds p105-Rb) transform established rat tsA SV40 T transformed cells at the restrictive temperature? Because these cells are already immortal, they should not require CR1. Would CR2 have any effect on the transformed phenotype? Would mutant p*53*?

The results of Shay and Pereira-Smith (submitted for publication) suggest that some oncogenes which have dominant effects in rodent cells are individually insufficient at altering the normal lifespan of human fibroblasts. When IMR-90 normal human fibroblasts are transfected with a steroid inducible (MMTV-LTR) SV40 T antigen, immortalized clones can be recovered which remain T antigen dependent for growth. In steroid free medium, the proliferative capacity of these cells can be restored with plasmids expressing SV40 T antigen, adenovirus E1A/E1B, human papilloma virus E6/E7, and intact HPV16. Plasmids expressing individual proteins such as adenovirus E1A, E1B, E6 or E7 could not restore growth.

In addition, when the SV40 T antigen deletion mutant dl A2433 (which can not bind p*53* protein) and the SV40 T antigen point mutant K1 (which binds cellular Rb poorly) were introduced separately, no proliferation or greatly reduced proliferation was observed. However, good complementation was achieved when both constructs were co-transfected into the recipient, thus implicating the involvement of both Rb and p*53* with SV40 T antigen induced immortalization. Similarly, the pairing of adenovirus E1A and E1B and E6 and E7 supplied those domains necessary for Rb and p*53* binding and subsequent restoration of cell proliferation.

It would be of great interest to this study to transfect K1 into the Ad-1 cells. These cells make E1A 12S and have grown at the restrictive temperature for an extended period of time but did not become immortal. The 12S protein of Ad-1 has been shown to bind Rb. It would be intriguing to see the effects of providing Ad-1 cells with the p*53* binding domain from SV40 (K1 mutant) or from adenovirus (E1B).

The Ad-1 cells in this study continued to thrive at 39°C for approximately 16 passages, after which time the cells experienced a 'crisis-like' episode and died. Protein extracts prepared during this period reveal that the 12S protein was still being expressed. Therefore, the possibility exists that human cells require additional events unique to the action of a particular oncogene for immortalization. One cannot exclude the possibility that other phenomena were occurring in the cell unrelated to the action of 12S oncogene.

Although much has been said about the contributions that this system has to offer to the understanding of cell transformation, the caveats also warrant attention. The presence of two dominant oncogenes in a cell can potentially influence each other or the cell in ways different from the effects of either oncogene alone. The 12S protein of E1A is capable of repressing transcription from the early SV40 promoter in transient assays in HeLa cells (Velcich and Ziff 1985). No documentation of the reverse situation has been reported. H/12s#1 was chosen for the shift-up experiments based on its expression of the 12S protein. However, after a few months of continuous growth at 33°C, the cells expressed reduced amounts of the 12S protein. These cells were maintained in selective media to prevent loss of the plasmid, so it is highly unlikely that the adenovirus E1A sequences were lost. Expression of the 12S protein was not vital to the cell at 33°C, because the SV40 T antigen was maintaining the transformed state. At 39°C, the Ad-1 cell line has continued to express the 12S protein which is presumably now maintaining the growth of these cells at the restrictive temperature. When the Ad-1 cells were shifted back to 33°C (to restore function of SV40 T antigen), cultures maintained in selective media began to look sickly until all the cells died. Companion cultures maintained in media without HAT suffered some cell losses, but a healthy population of cells continued to grow. These cells remain to be analyzed for both 12S protein and SV40 T antigen expression. However, one can speculate that at 33°C the presence of both SV40 T antigen and 12S protein were incompatible for the cell. Follow-up experiments will clarify this point. Such information poses technical problems for the investigator which may only be circumvented by the early characterization and shift-up of transfected colonies.

Despite these considerations, this system offers the opportunity to test in human cells that which is known in the rodent system. The inability of some oncogenes to transform or to transform far less efficiently suggests that other events in human fibroblasts, as yet undefined, exist. The information generated from studying mitosis in yeast, in conjunction with the identification of cellular factors found in association with oncogenes such as adenovirus E1A protein may very well converge to provide insights into the regulation of growth in human cells.

Table 1. Functional classification of cooperating oncogenes

| Cytoplasmic Oncogenes | | Nuclear Oncogenes |
|---|---|---|
| | rat embryo fibroblasts | |
| Ha-*ras* | SV40 LT | *myc* |
| Ki-*ras* | | N-*myc* |
| N-*ras* | | L-*myc* |
| *src* | | Adeno E1A |
| Polyoma MT | | Polyoma LT |
| | | Papillomavirus E7 |
| | avian bone marrow cells | |
| *erb*B | | *myc* |
| *src* | | *myb* |
| *fps* | | |
| *mil* | | |
| Ha-*ras* | | |
| *ros* | | |
| *yes* | | |
| *sea* | | |

Table 2. Partial List of Known Oncogenes and Associated Neoplasms.

| CATEGORY | ONCOGENE | HOMOLOGOUS CELLULAR GENE | ASSOCIATED NEOPLASM |
|---|---|---|---|
| Growth factors | *sis* | PDGF-2 | |
| | *int*-2 | FGF-like | Mammary carcinoma? |
| Transmembrane growth factor receptors | *erb*B | EGF receptor | |
| | *neu* (erbB-2, HER-2) | | Mammary carcinoma |
| | *fms* | M-CSF receptor | |
| | *ros*, *kit* and others | | |
| Membrane associated tyrosine kinases | *abl* | | Chronic myelogenous leukemia, acute lymphocytic leukemia, acute myelogenous leukemia |
| | *src* family* | | |
| | *fes*, *fps* | | |
| Membrane associated guanine nucleotide binding proteins | K-, N-, and *H-ras* | | Colorectal adenoma or carcinoma lung adenocarcinoma, prostate cancer |
| Cytoplasmic serine-threonine kinases | *raf/mil* | | |
| | *mos* | | |
| Cytoplasmic hormone receptors | *erb*A | Thyroid hormone receptor | |
| Nuclear factors | c-*myc* | | Burkitt's lymphoma |
| | N-*myc* | | Neuroblastoma |
| | L-*myc*, N-*myc* | | Small cell lung carcinoma |
| | *fos* | | |
| | *jun* | | |
| | myb, ets, ski | | |
| Antioncogenes | RB | | Retinoblastoma, soft tissue sarcoma, lung carcinoma, breast carcinoma, prostate cancer, and osteosarcoma |
| | p53 | | Colorectal carcinoma, lung carcinoma, Ductal breast carcinoma, Stomach carcinoma, and astrocytoma |
| Others | *bcl*-2 | | Non-Hodgkin's lymphoma |
| | *bcl*-1 | | |
| | *int*-1 | | Mammary carcinoma |

* The family includes *src, fgr, lck, hck, fyn, lyn, and tkl.*

Source: Druker et al. 1989.

Table 3. Plasmids.

| PLASMID | DESCRIPTION | SOURCE/REFERENCE |
|---|---|---|
| pPyLT1 | encodes PyLT antigen | R. Kamen/Treisman et. al (1981) |
| pPyLT-S | encodes PyLT antigen under the regulatory sequences of SV40 | L. Resnick-Silverman |
| pPSVE1-Bla | encodes the whole early region of polyoma under the regulatory sequences of SV40 | J. Hassell/Muller et. al (1984) |
| pZIPneoSVPyLT | retroviral vector encoding the genes for PyLT and neo resistance | P. Jat/Jat et. al (1986) |
| pKP45 | vector derived from pBR322 containing multi-cloning site polylinker | K. Peden |
| p4aA8 | encodes the human *hprt* gene | S. Weissman (unpublished) |
| pSH320-Py | encodes PyLT and the human *hprt* gene | S. Weissman (unpublished) |
| p12s-hprt | encodes AdE1A 12S gene and the human *hprt* gene | L. Resnick-Silverman |
| p12sWT | encodes AdE1A 12S gene | E. Moran/Moran et. al (1988) |
| pRNS-1 | encodes SV40 LT and the neo resistance gene under the control of the RSV LTR | H. Ozer/Litkzas et. al (1984) |
| pRSVneo | encodes the neo resistance gene under the control of the RSV LTR | C. Gorman/Gorman et. al (1982) |
| pJLPYO | encodes the origin of replication for polyoma-virus | J. Li/Murakami et. al (1982) |
| pRSVgpt | encodes *E. coli gpt* under the control of the RSV LTR | C. Gorman/Gorman et. al (1982) |

Table 4. Antibodies.

| ANTIBODY | DESCRIPTION | REFERENCE |
|---|---|---|
| PAb 419 | monoclonal antibody to the SV40 large T and small t antigens | Harlow et. al (1985) |
| PAb 421 | monoclonal antibody to p53 | Harlow et. al (1981) |
| PAb 210 E8 | monoclonal antibody β-subunit of *E. coli* RNA polymerase | Rockwell et. al (1985) |
| C36 | monoclonal antibody to the Rb protein | Whyte et. al (1988) |
| M73 | monoclonal antibody to E1A 12S and 13S polypeptides | Harlow et. al (1985) |
| F4 | monoclonal antibody to the amino terminus of the polyoma large T antigen | Pallas et. al (1986) |

Table 5. Comparison of SV40 and Polyoma Promoters in HS74

| Plasmid | µg/ml | G418$^R$ colonies/dish [a] | Foci |
|---|---|---|---|
| pRSVneo + pPyLT-S | 2, 10 | 15 | ND [b] |
|  | 2, 5 | 3-5 | ND [b] |
|  | 2, 2 | 1 | ND [b] |
| pRSVneo + pPyLT-1 | 2, 10 | 0 | ND [b] |
|  | 2, 2 | 0 | ND [b] |
| pPyLT-1 alone | 2 |  | 0 |
| pPyLT-S alone | 2 |  | 0 |
| pRSVneo alone | 2 | 1 | ND [b] |
|  | 10 | 10 | ND [b] |

[a] Dishes (100 mm) were seeded with $5x10^5$ cells; G418$^R$ resistant colonies were determined from triplicate dishes.

[b] ND, Not determined.

Table 6. Lifespan of G418$^R$ clones picked from HS74 transfected with pPyLT-S and pRSVneo.

Experiment 6 / Picked 17

Petri Dish Size

| | 15mm[a] | 30mm[b] | 60mm | 100mm | Subculture | FD |
|---|---|---|---|---|---|---|
| 1 | ▨ | ▨ | ▨ | | | - |
| 2 | ▨ | ▨ | ▨ | | | - |
| 3 | ▨ | ▨ | ▨ | | | - |
| 4 | ▨ | ▨ | ▨ | | | - |
| 5 | ▨ | ▨ | ▨ | | | - |
| 6 | ▨ | ▨ | ▨ | | | - |
| 7 | ▨ | ▨ | ▨ | | | - |
| 8 | NA[c] | | | | | |
| 9 | NA | | | | | |
| 10 | NA | | | | | |
| 11 | NA | | | | | |
| 12 | ▨ | ▨ | ▨ | | | - |
| 13 | ▨ | ▨ | ▨ | | | - |
| 14 | NA | | | | | |
| 15 | ▨ | ▨ | ▨ | | | - |
| 16 | ▨ | ▨ | ▨ | | | - |
| 17 | ▨ | ▨ | ▨ | | | - |

Experiment 10 / Picked 10

| | 15mm[a] | 30mm[b] | 60mm | 100mm | Subculture | FD |
|---|---|---|---|---|---|---|
| 1 | ▨ | ▨ | ▨ | | | - |
| 2 | ▨ | ▨ | ▨ | | | - |
| 3 | ▨ | ▨ | ▨ | | | - |
| 4 | ▨ | ▨ | ▨ | | | - |
| 5 | NA | | | | | |
| 6 | NA | | | | | |
| 7 | ▨ | ▨ | ▨ | ▨ | | - |
| 8 | ▨ | ▨ | ▨ | ▨ | | - |
| 9 | NA | | | | | |
| 10 | ▨ | ▨ | ▨ | ▨ | | - |

[a] This is the size of one well in a 24-well dish.

[b] This is the size of one well in a 12-well dish.

[c] NA, No attachment.

Table 7. AMPHOTROPHIC PRODUCER CELL LINES[a]

| Producer line | CFU/ml[b] |
|---|---|
| C1 | 0.5 |
| C3 | 2 |
| C7 | 1000 |
| C8 | 500 |
| C9 | 75 |
| C10 | 10 |

[a] Amphotropic producer cell lines are isolated as G418$^R$ clones from the transfection of PA317 packaging cells with pZIPneoSV(PyLT).

[b] CFU, G418$^R$ colony forming units per ml. of virus containing medium.

Table 8. Immunoblot Analysis of HAL and HAL Transfectants

| | Cell Line | | | | | |
|---|---|---|---|---|---|---|
| Protein | HAL 35°C | 39° C | H/12s#1 35°C | 39°C | Ad-1 39°C | H/RNS-1P 39°C |
| SVLT | + | + | + | + | + | + |
| E1A 12S | - | - | + → - (LP)[a] | + | + | - |
| Protein Complex | | | | | | |
| p53/SVLT | + | - | + | - | - | + |
| Rb/SVLT | + | weak + | + | NT[b] | NT[b] | + |
| Rb/E1A 12S | - | - | + → - (LP)[a] | + | + | - |

[a] LP, Late passage.
[b] NT, Not tested.

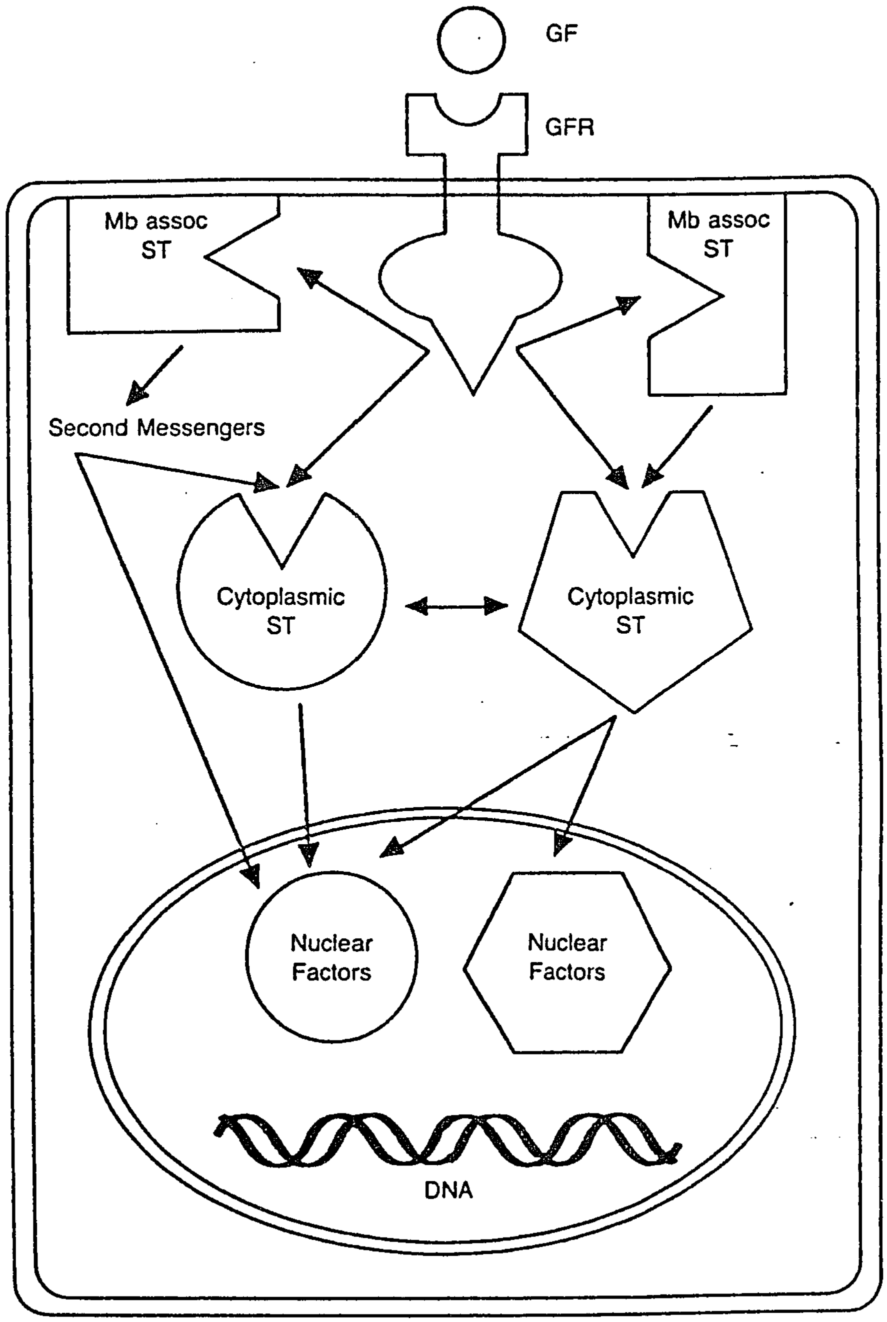


Figure 1. Signal transduction by a membrane-bound receptor.

A schematic depiction of the events which follow the binding of a growth factor (GF) to its membrane growth factor receptor (GFR). Mb assoc denotes membrane associated.
ST denotes signal transducer.

Source: Druker et al. 1989.

