Neufeld, David Samuel

IMMORTALIZATION AND CHARACTERIZATION OF HUMAN FIBROBLASTS TRANSFORMED WITH AN ORI(-) SV40 GENOME

City University of New York PH.D. 1986

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IMMORTALIZATION AND CHARACTERIZATION OF HUMAN FIBROBLASTS TRANSFORMED WITH AN ORI$^-$ SV40 GENOME

by

DAVID SAMUEL NEUFELD

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.

1986

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ABSTRACT

IMMORTALIZATION AND CHARACTERIZATION OF HUMAN FIBROBLASTS TRANSFORMED WITH AN ORI$^-$ SV40 GENOME

by

David S. Neufeld

Adviser: Professor Harvey L. Ozer

Human diploid cells derived from normal tissue undergo a characteristic pattern of growth in cell culture, ending in a non-replicative phase (Phase III), and, finally, cell death. Human diploid fibroblasts infected in vitro by the oncogenic DNA virus, SV40, become immortal at an enhanced frequency. Considerable viral DNA synthesis is also evident. To preclude viral DNA replication, HS74BM (a normal human diploid fibroblast) was transformed with an ori$^-$ mutant of SV40. Such ori$^-$ transformed human fibroblasts have an extended lifespan in culture, beyond that seen in human fibroblasts transformed with a viral DNA (ori$^+$), but eventually also senesce. However, a number of continuous cell lines were isolated with the ori$^-$ transformed human fibroblast. This could not be duplicated with ori$^+$ transformed human fibroblast. Three cell lines were obtained by blind passaging the cells and picking colonies which grew out of mass cultures. Another three cell lines were isolated by performing a cloning experiment. I then investigated the SV40 integrants, and found only one or two integrants for each line, one of which had apparently been conserved in all the immortalized

lines. I concentrated my efforts on Cl-39, which contained only one integrant. Southern analysis was performed to characterize the integrated sequences. Most of the late region and part of the early region were found to be missing. Consistent with absence of part of the early region, a truncated large T antigen was found.

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Ms. Sally Ripley, whose assistance with karyotyping and in-situ hybridization was invaluable.

My daughters, P'nina and Suri, who showed me the truely wonderful potential of human genetics.

Last, but not least, I would like to thank my mother and my in-laws, whose support, patience and understanding have facilitated the completion of this accomplishment.

This thesis is dedicated to my wife, Gittie, for her suggestions, understanding, support, assistance, encouragement, perseverance, editing, typing . . . the list is too long for the limited space available. Thanks ever so much -- I will try to reciprocate.

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CHAPTER 1: INTRODUCTION

This thesis will attempt to assess the relationship between simian virus 40 (SV40), transformation, and immortalization in human diploid cells in culture. Toward this goal, I will review relevant literature on SV40 as a prelude to examination of studies in the area of its effect on proliferation of human fibroblasts. The papovaviruses constitute one of the simplest groups of DNA tumor viruses. Of these, the best studied has been SV40. A review of the extensive work done with SV40 is beyond the scope of this thesis; I will therefore only summarize the salient features (see Tooze, 1981).

Monkey cells support productive, or lytic, infection with SV40. In contrast, mouse cells do not support the production of virus (or viral DNA) and the infection is said to be abortive. After surviving the infection, however, some mouse cells develop new properties and are then said to be "transformed." Rather than exhibiting the alternative responses of permissive or nonpermissive infection, virus production in human cells occurs at approximately 1% of the level produced by permissive monkey cells. Thus, human cells are said to be "semipermissive" for SV40 (Tooze, 1981; Ozer et al, 1981).

Infections begin with adsorption, penetration and uncoating of the virus. Although the exact pathway of penetration and uncoating is unknown, it is clear that

intact viral DNA must eventually reach the nucleus, where it is transcribed and replicated. The viral genome is a covalently closed superhelical circle (Form I) DNA. During the first few hours, the early promoter efficiently directs the synthesis of the various early mRNA species. Synthesis of the late mRNAs occurs sluggishly at this stage but becomes dominant as viral DNA replication gets underway, 12 to 18 hours after infection. Viral DNA replication then proceeds in a semi-discontinuous manner, with forks growing from the unique origin in opposite directions at approximately equal rates. The viral DNA is associated with host histones H1, H2A, H2B, H3, and H4, which form a chromatin-like structure.

The SV40 early transcription unit is transcribed in productively infected permissive cells into two mRNAs with identical 5' and 3' termini but different splice junctions (See Figure 1). The smaller 2.3 kilobase (kb) (16S) mRNA encodes large T antigen, a protein of 94,000 daltons apparent molecular weight (MW) 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 MW which is not essential for replication or transformation in culture, but which has an enhancing role in some circumstances (Topp and Rifkin, 1980).

The first major viral protein to accumulate in infected cells is the large T antigen (also termed the A

Figure 1. SV40 genome map showing various SV40 landmarks and key restriction enzyme recognition sites. (From Fiers et al, 1978).

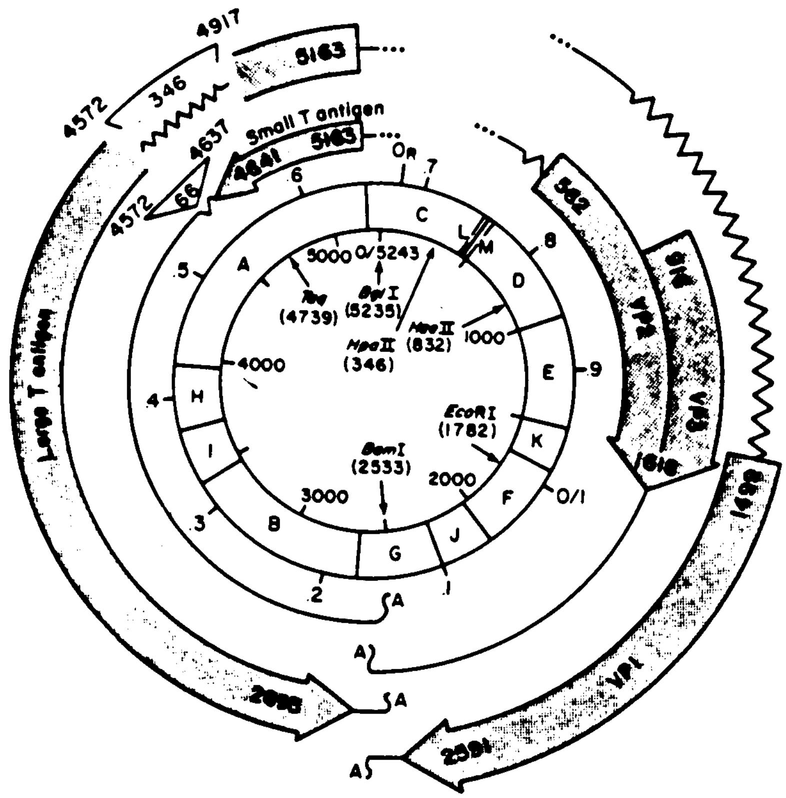
4917
5163
346
4572
Small T antigen
4637
5163
4841
4572
66
Large T antigen
2696
0R
.7
C
L
M
.8
D
.9
E
K
F
0/1
J
G
.1
B
.2
.3
I
H
.4
.5
A
.6
5000
0/5243
BglI
(5235)
(4739)
HpaII
(346)
HpaII
(832)
1000
4000
EcoRI
(1782)
BamI
(2533)
3000
2000
A
A
A
A
562
VP2
VP3
916
1616
1498
VP1
2591

Figure 1

protein). The protein is a pleiotropic effector with multiple biological and biochemical functions. It consists of 708 amino acids, and is required for various biological processes. These include (a)initiation and maintenance of cell transformation, (b)immortalization of primary rodent cells, (c)initiation of viral DNA replication, (d)induction of host cell DNA synthesis, (e)autoregulation of SV40 early region transcription, (f)stimulation of SV40 late region transcription, (g)stimulation of cellular mRNA and rRNA transcription, and (h)facilitation of growth of human adenoviruses in monkey cells. The T antigen's biochemical activities consist of (a)high affinity binding to a site in or near the origin region of replication, (b)low affinity binding to mammalian DNA, (c)ATPase activity, (d)association with a 53,000-dalton cellular protein (p53), and (e)formation of oligomeric forms. T antigen accumulates mainly in the nucleus but is also present in small amounts in cellular membranes. Essential coding regions for many of the biochemical and biological activities of T antigen are localized on different portions of the T antigen. This suggests that T antigen may be composed of several domains that can function independently (Sugano and Yamaguchi, 1984). In order to clarify this model of separate functional domains, which accounts for the various biological and biochemical activities attributed to this protein, a series of mutants of SV40 have been constructed with deletions in the coding sequence

for large T-antigen. The mutant DNAs were analyzed to define the mutational changes at the nucleotide sequence level, and were then tested for the various activities attributed to T antigen. At the biological level, the study of mutants has identified distinct domains for viral DNA replication, cellular DNA synthesis, activation of rRNA genes, cell transformation, helper function for adenoviruses and, possibly, autoregulation (Pipas et al, 1983; Soprano et al, 1981; Clark et al, 1981). These regions have been further dissected by a large number of base substitution mutants (Shortle and Nathans, 1978, 1979).

The SV40 virus late region codes for three proteins, VP1, VP2, and VP3, which are the structural components of the virions (Figure 1). These proteins are synthesized in appreciable amounts following viral DNA replication at late times during infection of permissive or semi-permissive cells. As with other viral proteins, the capsid proteins are synthesized in the cytoplasm. They are transported rapidly to the nucleus for assembly into progeny virus particles. Together, the three genes coding for these proteins occupy most of the remaining half of the viral genome and are arranged in opposite orientation to the early genes. The late region codes for mRNAs that are heterogeneous at their 5' ends. The major structural protein, VP1, is coded by an uninterrupted sequence of 1,092 nucleotides. An upstream leader of about 200

Figure 2. Structure and function of the control region of SV40. The T-antigen binding sites are indicated as T1, T2, and T3. The origin of DNA replication lies within the region spanned by nucleotides 34 and 5202. The AT-rich region related to the TATA box concensus sequence is indicated, as is the cleavage site of restriction enzyme Bgl 1. (From Tooze, 1980).

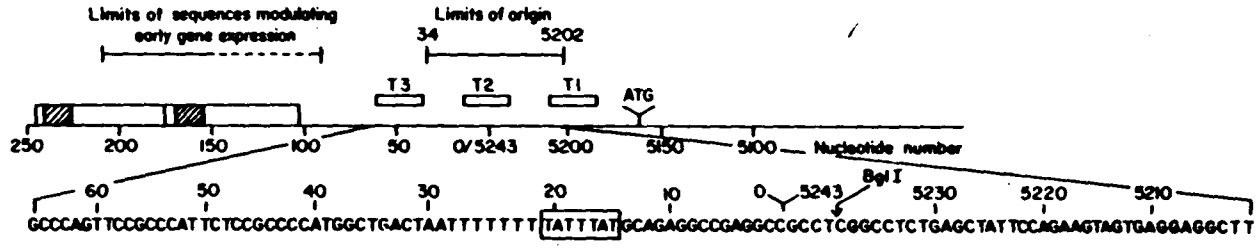
Limits of sequences modulating early gene expression
Limits of origin
34
5202
T3
T2
T1
ATG
250
200
150
100
50
0/5243
5200
5150
5100
Nucleotide number
60
50
40
30
20
10
0
5243
BglI
5230
5220
5210
GCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTT

Figure 2

nucleotides is added to these sequences. The coding sequences for VP2 and VP3 roughly correspond to the intervening sequences mapping between the leader and coding sequences of VP1. However, the common termination signal for VP2 and VP3 lies 110 nucleotides beyond the initiation codon for VP1. Hence the three genes overlap, and an examination of the sequence shows that this small region of the SV40 genome codes for parts of three proteins in two different reading frames. The entire sequence of VP3 is a subset of VP2. The exact structure at the 5' end of VP3 is not yet known. A small polypeptide, the agnoprotein, has been identified as encoded within the leader sequence. It has been shown to have properties relevant to virus assembly (interaction with VP1) and viral transcription (Jay et al, 1981).

The segment of the SV40 genome surrounding the origin of DNA replication is a complex regulatory region. It contains several characteristic features, including three tandem T antigen binding sites, two precise and one imprecise copy of a guanine-plus-cytosine-rich 21-base-pair (bp) repeat, and two copies of a 72-bp repeat (Figure 2). The specific sequences within this region that are required for various viral functions have been analyzed extensively both <u>in vivo</u> and <u>in vitro</u>. Briefly, most of the T antigen binding sites I and II are required for efficient DNA replication. A site for the restriction enzyme Bgl 1 lies within binding site II. The promoter for early gene

transcription lies largely within the third T antigen binding site and the two 21-bp repeats. It has also been shown that at least one intact copy of a 72-bp repeat is required for efficient early gene expression. The enhancing function of the 72-bp repeat occurs even when it is inserted at locations distant from the site of transcription initiation or in either orientation. In all cases, transcription was initiated at the same location as with the normal sequence organization. Although the sequences of the 72-bp repeats are not essential for late-region expression, their presence increases the level of late-region expression (Fromm and Berg, 1982; 1983).

Rather than the alternative responses of lytic infection or transformation, human cells undergo not only morphologic transformation but also continue production of infectious virus. In the initial stages of infection, 10 to 20% of the infected cells appear to lyse while the surviving cells continue to proliferate at about the same rate as that of uninfected cells. A second later stage is evident by rapid proliferation of cells ("transformed cells") in the cultures (Girardi et al, 1965). Unlike SV40-transformed cells of other species (e.g., mouse or rat), the proliferative capacity of human cells after initial transformation is often limited by a phase termed "crisis" (Girardi et al, 1965). Crisis is marked by abnormal mitotic figures, loss of attachments to the growth surface, reduced proliferative capacity, and the

occasional formation of multinucleated or giant cells. Transformed cells with an older *in vitro* passage history enter crisis sooner than those derived from younger lines. However, SV40 transformation increases the growth potential of human fibroblasts, and cells transformed near the end of their normal *in vitro* life span are again able to proliferate transiently. Some cells can survive crisis and grow with apparently unlimited life spans. Few studies have isolated individual transformants induced by SV40 and studied the progression of their growth properties (Sack, 1981).

Transformation by SV40 in both human and rodent cells in culture has been defined by several biologic criteria. These include loss of contact inhibition, loss of density-dependent inhibition of monolayer growth, reduced anchorage dependence (permitting growth in semisolid agars or methocel suspension), growth in media containing low serum concentrations, growth in a less-oriented manner than that seen in normal cells, growth on monolayers of normal cells, release of proteases, and the presence of SV40 T antigen in transformed cell nuclei. SV40-transformed cells commonly form tumors upon injection into susceptible animals-anchorage independence is the *in vitro* alteration which best correlates with this *in vivo* behavior.

Cells stably transformed by SV40 contain viral DNA covalently integrated into the chromosomal DNA of the host cell (Sambrook et al, 1968). Originally, Croce (Croce et

al, 1973; Croce and Koprowski, 1974) proposed the assignment of chromosome 7 as the unique site of integration of SV40 in human cells, based on somatic cell hybrids (between mouse cells and SV40-transformed human cells) which segregated human chromosomes. This conclusion was predicated on the fact that the expression of SV40 T antigen and the rescuability of the SV40 genome segregated concordantly with the presence of human chromosome 7. However, it was later shown by Croce and others (Croce, 1977; Kucherlapati et al, 1978) that integration could be demonstrated on different human chromosomes in different SV40 transformants, leading to the reverse hypothesis, that there is no specificity for integration. The Southern transfer hybridization technique has been used to analyze the structure of the integrated viral DNA in SV40-transformed rat (Botchan et al. 1976), human (Campo et al. 1978), hamster (Chepelinsky et al. 1980) and mouse (Ketner and Kelly 1976; Rigby, 1979) cells. The results indicate that there are no specific integration sites at the level of restriction endonuclease cleavage sites in the host or viral genome (Hwang and Kucherlapati, 1983; Clayton and Rigby, 1981). Moreover, integration patterns are frequently complex, and complete or partial tandem duplications of viral sequences are common (Clayton and Rigby, 1981). Analysis by DNA reassociation kinetics has shown that there is considerable variation between transformed cell lines in the number of copies of viral DNA

(Sambrook et al, 1975). In addition, the representation of different segments of viral genome is not necessarily uniform (Botchan et al, 1974).

Several reports have shown that two different gene products are required for the transformation of primary rodent cells by adenovirus (Ad) and polyomavirus (Py)(Houweling et al,1980; Rassoulzadegan et al, 1982; Treisman et al, 1981; Van den Elsen et al, 1983). Ruley (1983) and Land (Land et al, 1983) extended these findings and showed that two distinct classes of oncogenes are required for such transformation. One class (type A), including the Ad E1A region, myc, p53 and Py large T antigen genes, is required for colony formation and presumably immortalization or establishment of primary cells in culture. The other class (type B), including the Ad E1B region, activated ras, and Py middle T antigen genes, is required for the full expression of the transformed phenotype. The latter can transform a certain type of established cells (e.g., 3T3) in the absence of the former. Because T antigen is the sole product of the viral gene essential for transformation of established cell lines by SV40, and is capable of immortalizing rodent cells, both of the activities may be provided by T antigen (Sugano and Yamaguchi, 1984). Indeed, both type A and B phenotypes are simultaneously observed when SV40 virus is used to infect early passage rodent cells.

Furthermore, it can also be shown that maintenance, as

well as initiation, of transformation by SV40 clearly requires at least one of the early functions, namely, that of the A gene product, which codes for large T antigen (Brugge and Butel, 1975; Kimura and Itagaki, 1975; Clayton et al, 1982). Petit et al (1983), in studies in which rodent cells were "immortalized" with a mutant encoding a heat-labile temperature sensitive A protein (tsA mutant)showed that maintenance of the transformed state is dependent on T antigen. They demonstrated that a temperature shift to 39^{o} C (the restrictive temperature) caused the immortalized cells to revert to a "mortal" or senescent-like phenotype. Multiple studies have demonstrated that the transformed phenotype is dependent on T antigen when established rodent cells (e.g., 3T3) are transformed by tsA mutants and studied at different temperatures (Martin and Chou, 1975; Osborn and Weber, 1975; Tegtmeyer, 1975;).

No analogous studies have been reported on expression of oncogenes in early passage human fibroblasts. Sager et al (1983) reported no evident effect of introducing the EJ gene (a mutant form of the cellular Harvey ras gene) into such cells. Infection of human cells by tsA mutants of SV40 has been shown to be associated with continued cell growth and expression of the transformed phenotype in a temperature-dependent manner (Chou, 1978; Steinberg and Defendi, 1979); however, no established cell lines have been described in the literature containing such a viral

genome. Nonetheless, the fact that human diploid fibroblast cultures can be transformed by SV40 virus (Jensen et al, 1963; Girardi et al, 1965) into cultures that display an indefinite proliferative capacity, albeit at low frequency (see review by Sack, 1981), has been exploited in an effort to further clarify the limited life span of both normal and viral transformed human cells in culture. The interaction of such transformed cells with normal cells has been studied by cell fusion experiments. Initially, the transformed phenotype appeared to be dominant. Following the fusion of senescent normal cells with tumor-derived or SV40 transformed cells, DNA synthesis was reinitiated in the senescent nuclei (Norwood et al, 1975). Stein and Yanishevsky (1981) have shown that entry into DNA synthesis may be negatively controlled in both quiescent and senescent human diploid fibroblasts. They fused quiescent or senescent cells to replicating cells and found that while the initiation of DNA synthesis was temporarily inhibited, ongoing DNA synthesis was unaffected. Their results suggest that both quiescent and senescent cells possess an inhibitor to the initiation of DNA synthesis. In these studies, however, it is not possible to determine if the quiescent and senescent cells are blocked at the same point in the cell cycle, with the quiescent cells able to exit following an appropriate signal, while the senescent cells remain blocked.

Human cells transformed by the DNA tumor viruses SV40

and adenovirus were able to induce the initiation of DNA synthesis in senescent fibroblast nuclei in heterodikaryons. In contrast, however, carcinogen-transformed cells were unable to stimulate entry into S phase in the senescent nuclei, and in fact were themselves inhibited from synthesizing DNA in heterodikaryons (Stein et al, 1982). Fusing HeLa cells (Stein, 1983) or L cells (Nette et al, 1982) leads to DNA synthesis in the senescent nuclei of the hybrids, heterodikaryons, or cybrid fusion products.

Early studies involving the fusion of normal and transformed cells have reported the isolation of indefinitely proliferating hybrid clones (Goldstein and Linn, 1972; Croce and Koprowski, 1974; Stanbridge, 1976). However, Bunn and Tarrant (1980) and Muggleton-Harris and DeSimone (1980) have shown that the majority of hybrid clones isolated displayed a finite proliferative capacity. In these latter studies, however, the finite lifespan of most hybrid clones may have been due to the loss of specific HeLa cell chromosomes or SV40 sequences. This question was directly addressed by the work of Pereira-Smith and Smith (1981). They studied the proliferative potential of hybrid clones formed from the fusion of presenescent or senescent human cells with SV40-transformed human cells. Eighty-five percent of the hybrid clones isolated showed finite <u>in vitro</u> lifespans, while 15% displayed indefinite lifespans. Most significantly,

however, all of the hybrid clones expressed the viral T antigen. Therefore, even though the viral genome was being expressed in all of the hybrid clones, the senescent phenotype was often eventually dominant.

An early idea proposed by Weisman, Minot and others, stressed the possibility that aging might be the price paid for cellular differentiation, and that cells removed from the constraints of the animal body might be immortal. Carrel and Ebeling believed that they had demonstrated immortality in isolated cells by keeping cultures of chick heart fibroblasts alive and proliferating for 34 years, a period longer than the chick life span, before voluntarily terminating their experiment (Carrel and Burrows, 1911; Carrel, 1912, 1914 1935; Ebeling, 1913). Support for Carrel's observations came from the isolation of a number of cell lines, including "L" cells derived from mouse mesenchyme (Earle, 1943) and HeLa cells derived from a human cervical carcinoma (Gey et al, 1952), which could be grown continuously in culture without a decline in proliferative vigor. Thus, the notion that "cells were immortal, organisms mortal" seemed reasonable. It was not until 1957, when Swim and Parker reported a limited lifespan for human embryonic and adult tissue fibroblasts in culture, that there was indication that cells, like the parent organism, might actually age. In 1961, Hayflick and Moorehead's classic study demonstrated that cells from a variety of normal human tissue proliferated in culture for

various periods of time but eventually degenerated and died. After outgrowth from the explant, a period of rapid multiplication followed, during which frequent subcultivations were possible. Later, the proliferative capacity of the cells decreased, intracellular debris accumulated, nuclear abnormalities appeared, and the culture died. These workers later proposed that the limited replicative potential of human cells in vitro was an expression of senescence at the cellular level (Hayflick 1965). Hayflick's observations were confirmed by Hayakawa (1969) and subsequently by numerous laboratories for cells from a variety of tissues and organisms.

By contrast, attempts to reproduce Carrel's findings have not been successful. Even when techniques similar to those employed by Carrel were used (Gey et al. 1974), chick cultures could not be maintained for more than 44 months. Hayflick (1970, 1975, 1977) has suggested that Carrel, in preparing his chick embryo extract supplement to the culture medium, may have introduced new viable cells at each refeeding. All human cell lines that reportedly have an indefinite life span have been shown to express at least one of the characteristics of transformed cells (Cristofalo and Stanilus-Praeger, 1982).

Further support for the Hayflick view has come from the findings of several researchers who have shown that aging in vitro is reflected in cell culture. A number of investigators showed that the time between introduction of

embryonic tissue into culture and cell migration from the explant increased with age of the embryo (Carrel and Burrows, 1911; Cohn and Murray, 1925; Medawar 1940, Lefford 1964). In addition, several investigators (Soukupova and Holeckova, 1964; Waters and Walford, 1970) have reported that the outgrowth of cells, particularly fibroblasts, from rat and human tissue explants decreased with the increasing age of the donor. In 1975, Ooka et al reported a similar decrease in the rate of migration of rat epidermal cells in culture as the donor age increased. Hayflick (1965) additionally found that when mixtures of young and old populations of human fetal lung fibroblasts were grown together in the same culture vessel, the older population died first at the same number of population doublings as "old" matched controls. The young populations continued to grow vigorously until reaching approximately the same number of total population doublings, at which time they also died. This experiment seemed to rule out the possibility that lifespan limitation might be caused by deficiencies in the medium, microbial contamination, or toxic wastes produced by the cells, and indicated that it was, instead, intrinsic to cells. Additionally, when populations of cells were frozen in liquid nitrogen at a certain population doubling level and later thawed and recultured, the cells continued to divide until they had reached the average total number of doublings characteristic of that line (Hayflick, 1965). These

experiments collectively indicated that senescence in diploid cells in vitro seemed to be intrinsic to the cells and precisely timed.

It is possible to compare the proliferative capacities of cells grown in vitro from donors of different ages. Hayflick (1965) showed that an average of 20 (14-29) doublings occurred in cultures of adult-derived human lung fibroblasts, whereas an average of 48 (35-63) occurred in cultures derived from human embryo. Goldstein et al (1969) reported an inverse correlation between the age of the donor and the number of population doublings attained by cells from a series of skin. Martin's laboratory (Martin et al, 1970; Martin, 1977) also confirmed this observation by culturing fibroblasts from biopsies of upper arm tissue obtained from human donors ranging from embryos to persons 90 years of age. A significant inverse correlation between proliferative lifespan and donor age was found. Extensive studies by Schneider and Mitsui (1976) of the in vitro growth of upper arm skin fibroblasts from old and young human donors have shown a significant decrease in the rate of fibroblast migration, length of in vitro lifespan, rate of cell replication, and cell number at stationary phase when cells from old donors (63-92 years) were compared to cells from young donors (21-36 years). No change was found in cell volume or macromolecular content. The percentage of replicating cells and percentage of cells able to form colonies of more than 16 cells also decreased with

increasing donor age (Schneider, 1979). Similarly Bierman (1978) found a decrease in replicative capacity with increasing donor age for 17 lines of human arterial smooth muscle cells, as did LeGuilly et al (1973) for human liver cells, and Rheinwald and Green (1975) for epidermal cells. Ryan and coworkers (1981) reported findings that support the concept that cell lifespans are genetically determined. They found that skin fibroblasts from three pairs of monozygotic twins showed no significant difference in replicative lifespan within each twin pair, but did show such differences among pairs. It has also been suggested that the population doubling maxima of normal embryonic fibroblasts *in vitro* are proportional to the mean maximal lifespan of their donor species (Phillips and Cristofalo, 1985). Rohme (1981) has further demonstrated that the lifespans of circulating mammalian erythrocytes *in vivo* are also related to the maximum lifespan of the species.

A very interesting set of experiments has been reported from Daniel's laboratory. Some time ago they (Daniel et al, 1968), showed that small pieces of mouse mammary tissue could be serially transplanted into donor mice. Following transplantation, the tissue would proliferate to fill the host fat pad and become capable of most normal differentiated functions. With each successive transplant generation, however, approximately 15% of the proliferative capacity was lost until, after about six generations, the transplanted tissue was maintained but did

not grow. Berger and Daniel (1983) subsequently demonstrated that the ductal elongation in transplanted young proliferating mouse mammary gland is associated with a zone of DNA synthesis in the stroma surrounding the end bud. In contrast to this, however, serially aged tissue failed to elicit this stromal response. They point out the potential importance of these tissue interactions in the aging of mammary epitheliums in vivo. Most exciting, however, is their recent study (Daniel et al, 1984) involving cholera toxin. Mammary tissue was serially transplanted until it was senescent and then exposed in vivo to plastic implants that slowly released cholera toxin. New end buds developed, which indicated a temporary reinitiation of growth lasting about ten days. After this time, the effects were largely exhausted. The end buds regressed, and there was no evidence of abnormal growth states.

Several laboratories have focused their studies on various fusions between young and senescent cells and the general observation that the young cell nuclei are inhibited from entering S phase in such heterodikaryons. The inhibition of DNA synthesis also occurs in young cells when they are fused with cytoplasts prepared from senescent cells (Drescher-Lincola and Smith, 1983). Furthermore, this senescent-cell-dependent inhibition is sensitive to either cycloheximide or puromycin (Burmer et al, 1982) and can be optimally blocked by brief (one hour) cycloheximide

treatment during the first three hours after fusion. This implies a late G_1 block in senescent cells (Burmer et al, 1984).

Several lines of evidence from Cristofalo's laboratory also support a late G_1 block in senescent cells. First, they (Cristofalo, 1973) found that the level of thymidine kinase activity in old, slowly proliferating cultures of WI-38 cells was similar to that of young, rapidly dividing populations. This anomaly first raised the possibility that senescent cells retain the ability to phosphorylate thymidine and thus may be arrested in late G_1. Olashaw et al (1983) found that thymidine triphosphate (TTP) synthesis is not impaired in senescent cells. The addition of serum to density-arrested populations of young and old cells induced TTP synthesis to a similar extent after 12 hours in both populations. However, a far greater percentage of young cells subsequently initiated DNA synthesis as compared to old cells. Because induction of thymidine kinase activity and TTP synthesis are cell-cycle-dependent events that normally occur in late G_1, senescent cells may not be blocked in G_0, as are quiescent cells. Finally, additional support for a late G_1 block comes from examining nuclear fluorescence following staining with quinacrine dihydrochloride (Gorman and Cristofalo, 1984). As serum-stimulated cells traverse G_1 and reach S, the staining pattern changes from bright and homogeneous to dimmer and segregated. The fluorescence pattern of senescent cells is

typical of late G_1.

