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Patsalis, Philippos C., Ph.D.

City University of New York, 1993

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MOLECULAR AND CYTOGENETIC ANALYSIS OF HUMAN DIPLOID FIBROBLAST CELLS TRANSFORMED BY SIMIAN VIRUS 40; WHAT CAUSES IMMORTALIZATION?

by

PHILIPPOS C. PATSALIS

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.

1993

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

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Abstract

MOLECULAR AND CYTOGENETIC ANALYSIS OF HUMAN DIPLOID FIBROBLAST CELLS TRANSFORMED BY SIMIAN VIRUS 40; WHAT CAUSES IMMORTALIZATION?

by

Philippos C. Patsalis

Adviser: Professor Ann S. Henderson

Transformation of human diploid fibroblasts (HF) with SV40 can result in extension of life span beyond the normal limit of senescence and in a minority of cases, immortalization. This study used comparison of matched parental (diploid) and immortalized cell lines to determine if any single genetic factor could be related to the immortalization phenomena. The integration site of SV40 was shown to be at chromosome 5q21 by cytologic hybridization. A comparison of mortal and immortal cells showed no alterations involving the integrated SV40 genome *per se.* Karyotypic analysis of matched cell lines, identified a specific chromosomal breakpoint (6q21) in immortalized cells that was not present in the parental line. Hybridization analysis confirmed that sequences on the distal portion of 6q are lost in immortalized cells. Two single copy DNAs which flanks the breakpoint were identified and used to further define the exact breakpoint on 6q13. FISH analysis demonstrated the region 6q13->21 as belonging to another chromosome and confirmed the 6q13 breakpoint. A survey of random translocations and other anomalies occurring in the immortalized lines was also made. Some of these are

regions known to contain oncogenes and transforming proteins. The MCC tumor suppressor gene was rearranged and deregulated. The genes DCC, Bcl-2, APC were also found to be deregulated. We propose that deletion of specific sequences due to breakage of chromosome 6q represent one of the mutational events responsible for immortalization of SV40 transformed HF. In addition, the MCC and possibly other genes are involved in the progression of immortalization.

DEDICATION

I dedicate this book to my father Costas Patsalis, whose life was claimed by cancer in March 22, 1993.

Philippos C. Patsalis

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INTRODUCTION

A. Background

Diploid fibroblasts (HDFs) cells derived from normal human tissue have a limited life span *in vitro* and show characteristic growth patterns in cell culture (Hayflick *et al.*, 1961). After 40-60 population doublings, they cease to divide and reach a non-replicative phase, "senescence". Although senescence was originally described in human cells, similar observations have been made for many types of cells (Sack *et al.*, 1981). After an initial period of culture, HDFs undergo a rapid proliferation (Phase II), and at later stages, the culture becomes incapable of further proliferation with cell arrest occurring in G_1 (Phase III) (Goldstein, 1990).

Several hypotheses have been proposed to explain cellular senescence (rev. in Goldstein, 1990). One hypothesis is that senescence could be the consequence of errors introduced during the synthesis of major macromolecules, or the result of genetic damage. This hypothesis has been rendered unlikely since senescing HDFs derived from either normal persons or from persons with premature aging syndromes (Progeria and Werner syndromes), do not have increased protein and DNA synthetic infidelity (Goldstein, 1990). Another hypothesis is that cellular senescence resembles terminal differentiation, or a genetically programmed arrest of cell proliferation. This hypothesis is more likely on the basis of the following observations: (i) transformants of cells with an older *in vitro* (passage) age enter crisis sooner than those derived from younger lines;

therefore cells are committed to a limited *in vitro* life span (Sack *et al.*, 1981); (ii) the number of population doublings for the mass culture of a given cell strain is reproducible within relatively narrow limits (Hayflick *et al.*, 1961); cells appear to count the number of population doublings to a critical limit before they stop dividing, much like the replicative extinction that occurs during differentiation and (iii) genetic hybrids obtained from fusion of normal cells with immortal cells exhibit limited division potential (Pereira-Smith *et al.*, 1988). The latter point indicates that the phenotype of cellular senescence is dominant and that immortality results from recessive changes in normal growth regulatory genes. This also supports the hypothesis that cellular senescence is a genetically programmed process rather than the result of random accumulation of damage (Pereira-Smith *et al.*, 1988).

Transformation of HDFs with Simian Virus 40 (SV40) can extend the average life span of cells for 20-30 additional population doublings, at which time the cells enter crisis (Ide *et al.*, 1984). Crisis is marked by reduced proliferative capacity, abnormal mitotic figures, loss of attachment to the growth surface, and the occasional formation of multinucleated cell (Sack *et al.*, 1981). Crisis in SV40-infected human cells represents a period in which cell growth and cell death are initially balanced and the culture, as a whole, stops increasing. This is followed by a period in which cells stop dividing and the surviving cell number progressively declines (Shay *et al.*, 1989). In a minority of cases, a sub-population can overcome senescence and become immortal (Huschtscha *et al.*, 1983). When human diploid fibroblasts are transfected with SV40 ori- (defective origin of replication) instead

of wild type SV40, they are transformed at an enhanced frequency. (Small *et al.*, 1982). One explanation for this is that the transfected DNA is unable to excise and replicate and cause cell death through lytic viral infection. (Chang *et al.*, 1986). The immortalizing function of SV40 virus has been localized to the large tumor antigen (T antigen). Human cells transfected with a plasmid expressing only the SV40 early region are able to immortalize at much higher frequency than those transfected with the intact SV40 virus. Precisely how T antigen is facilitating immortalization is unknown. T antigen, however, is believed to be essential for both specific binding to SV40 DNA and binding to other cellular proteins (Colby *et al.*, 1982). Large T antigen is a 94 kDa protein (single polypeptide of 708 amino acids) and is responsible for several apparently unrelated genetic functions. It is required for viral DNA replication and for the regulation of viral gene expression. T antigen also affects several host functions, including the synthesis (or the activation) of enzymes involved in DNA metabolism, the stimulation of rRNA synthesis and the induction of cellular DNA synthesis. In addition, T-antigen alone is sufficient to induce and maintain the transformation of non-permissive cells, e.g., rodent cells (Stahl *ed al.*,1987).

Transformed permanent lines can emerge from crisis, forming foci of rapidly dividing cells. SV40-transformed permanent lines acquire new phenotypic features, including altered morphology, ability to grow in low concentrations of serum and solid agar, production of T antigen and an unstable karyotype (Sack *et al.*, 1980). The ease with which the permanent lines could be obtained after SV40 transfection

differs greatly between laboratories. Several labs (Girardi *et al.*, (1965); Todaro *et al.*, (1963); Radna *et al.*, 1987) have isolated lines at high frequency, while other studies showed that permanent transformation in HDFs is a rare event (Huschtscha *et al.*, 1983; Shay *et al.*, 1989; Moyer *et al.*, 1984). In one study, susceptibility to transformation was reported to increase with passage level of the diploid culture (Jensen *et al.*, 1963).

B. Two-step model of immortalization.

We, and others, have proposed a two-step model for immortalization with SV40 (Wright *et al.*, 1989; Radna *et al.*, 1989; Hubbard-Smith *et al.*, 1992). Cells expressing the SV40 large T antigen bypass the first stage, and proliferate until the second stage is initiated. The initiation of the second stage requires genetic change(s) in the cellular genome necessary for indefinite growth.

The requirement for large T antigen is supported by data which shows that cells cease to proliferate when large T antigen is removed or inactivated (Radna *et al.*, 1987; Resnick-Silverman *et al.*, 1991). Studies on the interaction between gene products encoded by the genome of oncogenic viruses with cellular proteins have provided evidence of how the SV40 virus overcomes the first step towards immortalization. Three different group of viruses, SV40, adenovirus type 5 and human papilloma viruses 16 and 18, encode oncogene products that bind to the cellular proteins of Rb, as well as p53 (Levine, 1990). Several studies suggests that SV40 and E1A adenoviruses perform their transforming functions by binding

and modulating the retinoblastoma gene (Rb) function. Like T antigen, Rb is a nuclear phosphoprotein with DNA binding activity. How it functions biochemically to regulate cell growth is not fully understood. SV40 mutants for T antigen which are defective in binding p53 do not extend the life span of HDFs (Lin *et al.*, 1992). These viruses inactivate Rb and p53 function (negative regulation) with a resulting stimulation of cell growth and division in infected cells (Levine and Momand, 1990). Alteration in the state of Rb phosphorylation has been found to correlate with entry of cells into S phase, therefore expanding the cell's life span (Ludlow *et al.*, 1989). Rb, which is unphosphorylated in G_0 or G_1 phase cells, become phosphorylated at the G_1/S boundary, suggesting that phosphorylation may be necessary for entry into S phage (Mihara *et al.*, 1989; Chen *et al.*, 1989). Senescing HDFs fail to phosphorylate the Rb protein which leads to inactivation (Stein *et al.*, 1990). Since SV40 T antigen binds only the unphosphorylated form of Rb (Ludlow *et al.*,1989), the simplest explanation is that the unphosphorylated Rb inhibits entry in S phase and that inhibition can be relieved by either phosphorylation or binding the T antigen protein (Stein *et al.*, 1990). On the other hand, an association of p53 protein with T antigen appears to stabilize the p53 protein. This protein normally has a short half-life (20-30 min.) when present in an uncomplexed form whereas T antigen-associated p53 has a half-life of more than 24 hours. In spite of much experimental effort, little is known about the role that the T antigen-p53 complex plays in SV40 infected or transformed cells, although circumstantial evidence implies that p53 could be important in regulating cell proliferation (Stahl *et*

al.,1987).

Complex formation of T antigen with p53 and Rb protein in a temperature dependent lineage, SVtsA/HF-A (AR-5 and HAL cell lines), at 35°C (temperature permissive for T antigen function) is further reduced under conditions of loss of T antigen function (39°C) (Resnick-Silverman *et al.*, 1991). In the same study, viral oncogenes (polyoma large T protein and adenovirus E1A-12S), known to bind Rb protein, were introduced into the clonal immortal AR-5 and HAL cell lines, which are derived from the temperature sensitive SVtsA/HF-A lineage. The sequences from these DNA tumor viruses resulted in complex formation with Rb at both 35°C and 39°C; however, the cells are still unable to proliferate at 39°C. These results are interpreted to mean that the SV40 large T antigen must affect other cellular processes in addition to its interaction with the Rb-1 protein to immortalize human cell in culture.

The second stage towards immortalization involves an entirely independent mechanism that limits proliferation. The onset of the second step produces crisis, and it is the inactivation of this step that results in the final immortalization event and the escape from crisis. The mechanism of inactivation is a very rare event, probably of mutational origin, and its inactivation represent a loss of a function. The T antigen in this stage induces extensive chromosome aberrations which might help eliminate or inactivate the gene(s) responsible for the second step (Steward *et al.*, 1991; Ray *et al.*, 1990). Therefore, the mutagenic function of T antigen may provide the genetic changes required for immortalization. The second

step is recessive to limited life span as assayed in genetic hybrids (Pereira-Smith *et al.*, 1985 and 1988). In these studies, fusion of diploid human fibroblast cells with SV40-transformed immortal cells demonstrated limited division potential. This suggests that cellular immortality is a result of recessive dysfunctions or alterations in the genetic program that limits the division of normal cells. The recessive alteration(s) in the cellular genome required for immortalization are hypothesized to be inactivation of growth suppressor genes and/or changes in gene regulation of a proto-oncogene or growth factor or growth factor receptor. These genetic changes also occur in carcinogenesis (Read *et al.*, 1991). Many genes and deviations from normal gene function have been associated with a cell's uncontrolled proliferation, indefinite growth and carcinogenesis (Read *et al.*, 1991). These include the aberrant expression of oncogenes (c-*myc*), loss of inactivation of growth suppressor genes (Wilm's tumor gene) which control cell differentiation, or the loss of control of cell cycle dependent genes (p53).

C. Rationale

The experimental goal of this research is to determine genetic factors involved in immortalization. Our study of immortalization was facilitated by^lcytogenetic and molecular analysis of genetically matched normal, preimmortal and immortal cell lines. Two independent lineages of SVori- were analyzed. The first lineage included immortalized derivatives from origin-defective SV40-transformed cells containing a wild type T-Antigen (SV/HF) (Neufeld *et al.*, 1987).

The second lineage was derived from SVtsA/HF-A, generated by using SVori(-) mutants of SV40 encoding a heat-labile large T antigen (pSVtsA58) (Hubbard-Smith *et al.*, 1992). In this lineage, at 35°C, or the temperature permissive for large T antigen function, the three immortalized derivatives AR5, tsA-A, and HAL had properties resembling those of cell lines transformed with wild type SV40. There were, however, unable to proliferate or form colonies at the temperature restrictive for large-T-antigen function (39°C) (Radna *et al.*, 1989; Resnick-Silverman *et al.*, 1991). These studies show that at the non-permissive temperatures, large T antigen is not functional. Since the immortal phenotype is suppressed, it strongly suggests that T antigen is required for the maintenance of the immortalization phenotype in HDFs. Other factors are necessary. HDFs transformed by SV40 become immortal only in a minority of cases, thus expression of large T antigen is necessary, but insufficient for immortalization. The preimmortal transformants derived from SVtsA/HF-A, as well as all the immortalized derivatires, failed to reveal any alteration involving the integrated SV40 genome (Hubbard-Smith *et al.*, 1992). Therefore, an additional cellular event is required for immortalization. This conclusion, along with the results of complementation experiments which place all SV40-immortalized cell lines into the same complementation group (Pereira-Smith *ed al.*, 1988), strongly suggesting that immortalization results from specific change(s) that have the same genetic basis.

Cytogenetic analysis was used to identify chromosomal aberrations in SV40-transformants as indications of genetic changes. A similar analysis has been used

with human tumors, to reveal non-random chromosomal aberrations. This has facilitated the identification of chromksomal regions containing oncogenes and tumor supprassor genes (Yunis *et al.*, 1984; Kinzler *et al.*, 1991).

D. Research findings

Our research used cytogenetic and molecular analysis for comparison of mortal and immortal cell lines of two genetically matched lineages, HStsA/HF-A and SV/HF-5. The initial cytogenetic research revealed a consistently deleted region on 6q, which is found only in the immortal derivatives. A critical role for chromosome 6q in immortalization was supported by studies reported during the course of this research. Ray *et al.* (1992) found non-random breaks at 6q21 in immortal SV40-transformed HF. Quantitative Southern hybridizations confirmed that the deletion was at 6q21->ter, by showing loss of one copy of the single copy DNA D6S116 (6q15) and the genes c-*mas*-1 (6q24-27), c-*ros* (6q22), int-gamma-R (6q21).

It is known that breakpoints of non-random chromosomal abnormalities in most human tumors host oncogenes or tumor suppressor genes (Kinzler *et al.*, 1991; Joslyn *et al.*, 1991; Cleary *et al.*, 1986). Therefore, further research was directed towards the specific breakpoint on chromosomal deletion 6q21->ter of the immortal cell line tsA-A, since all other immortal cell lines showed a missing chromosome 6. DNA analysis demonstrated that single copy DNA D6S122 (6q14) and D6S125 (6q12) flanked the breakpoint. The determination of the breakpoint

with these DNA loci unexpectedly showed a breakage on 6q13 position. Fluorescent *In Situ* Hybridization (FISH) was used with specific probes which stain chromosome 6. This confirmed the breakage point on chromosome 6 (6q13). This technique also demonstrated that DNA from 6q13->21 belongs to a chromosome other than chromosome 6. This resolved the problem of the exact location of the breakpoint.

Cytogenetic analysis of all immortal cell lines showed very few additional non-random abnormalities, other than those involving chromosome 6. These abnormalities involved chromosomal regions on 5, 7, 10, 11, 12, 18 and 21. In addition, hybridization *in situ* was used to localize the SV40 integration site in the human genome to region 5q21. Most of the chromosome regions involved in non-random abnormalities, other than deleted 6, are known to host genes involved in human neoplasia and other genetic diseases. DNA and RNA analysis was performed to assess whether there were rearrangements of genes associated with the chromosomal abnormalities found in immortalized cells. The only genetic region which showed obvious DNA rearrangements included the tumor suppressor gene MCC in the immortal cell line AR-5. This gene is localized in close proximity to the integration site of SV40 and is involved in a translocation t(5;18)(q21;q21) in the AR-5 immortal cell line. The RNA levels of MCC were found to be 100% elevated in AR-5 cells as compared with those in preimmortal cells. The rearrangement and altered expression of the MCC tumor suppressor gene suggests that it could be involved in the progression of the immortal phenotype of

the cell line AR-5. Since MCC gene on AR-5 cells was rearranged and deregulated, it is possible that other genes proximal to the MCC area, as APC, Bcl-2 and DCC, were also deregulated. All these genes were found to be significantly deregulated, which also suggests an association with the progression of the immortal phenotype.

This study, using genetically matched SV40-transformed preimmortal and immortal cell lines, shows that genetic specific changes in the cellular genome are required for immortalization.

MATERIALS AND METHODS

Most of the laboratory methods outlined are modification of those described in various laboratory manuals (Maniatis *et al.*,1982; Ausubel *et al.*, 1987)

Abbreviations

BMB (Boehringer Mannheim Biochemicals)

SDS (sodium dodecyl sulfate)

DME-M medium (Dulbecco's modified Eagle medium)

PFGE (Pulsed Field Gel Electrophoresis)

PBS (phosphate buffer saline)

UV (ultraviolet)

MOPS (morpholinopropanesulfonic acid)

FISH (Fluorescence *In Situ* Hybridization)

YAC (Yeast Artificial Chromosome)

CEPH (Centre d'Etute du Polymorphisme Humain)

PCR (Polymerase Chain Reaction)

S&S (Schleicher & Schuell)

RE: (Restriction enzyme)

EDTA: (EthyleneDiamine TetraAcidic disodium salt)

DDT: (dithiotreitol)

TEMED: (NNNN-tetramethylethylenediamine)

Reagents and Solutions

LB medium: (1.0% Bacto-tryptone [Difco], 0.5% Bacto-yeast extract [Difco], 100mM NaCl, adjusted to pH 7.5 with NaOH).

LB plates: (LB medium with 1.5% Bactoagar (Difco)).

HAM'S nutrient mixture F10: (w/ L-glutamine; w/o $NaHCO_3$, Gibco).

DME medium: (Dulbecco's modified Eagle medium containing glucose 4500 mg/L; w/ L-glutamine; w/ sodium pyruvate; w/o $NaHCO_3$, Gibco).

YPD: (2% dextrose; 2% bacto-peptone; 1% bacto yeast extract).

1% Agarose inserts: (1% SeaKem GTG agarose (FMC); 100ml 0.125M EDTA (pH 7.5).

SDS (20%): 20g in 100 ml of distill H_2O.

PBS (1X): (150mM NaCl, 150mM Na phosphate pH 7.2).

MOPS: (20mM 3-[N-morphkllino]propanesulfonic acid, 5mM sodium acetate, 1mM ADTA (pH 8.0)).

RE B buffer:(10mM Tris, 5mM $MgCl_2$, 100mM NaCl, 1mM 2-mercaptoethanol)

RE L Buffer: (10mM Tris, 10mM $MgCl_2$, 1mM Dithioerythritol)

RE M Buffer: (10mM Tris, 10mM $MgCl_2$, 50mM NaCl, 1mM Dithioerythritol)

RE H Buffer: (10mM Tris, 10mM $MgCl_2$, 100mM NaCl, 1mM Dithioerythritol)

DNA lysis buffer: (10mM Tris, 10mM NaCl, 10 mM EDTA (pH 8.0), 5% SDS, 50ug/ml proteinase K).

RNA lysis buffer: (0.8ml 0.1M Tris (pH9.0), 0.1M NaCl, 20mM EDTA, 0.1% sarkosyl)

RNase A: (10 mg RNase/ml 10mM Tris, 15mM sodium chloride pH 7.5. Boil for 15 minutes and allow to cool. Store -20°C).

Sevag's solution: (24:1 chloroform:isoamyl alcohol).

SSC (20x): (3M sodium chloride, 0.3M sodium citrate pH 7.0).

SSPE: (10mM Na_2HPO_4 pH 7.2, 0.18M NaCl, 1mM EDTA).

STE: (0.1M sodium chloride, 10mM Tris, 1mM EDTA (pH 8.0)).

TAE buffer (10x): (400mM Tris, 200mM sodium acetate, 1mM EDTA pH7.2).

TBE (10x): (0.89M Tris, 0.89M boric acid, 0.1mM EDTA).

TE: (10mM Tris, 1mM EDTA pH 8.0).

Bromophenol Blue: (40% Sucrose in H_20, 0.25% Bromophenol Blue).

1X trypsin-EDTA: (0.25% trypsin, 1mM EDTA , Gibco).

Gas mixture: (5.0% CO_2, 20% O_2, 75% N_2, T.W. Smith).