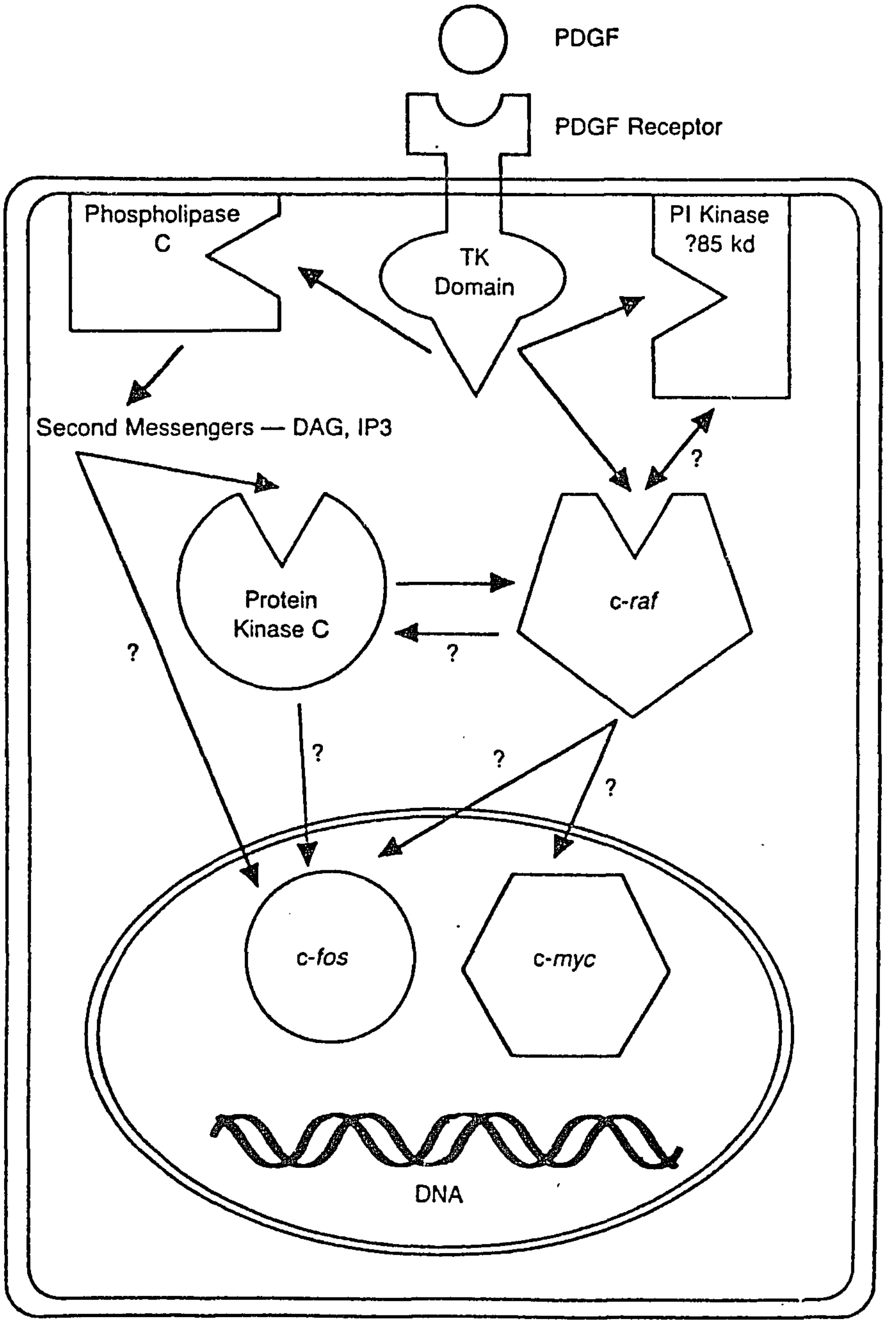


Figure 2. Signal transduction through the receptor for platelet-derived growth factor.

A schematic depiction of some known events which occur after platelet-derived growth factor (PDGF) binds to its membrane-bound receptor.
TK denotes tyrosine kinase, PI phosphtidylinositol, DAG diacylglycerol and IP3 inositol triphosphate.

Source: Druker et al. 1989.

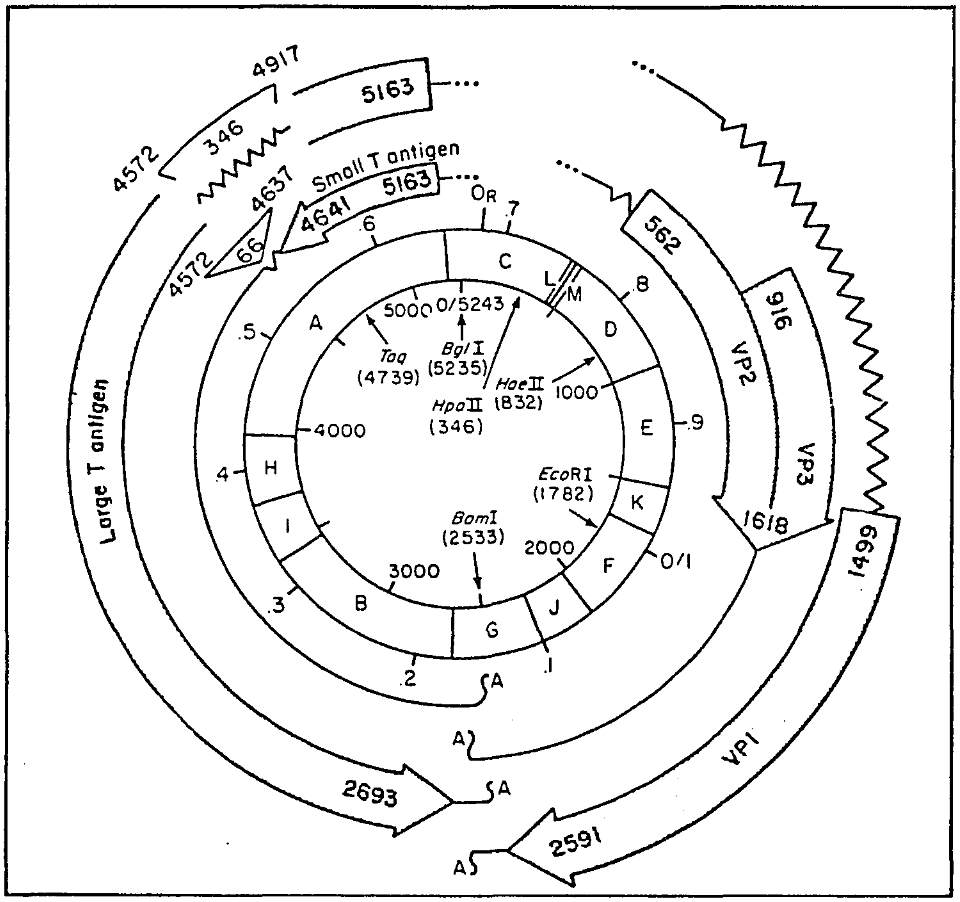


Figure 3. SV40 Genome.

Schematic representation of the SV40 genome, indicating positions of some salient features. The origin of replication ($O_R$) is located at the top. Numbers outside the circle refer to *Eco* RI cleavage site. The arcs with arrowheads (pointing in the 5'→3' direction) indicate coding segments within the viral mRNA.

Source: Tooze 1981.

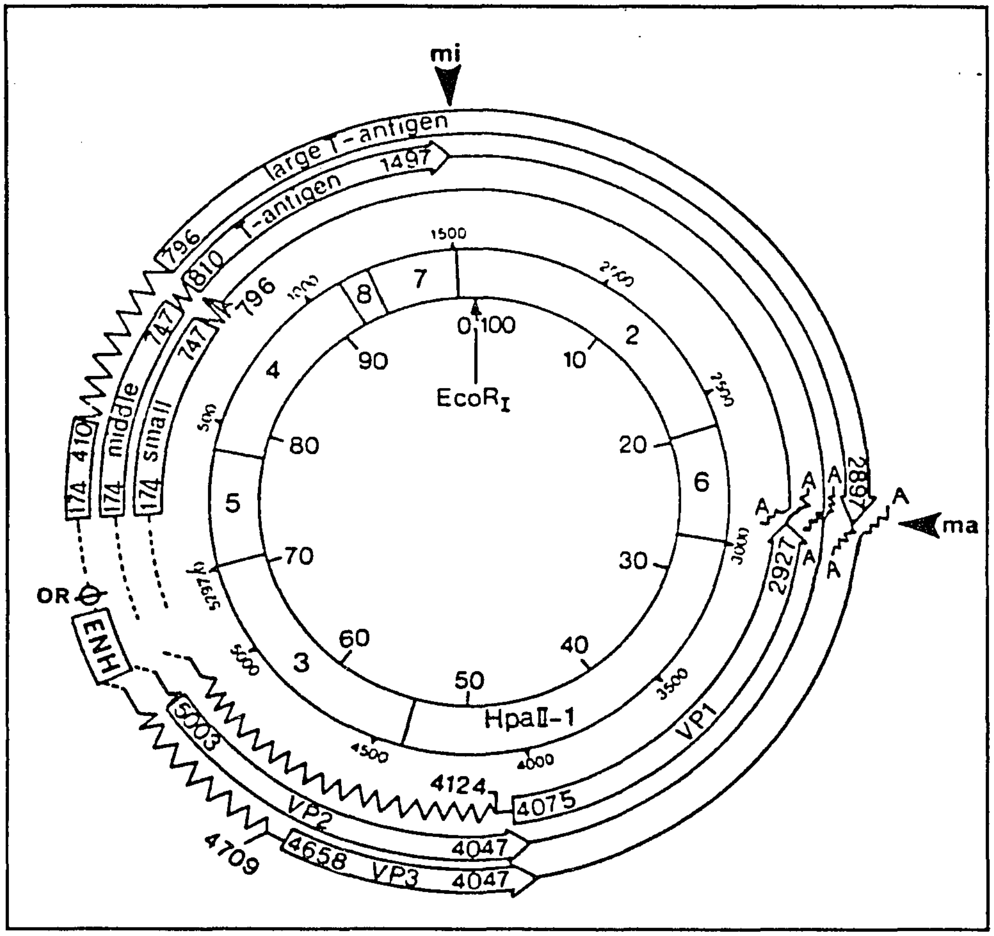


Figure 4. Polyoma Virus Genome.

Schematic representation of the polyoma virus genome, indicating various landmarks and key enzyme recognition sites. The coding portion or the DNA and its corresponding proteins are shown relative to the *Hpa* II physical map. A single translational reading frame is used for the amino-terminus domains of the three T antigens but three different frames determine their carboxy-terminal regions encoded 3' to the splices. The numbers within the arcs indicate initiation and termination codons.

Source: Tooze 1981.

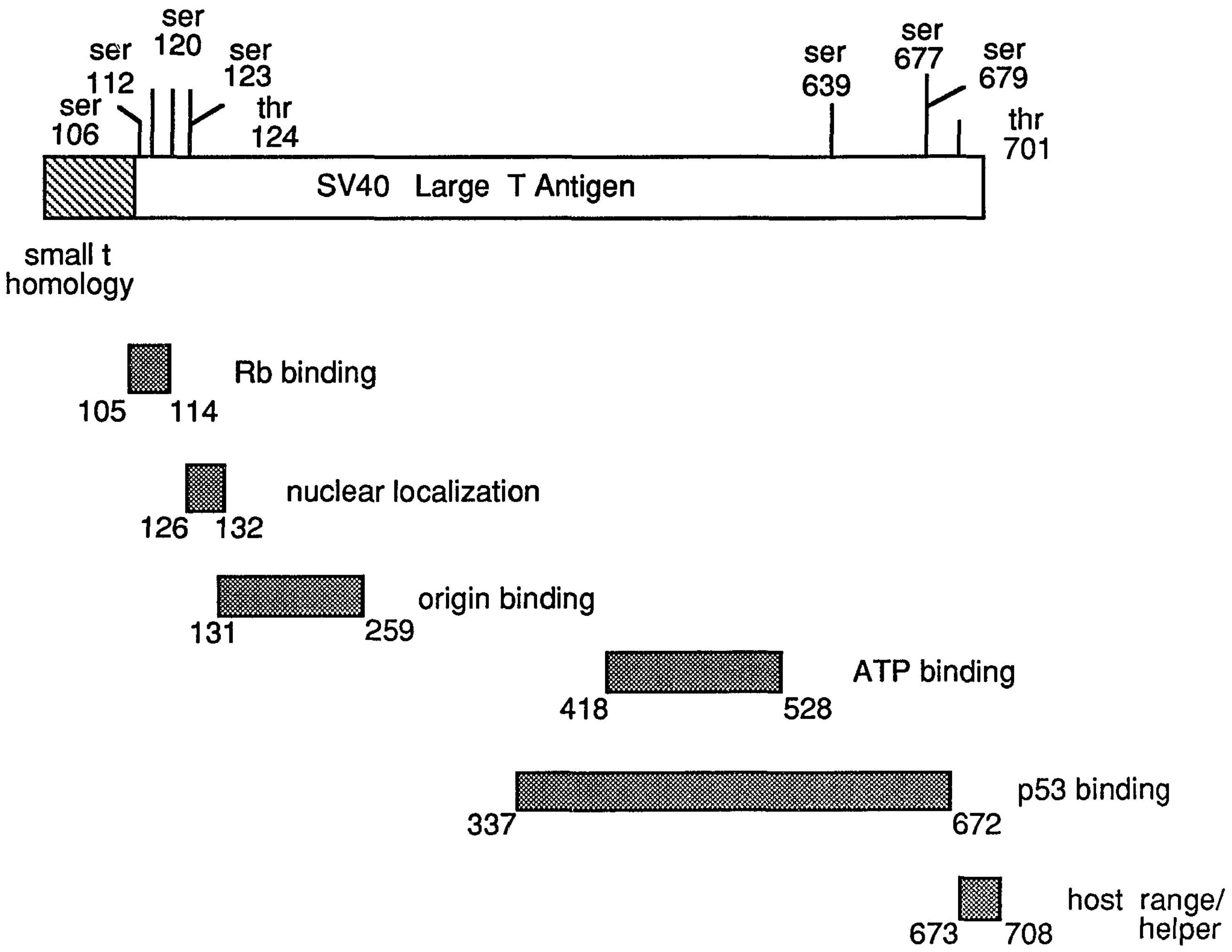


Figure 5. The Phosphorylation Sites and Domains of T Antigen

The phosphorylation sites of T antigen are indicated above box. Below T antigen are the estimated boundaries of the various domains related to replication and cell transformation.

Source: Prives, C. 1990.

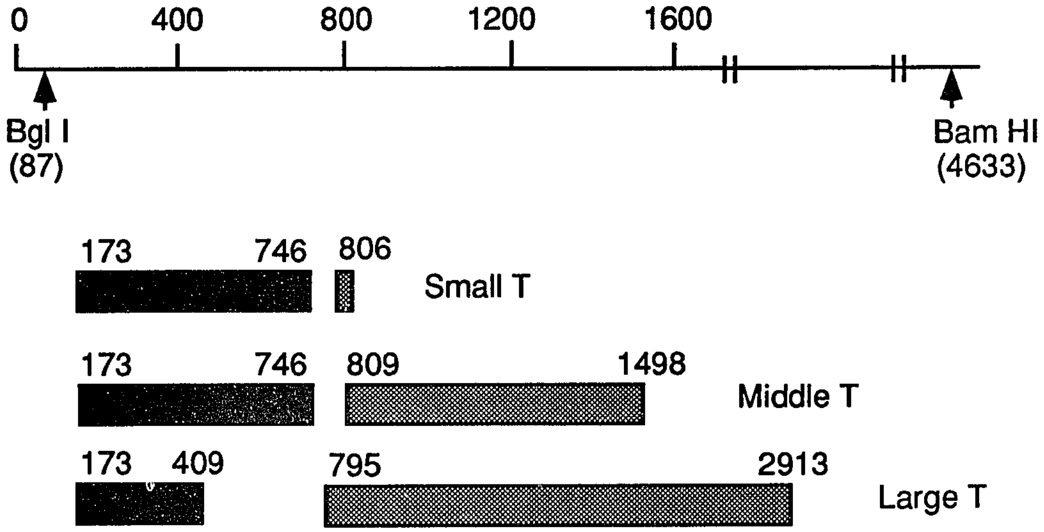


Figure 6. Polyoma Virus Early Region

Schematic representation of the organization of the polyoma early region. The segments of DNA which code for small, middle and large T antigen are depicted by the black and hatched rectangles.

Source: Jat et al. 1989.

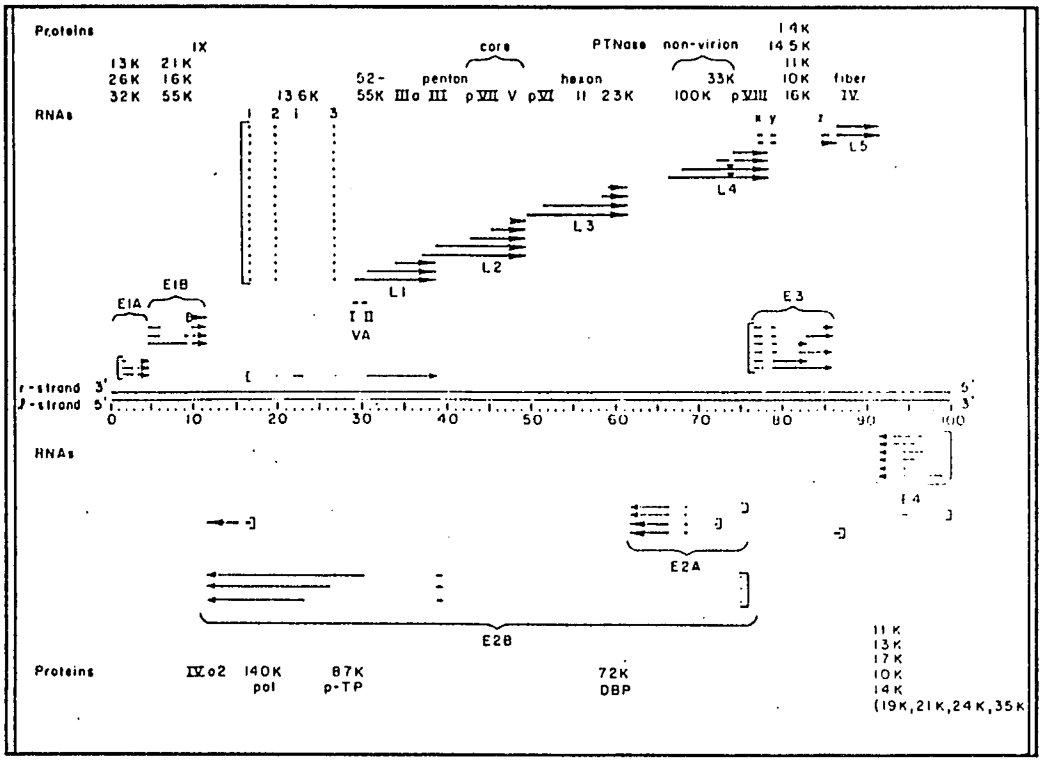
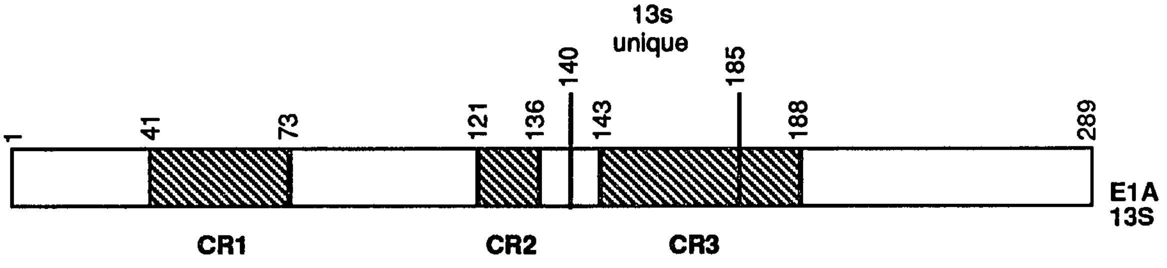


Figure 7. The Genome Organization of Adenovirus 2.