For those few cells in senescent cultures that are still capable of synthesizing DNA, the replicative system seems to work normally. Griffiths et al (1983) reported that the rate of DNA chain elongation was the same in young and old WI38 and MRC-5 cells. Kraus and Lin (1982) found the DNA polymerase-α (semiconservative DNA synthesis) decline with age in IMR-90 cells, as would be predicted. However, polymerase-β (DNA repair synthesis) was unchanged. Results essentially the same as these were reported by Fry et al (1984) for regenerating liver cells from young and old mice.

Several investigators have examined senescence by attempting to extend the proliferative potential of the cells. However, once the cells' basic nutritional requirements have been met, only two reproducible methods of extending the proliferative capacity of human fibroblasts have been described to date, namely, infection with a tumor virus as SV40, as discussed previously, or supplementation of medium with glucocorticoids (either hydrocortisone [HC] or the synthetic glucocorticoid dexamethasone [DEX]). In 1970, Cristofalo demonstrated a 40% extension in the proliferative capacity of WI-38 cells, as well as other fetal lung-derived fibroblasts, when HC or DEX were used. Rosner and Cristofalo (1981) have shown that the ability to translocate the temperature-activated hormone-receptor complex from the cytoplasm in the nucleus

is impaired in senescent cells. In addition, they showed that as the WI-38 cells age, they lose approximately 40% of their specific DEX receptors. These data are consistent with the fact that while longterm exposure to the glucocorticoid extends the proliferative lifespans of these fibroblasts, the cells eventually senesce in all cases.

Didinsky and Rheinwald (1981) studied the effects of several factors, including the addition of hydrocortisone to cultures that were passaged at low density and never allowed to reach densities that inhibit growth or promote a quiescent state in the cells. Under these conditions, the glucocorticoid had no effect on proliferative lifespan. While these results would appear to contradict the work of Cristofalo (1970), Cristofalo et al (1979) suggested a possible explanation for the discrepancy. They postulated that when subcultivated from high to low density in the presence of HC or DEX, WI-38 fibroblasts temporarily modify the medium in such a way as to increase the saturation density of the mass culture in a cumulative fashion across the cells' lifespan, rather than increasing the length of time that the cells are capable of proliferation. The mode of action appears to be stimulation of DNA synthesis in cells that might not otherwise be brought into the actively proliferating population (Phillips et al, 1982). This effect has now been studied in some detail. It appears to be mediated by the elaboration of a low molecular weight (less than 1,000 daltons) heat-stable, protease-resistant

molecule (Phillips and Cristofalo, 1985). Since Didinsky and Rheinwald (1981) intentionally did not allow their cultures to reach high densities, either this effect was not apparent or the medium was not modified.

Several investigators have examined the effects of the addition of various other growth stimulators to the medium. Didinsky and various associates (Didinsky and Rheinwald, 1981; Rheinwald and Green, 1977) studied the action of epidermal growth factor (EGF) and fibroblast growth factor (FGF) in modulating the proliferative capacity of fibroblasts in culture. Neither of these polypeptide growth factors had any significant effect on lifespan. Other investigators (Ohno, 1981; Didinsky and Rheinwald, 1981) examined the action of another polypeptide growth factor, platelet-derived growth factor (PDGF) and also found no modulation of lifespan. When somatomedin-c was used as the growth stimulator in human cells (Clemmens, 1983; Clemmens and Shaw, 1983), an inconclusive result was obtained. Recently, Carlin et al (1983) reported a defect in the autophosphorylation of the EGF-receptor in WI-38, although the level of receptor and its ability to bind EGF were unaltered.

A number of researchers (Philip and Cristofalo, 1980; 1981; Walthall and Ham, 1981; Bettger et al, 1981; Yamane et al, 1981; Kane and Yamane, 1982) have developed serum-free media and successfully passaged primary and secondary finite human fibroblast-like cell lines in culture.

Although three different culture media were used in these experiments, the value of all of these media lies in their use as a tool for studying the regulation of cell proliferation. Several laboratories have now begun systematic studies of the responsiveness of young and senescent cells to the specific mitogens in various serum-free media formulations. Cristofalo's laboratory (Phillips et al, 1984) has shown that as cultures of WI-38 cells senesce, they become progressively less responsive to mitogenic stimulation. However, there is no change as a function of age in the concentration of EGF, insulin (INS), transferrin (TRS), and DEX that elicited the maximum proliferative response. Chiger and Kaji (1983) found similar results with INS when working with IMR-90 cells. However, results with PDGF were not as clear, and may indicate that higher concentrations of mitogen are required for a maximum proliferative response.

Research in our laboratory has attempted to clarify the role of SV40 genes in transformation and immortalization of diploid fibroblasts. Although SV40 infection results in transformation of human cells, the frequency is lower than that observed upon infection of rodent cells. Furthermore, as already discussed, only a rare SV40 transformed human cell becomes established into a permanent cell line (i.e., becomes immortal), in marked contrast to rodent cells, where virtually all SV40 transformants become immortal. We postulated that a

significant factor in the inefficient transformation of human cells was the semi-permissive virus-cell interaction. Virus production could be relevant to cell lethality resulting in either a reduced incidence of transformation or the subsequent death of transformed cells. Our approach emphasized the use of defective viral genomes, necessitating DNA-mediated gene transfer rather than infection by virus particles. The effectiveness of DNA-mediated gene transfer for the stable expression of cell and virus sequences was initially demonstrated by several groups (Wigler et al, 1977 ; Maitland and McDougall, 1977; Bacchetti and Graham, 1977; Minson et al, 1978), who adapted the calcium phosphate coprecipitation transfection method of Graham and Van der Eb (1973). DNA is mixed in aqueous solution with calcium and phosphate ions which themselves form a precipitate when mixed; the DNA molecules are then co-precipitated in the matrix. In this physical state, they are more resistant than dissolved DNA to enzymatic degradation and mechanical shearing, and they appear to be readily taken up (via a poorly understood mechanism) by a variety of cells. Wigler and others (see above) reported success in using purified DNA from Herpes Simplex virus (HSV), reduced to noninfectious fragments by mechanical shearing or digestion with certain restriction endonucleases, to transfer the HSV-encoded thymidine kinase (tk) gene to mouse L cells ($LMtk^-$) lacking their own cell-encoded tk (Kit et al, 1963). The HSV tk gene product can

be biochemically and immunologically distinguished from the tk gene produced by mammalian cells. Gene transferrents were detected by selection in HAT (hypoxanthene-aminopterin-thymidine) medium, which permits only those cells making an active tk to grow. The presence of HSV DNA sequences in the cellular genome of these transformants was verified by nucleic acid hybridization techniques (Pellicer et al, 1978). Wigler et al (1978) demonstrated that HSV sequences were integrated into high molecular weight DNA, which could be sequentially transferred by DNA-mediated gene transfer to a second LMtk$^-$ cell. Subsequent studies demonstrated that the transformed sequences were actually integrated into the chromosome of the recipient cells (Robins et al, 1981). This approach has been extensively utilized for multiple examples of viral, recombinant, and cellular DNA's and the subject has been repeatedly reviewed (Pellicer et al, 1980; Scangos and Ruddle, 1981; Graf, 1982). Briefly, DNA may be transiently expressed in a variable proportion of cells as an extrachromosomal element. Stable expression is most commonly associated with integration of the input DNA in a much lower percentage of cells. In cases in which different viral and plasmid or cellular sequences (e.g. high molecular weight calf thymus or salmon sperm DNA as "carrier") are co-precipitated, the viral sequences can become associated with other viral sequences (e.g. concatamers through homologous recombination) and/or with heterologous DNA, as

an extrachromosomal intermediate. This intermediate typically becomes integrated into a chromosome as a single insert. This method was used to transform a human diploid fibroblast cell, HS74BM, using a fragment of SV40 DNA containing an intact early region and the origin of viral replication, but devoid of sequences encoding the late, viral structural proteins (Zouzias et al, 1980). From this study, a SV40-transformed human fibroblast was isolated in agarose. It showed properties typical of SV40 transformation. Virus production and virus spread was precluded. Biochemical analysis showed, however, that cells were synthesizing, on the average, 10,000 copies of the SV40 genome per cell. Subclones of the transformant were unable to be isolated because of the limited lifespan of the human cell despite the fact that a relatively early passage was used for the original transformation. No immortalized derivatives were obtained. It was felt that the high level of viral DNA replication was responsible for the limited viability. Therefore, another series of transformed cells were sought in HS74BM (Small et al, 1982). DNA transfection of SV40 sequences was performed utilizing pMK-SV40-Bgl I^R ($SVori^-$), isolated by Gluzman et al (1980a; 1980b), in which the origin for initiation of SV40 DNA synthesis was inactivated due to a 6 base pair (bp) deletion at the Bgl 1 site. A comparison between $SVori^-$ and virion, or pMK-SV40, containing an intact origin ($SVori^+$) as the transforming DNA in HS74 showed $SVori^-$ to

be the most efficient. This was particularly the case when transformants were identified by the most stringent criterion of anchorage independence (by colony formation in agarose). The frequency of transformants was lower whether a complete viral genome was used or a DNA fragment devoid in late sequences, as previously utilized by Zouzias et al (1980). These results taken together were consistent with the hypothesis that the relatively low frequency of SV40 transformation of human cells was due to the loss of some transformants because of virus-mediated cell death. Alteration of the capacity of autonomous replication of viral DNA was most relevant to the efficiency of transformation.

In this thesis, I propose to examine the growth properties of HS74 transformed by SV ori$^-$ as compared to those obtained with a wild-type genome. The availability of human cells transformed by a replication-deficient SV40 genome permits the assessment of the effects of the viral gene products independent of the complexities of extrachromosomal viral replication and cell killing, thus mimicking the state commonly studied in SV40 infected rodent cells. In particular, I propose to assess the "immortality" of such human transformants to determine whether the concordance of immortalization and SV40 transformation in rodent cells and their discordance in human fibroblasts is due to the semi-permissive SV40-cell interaction of the latter. Experiments will be performed

to determine whether they are already immortal, or more likely to become immortalized than wild-type transformants. If neither of these proves to be the case, I will attempt to develop a technique that will facilitate isolation of immortalized cell lines. I intend to compare non-immortal and immortal cell lines with regard to the integrated SV40 sequences. Such an analysis would test the hypothesis that immortalization of SV40 transformants is associated with a specific rearrangement of the viral sequences. No studies have described the comparison of immortalized derivatives from clonally isolated SV40-transformants. One prior attempt failed to isolate <u>any</u> immortalized derivatives from 90 SV40 transformants isolated as foci in monolayer (Gotoh et al, 1979).

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL CULTURE PROCEDURES

2.1.1. Cell lines and tissue culture conditions

Cells were grown in Dulbecco's modified Eagles medium (DME, M.A. Bioproducts) and Ham's F10 medium (F10, M.A. Bioproducts) in a 1:1 mixture. The medium was generally supplemented with 10% Fetal Bovine Serum (FBS, M.A. Bioproducts) or 10% Newborn Calf Serum (NCS, M.A. bioproducts), except when noted. The antibiotics penicillin "G" phosphate (Gibco, 0.05 gm/L) and streptomycin sulfate (Gibco, 0.05 gm/L) were also added to all media. Cultures were routinely incubated at 37° C in a 7.5% CO_2 atmosphere .

Cells were passaged by brief treatment with a trypsin-EDTA mixture (0.5 gm/L trypsin and 0.2 gm/L EDTA, M. A. Bioproducts). After the trypsin-EDTA was removed, the cells were removed from the dish in medium without serum.

Cell counts were obtained with a Royco Tissue Cell Counter after dilution in phosphate-buffered saline (PBS [150 mM NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, pH 7.4]).

Cell lines were stored in Nunc Cryotubes frozen in liquid nitrogen or in a -70° C freezer in medium containing 10% dimethyl sulfoxide (DMSO) and 20% serum.

HS74BM, a diploid human fibroblast derived from fetal bone marrow cells that displays a diploid karyotype (Smith

bone marrow cells that displays a diploid karyotype (Smith et al, 1976), was transformed by SV ori$^-$. Four isolates were chosen from a host of variously transformed human fibroblasts, which had been isolated as colonies in agarose. One was transformed with pSV ori$^-$ form I DNA (IF4A-1), a second was transformed with pSV ori$^-$ form III DNA (IF6A-1), a third had its plasmid sequences separated from the SV40 sequences prior to transformation (IF5A-2), and a fourth (an SV40 virally transformed human cell) was used as a control (IF1A-2). The separation of the transforming DNA of the third transformant (IF5A-2) was accomplished by restricting the molecule with Bam H1, (which is the enzyme that was used when the plasmid sequences were joined to the SV40 sequences) followed by agarose gel electrophoresis and electroelution.

It should be noted that all the cultures (IF1A-2, IF4A-1, IF5A-2, IF6A-1) had undergone a number of generations prior to frozen storage (14 generations before transforming the cells, 3 generations during their transformation, 8 generations while in agarose, and 17 generations from the time colonies were picked in agarose until they had grown to confluent 75cm^2 flasks). Reference cultures, were thawed to initiate my experiments and designated as P_0. Therefore, 42 generations should be added to all passage histories. In the case of the parent, HS74BM, one should add approximately 20 generations for the first ten passages prior to the beginning of its recorded

passage history.

2.1.2. Efficiency of colony formation in agarose

A solution of 2X DME/F10 with 20% serum and a 1.4% agarose solution were prepared. These two solutions were combined 1:1, so that the final solution consisted of DME/F10 with 10% serum and 0.7% agarose. 4.5 ml of this combined solution was added to each 60 mm petri dish as a basal layer and allowed to solidify before the next layer was added. The cells to be plated were suspended in 4 ml of normal growth media (see above) and added to 4 ml of the DME/F10, supplemented with 10% serum and 0.7% agarose. This 8 ml overlay was spread over the base layer and now consisted of 0.35% agarose. The plates were left overnight at 33° C to allow the overlay to solidify and then shifted to 37° C. The dishes were fed bi-weekly with 2 ml of overlay solution to prevent them from drying out.

2.2 DETERMINATION OF SV40 DNA SEQUENCES

2.2.1. Isolation of high molecular weight cellular DNA (Pellicer et al., 1978)

Cells were grown to confluence in 100 mm Petri dishes. Monolayers of 5 dishes were washed three times with PBS, and scraped with a rubber policeman, or a razor blade covered with teflon, into 2 ml of lysis buffer (50 mM Tris, pH 7.5, 20 mM Na_2EDTA, 100 mM NaCl). Sodium dodecyl

sulfate (SDS) was added to a final concentration of 0.1%, and tubes were immediately incubated at 65° C for 15 minutes to inactivate endogenous nucleases. Tubes were cooled to room temperature and Proteinase K (Boehringer Mannheim, 5 mg/ml stock) was added to a final concentration of 50-100 ug/ml. Tubes were then inverted to mix, and incubated at 37° C overnight. The cell lysate was extracted with an equal volume of buffer-saturated redistilled phenol; phases were separated by centrifugation at 2000 rpm for 15 minutes at room temperature. The aqueous phase was removed with a wide-bore pipette and re-extracted with phenol:chloroform:isoamyl alcohol (48:48:2), followed by an extraction with chloroform:isoamyl alcohol (24:1). The viscous aqueous phase was transferred to a new tube; 2.5 volumes of ice cold 95% ethanol were added, and the mixture was inverted several times until the DNA precipitated into a white mass. The DNA was picked up with a hooked Pasteur pipette and washed once with 70% ethanol. It was then transferred to a new tube containing 2 ml of sterile TE (10 mM Tris pH 7.5, 1 mM EDTA). Pancreatic RNase (Worthington), which had been boiled for 10 minutes to inactivate trace amounts of DNase, was added to a final concentration of 10 ug/ml, and the solution incubated at 37° C overnight. The RNase was removed by extraction with organic solvents as above. DNA was precipitated with 2.5 volumes of ice-cold 95% ethanol by inverting the tube a number of times until the DNA began to precipitate as a

white mass, and it was recovered by centrifugation at 2000 rpm for fifteen minutes at room temperature. The DNA concentration was determined by diluting the sample 1:50 in TE and reading the UV absorption at 260 nm (1 O.D. is equivalent to 50 ug/ml double-stranded DNA).

2.2.2. Viral DNA

Purified SV40 virus was provided by Dr. T. L. Ozer. The viral DNA was isolated following his procedure (Ozer, 1972), using a low multiplicity of infection. The viral DNA was then purified by banding on a cesium chloride gradient. Separation of the form I DNA on an agarose gel further purified the DNA. Recombinant SV40 DNA was obtained from Dr. Y. Gluzman (Gluzman et al, 1980a; 1980b), and maintained in accordance with NIH guidelines. Plasmid sequences were purified by conventional methodology (Maniatis et al, 1982).

2.2.3. Isolation of low molecular weight DNA

The method of Hirt (1967) was used in an attempt to isolate covalently closed circular DNA from the immortalized cell lines. The medium from each infected culture was aspirated and monolayers were washed once with PBS. One milliliter of Hirt lysis buffer (HLB [0.6% SDS, 10 mM EDTA, 10mM Tris-Hcl, pH 7.6]) was added to each dish, and the cells were lysed by gentle rocking. The cell lysate was poured into Oak Ridge polycarbonate centrifuge

tubes. Sodium chloride (as a 5 M solution) was added to a final concentration of 1 M; tubes were gently mixed by inversion and incubated at 4° C overnight. The lysate was centrifuged at 25,000 rpm for 40 minutes at 4° C in a Beckman Type 50 Ti rotor, and the supernatant was transferred to polypropylene centrifuge tubes. The supernatant was extracted with HLB-equilibrated phenol and extracted once with chloroform:isoamyl alcohol (24:1), followed by an extraction using isoamyl alcohol alone. Sodium chloride was added to a final concentration of 200 mM, and DNA was precipitated with two volumes of 95% ethanol overnight at -20° C. DNA was recovered by centrifugation in a Sorvall SS34 rotor at 15,000 rpm. for 45 minutes and resuspended in TE.

2.2.4. Restriction endonuclease digestion and agarose gel electrophoresis (Southern, 1980)

Restriction endonucleases used in these studies were supplied by New England Biolabs, Bethesda Research Laboratories, and IBI. The optimum buffer conditions for each enzyme, as described in the manuals of these three companies, are given in Table 1. Low salt buffer contained 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 mM Tris-HCl pH 7.5. Medium salt buffer contained the above plus 50 mM NaCl. High salt buffer contained 100 mM NaCl, 10 mM $MgCl_2$,1 mM DTT, and 50 mM Tris-HCl pH 7.5. Very high salt buffer contained 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 mM

Tris-HCl pH 7.5. Reactions were generally performed in volumes of 20-100 ul containing 10 ug of DNA in TE, 0.1 volume of 10X reaction buffer (low, medium, high, or very high) and 1-2 units of enzyme per ug DNA. Two ug of lambda DNA were added to each reaction tube to insure that the reaction had proceeded to completion. Reactions were performed at 37° C overnight and terminated by the addition of EDTA to a final concentration of 10 mM, unless otherwise indicated.

For preparation of 0.6-1.0% agarose gels, the correct amount of powdered agarose (Seakem, ME) was added to the measured amount of Tris phosphate (TPE) electrophoresis buffer (0.08 M Tris-phosphate, 0.002 M EDTA pH 8.0). Agarose was melted by brief autoclaving in a pressure cooker, allowed to cool to 50° C and poured into the agar-sealed bed of a horizontal electrophoresis apparatus. A well-forming comb was immediately set into position and the gel allowed to harden at room temperature, after which enough TPE was added to the apparatus to cover the gel to a depth of 1 mM (ethidium bromide, 0.5 ug/ml, was added to the gel solution of TPE in some cases). The well-former was carefully removed and the gel was connected to a power supply and pre-run at 50 V (constant voltage) for at least 15 minutes. DNA samples were mixed with 0.1 volume of 10X gel loading buffer (0.25% bromophenol blue, 5% SDS, 25% glycerol) and applied to the slots of the submerged gel at 5 V with a micropipetter. 10 ug of cellular DNA were

Table 1. Description of Restriction Enzymes

Enzyme	Salt concentration	Incubation temperature	Recogniton sequence
Bam HI	medium	37° C	G`GATCC
Bcl I	medium	50° C	T`GATCA
Bgl I	medium	37° C	GCCNNNN`NGGC
Eco RI	high	37° C	G`AATTC
Hae II	low	37° C	PuGCGC`Py
Hind III	medium	37° C	A`AGCTT
Kpn I	low	37° C	GGTAC`C
Pst I	medium	37° C	CTCGA`G
Pvu II	medium	37° C	CAG`CTG
Sst I (Sac I)[1]	low	37° C	CCGC`GG
Xho I	high	37° C	C`TCGAG

[1] an isoschizomar

loaded per slot. Electrophoresis was conducted at 40 V for sixteen to twenty hours at room temperature, unless otherwise noted. Lambda phage DNA cleaved by Hind III was used as a molecular weight marker. Gels run in the absence of ethidium bromide were stained in the running buffer containing 0.5 ug/ml ethidium bromide for 45 minutes at room temperature. DNA in the gel was visualized on an ultraviolet transilluminator light box and photographed with a Polaroid camera using Type 57 or Type 667 film (Kodak).

2.2.5. Transfer of DNA from agarose gels to nitrocellulose (Southern, 1975)

After electrophoresis, gels were soaked in 250 ml of 0.25 M HCl for fifteen minutes at room temperature with constant shaking to partially depurinate the DNA and facilitate subsequent transfer of large fragments. The gels were rinsed twice with distilled water and the DNA denatured by adding a 250 ml solution of 0.5 M NaOH and 1.5 M NaCl for one hour at room temperature with constant shaking. Following this, the gels were rinsed twice with distilled water and neutralized by soaking in a 250 ml solution of 0.5 M Tris-HCl (pH 7.5-8.0) and 1.5 M NaCl for one hour at room temperature with constant shaking. A sheet of Whatman 3MM paper, cut to size, was placed over a raised plastic support sitting in a reservoir filled with 20X SSPE (3.6 M NaCl, 20 mM NaH_2PO_4, 20 mM EDTA, pH 7.4),

with the ends of the filter in contact with the salt solution. The gel was then placed on top of the saturated filter paper, and any air bubbles were carefully removed by rolling a glass tube over it. A piece of nitrocellulose paper (Shleicher and Schuell BA 85), cut to the size of the gel, was pre-wet in H_2O for at least fifteen minutes and placed with forceps on top of the gel, removing air bubbles as described previously. Saran Wrap was placed around the filter, starting from the edges of the nitrocellulose filter to the outside of the reservoir, to ensure an even flow of SSPE throughout the filter. Four pieces of 3MM paper, also cut to size, were placed on top of the nitrocellulose. The piece closest to the nitrocellulose had been pre-wet in water. A stack of paper towels (one to two inches high), cut to the size of the gel, was placed on top of the 3MM paper. A glass plate was put on top of the stack and weighed down with a one-kg weight. Transfer of the DNA was allowed to proceed for fourteen to twenty hours. The stack was then disassembled; the nitrocellulose filter was peeled off the dehydrated gel and rinsed for ten minutes in 2X SSPE. The wet filter was sandwiched between two pieces of 3MM paper and baked for two hours at 80° C under vacuum. The baked filter was placed in a heat-sealable plastic bag ("Seal-a-Meal," Dazey).

2.2.6. Nick translation of DNA

^{32}P-labelled DNA probes with a specific activity greater than 10^8 cpm/ug were prepared by the method of Rigby et al (1977), as modified by Maniatis et al (1982). The nick-translation reaction was set up by the addition of the following reagants to a 1.5 ml Eppendorf tube: 1 nmole of each unlabelled dNTP (dGTP and dTTP), 180 pmoles of each (- ^{32}P) dNTP (New England Nuclear, specific activity 800 Ci/mmole [dCTP and dATP]), 1 ug of DNA, 5 ul of 10X nick-translation buffer (0.5M Tris-HCl ph 7.2, 0.1M $MgSO_4$, 1mM EDTA, 500 ug/ml BSA), and deionized H_2O to final volume of 43 ul. One ul of a 0.1 ug/ml solution of DNase I (Worthington) in 50% glycerol was added, followed one minute later by 1 ul (6.7 units) of E. coli DNA polymerase I (Boehringer Mannheim). The nick-translation reaction was performed at 16^{o} C for 60 minutes. The reaction was terminated by the addition of 2 ul of 0.5 M EDTA. The nick-translated DNA was separated from unincorporated dNTP's by "spun-column" chromotography, as described in Maniatis et al (1982). The bottom of a 1 ml tuberculin syringe was plugged with small amount of glass wool. Sephadex G-50 (Pharmacia) which had been swelled in STE (100 mM NaCl, 10 mM Tris-HCl ph 8.0, 1 mM EDTA) was packed in the syringe by centrifugation in a 15 ml conical centrifuge tube at 1500x g for four minutes until the tube packed column volume was 0.9 ml. One hundred microliters of STE were added to the column and recentrifuged under

identical conditions. This step was repeated once. The nick-translation reaction was brought to a volume of 100 ul with STE, and was then applied to the column which was then centrifuged exactly as before, collecting the 100 ul of effluent from the syringe in a decapped Eppendorf tube (the unincorporated [^{32}P] dNTP's remained in the syringe, which was discarded). The recovered probe DNA was diluted with TE to a final volume of 0.3 ml; 1 ul was used to determine specific activity of the probe by scintillation counting.

2.2.7. Hybridization and autoradiography of Southern filters

The hybridization procedure described by Wahl et al. (1979) was followed with slight modifications. A heat-sealable plastic bag containing the dried filter was opened and 10 ml of 2X SSPE was added for two minutes. This solution was decanted and 10 ml of prehybridization solution (consisting of 50% formamide [MCB], 5% dextran sulfate [Pharmacia], 2.5X SSPE, 0.1% SDS, 1X Denhardt's reagent [0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA] and 150 ug/ml sonicated, sheared salmon testis DNA) were added to the bag, which was then resealed. It was then incubated for at least three hours in a water bath at 45^{o} C. The corner of the bag was cut off and the prehybridization solution was decanted. Five ml of hybridization solution (consisting of 50% formamide (MCB) 10% dextran sulfate, 2X SSPE, 0.1% SDS, 5 mM Na_2EDTA, 50

ug/ml sonicated salmon testis DNA, and 0.5-1.0x10^7 cpm of heat-denatured ^{32}P-labelled probe DNA) were added to the bag. Excess air was removed from the bag, which was the heat-sealed and incubated for 16 to 20 hours by submersion in a water bath at 45° C. The hybridization solution was squeezed out of the bag and the filter was placed for five minutes in a glass tray containing a 250 ml solution of 2X SSPE and 0.5% SDS at room temperature. The filter was then transferred for fifteen minutes to a new glass tray containing 250 ml of 2X SSPE and 0.1% SDS at room temperature, and occasionally agitated. Finally, the filter was transferred for two hours to a tray containing 400 ml of 0.1X SSPE and 0.1% (or 0.5%) SDS at 50° C with constant gentle agitation.

The filter was blotted on a sheet of 3MM paper, wrapped in Saran Wrap, and mounted in an X-ray film holder; a single sheet of Kodak X-Omat AR-2 film was placed between the filter and the intensifying screen (DuPont Cronex Lightning-Plus), following the method of Laskey and Mills (1977). The film holder was placed at -70° C for the duration of exposure. The X-ray film was developed using conventional photographic processing methods.

2.2.8. Removing old probe for hybridization

Filters were soaked for 10 minutes at room temperature, with slight agitation, in 0.2 M NaOH and 0.1% SDS, rinsed with deionized H_2O, neutralized for three

minutes in 0.5 M Tris-HCl, pH 7.0, and washed in 0.1% SDS. Filters were dried at room temperature on 3MM filter paper, and either used immediately or stored indefinitely in heat-sealable plastic bags.

2.3 CYTOLOGICAL HYBRIDIZATION (Henderson, 1982)

2.3.1. Slide preparations

Cells were seeded on ten to twenty 100 mm plates and allowed to grow to sub-confluence. One day prior to the addition of Colcemid (Gibco), the cultures were re-fed to ensure vigorous growth. The cultures were exposed to 0.5 ug Colcemid for one to 1-1/2 hours, after which the cells were dislodged, collected with the media from the plates, and centrifuged for five minutes at 1000 g. The medium was aspirated and 8 ml of 0.075 M KCl were gently added to the test tube to swell the cells. The tube was then incubated for 45 minutes at 37^{o} C, with occasional shaking. Subsequently, 2 ml of fixative (3 parts methanol: 1 part glacial acetic acid) were added to the test tube followed by centrifugation for five minutes at 1000 g. The liquid was removed and the pellet resuspended in 3 ml of fixative. This was allowed to stand for five minutes, after which the preparation was centrifuged for five minutes at 1000 g. The fixative was removed and new fixative was added to resuspend the pellet. The preparation was stored at 4^{o} C. At least three times during the next week, the preparation

was centrifuged at 1000 g for five minutes and freshly made fixative was added to resuspend the pellet, thereby removing as much protein as possible.