NDSK: (2mg/ml proteinase K; 1% N-lauroyl sarcosine; 0.01M Tris-HCl/0.5M EDTA, pH 9.5.).

NDS: (1% N-lauroyl sarcosine; 0.01M Tris-HCl/0.5M EDTA, pH 9.5).

PMSF-TE: (1 microliter of 0.1M PMSF (in isopropyl alcohol) par 1ml of TE buffer pH 7.6).

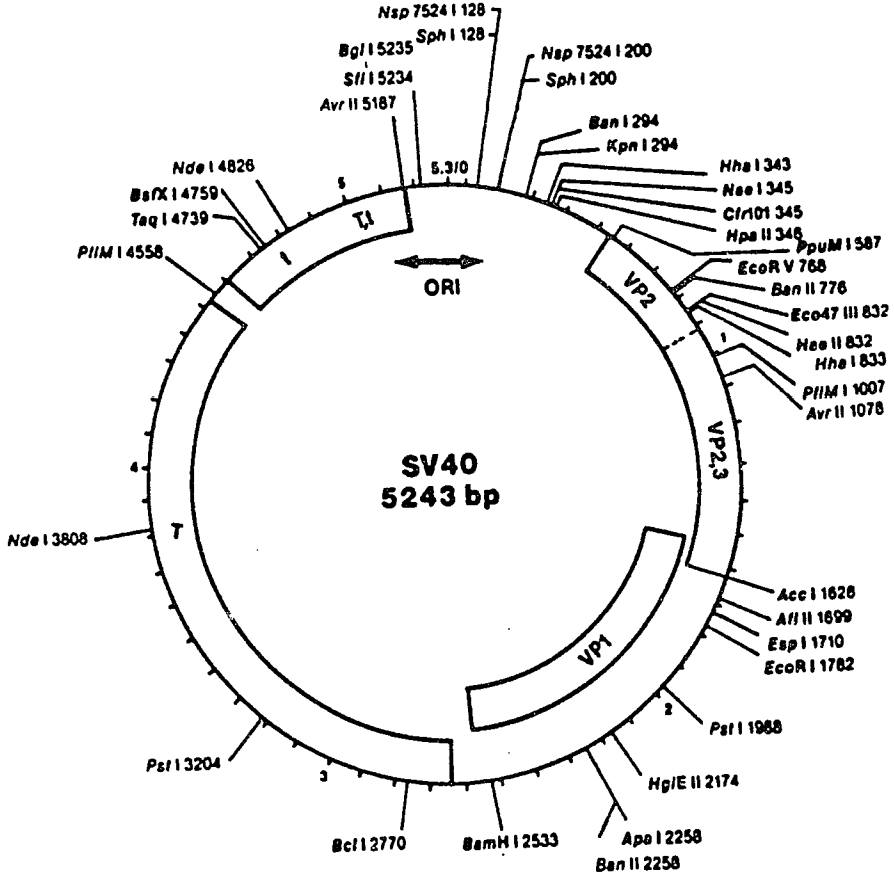
Fix solution: (3:1 methanol:acetic acid (allways prepared fresh) and then placed at 4^0C for 30 min before use).

Cell lines and tissue culture

Our model includes genetically-matched normal, preimmortal and immortal cell lines. The cells include immortalized derivatives from origin-defective mutants of SV40-transformed cells containing either a wild type T-Antigen (SV/HF), or a temperature sensitive SV40 genome [tsA/58 (SVtsA/HF)].

Six immortalized derivatives, SV/HF-5/2,SV/HF-5/3, SV/HF-5/5, SV/HF-5/37, SV/HF-5/38 and SV/HF-5/39, were originally obtained from a single SV/HF transformant. Each of the immortal cell lines conserved the wild type SV40 sequence with some part of the large T antigen-coding region missing as a result of transformation.

Another set of immortalized transformants (SVtsA/HF-A) were generated by using SVori(-) mutants of SV40 encoding the a heat-labile large T antigen (pSVtsA58). The vector pSVtsA58 was derived by cloning the ori(-) temperature sensitive SV40 tsA58 genome (pSVtsA) at the EcoRI site in a Bgl I and BamHI-resistant derivative of pMK16 *(Table 1).* Immediately after transformation of HS74BM the derived cell line was called tsA-0. This cell line is considered as preimmortal; 7 passages later cells slowed down, changed morphology and resume grow indefinitely. This nonclonal immortal cell line named tsA-A. These cells were then mass cultured for 40 population doubling and treated with EMS. The cells were subjected to thioguanine resistant selection, following subcloning after 5 population doublings. This cell line was called HAL. AR-5 cell line is another immortalized derivative after clonal isolation from the preimmortal cell line tsA-0.



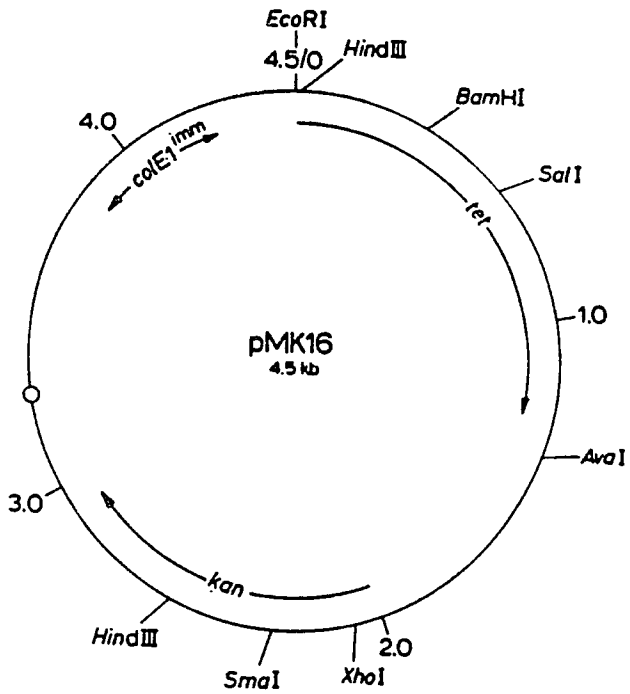


Table 1.
SV40 DNA and pMK16 plasmid maps.

At 35°C, or the temperature permissive for large T antigen function, the three immortalized derivatives AR5, tsA-A, and HAL had properties resembling those of cell lines transformed with wild type SV40. There were, however, unable to proliferate or form colonies at the temperature restrictive for large-T-antigen function (39°C).

A. Media and Tissue Culture

All cell lines were grown in the presence of 1:1 mixture of the two following media: 1X D-MEM medium and 1X HAM'S nutrient mixture F10 with 10% fetal calf serum (Hyclone) and 1.0% antibiotic -antimycotic solution (Gibco).

All cells were fed three times a week. When fibroblast cells reached confluence (about a week), the medium is removed and the cells are trypsinized for 2-3 min (minutes) in the presence of 1X trypsin-EDTA (Gibco). The cells were transferred to a sterile 15 ml polypropylate tube and centrifuged at 1,000xg for 7 minutes. The trypsin solution was discarded and the pellet was washed and suspended in fresh medium. The suspended cells were transferred into new flasks and diluted with fresh medium. The cell lines were grown in the presence of a gas mixture in the appropriate temperature.

Nucleic Acids Isolation

A. Total genomic DNA Extraction

Genomic DNA was obtained using a modification of the method described

by Maniatis *et al.* (1982). Nucleic acids were extracted twice with an equal volume of saturated phenol:chlorophorm:isoamyl alcohol (25:24:1), and once with chlorophorm:isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous phase by adding 150mM NaCl and 2 volumes of ice-cold 95% ethanol. The above mixture was stored at -70°C for 1 hour and then are pelleted by centrifuging at 10,000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was air dried for 30 min and then dissolved in 0.6 ml 1X SSC buffer. The RNA and proteins were digested with 50 µg/ml RNase A for 60 minutes at 37°C and by proteinase-K (BMB) (50 ug/ml for 60 min at 37°C). The DNA is then extracted using phenol:chlorophorm:isoamyl alcohol (25:24:1) phase extraction, and once with chloroform:isoamyl alcohol(24:1). The DNA is precipitated with ice cold 95% ethanol and centrifuged at 10,000 rpm for 30 min at 4°C. The concentration of the DNA is determined by spectrophotometric readings at 260 nm (nanometers). This is based upon the standard, OD_{260} (1.0) = 50 ug/ml double stranded DNA. The purity of the sample is determined by the ratio of readings at 260 nm and 280 nm (OD_{260}/OD_{280}). DNA sample is stored at 4°C in TE buffer.

B. Total DNA isolation in agarose inserts for PFGE

Isolation of large fragments of genomic DNA (greater than 2000Kb) was accomplished by PFGE. About 1-3x10^8 cells are needed for the preparation of 20 agarose inserts which contain 6-30 ug of DNA. After the final washing, the cell pellet is warmed to 50°C in a water bath for 15 min, and then mixed very well with

2 ml of insert agarose (also prewarmed at 50°C). The mixture was quickly loaded into the insert molds. The inserts are refrigerated for 5-10 minutes and placed in NDSK (20 inserts/ 3 ml buffer). The inserts were incubated for 2 hours at room temperature and then for two days at 50°C to lyse the cells. The inserts were then placed in PMSF-TE to inhibit the effects of proteinase-K and shaken at room temperature overnight. The inserts were washed twice more in TE pH 7.6 at room temperature overnight and stored at TE pH 7.6 at 4°C.

C. Total Yeast DNA isolation in agarose inserts for PFGE

Culture tubas containing 50 ml of YPD medium were inoculated with a single colony from a yeast plate and grown overnight at 30°C with vigorous shaking. Cell were harvested by centrifugation at 1,500g for 7 minutes at room temperature. The supernatant was discarded an' the precipitant was washed twice in ice cold 0.05M EDTA (pH 7.5). The pellet was then placed in 50°C water bath for 30 minutes. Molten agarose (equilibrated to 50°C) was added as follows: 1 ml agarose/1 x 10^9 cells and 15.5 microliter zymolase (20,000 units/ml of agarose). This solution was used to fill the inserts slots of insert makers. When the agarose solidified, the agarose plugs were placed in 7.5% solution of 2-mercaptoethanol in 0.5M EDTA (pH 7.5) and incubated in a 37°C water bath overnight. After 24 hours, the inserts were drained to reduce the mercaptoethanol concentration and placed in NDSK (10 ml per 100 inserts). The inserts are incubated at 50°C overnight. After this the inserts can be stored in NDSK or in TE buffer (pH 7.6) at 4°C.

D. Total RNA extraction

This is a modification of method described by Maniatis *et al.*, 1982. The cells were lysed with 3-5 ml of RNA lysis buffer and passed 5-6X through a 5cc #21G needle syringe for complete lysis. The nucleic acids are extracted three times with an equal volume of saturated phenol:chloroform:isoamyl alcohol(25:24:1), and once with chloroform:isoamyl alcohol(24:1). Ice-cold 100mM LiCl in 95% ethanol is added to the aqueous phase to precipitate the nucleic acids. The sample was stored for 30 min at -70°C and centrifuged at 10,000g for 30 min at 4°C. The pellet was washed once with 95% ethanol, air dried for 5-10 min and dissolved by gentle vortexing in 600 ul 50mM Tris-Cl, pH 8.0. The dissolved pellet, 6 ul 1M $MgCl_2$ (10mM final concentration) and 20 ul DNase (BMB) at 100 ug/ml (40 units) are added. The sample was digested for 60 min on ice, followed by the addition of 12 ul 0.5M EDTA (10mM final concentration) and 60 ul (1/10 volume) 3M NaOAc, pH 5.2. After mixing, the sample was extracted twice with an equal volume of saturated phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1). The aqueous phase was precipitated with 95% ethanol and kept in -70°C for overnight. The RNA was centrifuged at 10,000 rpm for 30 min at 4°C and dissolved in 50-100 ul TE buffer. Spectrophotometric readings at 260 and 280 nm are used to measure the concentration and purity of the RNA. Concentration is determined using the standard, OD_{260} of 1 = 40 ug/ml RNA. The ratio of OD_{260}/OD_{280} is determined. A sample with a ratio of 1.9-2.0 was used. Concentration and purity are also determined through agarose gel

electrophoresis. The RNA sample is stored in TE buffer at -70°C.

Restriction Endonuclease digestion

A. Restriction enzyme digestion of DNA

DNA is digested at 37°C overnight in the presence of the appropriate buffer supplied by the manufacturer (BMB). To ensure complete digestion, most reactions are incubated at longer periods of time than suggested; total genomic DNA and plasmids are digested approximately 16-20 hours. Reactions involving digestion with more than one enzyme proceeded with the digestion of a low salt buffer enzyme, followed by adjusting the salt concentration to either a medium or a high value and by the addition of the second enzyme.

B. Restriction enzyme of DNA in agarose inserts

Agarose inserts containing genomic DNA of sizes larger than 2000 Kb were digested with restriction enzymes as follows: the reaction mixture of 200 ul per insert was incubated with 50 units of enzyme, 1X of the recommended buffer and 0.1% Triton X-100. The volume of each insert was estimated 80-100 ul. The mixture was mixed at the recommended temperature for maximum enzyme efficiency for 30 hours. The reaction was stopped with NDS (2 ml/insert), shaking on ice for 2 hours, and then stored at 4°C.

Agarose Gel Electrophoresis

A. DNA gel electrophoresis

Agarose gels were prepared by dissolving agarose (BRL) into 1X electrophoresis buffer TAE buffer and heating in a microwave until the agarose dissolves. The agarose solution was poured into a horizontal gel plate and then the well-forming comb is positioned in the tray.

Gel-loading (TAE) buffer was added to the DNA samples and heated for 10 minutes in a 68°C water bath. The voltage and run times for gels depended on the size of the DNA to be analyzed. Analysis of small DNA fragments used 12-15 V/cm (volts per centimeter), whereas gels with large DNA fragments were run at 2-3 V/cm. Gels with genomic DNA were run at 1.5V/cm. In all cases, the gels run until the bromophenol blue migrated 3/4 down the gel.

After electrophoresis was completed, the gels were placed in a solution of 0.5 ug/ml ethidium bromide (EtBr) for 15 min at RT and washed 20 minutes with water to remove the extra EtBr. The DNA was visualized on a long wave UV (ultraviolet) transilluminator (Spectroline) and the gels were photographed using a Konica instant camera with Polaroid type 667 Land film.

A computer program (using bacteriophage lambda Hind III fragments) as a standard is used to calculate each DNA fragment size.

B. RNA gel electrophoresis

RNA agarose gels were stained and photographed exactly the same way

as those for DNA. For gels, RNase free powdered agarose (BRL) is dissolved in distilled water and heated until it is totally dissolved. The gel is allowed to cool to 60°C and 1X MOPS buffer; 6.48% (2.2M) formaldehyde are added. The total volume was 200 ml for 1% gels.

The samples are prepared in a sample buffer with final concentration of 50% formamide, 6.48% (2.2M) formaldehyde and 1X MOPS buffer. The samples were heated for 10 minutes in a 65°C water bath, followed by the addition of loading buffer. The gels were run at 3.5 V/cm for 3 hrs. To visualize the RNA, the gel was stained with 0.5 ug/ml EtBr for 20 min and washed 20 min with water to remove the extra EtBr.

C. Pulsed Field Gel Electrophoresis [PFGE]

PFGE gels are prepared by dissolving low temperature melting agarose (SeaKem-GTG, FMC) into 0.5X TBE electrophoresis buffer and by heating in a microwave until the agarose dissolves. DNA was isolated in agarose inserts is placed in the wells and run with the following conditions: Voltage 5.5 V/cm; forward pulse time 2.4 sec; reverse pulse time 0.8 sec; ramp factor 1.6. on a PC 750 Pulse Controller (Hoefer Scientific Instruments) for 24 hours in 10°C. After electrophoresis was completed the gels were placed in a solution of 0.5 ug/ml ethidium bromide (EtBr) for 15 minutes at RT and washed 20 min with water to remove the extra EtBr. The DNA is visualized on a long wave UV (ultraviolet) transilluminator (Spectroline) and the gels are photographed using a Konica instant

camera with Polaroid type 667 Land film.

Radioactive Labelling of DNA Probes

A. Random primer extension

The random primer extension method for labeling DNA was developed by Feinberg and Vogelstein, (1984). A kit (BMB) was used for labelling of DNA probes. One microliter of labelled DNA is mixed with 10 ml Ecoscint and placed in the preset ^{32}P channel of the scintillation counter to determine incorporation of radioactive label. The specific activity (SA) of the probe was determined by multiplying the number of counts by the total volume of the probe, divided by the number of ng.

B. Nick translation

Nick translation also used a kit (BMB) and the accompanying instructions were followed. 50-100 ng DNA were used for each labelling.

Nucleic Acid Blotting

A. Southern blotting

DNA from agarose gel were transferred to a positively charged nylon membrane (S&S) as described by Southern (1975).

Direct transfer was as described (S&S manual). If the fragments of interest are larger than 15 Kb, the DNA was nicked by depurination in 500 ml of 0.2N HCl

acid and rinsed with deionized water. The transfer was allowed to proceed for 18 hrs. The membrane with the bound DNA is baked for 2-4 hours in a vacuum oven at 80°C and subjected to prehybridization and then, hybridization conditions.

The membrane was placed in a heat-sealed plastic bag and prehybridized for 3-4 hrs at 42°C in a solution containing 50% formamide and 5X SSPE, 0.2% SDS, 2X Denhardt's and 100 ug/ml sheared salmon sperm DNA (Southern, 1975). After prehybridization, the radiolabelled probe is added, the bag is resealed and hybridization is carried out in a shaking water bath for 12-18 hrs at 42°C. The labelled probe was denatured by boiling for 10 minutes, cooled on ice for 5 minutes and then added to the hybridization solution. For higher stringent conditions, hybridization was performed at 65°C with the same conditions.

Following hybridization, the membrane was washed as given (S & S Manual). The X-ray films were exposed to the filters for appropriate times. They were developed for 1-5 min in Kodak GBX developer, immersed 1 min in a solution of 3.0% acetic acid to arrest development and then fixed in rapid fixer (Kodak) for 10 min. The films are rinsed in cold water for 15 min and air dried.

B. Northern blotting

RNA formaldehyde gels were transferred to S&S nylon membrane as described for DNA. Following electrophoresis, the gel is stained with 0.5 ug/ml EtBr for 15-20 min, and it is rinsed 2-3X with distilled water and with 10X SSC for 30 min with gentle agitation at RT. The prehybridization, hybridization and washing

conditions used are identical to those for Southern blots.

C. DNA dot blots

Twenty ug of DNA were denatured in 0.1 volume of 3M NaOH and incubated at 60-70°C for 1 hour (Ausubel *et al.*, 1987). The DNA was cooled to room temperature, adjusted to neutrality with HCl and one volume of 12X SSC was added (final 6XSSC). The nytran membrane (S&S) was cut to the exact size of the dot blot apparatus (Bio-Rad) and moistened in 6X SSC for 10 minutes at room temperature. The membrane must be moist at the time of DNA dotting. The membrane was placed in the dot blot apparatus and the samples are applied in four consecutive dots with decreasing dilutions of 1:1. The membrane was baked for 2-4 hours in 80°C.

The hybridization, washing and film exposing and developing procedures were identical to those used for Southern hybridizations.

D. RNA dot blots

Twenty ug of RNA was diluted into 100 ul of distilled autoclave H_2O and 100 ul of 20X SSC. Other conditions are as given for dot blotting of DNA. The membrane was baked for 10 min and crosslinked on a long wave UV (ultraviolet) transilluminator (Spectroline) for 10 min.

The membrane was prehybridized for 2-4 hours at 45°C, in 10% dextran sulfate; 50% formamide; 1% SDS; and 1M NaCl. The prehybridization solution is

mixed well by vortexing and then prewarmed for 30 minutes at 45°C. Denatured salmon sperm DNA was added to the prehybridization solution with probe to a final concentration of 100 ug/ml. The membrane was hybridized in 45°C for overnight. The membrane was washed twice in 2X SSC for 5 min at room temperature and twice in 2X SSC; 1% SDS for 30 min at 52°C. After washing, the membrane was exposed with an X-Ray film and then it is developed as described above.

Nucleotide Sequencing

A. Sequencing Reaction

The following technique gave excellent results with double stranded DNA sequencing (Bodduluri, personal communication). 3-5 ug plasmid template DNA was adjusted to a volume of 12 ul. The DNA was denatured in 3 ul fresh 2N NaOH. Following denaturation, 12 ul of primers (500 ng total) were mixed and immediately precipitated by adding 5 ul of NaOAC (pH 5.0) and 125 ul of ice cold 95% ethanol and placing in dry ice-ethanol bath for 10 min. The sample was centrifuged in 14,000 rpm and washed with 70% ethanol. The pellet was dried and suspended in 8 ul of 10mM Tris-HCl pH 8.0.