Schematic representation of the adenovirus chromosome and RNA transcripts as originally mapped by electron microscopy of RNA:DNA heteroduplexes. The chromosome is a linear duplex DNA molecule consisting of 35,937 nucleotide pairs. The genome is divided into 100 map units.

The r-strand of DNA is transcribed rightwards into RNA and the l-strand is transcribed leftwards. Early RNAs are depicted by thin arrows and late RNAs by thick arrows. Gaps in the arrows represent intervening sequences removed by splicing. The dots signify short leader segments joined to the 5' ends of some early and most late mRNAs.

Source: Petterson et al. 1986.



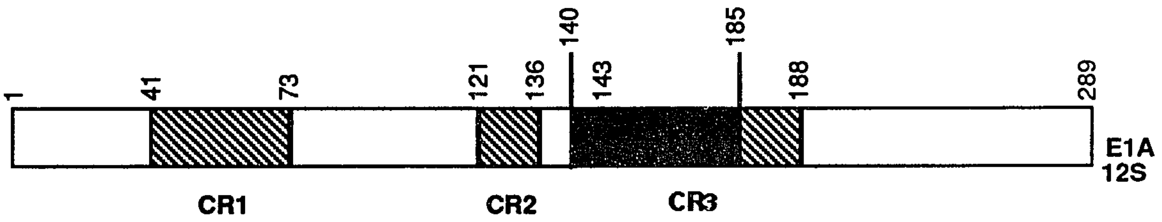


Figure 8. Schematic representation of the products of the E1A region of Adenovirus 2.

The nucleotide sequence of the coding region of the 13S E1A cDNA, which encodes a 289 amino acid product is represented by the top bar. The 243 amino acid 12S E1A cDNA is represented below as an in-frame deletion of 46 amino acids from the 13S product. Nucleotide positions of selected restriction sites are indicated above the line. CR1, CR2 and CR3 denote conserved regions among the different serotypes of Adenovirus.

Source: Moran, E. 1988

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SV40 large T | 99 | N | E | E | N | L | F | C | S | E | E | M | | P | S | S | D | D | E | A | T | 117 |
| Ad5 E1A | 118 | E | V | I | D | L | T | C | H | E | A | G | F | P | P | S | D | D | E | D | E | 137 |
| HPV-E7 | 18 | E | T | T | D | L | Y | C | Y | E | Q | L | N | D | S | S | E | E | E | D | E | 37 |

Figure 9. Amino acid homology amongst SV40 large T, Ad5 E1A, and HPV-E7 proteins.

Alignment of domain 2 sequences from human adenovirus serotype 5 with homologous sequences from SV40 T large T antigen and human papillomavirus (HPV) E7.

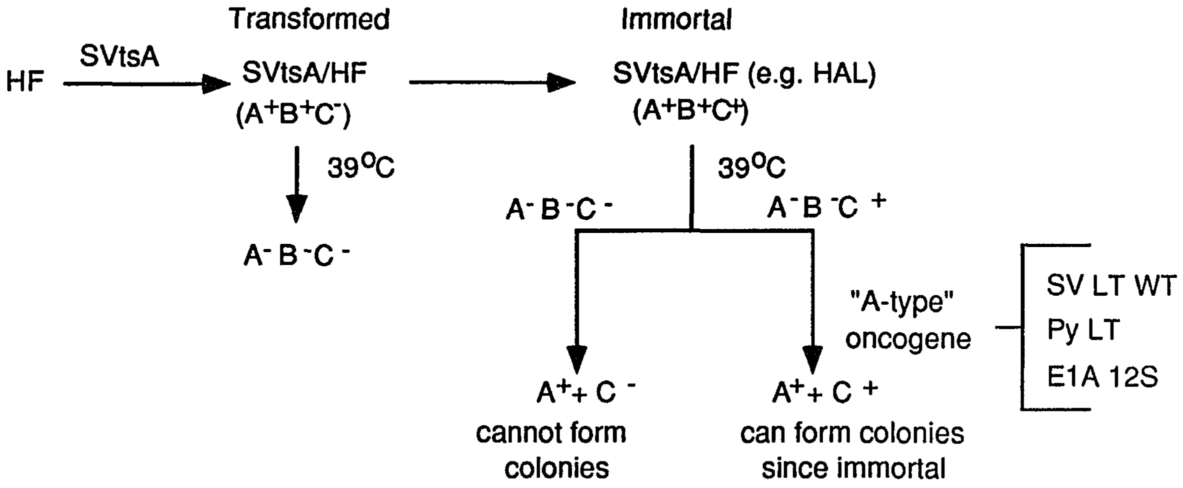


Figure 10. Model for rescue of SVtsA/HF transformants

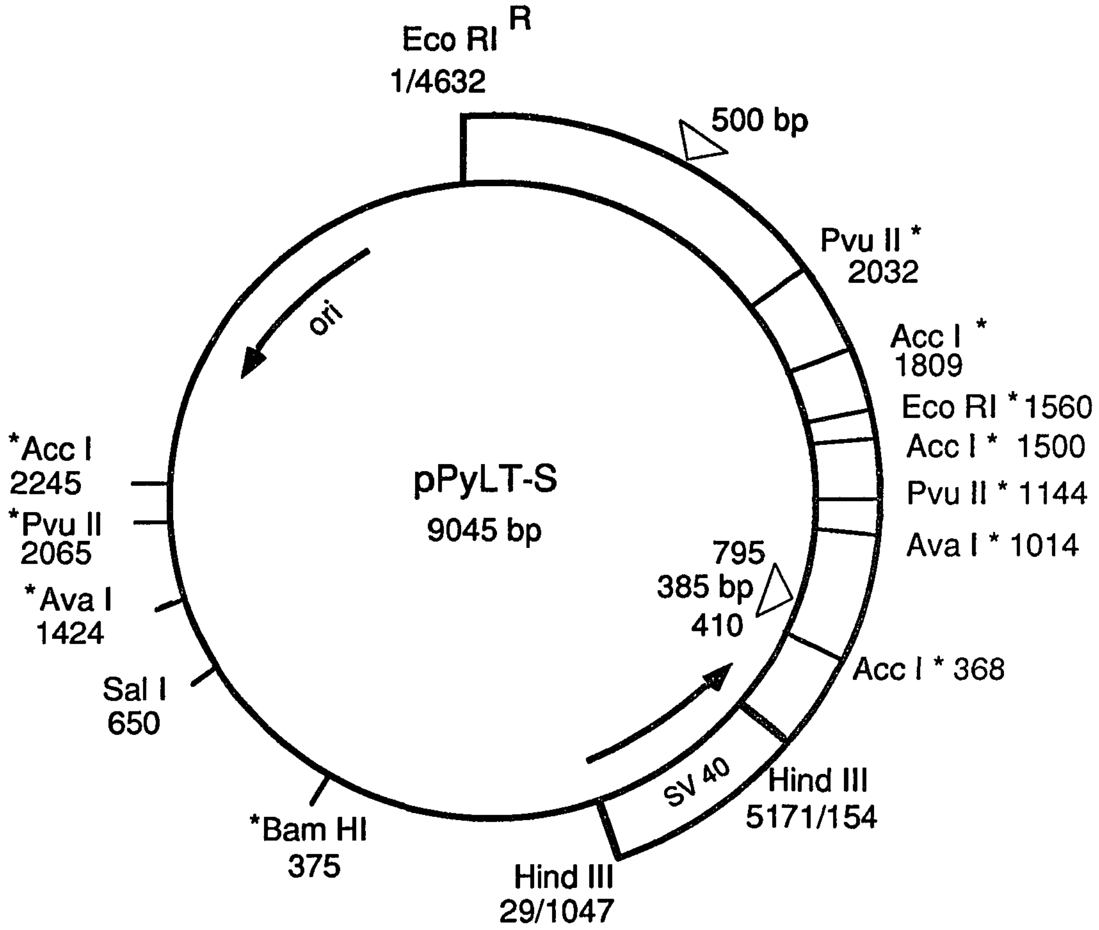


Figure 11. The structure of pPyLT-S DNA (ca. 9045 b.p.).

This plasmid resembles pPSVE1Bla (described in figure13) in all respects except for a deletion of 385 b.p in the intron of the polyoma large T sequences, as indicated by the triangle within the circle. This precludes the expression of PyST and PyMT, allowing only the expression of PyLT antigen.

* denotes sites which have been verified by restriction enzyme analysis.

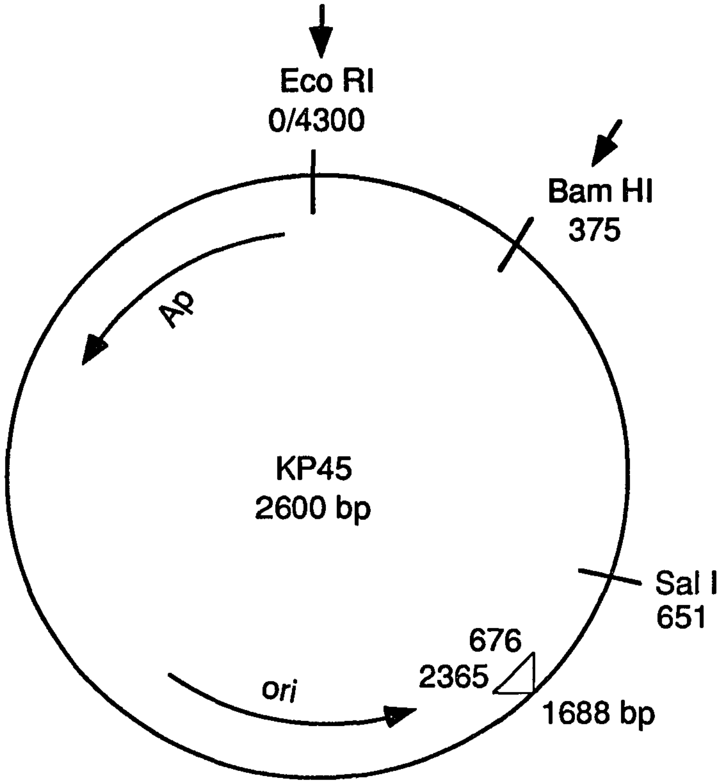


Figure 12. The structure of KP45 DNA (ca. 2600 b.p.).

This plasmid is derived from pBR322. It contains a deletion of 1688 b.p. which extends from nucleotide 676 to nucleotide 2365. The arrows indicate the sites into which a 2.9 kb Eco RI-Bam HI fragment of pPSVE1Bla is cloned.

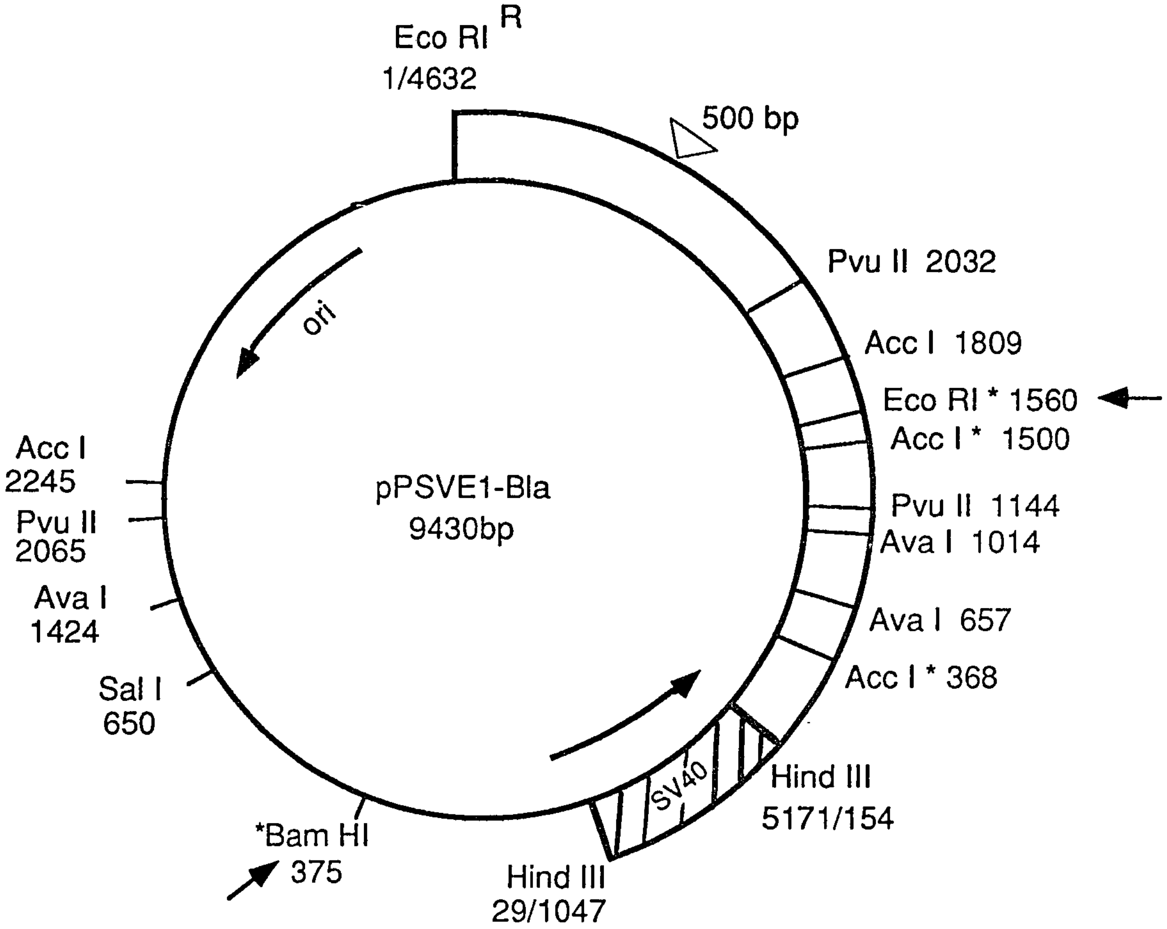


Figure 13. The structure of pPSVE1-Bla DNA (ca. 9430 b.p.)

This plasmid is composed of pBR322 DNA (4333 b.p.), the Hind III C fragment of SV40 DNA (1119 b.p; hatched box) and polyomavirus DNA (ca. 3978 b.p.; open box). The sequences in pBR322 DNA between its Eco RI (nucleotide 1) and Hind III (nucleotide 29) sites were replaced with the SV40 -polyoma virus hybrid transcription. unit. The pBR322 Eco RI (nucleotide 1) and the polyomavirus Bam HI (nucleotide 4632) sites were destroyed but the Hind III site was preserved. The early promoter of SV40 drives the transcription of the polyomavirus early region. There is a deletion (denoted by the triangle) of about 500 b.p. in the polyomavirus sequences which encompass the Hind III site at nucleotide 3918. Transcription of the three (PyST, PyMT and PyLT) co-terminal mRNAs should originate in SV40 DNA and terminate in polyomavirus DNA.
* denotes sites verified by enzyme analysis.

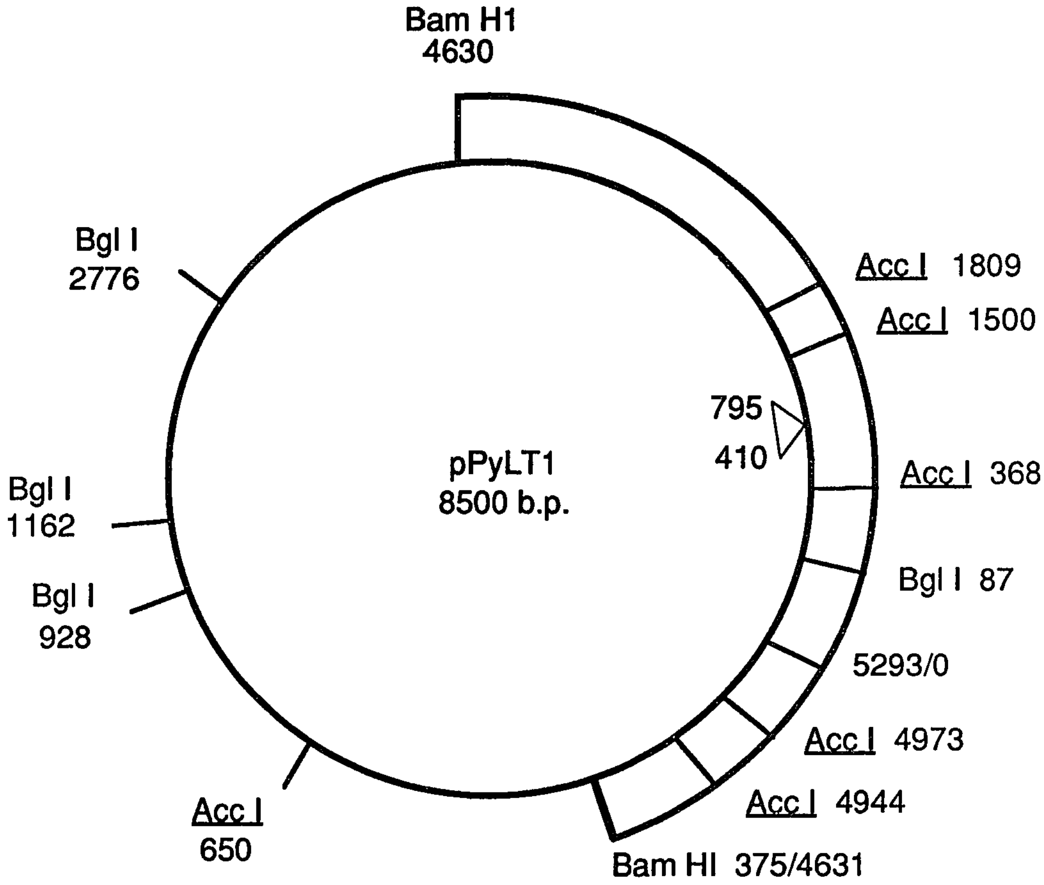


Figure 14. The structure of pPyLT1 DNA (ca.8500 b.p.)