2.3.2. G-Banding and chromosome identification

At the end of the week, the cells were resuspended in freshly-made fixative, and slides were prepared. These slides were allowed to dry by placing them in a vacuum at 37° C. At the end of one week, the slides were removed for banding. The staining solution (26 ml 0.02 M K_2PO_4 pH 8.6-8.9, 7 ml Methanol, 3 ml Trypsin-Versene [1X EDTA], 0.55-0.6 ml Giemsa stain [Fisher Scientific]) was added to the slide for five to six minutes and then rinsed off. If the slides were not satisfactory, the stain could be removed with 70% ethanol for one minute, and the sample restained. When the metaphase plates were properly banded, they were photographed.

2.3.3. Removal of endogenous RNA

The stain was initially removed by washing the slides twice in 70% ethanol (three minutes for each washing), and once in 95% ethanol for five minutes. The slides were allowed to air-dry. They were then treated for one hour at 37° C with 100 ug/ml RNase A (protease free) in 2X SSC (0.3 M NaCl, 30 mM sodium citrate pH 7.0). The slides were then washed in 2X SSC, followed by three washes in 70% ethanol for three to five minutes each, and two washes of 95%

ethanol for three minutes each. The slides were dessicated for at least 48 hours.

2.3.4. Nick translation of DNA for hybridization

^{125}I-labelled DNA probes with a specific activity greater than 10^8 cpm/ug were prepared by the method of Rigby et al (1977). The nick-translation reaction was set up by the addition of the following reagants to a 1.5 ml Eppendorf tube: 20 uM of each unlabelled dNTP (dATP, dGTP and dTTP), 4uM of (^{125}I) dCTP (New England Nuclear, specific activity 2,200 Ci/mmole [dCTP]), 200 ng of DNA, 4 ul of 10X nick-translation buffer (0.05M Tris-HCl pH 7.9, 0.005M $MgCl_2$, 0.01M Mercaptoethanol, 50 ug/ml BSA), and deionized H_2O to a final volume of 28 ul. One ul of a 0.1 ng/ml solution of DNase I (Worthington) in 50% glycerol was added, followed five minutes later by 120u/ml of *E. coli* DNA polymerase I (Boehringer Mannheim). The nick-translation reaction was performed at 15^{o} C for 2 hours. The reaction was terminated by the addition of EDTA to a final concentration of 10mM EDTA. The protein in the reaction mixture was extracted by adding an equal volume of phenol-chloroform-isoamyl (48:48:2) saturated with column buffer (0.01 M Tris-HCl [pH 7.8], 0.15 M NaCl, 0.01 M EDTA). After centrifugation, the aqueous phase was applied to a Sephadex G-25M column equilibrated in the same buffer. Carrier (bacterial) RNA was added to eluant [^{125}I] DNA, recovered by precipitation with 2 volumes of ethanol,

centrifuged at 9000 rpm for 30 minutes, dried and redissolved in column buffer.

2.3.5. In-Situ hybridization

The slides were incubated for two hours in formamide (Fisher) at 65-70° C. They were then immediately plunged into freezer-cold 70% ethanol, where they were soaked for ten minutes, and then washed in 70% ethanol for ten minutes. They were washed twice, for five minutes per washing, with 70% ethanol. Following this, the slides were washed in 95% ethanol twice, with each washing lasting 5 minutes. They were then blotted before adding the probe. The slides were then covered with a cover slip (22 mm x 30 mm), and incubated at 38° C for approximately 18 hours. At the end of this period, slides were put into a dish containing 50% formamide and 3X SSC, and washed in 2X SSC and 0.1 M KI. The wash solution was changed three to four times, with each wash lasting 15-30 minutes. The slides were washed in 2X SSC eight to ten times, followed by three washes in 70% ethanol (five minutes each), and, finally, two washings in 95% ethanol (five minutes per washing). The slides were allowed to air dry for thirty minutes, and then dipped in 50% NTB-2 emulsion (Kodak). They were sealed in a light-proof box and stored at -20° C until developing. They were developed for 2-1/2 minutes in D-19 developer, followed by a 15-second immersion in 1% acetic acid, and a 4-minute immersion in fixer. The slides were

washed in running water for thirty minutes and stained (5 ml 10X E buffer [40mM Tris, 20mM NaOAC, 1mM EDTA, pH 7.7], 40 ml dH_2O, 5 ml Giemsa stain) for two hours. The control slides were stained for thirty minutes.

2.4 ASSAYS FOR VIRAL PROTEINS

2.4.1. Immunofluorescent staining of monolayer cells

Cells were seeded on rectangular coverslips (11 mm x 22 mm No. 2, Cat. no. 6663-Q10, Thomas Scientific). Subconfluent coverslips were fixed by the method of Solomon et al (1979) in the following manner. Medium was removed and the coverslips were washed twice with PBS at room temperature. The coverslips were then incubated at room temperature overnight in a freshly prepared 3.7% formaldehyde solution. The formaldehyde solution was then removed, and the coverslips were stored in PBS at 4^{o} C. To permeabilize the cells, the coverslips were incubated for two minutes at -20^{o} C in a cold 50% PBS:Acetone solution, followed by a five minute incubation in cold 100% acetone at -20^{o} C and again 2 minutes in the 50% mixture. To stain the coverslips, they were transferred to a dry 150 mm Petri dish which served as a holder. The appropriate primary antibody (see Table 2) was applied to the coverslips and incubated for 45 minutes in a humidified chamber at 37^{o} C. Subsequently, the coverslips were washed three times in PBS before the appropriate fluorescein conjugated antiglobulin

Table 2. Description of Antibody Source and Specificity

Antibody	Source	Specificity	Laboratory
H T	polyclonal (serum)	SV40 T antigen	N.I.H.
M T (pAb 101)	monoclonal (purified)	SV40 T antigen (carboxyl terminal region)	E. Gurney
M T (pAb 108)	monoclonal (purified)	SV40 T antigen (amino terminal region)	E. Gurney
M T (pAb 416)	monoclonal (ascites)	SV40 T antigen (amino terminal region)	C. Prives R. Pollack
M T (pAb 419)	monoclonal (ascites)	SV40 T antigen (amino terminal region)	T. Kelly
R SDS disrupted virus	polyclonal (serum)	SV40 VP1 and VP3	H. L. Ozer

H = Hamster
M = Mouse
R = Rabbit

and Rhodamine-Bovine Serum Albumin (NIH Reagent) were applied to the coverslips, and again incubated for 45 minutes. The coverslips were then washed three times in PBS and once in distilled H_2O before being mounted, cell side down, onto a slide using 50% glycerol as mounting medium. The slides were viewed on a Leitz Microscope Ortholux Model, equipped for fluorescence microscopy under epi-illumination.

For photographic purposes, coverslips were mounted as described by Johnson et al (1981) [August-Waeldin method] to minimize bleaching. This mountant was composed of 10 ml of PBS containing 100 mg of p-phenylenediamine and 90 ml of glycerol. The final pH was adjusted to approximately 8.0, with 0.5 M carbonate-bicarbonate buffer at pH 9.0. Solutions containing p-phenylenediamine rapidly become brown on standing at room temperature and are then unsatisfactory for use in critical microscopy. This problem was overcome by storing the mixture in the dark at -20° C, in aliquots sufficient for one day's use.

2.4.2. Immunoprecipitation

Immunoprecipitates of viral proteins to be analyzed by polyacrilamide gel electrophoresis (PAGE) were prepared using either equal volumes of whole lysates or varied volumes containing equal TCA-precipitable radioactivity. These were added in a 1:3 ratio to the extraction buffer and then incubated for 1 hr at 4° C with 15-20 ul anti-T

serum. After the hour, 75-100 ul Staph A (Calbiochem 10% suspension) were added and re-incubated for 30 minutes on ice. The pellets were collected by centrifugation for 1 minute in a microfuge and washed vigorously 3 times in pellet washing buffer (1 M NaCl, 0.002 M EDTA, 1% NP40 and 0.001 M Tris pH 7.5). After the last wash, 20 ul of 3X sample buffer (30% glycerol, 1.5% B-mercaptoethanol, 0.95% SDS, 0.18 M tris, pH 6.8, and a few grains of Bromophenol Blue) and 30 ul of distilled deioinized H_2O was added to each sample. The samples were vortexed and heated at 90^{o} C for 2 minutes to disassociate the immunoprecipitate. The sample was then centrifuged in a microfuge and was loaded on a gel. Typically, one-half of the sample was used per lane.

2.4.3. Polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970)

For the preparation of a vertical 12.5% polyacrylamide gel, 20 ml of a separating gel solution was made up in the following manner. A 30% acrylamide (Biorad) and 0.8% bis (Biorad) stock solution was appropriately diluted in lower tris buffer (0.4% SDS, 0.015 M Tris-HCl, pH 8.8) plus 60 ul freshly made 10% ammonium persulfate (Biorad). The solution was put into a 125 ml side arm flask and then degassed. Nine microliters of Temed (Biorad) were added and mixed thoroughly. Two glass plates were clipped together with spacers (1mm in width) on 3 sides and sealed

with hot 1.4% agar (Nobel, Difco). The solution was poured into this space. A 0.1% SDS solution was layered on top of the gel in order to have a smooth interface. After the gel was polymerized (about 30 minutes), a 10ml stacking gel solution was prepared. The bis-acrylamide stock solution was diluted to a final concentration of 3% acrylamide in upper tris buffer (0.005 M Tris-HCl, pH 6.8, 0.4% SDS) plus 60 ul 10% ammonium persulfate and 20 ul Temed. A 12-well (1 mm width) teflon comb was placed into the stacking gel after it had been poured on top of the separating gel. It was then allowed to polymerize undisturbed. After polymerization had taken place (about 30 minutes), the bottom spacer was removed and the glass plates were clipped into a vertical gel apparatus (Aquabogue, L.I.). The space between the glass plate and the wall of the apparatus was sealed with hot agar. Gel running buffer (0.025 M Tris, 0.19 M Glycine, 0.1% SDS) was added into the upper chamber and checked for leakage into the lower chamber. The lower chamber was then filled and a syringe with a bent needle was used to remove air bubbles at the bottom of the gel. The comb was removed and the samples loaded. Twenty five microliter samples were loaded per well and electrophoresed for 3-5 hours at 150 volts.

2.4.4. Immunoblotting Procedure (Towbin et al., 1979)

After the completion of electrophoresis, the gel was removed and placed into electroblot buffer (192 mM glycine,

20% methanol, 24 mM Tris, pH 8.3). A piece of 3MM filter paper was placed on top of the electrophoretic transfer grid. The gel was placed on top of the 3MM filter paper and a piece of prewet nitrocellulose filter (BA 85, Schleicher and Schuell) was placed with forceps on top of the gel. Another piece of 3MM filter was placed on top of the nitrocellulose paper. This entire "sandwich" was smoothed out to remove trapped air bubbles. The electrophoretic transfer grid was placed in the Electroblot apparatus (Hoefer). The transfer was completed in 16 hours at 0.1 amps.

For the blotting reaction, the filter was placed in a heat-sealable plastic bag ("Seal-a-Meal", Dazey) and incubated for one hour in blocking buffer containing 3% gelatin in Tris buffered saline (TBS) (50 mM NaCl, 20 mM Tris, pH 7.5). At the end of the incubation period, the bag was opened at a corner and the blocking buffer was squeezed out and replaced with a primary anti-T antibody solution in 1% gelatin. The bag was resealed and incubated on a shaker for 1 hour at room temperature. The filters were then washed four times in TBS over a period of 20 minutes, placed into a new bag and incubated with Horse Radish Peroxidase (Bio Rad) for 1 hour on a shaker at room temperature. The filter was then washed four times and developed in a solution of 0.05% 4 chloro-1-napthol, 0.01% H_2O_2 in TBS. When the filter was fully developed (approximately 15 minutes), it was rinsed with distilled

H_2O and photographed. It was then possible to store the filter in the dark at 4° C (in H_2O) for several months.

CHAPTER 3: RESULTS

ISOLATION OF IMMORTALIZED LINES

Human diploid cells derived from normal tissue undergo a characteristic pattern of growth in cell culture. After an initial period in culture (phase I), fibroblasts undergo a stage of rapid proliferation (phase II). At later stages, cells show an increased cell volume, a lengthening of interdivision time, and a decline in saturation density following plating, culminating in a non-replicative phase (phase III) and, ultimately, cell death (Hayflick and Moorehead, 1961). After approximately 50-60 population doublings (PD), the culture becomes incapable of further proliferation. The cells can, however, remain viable for several weeks in phase III. This pattern has been consistently demonstrated for many cell types. Immortalization of normal human diploid fibroblasts in culture has not been reported. When human diploid fibroblasts are infected with SV40, transformed foci that are capable of extended proliferation appear. Although these SV40-transformed cells have several transformed properties, they still have a finite proliferative lifespan (Sack and Obie, 1981). After a period of proliferation, which is often 20-40 population doublings longer than the lifespan of the uninfected control cells, the SV40-transformed human diploid fibroblasts cease to increase in

cell number and enter the crisis stage. Over the ensuing weeks, the number of cells in the population decreases, and, usually, the culture is lost (termed "crisis"). On rare occasions ($<10^7$ [Huschtscha and Holliday, 1983]), a variant cell type survives crisis and gives rise to a post-crisis cell line with unlimited proliferative potential. As discussed in the Introduction, this phenomenon is the result of a complex series of virus-cell interactions. The complexity is further complicated by the fact that the SV40-transformant is capable of supporting extrachromosomal viral DNA synthesis and virion production, either of which might be expected to be associated with decreased cell growth and/or viability. Although transformation functions of the genome are not affected by such SV40 molecules, it would therefore be beneficial to study SV40-mediated immortalization in a system in which viral DNA replication and lytic infection are eliminated. Such a possiblility was provided when HS74BM (a female, normal human fetal bone marrow fibroblast) was transformed by a recombinant SV40 DNA molecule, pMK-SV40-Bgl 1^r (SV ori^-), in this laboratory (Small et al, 1982). In this construction, a 6 base pair deletion was introduced within the origin of DNA replication (0.67 map units) by <u>in vitro</u> site-specific mutagenesis (Gluzman and Sambrook, 1979). Thus, it has an efficient transforming early region but is unable to excise and replicate its DNA extrachromosomally or to produce infectious virus in semipermissive human cells, as

previously demonstrated in established monkey cells (COS [Gluzman, 1980]). Consequently, such cells are advantageous for studying the mechanisms of immortalization for several reasons. First, they are clonal isolates (as colonies in agarose); secondly, they are genetically unable to replicate viral DNA, minimizing the complication due to viral cytopathic effects (viral DNA or particle formation); and thirdly, the pattern of integration of viral DNA would be expected to be more stable due to restriction of viral excision, transient extrachromosomal replication, and reintegration. Pursuing this line of reasoning, four isolates were selected from human fibroblasts transformed by various SV40 sequences, which had been previously isolated as colonies in agarose (Small et al, 1982). One was transformed with recombinant pSVori$^-$ form I DNA (IF4A-1), a second was transformed with pSVori$^-$ DNA linearized within the plasmid sequences (IF6A-1), and a third had the plasmid sequences separated from the SV40 sequences prior to transformation (IF5A-2); the fourth, transformed by an intact viral genome, was used as a control (IF1A-2). (See Materials and Methods for a more detailed description of the sequences involved.)

Previous attempts at immortalizing human cells with SV40 were relatively unsuccessful. Although SV40 transformation increases the growth potential of human fibroblasts, the frequency at which cell lines survive crisis has not been found to exceed 10^{-6} (Sack, 1981). The

fact that an intact SV40 genome (designated SVori$^+$) had been used may have been the reason for their failures, since this could have allowed for virus production. Since the human cells I planned to use were transformed with SVori$^-$, I felt that I would have a better chance at acquiring an immortalized line, as virus production was precluded. With this in mind, the four isolates were subcultured (1:5 dilution weekly when confluent) to determine if they had unlimited lifespans in culture. The culture medium was supplemented in separate cultures with 10% FBS or 10% NCS for each transformed fibroblast. IF1A-2, the virally transformed cell strain, could be passaged once weekly in medium supplemented with 10% FBS for the first five passages (see Table 3), but required 11 days until it could be passaged again, and could not be passaged for a seventh and final time until 28 days had elapsed. After this last passage, no colonies became visible although the dish was re-fed for almost two months. When IF1A-2 was passaged in medium supplemented with 10% NCS, a slightly better result was obtained (see Table 4). The culture was able to be passaged once a week or more frequently for the first nine passages; however, almost two weeks elapsed between the next two passages, and more than three weeks for the last passage (#12). With this experiment, clonal growth was found after the last passage, but once again, the culture did not survive "crisis." It should be noted that all the cultures (IF1A-2, IF4A-1,

Table 3. Passage History of IF1A-2 in 10% FBS[a]

Passage Number	Cells (x 10^6)[b] Per Dish	Days Per Passage	Total[c] Generations
0	3.3	---	---
1	5.0	7	3
2	3.9	7	5
3	6.8	7	8
4	3.3	7	9
5	2.9	7	11
6	3.2	11	14
7	4.4	28	17

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.
[b] Corresponds to the number of cells on the trypsinized dish.
[c] Generation number was calculated to the nearer integer.

Table 4. Passage History of IF1A-2 in 10% NCS[a]

Passage Number	Cells ($\times 10^6$)[b] Per Dish	Days Per Passage	Total[c] Generations
0	3.3	---	---
1	5.0	7	3
2	3.7	7	5
3	6.2	4	8
4	5.5	6	10
5	3.5	4	12
6	3.4	7	15
7	5.9	7	17
8	4.9	7	19
9	3.3	7	21
10	2.9	12	24
11	4.0	13	26
12	3.1	24	28

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

IF5A-2, IF6A-1) had undergone a number of generations prior to these manipulations. Therefore, 42 generations should be added to all passage histories. The parent, HS74BM, senesces when it reaches approximately 60 generations as shown in Table 5 (one should add approximately 20 generations for the first ten passages prior to the beginning of the recorded passage history of HS74BM). Thus, both cell lines show passage histories consistent with the expected number of generations for a normal human diploid fibroblast (sixty) as previously reported (Hayflick and Moorehead, 1961; Hayflick, 1980), and no extension was observed for the SV40 transformant generated by the wild-type viral genome. These data are graphically depicted in Figure 3.

With the other three transformants, somewhat better results were observed. IF4A-1, supplemented with FBS, was passaged within a week for most of its first 16 passages, but the seventeenth passage required almost two weeks (see Table 6). After incubating the culture for six weeks following the seventeenth passage, it was discarded, since no living cells were evident on the dish. When this transformant was supplemented with 10% NCS, a poorer result was obtained (see Table 7). Most of the initial 11 passages were performed within a week. The following three passages took eight to ten days, and the final (15th) passage required two weeks. Once again, this last dish was carried for nine weeks, at which point it was discarded.

Table 5. Passage history of HS74BM[a]

Passage Number	Cell ($x10^6$)[b] Per Dish	Days Per Passage	Total[c] Generations
5	5.9	3	---
6	3.9	7	2
7	4.2	7	5
8	5.0	7	8
9	3.5	7	10
10	6.1	11	13
11	5.2	6	15
12	2.2	4	16
13	4.1	8	19
14	6.7	10	22
15	6.4	7	24
16	5.1	6	26
17	4.4	8	28
18	3.8	10	30
19	2.4	7	32
20	2.9	11	35
21	4.0	16	38
22	4.0	13	40
23	2.8	22	42

[a] Cells were acquired at passage 5 (designated P_0), passaged three times for expansion, frozen, thawed, passaged twice and refrozen (P_5). These cells were thawed and used for this experiment.

[b] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[c] Generation number was calculated to the nearer integer.

Figure 3. Comparison of growth patterns of HS74BM (represented by closed circles) and IF5A-2 (represented by open circles).

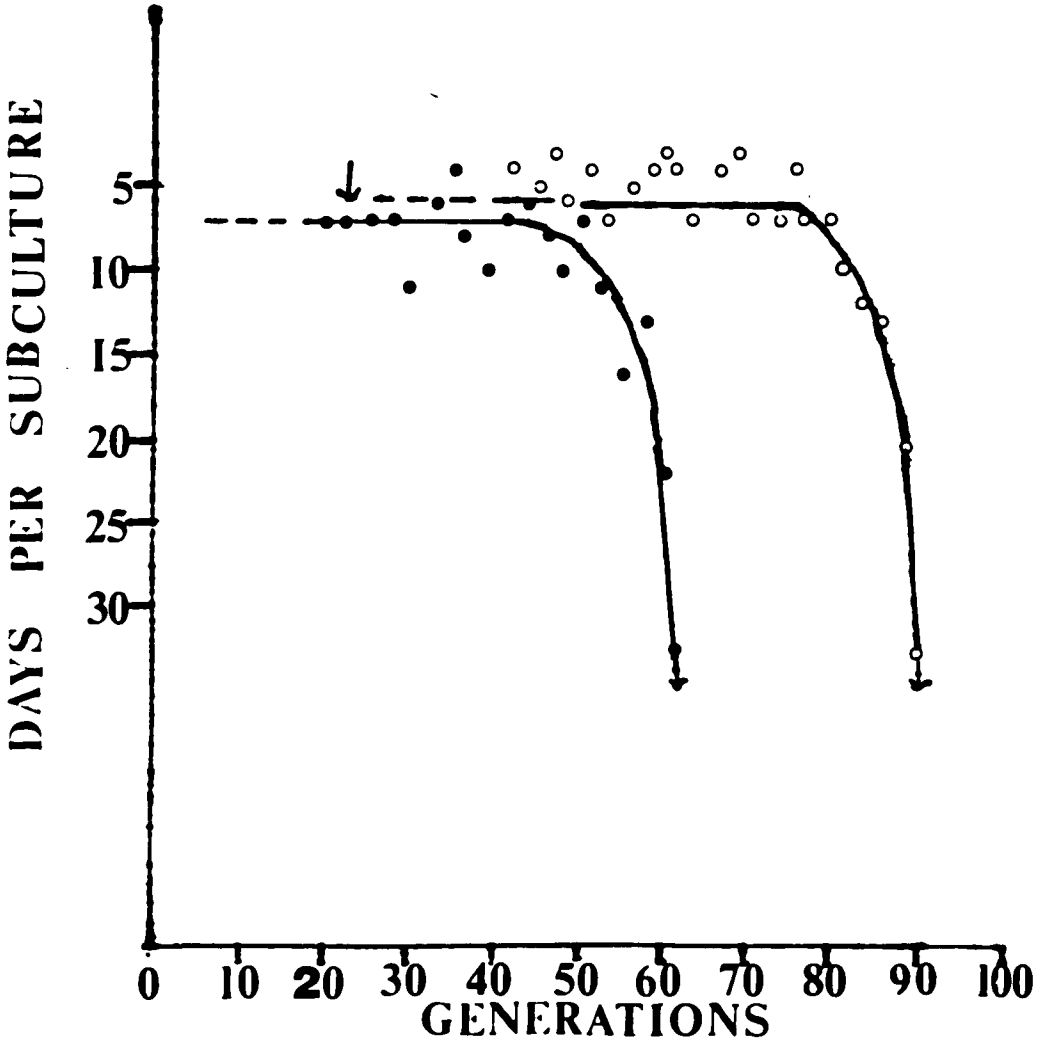
DAYS PER SUBCULTURE
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
90
100
GENERATIONS

Figure 3

Table 6. Passage History of IF4A-1 in 10% FBS[a]

Passage Number	Cells (x 10^6)[b] Per Dish	Days Per Passage	Total[c] Generations
0	4.1	---	---
1	11.0	6	4
2	10.0	6	6
3	5.3	7	7
4	5.3	3	9
5	8.6	7	12
6	13.0	6	15
7	5.7	3	16
8	4.7	4	18
9	2.9	7	20
10	6.9	11	24
11	4.3	7	26
12	5.8	10	29
13	4.3	11	31
14	5.0	10	34
15	6.6	7	37
16	5.5	7	39
17	2.5	13	40

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Table 7. Passage History of IF4A-1 in 10% NCS[a]

Passage Number	Cells ($x10^6$)[b] Per Dish	Days Per Passage	Total[c] Generations
0	4.1	---	---
1	11.0	6	4
2	6.5	6	6
3	9.3	6	9
4	5.3	3	11
5	5.8	4	13
6	2.5	3	14
7	5.6	6	17
8	8.8	10	20
9	5.0	4	22
10	6.0	7	25
11	6.3	7	27
12	5.3	10	29
13	3.6	8	31
14	4.1	10	33
15	3.5	14	35

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Although an extended life span (18 generations beyond the expected sixty) was obtained, the cultures succumbed to crisis.

The best results were obtained with IF5A-2. The culture supplemented with NCS fared better than the one supplemented with FBS. The NCS-supplemented culture was passaged once a week or more frequently until passage 18 (see Table 8). The next three passages required 10, 12, and 13 days respectively, and the final (22nd) passage, three weeks. This last culture was re-fed for more than a month and rare small colonies were observed, but these were never able to be grown to mass culture. This is graphically represented in Figure 3. When FBS was used as the supplement, the cells were passaged within one week for the first 17 passages, but required 1-1/2 weeks for the subsequent two passages (see Table 9). Although these cultures were maintained for more than two months and many small colonies were observed, the dishes never became confluent, nor could the cells be passaged.

The last of the transformants, IF6A-1, had a history similar to that of IF5A-2. When the culture was supplemented with FBS (see Table 10), 14 passages occurred before the growth of the culture slowed. The next four passages required from 1-1/2 to three weeks before the culture was able to be passaged. Although many colonies appeared three weeks after the last subculture, they did not became sufficiently abundant to form a mass culture.

Table 8. Passage History of IF5A-2 in 10% NCS[a]

Passage Number	Cells ($x10^6$)[b] Per Dish	Days Per Passage	Total[c] Generations
0	6.0	---	---
1	9.8	4	3
2	11.0	5	5
3	6.2	3	6
4	8.0	7	9
5	7.6	4	11
6	16.0	7	14
7	18.0	5	16
8	13.0	4	18
9	7.4	3	19
10	6.5	4	21
11	12.0	7	24
12	8.7	4	26
13	5.6	3	28
14	10.0	7	31
15	7.4	7	33
16	3.0	4	34
17	4.5	7	37
18	4.4	7	39
19	4.8	10	41
20	3.3	12	43
21	4.0	13	46
22	2.6	21	48

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Table 9. Passage History of IF5A-2 in 10% FBS[a]

Passage Number	Cells($\times 10^6$)[b] Per Dish	Days Per Passage	Total[c] Generations
0	6.0	---	---
1	15.0	5	4
2	8.4	4	5
3	15.0	7	8
4	17.0	4	10
5	7.9	3	11
6	7.0	4	13
7	5.4	3	15
8	10.0	7	18
9	4.3	4	19
10	3.8	3	21
11	5.8	4	24
12	10.0	7	27
13	8.3	7	29
14	8.0	7	31
15	5.5	8	33
16	6.8	6	36
17	3.7	7	38
18	4.7	10	41
19	5.5	11	44

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Table 10. Passage History of IF6A-1 in 10% FBS[a]

Passage Number	Cells (x 10^6)[b] Per Dish	Days Per Passage	Total[c] Generations
0	5.7	---	---
1	5.3	3	2
2	5.0	4	4
3	12.0	5	8
4	4.6	3	9
5	7.8	4	12
6	13.0	7	15
7	7.4	4	16
8	13.0	7	19
9	8.2	4	21
10	9.0	7	23
11	12.0	10	25
12	8.5	7	27
13	3.8	4	28
14	7.0	7	27
15	7.8	10	33
16	11.0	21	36
17	10.0	14	38
18	5.3	11	39

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Table 11. Passage History of IF6A-1 in 10% NCS[a]

Passage Number	Cells (x 10^6)[b] Per Dish	Days Per Passage	Total[c] Generations
0	5.7	---	---
1	5.3	3	2
2	9.3	4	5
3	12.0	5	8
4	5.4	3	9
5	3.1	4	10
6	11.0	7	14
7	4.5	3	15
8	12.0	6	19
9	8.2	3	21
10	9.0	4	23
11	14.0	7	26
12	11.0	7	28
13	6.1	4	29
14	9.2	7	32
15	7.4	6	34
16	6.5	8	36
17	5.2	10	38
18	6.2	11	40
19	6.3	17	42
20	5.0	18	44
21	2.5	14	45

[a] Cultures were thawed, allowed to reach confluence and then trypsinized. Subsequently, cultures were passaged 1:5 when confluent, regardless of cell number.

[b] Corresponds to the number of cells on the trypsinized dish.

[c] Generation number was calculated to the nearer integer.

Cells were passaged with medium containing NCS at least once weekly until passage 16 (see Table 11). The next two passages required a week and a half, and the last three passages required 2 to 2-1/2 weeks each. This last culture contained only rare, small colonies after several weeks. In summary, we see that the SVori$^-$ transformants showed an increased longevity (from 9 to 24 generations) beyond that found for normal human fibroblasts. However, they all succumbed to crisis and cell death. The results of all these Tables, summarized in Table 12, indicated that transformation by an SV40 genome containing the ori$^-$ mutation was not sufficient to immortalize these cells. Therefore, crisis could not be due simply to virus replication but, rather, involved changes in the cells themselves; more extensive work would have to be done in order to immortalize the cultures. I decided to concentrate my efforts on IF5A-2 for three reasons: first (and most importantly), the transformation was accomplished with SV40 sequences only; secondly, it grew the greatest number of generations; and, thirdly, karyotype analysis indicated that at least 40% of the metaphase plates contained a pseudodiploid number of chromosomes. The other two transformed cell lines showed rare cells with a pseudodiploid number of chromosomes.