The reaction mixture was prepared as follows: 1 ul DTT (0.1M); 1 ul of ^{32}P-dATP (>800 Ci/mmole, NEN); 2 ul dGTP (1:5 diluted) (USB); 2 ul of 5X reaction buffer (USB); and 2 ul of sequenase enzyme (USB). 8 ul of the above mixture was mixed and incubated for 10 min at room temperature. The dideoxy termination mixtures were prewarmed at 37°C for 1 min. After the sequencing reaction was

completed, 3.5 ul of labelled template was added to the dideoxy termination mixtures, mixed and incubated for 5 min at 37°C. The termination reaction was stopped with 4 ul stop solution and placed in the ice.

B. Sequencing Gel Electrophoresis

Two gel plates measuring 33 x 38 cm and 33 x 40 cm, respectively, were cleajed with a 0.1% SDS solution, rinsed with distilled water and rubbed with 95% ethanol. The ethanol was allowed to evaporate and the gel plates are again cleaned with Sigmacode. The gel sandwich was assembled using 0.4 mm thick plastic side and bottom spacers. Dabs of vacuum grease were applied to the junctures of the side and bottom spacers to prevent leakage. The sides and bottom of the assembled gel plates were sealed with electrical tape and large clips.

6% polyacrylamide/7M urea gels were made by mixing 14.5 ml 40% (w/v) acrylamide (19:1 acrylamide: N,N methylenebisacrylamide), 10 ml 10X TBE buffer, 1mM EDTA (pH 8.3) and 40.5 ml distilled water. 42 g ultra pure solid urea (BRL) is added and dissolved into the mixture with stirring over low heat.

The gel mixture was degassed by stirring *in vacuo* 15-30 minutes. 2 ml of the gel mixture was removed, adjusted with 16 ul 10% (w/v) ammonium persulfate (BioRad) and 1 ul TEMED (BioRad), vortexed and pipetted into the gel sandwich. The gel sandwich was laid at a 30° angle to the horizontal and the 2.0 ml acrylamide plug allowed to polymerize 15-30 min. The rest of the gel mixture was adjusted with 0.8 ml 10% (w/v) ammonium persulfate and 60 ul TEMED, mixed,

and quickly injected between the gel plates using a 30cc syringe. The 0.4 mm comb spacer (flat side of comb) (BRL) was positioned at the top of the gel. The gel sandwich was laid at a 30° angle to the horizontal, excess gel mixture was pipetted across the comb spacer to seal the gel and the acrylamide is allowed to polymerize 30-60 minutes.

After polymerization was complete, the comb and bottom spacer were removed. The top of the gel was flushed with 1x TBE and the gel sandwich clipped into the vertical gel electrophoresis apparatus (BRL). The top and bottom electrophoresis tank reservoirs were filled with 1X TBE and air bubbles trapped at the bottom of the gel are removed using a syringe filhed with 1X TBE with a bent 20 gauge needle. The comb was inserted and the gel was run at 15-20 mA (1500-2000V) for 15-30 minutes.

After the pre-run completion, the sequencing reaction samples were heated at 75-80°C for 5 min. 3-4 ul of each sample was loaded quickly into separate adjacent wells and the gel run at 20-25 mA (2000-2500 Volts) until the bromphenol blue dye reached the bottom of the gel (2-4 hours). The remainder of the samples, which had been kept on ice during this time, were heated at 75-80°C for 5 minutes and 3-4 ul of each sample were loaded into separate adjacent wells. Electrophoresis used 20-25 mA (200-2500 volts).

The orientation of the gel is marked and the gel is wrapped in plastic wrap and exposed directly or after drying under vacuum to Kodak X-Omat AR film at 80°C for 3 hours. The X-ray film is processed as previously described.

Sequences are read from the bottom to the top of the gel, so that the nucleotide base sequences were obtained in the 5' to 3' direction. Overlaps in the base sequence at the bottom of the first "run" and top of the second "run" provided sequence continuity.

YAC library screening

The YAC (Yeast Artificial Chromosome) library was obtained from CEPH (Centre d'Etute du Polymorphisme Humain) in France, Paris. DNA from all the YAC library clones was sent from the institute to our laboratory in 115 DNA pools. The screening of the library was done with PCR (Polymerase Chain Reaction). A first screening determined the positive DNA pool(s) and the results were sent to CEPH Institute. Each DNA pool contained DNA from hundreds of YAC clones. Following the first screening, another set of subpools was sent. The results from the second screening of the subpools was again reported to CEPH institute. Based on the CEPH Institute's initial arrangement of YAC clojes in subpools, they send positive YAC clones. All PCR amplification of DNA pools and subpools were repeated twice or more.

A. PCR Procedure

For every PCR reaction 10 ng of DNA from a pool or subpool was mixed in a 500 ul polypropylate tube with the following: 100 ng of each primer; 3 mM of dNTPs (Pharmasia); 1X PCR buffer (10X Buffer: 500 mM KCl; 100 mM Tris-HCl

pH 8.3; 25 mM $MgCl_2$; BSA 1.7 mg/ml). Genomic (100 ng) and plasmid DNA (15 ng) is used as controls. The total volume was 30 ul. The above solution was incubated at 95°C for 5 min and was placed in ice for another 5 min. Following the abova denaturation step, 0.5-1 units of Vent polymerase (Biolabs) was added, mixed very well and 1 drop of oil placed on the top of the mixture to prevent elaboration. The reaction was run in a Savant PCR machine with the following conditions: 94°C for 60 sec; 60°C for 60 sec; 72°C for 60 sec; for 35 cycles.

Following PCR, oil was removed and 5 ul of Bromophenol blue was added. The sample was denatured in 95°C for 5 min and put in ice for another 5 min. A 2% agarose gel was prepared and the complete volume of the sample was run using 12-15 V/cm. After electrophoresis was completed, the gels were placed in a solution of 0.5 ug/ml ethidium bromide (EtBr) for 15 minutes at RT and then washed 20 min with water to remove the extra EtBr. The amplified DNA was visualized on a long wave UV (ultraviolet) transilluminator (Spectroline) and the gels were photographed using a Konica instant camera with Polaroid type 667 Land film.

B. Designing and purifying primers

The annealing temperature of the primers was determined using the following formula: T°C=[(4 X GC) + (2 X AT)] - 5°C. Primers were chosen which were 24 bp, with an annealing temperature at 60°C, which form no secondary structures and amplify about 300 bp from the locus of interest. The primers were

constructed in the Sequencing and Synthesis Facility at Hunter College.

One volume of 30% NH_4OH was added to the primers and the total volume was passed through a 20 ml G-50 column. Ten fractions of 1.5 ml were collected from the column and the concentration measured. Fractions which had an OD_{260} greater than 1.0 and pH<7.5 were selected.. The concentration of primers was calculated on the basis of the formula OD_{260}=1 for 30ug/ml primer.

Transformation experiments and preparation of the probes

All genes and single copy DNAs used in this study are described in *Table 2.* To obtain these probes, the plasmids were transformed in the HB101 strain of *Escherichia coli.* This was followed by a large plasmid preparation and finally isolation of the insert from the plasmid with Geneclean.

A. Preparation of competent cells

HB101 cells were grown in 50 ml LB broth for 24 hrs at 37°C. The next day, 50 ml of LB media was inoculated from the overnight culture and grown for 2-4 hrs at 37°C. Three ml of this culture were placed on ice for 10 min, and then the cells were collected by centrifugation at 4,000g for 5 min at 4°C. The cells were suspended in 1.5 ml of cold sterile solution of 50mM $CaCl_2$, 10mM Tris-HCl pH 8.0 and incubated in an ice bath for 15 min. Cells were obtained by centrifugation at 4°C and suspended again in 0.2 ml of cold-sterile solution of 50mM $CaCl_2$, 10mM Tris-HCl (pH 8.0). These cells were placed at 4°C for 24 hrs and then in the -70°C.

Clone Designation	Map Location	Locus	Name	G (Genomic) c (cDNA)	Vector	Flanking Enzyme Size	Insert Size kb	Obtained from:
40cl	5q21	MCC	Mutated in Colorectal Cancer	c	Bluescript	EcoRI	2.3	Dr. B. Vogelstein
pDCC	18q21	DCC	Deleted in Colorectal Cancer	c	Bluescript	EcoRI	1.65	Dr. B. Vogelstein
FB 70B	5q21	APC	Adenomatous Polyposis Coli	c	Bluescript	EcoRI	3.6	Dr. B. Vogelstein
c11p11	5q21	FAP	Familial Adenomatous Polyposis	G		EcoRI	3.6	Dr. R. Williamson
pDy-1	11q13	D11S287	Parathyroid tumor gene	c	pUC18	BamH1+HindIII	1.8	Dr. A Arnold
p-Cyclin D-1	11q13	Cyclin D-1	Cyclin D-1	c	pUC118	BamH1+HindIII	1.5	Dr. D. Beach
p-135	15q11	D15S16	Single copy DNA	G	pBR322	Hind III	1.3	ATCC
pV3S	7p15	TCRGV3	T Cell Receptor	G	pUC	Sac I	1.1	ATCC
Bcl-2	18q21	Bcl-2	B cell lymphoma 2	c				Oncor Inc
pbcr	22q11	bcr	Breakpoint Cluster Region	G	pUC12	HindIII	1.95	ATCC
pCH6	6p12	D6S10	Single copy DNA	G	pBR325	EcoRI	1.9	ATCC
p-900	11p11	oligo-A	Oligo A Synthetase	c	pUC8	Pst I	0.9	Dr. M. Revel
pc-myb E-26	6q22	c-myb	c-myb oncogene	c	pUC8	EcoRI	2.6	Dr. S. R. Tronick
pmcf 3-5.2	6q21	c-ros-1	c-ros oncogene	c	pUC8	Sal I	2	Dr. M. Wingler
pmasF	6q24	c-mas-1	c-mas oncogene	c	pUC8	BamH1+HindIII	0.7	Dr. M. Wingler
pHuIFN-gammaR8	6q21	c-Int-R	Interferon gamma receptor	c	pUC8	Xba I	2	Dr. S. Pestka
p21.6A	6q15	D6S116	Single copy DNA	G	pUC8	HindIII	4.8	Dr. M. Golubic
p21.14	6q14	D6S122	Single copy DNA	G	pUC8	HindIII	4.2	Dr. M. Golubic
p327A	6q12	D6S125	Single copy DNA	G	pUC8	HindIII	4.4	Dr. M. Golubic
EGFR	7p13	EGFR	Epidermal Growth Factor Receptor	c	pBR322	Cla I	2.4	ATCC
KRAS-2	12p12	KRAS-2	Kirstein rat sarcoma v-onc	c	pUC13	Pst I	1.1	ATCC
SV40			SV40 Virus					Dr. H. Ozer
pMK16			pMK16 Plasmid					Dr. H. Ozer

Table 2.
Probes used in this study.

B. Transformation of bacteria cells

Compepent cells were kept at -70°C and placed in ice before transformation. For each transformation, three tubes are prepared; one experimental and two controls. The controls were a negative control in which no plasmid is added and a positive control in which a another plasmid known to give transformants is added. For each transformation, 0.2 ml competent cells are mixed with DNA and placed on ice for 30 minutes. The samples are heat-shocked at 42°C for 2 minutes followed by the addition of 0.8 ml LB broth (75 µg/ml ampicillin) to each tube. The cells were incubated for 2 hr at 37°C with shaking. They were then plated on LB plates (75 ug/ml ampicillin) and incubated at 37°C for 24 hrs.

C. Large scale plasmid DNA isolation

The method for large scale plasmid DNA isolation is described as "maxi-prep" in Qiagen Plasmid Handbook, Spring 1992 (Qiagen). The protocol is followed exactly as described in the handbook.

D. Probe purification

The plasmid were digested with endonucleases which excise the insert. The samples were run on a 1% agarose gel. The band was identified with EtBr and the appropriately sized band was excised and cleaned using a GeneClean Kit (Bio 101) with conditions described by the company. This method is proved to be a very effective way of retrieving 80-90% of the DNA fragment from the agarose gel.

Quantitation of DNA used known markers on the gels, as well as direct measurements with a TKO 100 Fluorometer (Hoefer Scientific Instruments).

Cytogenetic Analysis

A. Preparation of slides

15 ul/ml of colcemid (Gibco) was added to culture media of cells for 4 hours. The medium was removed and the cells were trypsinized for 2-3 minutes in the presence of 1X trypsin-EDTA (Gibco). After centrifugation, tha cells were washed with PBS and suspended very gently in a hypoponic solution of 75 mM KCl, and incubated in a 37^{0}C water bath for 20 minutes. Following incubation, five drops of fixative solution was added to the cells. The fixative was replaced after 20 min and the cells were stored at 4^{0}C. The fixative was changed three times a week for one to two to weeks.

Cells were pelleted and suspended in one to two ml fresh fixative before dropping on slides. The slides were observed immediately under phase microscope. If the metaphase spreads were not satisfactory (overlapping chromosomes), the slides were exposed to humidity immediately after the dropping. Slides were stored in a desiccator at 37^{0}C for a 2-7 days before G-banding or *in situ* hybridization.

B. G-banding of chromosomes & Chromosomal analysis

All solutions were prepared fresh. Slides were immersed in 30% H_2O_2

(Fisher) for 10 seconds to aid in the removal of cytoplasm. The slides were immediately washed in double distilled H_2O 2-3 times, partially dried and placed in 1X Trypsin for 2-4 min. Trypsin is prepared by diluting 2ml 10x trypsin (Gibco) with 18ml isoton pH 6.8 (Isoton 11 - balanced electrolyte solution (azide free)) (CMS). The excess trypsin was drained and slide 3 times in isoton solution containing 4% FCS (FCS inactivates the trypsin). The slide was briefly washed again in isoton and stained for 4-5 min in Giemsa. Giemsa was prepared by adding 2ml Gurrs Giemsa (BDH chemicals LTD Poole England) to 50 ml isoton. The slide was rinsed in double distilled H_2O followed by tap water and dried. The quality of banding is determined by the use of a microscope. Incubation times in trypsin and giemsa are altered accordingly.

The slides were screened under a microscope and pictures of the well spread stained metaphases were taken. High contrast Technical pan film (Kodak) was used and developed by manufacturer's instructions. Following the chromosomal analysis of 50-150 cells, several karyotypes are prepared for every cell line.

Hybridization *in situ*

A. Isotopic Hybridization *in situ*

Slides for *in situ* hybridization should be stained with G-banding and metaphase plates should be prephotographed. The coordinates of those metaphases must be recorded precisely. Following this the slides were de-stained

in 70% EtOH (2X), 95% EtOH (1X) for 3-5 minutes each. Slides were placed in 2XSSC at 37°C with a final concentration of 100 ug RNase A/ml. They were then washed with three changes of 2XSSC, 70% ethanol (2X) and 95% ethanol (1X) for 3-5 minutes each and stored desiccated at 37°C.

The probe was prepared with the nick translation method using 100uCi ^{125}I dCTP (specific activity = 2200Ci/mmole) (NEN), and ethanol precipitated in the presence of 3 ug tRNA as carrier. The pellet was suspended in 100ul distilled H_2O, and denatured by boiling for 10 minutes and placing on ice for 5 minutes. Finally, the probe was diluted into the hybridization solution which has a final concentration of 0.01M Tris pH 7.6 50% formamide, 3X SSC. The final volume was 50 ul/slide.

Chromosomal DNA was denatured by placing the slides in Coplin jar containing 95% formamide and 1X SSC pH 7.0-7.2 for 1.5 hours at 70°C. The slides were placed in cold 70% ethanol for 10 minutes to prevent renaturation of the DNA and washed for an additional two times in ice-cold 70% ethanol and two times in 95% ethanol for 5 minutes. The washing continued at room temperature with 95% ethanol for 3 minutes. The probe was placed on the slides and covered with a 22X40 mm coverslip. Hybridization was overnight at 43°C in moisturizing chambers containing 50% formamide and 3X SSC. About 20 hours later, the coverslips were removed into 50% formamide and 3X SSC. The slides were washed as follows: once in 2XSSC at 45°C for five minutes; twice with 2X SSC at room temperature; once for one hour with 0.1M potassium iodide in 2X SSC; in 2X SSC for 2-3 hours with three to four changes of buffer; a total of 2L 2X SSC for

every 10 slides are used. The slides were then washed in 70% ethanol (3X), followed by 95% ethanol (2X) for 3-5 minutes each.

The slides were coated with emulsion (Kodak NTB2) and placed in light tight boxes containing drierite for 4-6 weeks. Metaphase plates which had been previously photographed were relocated and another photograph made using technical pan film.

Statistical analysis. A statistical analysis of the data was used to determine the insertion site. The entire chromosomal complement of a cell is divided into 95 sections of approximately equal length. The number of grains over each chromosomal region is compared with the total number of grains present. The region containing a statistically significant number of grains is accepted as the site of integration.

B. FISH: Fluorescence *In Situ* Hybridization

Whole chromosome painting system (Gibco BRL) was used for the identification of human chromosome 6 or human chromosome fragments by FISH. The labelled probe and all reagents was obtained with the kit. The procedures for completing this experiment is followed exactly from the instruction manual.

FISH was also applied in other experiments where a plasmid containing SV40 and other loci were localized. In these cases, probes were labelled with biotin using Oncor Non-isotopic probe labeling kit or with digoxigenin using BMB Non-isotopic probe labeling kit. The DNA labelling was done exactly as

recommended by the manufacturers.

The protocol and reagents for the non-isotopic method of cytological hybridization were supplied by the manufacturer (Oncor) in "Single copy sequence detection in metaphase chromosomes or nuclei fluorescence microscopy" kit. The procedure is followed as outlined by the manufacturer.

Film Development and printing the negatives

A. Technical pan film

Technical pan film (Kodak) was developed for 14 minutes in Microdol-X developer at 22°C (Kodak) with constant agitation. The film was fixed with Rapid Fix solution-A (Kodak) diluted 1:3 with water for 7 minutes. Finally, the film is rinsed with photoflo-200 (10 ml/L)(Kodak) in order to product film from water spots.

B. Printing the negative

Positive prints were produced using an Omega Pro Lab B66 enlarger. The printing was done on the Rapidoprint DD 37 E machine (AGFA), using the P1-4 Rapidoprint paper, rapidone developer and fix solution.

C. X-ray film

X-ray film was developed in GBX developer (Kodak) for a few minutes, rinsed in tap water and fixed in Kodak rapid fix for 10 minutes. Excess rapid fix was removed by washing the filter for 20 minutes in tap water.

D. Autoradiographic Emulsion from slides hybridized *in situ*

The slides were dipped in melted (38^{0}C) emulsion (Kodak NTB2) diluted with 1:1 distill H_20. The slides were dried for 30 minutes prior to being placed in light tight boxes containing drierite. The autoradiograms were exposed at 4^{0}C for about 3-4 weeks. After that, periodically, a slide was developed in D-19 developer for 2.5 minutes. Development was stopped with 1% acetic acid and the slides are then fixed for four minutes with Rapid Fix (Kodak). Slides were stained in 10% giemsa for 18 minutes (if not G-banded) or for 2 hours (ib G-banded), and photographed using technical pan film.

RESULTS

Overview.

The experimental goal of this research was to determine genetic factors involved in immortalization. The approach was based on previous findings that SV40-transformed cells can undergo immortalization at low frequency. This allowed us to compare characteristics between mortal, "preimmortal" and immortalized cell lines that were derived from a single parental cell line. Four experimental approaches were used to determine differences between mortal cells and resultant matched SV40-transformed preimmortal and immortal cell lines. The first approach compared cytogenetic characteristics between the parental and the derived cell lines. This analysis identified a consistent chromosome aberration on the long arm of chromosome 6 that could be positively associated with the immortalized state. As progression within the cell lines occurred, other chromosome abnormalities could be identified. The second approach determined the relevance of the integration site of the SV40 genome to the immortalized phenotype. This analysis determined that the immortal phenotype cannot be simply attributed to alterations resulting from viral integration. It is possible that the relatively high frequency of immortalization of SVtsA/HF-A (without evident crisis) set in motion another series of events that included a consistent chromosomal abnormalities and later progression on further aberrations. The third experimental approach analyzed the DNA in identified chromosomal aberrations. This goal was based on the presence

of rearrangements of specific regions known to be involved in neoplasm and other genetic diseases as the cell lineages progressed. Rearrangement and changes in expression of genes, which are located at or near the SV40 integration site, was evident in the progression of the immortal phenotype. Thus, one possibility is that immortalization could be the result of the multiple cooperation of one or many genes, in addition to cellular changes on chromosome 6. The fourth approach was based on the observation that a deletion of chromosomal material on chromosome 6q was the most consistent first step in the immortalization process. Molecular analysis confirmed loss of sequences on chromosome 6q21->ter. Fluorescence *In situ* Hybridization (FISH) and molecular analysis determined that the deleted chromosome 6 breakpoint was at 6q13 on the basis of homologies to two single copy DNA probes which were shown to flank the region of the breakage. Further analysis showed that the region 6q13 to 6q21 resulted from translocated DNA from another chromosome.