This plasmid consists of pAT153 sequences into which a modified polyoma genome has been cloned (Bam HI 375/4631). The restriction fragment encompassing the large T intron (410-795) has been replaced with the corresponding shorter fragment from the cDNA clone. This deletion is denoted by the triangle. As a result of this deletion pPyLT1 expresses only polyoma large T antigen. The Acc I and Bgl I restriction sites have been verified by restriction enzyme analysis.

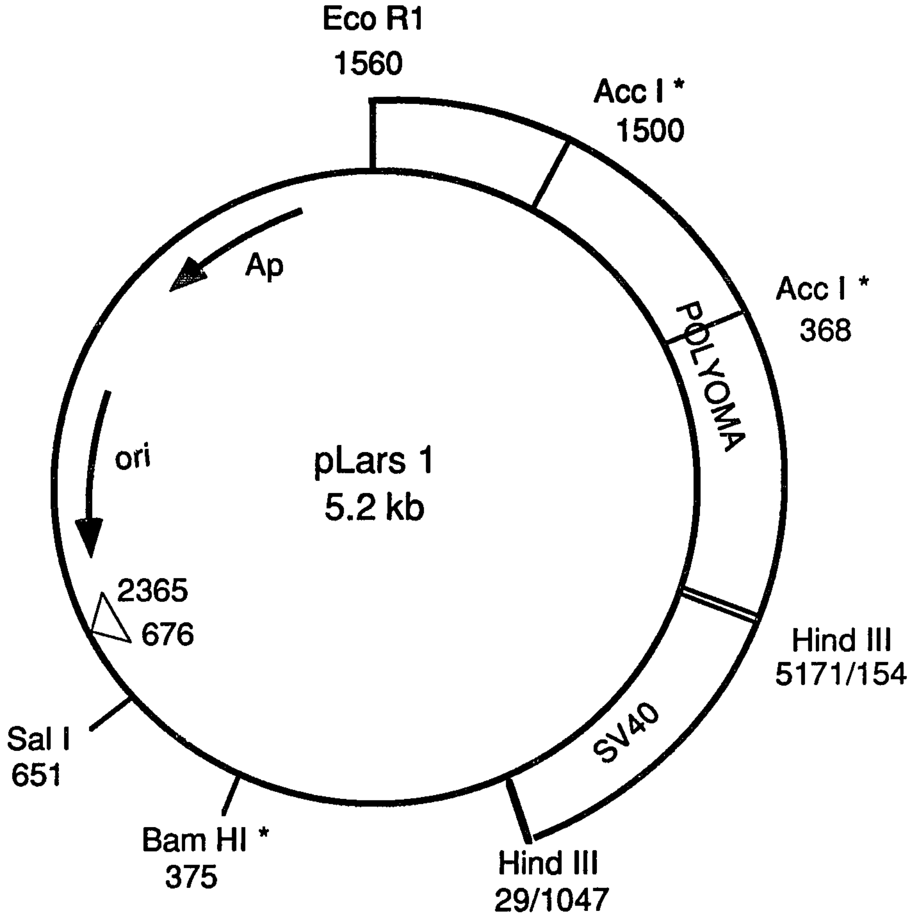


Figure 15. The structure of pLars 1 DNA (5.2 kb).

This plasmid consists of the 2.3 kb Eco RI-Bam HI fragment of KP45 ligated to the 2.9 kb Eco RI-Bam HI fragment of pPSVE1Bla. This fragment encompasses the SV40-polyomavirus hybrid unit up to and including the Eco RI restriction site at 1560 b.p. in the polyoma sequences.
* denotes sites verified by restriction enzyme analysis.

| 1<br>*Acc* I | 2<br>*Acc* I +<br>*Bgl* I | 3<br>*Hind* III I |
|---|---|---|
| | | 23.7 kb |
| | | 9.5 kb |
| | | 6.6 kb |
| 6.2 kb | | |
| | | 4.4 kb |
| | 4.02 kb | |
| | | 2.25 kb |
| | | 1.96 kb |
| | 1.614 kb | |
| | .800 kb | |
| → .748 kb | .748 kb | |
| .688 kb | | |
| .585 kb | .585 kb | .59 kb |
| | .407 kb | |
| .300 kb | .300 kb | |
| | .281 kb | |
| | .234 kb | |

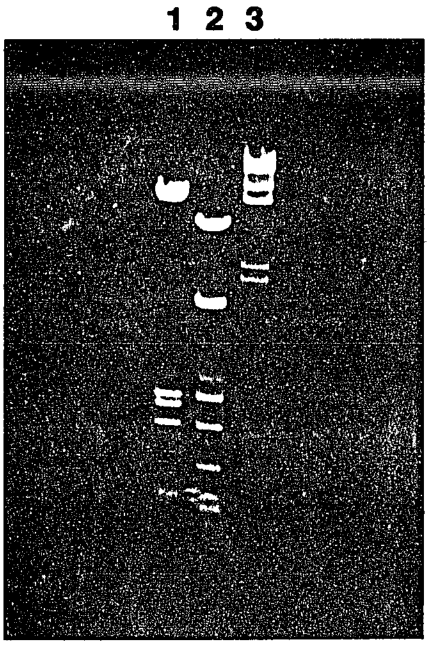


Figure 16. Restriction enzyme digestion of pPyLT1.

pPyLT1 DNA was digested with either *Acc* I (lane 1) or *Acc* I + *Bgl* I (lane 2) before electrophoresis in a 1% agarose gel. *Hind* III cut I DNA is included in lane 3 for molecular weight markers. The arrow highlights the 748 b.p. fragment to be gel purified.

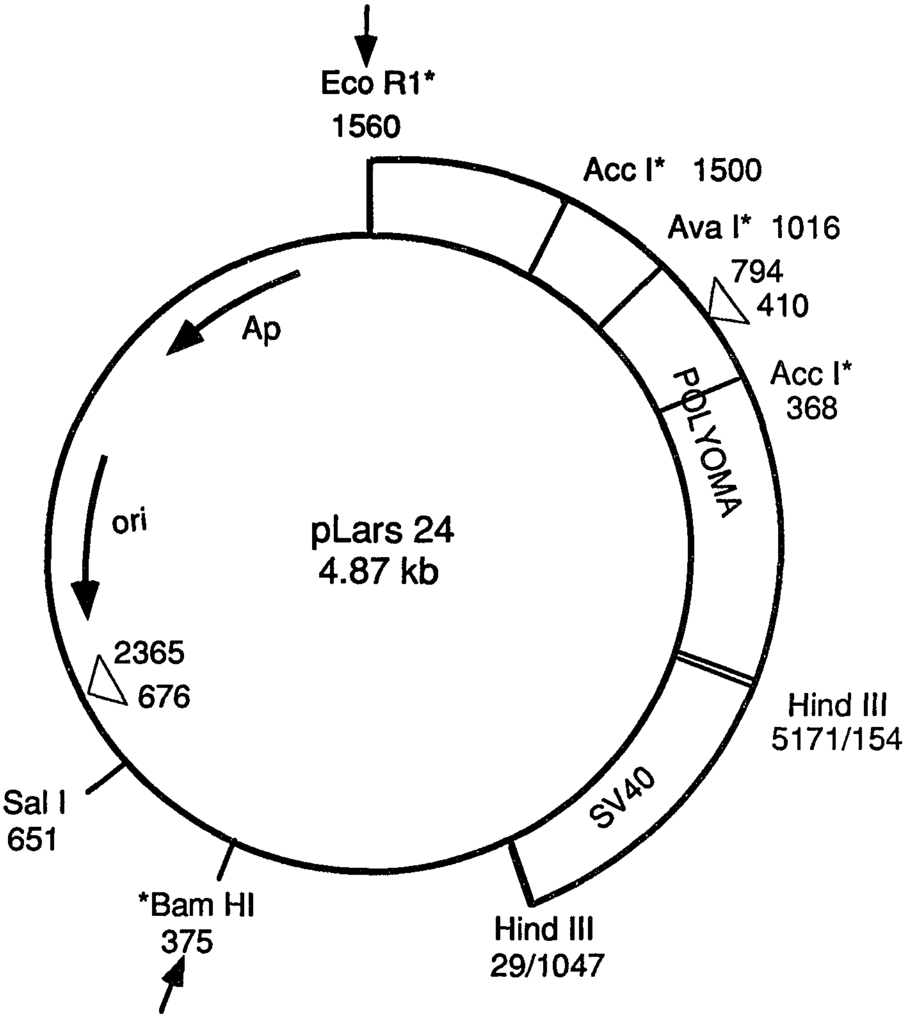


Figure 17. The structure of pLars 24 DNA (4.87 kb).

This plasmid is a modification of pLars 1. Replacement of the 1130 b.p. Acc I fragment with its corresponding shorter 748 b.p. Acc I fragment from pPyLT1 yields a deletion of 384 b.p. in the large T intron. This is denoted by the triangle outside the circle.
*sites have been verified by restriction enzyme analysis.

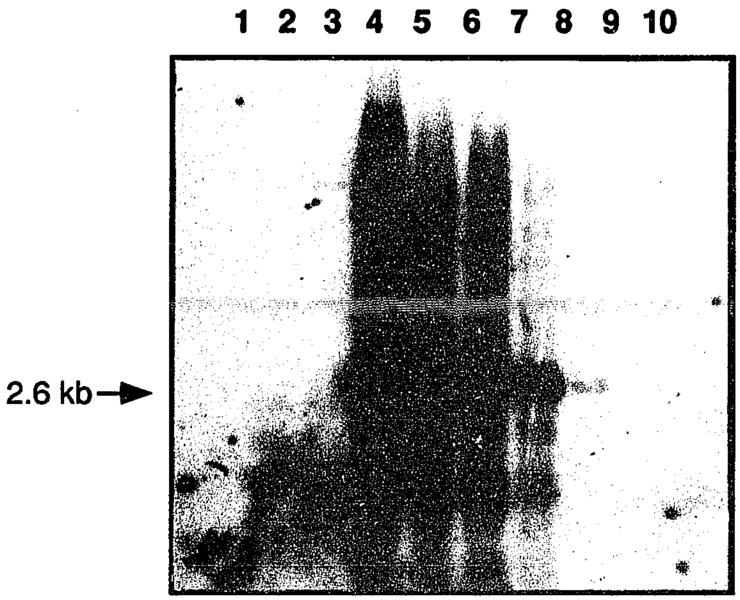


Figure 18. The replication of pJLYO and pPyLT-S in 3T3 cells.

3T3 cells were seeded at 37° C and transfected for 4 hours on the following day with 1mg pJLPYO (lanes 1 and 2), 1 mg pPSVE1Bla (lanes 3 and 4), 1mg pJLPYO and 1 mg pPSVE1Bla (lanes 5 and 6), 1 mg pJLPYO and 1 mg pPyLT-S (lanes 7 and 8) or calf thymus DNA (lanes 9 and 10). All transfections included 10 mg of calf thymus DNA. Low molecular weight DNA (Hirt extraction procedure) was either harvested 48 hours (lanes 1,3,5,7) or 72 hours (lanes 2,4,6,8) after transfection. The DNAs were analyzed for the replication of pJLPYO by digestion with *Dpn* I and *Sal* I followed by the Southern blot procedure. pJLPYO labeled with $[^{32}P]$ dCTP by the random - primer method was used as a probe. The arrow indicates the 2.6 kb linearized form of pJLPYO.

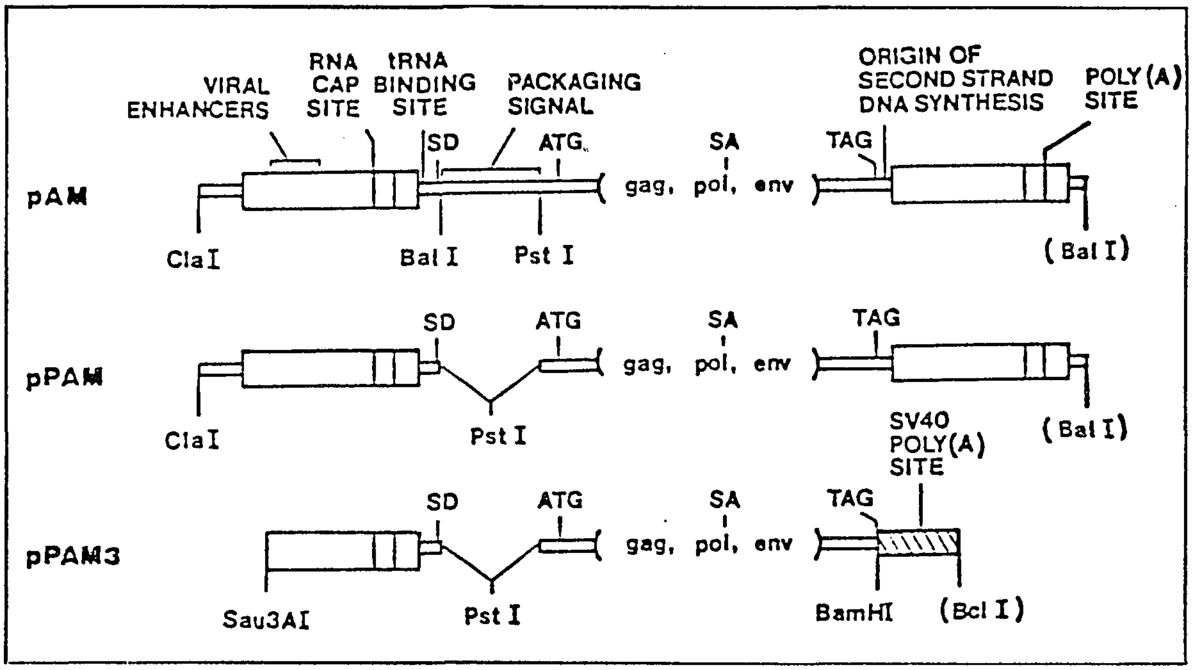


Figure 19. Structure of Packaging Construct used to Generate the Amphotropic Packaging Cell Line PA317.

Large open boxes represent retroviral LTRs; small open boxes represent other retroviral sequences; and hatched boxes denote simian virus 40 sequences. Landmark restriction sites are indicated. The plamid pPAM is a derivative of a recombinant amphotropic helper virus (pAM) in which the packaging signal has been deleted. pPAM3 was one of a series of constructs which contained modifications of pPAM. Important features of pPAM3 include: replacement of the 3' LTR with the late polyadenylation signal from SV40 and removal of viral sequences 5' of the viral enhancers in the 5' LTR. SD, splice donor, SA,splice acceptor.

Source: Miller and Buttimore 1986.

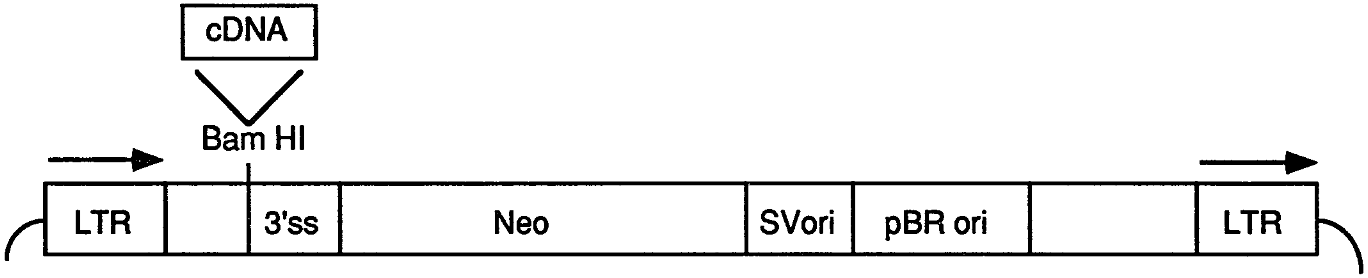
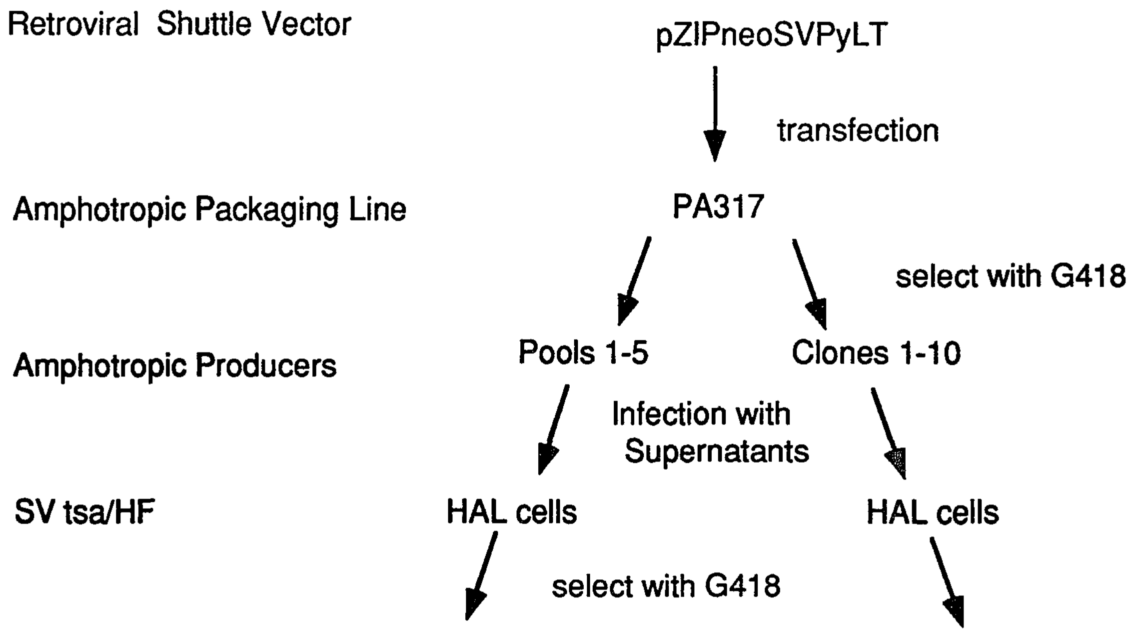


Figure 20. The structure of the retroviral vector pZIP-Neo SV(X)1.