There were several possible combinations of rate of immortalization (the ability of an individual cell to overcome senescence) and rate of rescuability (the ability

Table 12. Comparison of Lifespans of Various Cell Lines[a]

Cell Line	Total Generations
HS74BM	62
IF1A-2	59, 70
IF4A-1	77, 82
IF5A-2	86, 90
IF6A-1	81, 87

[a] This Table summarizes Tables 3,4, and 6 through 11.

to isolate such a cell and subsequently establish a cell line) to explain why establishment of cell lines did not occur -- high frequency of immortalization/low rescuability (HI/LR), low frequency of immortalization/low rescuability (LI/LR), and low frequency of immortalization/high rescuability (LI/HR). High frequency of immortalization/high rescuability (HI/HR) would, obviously, result in an established cell line and did not occur in this system, based on the above results.

The first model (HI/LR) was investigated by supplementing the standard growth medium (see Materials and Methods) with 20% NCS or 20% FBS, in the event that the transformants have a progressively decreasing ability to respond to serum as they age (as later reported by Stein, [1985]). Since dexamethasone (DEX [550ng/ml]), was shown to extend the lifespan of human diploid fibroblasts, it, too, was used as a supplement for growth (Phillips and Cristofalo, 1981; Walthall and Ham, 1981). A combination of additives (epidermal growth factor [100ng/ml], insulin [5ug/ml], transferrin [5ug/ml], and DEX [55ng/ml]) was also used as a supplement to the standard medium because Macieira-Coelho (1966) and Cristofalo (1970) showed that these factors support the proliferation of normal human diploid fibroblasts at a rate and to a density similar to that of medium supplemented with 10% FBS. Sets of three 60mm dishes, containing each one of the medium variations described above, were seeded with $2x10^5$ cells. The results

are summarized in Table 13. The experiment was started at passage 21 since it was felt that a short exposure to one of these conditions should suffice for an effect on recovery. However, even though the cells were passaged once for most conditions (except 20% NCS), these conditions did not extend the cells' lifespan. Although culture incubated with 20% FBS or serum free medium (SFM, see Table 13) did show an increase in replicative capacity, none of these parameters resulted in culture which was able to be passaged a second time, despite having been maintained in culture for at least a month. This result indicated that either these additives had no effect on senescence, or, alternatively that the cells were exposed to these conditions at too late a stage to be effective. To explore the latter possibility, a duplicate experiment was set up at an earlier passage (as shown in Table 14). Once again, $2x10^5$ cells were innoculated per dish in triplicate, beginning at passage 15. None of the cultures reached passage 19, a passage that IF5A-2 had reached in an earlier experiment (see Table 7). (One should note that unlike WI-38, which always reached the same passage after thawing [Hayflick, 1965], when IF5A was thawed, it did not always reach precisely the same passage [see also Table 20]. We attribute this variability to technical considerations.)

Concurrently, a similar experiment was set up to explore the second model (LI/LR). $7.5x10^5$ cells were seeded on each of three 60mm dishes (see Table 15) under

Table 13. Passage History of IF5A-2 in supplemented media[a]

Cells (x 10^5) Per Dish in [b]					Passage	
NCS 10%	FBS 20%	NCS 20%	DEX.[c]	SFM[d]	Days[e]	Number
1.9	4.6	<1.0	1.9	3.4	14	1
0	0	0	0	0	>14	2

[a] Cultures were trypsinized and counted, and $2x10^5$ cells were seeded on each of three dishes. If possible, $2x10^5$ cells were seeded in subsequent passages. The standard growth medium (10% NCS) was supplemented with the additives listed below.

[b] Average number of cells per dish

[c] 550 ng/ml dexamethasone.

[d] Additives commonly used in serum-free medium (100 ng/ml epidermal growth factor, 5 ug/ml insulin, 5 ug/ml transferrin, 55 ng/ml dexamethasone).

[e] Time elapsed from last passage.

Table 14. Passage History of IF5A-2 in Supplemented Media[a]

Cells (x 10^5) Per Dish in [b]					Passage	
NCS 10%	FBS 20%	NCS 20%	DEX.[c]	SFM[d]	Days[e]	Number
22.0	22.0	30.7	33.3	56.7	15	1
13.5	22.3	13.7	16.3	5.0	19 -- 27 41 --	--2
N.D.	N.D.	0.4	2.0	N.D.	28 -- 29 --	--3

[a] Cultures were trypsinized and counted, and $2x10^5$ cells were seeded on each of three dishes. If possible, $2x10^5$ cells were seeded in subsequent passages. The standard growth medium (10% NCS) was supplemented with the additives listed below.

[b] Average number of three dishes.

[c] 550 ng/ml dexamethasone.

[d] Additives commonly used in serum-free medium (100 ng/ml epidermal growth factor, 5 ug/ml insulin, 5 ug/ml transferrin, 55 ng/ml dexamethasone).

[e] Time elapsed from last passage.

[f] N.D.= not determined

Table 15. Passage History of IF5A-2 in supplemented media[a]

Cells (x 10^5) Per Dish in [b]					Passage	
NCS 10%	FBS 20%	NCS 20%	DEX.[c]	SFM[d]	Days[e]	Number
12.3	40.0	30.0	40.0	48.7	9	1
			31.2	31.0	14----	
		19.7			20	---2
18.0	20.3				30----	
			22.0		12----	
8.7					14	
		14.5			17	---3
				60.0	20	
	8.7				24----	
			20.0		12----	
		4.7			17	---4
				1.6	20	
NR[f]	NR				>100----	
				4.6	7----	
			2.6		24	---5
NR	NR	NR			>100----	

[a] Regardless of cell number, 7.5 x 10^5 cells were seeded on each of three dishes. The standard medium was supplemented with the additives listed below.

[b] Average number of three dishes in each condition.

[c] 550 ng/ml dexamethasone.

[d] Additives commonly used in serum-free media (100 ng/ml epidermal growth factor, 5 ug/ml insulin, 5 ug/ml transferrin, 55 ng/ml dexamethasone).

[e] Time elapsed from last passage.

[f] These cultures never reached this passage number.

the different medium conditions. Once again, most of the cells in most of the conditions became senescent by passage 20. An exceptional result was obtained with the cells treated with 20% NCS. The cells, which were in a 60mm dish for more than 100 days after passage 19, were transferred to a 35mm dish to maintain their density, and three weeks later were transferred back to a 60mm dish. Sixteen days later, the contents of the 60mm dish were passaged into two 100mm dishes. Five days later, each dish was split 1:5 and this regimen was continued for another 40 passages. This line was called Cl-5. A few colonies were evident in a 100mm dish treated with 20% NCS after the cells had been in culture for almost 2 months. These were picked and put into 24-well dishes. After 15 weeks had elapsed, all the cells from two wells were transferred to two separate 35mm dishes. A week later, they were transferred to a 60mm dish. After slightly more than a week, the cells were tranferred to a 100mm dish. Counting of passage number began at the transfer from 24-well dishes to 35mm dishes. Following this, they were passaged approximately once a week (1:5 split) until passage 30, and subsequently once weekly (1:10 split) from passage 30, until at least passage 80 (Cl-2) or passage 100 (Cl-3). Although I had thus succeeded in establishing immortalized lines, the process of acquiring these lines was not very noteworthy as this work was reminiscent of work done in other laboratories (Hayflick, 1977). These results indicated that the various

additives had no effect on the lifespan of these cells.

The third model (LI/HR) was explored by seeding $3x10^7$ cells under cloning conditions. Since the results to date were all negative, another transformant, IE52 (HS74BM transformed with linearized pSVori$^-$ DNA), was used for this experiment. I considered that my lack of success was possibly due to the particular transformant with which I was working, rather than to the technique itself. The isolate IE52 differs from IF6A-1 in that IF6A-1 was picked as a discrete colony in agarose, whereas IE52 came from a culture with multiple foci in monolayer whose cells were pooled and frozen down. Since IE52 was not passaged through agarose, it had attained fewer generations than IF6A-1 prior to the initiation of this procedure. The IE52 cells were therefore passaged to attain 49 generations (see Table 16) before the beginning of the experiment, so as to have the the cells at a stage similar to that of the other transformant (IF5A-1) when the procedure was implemented. Ten T-150 cm^2 flasks were each seeded with $1x10^6$ cells and twenty 100mm dishes were seeded with $5x10^5$ cells, for a total of $3x10^7$ cells. Twenty six colonies were picked from these plates and put into the wells of two 24-well dishes, but none of the colonies could be subcultured beyond this point. After 10 weeks in culture, the dishes were stained, and no colonies were observed on these plates.

At this point, I considered a new model for the immortalization phenomenon. I postulated that

Table 16. Passage history of IE52

Passage Number	Days Per Passage	Total Generations
1	8	0[a]
2	5	8
3	14	13
4	6	13
5	7	16
6	4	17
7	12	22
8	22	25
9	12	28
10	15	29
11	14	32

[a] Prior to this, the cells had gone through approximately 17 generations prior to and during transformation.

immortalization was a rare selective event that could occur soon after transformation of the cells by SV40. However, time (and passaging) was needed to eliminate the other cells that were not immortal. Although this hypothesis was very similar to the low immortalization-high rescuability (LI/HR) model described above, the major difference was the timing; the procedure must be initiated at an early point in the lifespan of the cell. However, one could not start too early in the course of events, since there would be too many cells with which to deal. To establish when the appropriate point might be (i.e., just prior to the beginning of senescence of the population), an efficiency of colony (EOC) formation in monolayer and agarose was determined for IF5A-1 (see Figure 4). Starting at passage 10 (which had an EOC of approximately 10%), the efficiency fell from 3.0% at passage 11 to less than 0.01% at passage 17 in monolayer, and from 0.03% to below 0.001% for agarose. The same experiment was carried out concurrently on immortalized LNSV (a cell transformed with SV40 derived from a Lesch-Nyhan patient), as a control. This indicated that the best time to attempt to immortalize the cells would be at passage 11, just prior to the decline in efficiency of colony formation. This would give us the opportunity to work with fewer cells (but not too few), so that we would be able to pick a significant number of colonies that would allow us to identify an immortal cell line. Ten dishes were each seeded with 10^3 cells (IF5A-2).

Figure 4. Graphs of efficiency of colony formation in monolayer and agarose. 60mm dishes were seeded with 1 x 10^2, 1 x 10^3, and 1 x 10^4 cells, refed biweekly and colonies were counted at the end of two weeks for monolayer (Figure 4A). 1 x 10^3, 1 x 10^4, and 1 x 10^5 cells were seeded on 60mm dishes for agarose (Figure 4B) (as described in Materials and Methods). Numbers on graph represent larger, macroscopic colonies. A large number of very small, macroscopic colonies was also observed at passages 11 and 12 and, to a lesser extent, in passage 13. The overall colony frequency was approximately 380 per 1 x 10^5 cells at passage 11.

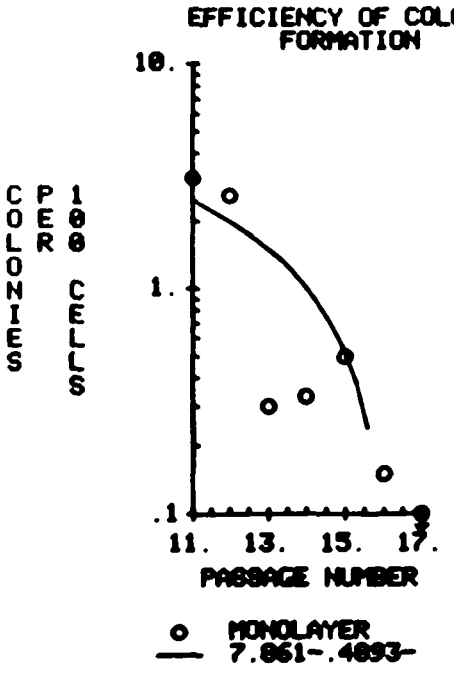

Figure 4A

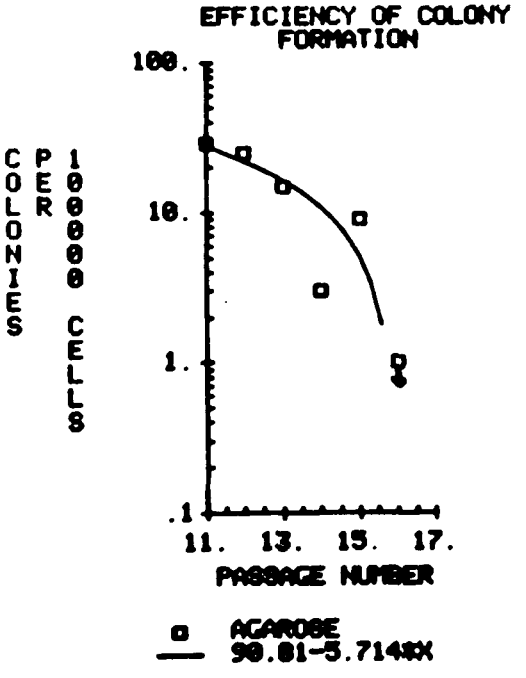

Figure 4B

After two to three weeks, individual colonies were picked with sterile wooden dowels and transferred to a 24-well dish which contained 20% FBS. The cells were not expected to be well-dispersed. After several days to a week, cells were dispersed and/or transferred using the following procedure. The medium was removed, and 1 ml of trypsin-EDTA was added for 1/2 to 1 minute, and then removed. The cells were suspended in 1 ml of medium without serum with gentle trituration, and innoculated into a 30mm well containing medium supplemented with 20% FBS. After one to four hours, cultures were re-fed with medium supplemented with 20% FBS. Subsequently, the dishes were subcultured into 60mm and 100mm dishes. After picking approximately 100 colonies, three immortal cell lines were obtained (see Table 17). These cell lines are considered immortal since they have been subcultured (at 1:5 until reaching passage 30 and 1:10 from passage 30 onwards) more than 100 times. Concurrently, another experiment was initiated using IE51 (another cell line started from a culture which contained multiple transformed foci following transformation with linearized pSVori$^-$ DNA). These cells were passaged prior to the beginning of the experiment in order to approximate a similar passage number as that of IF5A-2 (see Tables 7 and 8), although one should note that the generation number is not identical, since IF5A-2 was cloned in agarose, and IE51 was not. The passage history of IE51 is summarized in Table 18. Ten dishes were each seeded with $1x10^3$ cells and

Table 17. Lifespan of clones picked from IF5A-2P_{10}/P_1

	Petri Dish Size				Subculture	
	15mm[a]	35mm	60mm	100mm	1:3	1:5
1	----------					
2	----------					
3	----------					
4	----------	----------				
5	----------	----------				
6	----------					
7	----------	----------				
8	----------					
9	----------					
10	----------					
11	----------					
12	----------					
13	----------					
14	----------					
15	----------	----------				
16	----------					
17	----------					
18	----------					
19	----------					
20	----------					
21	----------	----------				
22	----------					
23	----------	----------				
24	----------					
25	----------	----------				
26	----------					
27	----------					
28	----------					
29	----------					
30	----------					
31	----------					
32	----------					
33	----------					
34	----------					
35	----------					
36	----------					
37	----------	----------	----------	----------	----------	FD[b]
38	----------	----------	----------	----------	----------	FD
39	----------	----------	----------	----------	----------	FD
40-103	--[c]					

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

[b] Some of the cells were frozen down at this point.

[c] The last 63 colonies could not be transferred from a 24-well dish to a 35mm dish.

Table 18. Passage history of IE51

Passage Number	Days Per Passage	Total Generation
1	8	2[a]
2	5	10
3	14	14
4	8	18
5	12	23

[a] Prior to this, the cells had gone through approximately 17 generations prior to and during transformation.

115 colonies were picked and innoculated into 24-welled dishes using wooden dowels as above. Of these, 109 colonies were transferred from the 24-well dishes to 35mm dishes (see Table 19, experiment 2). From Table 19, one can see that if the experiment is started at too early a passage, many more colonies of the picked colonies can be transferred to a 100mm dish and subsequently frozen down. A very low percentage, if any, of these colonies should be considered immortal. Thus, if the colony isolation method (C.I.M.) experiment is started too early, further subculturing must be performed in order to identify and recover a continuously growing cell line, thus obviating the premise of this approach. However, 37 colonies were transferred a second time under cloning conditions, since the total amount was too cumbersome to work with. Unfortunately, even though 126 colonies were able to be picked from this second set of dishes, the experiment had to be aborted due to an incubator failure. This part of the experiment, I believe, is comparable to the experiment described in Table 14 in which three immortal cell lines were isolated. Therefore, it is my belief that, if not for the incubator failure, another set of immortal cell lines would have been isolated.

The strikingly high frequency of established cell lines obtained by this colony isolation method suggested that this method could be exploited to quantify the phenomenon of immortalization. I therefore attempted to

Table 19. Comparison of lifespan of clones picked in colony assay

		Petri Dish Size				
Experiment	Picked	15mm	35mm	60mm	100mm	FD[a]
1	52	52	39	23	10	3
2	115	115	109	105	79	56

[a] The number of colonies that were frozen down from each experiment.

repeat the above experiment using IF5A-2 at a number of different passages. I was, however, unsuccessful. Although many colonies were picked, most experiments involved less than 100 colonies which I considered the minimum number needed to acquire an immortalized line. The one exception to this was experiment 2, begun at early passage (see Table 20). However, since this procedure was initiated at a very early passage, it is likely that these colonies were not immortal. Thus, it seems that this technique is extremely difficult to reproduce.

Table 20. Comparison of lifespan of clones picked in colony assay from IF5A[a]

Experiment Number	Cells Seeded	Passage Number	Petri Dish Size 15mm	35mm	60mm	100mm
1	$1.1x10^5$	P_{11}/P_2	71	9	1	--
2	$1.0x10^4$	$P_1/P_1/P_2$	161	67	26	3
3	$6.0x10^5$	$P_{11}/P_1/P_3$	56	1	--	--
4	$1.1x10^5$	$P_1/P_1/P_8$	87	15	2	--
5	$1.1x10^5$	$P_1/P_1/P_8$	52	16	1	--
6	$2.0X10^4$	$P_1/P_1/P_7$	84	13	2	1

[a] Although some of the colonies picked were able to be passaged to 100mm dishes, none survived long enough to be frozen down.

CHAPTER 4: RESULTS

CHARACTERIZATION OF THE IMMORTALIZED LINES

In the preceding Chapter, I described the isolation of six immortalized lines from IF5A-2. These cell lines were studied further in an effort to develop possible insights into the mechanism of immortalization. The fact that these cell lines were immortal was concluded on the basis of the fact that these rare derivatives have been cultivatable continuously for many additional months or years (more than 50-100 passages before they were discontinued), and have been successfully frozen and recultured. As a prelude to detailed analysis, I felt it appropriate to first verify that the cell lines were indeed derived from IF5A-2. This concern was based on the low frequency with which three cell lines (Cl-2, Cl-3, and Cl-5) were obtained following prolonged incubation, as well as the non-reproducible high frequency with which the three other cell lines (Cl-37, Cl-38 and Cl-39) were obtained, and the known history of the cell-line cross-contamination in the literature (Nelson-Rees et al, 1981). The first area of investigation was to determine if the cells were really human, or whether a mouse line had been inadvertently isolated. Towards that end, metaphase plates for all six lines were studied to determine whether mostly metacentric and submetacentric chromosomes could be found, as is typical of human cells,

or were the majority acrocentric chromosomes, typical of mouse cells (commonly used in this laboratory). The majority of the chromosomes were found to be metacentric. G-banding was subsequently performed; the banding pattern confirmed that these were human cells (discussed in detail later).

Once I had determined that I was dealing with a human cell, the next parameter to be studied was production of large T-antigen. This would indicate if the SV40 genome, or at least part of it, was present in these cells. Cells from each of the isolated lines (Cl-37, Cl-38, Cl-39, Cl-2, Cl-3, Cl-5) and their parent (IF5A-2) were examined by indirect immunofluorescence with several antibodies (see Table 2, Materials and Methods). Initially, a hamster anti-T antibody was used, but it was found to show a high background for HS74 cells, as well. Monoclonal antibodies (pAb), which are more specific, were used for all subsequent studies. pAb 416 was utilized most often, as it produced a clear negative reaction in HS74 (see Figure 5), a cell line that contains no T-antigen. With all four of the antibodies, a positive reaction (presence of faint green nuclei) was observed, which was most clearly visualized in the presence of a rhodamine-albumin counterstain. At later passages (>70), the nuclei appeared brighter, suggesting more T was being produced (see Figure 6). At an early passage (P15) after the isolation of the immortalized derivatives, 100% of the

Figure 5. Photographs of immunofluorescence assay for HS74BM. Cells were seeded on cover slips, allowed to become subconfluent, harvested, stained with antibody pAb 416, and mounted for photography and photographed for 20 seconds as described in Materials and Methods.

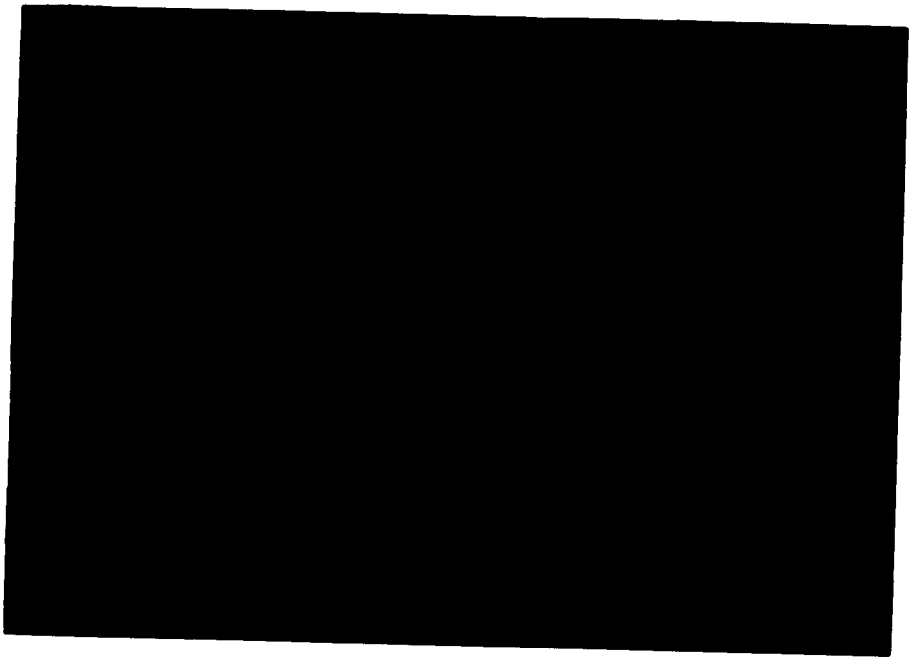

Figure 5

Figure 6. Photographs of immunofluorescence assay:
(A) IF5A photographed for 10 seconds,
(B) Cl-37 photographed for 30 seconds,
(C) Cl-38 photographed 10 seconds,
(D) Cl-39 photographed for 20 seconds,
(E) Cl-2 photographed for 5 seconds,
(F) Cl-3 photographed for 20 seconds,
(G) Cl-5 photographed for 30 seconds.
Cells were seeded on cover slips, allowed to become subconfluent, harvested, stained with antibody pAb 416, and mounted for photography as described in Materials and Methods.