PART ONE: CYTOGENETIC ANALYSIS OF ALL CELL LINES

A. Properties of the cells and cell lineages.

The finding that SV40-transformed human cells undergo immortalization at low frequency allowed us to compare characteristics between mortal, "preimmortal", immortal and "progressed" immortalized cell lines that were derived from a single apparently normal parental cell line. The clonal immortal cell lines were derived following transformation with origin defective SV40 genome. The cells include

immortalized derivatives from two independent transformants of the cell line HS74BM (human diploid fetal bone marrow fibroblast cell line), obtained by H. Smith (Smith *et al.*, 1976). The first cell lineage was obtained by transfection with an origin-defective SV40 genome containing a wild type T-Antigen (SV/HF) (Neufeld *et al.*, 1987). The second lineage was produced as the result of transfection of HS74 with a temperature sensitive SV40 genome [tsA58 (SVtsA/HF)] (Radna *et al.*, 1989). The lineage of the two independent transformations is showed on *Table 3* (see Materials and Methods)

In the first lineage, six immortalized derivatives SV/HF-5/2, SV/HF-5/3, SV/HF-5/5, SV/HB-5/37, SV/HF-5/38 and SV/HF-5/39 were originally obtained by clonal isolation after transfecting SV40(ori-) into HS74 cells (Radna *et al.*, 1987). Each of the immortal cell lines conserved the wild type SV40 sequence with a portion of the large T antigen-coding region missing (Neufeld *et al.*, 1987). It was therefore expected that T-antigen protein would be truncated. The T-antigen was observed by immunoblot, as a single protein at 80,000 Daltons (wild type protein is 94,000 Daltons), confirming the truncation (Neufeld *et al.*, 1987). To clarify the role of large T antigen, new SVtsA/HF-A immortalized transformants were generated by using SVori(-) mutants of SV40 encoding a heat-labile large T antigen (pSVtsA58) (Radna *et al.*, 1989). The vector pSVtsA58 was derived by cloning the ori(-) temperature sensitive SV40 tsA58 genome (pSVtsA) into the EcoRI site in a Bgl I and BamHI resistant derivative of PMK16. At 35°C, or the temperature permissive for large T antigen function, the three derived immortal cell

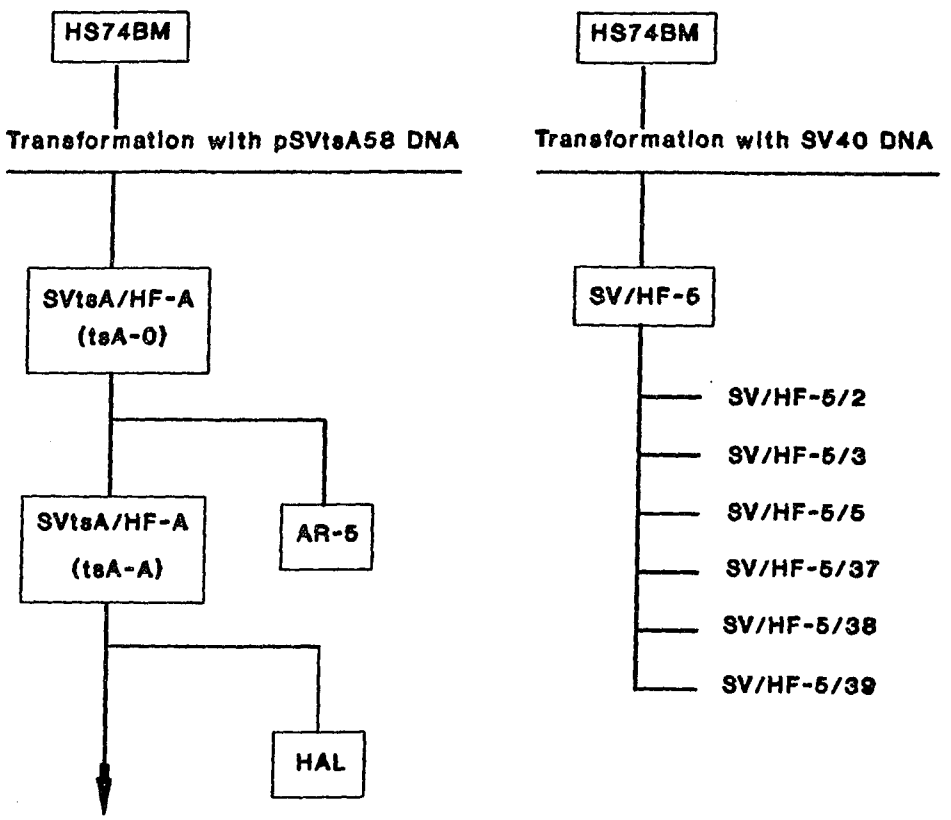


Table 3.
Lineage of the Cell Lines.

lines (AR5, tsA-A, and HAL) from this lineage have been in continuous culture for more than 200 generations (SVtsA/HF and SV/HF typically senesce at 60-90 generations). They were, however, unable to proliferate or form colonies at the temperature restrictive for large-T-antigen function (39°C). In contrast, HS74 and two SV/HF-5 immortal cell lines (non-temperature dependent) grew as well at 39°C as at 35°C as expected (Radna *et al.*,1987 and Resnick-Silverman *et al.*, 1991). These data demonstrate the persistent requirement for large T-antigen function for growth and viability of the immortalized cell line.

B. Determination of copy number and integration pattern of SV40.

Based on the requirement of T-antigen in immortalization, the following question could be addressed. Could the immortal phenotype be attributed to alterations resulting from viral integration? To approach this question, an analysis was carried out to determine the copy number and the integration pattern of SV40. DNA from the normal, preimmortal and immortal cell lines was digested with EcoRI and BglII and analysed with Southern hybridization *(Figure 1).* The DNA patterns in both experiments were similar in all cell lines. The immortal cell lines, however, showed a higher level of hybridization to the SV40 probe than did the preimmortal tsA-0. Densitometry measurements and normalization to an internal control (oligo-A synthetase located on chromosome 11) demonstrated that the immortal tsA-A and AR5 had approximately 2-fold the amount of SV40 DNA as compared to tsA-0 *(Figure 1).* One of the subclones, HAL, had a still higher level of SV40 sequences,

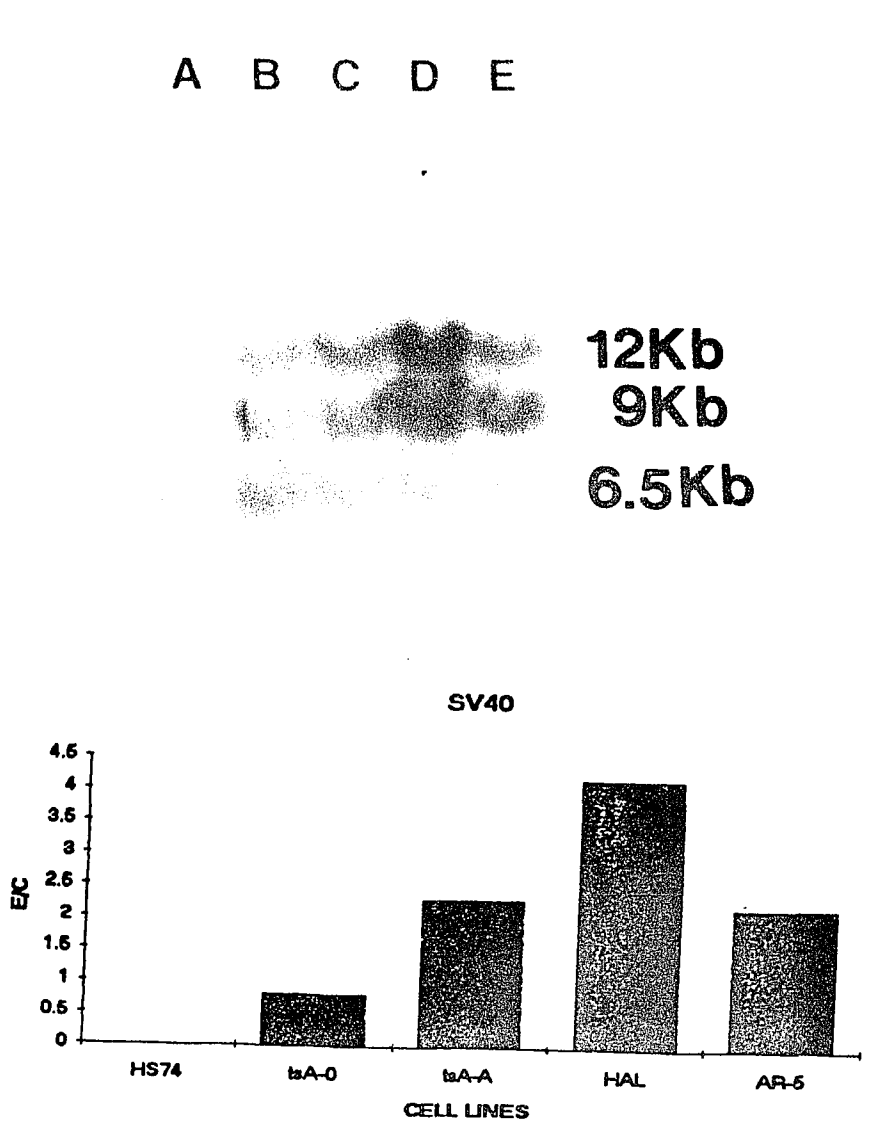


Figure 1.

Comparison of DNA from normal HS74BM, preimmortal SVtsA/HF-A and the other immortal cell lines.

DNA was prepared from normal HS74, preimmortal tsA-0 and the immortal cell lines from SVtsA/HF-A lineage; tsA-A, HAL, AR-5. 1: The DNA was digested with EcoRI endonuclease which excised the SV40 sequences from the vector pMK16 (pSVtsA58) and cuts once within SV40 sequence, and analyzed by Southern procedure. ^{32}P-labeled probes for SV40 and oligo-A synthetase DNAs were radiolabeled separately and mixed together for simultaneous hybridization to the blot. The 6.5Kb band of oligo(A) synthetase provides an internal control for quantitation. A:(HS74BM/p7); B:(preimmortal tsA-0/p9); C:(immortal tsA-A/p25); D:(immortal HAL/p24); E:(immortal AR-5/p27). Densitometry measurements shows the level of hybridization to the SV40 probe after normalization to the internal control (oligo-A synthetase). The immortal tsA-A (p21) and AR5 (p20) had approximately 2-fold the level of tsA-0 (p5). The subclone HAL had a still higher level of SV40 sequences, approximately 2-fold the level of tsA-A and AR-5.

or approximately 4-fold the level of tsA-0. The higher level of hybridization to the SV40 DNA in the immortal cell lines could be explained by SV40 amplification. Cytogenetic analysis supported this hypothesis, since double minutes (DM) were seen in the immortal cell lines. The presence of DMs is often diagnostic of DNA amplification, and characteristic of tumors in well-advanced stages (Helm *et al.*, 1987). This was confirmed in that the DMs showed positive hybridization with an SV40 DNA probe following hybridization *in situ (Figure 2).* The increase in SV40 DNA, however, was not associated with any consistent alteration in the level of viral large T and/or small t proteins as quantified by immunoblot (Hubbard-Smith *et al.*, 1992). This analysis suggests that the immortal phenotype can not be attributed to alterations of the level of T or t-antigens resulting from viral integration. It is possible that the SV40 integration event *per se*, could set in motion another series of events that included chromosomal abnormalities and later progression in further aberrations.

Dot blot analysis using DNA of the parental and immortal cell lines against pMK16 DNA, the original vector in transfection, and SV40 DNA, verified that the pSVtsA58 genome was present in all cell lines with the expected exception of the normal, non-transfected cells *(Figure 3).* Consistent with other investigator's findings, these experiments also determined that SV40 has minor homology with the human genome. On this basis, it was determined that PMK DNA would be a better probe in future experiments.

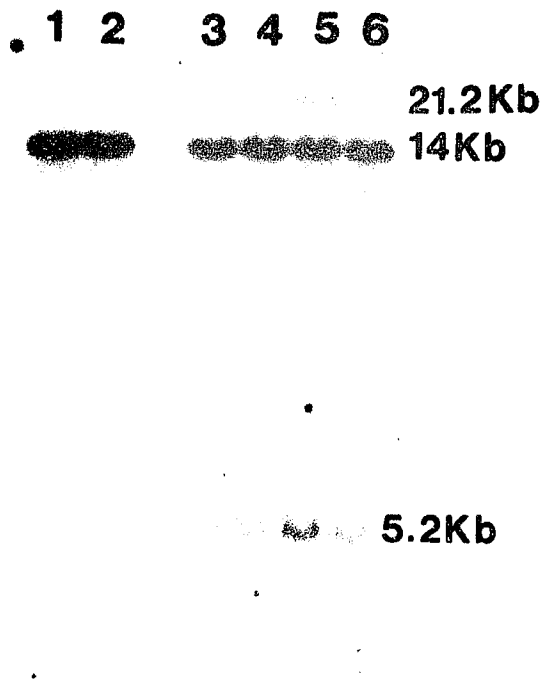


Figure 2.

Comparison of Bgl II digested DNA from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines.

DNA was prepared from normal HS74, preimmortal tsA-0 and the other SVtsA/HF-A lineage immortal cell lines. DNA was digested with bgl II which does not cut SV40, but cuts once pMK16 sequences, and analyzed by Southern procedure. ^{32}P-labeled probes for SV40 and rDNA were radiolabeled separately and mixed together for simultaneous hybridization to the blot. A 21.2Kb band for rDNA (B-fragment) provided an internal control for quantitation. 1:(HS74BM/p7); 2:(HS74BM/p7); 3:(preimmortal tsA-0/p9); 4:(immortal tsA-A/p25); 5:(immortal HAL/p24); 6:(immortal AR-5/p27).

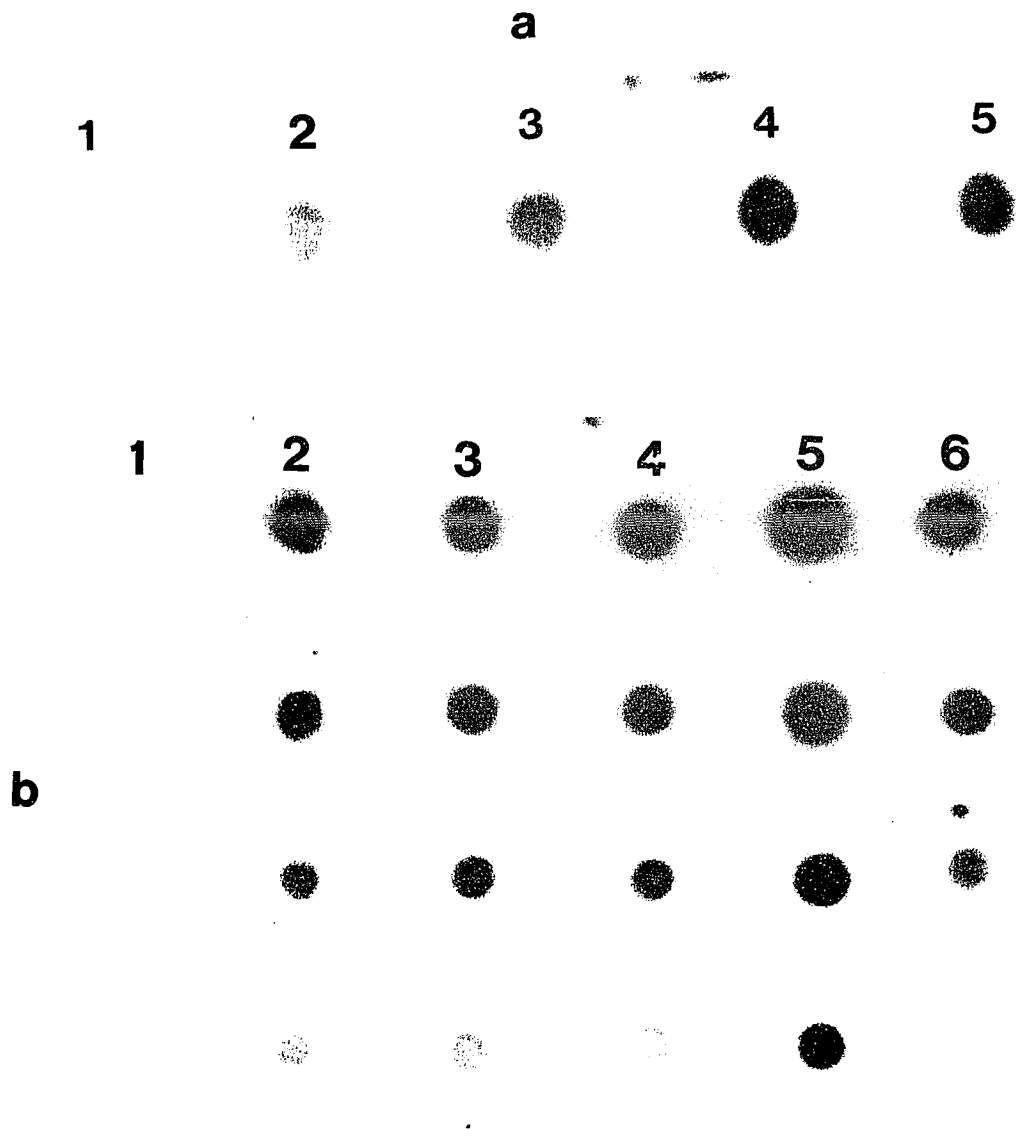


Figure 3.
pMK16 and SV40 as probes verified that the pSVtsA58 genome is present in all cell lines except the normal non-transfected cell.
DNA from normal HS74BM, preimmortal tsA-0 and the other immotral cell lines, was used for dot blot analysis. **a**:DNA from all cell lines was hybridized with ^{32}P-labeled probe for SV40. **b**:Different quantities of DNA from all cell lines was hybridized with ^{32}P-labeled probe for pMK16. For a: 1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25); 4:(Immortal HAL/p24); 5:(Immortal AR-5/p27). For b: 1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25): 4:(Immortal HAL/p24); 5:(Immortal AR-5/p15); 6:(Immortal AR-5/p27).

C. Localization of integration site of SV40.

One possible association between the viral integration event and immortalization is that the integration event *per se* affected other gene(s) in the same region of the chromosome that could be involved in immortalization. The first approach to this question was the localization of SV40 in the human genome.

Following isotopic *in situ* hybridization with PMK DNA, statistical analysis of grain density over chromosomes in all cell lines revealed the SV40 integration site. The preimmortal and immortal derivatives showed one integration site on chromosome 5q15-21. A graph of the statistical analysis for the normal non-transfected HS74BM and the cell line AR-5 is given in *Figure 4. Figure 5* shows the localization of the SV40 DNA on an idiogram of chromosome 5 and a picture of a metaphase plate before and after hybridization *in situ*. Interestingly, two tumor suppressor genes, APC (Adenomatous Polyposis Coli) (Croden *et al.*, 1991) and MCC (Mutated in Colorectal Cancer) (Kinzler *et al.*, 1991), have been localized in the proximity of the integration site region, 5q21-22. This findings suggested a possible association between the viral integration event and an effect of known tumor associated genes. The integration event *per se* could effect MCC or APC or other unknown gene(s) in the 5q21-22 region, in a direct (cause deletion) or an indirect (deregulate) manner.