This replication defective retroviral vector contains the neo gene in place of the env gene and a unique *Bam* H1 site, created for a second gene in place of the gag-pol genes. This vector can express both genes from the LTR. It is capable of replicating in *E. coli* because it contains the pBR322 origin of replication and in COS cells because it contains the SV40 origin of replication.



| Infectants | HP1 | HP2 | HP3 | HP4 | HC7 | HC8 | HC9 | HC10 | HC1-1 |
|---|---|---|---|---|---|---|---|---|---|
| Western Analysis SV40 LT | + | + | + | + | + | + | + | + | + |
| PyLT | - | - | - | - | - | - | + | + | + |
| Southern Analysis PyLT | + | + | + | N.D. | N.D. | N.D. | + | N.D. | + |

Figure 21. The generation of HAL infectants and the analysis for expression of PyLT antigen.

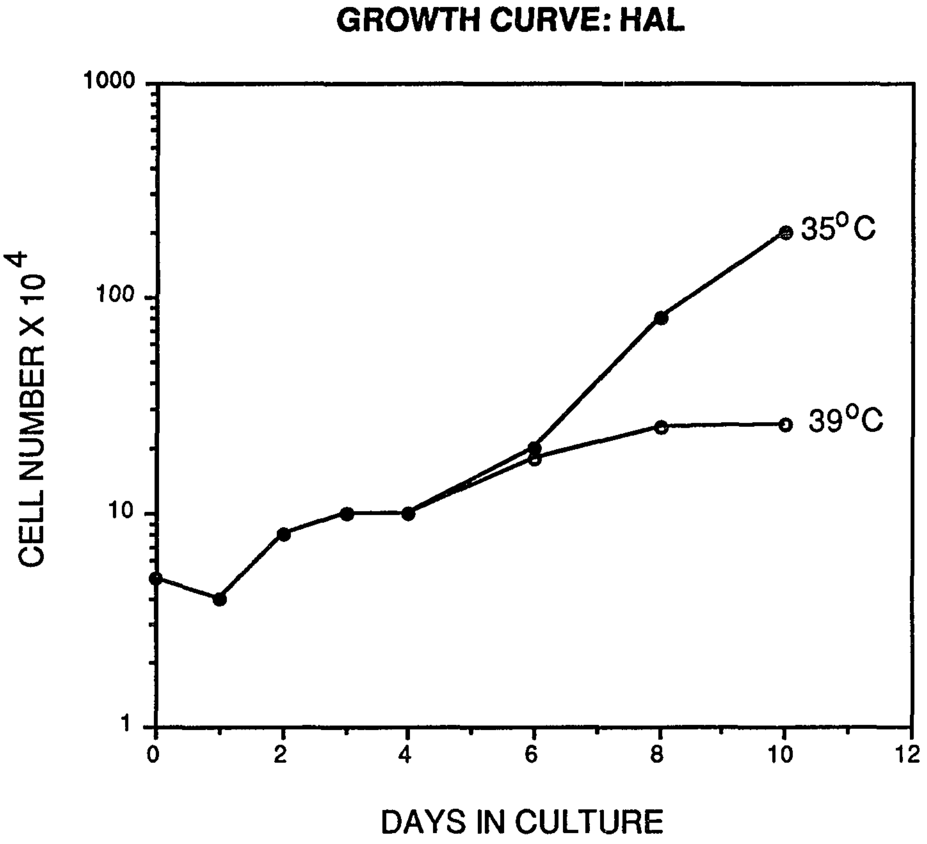


Figure 22. Growth curve of HAL.

$1 \times 10^5$ cells were seeded per 60mm dish at 35°C and subsequently shifted to 39°C or maintained at 35° C for determination of cell number.

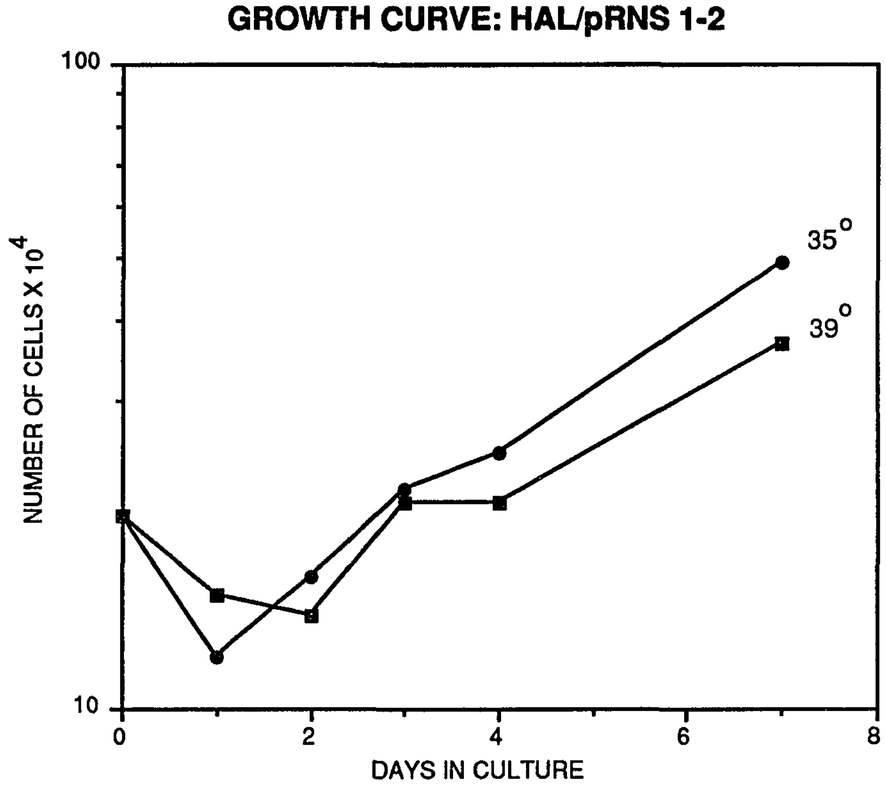


Figure 23. Growth curve of HAL/pRNS 1-2.

$2x10^5$ cells were seeded per 60mm dish at 35°C and subsequently shifted to 39° C or maintained at 35° C for determination of cell number.

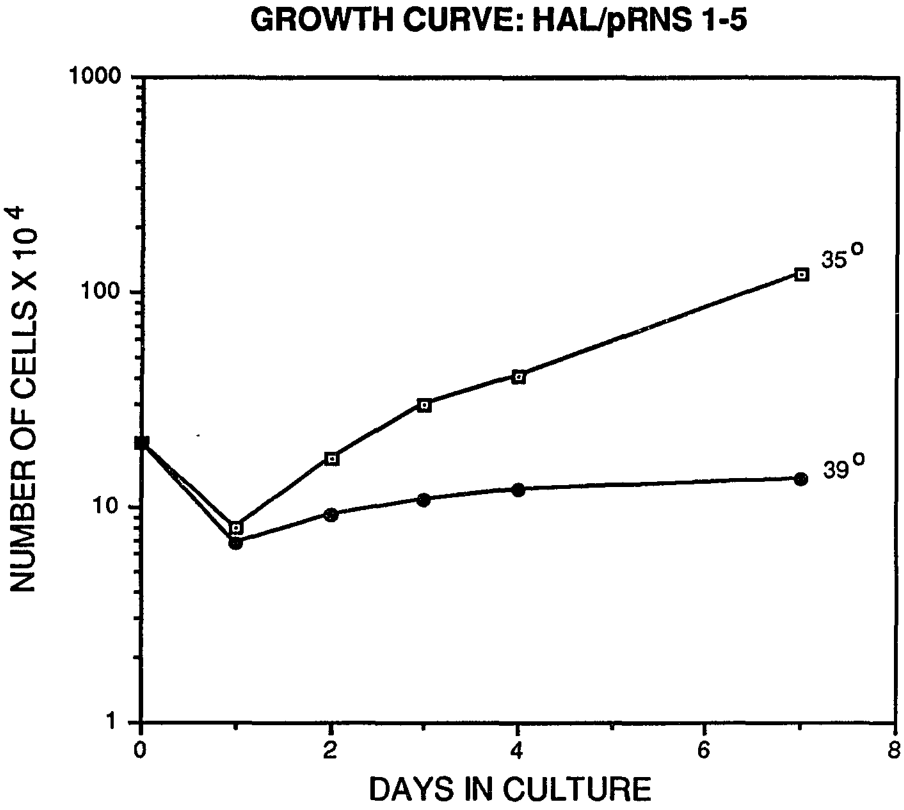


Figure 24. Growth curve of HAL/pRNS1-5.

$2x10^5$ cells were seeded per 60mm dish at 35°C and subsequently shifted to 39°C or maintained at 35°C for determination of cell number.

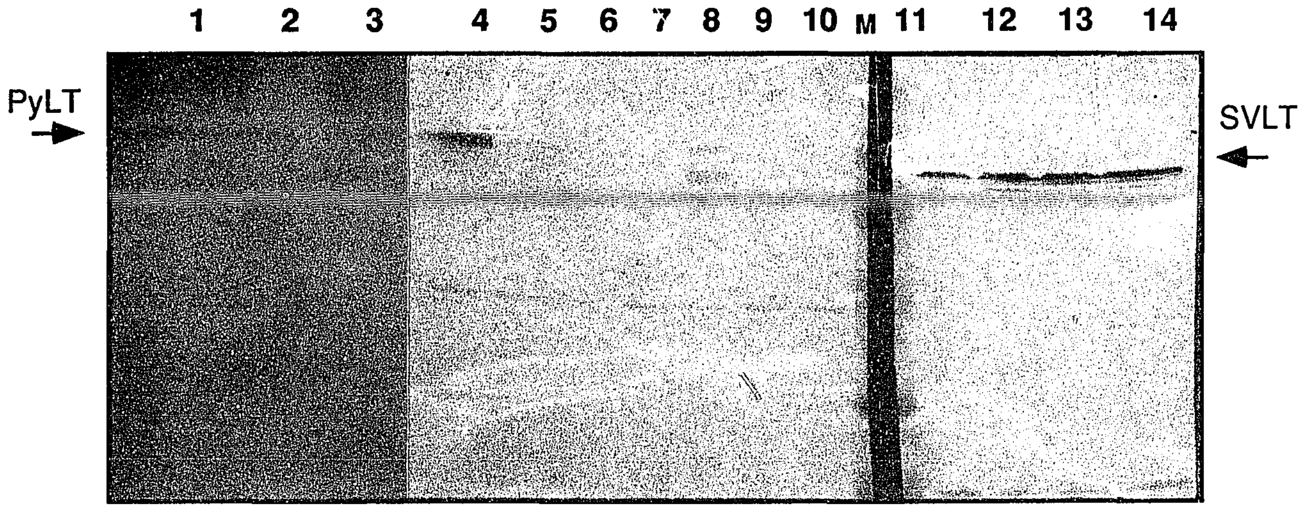


Figure 25. Immunoblot for PyLT and SVLT antigens in retrovirus infected HAL cells.

Protein extracts were electrophoresed in lanes 1-3 or immunoprecipitated with monoclonal antibody F4 to PyLT and then electrophoresed (lanes 4-10). Both immunoblots were reacted with F4 antibody followed by horse radish peroxidase conjugated antiglobulin. Lanes 11-14 were protein extracts which were electrophoresed and probed for SVLT with PAb419 followed by horse radish peroxidase conjugated antiglobulin. Lane 1: PyT54; lane 2: HC1-1; lane 3: HC9-1; lane 4: C9; lane 5: C7; lane 6: HAL/p4aA8; lane 7 and lane 13: HC7; lane 8 and 11: HC9; lane 9 and 14: HC8; lane 10 and 12: HAL and lane M: prestained molecular weight standards for 200, 97, 64, 43, 29, and 18 kD. C7 and C9 are producer cell lines making retrovirus containing PyLT sequences; HC1-1, HC7, HC8, HC9, and HC9-1 are retroviral infected HAL cell lines; HAL/p4aA8 is a transfected HAL cell line expressing the *hprt* gene and PyT54 is a transfected hamster cell line expressing PyLT antigen.

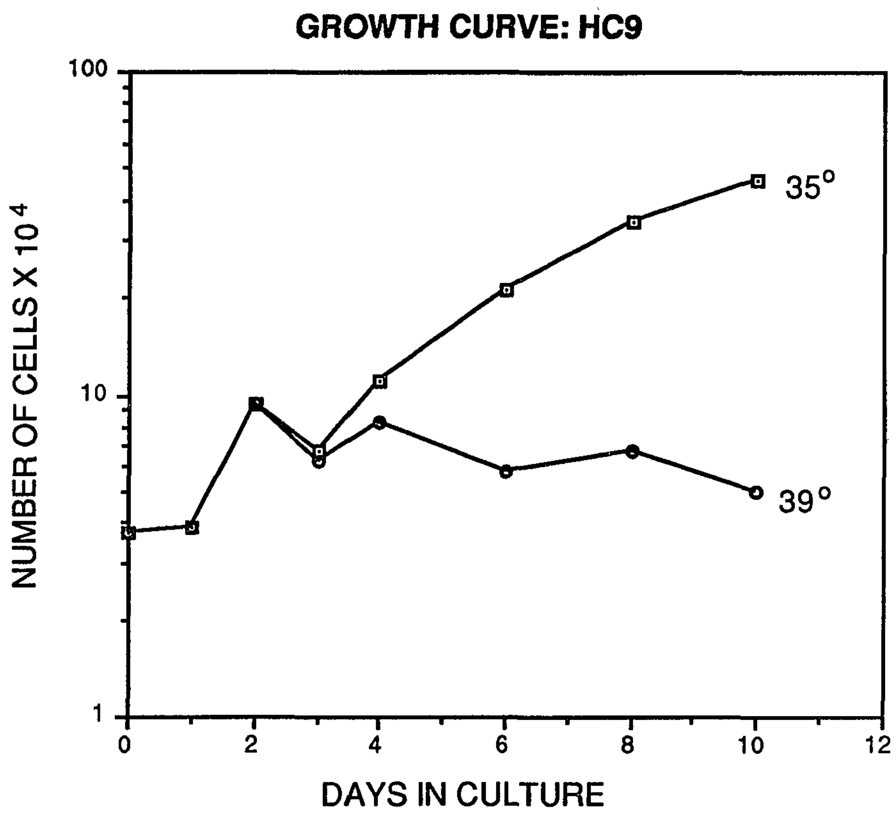


Figure 26. Growth curve of HC9.

$5 \times 10^4$ cells were seeded per 60mm dish at 35°C and subsequently shifted on day 3 or when the cells have reached a density of $1 \times 10^5$ cells to 39°C or maintained at 35°C for determination of cell number.

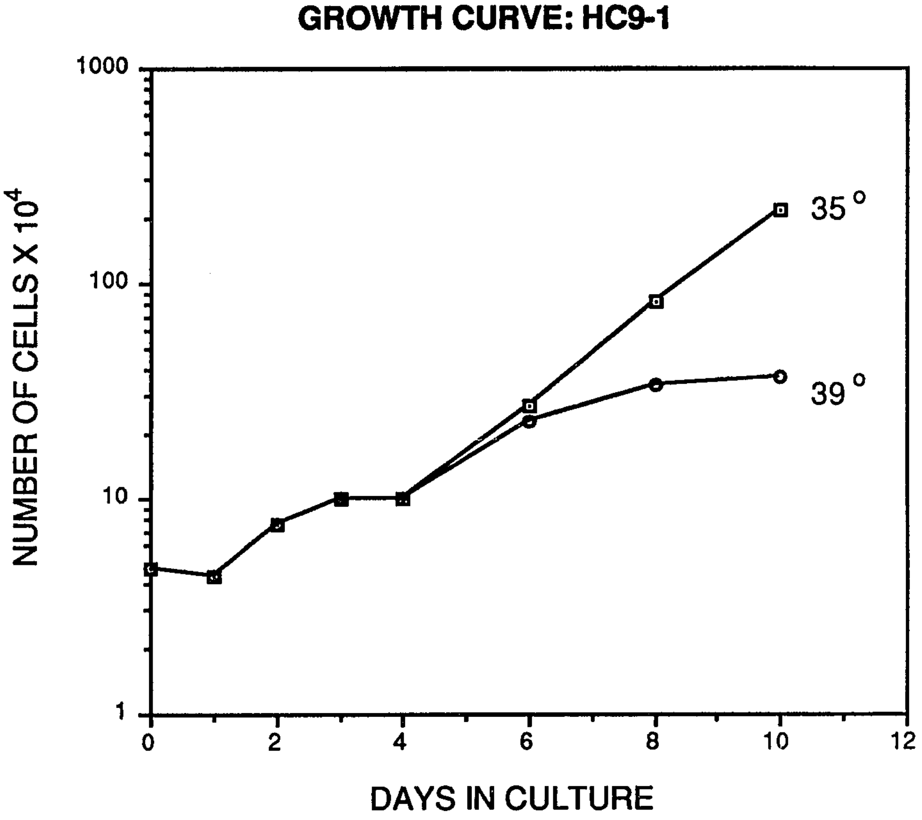


Figure 27. Growth curve of HC9-1.