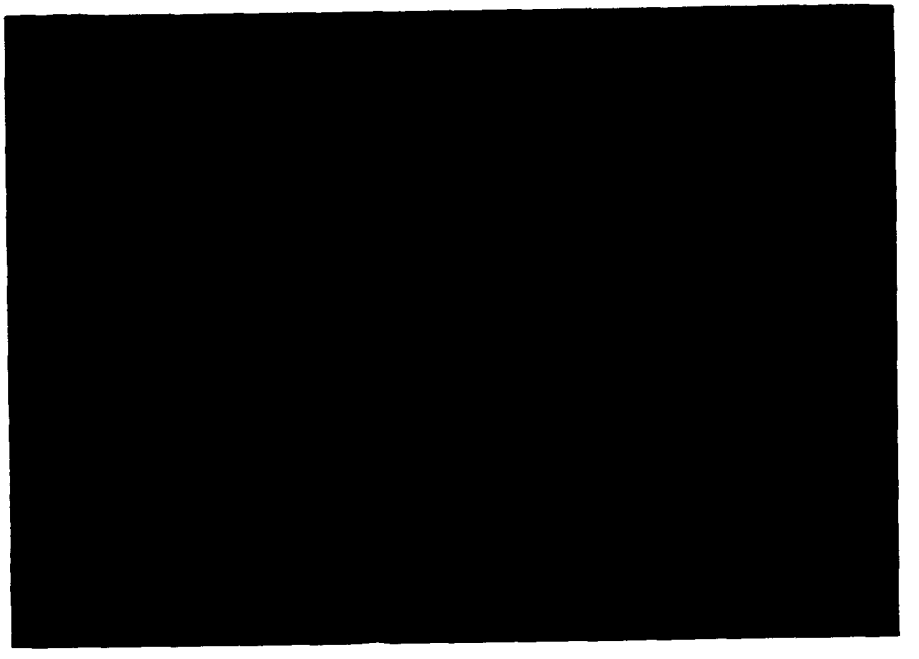

Figure 6A

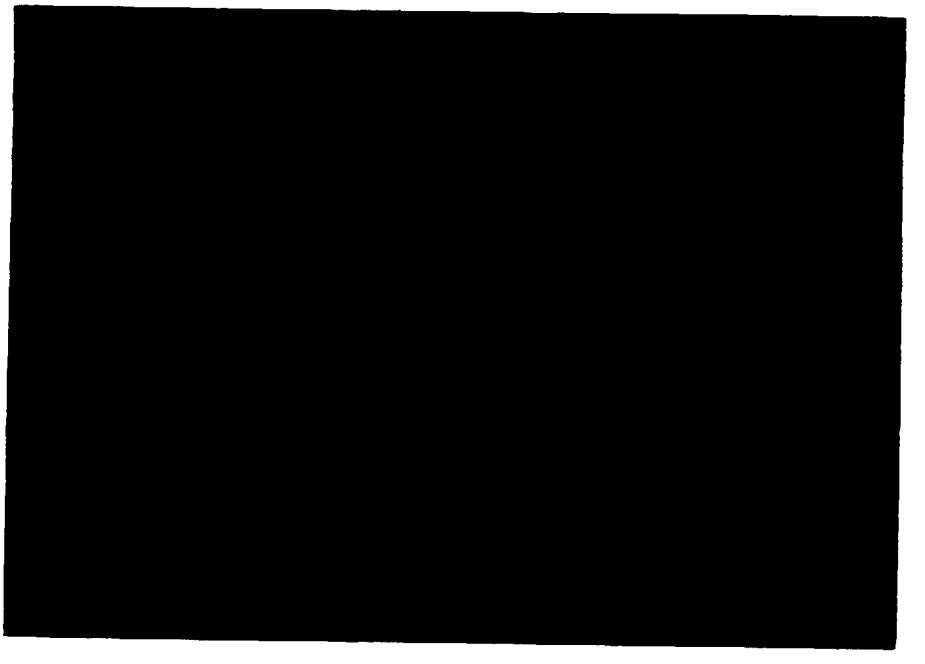

Figure 6B

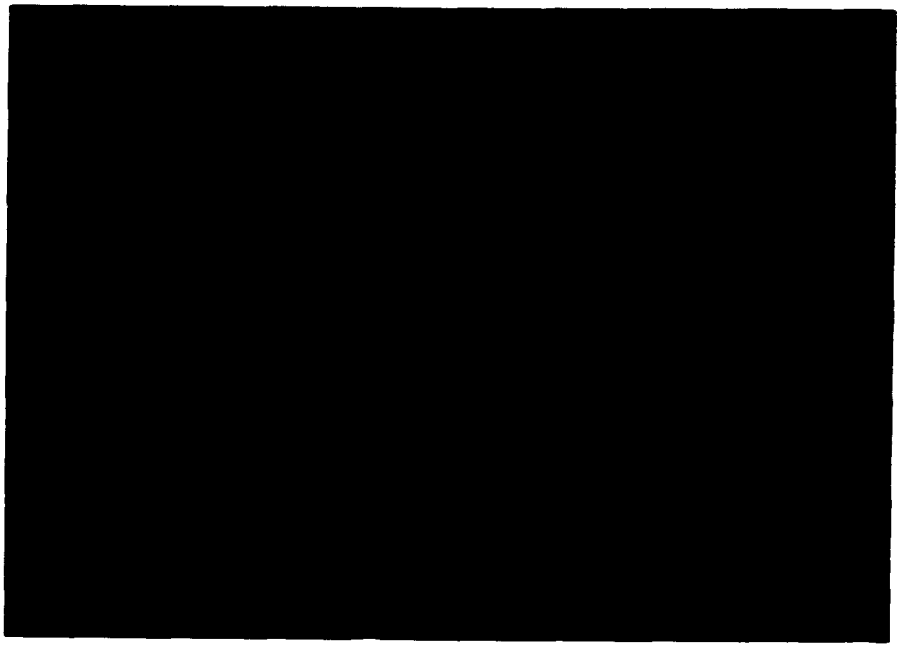

Figure 6C

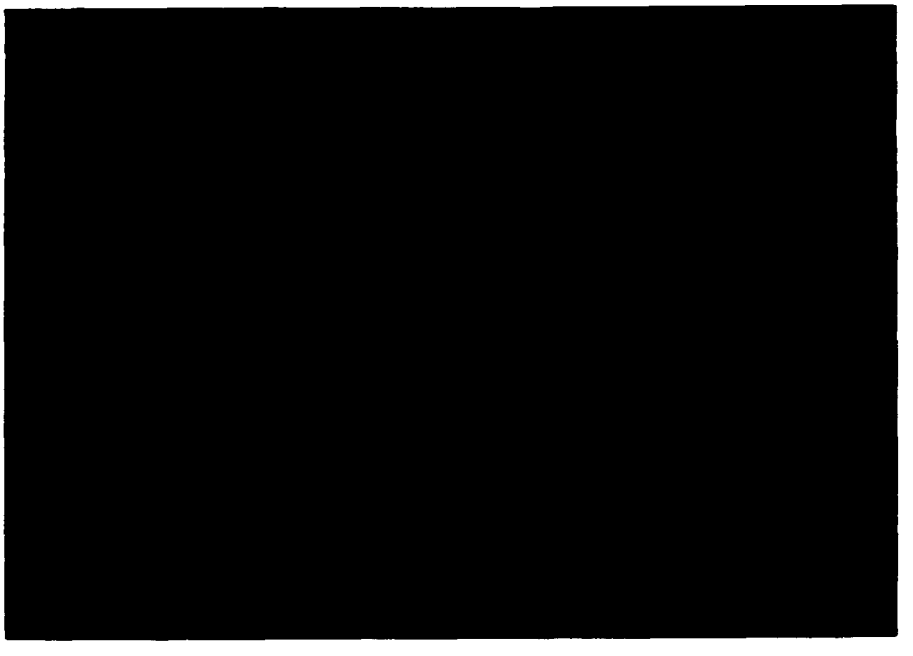

Figure 6D

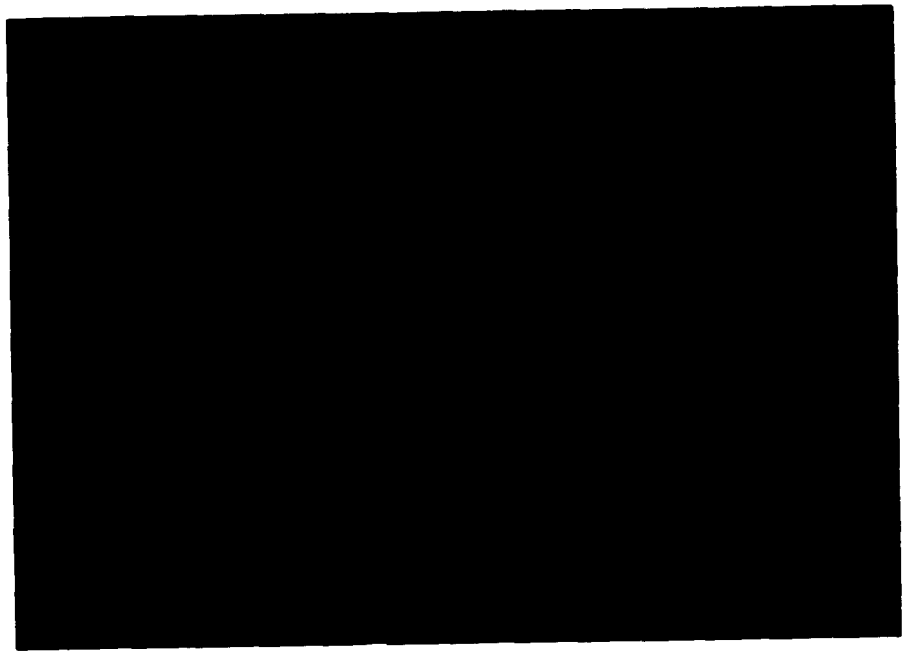

Figure 6E

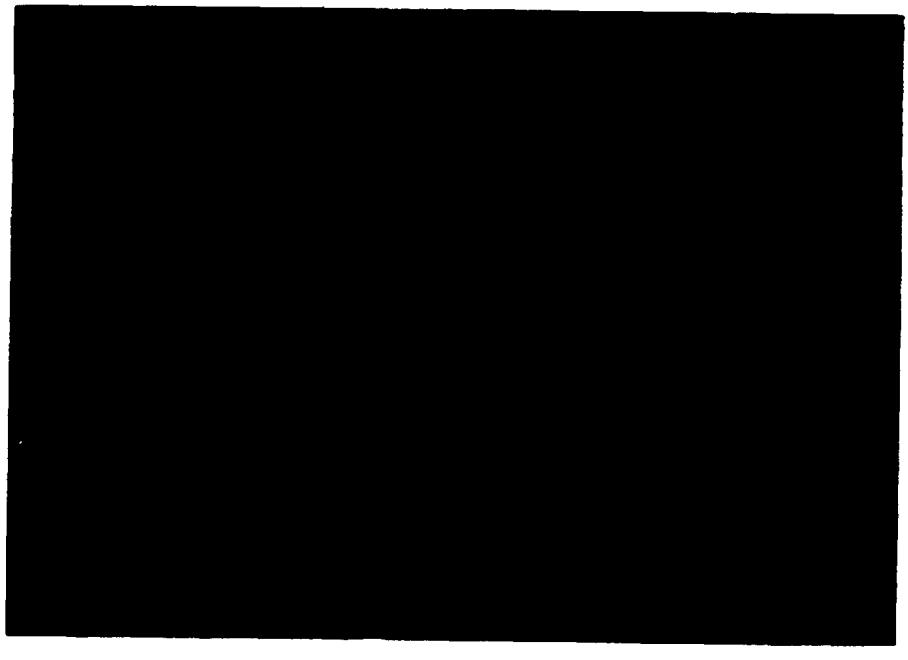

Figure 6F

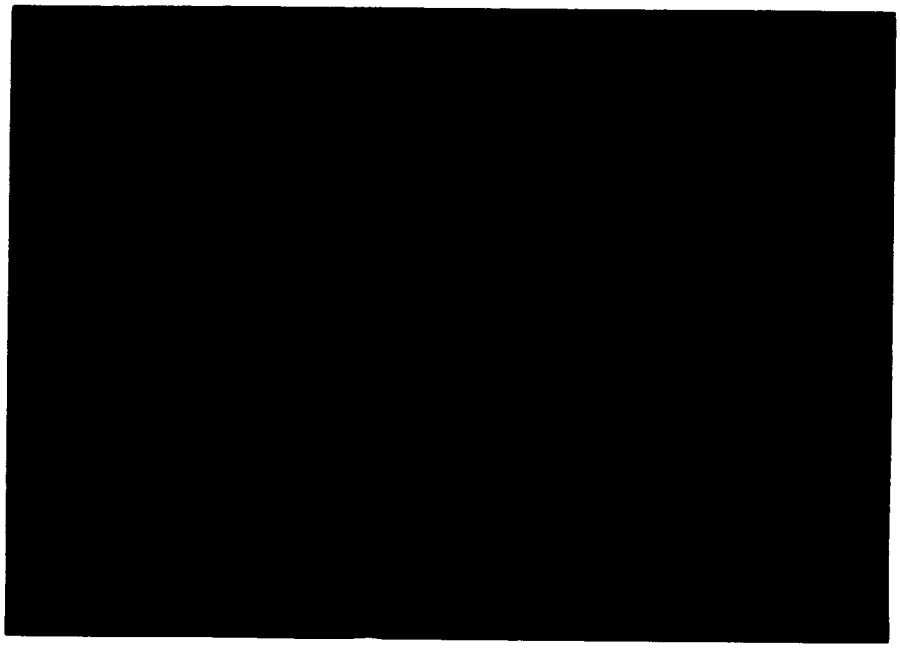

Figure 6G

cells displayed positive reactions. One interesting phenomenon, that did not seem to be of special significance with relation to immortality, was the stippled effect found in some cells (see Figure 7). Throughout these studies, a Chinese Hamster Ovary cell line containing an integrated SV40 genome or COS 7 (a monkey cell line transformed with SVori$^-$) was used as a positive control; but always produced 100% bright positive nuclei (see Figure 8). The presence of T-antigen (albeit at low levels) indicated the presence of an SV40 genome in these cells. The cells were also tested for the production of viral structural proteins by immunofluorescence, but no positive results were obtained, as expected (see Materials and Methods).

There was one other human cell line with which I was working, a Lesch-Nyhan cell transformed with SV40 (LNSV), which had to be ruled out. LNSV is a thioguanine (TG) resistant cell line. Using this characteristic, the isolates could be distinguished from LNSV by incubating them in media containing TG and noting if growth occurred, as would be expected of LNSV. Table 21 indicates that these isolates were not derivatives of LNSV, as they were all sensitive to thioguanine. I therefore concluded that these immortal cell lines were not derived from known contaminants, and were therefore derivatives of IF5A-2.

I initiated characterization of these cells by examining their growth properties. No studies have been reported of an SV40-transformed human fibroblast and

Figure 7. Photographs of immunofluorescence assay for Cl-3. Cells were seeded on cover slips, allowed to become subconfluent, harvested, stained with antibody pAb 416, and mounted for photography as described in Materials and Methods. (A) photographed for 30 seconds (B) photographed for 10 seconds.

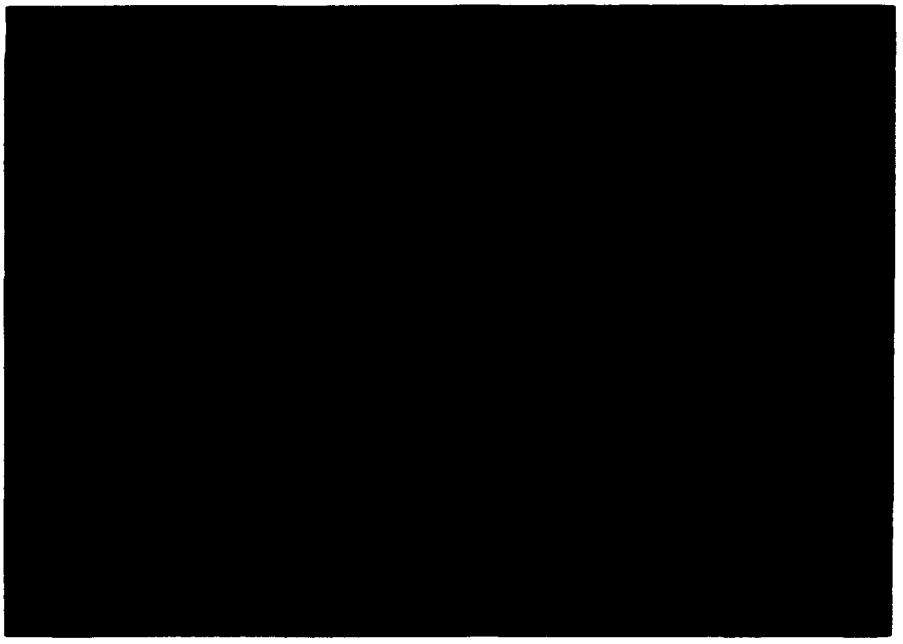

Figure 7A

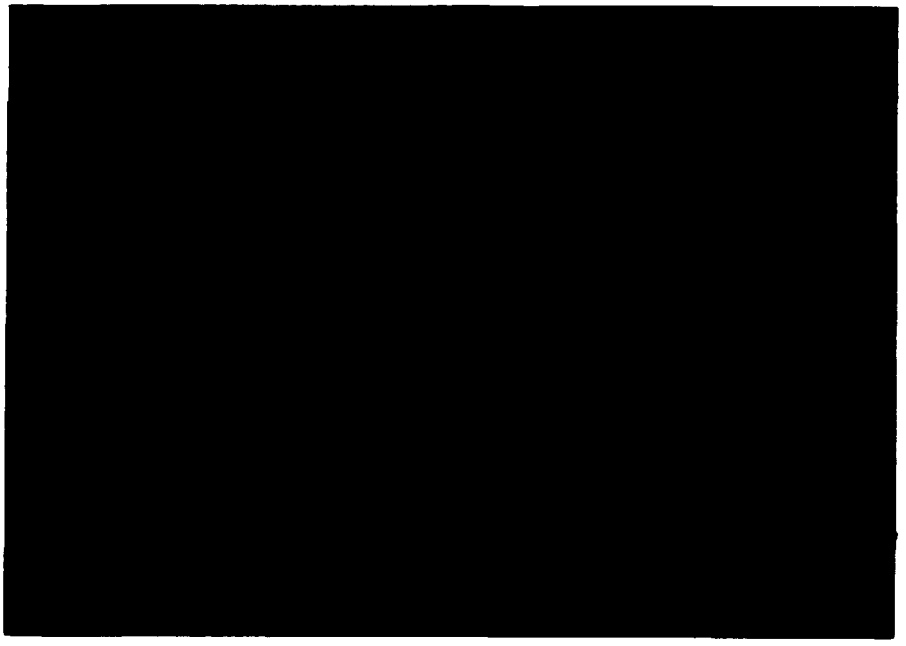

Figure 7B

Figure 8. Photographs of immunofluorescence assay for COS 7 cells. Cells were seeded on cover slips, allowed to become subconfluent, harvested, stained with antibody pAb 416, and mounted for photography and photographed for 20 seconds as described in Materials and Methods.

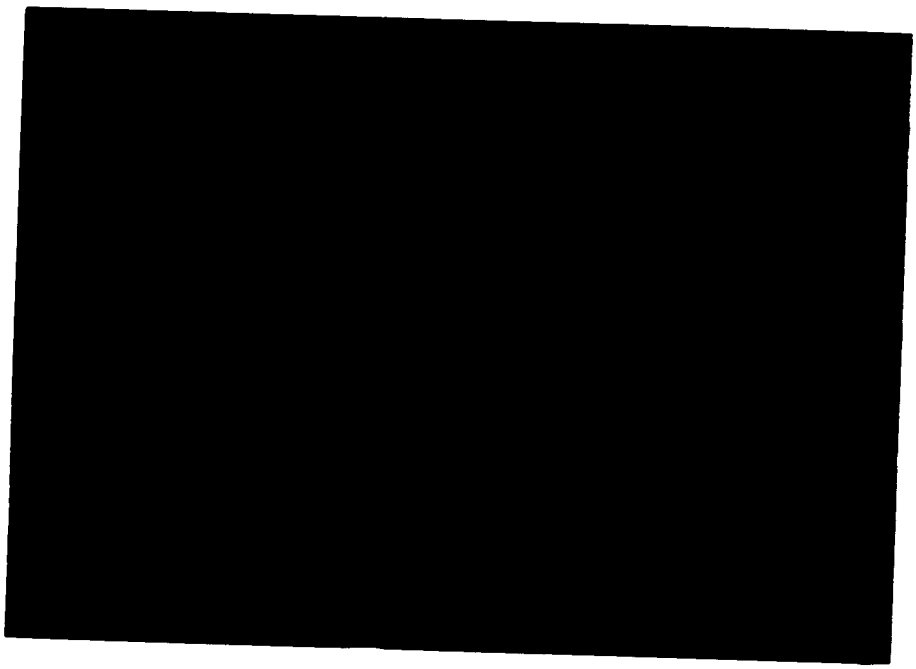

Figure 8

Table 21. Efficiency of colony formation in different types of media[a]

Cell Line	Type of Media DME	10^{-5}M TG
Cl-2	confluent	0
Cl-3	confluent	0
Cl-5	confluent	0
Cl-37	confluent	0
Cl-38	confluent	0
Cl-39	confluent	0
LNSV	confluent	confluent
LNSV	---	7%[b]

[a] For each parameter, $5x10^4$ cells were seeded on a 60mm plate in triplicate.

[b] One set was seeded with $2x10^2$ cells and 14 colonies were found.

clonally isolated immortalized derivatives. The results are shown in Table 22 and Figures 9 to 12. As IF5A-2, all the cell lines were capable of colony formation at low cell density in monolayer. The efficiency of colony formation (EOC) varied among the cell lines. Interestingly, no relationship with the method of isolation was observed. The EOC of Cl-2, for example, was only 5%. Indeed, HS74 and IF5A-2, early in their respective passage histories, have as high or higher EOC. It should be emphasized that all the immortalized cell lines, of course, gave considerable higher EOC than IF5A-2 at the time at a comparable number of generations. The apparent paradoxical EOC among the different cell lines may be explained, in part, by possible variability at different passages as indicated by the comparison of Cl-39 at two points post-immortalization; the EOC increased from 1% to 26% between passage 20 and 90. High EOC is, therefore, not a necessary consequence of immortalization. It should also be noted that Cl-39 also retained the transformed phenotype of anchorage-independence expected for a descendant of IF5A-2, which had been isolated in agarose. When tested at later passage (P_{80}-P_{90}), the EOC in agarose was approximately 0.3% (30 colonies per 10^4 cells). Systematic EOC determinations were not performed.

Growth curves were performed at different passage levels for each of the immortalized cell lines. The population doubling times varied between 26 and 48 hours;

Table 22. Comparison of efficiency of colony formation

Cell Line	Passage Number	Efficiency of Colony Formation (Percent)	Doubling Time[a] (37° C)
Cl-2	78	5[b]	42 hrs.
Cl-3	52	48[b]	43 hrs.
Cl-5	51	29[b]	26 hrs.
Cl-37	42	N.D.[c]	48 hrs.
Cl-38	42	59[b]	48 hrs.
Cl-39	20-23	1[d]	42 hrs.
Cl-39	80-90	10-26[d]	30 hrs.
IF5A-2	10	3-10[d]	42 hrs.
HS74	10	5-20[d]	38 hrs.

[a] 60mm dishes were seeded with 5×10^4 cells and cell number determined over several days for triplicate dishes. Doubling time was calculated from the slope of the fit curve generated by inspection as shown in Figures 9 and 10.

[b] Dishes were seeded with 2×10^2 cells in triplicate and counted two weeks later.

[c] N.D.= Not Determined

[d] Dishes were seeded with 1×10^2 cells in triplicate and counted two weeks later.

Figure 9. Comparison of growth curves for Cl-2, Cl-3 and Cl-5 cells at 37° C. Cultures were seeded with 5×10^4 cells per dish and incubated at 37° C. After the initial seeding, at each time point indicated, the cells in triplicate cultures were counted as described in Materials and Methods.

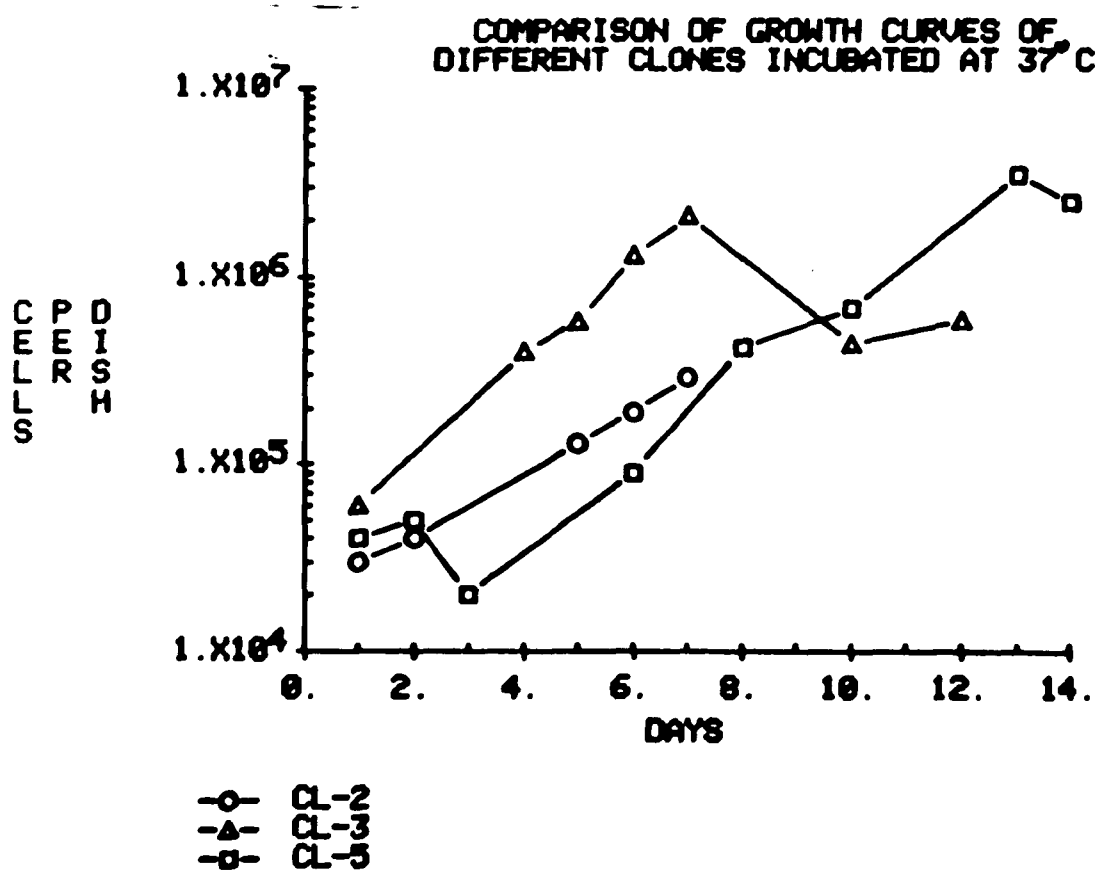
COMPARISON OF GROWTH CURVES OF
DIFFERENT CLONES INCUBATED AT 37°C
1.X10⁷
1.X10⁶
1.X10⁵
1.X10⁴
CELLS PER DISH
0.
2.
4.
6.
8.
10.
12.
14.
DAYS
CL-2
CL-3
CL-5

Figure 9

Figure 10. Comparison of growth curves for Cl-37, Cl-38, Cl-39P_{12} (early passage) and Cl-39P_{80} (late passage) at 37° C. Cultures were seeded with 5 x 10^4 cells per dish and incubated at 37° C. After the initial seeding, at each time point indicated, the cells in triplicate cultures were counted as described in Materials and Methods.

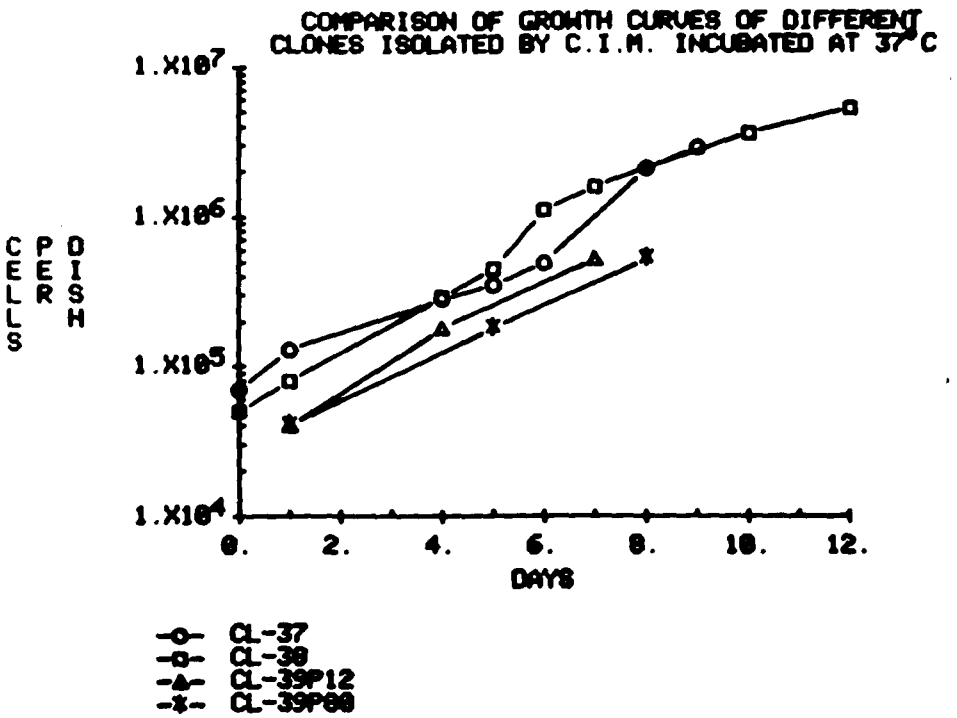
COMPARISON OF GROWTH CURVES OF DIFFERENT CLONES ISOLATED BY C.I.M. INCUBATED AT 37°C
1.X10^7
1.X10^6
1.X10^5
1.X10^4
CELLS PER DISH
0.
2.
4.
6.
8.
10.
12.
DAYS
CL-37
CL-38
CL-39P12
CL-39P88

Figure 10

Figure 11. Comparison of growth curves of Cl-39 (early passage) at 35° C, 37° C and 38.5° C. Cultures were seeded with 5 x 10^4 cells per dish and incubated at the various temperatures. After the initial seeding, at each time point indicated, the cells in triplicate cultures were counted as described in Materials and Methods.

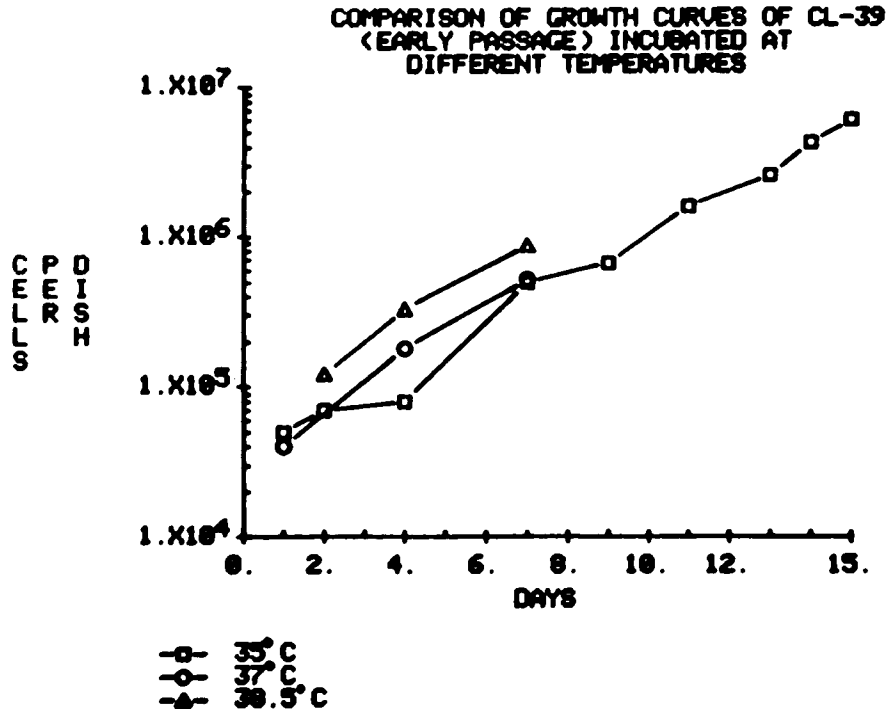
COMPARISON OF GROWTH CURVES OF CL-39
(EARLY PASSAGE) INCUBATED AT
DIFFERENT TEMPERATURES
CELLS PER DISH
1.X10^7
1.X10^6
1.X10^5
1.X10^4
0.
2.
4.
6.
8.
10.
12.
15.
DAYS
35°C
37°C
38.5°C

Figure 11

Figure 12. Comparison of growth curves of Cl-39 (late passage) at 33° C, 35° C, 37° C and 38.5° C. Cultures were seeded with 5 x 10^4 cells per dish and incubated at the proper temperatures. After the initial seeding, at each time point indicated, the cells in triplicate cultures were counted as described in Materials and Methods.