D. Cytogenetic Analysis

Since the immortalized phenotype can not be fully explained by alteration

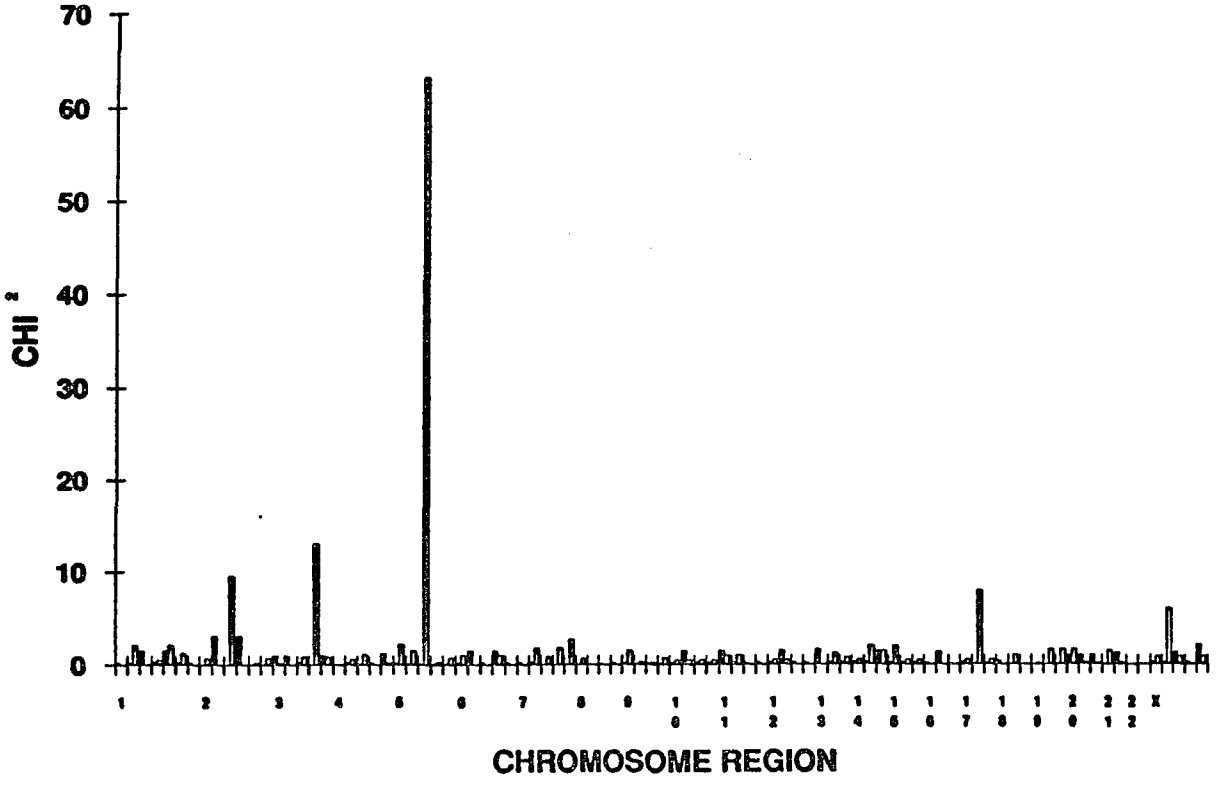


Figure 4.

Localization of SV40 intergration site with isotopic *In Situ* hybridization analysis. Statistical analysis of the silver grain density over all chromosomes. Hybridization was with probe pMK16 DNA (vector). Distribution of X^2 value of each chromosomal region. The only region with a statistically significant X^2 is on chromosome 5/Region 3. This region is 5q15-21. The abnormal chromosomes of this cell line which have X^2>or=0 are not shown on the graph. The symbol ◘ represent the X^2 values for the non-transfected cell parental cell line HS74BM and the ■ represent the X^2 value for the cell line AR-5.

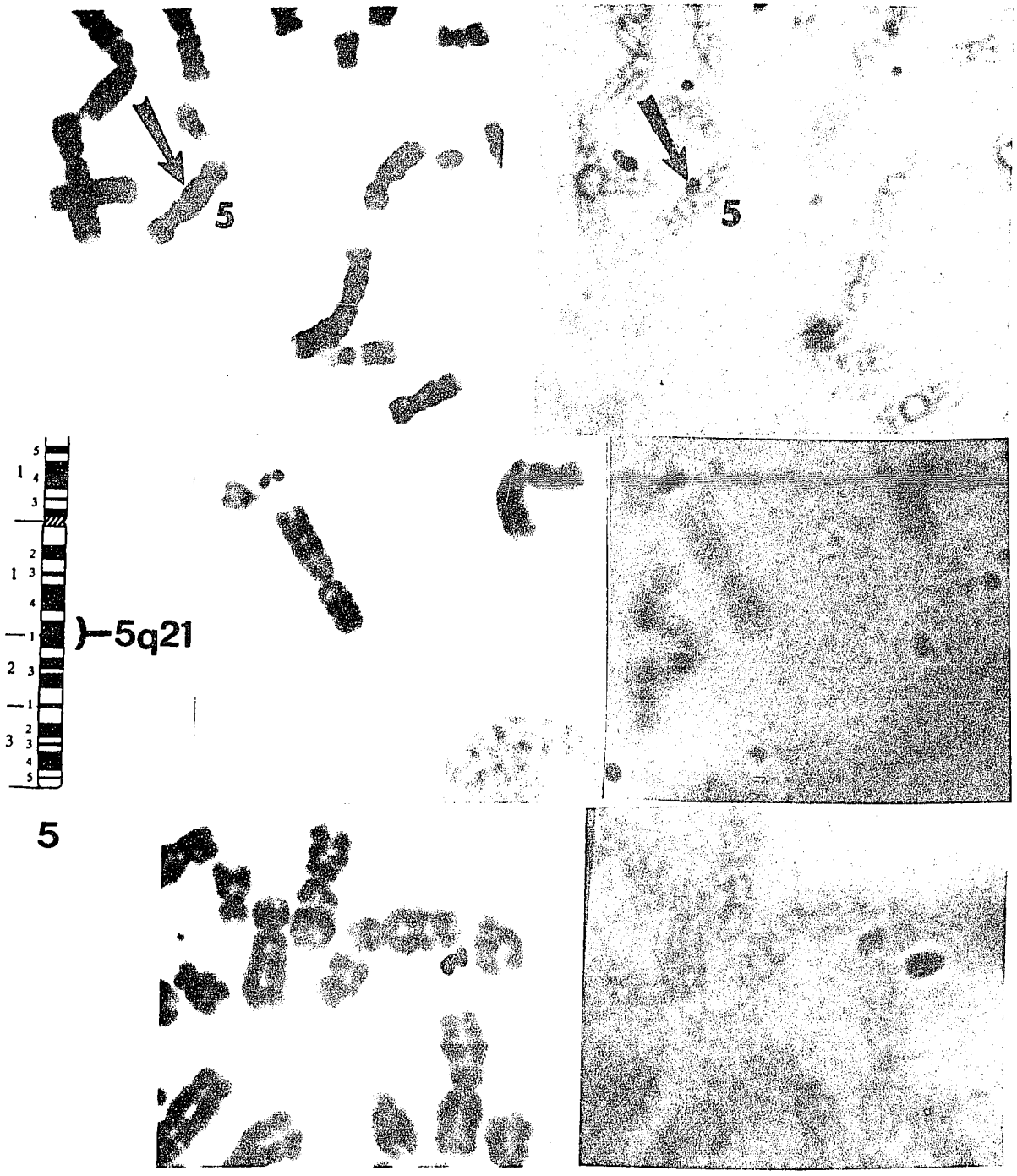


Figure 5.
SV40 integration site on chromosome 5q15-21.
The ideogram of human chromosome 5, showing approximate location of the SV40 DNA on the region 5q21. Pictures of a metaphase plate before **(a)** and after *in situ* hybridization **(b)** with a probe pMK16 (vector) detecting the pSVtsA58 intergration site. DMs showed positive hybridization with the pMK16 DNA probe following hybridization *in situ*

in SV40 function, nor at the first level of analysis, on the basis of integration site, other changes in the genome of immortalized cells were determined. Extensive cytogenetic analysis was done in order to identify chromosomal aberrations as indicative of genetic changes. The results of cytogenetic analysis of all normal, preimmortal and immortal cell lines are reported in *Table 4.* The parental cell line HS74 (Smith *et al.*, 1976) shows a 100% normal diploid karyotype 46,XX. *(Figure* 6). More than 50 cells each from preimmortal and early immortal SVtsA/HF-A, as well as the cloned immortal derivatives HAL and AR5, were analyzed. Analysis of the early preimmortal cells tsA-0 (p5) showed that 65% of the cell population was missing one chromosome 16, but there were no other obvious chromosomal changes (45,XX,-16) *(Figure 7).* The missing chromosome 16 was consistently found in all cell lines of the lineage and substantiates (in addition to the consistent integration site for SV40 DNA) that all cell lines were derived from the parental line. The uncloned immortalized tsA-A cells showed, in addition to the missing chromosome 16, a deletion of the long arm of chromosome 6 with loss of the entire portion of 6q distal to 6q21 *(Figure 8).* The deletion of 6q21 was a non-random event since all uncloned and cloned immortal SVtsA/HF-A shared changes on chromosome 6 and 16 only. In addition, different passages of the respective sub-lines showed characteristic changes which suggested progression of karyotypic alterations. All other clonal abnormalities found in SVtsA/HF-A, HAL, AR5 (early passage) and AR5 (late passage) are summarized in Table 4. Representative karyotypes for HAL, AR5 early and AR5 late passage are shown

Unage	# cells	-13	-16	-6	del(6)(q21)	15p+	t(11;22)	DM	del(1)	4q+	t(8;11)	14p+	14q+	18p+	-X	t(5;18)	t(10;12)	t(2;14;19)	ncra	der(7)	der(7)	del(13)
HS748M																						
SVtsA/HF-A	150																					
p5																						
sub1	80		X																			
sub2	70																		X			
SVtsA/HF-A	50																					
p22																						
sub1	10		X																			
sub2	47		X		X			x	X	X							X		X	X		
sub3	3																		X			
HAL	130																					
p12																						
sub1	68	X	X	X			X	x		X	X			X	X		X		X	X	X	X
sub2	62	X	X	X			X	x		X	X			X	X		X		X	X	X	X
AR5	150		X	X		x	X	x								X			X			
p15																						
AR5	125																					
p31																						
sub1	75	X	X	X		x	X	x							X	X		X	X			
sub2	24	X	X	X		x	X	x							X	X			X			
sub3	16	X	X	X		x	X	x				X	X		X	X			X			
sub4	11	X	X	X		x	X	x							X	X			X			

Unage	# cells	-13	-16	del(3)	del(6) (q21)	del(6) (q15)	del(6) (q23)	i(14q)	18q+	del(1)	del(7)	der(12)	del(12)	15p+	t(11;22)	del(11)	1p+	DM	der(11)	del(11)	der(5)	19q+	ms	14q+	ncra
SV/HF-S /37 p85	30	X	X	X	X			X	X	X	X	X		X	X	X	X	X	X			X	X		X
SV/HF-S /39 p40	20	X	X	X	X	x	x	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	x	X
SV/HF-S /39 p60	45	X	X	X	X	x	x	X	X	X	X		X	X		X	X	X	X	X	X	X	X	x	X

Table 4.
Summary of the cytogenetic analysis of all cell lines.

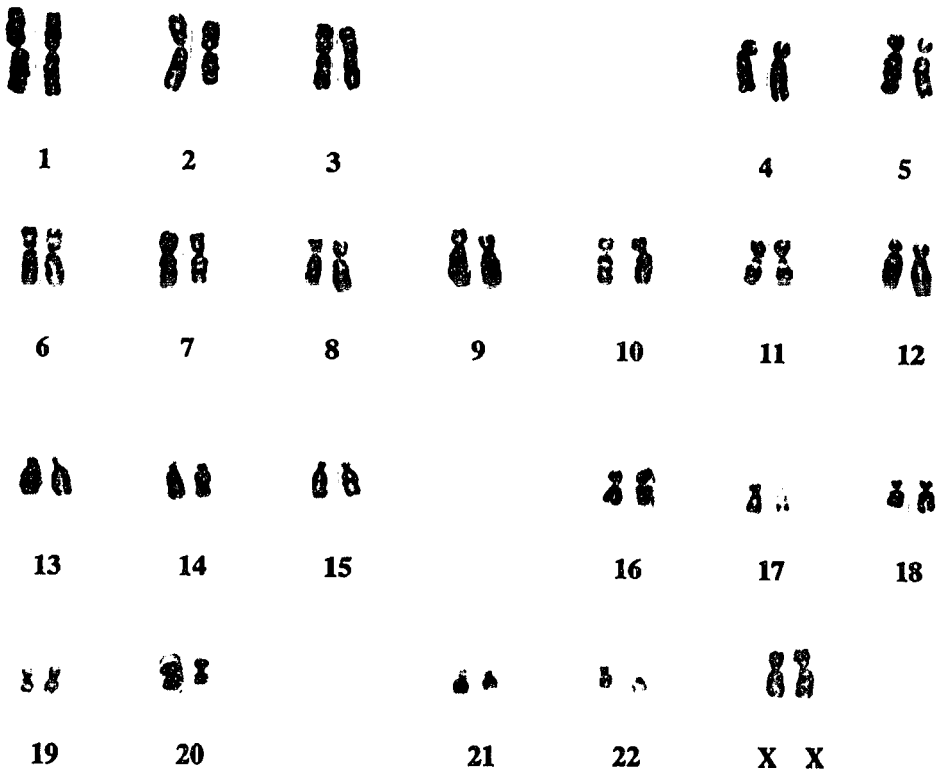


Figure 6.
G-banded karyotype of typical metaphase plates of the non-transformed human bone marrow fibroblast cells HS74.
All cells show normal karyotype, 46,XX.

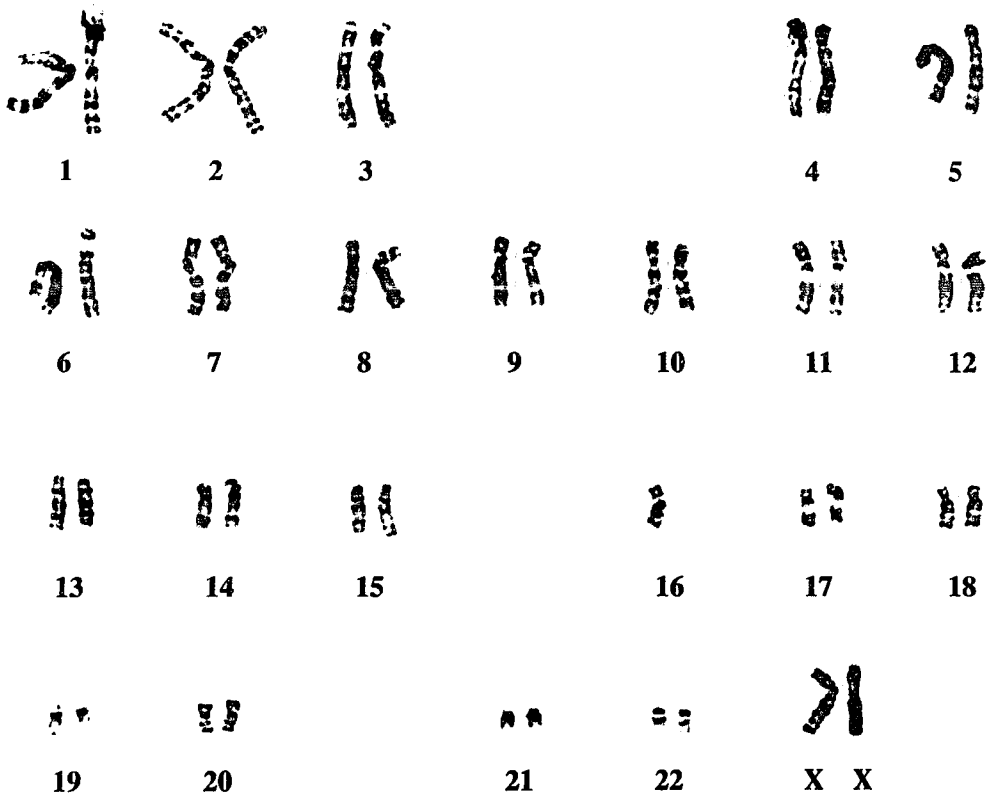


Figure 7.
G-banded karyotype of typical metaphase plates of early passage tsA-0 cells.
Representative G-banded karyotype cell line tsA-0 at passage 5 showing in 65% of the cell clonal deletion of chromosome 16. The karyotype of these cells is: 46,XX,-16.

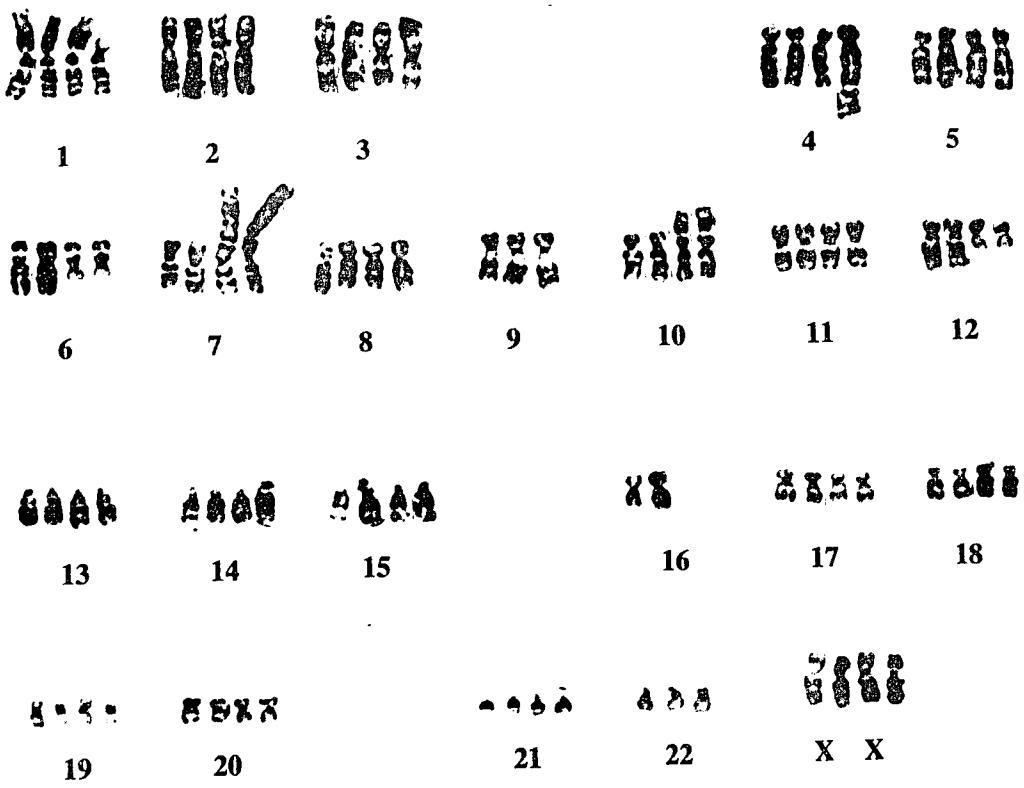


Figure 8.

G-banded karyotype of typical metaphase plates of immortal tsA-A cells.

G-banded karyotype from predominant population of immortal cells in the cell line tsA-A at passage 22. The karyotype of these cells is:70-90,XXXX,-16,-16,del(1)(p21),del(6)(q21),del(6)(q21),4q+,der(7)t(5;7)(p13;p22->p15::p22->qter),der(7)t(5;7)(p13;p22->p15::p22->qter),t(10;12)(p11;q11),t(10;12)(p11;q11),+dmin,+NCRA.

in *Figures 9-11.* The cytogenetic analysis of this lineage indicated that loss of sequences on chromosome 6q21->ter is involved in immortalization.

The immortal cell lines SV/HF-5/37 and SV/HF-5/39, which were transformed with the wild-type T antigen (SV/HF), were also analyzed cytogenetically. These cell lines showed multiple rearrangements. Among the aberrations was a non-random deletion of chromosome 6 with breakage at region 6q21. Thus, this was a consistent finding among all immortal cell lines *(Figure 12-13).*

Partial karyotypes comparing the chromosomal aberrations from transformants with the temperature sensitive SVor(-) (SVtsA/HF-A) cells are given in *Figure 14. Figure 15* illustrates chromosomal abnormalities arising from the transformants from the wild type SV/HF-5 cells.