$5 \times 10^4$ cells were seeded per 60mm dish at 35°C and subsequently shifted on day 3 or when the cells have reached a density of $1 \times 10^5$ cells to 39°C or maintained at 35°C for determination of cell number.

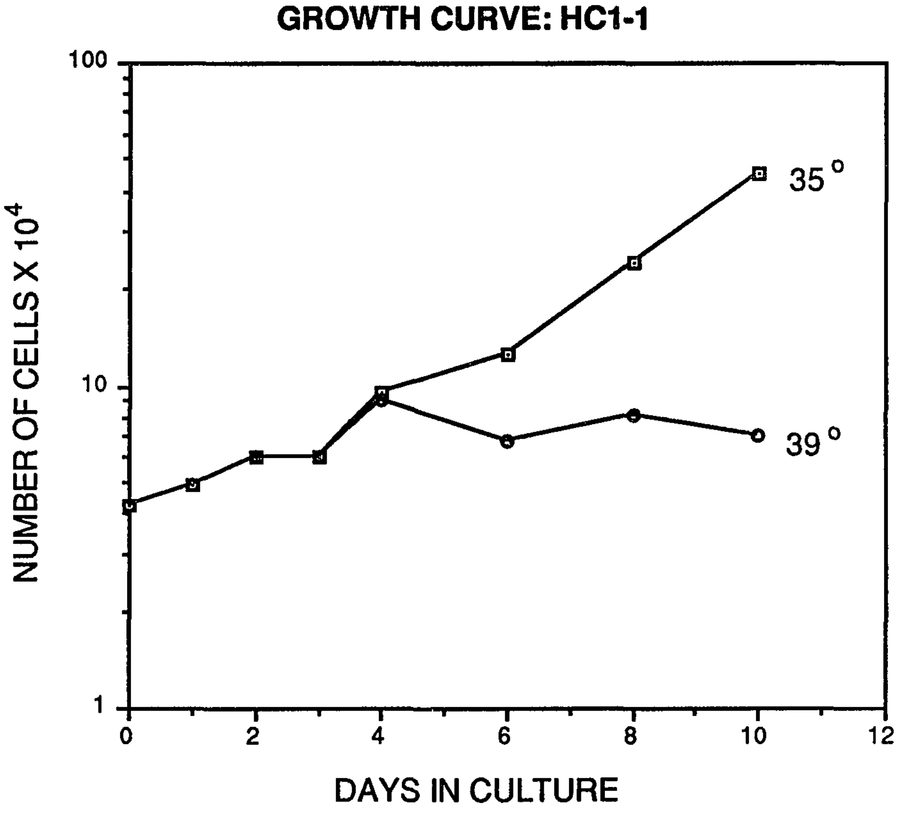


Figure 28. Growth curve of HC1-1.

$5x10^4$ cells were seeded per 60mm dish at 35°C and subsequently shifted on day 3 or when the cells have reached a density of $1x10^5$ cells to 39°C or maintained at 35°C for determination of cell number.

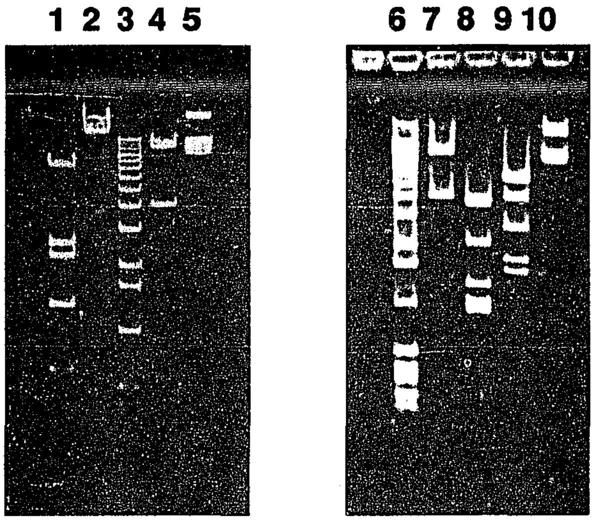


Figure 29. Restriction enzyme digest of pZIPneoSV(PyLT) DNA.

DNA samples are either left uncut (lane 5 and 10), digested with *Hind* III and *Sal* I (lane 1), *Sal* I (lane 2), *Bam* HI (lanes 4 and 7), *Ava* I (lane 8) or *Xba* I (lane 9). A 1kb ladder is shown in lanes 3 and 6.

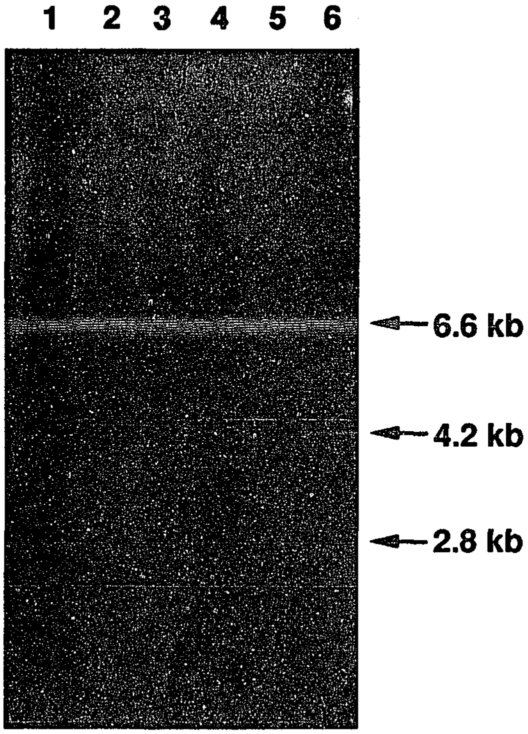


Figure 30. Southern analysis of cellular DNAs from retrovirus infected HAL cells.

10 µg of DNA were digested with *Bam* HI (lanes 1,3,5) or *Xba* I (lanes 2,4,6,) and electrophoresed in a 1% agarose gel at 30 V for 17 hours. The DNA was transferred to nitrocellulose and hybridized to 1x$10^7$cpm $^{32}$ P-labeled probe to polyoma (made from a gel purified 5.2 kb *Bam* HI fragment of pAT153-Py). This autoradiograph was exposed for 3 hours. HP3 (lanes 1 and 2) and HP1 (lanes 3 and 4) are retrovirus infected HAL cell lines. P3 (lanes 5 and 6) is a producer cell line making retrovirus encoding PyLT sequences.

Figure 31. Southern analysis of cellular DNAs from retrovirus infected HAL cells.

All DNA was left uncut and either 2μg (lanes 1-4) or 10μg (lanes 5-8) were electrophoresed in a 1% agarose gel at 30 V for 17 hours. The DNA was transferred to nitrocellulose and hybridized to $1\times10^7$ cpm $^{32}$P-labeled probe to polyoma (made from a gel purified 5.2 kb *Bam* HI fragment of pAT153-Py). This autoradiograph was exposed for 20 hours. P3 (lanes 1 and 5) is the producer line. HP3 (lanes 2 and 6); HP1 (lanes 3 and 7) and HP4 (lanes 4 and 8) are retrovirus infected HAL cell lines.

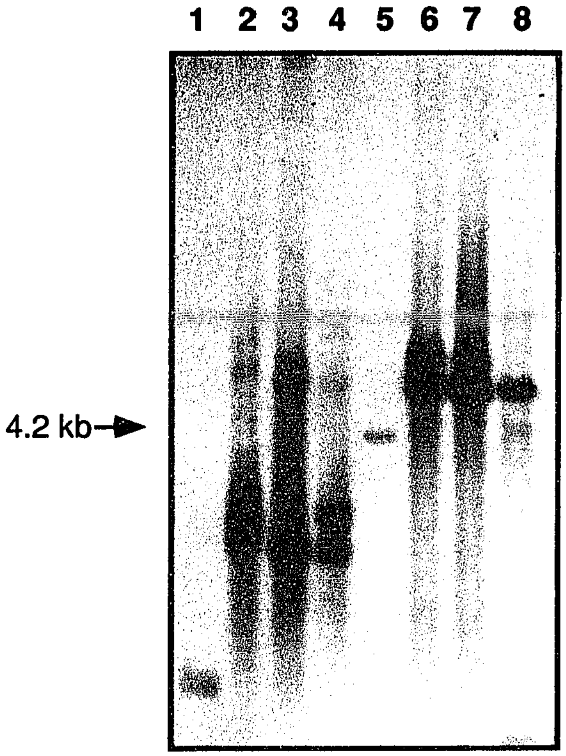


Figure 32. Southern analysis of cellular DNAs from retrovirus infected HAL cells.

10 µg of DNA was either left uncut (lanes 1-4) or digested with *Bam* HI (lanes 5-8) and electrophoresed in a 1% agarose gel at 30 V for 17 hours. The DNA was transferred to nitrocellulose and hybridized to $1 \times 10^7$ cpm $^{32}P$-labeled probe to polyoma (made from a gel purified 5.2 kb *Bam* HI fragment of pAT153-Py). This autoradiograph was exposed for 20 hours. C9 (lanes 1 and 5) is a producer cell line. HC9-1 (lanes 2 and 6); HC9-2 (lanes 3 and 7); and HC9 (lanes 4 and 8) are retrovirus infected HAL cell lines. A 1kb molecular weight ladder has been included in lane 1.

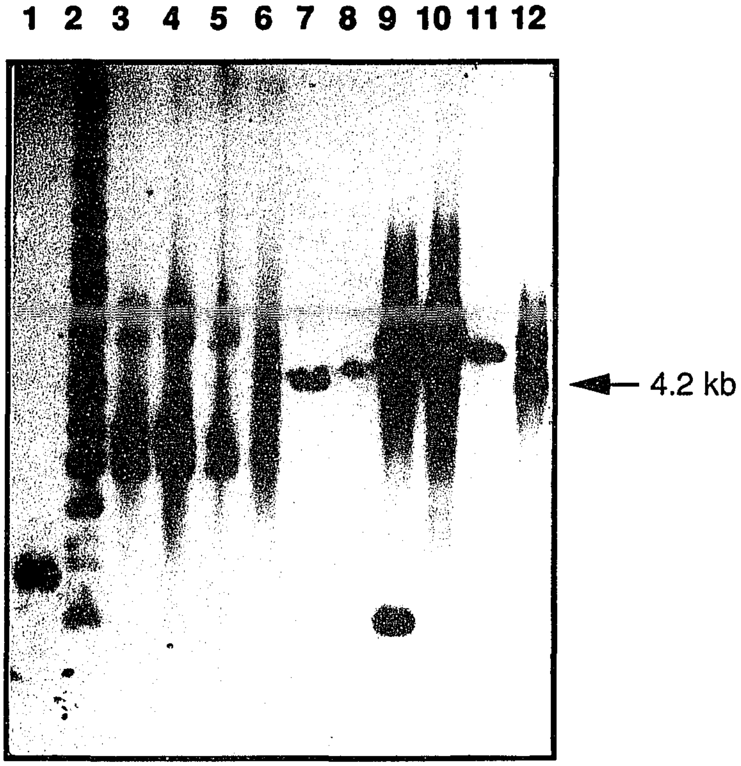


Figure 33. Southern analysis of cellular DNAs from retrovirus infected HAL cells.

10 $\mu$g of DNA was either left uncut (lanes 1-6) or digested with *Bam* HI (lanes 7-12) and electrophoresed in a 1% agarose gel at 30 V for 17 hours. The DNA was transferred to nitrocellulose and hybridized to $1 \times 10^7$ cpm $^{32}$P-labeled probe to polyoma (made from a gel purified 5.2 kb *Bam* HI fragment of pAT153-Py). This autoradiograph was exposed for 20 hours. C9 (lanes 1 and 7) is a producer cell line. HC9A (lanes 2 and 8); HC9 (lanes 3 and 9); HC9-1 (lanes 4 and 10); HC9-3 (lanes 5 and 11) and HC1-1 (lanes 6 and 12) are retrovirus infected HAL cell lines. A 1kb ladder is included in lane 1 and a ladder for supercoiled DNA is included in lane 2.

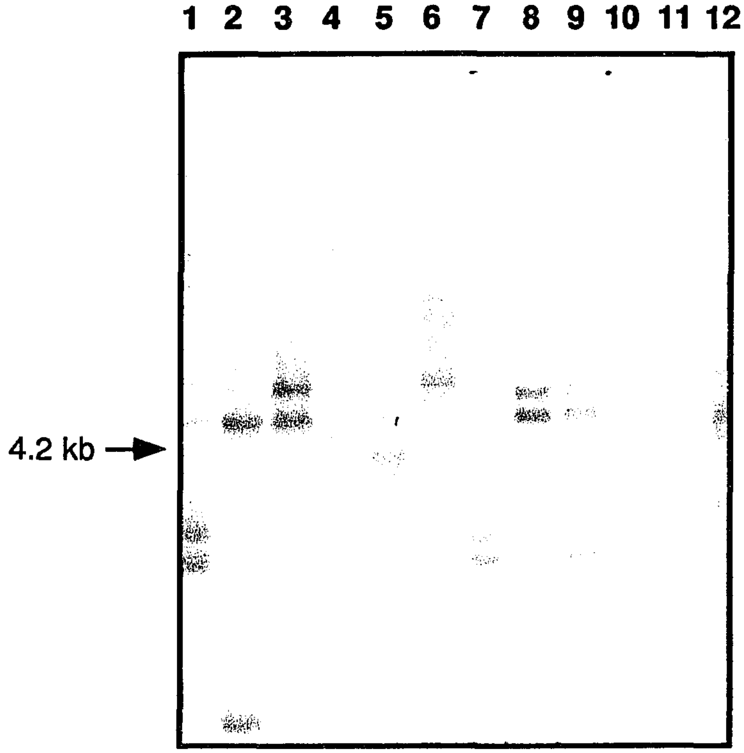


Figure 34. Southern analysis of Hirt supernatants and pellets from retrovirus infected HAL cells.

Hirt supernatants were left uncut (lanes 1,4,7), *Bam* HI digested (lanes 2,5,8) or *Xba* I digested (lanes 3,6,9). The Hirt pellets were *Bam* HI digested (lanes 10,11,12). The DNA was electrophoresed in a 1% gel at 30 V for 17 hours and subsequently transferred to nitrocellulose and hybridized to $1 \times 10^7$ cpm $^{32}$P-labeled probe to polyoma (made from a gel purified 5.2 kb *Bam* HI fragment of pAT153-Py). This autoradiograph was exposed for 20 hours. HC9 (lanes 1,2,3,11); HC1-1 (lanes 4,5,6,12) and HC9-1 (lanes 7,8,9,10) are retrovirus infected HAL cell lines.

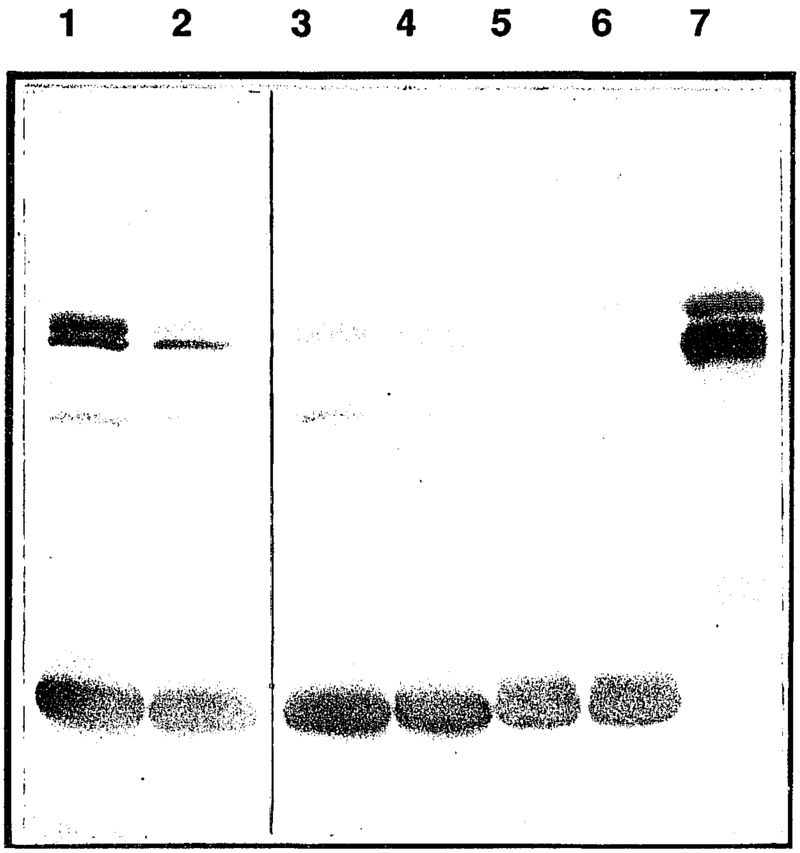


Figure 35. Rb-PyLT complexes in retrovirus infected HAL cells.

HC1-1 cells were cultured for 4 days at 35° C (lanes 1,3,5) or 39° C (lanes 2,4,6) and analyzed after immunoprecipitation with PAb C36 to Rb-1 (lanes 1-4) or control PAb M73 to AdE1A (lanes 5 and 6). Lane 7 contains 200 ng of immunoaffinity purified PyLT. The immunoblot was separated and reacted with either C36 (lanes 1 and 2) or monoclonal antibody F4 to PyLT (lanes 3-7) followed by alkaline phosphatase conjugated antiglobulin.