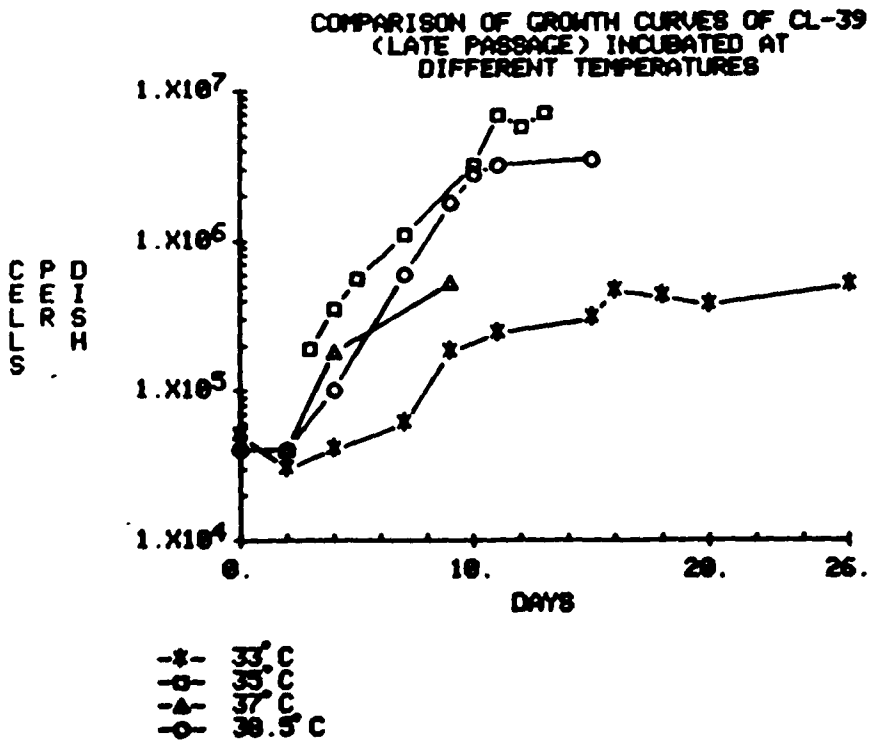
COMPARISON OF GROWTH CURVES OF CL-39
(LATE PASSAGE) INCUBATED AT
DIFFERENT TEMPERATURES
1.X10^7
1.X10^6
1.X10^5
1.X10^4
CELLS PER DISH
0.
10.
20.
25.
DAYS
33°C
35°C
37°C
39.5°C

Figure 12

the pattern was consistent with the results observed for the EOC. The immortalized cell lines overlapped the values observed for HS74 and IF5A-2, which were 38 hours and 48 hours respectively. The growth rate improved with passage for C1-39 (from 42 hours to 30 hours between passages 20 and 90). It could therefore be concluded that immortalization was not always associated with an increased growth rate. All the above results were obtained at 37° C. C1-39, at two different passages, was also examined at different temperatures; the results are summarized in Table 23. At later passage, the growth rate was reduced at 33° C (48 hours) and 35° C (38 hours) as compared to 37° or 38.5° C, which were indistinguishable. The EOC was similarly unaffected at 38.5° C, as compared to 37° C. At early passage, the growth rate was reduced at all temperatures. C1-39 also showed an increased saturation density at confluence for cell growth (as expected for an SV40-transformed cell line), as shown in Table 24. Similar results were obtained at 35° C, 37° C, and 38.5° C (data not shown). The overall behavior is consistent with that expected for IF5A-2, since a wild-type T antigen was involved in the initial transformation. These data also served as a control for growth studies with a series of transformants subsequently isolated in the laboratory. These were obtained after transfection with an origin-defective derivative of tsA 58, a mutant which encodes a heat-labile T antigen.

Table 23. Doubling times of Cl-39 at different temperatures[a]

Passage Number	Temperatures (°C) 33	35	37	38.5
P12/P4	N.D.[b]	45.6	42	43
P80/P4	48	38	28	30

[a] 60mm dishes were each seeded with 5 x 10^4 cells. Three dishes were counted at each time point. The average of the three cell counts was used to plot the curve as shown in Figures 11 and 12.

[b] N.D.= Not Determined

Table 24. Saturation density of human cell lines

Cell line	Cell number
HS74	1.5×10^6
IF5A-2	4.0×10^6
Cl-3	5.9×10^6
Cl-37	$>2.9 \times 10^6$
Cl-38	5.3×10^6
Cl-39	5.5×10^6

[a] 60mm dishes or 12-well dishes (Cl-39) were seeded with 5×10^4 cells. Dishes were incubated until confluence was obtained and cell number was determined in triplicate several days after confluence.

The SV40 sequences in IF5A-2 and the immortalized cells were examined to determine the number and stability of the viral genome (and associated non-viral sequences). Typically, cell lines that are transformed with SV40 contain multiple partial copies of the viral genome integrated into the cellular genome (Sack, 1981). However, such transformed human fibroblasts had been obtained after infection by SV40, which had an intact origin of replication and T antigen (usually as infectious virions). On the other hand, the transformation in these experiments was done with SVori$^-$, thus excluding replication, so the possibility existed that there would not be multiple copies of the SV40 genome. Conversely, since the transformation was done by the calcium phosphate co-precipitation technique, there was a chance that concatamers were formed which involved multiple copies of SV40. Finally, since the majority of the DNA taken up by calcium phosphate co-precipitation suffers degradation, the probability of loss of the terminal sequences of the integrating DNA also existed. To assess these possibilities, experiments were done in which the same sequence-specific endonuclease was used to digest high molecular weight DNA from each of the cell lines and Southern analysis performed.

A series of enzymes which do not cut the input SV40 -- specifically, Bgl 1 (Figure 13), Xho 1 (Figure 14), Sst 1 (Figure 15) and Ava I (Figure 16) -- were used. Although Bgl 1 digests wild type SV40 DNA, the SVori$^-$ mutant c-6

Figure 13. Southern analysis of cellular DNAs from (3) IF5A, (4) Cl-39, (5) Cl-5, (6) Cl-37, (7) Cl-38, (8) Cl-2, (9) Cl-3. Each DNA sample was digested with Bgl 1 and electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 1 day. (1) SV40 digested with Eco Rl. (2) pMKSV40ori$^-$ digested with Xho 1.

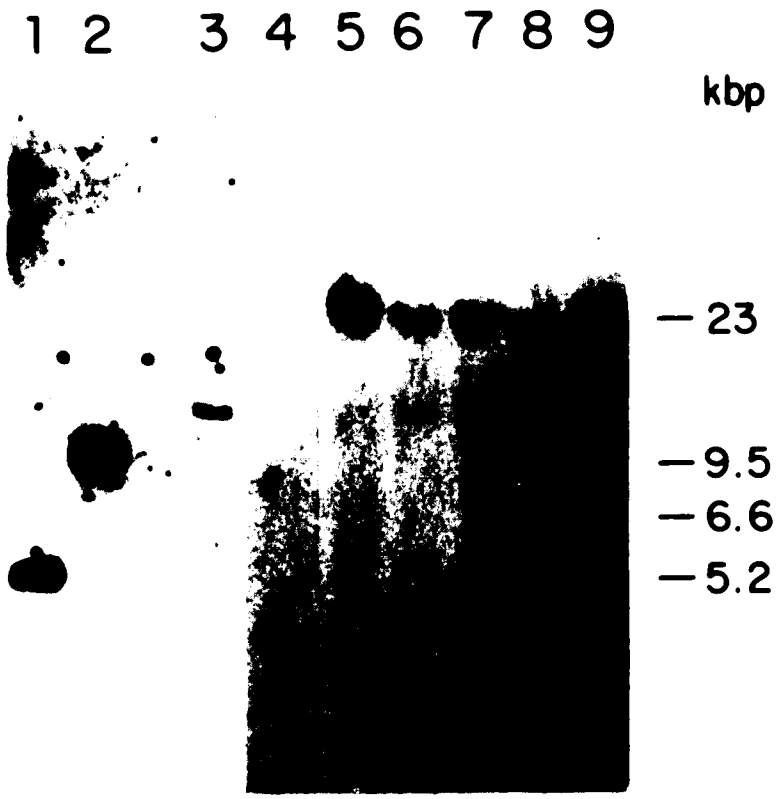

Figure 13

Figure 14. Southern analysis of cellular DNAs from (3) IF5A, (4) Cl-39, (5) Cl-5, (6) Cl-37, (7) Cl-38, (8) Cl-2, (9) Cl-3. Each DNA sample was digested with Xho 1 and electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for a week. (1) SV40 digested with Eco R1. (2) pMKSV40ori$^-$ digested with Xho 1.

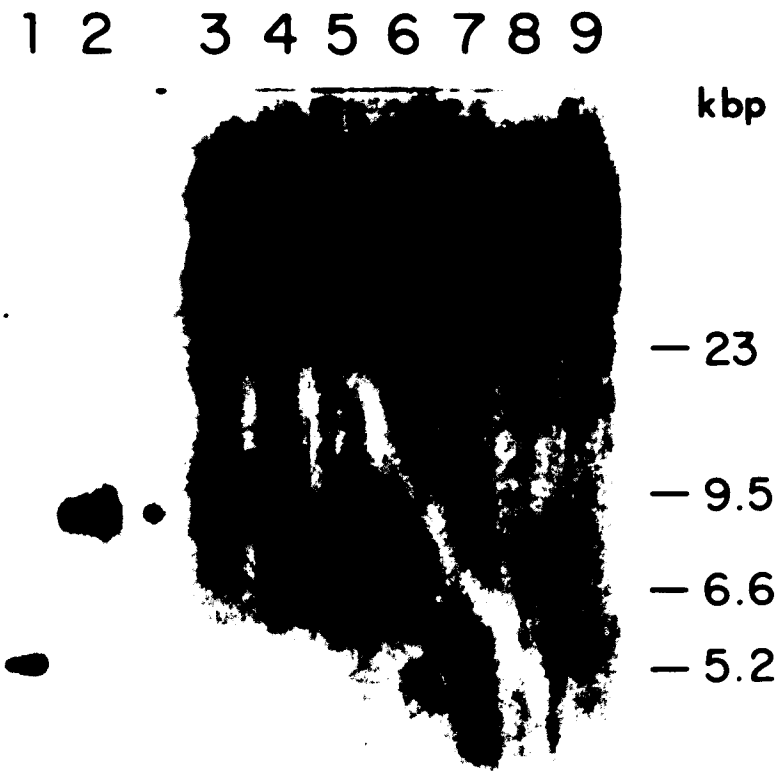

Figure 14

Figure 15. Southern analysis of cellular DNAs from (3) Cl-37, (4) Cl-38, (5) Cl-39, (6) Cl-2, (7) Cl-3, (8) Cl-5, (9) IF5A. Each DNA sample was digested with Sst 1 and electrophoresed in a 1.0% agarose gel at 30 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 3 days. (1) pMKSV40ori$^-$ digested with Xho 1. (2) SV40 digested with Sst 1.

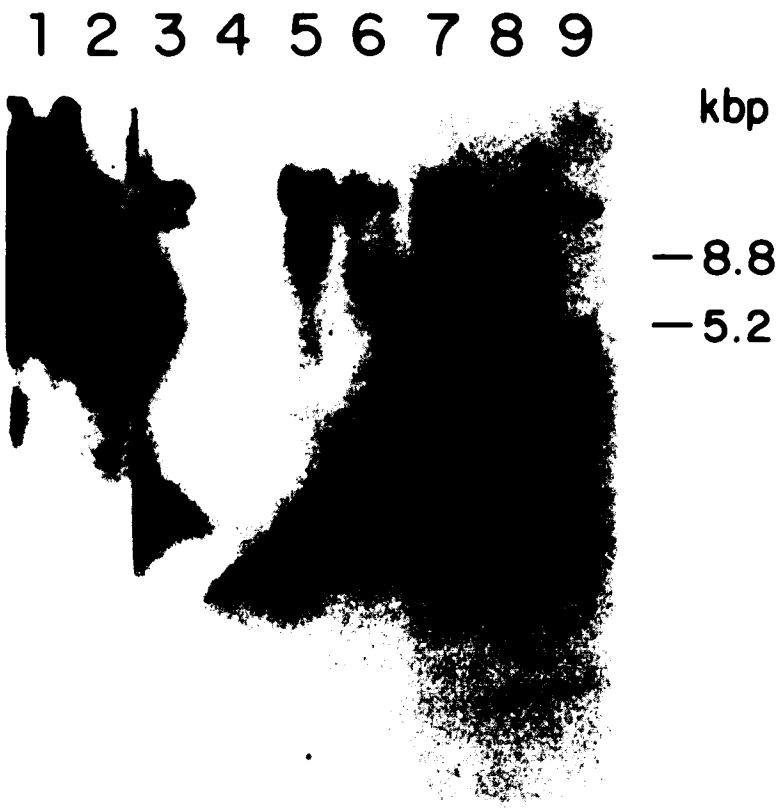

Figure 15

Figure 16. Southern analysis of cellular DNAs from (3) IF5A, (4) Cl-39, (5) Cl-37, (6) Cl-3. Each DNA sample was digested with Ava 1 and electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 3 days. (1) SV40 digested with Eco Rl. (2) pMKSV40ori$^-$ digested with Xho 1.

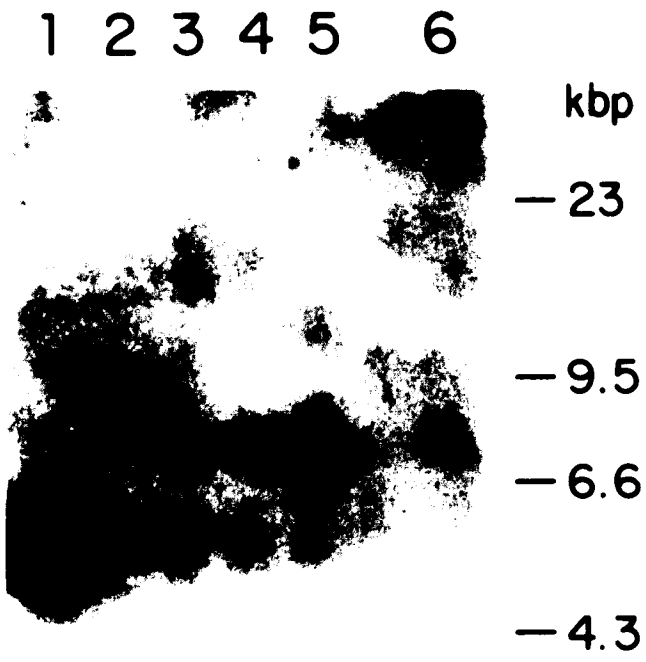

Figure 16

(which was used in the initial transformation studies) contained a deletion at the Bgl I site and, therefore, that site could not be present in the resident viral genome. These enzymes digest cellular sequences adjacent to the putative integrated viral genome(s), such that each discrete band of viral sequences would be expected to reflect a separate site of integration. The results indicate that the SV40 sequences are in two sites. Figure 13 shows two DNA fragments not only for IF5A (lane 3) but also for Cl-37, Cl-38 and Cl-3 (lanes 6, 7 and 9 respectively). Figure 14 also indicates two DNA fragments for IF5A; however, it is difficult to be certain of the presence of more than one clearly defined DNA fragment for any of the immortalized cell lines, as indicated in lanes 4 through 9. In Figure 15, lane 3, one finds two DNA fragments for Cl-37, whereas lanes 4 through 9 indicate one DNA fragment for all other lines, including the parent IF5A. Although IF5A appears to contain only one integration site in this gel, there is a possibility, in view of the overall faint intensity of the viral sequences, that the lanes were underloaded and therefore mistakenly indicate only one DNA fragment. The presence of two DNA fragments with Bgl 1, Xho 1, and Sst are confirmed (and better documented) as discussed later (see Figure 20). The behavior of Cl-38 (Figure 15, lane 4) is aberrant, as evidenced from the ethidium bromide staining of the gel (data not shown). Digestion of the DNA preparations with

Ava I (Figure 16) shows one integration site for IF5A, Cl-39, Cl-37 and Cl-3 (lanes 3, 4, 5 and 6 respectively). Differences among the various enzymes for IF5A-2 are consistent with the findings of others, since the locations of sites for digestion with any given enzyme would be expected to be fortuitous, and not evident in all cases. These results are consistent with a minimum of two integration sites for SV40 in IF5A-2. One should note that lambda-DNA was added to each of the reaction tubes for Figures 13 and 16 to verify that complete digestion had taken place. This is observed on an ethidium-bromide-stained gel; i.e., DNA fragments corresponding to those seen when only lambda-DNA was digested were noted (data not shown). One should note that Cl-3 also contained two copies of SV40; however, one of its copies must have moved to a different position than that of Cl-37 and Cl-38. This was shown to be true because one of the DNA fragments upon Bgl 1 digestion did not match with either of the DNA fragments of Cl-37 and Cl-38. In all the lines, one of their respective DNA fragment(s) matched those of IF5A (see Figure 13).

I next examined the DNA from each cell line with enzymes that cleave wild type SV40 DNA once (Figure 17). Such an analysis would be expected to result in two DNA fragments per integrated viral sequences, assuming an intact genome. The enzymes used were Bam Hl (Figure 18) and Eco Rl (Figure 19). Bam Hl did not appear to cut

Figure 17. A linear restriction map of the SV40 genome showing relevant restriction enzyme sites.

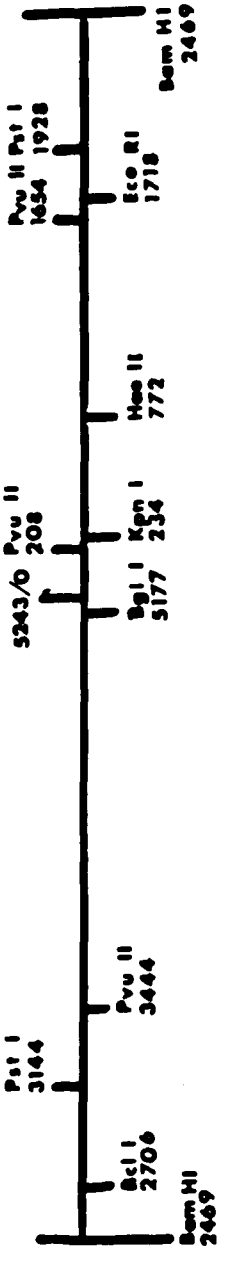
BamHI
2469
Pvu II PstI
1654 1928
EcoRI
1718
HaeII
772
Pvu II
208
KpnI
234
5243/0
BglI
5177
Pvu II
3444
PstI
3144
BclI
2706
BamHI
2469

Figure 17

Figure 18. Southern analysis of cellular DNAs from (1) Cl-37, (2) Cl-38, (3) Cl-39, (4) Cl-2, (5) Cl-3, (6) Cl-5, (7) IF5A. Each DNA sample was digested with Bam Hl and electrophoresed in a 1.0% agarose gel at 30 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography overnight.

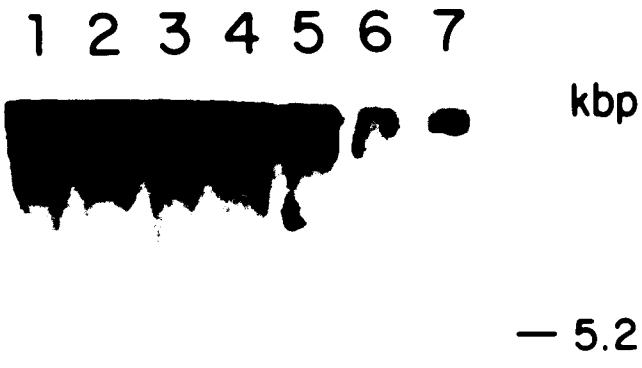

Figure 18

Figure 19. Southern analysis of cellular DNAs from (2) Cl-39, (3) Cl-2, (4) Cl-3, (5) Cl-5, (6) IF5A. Each DNA sample was digested with Eco Rl electrophoresed in a 1.0% agarose gel at 30 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 1 week. (1) SV40 digested with Eco Rl.

Figure 19

within the SV40 sequences, since only very high molecular weight DNA fragments were found: two in Cl-37 (lane 1) and Cl-38 (lane 2), and a single DNA fragment in the others. It is difficult to comment on IF5A because of apparent poor transfer. This was not surprising per se, as the SV40 DNA had been linearized at the Bam Hl site before it was introduced into the human cell. Since recombination takes place upon integration, one would expect both ends of the molecule to have lost some sequences, thus eliminating the Bam Hl site (Ketner and Kelly, 1976; Clayton and Rigby, 1981). Alternatively, exonuclease digestion of the terminal nucleotides could have resulted in loss of the Bam Hl recognition sequences prior to integration (see Figure 17). More surprisingly, Eco Rl (Figure 19) did not seem to digest within the SV40 sequences either. Only one DNA fragment was found for IF5A, Cl-39, Cl-2, Cl-3 and Cl-5 (lanes 6, 2, 3, 4, and 5 respectively). The DNA fragments obtained with these enzymes (Bgl 1, Xho 1, Sst 1, Bam Hl and Eco Rl) were much larger (10kb-23kb) than one would expect with an enzyme that cleaves internally. Data will be presented later which bear further on this point.

Comparison of the pattern of the viral sequences among the DNA from the different cell lines digested with the several enzymes indicated that one copy of viral sequences was conserved, while the second copy in IF5A could be altered. For example, Cl-3 (Bgl 1, Figure 13) showed an altered position of the lower molecular weight DNA fragment

in IF5A; it was not detectable in Cl-2, Cl-5 or Cl-39. By inference, therefore, the presence (or position) of that DNA fragment was irrelevant to the immortalization of IF5A. I consequently, focused my attention on the structure of the other viral sequence (within the higher molecular weight Bgl 1 DNA fragment). I decided to work only with Cl-39, as it had a single integrated copy. I sought to determine whether this copy had moved to a new position in the cellular genome or if it had remained in the same position as one of the copies of IF5A. If it had moved to a new site, the possibility existed that immortalization was a direct cause of this move, and that, upon reintegration, a gene was disturbed in such a fashion that the cell now became immortal. However, if the initial copy remained in the original position, this would indicate that the position of integration has no significance with relationship to immortalization. If this were not the case, all IF5A cells would be immortal, contradicting the results obtained in the preceding Chapter. Therefore, if the DNA fragment of Cl-39 matched up to one of the DNA fragments of IF5A, and the only apparent difference between the parent and Cl-39 was that Cl-39 had lost one SV40 copy, one would be hard pressed to use a loss such as this as a specific mechanism for immortalization. To assess this, DNAs from both Cl-39 and IF5A were directly compared after digestion with a variety of enzymes that either did not cleave wild type SV40 DNA, or only cleaved it once. These

included Sst 1, Xho 1, Bgl 1, Bam Hl, Eco Rl (already discussed) and Kpn 1. These two gels (see Figure 20 [IF5A] and Figure 21 [Cl-39]) were electrophoresed under identical conditions (0.7% agarose, 40V, for 16 hours). If one compares the marker DNA's of the two gels (Figure 20, lane 2, and Figure 21, lane 1 [wild type SV40 digested with Eco Rl] and Figure 20, lane 4, and Figure 21, lane 2 [pMKSVori$^-$ digested with Xho), one finds an excellent agreement. When lanes 4 and 5 of Figure 21 are compared (both digested with Sst 1), one sees that one of the two DNA fragments of IF5A found in lane 4 matches exactly to the single CL-39 DNA fragment in lane 5. In addition, the pattern in Figure 21 corresponds to the one seen in Figure 20, lane 5. Similarly, in all cases, the Cl-39 DNA fragment matches one of the IF5A DNA fragments, thus enabling me to be reasonably certain that the insert in Cl-39 had not moved from its place in IF5A. This conclusion was based on a comparison of the following: Xho 1 (Figure 20, lane 6 with Figure 21, lane 6), Kpn 1 (Figure 20, lane 8 and Figure 21, lane 10), Bam Hl (Figure 21, lane 9 with Figure 21, lane 8), and Eco Rl (Figure 20, lane 10 and Figure 21, lane 9). Instead, Cl-39 had only lost one of the SV40 copies found in IF5A. One apparent exception to this was the Bgl 1 DNA fragment (Figure 20, lane 7; Figure 21, lane 7) which was <u>not properly cut in Figure 21</u> (Cl-39), as can be seen from another gel (data not shown) where lambda-DNA was added as an internal control. When the lambda-DNA from this

Figure 20. Southern analysis of cellular DNAs from IF5A. Ten micrograms of DNA were digested with (5) Sst 1, (6) Xho 1, (7) Bgl 1, (8) Kpn 1, (9) Bam Hl, (10) Eco Rl, electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 1 week. (1) SV40 undigested. (2) SV40 digested with Eco Rl. (3) pMKSV40ori$^-$ undigested. (4) pMKSV40ori$^-$ digested with Xho 1.

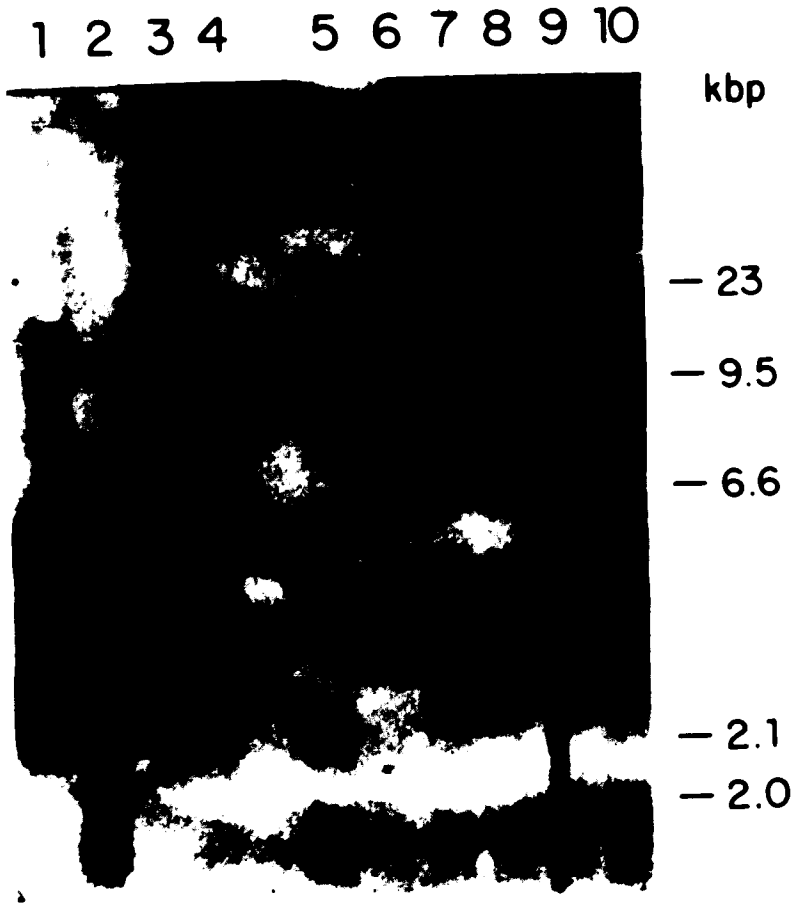

Figure 20

Figure 21. Southern analysis of cellular DNAs from Cl-39 and IF5A. Ten micrograms of IF5A DNA were digested with (4) Sst 1. Ten micrograms of Cl-39 DNA were digested with (5) Sst 1, (6) Xho 1, (7) Bgl 1, (8) Bam Hl, (9) Eco Rl, (10) Kpn 1, electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 5 days. (1) SV40 digested with Eco Rl. (2) pMKSV40ori$^-$ digested with Xho 1. (3) SV40 digested with Taq 1 and Hae II.

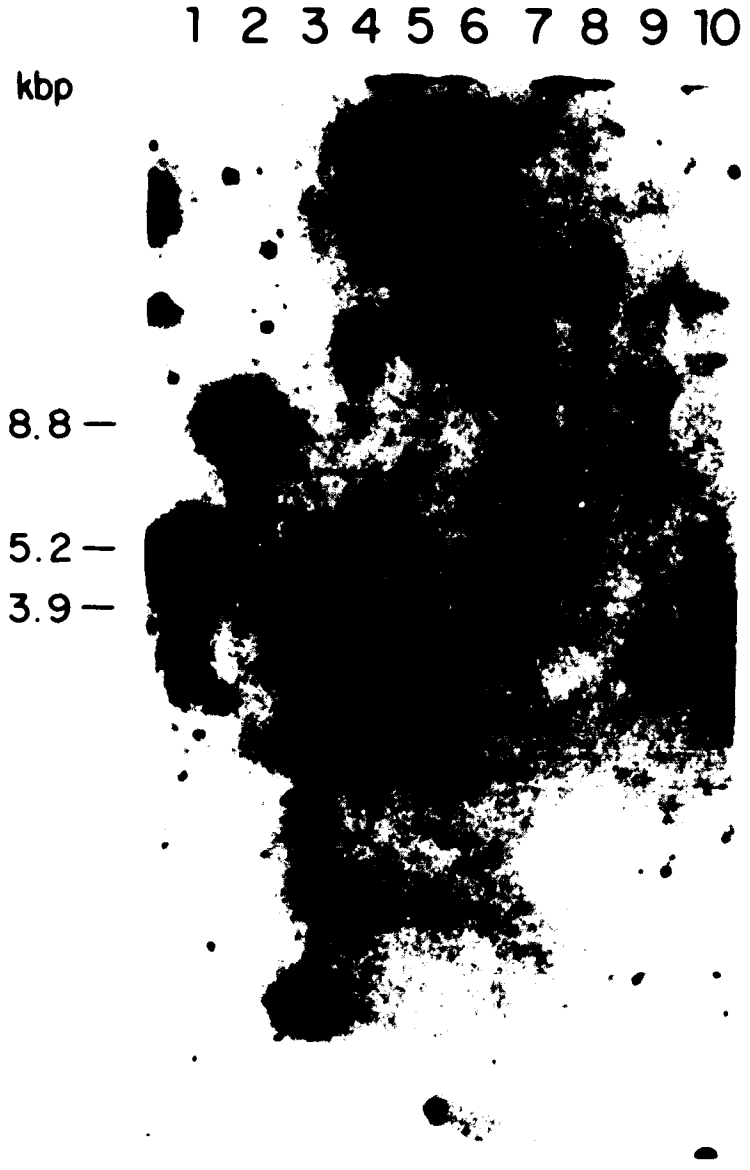

Figure 21

digestion (data not shown) is observed in an ethidium-bromide-stained gel, one finds the expected molecular weight DNA fragments, which would imply that the high molecular weight DNA was digested properly. If the DNA was digested properly, the molecular weight of the DNA fragment can be assumed to be correct. Since the DNA fragment in the other gel matches only the DNA fragment in Figure 20 and not the DNA fragment in Figure 21, one can assume that only the DNA fragments in Figure 21 are correct. I therefore concluded that no rearrangement had occurred in the conserved SV40 sequences in the immortalization of IF5A.