Other significant findings among the SVtsA/HF-A lineage were the following. The translocation t(11;22)(q13;p11) was present in the more progressed cells, HAL and AR-5, but was not observed in SVtsA/HF-A non-clonal immortals (tsA-A). This could be of importance since the chromosomal region 11q13, involved in t(11;22), is the bcl-1 (B-Cell Leukemia-Lymphoma gene 1) region, often associated with B-cell lymphomas and other leukemias (Koduru *et al.*, 1989; Showe *et al.*, 1986). There are two candidate genes for in the bcl-1 region that could be responsible for the phenotype, PRAD-1 (Parathyroid Adenomatosis) (Amold *et al.*, 1989; Rosemberg *et al.*, 1991) and Cyclin D1 (Motokura *et al.*, 1991; Xiong *et al.*, 1991). Another translocation of possible importance was t(5;18)(q21;q21). This

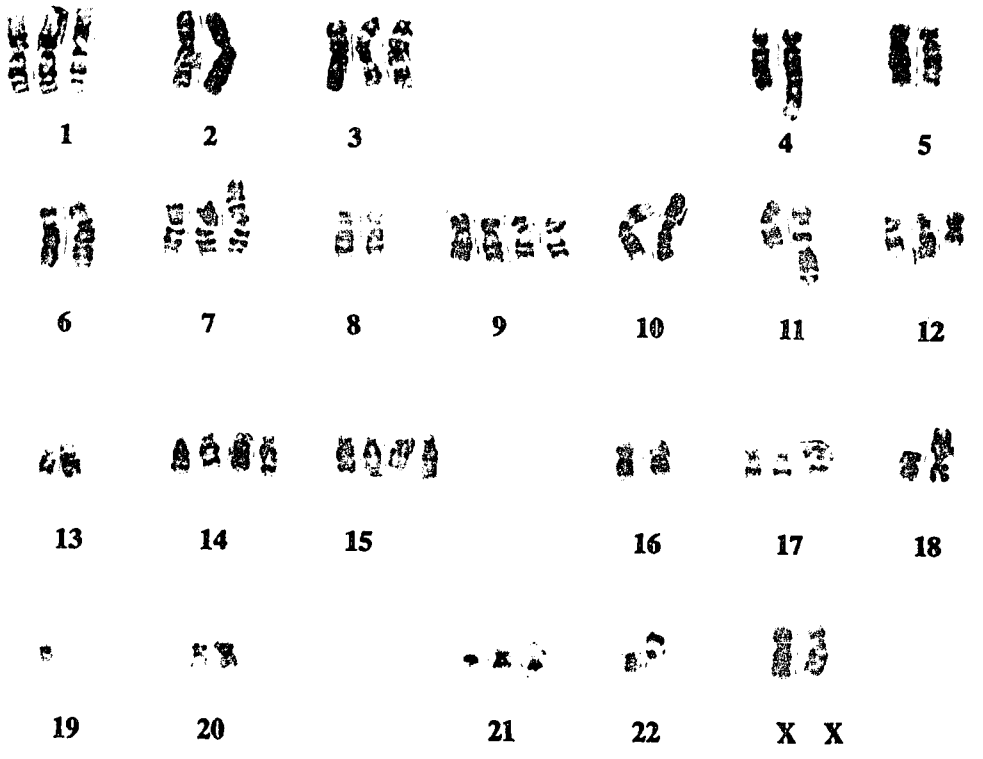


Figure 9.
G-banded karyotype of typical metaphase plates of the clonal immortal HAL.
G-banded karyotype from predominant population of the cell line HAL at passage 12. The HAL cell line is derived after subclonic the immortal HStsA/HF-A cells at passage 40. The karyotype of these cells is:
Sub1,53%; 42,XX,-6,-13,-16,-X,del(13)(q14;q22),4q+,der(7)t(5;7)(q11;p22->p15::p22->q31), t(10;12)(p11;q11),18p+,der(11)t(8;11)(p11->qter;q25), t(11;22)(q13;p11),+NCRA.
Sub2,47%; 70-90,XXXX,-6,-6,-13,-13,-16,-16,-X,-X,del(13)(q14;q22), del(13)(q14;q22),4q+,4q+,der(7)t(5;7)(q11;p22->p15::p22->q31), der(7)t(5;7)(q11;p22->p15::p22->q31),t(10;12)(p11;q11),t(10;12) (p11;q11),18p+,18p+,der(11)t(8;11)(p11->qter;q25),der(11)t(8;11) (p11->qter;q25),t(11;22)(q13;p11),t(11;22)(q13;p11),+NCRA.

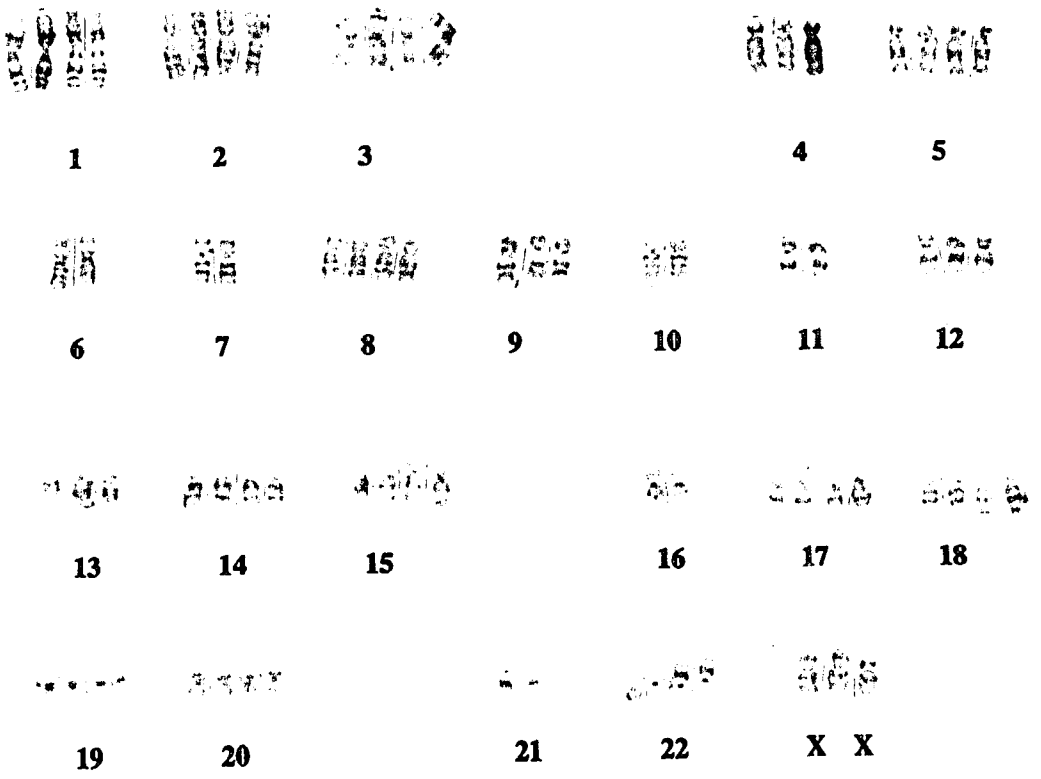


Figure 10.
G-banded karyotype of typical metaphase plates of the early passage of the clonal immortal SVtsA/HF-A cells, called AR5.
Representative G-banded karyotype from the clonal immortal cell line AR5 at early passage 15. All the cell show the following karyotype: 70-90,XXXX,-16,-16,del(6)(q21),del(6)(q21),der(18)t(5;18)(q22;q21),der(18)t(5;18)(q22;q21), t(11;22)(q13;p11),t(11;22)(q13:p11),+NCRA.

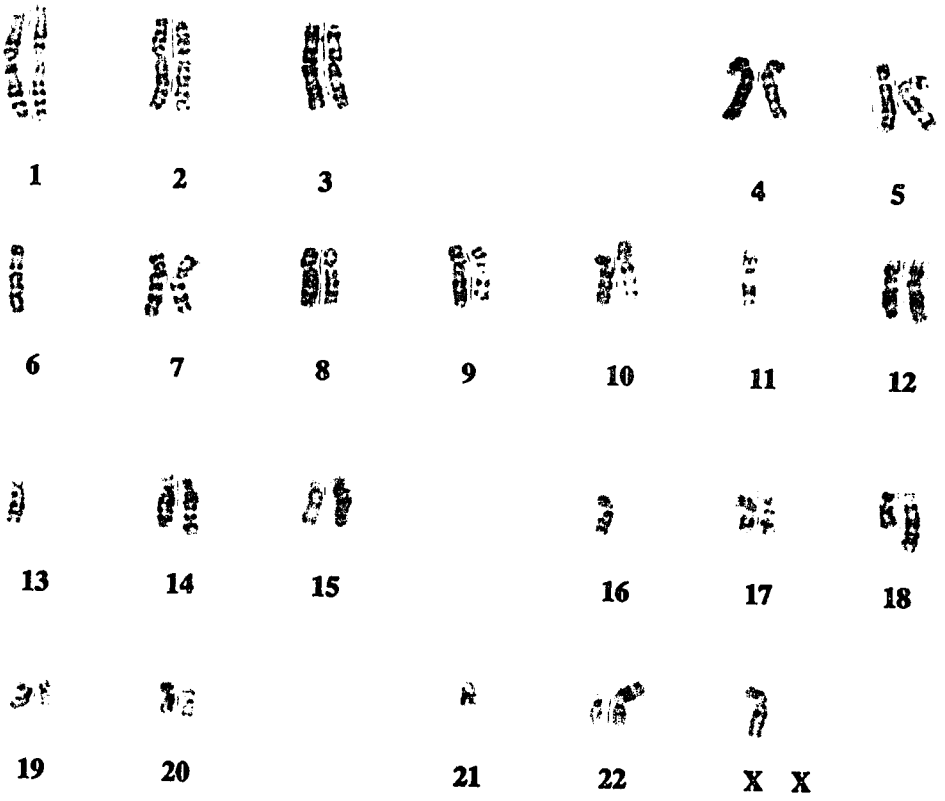


Figure 11.

G-banded karyotype of typical metaphase plates of the late passage of the clonal immortal SVtsA/HF-A cells, called AR5.

Representative G-banded karyotype from the clonal immortal cell line AR5 at early passage 31. There are four sublines and they show the following karyotype:
Sub. 1; 60% 43,XX,-13,-16,-X,del(6)(q21),t(2;14;19)(q21;q11;p13),der(18) t(5;18)(q22;q21), (11;22)(q13;p11),+NCRA.
Sub. 2; 19% 43,XX,-13,-16,-X,del(6)(q21),der(18)t(5;18)(q22;q21),t(11;22) (q13;p11),+NCRA.
Sub. 3; 12% 44,XX,-13,-16,-X,del(6)(q21),+14p+,14q+,der(18)t(5;18)(q22;q21), t(11;22)(q13;p11),+NCRA.
Sub. 4; 9% 70-90,XXXX,-13,-13,-16,-16,-X,-X,del(6)(q21),del(6)(q21),der(18)t(5;18) (q22;q21),der(18)t(5;18)(q22;q21),t(11;22)(q13;p11)+NCRA

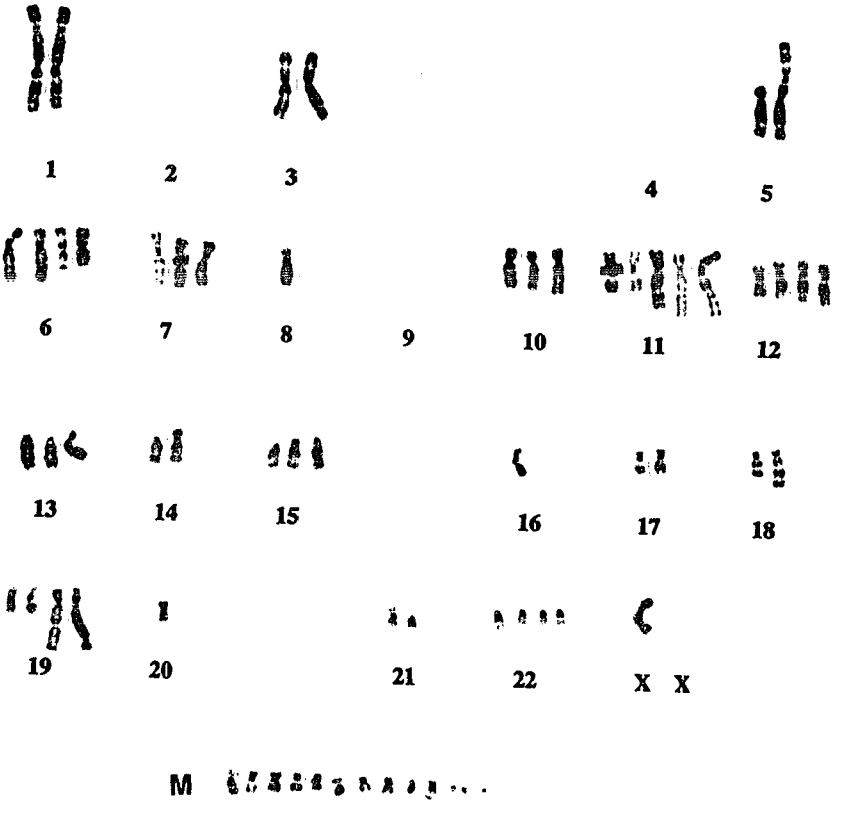


Figure 12.
G-banded karyotype from a metaphase plate of the immortal cell line SV/HF-5/37.
G-banded karyotype from the clonal immortal cell line SV/HF-A/37 at passage 85. The karyotype of this cell line is very abnormal and not all of the abnormalities are shown.

Figure 13.
G-banded karyotype from a metaphase plate of the immortal cell line SV/HF-5/39.
G-banded karyotype from the clonal immortal cell line SV/HF-A/39 at passage 60. The karyotype of this cell line is very abnormal and not all of the abnormalities are shown.

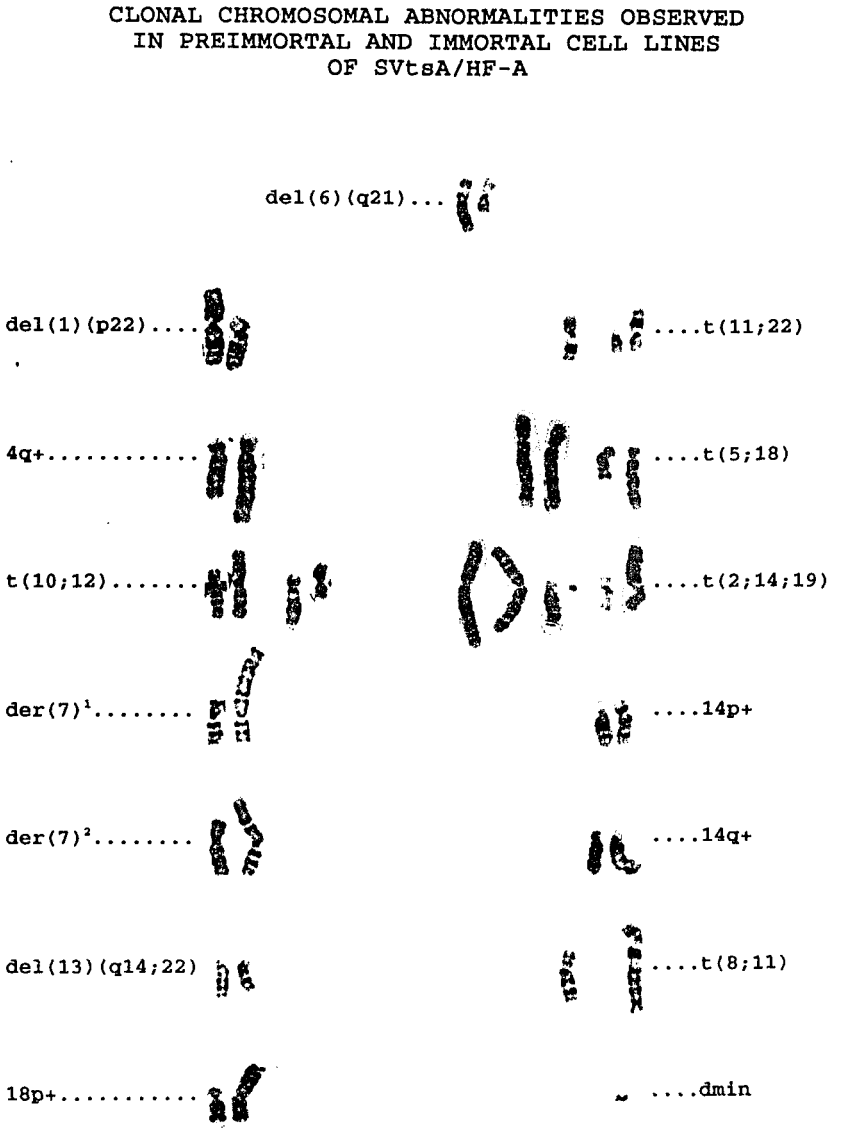


Figure 14.

Clonal chromosomal abnormalities observed in preimmortal and immortal cell lines of SVtsA/HF-A.

G-banded partial karyotype show all the clonal aberrations from SVtsA/HF-A preimmortal cell line and its immortalized derivatives, after transformation with pSVtsA58 plasmid.

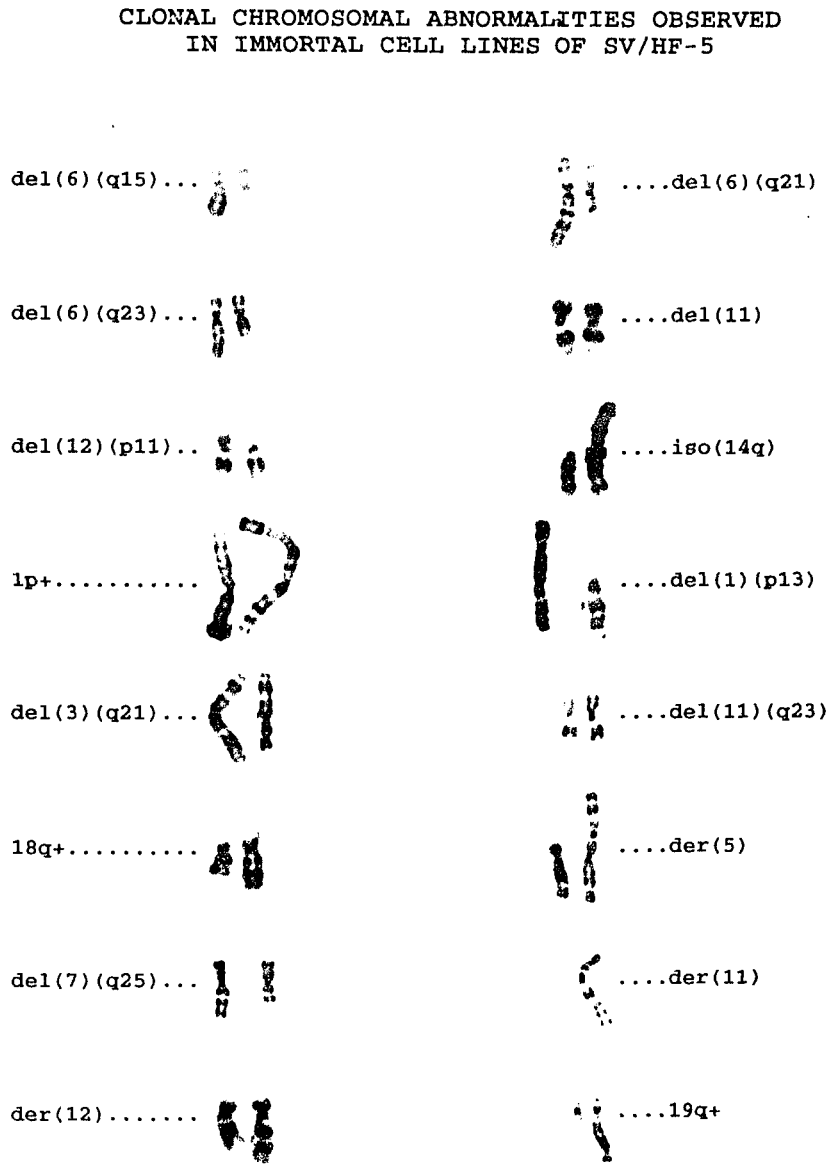


Figure 15.
Clonal chromosomal abnormalities observed in all immortal cell lines of SV/HF-5. G-banded partial karyotype show all the clonal aberrations from SV/HF-A immortalized derivatives, after transformation with wild type SV40 genome.

translocation was consistently present in all early and late passages of AR-5. The 18q22, involved in t(5;18) is the genetic region for bcl-2 (B-Cell Lymphoma/Leukemia gene 2) which is highly rearranged with Burkitt's Lymphoma and other leukemias (Gauwerky *et al.*, 1988; Nunez *et al.*, 1989). The same region contains the tumor suppressor gene DCC (Deleted in Colon Carcer) which is involved in later stages of sporatic colorectal cancer (Fearon *et al.*, 1990). The region of chromosome 5 involved in this translocation is 5q21, or a region in close proximity to the integration site for the SV40 DNA. This region contains two tumor suppressor genes, APC (Adenomatous Polyposis Coli) (Croden *et al.*, 1991), and MCC (Mutated in Colorectal Cancer), (Nishisho *et al.*, 1991; Kinzler *et al.*, 1991). Both genes are involved in familial adenomatous coli and colorectal cancer (Nishisho *et al.*, 1991). A third chromosome rearrangement of importance was the translocation t(10;12). This translocation, along with a derived chromosome 7 [der(7)], was consistent among the non-clonal immortal cells, SVtsA/HF-A and HAL. The KRAS-2 (Kirsten Rat Sarcoma 2 viral oncogene) on chromosome 12 and EGFR (Epidermal Growth Factor Receptor) and/or TCR (T-Cell Receptor) on chromosome 7 are known to be involved in neoplasms. A more detailed analysis of these findings is summarized in *Table 5*. The significance of these regions, which were often involved in chromosomal rearrangements formed the subject for further research.