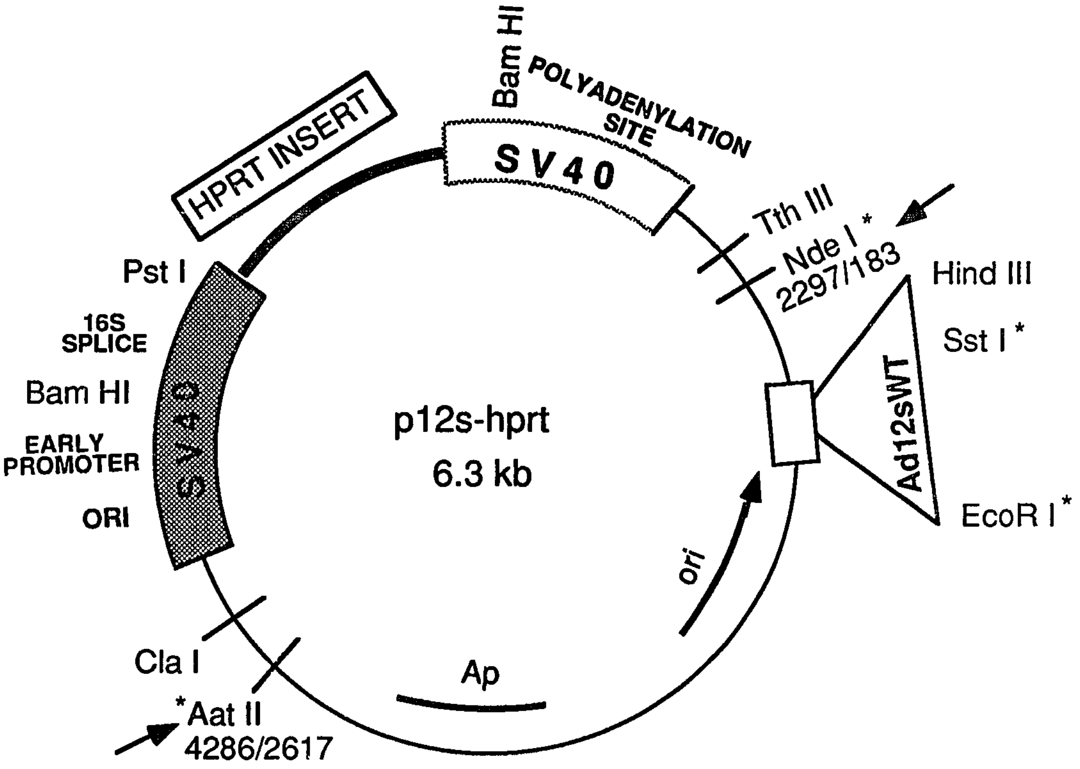


Figure 36. The structure of p12s-hprt DNA (ca 6.3kb).

This recombinant plasmid contains a 2.5 kb *Aat* II-*Nde* I fragment derived from p4aA8, an Okayama-Berg derived pCD1 vector (Okayama and Berg 1983) encoding the cDNA for human *hprt* and a 3.8 kb *Aat* II-*Nde* I fragment of a pUC18 vector containing the sequences encoding the 12S E1A gene cloned into the *Eco* RI and *Sst* I sites of the polylinker ( Moran l988).
*denotes sites which have been verified by restriction enzyme analysis.

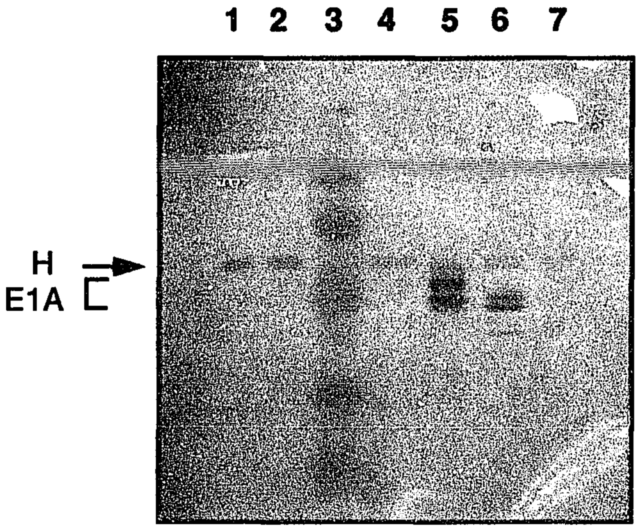


Figure 37. Immunoblot analysis of E1A 12S protein in HAL transfectants.

Extracts containing equal amounts of proein were immunoprecipitated with monoclonal antibody M73 to AdE1A and analyzed by immunoblot (as described in Methods) with M73 and horse radish peroxidase conjugated antiglobulin. H/12s#4 (lane 1); H/12s#5 (lane 2); H/12s#1 (lane 6); and H/12s#7 (lane 7) are cell lines isolated from the transfection of HAL cells with p12s-hprt. HAL cell extract is included in lane 4 and 293 cell extract (adenovirus transformed epithelial cells) in lane 5. Prestained molecular weight standards for 200, 97, 64,43, 29 and 18kD proteins are included in lane 3. Markers are indicated for the mobility of IgG heavy chains (H) and AdE1A proteins (E1A).

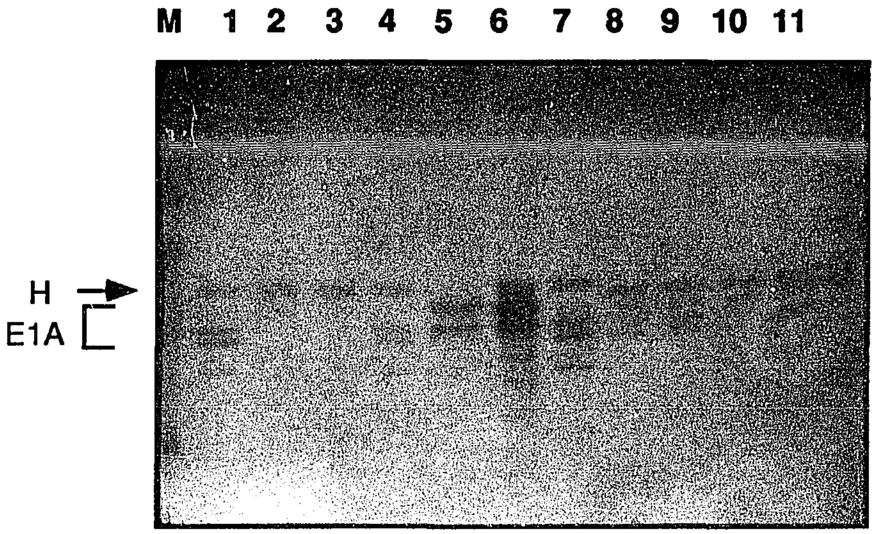


Figure 38. Immunoblot analysis of AdE1A 12S protein and AdE1A-Rb complexes in HAL transfectants.

Extracts containing equal amounts of protein were either immunoprecipitated with monoclonal antibody M73 to AdE1A protein (lanes 1-7) or monoclonal antibody C36 to Rb protein (lanes 8-11). The immunoblot was probed with M73 and horse radish peroxidase conjugated antiglobulin. H/12s#3 (lanes 1 and 9); H/12s#14 (lanes 3 and 10); H/12s#10 (lanes 4 and 8) and H/12s#11 (lane 7) are cell lines isolated from the transfection of HAL cells with p12shprt. HAL cell extract is included in lane 2 and 293 cell extract (adenovirus transformed epithelial cells) in lanes 6 and 11. Prestained molecular weight markers and markers for the mobility of IgG heavy chains (H) and AdE1A proteins (E1A) are indicated.

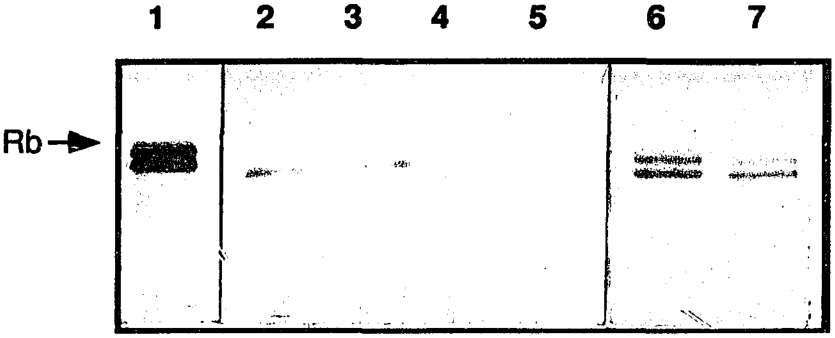


Figure 39. Rb-AdE1A complexes in transfected HAL cells.

293 cells (lane 1) or H/12s#1 cells cultured for 3 days at 35° C (lanes 2,4,6) or 39° C (lanes 3,5,7) were analyzed after immunoprecipitation with different monoclonal antibodies. The composite immunoblot was reacted with monoclonal antibody C36 to Rb protein followed by alkaline phosphatase conjugated antiglobulin. Lane1: extract from 293 cells immunoprecipitated with M73 antibody to AdE1A; lane 2 and 3: extract immunoprecipitated with M73 antibody to AdE1A; lane 4 and 5: extract immunoprecipitated with control antibody; lane 6: residual supernatant from immunoprecipitation in lane 2 reprecipitated with C36; lane 7: residual supernatant from immunoprecipitation in lane 3 reprecipitated with C36.

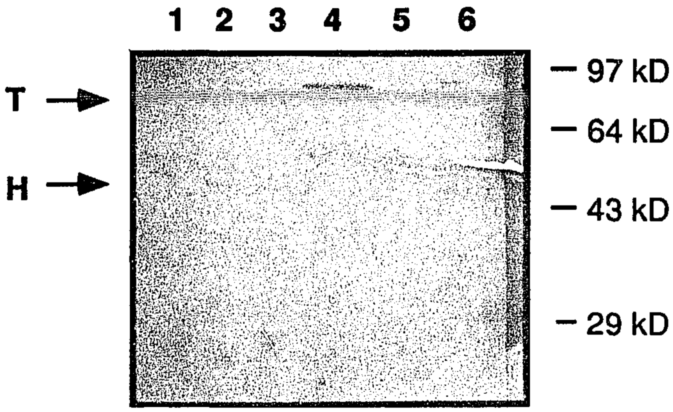
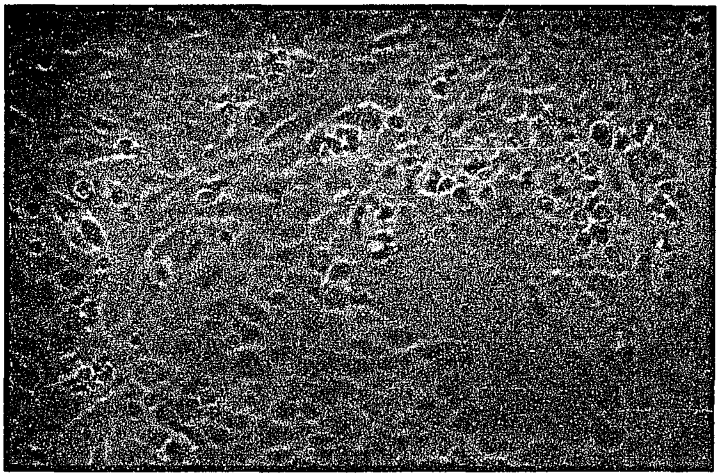
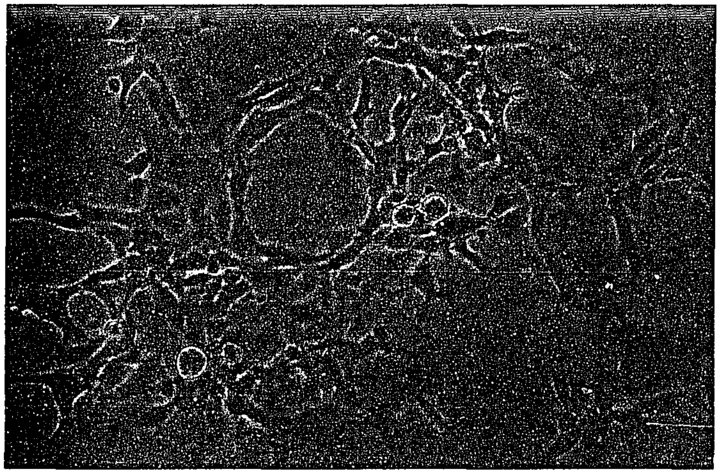


Figure 40. SVLT-Rb complexes in transfected HAL cells.

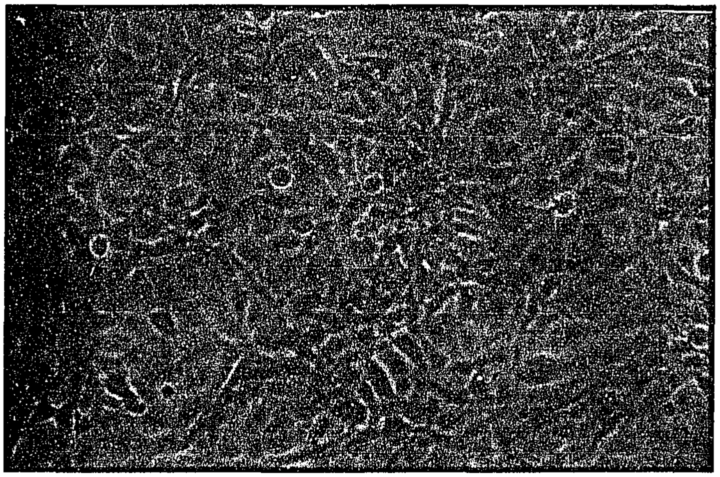
Extracts containing equal amounts of protein were immunoprecipitated with monoclonal antibody C36 to Rb-I and analyzed by immunoblot with PAb419 to SV40 T antigen and horse radish peroxidase conjugated antiglobulin. H/RNS-1P (35° C) (lane 1) and H/RNS-1P (39° C) (lane 2) are mass cultures of HAL transfected with wild type SV40 LT. H/12s#13 (lane 3) and H/12s#1 (lane 6) are cell lines isolated from the transfection of HAL with p12s-hprt. An extract of HAL cells is included in lane 5. An extract of a HAL cell line transfected with PyLT is included in lane 4. Prestained molecular weight standards and markers for the mobility of IgG heavy chains (H) and SVLT (T) are indicated.



a. Ad-1 cells grown at 39° C.



b. H/12s#1 cells grown at 35° C.



c. HAL cells grown at 35° C.

Figure 41. Morphology of a: Ad-1, b: H/12s#1 and c: HAL cell lines.

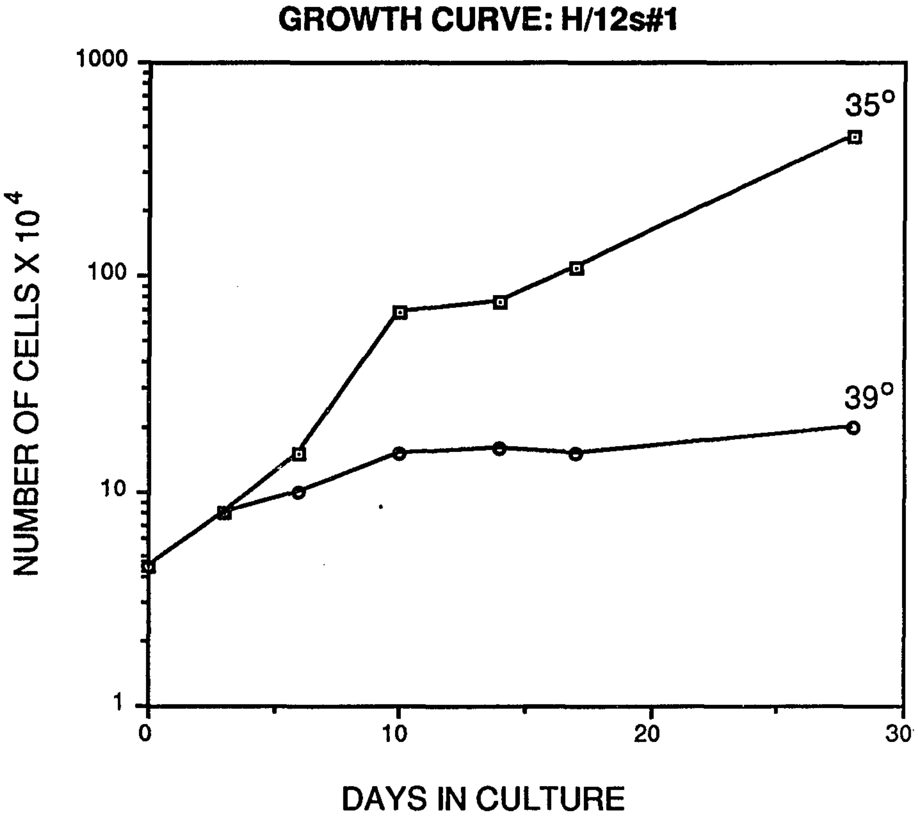


Figure 42. Growth curve of H/12s#1.

5x10$^4$ cells were seeded per 60mm dish at 35° C and subsequently shifted on day 3 to 39° C for determination of cell number.

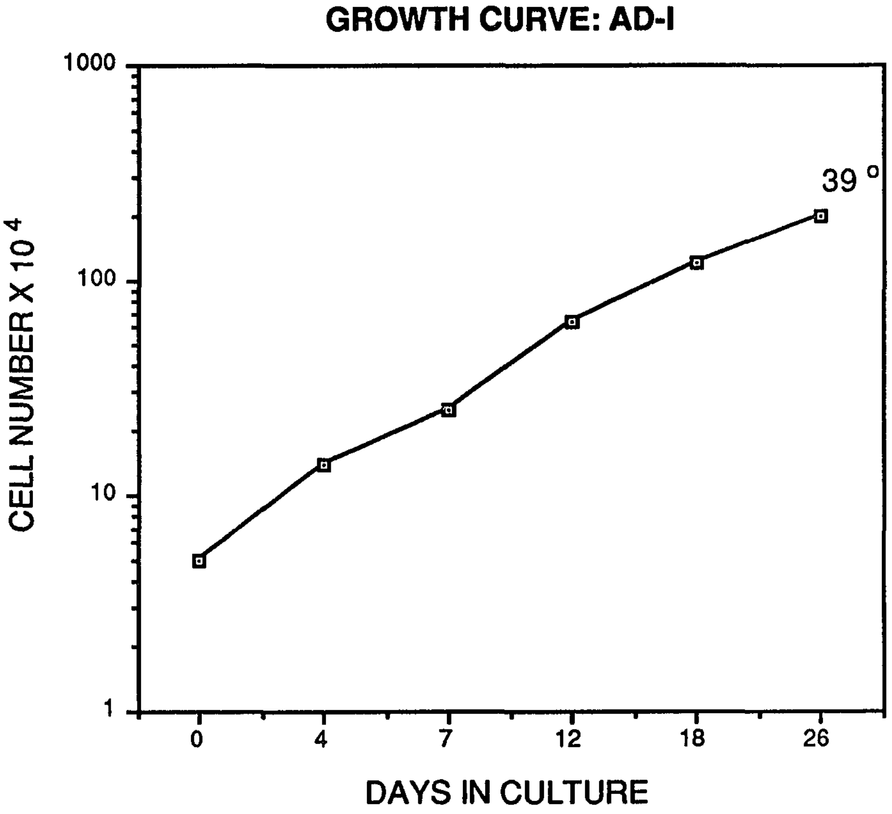


Figure 43. Growth curve of Ad-1.