I then tried to more completely map the SV40 insert in C1-39. From the Southern analysis previously described, digestion of C1-39 DNA with Eco R1 or Bam H1 resulted in large (>10 kb) DNA fragments. Since only one DNA fragment was found when Bam H1 and Eco R1 were used to digest C1-39 DNA, Bam H1 and Eco R1 probably did not cut internally. Kpn 1, on the other hand, seemed to cut internally, as two DNA fragments were found (Figure 21, lane 10). Speculating that a small part of the SV40 genome was missing, I digested the DNA with Bcl 1 and Eco R1 as a double digestion (Figure 22). When the DNA was digested with Bcl 1 alone, one large (~15kb) single DNA fragment containing SV40 sequences was found (Figure 22, lane 2). When the high molecular weight DNA was digested with Eco R1 alone (Figure 22, lane 10), a single DNA fragment was found of

Figure 22. Southern analysis of cellular DNAs from Cl-39. Ten micrograms of DNA were digested with (2) Bcl 1, (3) Bcl 1 and Kpn 1, (4) Kpn 1, (6) Bcl 1 and Bam Hl, (7) Bam Hl, (9) Bcl 1 and Eco Rl (10) Eco Rl, electrophoresed in a 0.7% agarose gel at 50 volts for 8 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 1 week. (1) SV40 digested with Eco Rl and pMKSV40ori$^-$ digested with Xho 1. (5) SV40 digested with Bcl 1 and Bam Hl. (8) SV40 digested with Bcl 1 and Eco Rl.

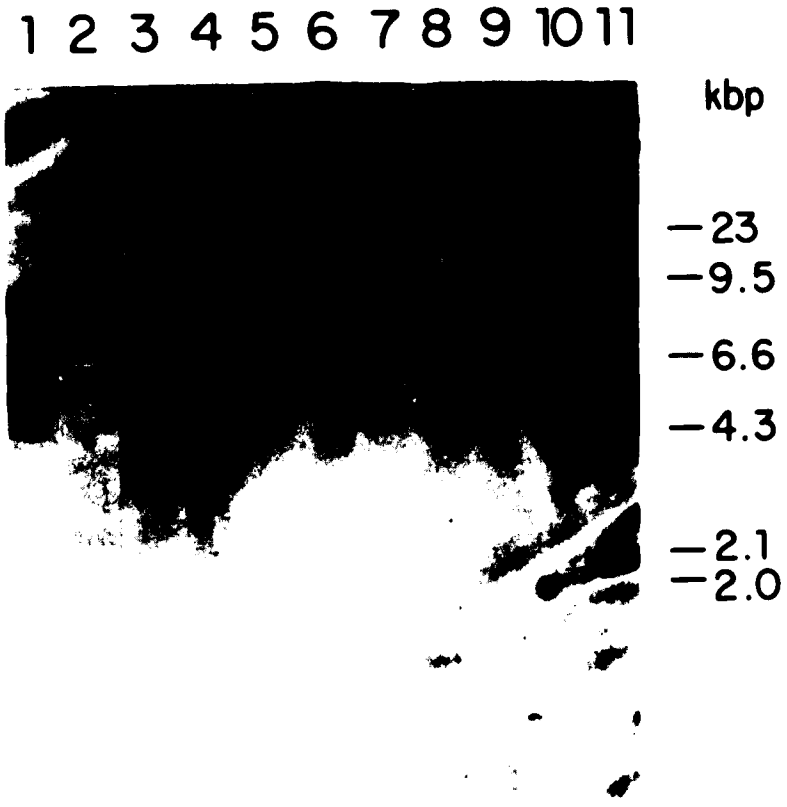

Figure 22

approximately the same size (~15kb). When a double digestion was performed with both Bcl 1 and Eco R1, a single DNA fragment of about 9.0 kilobases was found (Figure 22, lane 9), instead of the expected fragment of subgenomic size (SV40 genome in Figure 22, lane 8). This suggested that at least one, if not both, of the sites was missing. One would expect more than one DNA fragment (two or possibly three), if one or both of the enzymes digested the expected viral sequences. Additionally, instead of finding the expected 4.3 kb DNA fragment (SV40 genome in Figure 22, lane 8), the observed DNA fragment was much larger (8kb). Analogous results were obtained when I did a double digestion with Bcl 1 and Bam H1 (see Figure 22, lane 6). The size of the single DNA fragment was still much larger than the 5.0 kb piece one would expect from such a digestion (Figure 22, lane 5), although the fragment was smaller than either Bam H1 alone (lane 7) or Bcl 1 alone (lane 2). This result would indicate that possibly neither of the restriction sites were present in this SV40 insert. To clarify this possibility further, a number of additional double digestions were performed: first, Bcl 1 and Kpn 1; second, Pst 1 and Kpn 1; third, Pst 1 and Hae II, and finally, Pvu II. When a double digestion of C1-39 DNA was performed with Bcl 1 and Kpn 1 (see Figure 22, lane 3), only one DNA fragment of 4.0kb length was found. Once again, the expected DNA fragment (2.8kb) was not found. Figure 23 shows another series of double digestions. When

Figure 23. Southern analysis of cellular DNAs from Cl-39. Ten micrograms of DNA were digested with (3) Pst 1, (5) Pst 1 and Kpn 1, (7) Pst 1 and Hae II, (9) Pvu II, electrophoresed in a 0.6% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 3 days. (1) pMKSV40ori$^-$ digested with Xho 1. (2) SV40 digested with Pst 1. (4) SV40 digested with Pst 1 and Kpn 1. (6) SV40 digested with Pst 1 and Hae II. (8) SV40 digested with Pvu II.

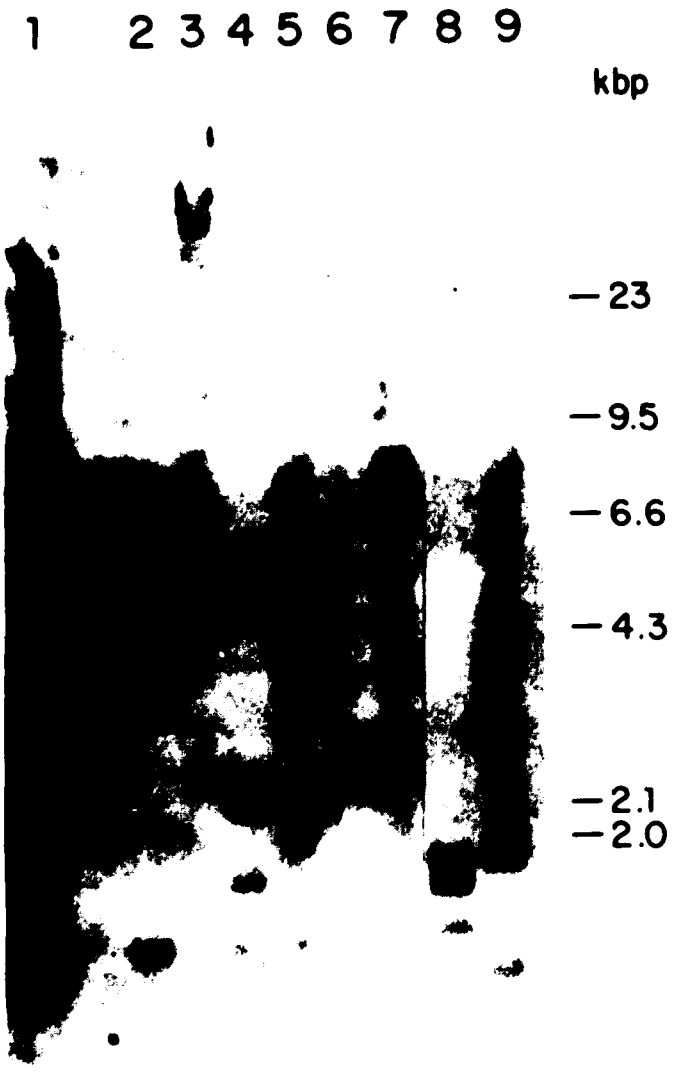

Figure 23

a double digestion was performed using Pst 1 and Kpn 1 on wild type SV40 (Figure 23, lane 4), three DNA fragments are found (2.3kb, 1.7kb and 1.2kb), as expected. DNA from Cl-39 was digested with these enzymes (see Figure 23, lane 5), and only the 2.3kb fragment was found. Digestion with both enzymes is verified by comparison with Pst 1 alone (see lane 3). This fragment covered most of the early region from nucleotide 3144 to nucleotide 234. A similar result was found with another combination of enzymes, Pst 1 and Hae II, which in wild type SV40 (see Figure 23, lane 6), generated 2.87kb, 1.2kb and 1.16kb fragments. When this double digestion was carried out, only the 2.87kb fragment was found in Cl-39 (Figure 23, lane 7). When Cl-39 DNA was digested with Pvu II (see Figure 23, lane 9), only the 2.0kb fragment was found; neither the 1.45kb nor the 1.8kb was present (see lane 8). For comparison, SV40 DNA was also digested with other enzymes to substantiate the size of the relevant fragments. All the above results indicated that the majority of the early region was present but the greater part of the late region was absent. This finding was not difficult to understand, since only the early region is needed to transform the cells. The more significant finding was that the Bcl 1 site was not present. This was determined by the fact that a 2.8kb fragment, which should be generated by cutting Cl-39 DNA with Bcl 1 and Kpn 1, was not found (Figure 22, lane 3). Lambda DNA had been added to this lane, verifying that the

DNA was cut properly (data not shown). Since the Bcl 1 site (bp 2706) is only a few base pairs from the end of the large T-antigen, we can be certain that at least part of the carboxyl end of the protein was missing. The nearest convenient restriction site is that of Pst 1 at 3144. Pst 1 has two restriction sites in wild type SV40, which produce a 4.0kb fragment and a 1.2kb fragment. When DNA from all six of the immortalized cell lines and IF5A-2 were digested with Pst 1 alone (see Figure 24), a DNA fragment which matched one of the IF5A DNA fragments was detected in all cases (compare lane 3 with lanes 4 through 9). When the Pst 1 digestion was repeated for Cl-39 alone, the size of this DNA fragment was approximately 4.2kb (see Figure 23 lane 3). If this information is taken together with previous data from the above double digestions, it would appear that the Pst 1 site being detected is that in the early region, since the one in the late region (at base pair 1988) is distal to the Eco Rl site and should have been deleted. The size of the Pst 1 DNA fragment found in the immortalized cell lines is fortuitously similar to the wild type Pst 1 fragment.

I could not ascertain how much of T-antigen is missing by such a restriction enzyme analysis, since there are no convenient restriction sites between the Pst 1 site at 3144 and the Bcl 1 site at 2706. An alternate approach would be to directly examine the size of the T antigen synthesized in Cl-39 and IF5A. A Polyacrylamide gel

Figure 24. Southern analysis of cellular DNAs from (3) IF5A, (4) Cl-39, (5) Cl-5, (6) Cl-37, (7) Cl-38, (8) Cl-2, (9) Cl-3. Each DNA sample was digested with Pst 1 and electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 5 days. (1) SV40 digested with Eco Rl. (2) pMKSV40ori$^-$ digested with Xho 1.

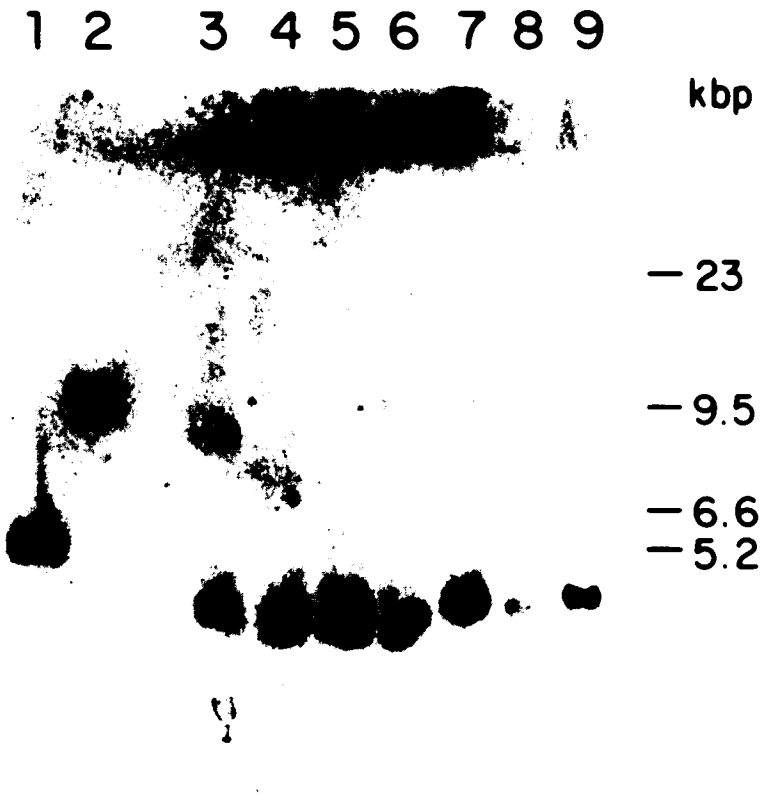

Figure 24

electrophoresis (PAGE) analysis would be expected to reflect the fact that a significant deletion had occurred. Upon immunoblot analysis I indeed found a significantly truncated large T molecule (as described in the next paragraph), indicating that there is a sizable portion of the SV40 genome missing at the end of the early region; it is possible the early region ends just after the Pst 1 site.

I attempted to determine the size of the T antigen synthesized in C1-39 and IF5A. Cells were harvested, immunoprecipitated, and subjected to polyacrilamide gel electrophoresis. The proteins were transferred from the gel to a nitrocellulose filter and T was detected using the monoclonal antibody 416 (immunoblot). Cos 7 cells, which are origin-defective but produce a normal size T-antigen, were used as a positive control. I found that all the mutants produced a large T-antigen that was considerably smaller than the wild-type protein; its size was approximately 80,000 daltons (data not shown). The parent of all these mutants, IF5A, produced two different sized T-antigens, neither of which was of normal size. However, one of these antigens matched the T-antigen found in all the immortalized cell lines. The other band of IF5A corresponded to a protein with an approximate molecular weight of 85 kilo daltons. Thus, the western blot confirmed what had been surmised from the Southern analysis, namely, that C1-39 contained a truncated large T.

To be sure that a human protease was not partially degrading the large T-antigen (and thus reducing its size), extracts of both Cos 7 and Cl-39 cells were mixed together, immunoprecipitated, and analyzed by immunoblot (Figure 25, lane 4). Two bands were found -- one which matched the Cos 7 polypeptide (lane 5), and another which matched the Cl-39 band (lane 3). This indicated that the T-antigen product in the immortalized lines was truly smaller than wild type T-antigen. Another immunoblot analysis was performed using monoclonal 419, which reacts with both large T and small t-antigen. Figure 26 shows that IF5A (lane 2), as well as Cl-39 (lane 3), did indeed produce a normal sized small t-antigen, since the bands of Cl-39 and Cos 7 co-migrated (see Figure 26, lanes 3 and 4 respectively). All this information fit well with the data obtained from Southern analysis described above. Specifically, the early region was incomplete and ended somewhere between the Pst 1 site (3144) and the Bcl 1 site (3444), thus producing a truncated protein.

To see whether or not any of the plasmid sequences of pMK-16 were incorporated in the immortalized cell lines, Southern blots were probed with pBR 322, the parental plasmid of pMK-16 (see Figure 27). No plasmid sequences were detected although the control lanes (lanes 3 and 4) hybridized quite well. From previous data, it also became obvious that there were no tandem repeats. Tandem repeats would generate a fragment with a molecular weight between

Figure 25. Immunoblot analysis of Cl-39 and IF5A for SV40 large T antigen. 50 ul of extract were loaded on a gel. Proteins were transferred onto a nitrocellulose filter and immunoblotted, as described in Materials and Methods, with monoclonal antibody pAB 416 against SV40 large T antigen. Lane (1) HS74BM, (2) IF5A, (3) Cos 7, (4) Cl-39 and Cos 7, (5) Cl-39, (6) molecular weight markers.

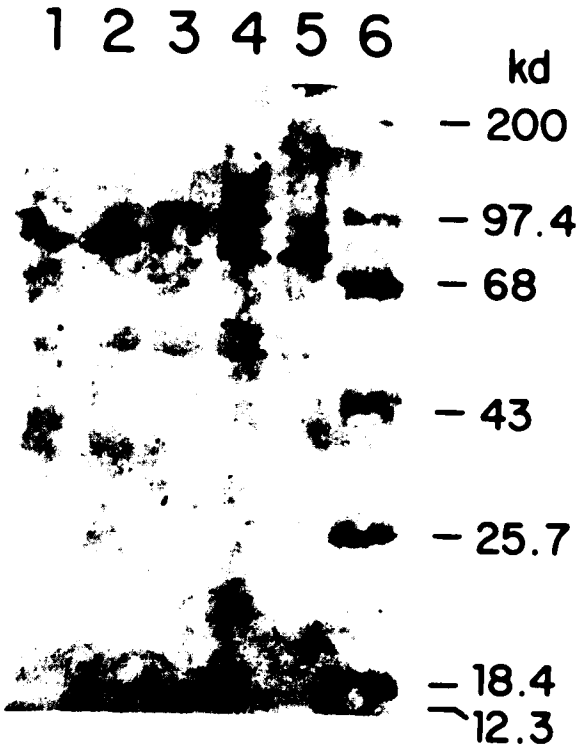

Figure 25

Figure 26. Immunoblot analysis of C1-39 and IF5A for SV40 small t antigen. 50 ul of extract were loaded on a gel. Proteins were transferred onto a nitrocellulose filter and immunoblotted, as described in Materials and Methods, with monoclonal antibody pAB 419 against SV40 small t antigen. Lane (1) molecular weight markers, (2) IF5A, (3) C1-39 (4) Cos 7.

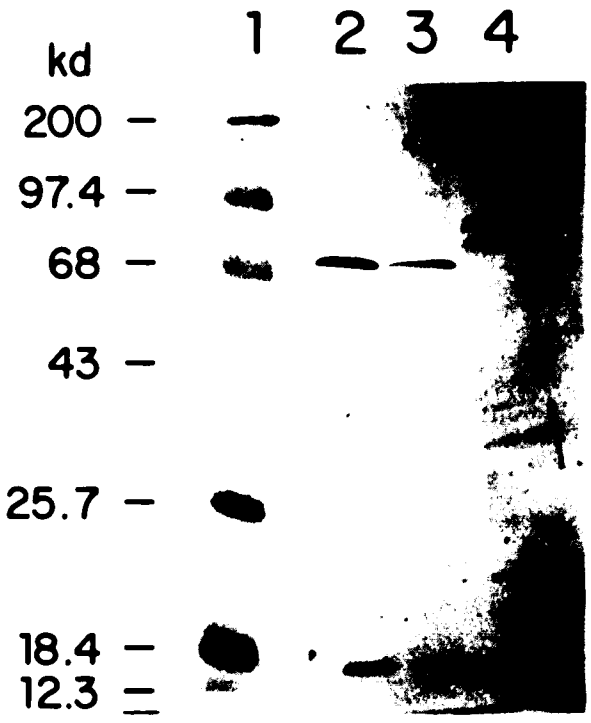

Figure 26

Figure 27. Southern analysis of cellular DNAs from IF5A. The filter was dehybridized, incubated with 1×10^7 cpm of ^{32}p-labeled pBR322, and exposed for autoradiography for 1 day. (1) SV40 undigested. (2) SV40 digested with Eco Rl. (3) pMKSV40ori$^-$ undigested. (4) pMKSV40ori$^-$ with Xho 1 (5) Sst 1, (6) Xho 1, (7) Bgl 1, (8) Kpn 1, (9) Bam Hl, (10) Eco Rl.

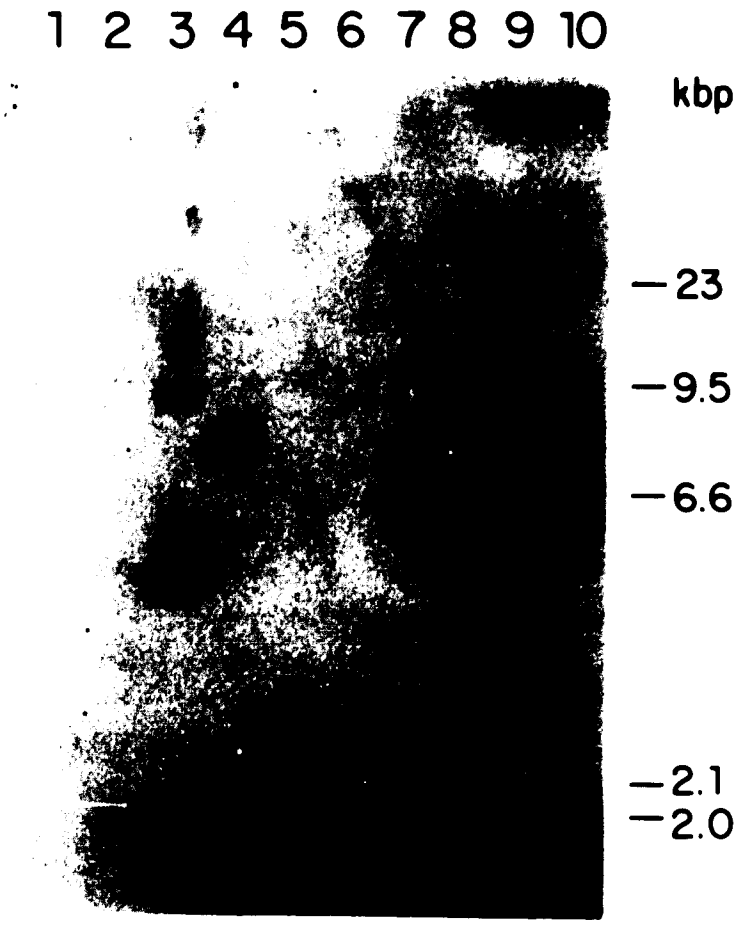

Figure 27

2.87kb (the size of the Pstl/Hae II fragment) and 4.1kb, the size of the fragment that would span the distance from the Bcl 1 site to the Pvu II site at 1654, both of which are absent from the proposed SV40 integrant. Figure 21, lane 10, in which DNA from Cl-39 was digested with Kpn 1, confirms this, as does Figure 23, lane 4, where DNA from Cl-39 was digested with Pst 1. Both of these enzymes cut once within the SV40 DNA contained in Cl-39, and would thus be expected to reveal tandem repeats.

Hirt lysates were also made for the parent, IF5A (see Figure 28, lane 3), and all of the immortalized cell lines (lanes 4 through 10) to determine if free viral DNA was being produced. This proved not to be the case, and agrees with the fact that the transforming DNA was origin defective. The DNA fragment seen in the high molecular weight region is consistent with contaminating integrated (cellular) DNA.

The possibility existed that a large piece of DNA had moved upon immortalization which included the integrated SV40 sequences and a large portion of cellular DNA. Such an event might not be expected to be detected by Southern analysis. Therefore, karyotype analysis, G-banding and, ultimately, <u>in-situ</u> hybridization were performed on IF5A, Cl-39, Cl-37 and Cl-3. Cl-39 was chosen to represent the immortalized lines with one integration site. Cl-37 was chosen as a representative of those with two integration sites, and Cl-3 was chosen because (based on Southern

Figure 28. Southern blot analysis of Hirt lysates from (3) IF5A, (4) Cl-37, (5) Cl-38, (6) Cl-39, (7) Cl-2, (8) Cl-3, (9) Cl-5 (10) Cl-5. The gel was electrophoresed in a 0.7% agarose gel at 40 volts for 16 hours. The DNA was transferred to nitrocellulose, hybridized to 1 x 10^7 cpm of ^{32}p-labeled SV40 and exposed for autoradiography for 1 day. (1) SV40 digested with Eco Rl. (2) pMKSV40ori$^-$ digested with Xho 1.

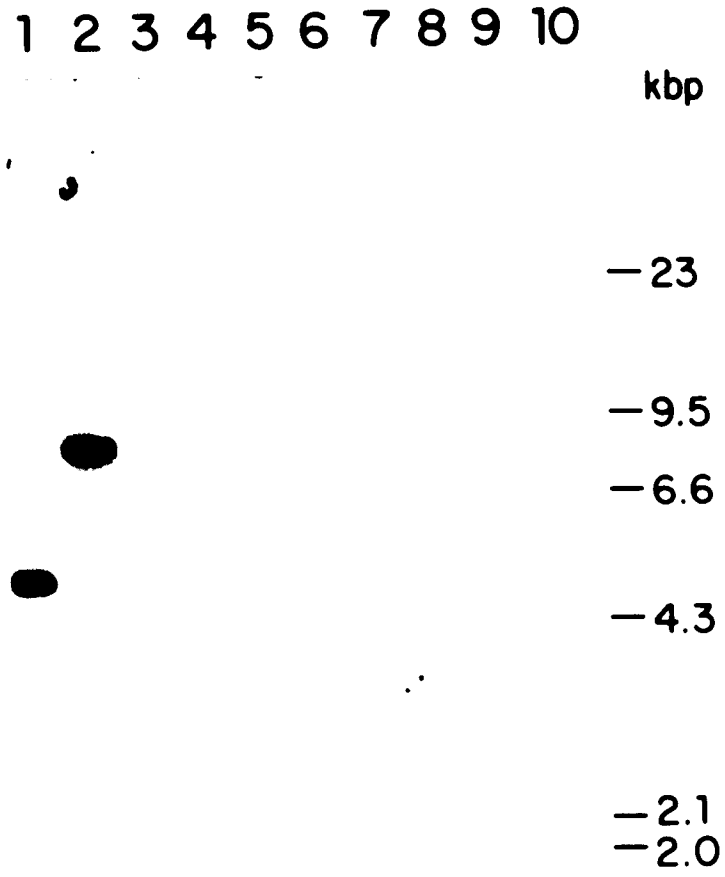

Figure 28

analysis) it contained an insert that had moved. These were all compared to the parent, IF5A. Karyotype analysis of IF5A indicates they are a bimodal cell population, with a group of pseudodiploid cells ([Figure 29] chromosome range 42-46, modal # =44) and a second group of heteroploid cells (Figure 30). The three continuous cell lines studied (Figures 31, 32 and 33) do not contain a stable karyotype; random breakage and telomeric chaining is found. We found joining of intact chromosomes in end-to-end associations by diffuse non-banding material forming pseudostructural abnormalities, consistent with the findings reported by Nichols et al (1985). Changes in chromosome number and structure were found in these cells, as has been found by other investigators (Koprowski et al, 1962; Nichols et al, 1978). Chromosomal abnormalities such as those shown in Figure 34 are typical of each of the continuous cell lines studied. It would seem that the heteroploid group of cells found in IF5A give rise to the immortal lines, because some of the heteroploid IF5A cells contain a 1p+ rearrangement which is found in all of the immortalized lines. Karyotype analysis of these immortal cell lines has also determined that one chromosome 16 is absent from most of the immortal cells, although a low frequency occurrence of the intact chromosome in isolation or in combination with another chromosome is found. This is in good agreement with the literature which has found that SV40 immortal cells do not contain a normal (diploid) karyotype

Figure 29. A G-banded karyotype of IF5A with a pseudodiploid chromosome number. Unidentifiable abnormal chromosomes shown at the top of the Figure.

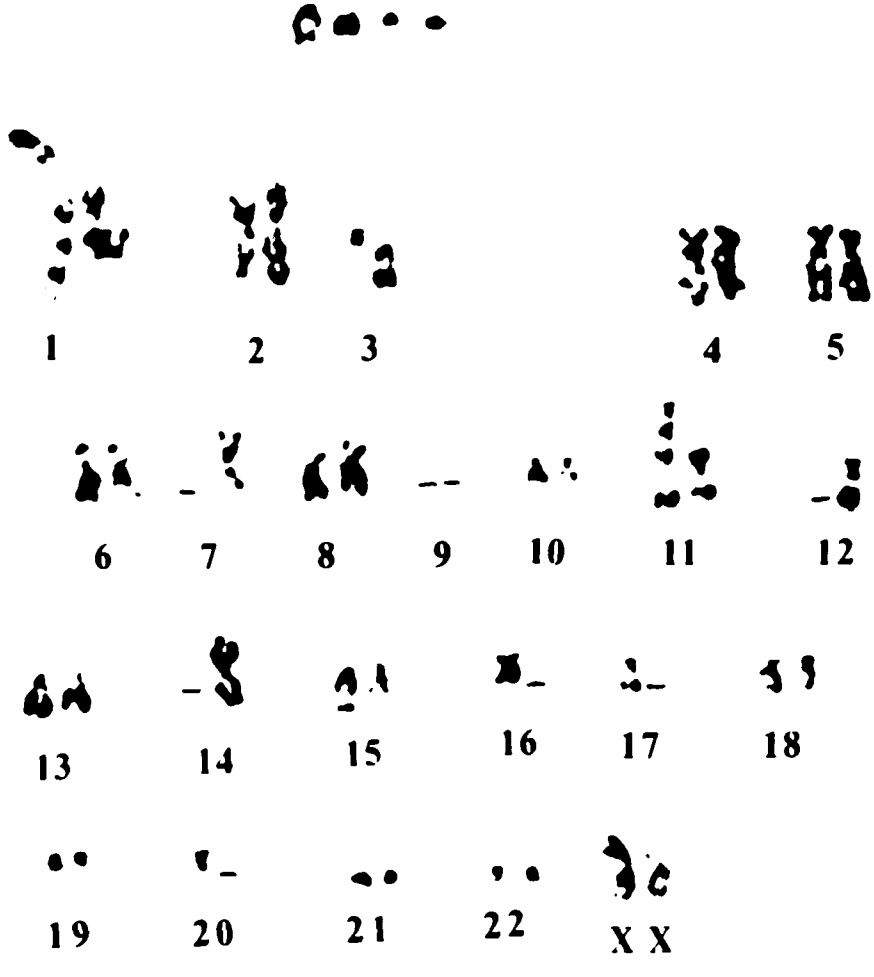

Figure 29

Figure 30. A G-banded karyotype of IF5A showing a heteroploid chromosome number. Unidentifiable abnormal chromosomes typically found in the heteroploid cells shown in the upper part of the Figure.