In summary, the cytogenetic analysis showed a series of defined chromosomal aberrations in different lineages of immortalized cells. The deletion

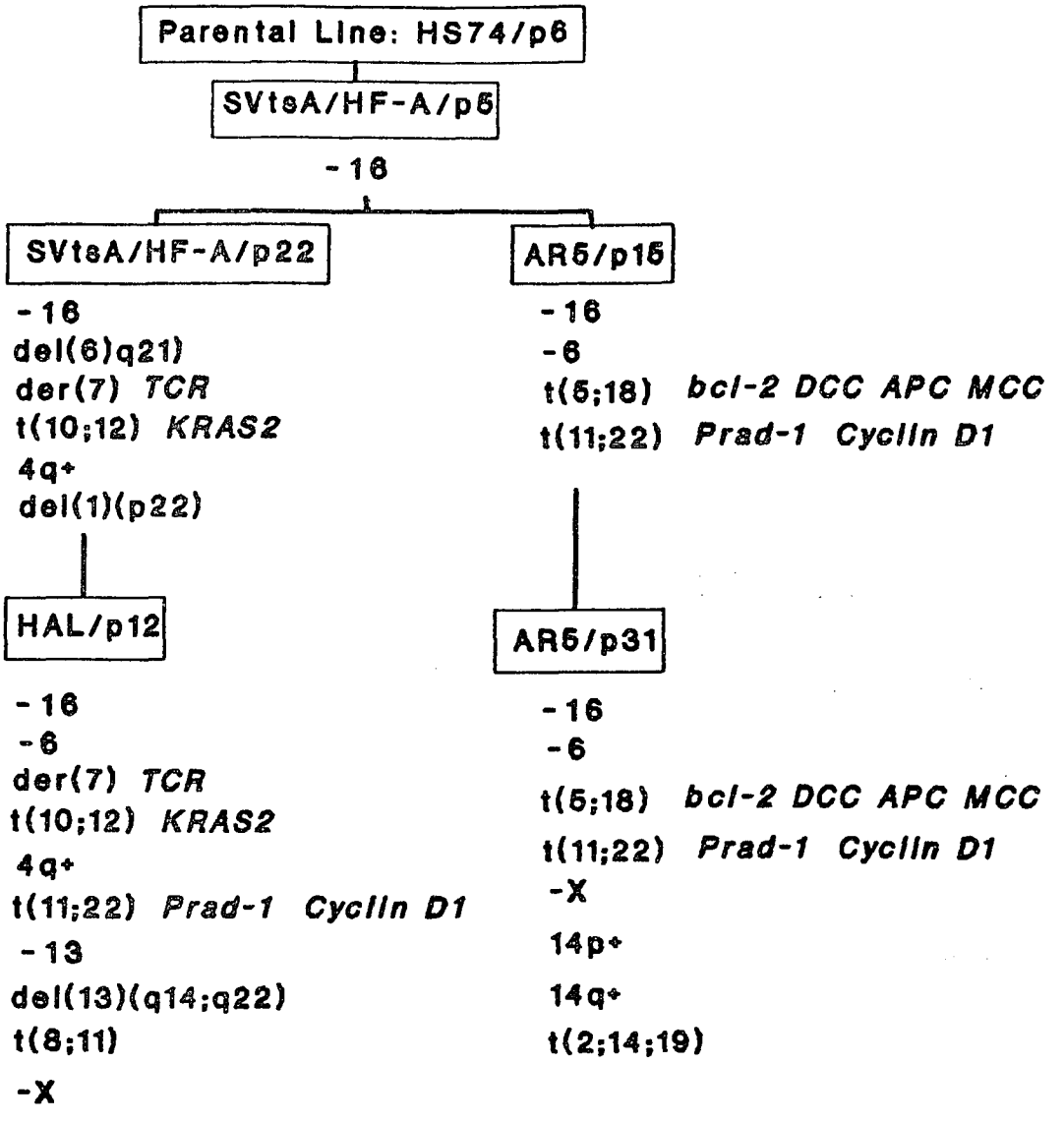


Table 5.

Chromosomal abnormalities involve regions that code genes known to be involved in neoplasm.

These aberrations are consistent among the preimmortal SVtsA/HF-A/p5, the non-clonal immortals SVtsA/HF-A/p22, and clonal immortals HAL and AR-5 cell lines. The genes showed in *italics* are known to be involved in human tumors and localized on the reagion of chromosomal rearrangement.

of chromosome 6q21->ter was a non-random chromosomal abnormality, consistently observed as a common aberration among all immortal cell lines. This analysis indicated that loss of sequences of chromosome 6q21->ter was involved in immortalization. Other non-random chromosomal abnormalities were found in presumably more progressed immortal cell lines. This suggested that immortalization could be the result of a multiple cooperation of genes, in addition to cellular changes on chromosome 6.

PART TWO: ANALYSIS OF CHROMOSOMAL ABERRATIONS OTHER THAN CHROMOSOME 6.

These experiments analyzed the DNA in chromosomal aberrations for rearrangement of specific regions known to be involved in neoplasm and other genetic diseases. The possible significance of the regions 5q21, 11q13, 18q22, 7p13-15, 12p12 (discussed above) in the immortalization phenotype was determined. *Table 5* shows the non-random chromosomal abnormalities in all cell lines and the protooncogenes, tumor suppressor genes and transforming proteins that were localized in this regions. The approach was two-fold. The first addressed the following question: (i) can the protooncogenes, tumor suppressor genes and transforming proteins be localized in regions involved in the observed chromosomal abnormalities, and if so, are they rearranged and therefore, possibly related to or involved in the phenomenon of immortalization? The second approach was designed to determine if there were changes in RNA expression at loci known

to be in regions associated with chromosome rearrangements.

A. Determination of rearrangements using hybridization analysis.

Southern blot analysis was performed to assess whether rearrangements of genes associated with the chromosomal abnormalities was involved in immortalization. Several restriction enzymes were used in this analysis. The following genes were screened for possible rearrangements: KRAS2, APC, FAD, TCR, PRAD-1, MCC, DCC, bcl-2 and Cyclin D-1.

DNA from all five cell lines (HS74, tsA-0, tsA-A, HAL and AR-5) was analyzed following digestion with a group of five different restriction endonucleases (average cutters). There were no rearrangements in the Cyclin D-1 DNA *(Figure 16, 17)*. The experiment was designed and performed in the same manner for the genes bcl-2 *(Figure 18, 19)*, DCC *(Figure 20, 21)*, PRAD-1 *(Figure 22, 23)*, TCR *(Figure 24, 25)*, APC and FAD *(Figure 26, 27)*, and KRAS-2 *(Figure 28, 29)*. None of these genes showed obvious DNA rearrangements. It is concluded that the genes putatively associated with these identifiable chromosome rearrangements were unaffected by and did not effect the immortalization process.

The DNA of the tumor suppressor gene MCC (5q21), was localized in close proximity to the integration site of SV40, and on the translocation t(5;18)(q21;q21) which was found as one of the few non-random aberrations in the AR-5 immortal cell line. MCC was analyzed following digestion with a group of seven different restriction endonucleases (average cutters). No MCC rearrangements were

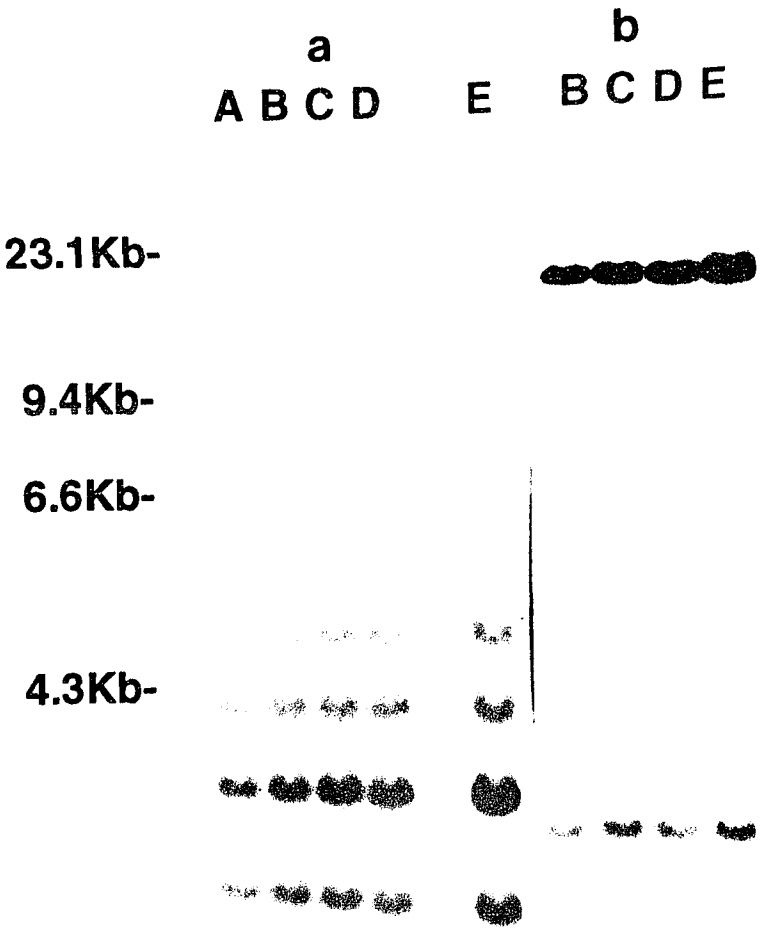


Figure 16.
Southern blot analysis of Cyclin D-1 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for Cyclin D-1 gene was hybridized to the blot. All cell lines show the same hybridization pattern, demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with Taq I endonuclease.
b: Restriction enzyme digestion with Hind III endonuclease.

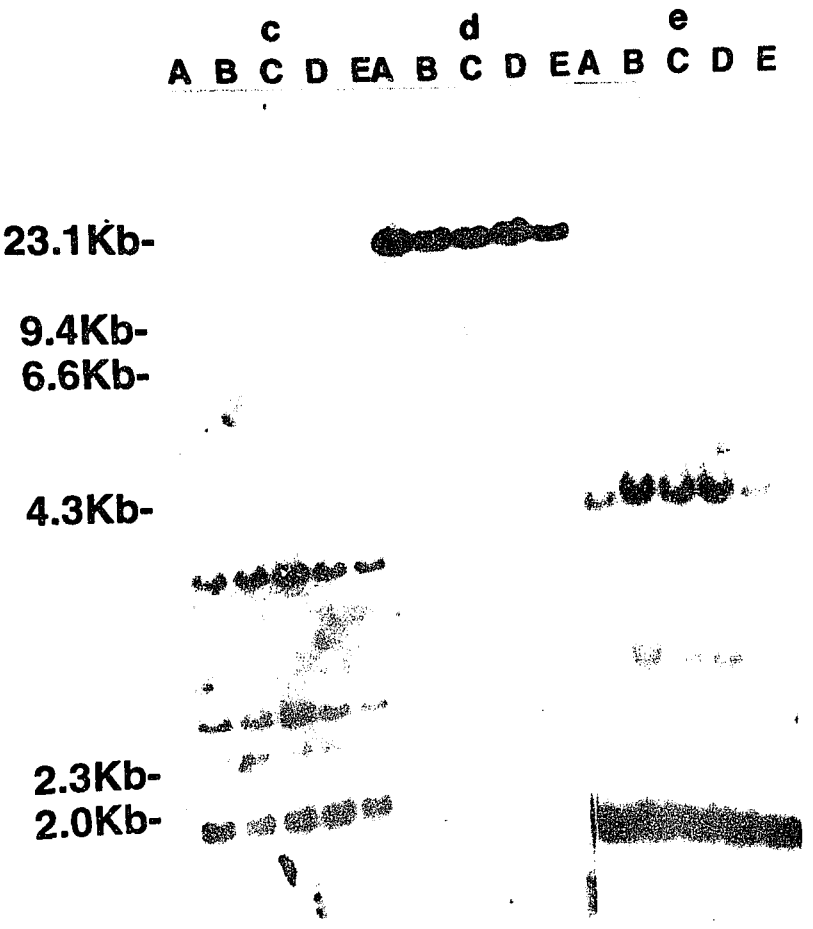


Figure 17.
Southern blot analysis of Cyclin D-1 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for Cyclin D-1 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
c: Restriction enzyme digestion with Pvu II endonuclease.
d: Restriction enzyme digestion with Bgl II endonuclease.
e: Restriction enzyme digestion with EcoRI endonuclease.

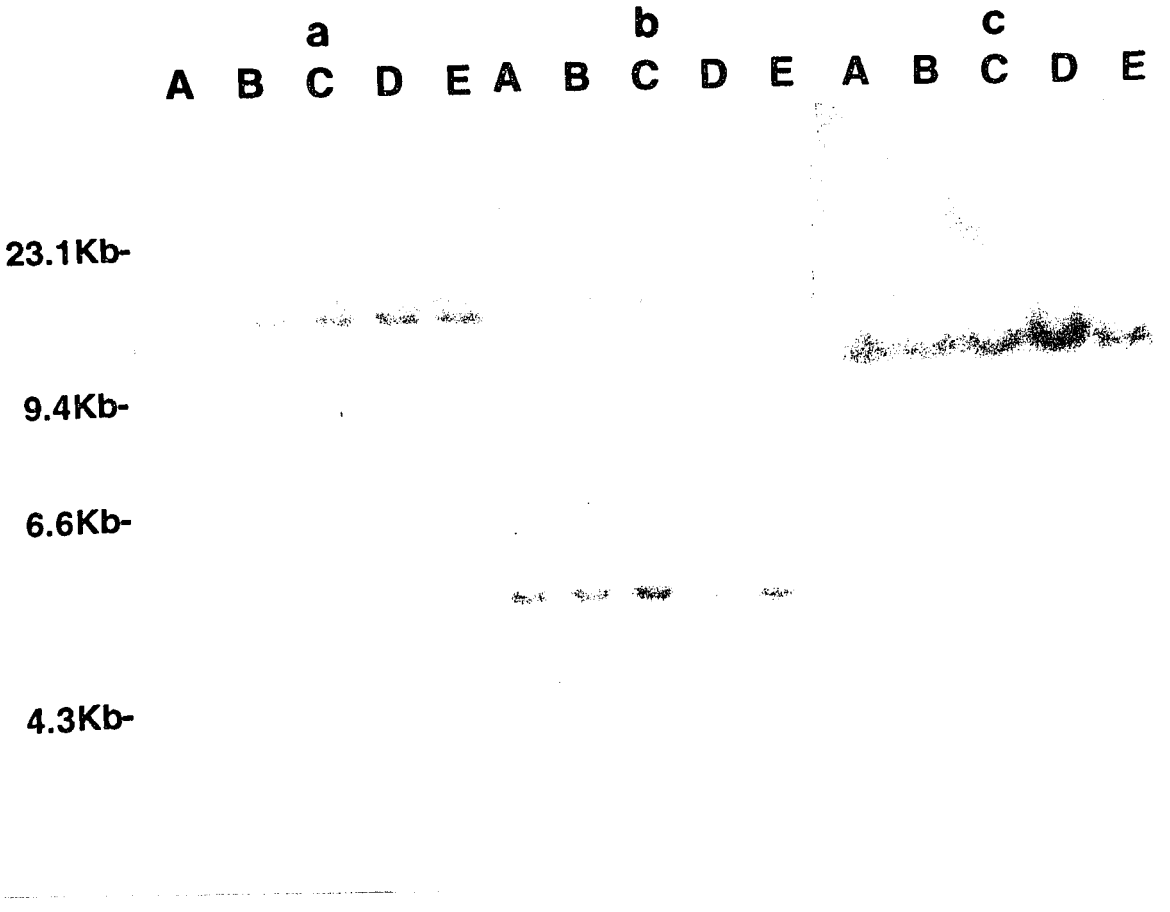


Figure 18.
Southern blot analysis of bcl-2 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for bcl-2 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with Hind III endonuclease.
b: Restriction enzyme digestion with EcoRI endonuclease.
c: Restriction enzyme digestion with Bgl II endonuclease.

Figure 19.
Southern blot analysis of bcl-2 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for bcl-2 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
d: Restriction enzyme digestion with Pvu II endonuclease.
e: Restriction enzyme digestion with Pst I endonuclease.

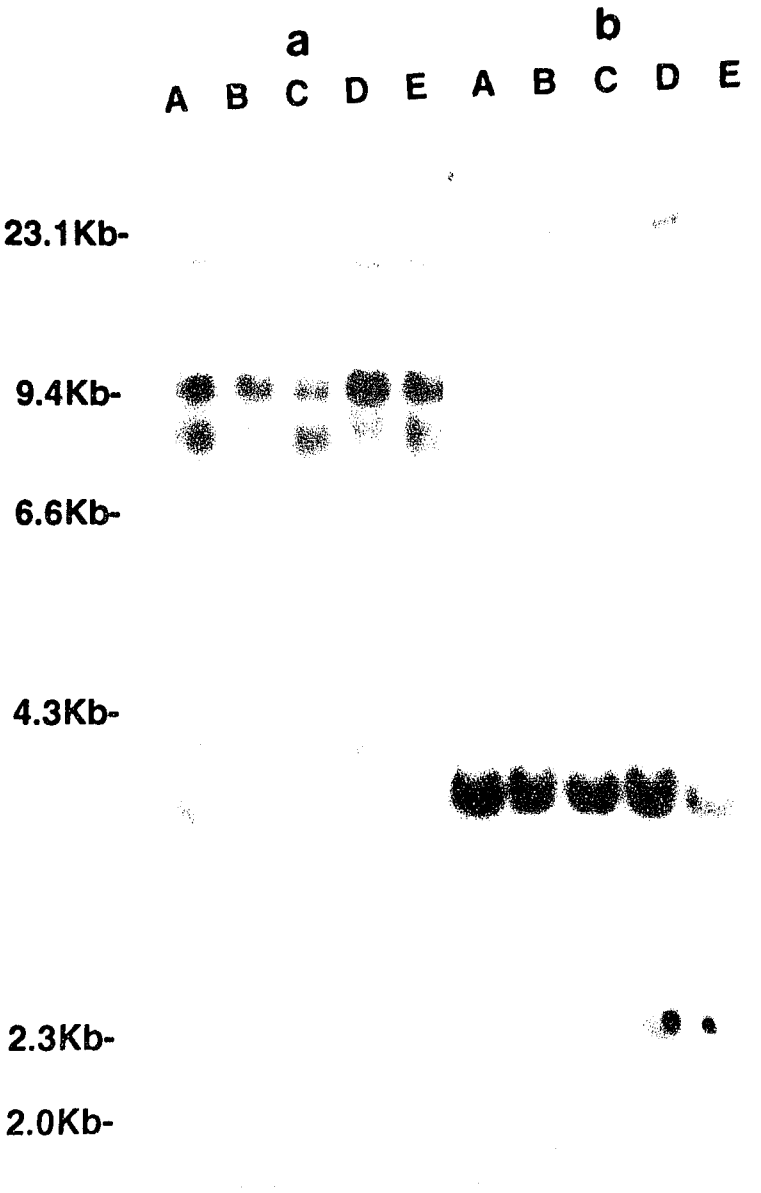


Figure 20.
Southern blot analysis of DCC gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for DCC gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with Bgl II endonuclease.
b: Restriction enzyme digestion with EcoRI endonuclease.

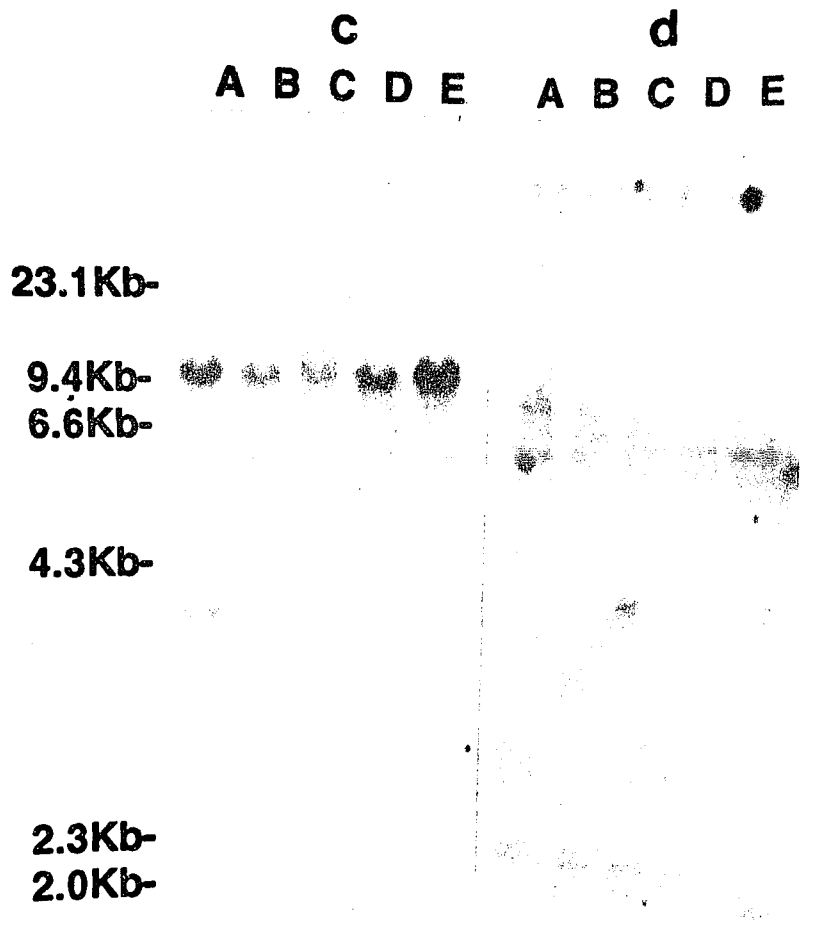


Figure 21.
Southern blot analysis of DCC gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for DCC gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
c: Restriction enzyme digestion with Hind III endonuclease.
d: Restriction enzyme digestion with Pst I endonuclease.