$5x10^4$ cells were seeded per 60mm dish at 39° C and maintained for determination of cell number.

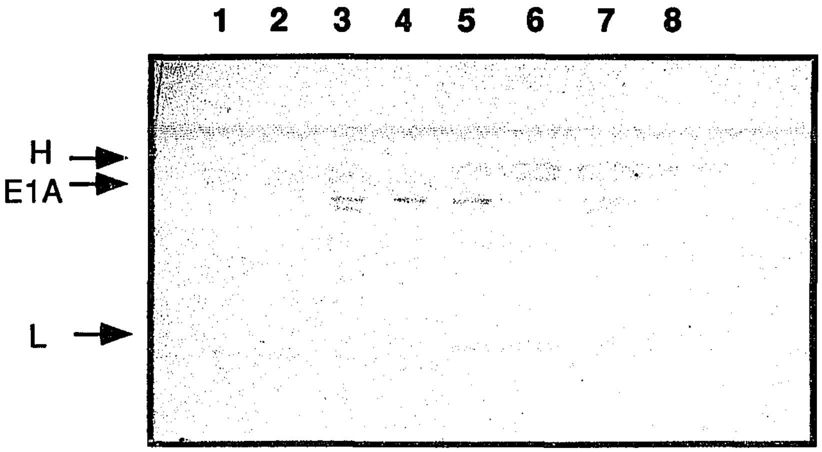


Figure 44. Immunoblot analysis of AdE1A 12S protein and AdE1A-Rb complexes in Ad-1 and H/12s#1.

Equal amounts of protein extract were immunoprecipitated with monoclonal antibody M73 to AdE1A (lanes 5-8) or monoclonal antibody C36 to Rb-1 (lanes 1-4) and analyzed by immunoblot with M73. Lanes 1 and 8: H/12s#11; lanes 2 and 6: H/12s#1 (late passage); lane 7: H/12s#1 (freeze down of early passage); lanes 3 and 5: Ad-1 39° C; lane 4: Ad-1 35° C. Markers are indicated for the mobility of IgG heavy chains (H), IgG light chains (L) and AdE1A (E1A).

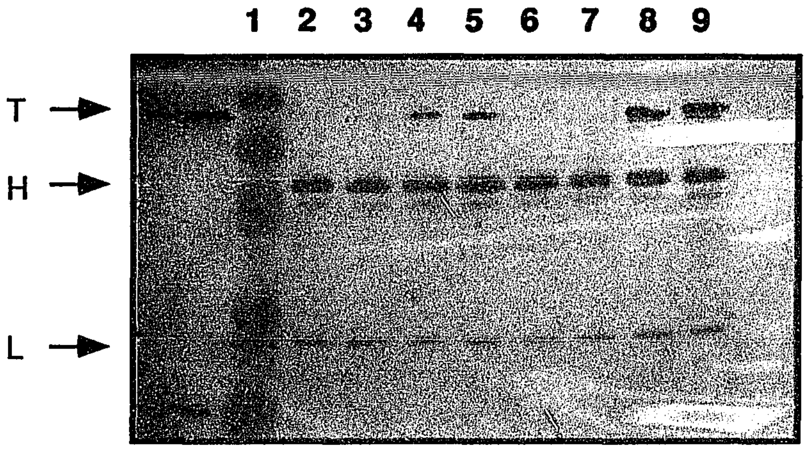


Figure 45. SVLT-p53 complexes in transfected HAL cells.

HAL and transfected HAL cell lines were cultured for 3 days at 35° C (lanes 4,5 and 7) or 39° C (lanes 2,3,6, and 8). After immunoprecipitation with monoclonal antibody PAb421 to p53 the immunoblot was reacted with PAb419 to SVLT and horse radish peroxidase conjugated antiglobulin. Lane 1: Prestained molecular weight standards; lane2: Ad-1 39° C; lane3: HAL 39° C; lane 4: HAL 35° C; lane 5: H/12s#1 35° C; lanes 6 and 7: H/12s#1 39° C; lane 8: H/RNS-1P 35° C and lane 9: H/RNS-1P 39° C. Markers are indicated for the mobility of IgG heavy chains (H), IgG light chains (L) and SVLT (T).

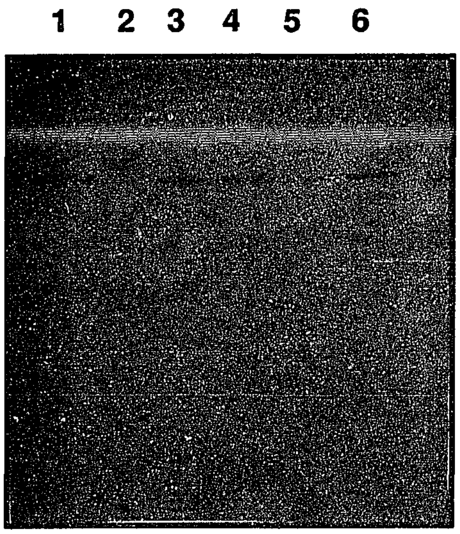
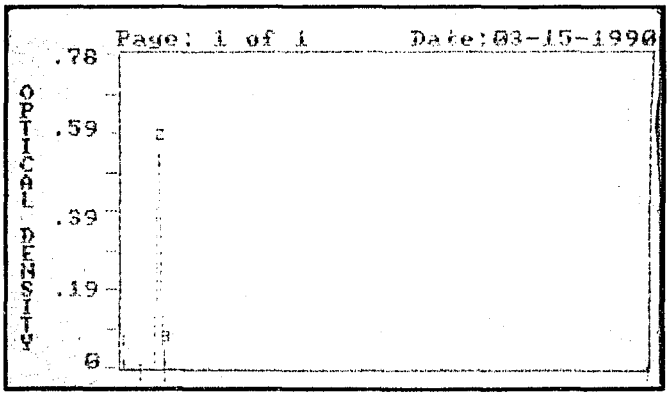
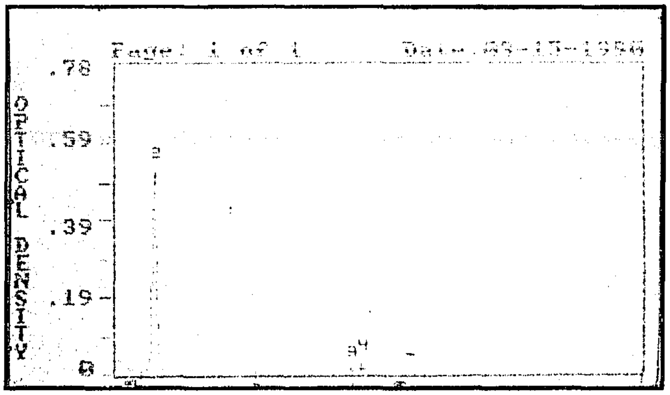


Figure 46. Immunoblot analysis of SVLT in Ad-1 cells.

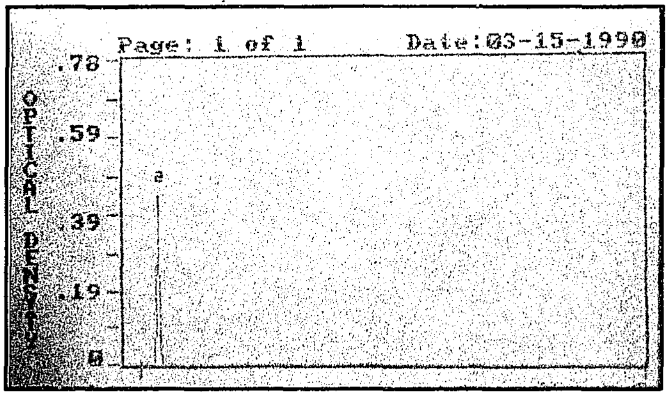
Protein extracts of HAL (lanes 1 and 3), H/12s#1 (lane 4), Ad-1 39° C (lane 5) and Ad-1 35° C (lane 6) were analyzed by immunoblot with monoclonal antibody PAb419 to SVLT. Prestained molecular weight standards are indicated in lane 2. The protein extracts in lanes 5 and 6 were comparable but half the amount contained in lanes 1,3 and 4.



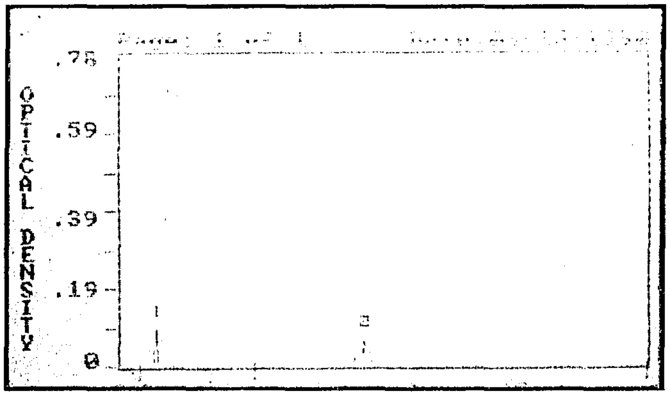
HAL (lane 3)



H/12s#1 (lane 4).



Ad-1 35°C (lane 6).



Ad-1 39° C (lane 5).

Figure 47. Scanning densitometry of SVLT immunoblot analysis in Ad-1 cells (Figure 46).

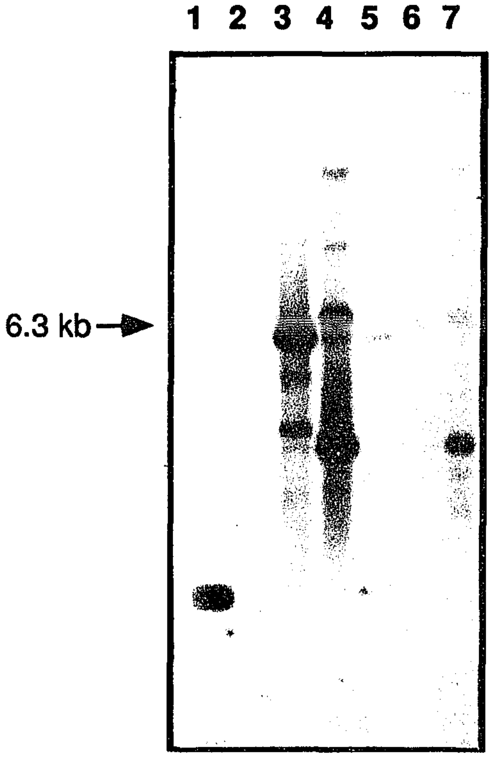


Figure 48. Southern analysis of Hirt supernatants from Ad-1 and H/12s#1.

Hirt supernatants were uncut (lanes 4 and 6) or *Aat* II-*Nde* I digested (lanes 3 and 5), electrophoresed in a 1% agarose gel and probed by Southern blot with a $^{32}$P-labeled 1.7 kb *Eco* RI-*Pst* I fragment of E1A WT sequences from pE1AWT. Lane 1: 1kb molecular weight ladder; lane 2: 10 pg of a gel purified 1.7 kb *Eco* RI-*Pst* I fragment of pE1AWT; lane 3 and 4: Ad-1 35° C; lanes 5 and 6: Ad-1 39° C and lane 7: uncut HMW preparation of H/12s#1.

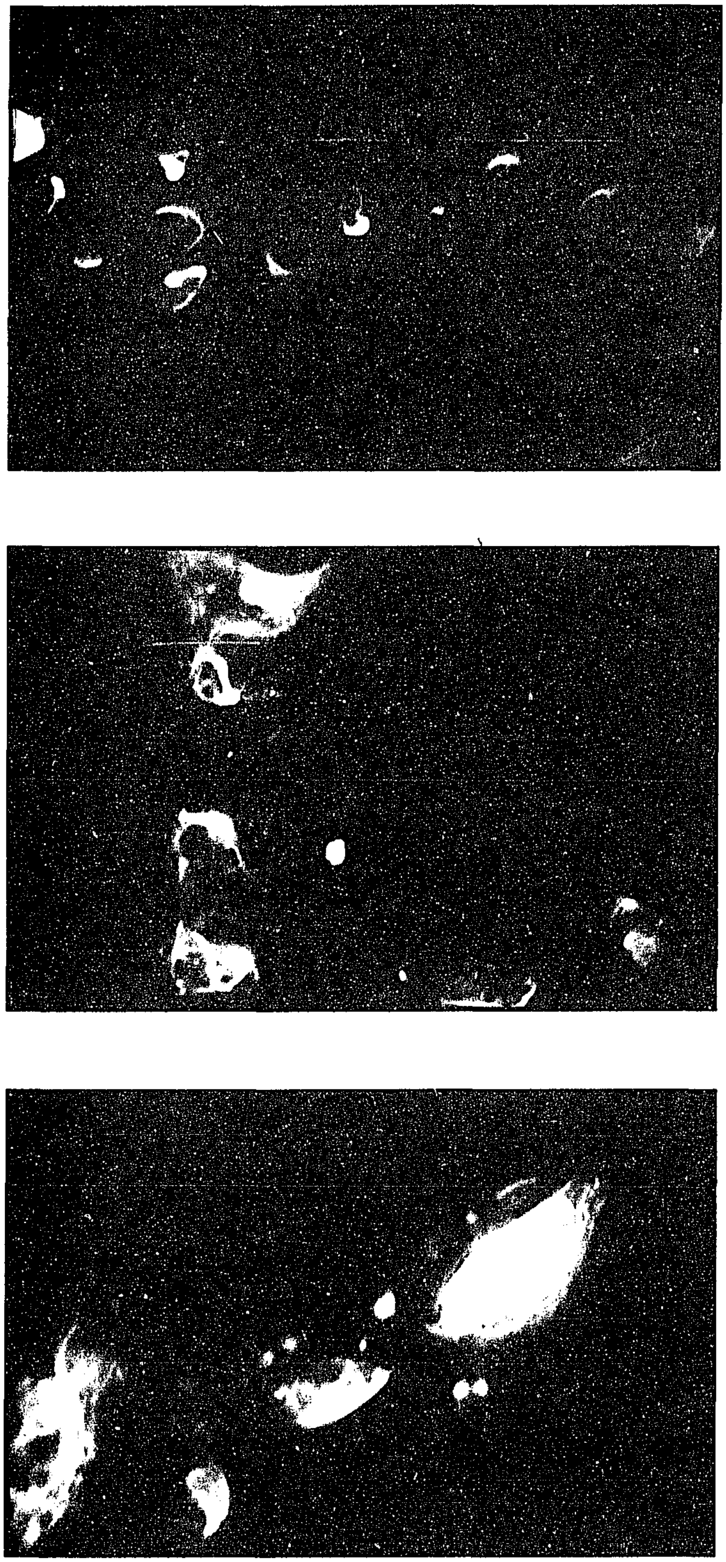


Figure 49. Immunofluorescent staining of Ad-1 cells with polyclonal antibody to cytokeratins.

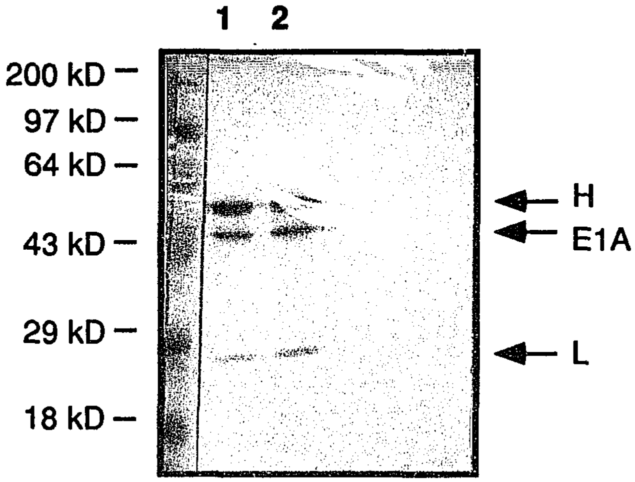


Figure 50. Immunoblot analysis of AdE1A protein in early and late passage Ad-1 cells.

Protein extracts of Ad-1, early passage (lane 1) and late passage (lane 2) are immunoprecipitated with monoclonal antibody M73 and analyzed by immunoblot with M73. Prestained molecular weight standards and markers for the mobility of IgG heavy chain (H), IgG light chain (L) and Ad-E1A (E1A) are indicated.

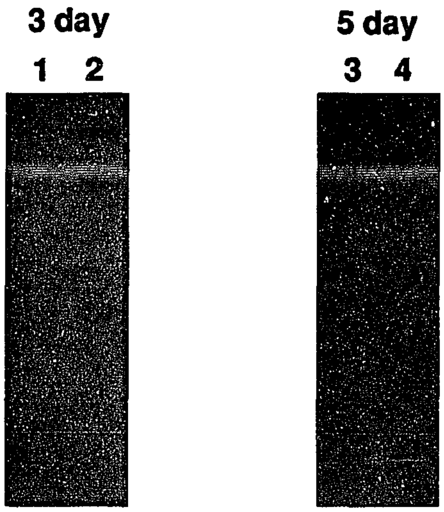


Figure 51. Autoradiograph of $^{35}$S Methionine-labeled immunoprecipitates in Ad-1 and H/12s#1.

Protein extracts from Ad-1 (lanes 2 and 4) and H/12s#1 (lanes 1 and 3) were prepared from 100mm dishes of cells incubated with Methionine-free medium containing 100 μCi/ml of $^{35}$S Methionine for 2 hours. The protein extracts were immunoprecipitated with monoclonal antibody M73 to AdE1A. The autoradiograph was exposed for 3 days (lanes 1 and 2) and 5 days (lanes 3 and 4). Molecular weight markers are indicated for each exposure.

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