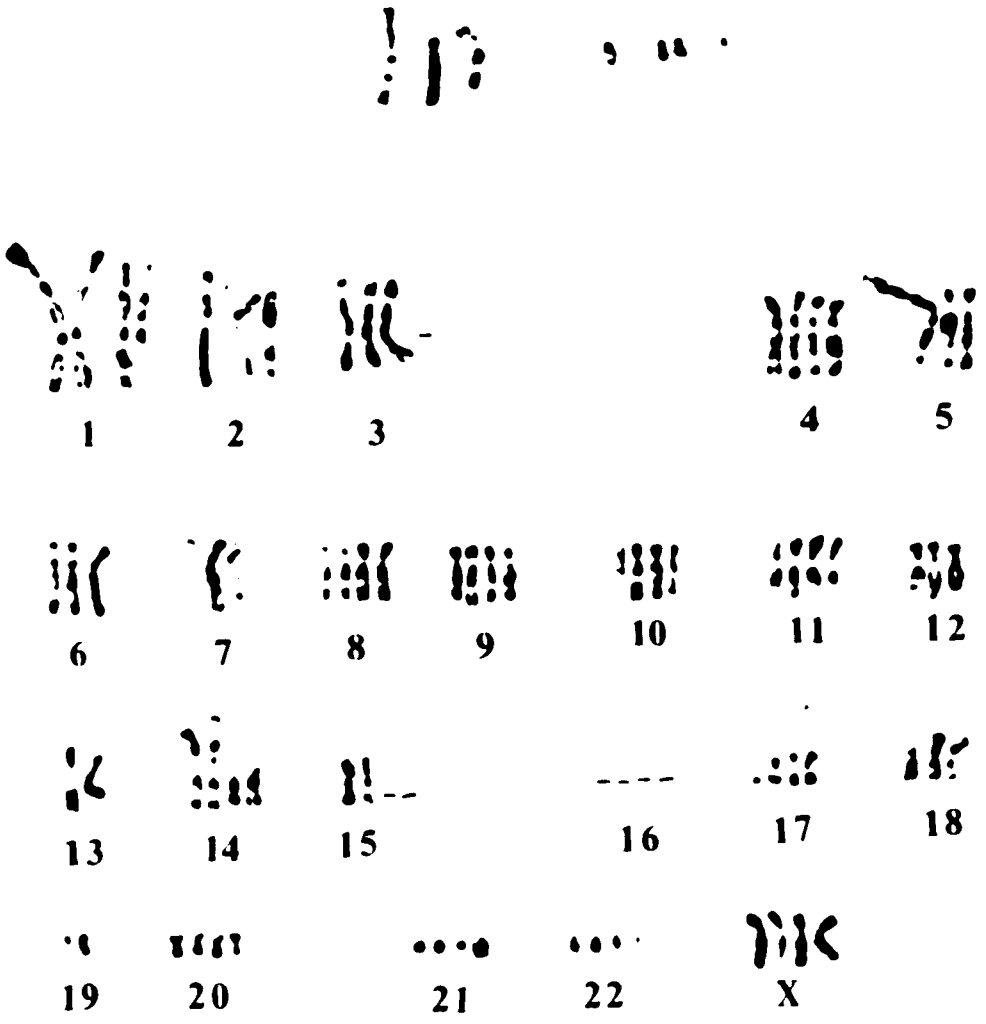

Figure 30

Figure 31. A G-banded karyotype of C1-39 showing a heteroploid chromosome number. Unidentifiable abnormal chromosomes typically found in the heteroploid cells shown in the upper part of the Figure.

Figure 31

Figure 32. A G-banded karyotype of Cl-37 showing a heteroploid chromosome number. Unidentifiable abnormal chromosomes typically found in the heteroploid cells shown in the upper part of the Figure.

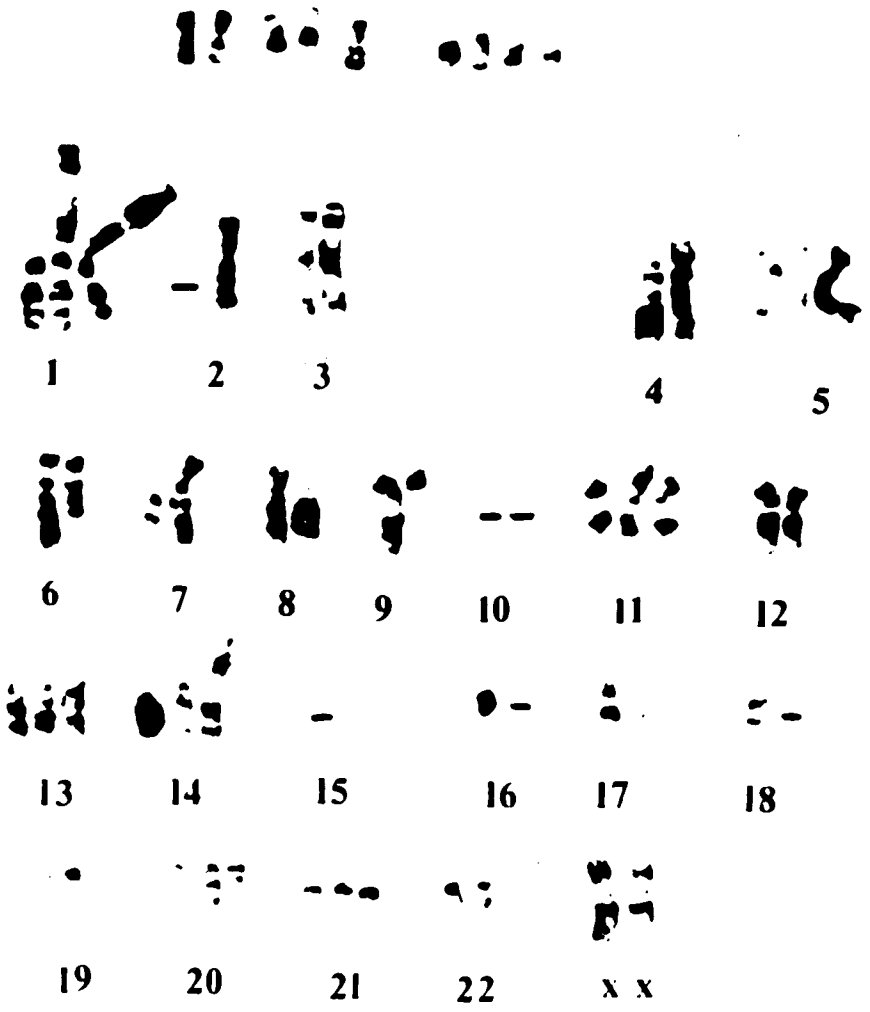

Figure 32

Figure 33. A G-banded karyotype of Cl-3 showing a heteroploid chromosome number. Unidentifiable abnormal chromosomes typically found in the heteroploid cells shown in the upper part of the Figure.

Figure 33

Figure 34. G-banded mitosis showing various apparent chromosome aberrations including double minutes.

Figure 34

(Hayflick, 1977; Cristofalo, 1982).

When these cell lines were subjected to in-situ hybridization, no significant grain counts were found at the telomeric junctions. This indicated that the virus was not involved with these end-to-end associations. Nichols et al (1985) suggested that virus-induced uncoiling and stickiness of the telomeric areas may be a possible explanation for these pseudostructural abnormalities. The only possible evidence of cytological significance that could be determined from the in-situ hybridizations was on chromosome 15; however, since an insufficient number of metaphase plates were examined, no definite conclusion can be drawn from these data.

CHAPTER 5: DISCUSSION

Human diploid cells derived from normal tissue undergo a characteristic pattern of growth in cell culture, ending with a non-replicative phase and, finally, cell death. Transformation with SV40 can alter this pattern in two ways -- first, extending the normal lifespan and secondly, in rare cases, resulting in the appearance of immortalized derivatives. I have examined both phenomena in a normal diploid fibroblast derived from fetal bone marrow, HS74BM, and a series of transformants generated with non-defective and replication defective viral genomes. The results confirm and extend many of the findings in the literature. Furthermore, my data support the premise that the capacity of human cells to support SV40 DNA replication is relevant to the efficiency with which immortalized SV40-transformed human cell lines can be obtained. One of the goals of this thesis was to determine whether cells transformed with SVori$^-$ were at an advantage over cells transformed with SV40 with a complete genome with regard to their ability to become immortal. As previously mentioned in the Introduction, cells transfected with SVori$^-$ show an enhanced transformation efficiency over wild-type SV40 (Small et al, 1982). My results demonstrate that the lifespan of cells transformed with an origin-defective SV40 molecule is also increased. Although other investigators (Jensen et al, 1963; Girardi et al, 1966; Gotoh et al,

1979) have shown that SV40 transformation increases the growth potential of human fibroblasts, my results indicate that this increase can be extended even further if SVori$^-$ is used as the transforming DNA. When the lifespans of HS74 transformed with SVori$^+$ (Tables 1 and 2) and HS74 transformed with SVori$^-$ (Tables 4-9) are compared, nearly a doubling of the number of generations is evident. Tables 1 and 2 show that the greatest number of generations that can be achieved with SVori$^+$ as the transforming agent is 27 generations. When one compares this to at least 35, and as many as 48, generations that can be attained with SVori$^-$, it is clear that SVori$^-$ is the transforming agent of choice to increase the lifespan of the cell line. The lack of difference in lifespan between the SVori$^+$ transformant and HS74 may be artifactual, because of the inherent difficulty in estimating generation number involving very different passage methodologies. This difficulty would be less relevant to the comparisons among the transformants since they were passaged similarly and in parallel. Transformation with SVori$^-$ also seems to facilitate the establishment of cell lines. This conclusion is based on the relative ease with which our laboratory was able to isolate not one, but several, immortal cell lines. The first immortalized cell lines (Cl-2, Cl-3 and Cl-5) were developed by simply leaving the cells in culture for an extended period of time. The clones that grew out were picked and passaged indefinitely. This experiment was

unable to be duplicated with cells transformed with SVori$^+$ (SVHF-A), although an equal or greater number of cell was used in the experiment (Zouzias et al, 1980; data not shown). The second set of immortalized lines were obtained by performing a cloning experiment involving 10^4 cells (see Table 17); three of the colonies picked became immortal. This calculates to an extraordinarily high frequency of 0.03% of the cells which were plated for colony isolation. This would again concur with the notion that SVori$^-$ is superior over SVori$^+$ in its ability to establish cell lines. Although I was unable to reproduce that result, two other successful experiments executed in our laboratory support the overall conclusion. In one experiment, IF5A was treated with $5x10^{-6}$ M griseofulvin. Six immortal colonies were recovered after treating $2.2x10^6$ cells and subsequent blind passage. In the second type of experiment, HS74 was transfected with pSVori$^-$tsA58. Transformed foci were identified at 35^o C, picked and grown into mass cultures. In the case of one transformant which has been studied extensively, apparently immortalized derivatives have been repeatedly isolated. My experiments (and those which were performed subsequently by others in the laboratory) are to be contrasted with prior experience in the literature. Blind passage of cultures containing multiple transformants yields variable, and typically very low, success rates (Huschtscha and Holliday, 1983). Two studies involving cloned SV40 transformed fibroblasts were

completely unsuccessful, including one from this laboratory (Gotoh et al, 1979; Zouzias et al,1980) as indeed were my own experiments with IF1A. Interestingly, the SV40 genome recovered from immortalized SV40 transformed human fibroblasts is often defective due to a mutation in the A gene sequence resulting in a replication deficient T antigen which is still able to transform cells (Gluzman and Ahrens, 1982; Manos and Gluzman, 1984; 1985;). It is not known whether the original transformation event involved such a defective genome or whether the alteration occurred subsequently. It should be noted that SV40 virus stock used for transformation studies in the 1960's commonly contained defectives, since high multiplicity infection was typically used to generate and maintain virus stocks (Lavi and Winocour, 1972; Winocour et al, 1975). It should, however, be emphasized that transformation by an origin-defective but otherwise competent SV40 genome does not invariably result in the recovery of immortalized cell lines. Indeed, I was unsuccessful in any experiment with IF4A or IF6A. However, my conclusion that SVori$^-$ transformants are a valuable approach has recently been also noted by three other laboratories. Murnane et al, (1985) successfully established cell lines from fibroblasts with the hereditary human disease ataxia-telangiectasia (AT). Immortalized derivatives were obtained in one of four individual transformants. Canaani et al (1986) reported that immortalized cell lines were obtained with

two of seven transformants in fibroblasts with Xeroderma pigmentosum (XP). Gallimore (personal communication) has made the most extensive series of studies over several years. Only one of 200 individual transformants generated with wild-type SV40 became established, whereas 20 of 200 transformants generated with SVori$^-$ have been successfully established. Other laboratories have documented the fact that pSVori$^-$ can be an effective vehicle for the development of permanent monocyte/macrophage cell lines (Nagata et al, 1983) or human fetal glial cells (Major et al, 1985). The enhanced lifespan and subsequent establishment of the transformed cells is probably due to the viral DNA's inability to excise and replicate in transformed cells. This property should increase the stability of the transformed phenotype. Furthermore, it prevents the possibility of cell death mediated by uncontrolled viral DNA synthesis or production of virus particles.

An interesting phenomenon that was found with SVori$^-$ was that, unlike the typical integration patterns found with Svori$^+$ (tandem repeats, extensive amplifications and rearrangements), a simpler arrangement was found. I observed this with IF5A and the immortalized cell lines (see Figure 13), where one or two integration sites were found. Other investigators also found one or two integration sites in their cell lines transformed with SVori$^-$ (Van Doren and Gluzman, 1984; Murnane et al, 1985;

Canaani et al, 1986).

The biochemical and genetic basis for immortalization could not be determined, although the data which I obtained bear on many possible hypotheses. Investigators have speculated that "crisis" in SV40-transformed human fibroblasts is related to senescence. Stein (1985) demonstrated that AG3204, a pre-crisis SV40-transformed IMR-90 (human fetal lung fibroblast), showed an age-related increased serum requirement, as Ohno (1979) had previously shown for IMR-90 itself. Such findings were used to support the model that changes are occurring which are obscured by conventional mass cultivation. I have found additional evidence to support that view, since the efficiency of colony formation in 10% serum of IF5A fell progressively between passage 11 and passage 15 (i.e., generations 24 to 33), even though the overall growth pattern appeared unchanged until 41 generations. Moreover, this pattern of EOC was observed whether cells were plated in monolayer or in semi-solid agarose. Since the latter is a stringent criterion for the transformed phenotype, the results would appear to be applicable to all members of the population. Although EOC is a complex phenotype which is dependent on additional factors, the results are consistent with the increased requirements for growth factors, as described by Stein (1985).

In addition, I had initially considered several possible models for the incidence and identification

(rescuability) of immortalized derivatives within IF5A. Since no reproducible method was obtained, it was not possible to conclude that any of the models (HI/LR, LI/HR, LI/LR) was correct. However, my observations support the idea that the cell density at the onset of senescence within the culture was important. If the cell density is too low, one may not have sufficient cells for an immortalized derivative to be present. If the density is too high, the presence of a large number of dead or dying cells might be expected to be toxic for the fledgling immortal cell. I similarly strongly suspect that trypsinization resulting in cell damage and (often) significant decrease in cell density may be important variables in recovery. Both considerations were relevant to the clonal isolation method (C.I.M.), as described, and the recovery of the rare immortalized derivatives. Rapidly growing immortalized derivatives have been only rarely observed in this laboratory (Ozer and Caton, unpublished data). It is unclear whether the C.I.M. (see experimental procedure for Table 17) is a technique that can be used to consistently isolate immortalized cell lines. Although the technique seemed effective for the experiment described in Table 17, the unsuccessful experiments described in Table 20 indicate that this is not a reliable method. Another set of immortalized cell lines were isolated with pSVori$^-$ tsA (tsA/HF) with this method; however, other immortalized lines were isolated by simply passaging the cells of this

transformant (Radna, Caton and Ozer, unpublished data). Several attempts were made with IF5A over more than a one year period. In each case, a new culture was started from a frozen stock. The passage history prior to frozen storage varied, but most studies were performed with cells predicted to be at a comparable stage in their lifespan as initially used. It must be emphasized, however, that the number of generations estimated by continuous passage can be significantly different from that involving intervening freezing and thawing due to variable recoveries. One must at least consider that the three immortalized cell lines derived by the C.I.M. were the result of a single (rarer) event, analogous to the results obtained in a fluctuation test (Luria and Delbruck, 1943). Such immortalized cells would be absent in other cultures of IF5A. No definitive statement can be made. The cell lines Cl-37, Cl-38 and Cl-39 share several features but are also distinguishable. First, Southern analysis shows conservation of one of the original integration sites for Cl-37, Cl-38 and Cl-39, consistent with a common origin (see Figures 13-15). Two copies are found in Cl-37 and Cl-38; however, Cl-39 has lost one copy. This loss could have occurred subsequently; however, both copies in Cl-37 and Cl-38 are stable to repeated passage, even though one of the copies in Cl-37 and Cl-38 moved sometime between the transformation of IF5A and the time the cells became immortal (see Figure 14). Second, IF5A and the immortalized derivatives have been

examined for multiple surface antigens using monoclonal antibodies in collaboration with Dr. E. Klein at Sloan-Kettering Institute. A number of antigens have been identified as increased in IF5A as compared to the normal parent HS74. An additional antigen has been found to be expressed in Cl-37, Cl-38, and Cl-39, but not in IF5A or HS74. However, it is also expressed in the other immortalized clones that I have isolated independently (i.e., Cl-2, Cl-3, and Cl-5). It is not expressed in all immortal clones of HS74 transformed by SV40 (e.g., tsA/HF), and is also expressed in some, but not all, immortal SV40-transformed in other cell types. We have tentatively concluded that expression of this antigen is a common but secondary consequence of SV40 transformation. Thus, although SVori$^-$ confers immortality to cells at a higher frequency than does wild type SV40, the C.I.M. does not reliably establish a continuous cell line. However, I remain optimistic that the C.I.M. can be used to enhance the isolation of immortal cell lines, provided that the experiment is started at the right point in time allowing greater than 100 colonies to be picked.

Since I had isolated multiple, putatively independent, clones from IF5A, it was also possible to assess whether any common features of growth were shared by the immortalized derivatives. All immortalized cell lines had, of course, acquired the ability to grow indefinitely, in contrast to IF5A or HS74. However, when the growth rate

and EOC of the immortalized cell lines were compared with the non-immortal parents at early passage, no dramatic differences were evident. Differences among the cell lines with respect to their growth rate and EOC were observed. It was somewhat surprising that when Cl-39 was initially isolated as a colony, it had a relatively low EOC, as well. This further suggests that the method of isolation did not result in a particular growth phenotype. More striking were secondary changes which were evident on continued passage of the already immortalized cell lines (e.g., Cl-39).

Cells stably transformed by SV40 contain viral DNA which is covalently integrated into the chromosomal DNA of the host cell (Sambrook et al, 1968). Analysis by DNA reassociation kinetics has shown that there is considerable variation between transformed cell lines in the number of integrated copies of viral DNA (Sambrook et al, 1975), and that the representation of different segments of the viral genome is not necessarily uniform (Botchan et al, 1974). The Southern transfer hybridization technique has been used to analyze the structure of the integrated viral DNA as reviewed in the Introduction, and the results indicate that there are no specific integration sites on either cellular or viral DNA. Integration can also occur in different chromosomes. More detailed analysis might reveal preferred sites of integration or nucleotide sequence specificity, as suggested by the findings of Stringer (1982) in which he

reported the existence of five base-pairs of homology at the virus-host junction. This same sequence (T-G-T-G-T) was found by Mounts and Kelly (1984), four nucleotides from the right virus-host junction of their mouse transformant (SVB400). Hasson et al (1984) found short direct repeats are preferential sites for illegitimate recombination in SV40 DNA.

Comparison of the integration pattern of IF5A and the immortalized cell lines permitted a test of the hypothesis that rearrangement of the SV40 (and adjacent cellular) sequences was a necessary step in immortalization. This did not appear to be the case, since all cell lines showed conservation of one of the SV40 copies and adjacent sequences within the limitation of the Southern analyses. An effort was made to extend the analysis by the in situ hybridization technique (in collaboration with Ms. Sally Ripley) to assess whether a rearrangement of a large subchromosomal segment was associated with immortalization. This analysis was made exceedingly difficult by the number of chromosomal abnormalities in IF5A at the time at which the immortalized derivatives were isolated. The results strongly indicate that the site of integration cannot be determined unambiguously in IF5A, a necessary prerequisite to further analyses.

The results I obtained are consistent with the findings of others that multiple patterns of integration can be observed. I also detected differences among my

immortalized cell lines, even though they were all derived from a single cloned transformant. They varied in the number of integration sites; one site was found for Cl-2, Cl-5 and Cl-39 and two integration sites were noted for Cl-3, Cl-37 and Cl-38 (Figure 13). In addition, one of the SV40 integrants in Cl-3, seemed to have moved to a new position different from that of one of the integrants of Cl-37 and Cl-38. When one examines the lower molecular weight DNA fragments in Figure 13 (lane 9), one finds one in Cl-3 that does not have the same molecular weight as that fragment of IF5A (lane 3), whereas the lower molecular weight DNA fragments of Cl-37 and Cl-38 (lanes 6 and 7 respectively), as well as the higher molecular weight DNA fragments of all the continuous cell lines, are indistinguishable from that of IF5A. However, Figure 14 indicates that one of the DNA fragments has also moved for Cl-37 and Cl-38 (see Figure 14, lane 6 and lane 7 respectively) because if one examines the lower molecular weight DNA fragment of IF5A (lane 3) one does not find a matching DNA fragment for either Cl-37 (lane 6) or Cl-38 (lane 7). Although Botchan et al (1976) reported that in several established lines the integration pattern was stable over several hundred cell generations, Hiscott et al (1980) found both amplification and rearrangement of the integrated SV40 DNA in a transformed mouse line. This latter observation has been confirmed by other investigators (Clayton and Rigby, 1981; Mounts and Kelly,

1984). Thus the results of my research agree with previously reported data; namely, I also found rearrangements to be related to the number of generations in tissue culture. These rearrangements included loss of one of the integration sites as well as movement of a site to a new location.

Southern analysis was also used to determine in detail the integration pattern of one of the copies. From the information derived from Figures 22 and 23, it is apparent that at least the SV40 sequences from base pair 1654 (a Pvu II site) to 2770 (a Bcl 1 site) are missing. The restriction enzyme analysis is interpretable as a colinear map of a subgenomic region, as shown in Figure 35, extending from the Hae II site (772 bp) in the late region through the origin to the Pst 1 site (3144 bp) in the early region. Thus, although a complete linear SV40 molecule (which was linearized at the Bam Hl site) was used for the initial transfection, the insert must have undergone changes prior to its present state. Other investigators (Clayton and Rigby, 1981; Mounts and Kelly, 1984) have found that integration patterns are frequently complex, and complete or partial tandem duplications of viral sequences are common. These researchers have also found that loss of sequences occur in both the integrating DNA and the host DNA upon integration. Exonuclease digestion is known to occur when calcium phosphate coprecipitation is used as the

Figure 35. Linear restriction map of the possible SV40 genome sequences integrated in Cl-39. The solid line indicates sequences integrated in Cl-39 which have been confirmed by Southern analysis. The wavy line indicates sequences whose presence in Cl-39 has not yet been confirmed.

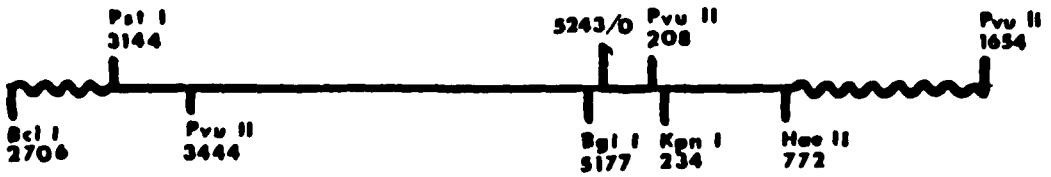
Pst I
3144
5243/0
Pvu II
208
Pvu II
1654
Bcl I
2706
Pvu II
3444
Bgl I
5177
Kpn I
234
Hae II
772

Figure 35

mode of DNA transfer. Either of these two possibilities would have resulted in a different piece of DNA integrating than was originally used in the transformation. The easiest way to explain its integration pattern in light of these findings is that a "double crossover" occurred; there was one recombinational event in the early region and a second one in the late region, thereby deleting the sequences between 1716 and the Bcl 1 site at 2770. However, one may also postulate that exonuclease digestion occured prior to integration. The extent of this digestion may be as little as a few nucleotides (enough to destroy the Bam Hl site) or as many as 1054 nucleotides (the entire region that is missing).

Regardless of the mechanism, some part of the large T antigen coding region is missing. Thus, if we look at the size of the large T, we would expect to find a truncated T (provided enough of the T is missing). This can be seen from the immunoblots (Figure 25) where one finds two bands for IF5A (Figure 25, lane 2) corresponding to the two SV40 integrants which provide two different sized molecular weight proteins and Cl-39 (lane 5), where only one band is found co-migrated with one of the IF5A bands, and seemingly corresponding to one integrant and one protein. Also confirming the Southern blot analysis is the fact that the Cl-39 band corresponds to a truncated T antigen protein of approximately 80,000 daltons. The molecular weight of the wild-type protein is 94,000 daltons. Thus, the protein of

the immortalized lines is 14,000 daltons smaller than the wild type protein. The maximal deletion of the SV40 sequences would predict a smaller polypeptide; however, it falls within the range of possibilities. T antigen has 708 amino acids of which 558 are proximal (amino end) to the Pst I site. There are only 26 amino acids between the Bcl I site and the carboxyl end. The deletion would be expected to include the termination sequences and polyadenylation sites of the T polypeptide. Consequently, the protein synthesized in Cl-39 (or IF5A) would be expected to be a fusion protein with amino acids at its carboxyl end contributed by the adjacent cellular sequences. Such would not be of any predictable length. Accurate assessment would require sequencing the integrated SV40 and cellular sequences using suitable recombinant DNA cloning methodology. Such experiments have not yet been performed. One should also note that these putative cellular sequences need not be human-derived. The initial transformation involved SV40 DNA and calf thymus DNA (as carrier). The viral sequences could have become integrated into the latter DNA and that intermediate subsequently became integrated into the host chromosome. As mentioned in the Introduction, T antigen is believed to be composed of several domains that can function independently. Lewis et al (1983) isolated a mutant in which the last 9,000 daltons were incorrectly transcribed because of an incorrect reading frame. This mutant could not transform

mouse cells; however, the incorrect amino acids may have been deleterious to the protein and not allowed it to transform the cells. Colby and Shenk (1982) reported that the immortalization activity may be localized on the amino-terminal half of the T antigen. Pipas et al (1983) showed that portions on both of the amino- and carboxy-terminal halves are required for the transformation of a rat line (REF 52). However, Clayton et al (1982) and Sompayrac et al (1983) showed that the amino-terminal half is sufficient for the transformation of established rat and mouse cells, although at reduced frequency (~1% of the wild-type cells). These results may be interpreted as indicating that different functional domains of the T antigen induce different growth phenotypes in different cells. In light of these findings and those reported by Petit et al (1983), in which they concluded that an active A gene product is needed to maintain immortalization, I believe that the large T antigen found in Cl-39 is sufficient to maintain the transformed phenotype of these cells. Our laboratory has continued to investigate this area by transfecting HS74 with SV40 sequences which contain double mutations: origin-defective for replication and a point mutation resulting in a temperature-sensitive T antigen (tsA58). Transformed foci were picked, propagated, and passaged continuously at 35° C. These transformants have been shown to be temperature-sensitive for cell growth, since they are capable of continuous growth at 35° C and are unable to

grow at 39° C. Thus these continuous cell lines can be used to investigate the role of T in immortalization of human fibroblasts.

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