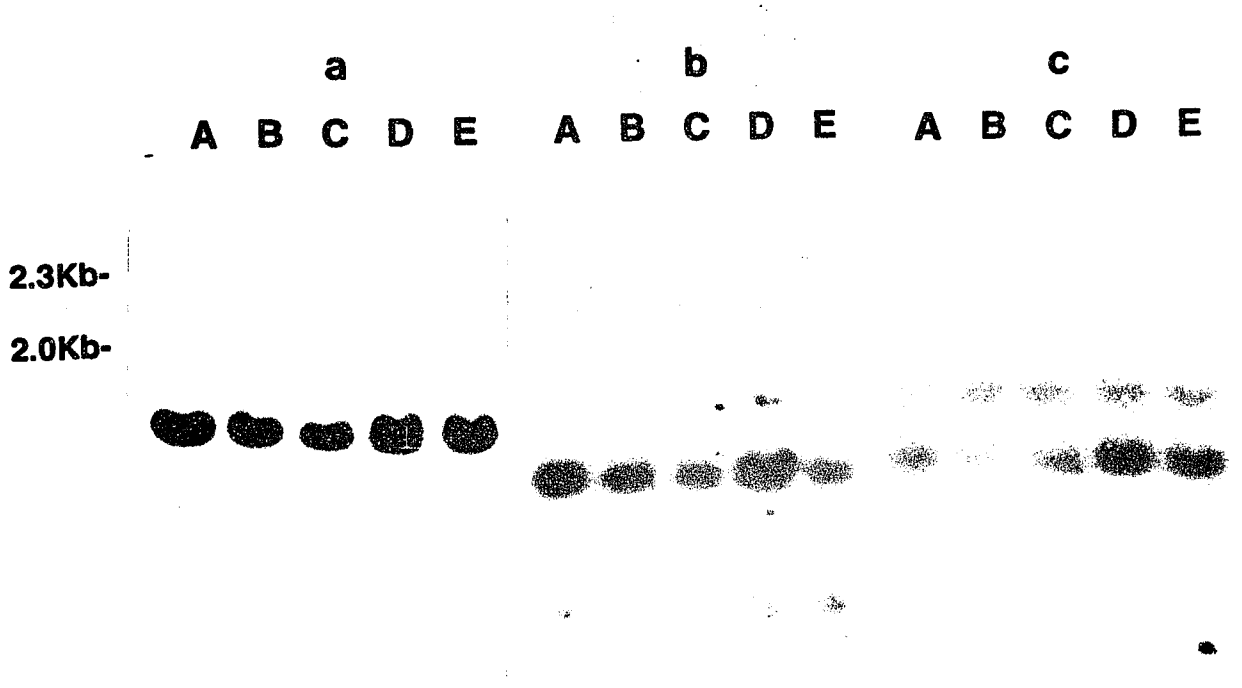


Figure 22.
Southern blot analysis of PRAD-1 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for PRAD-1 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with EcoRI endonuclease.
b: Restriction enzyme digestion with Pvu II endonuclease.
c: Restriction enzyme digestion with Pst I endonuclease.

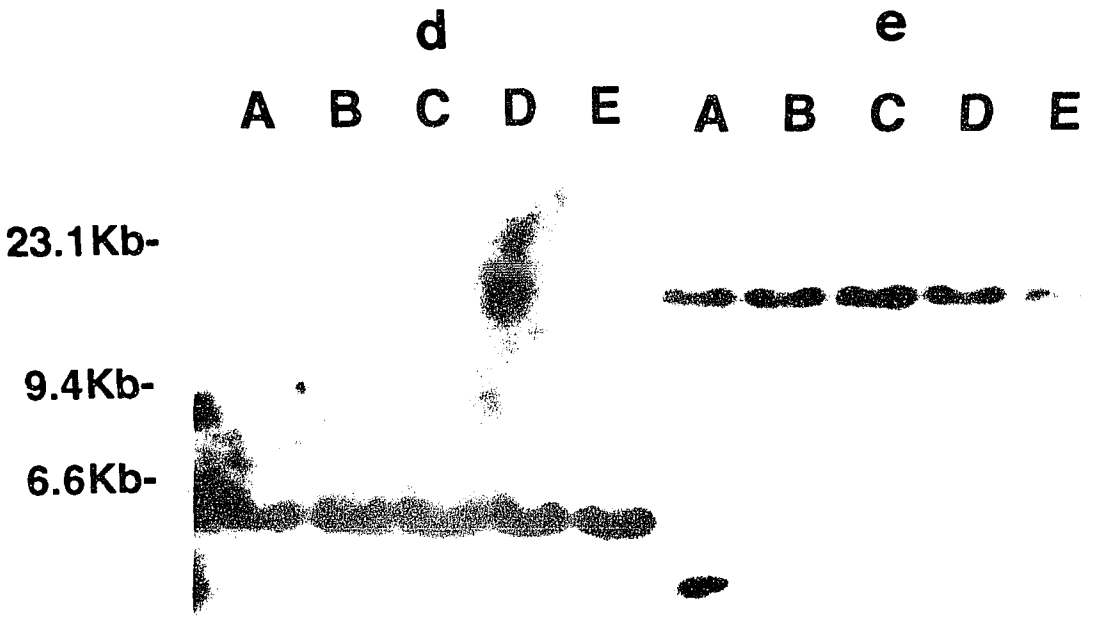


Figure 23.
Southern blot analysis of PRAD-1 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for PRAD-1 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
d: Restriction enzyme digestion with Bgl II endonuclease.
e: Restriction enzyme digestion with Hind III endonuclease.

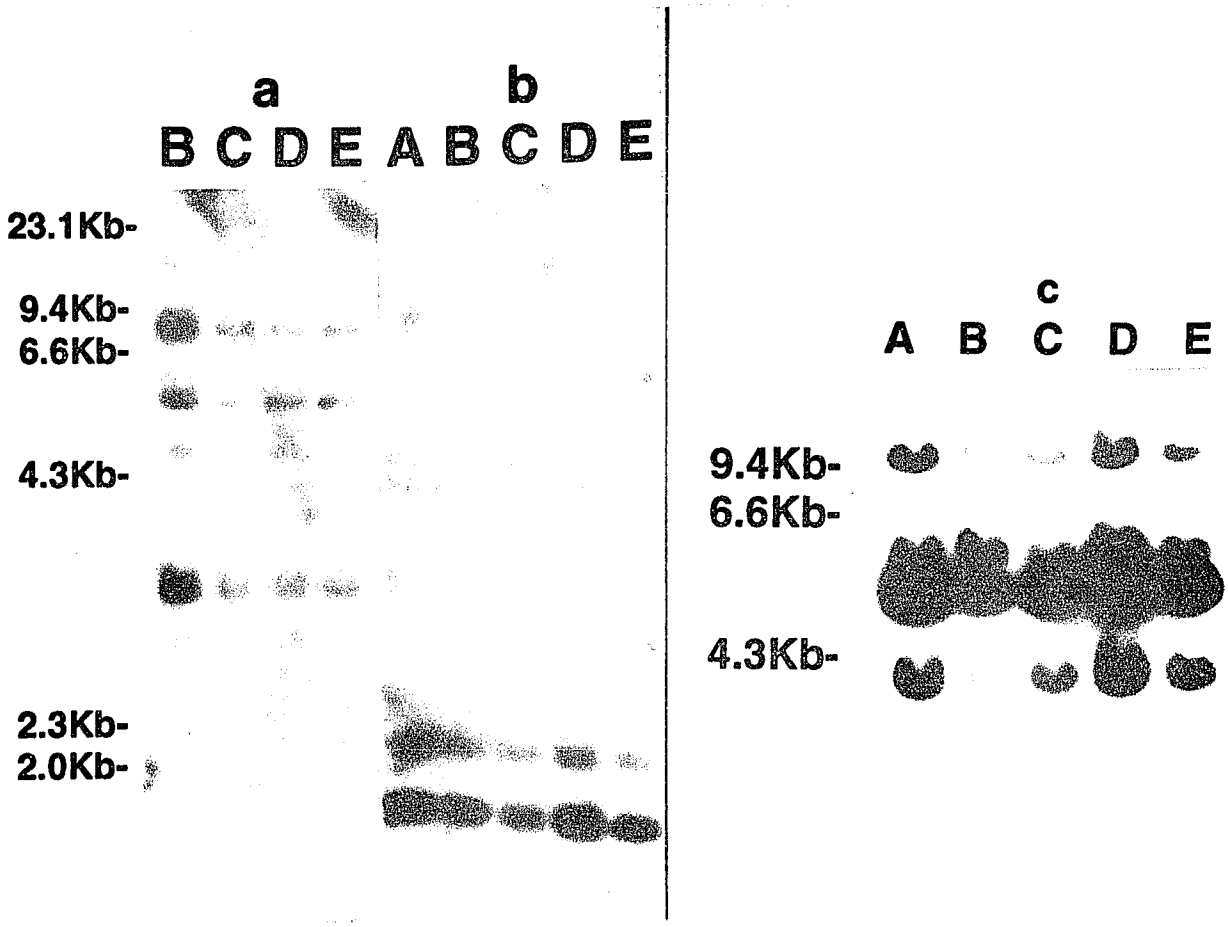


Figure 24.

Southern blot analysis of TCR gene in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for TCR gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

a: Restriction enzyme digestion with Hind III endonuclease.

b: Restriction enzyme digestion with Pvu II endonuclease.

c: Restriction enzyme digestion with BamHI endonuclease.

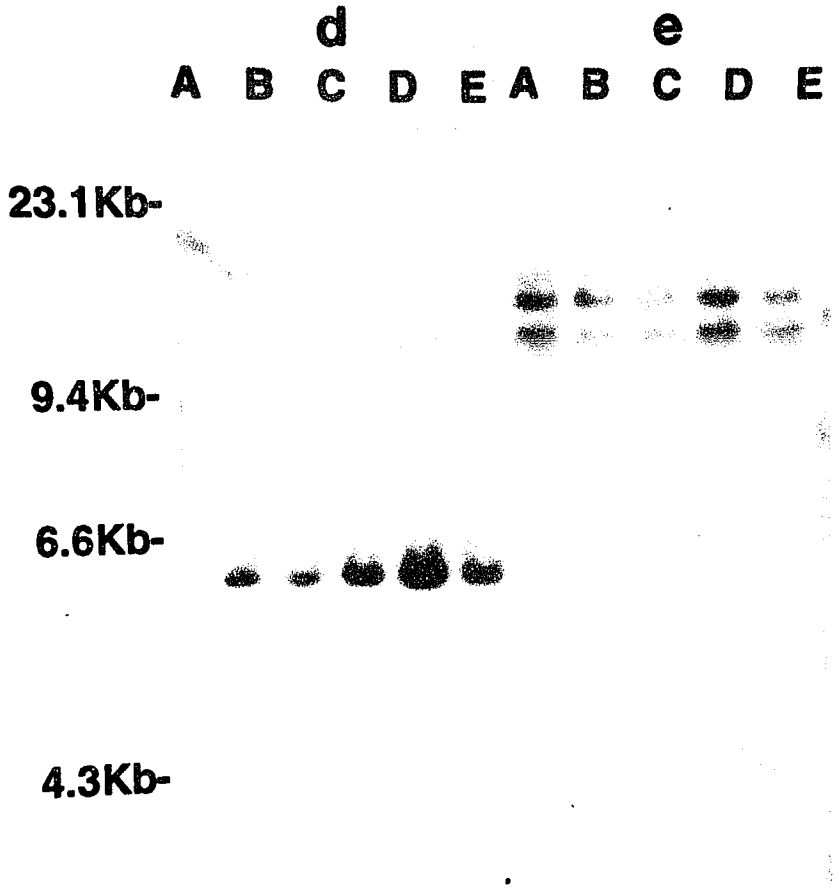


Figure 25.
Southern blot analysis of TCR gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for TCR gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
d: Restriction enzyme digestion with EcoRI endonuclease.
e: Restriction enzyme digestion with Bgl II endonuclease.

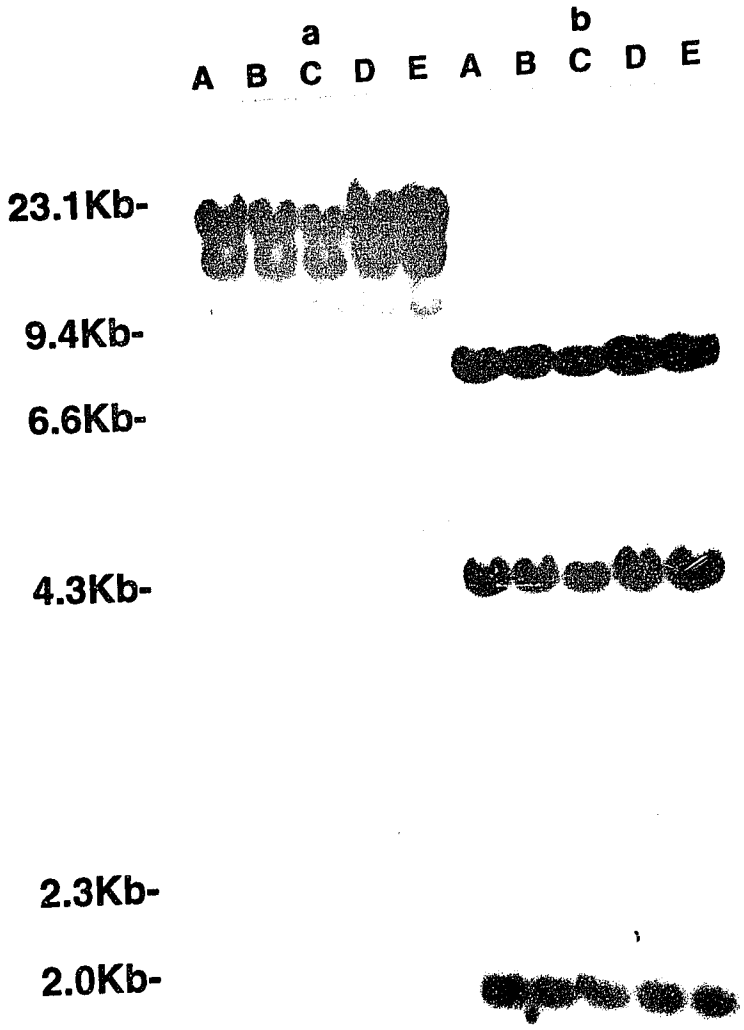


Figure 26.
Southern blot analysis of APC and FAD genes in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for APC and FAD gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with BamHI endonuclease.
b: Restriction enzyme digestion with Pvu II endonuclease.

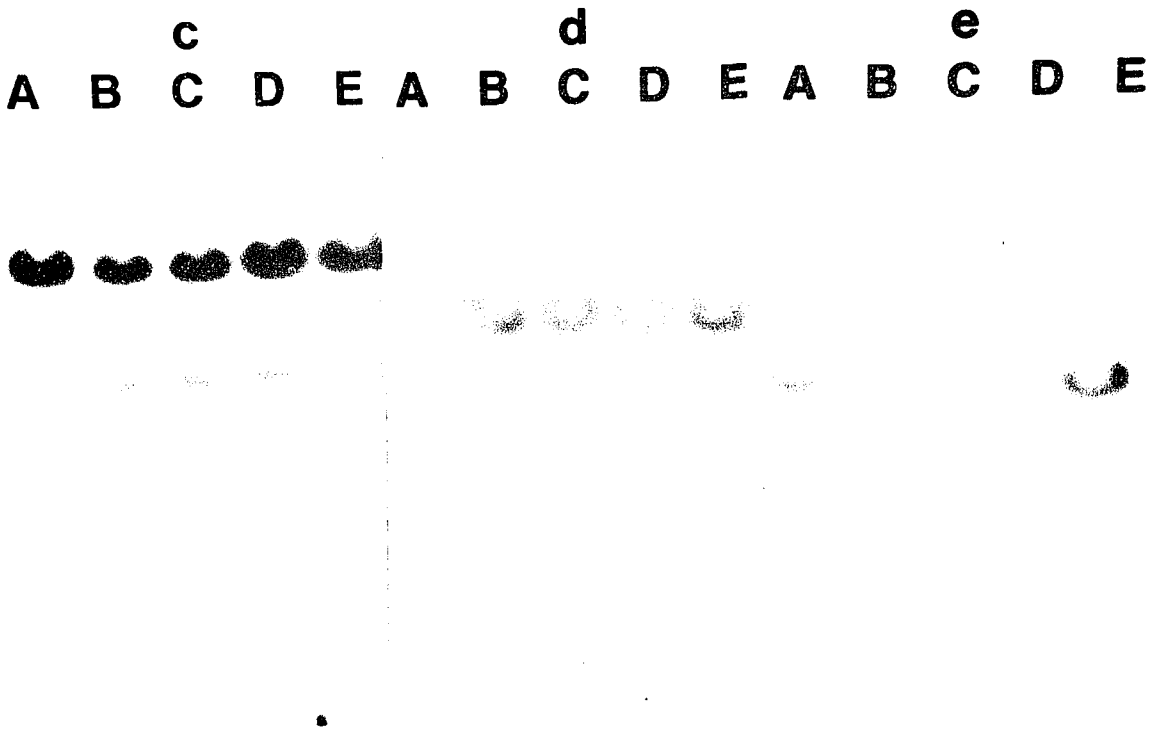


Figure 27.
Southern blot analysis of APC and FAD genes in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for APC and FAD gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
c: Restriction enzyme digestion with EcoRI endonuclease.
d: Restriction enzyme digestion with Hind III endonuclease.
e: Restriction enzyme digestion with Bgl II endonuclease.

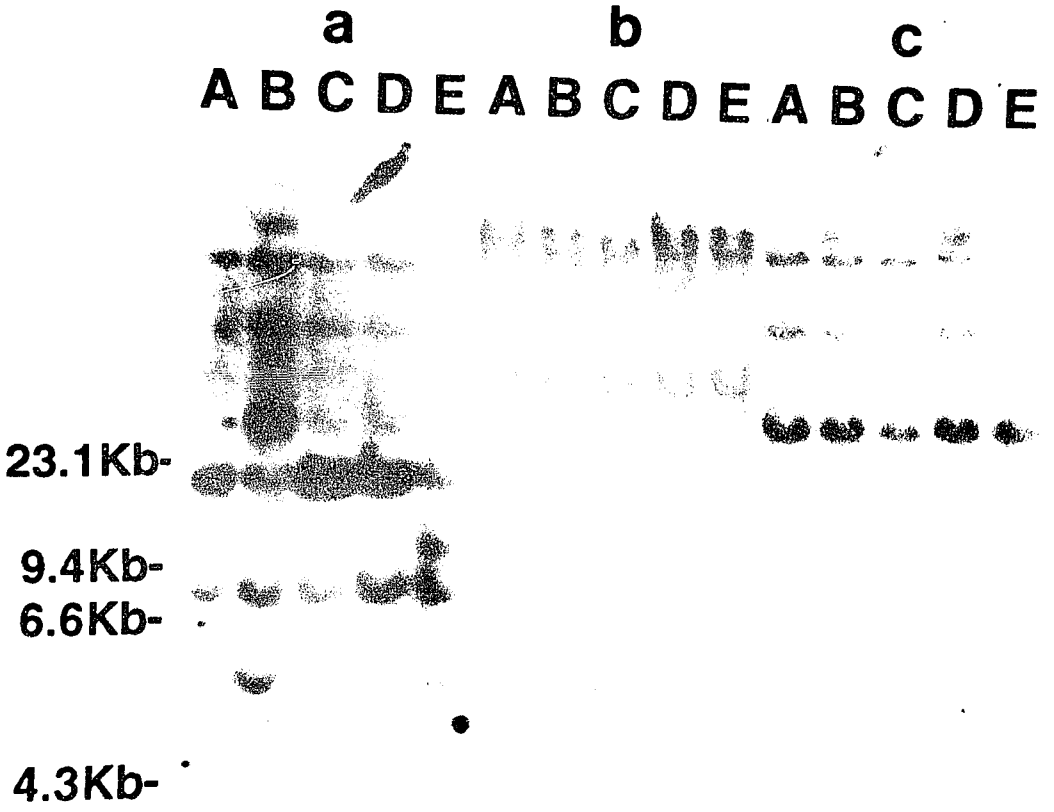


Figure 28.
Southern blot analysis of K-RAS-2 gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for K-RAS-2 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with Bgl II endonuclease.
b: Restriction enzyme digestion with BamHI endonuclease.
c: Restriction enzyme digestion with Pvu II endonuclease.

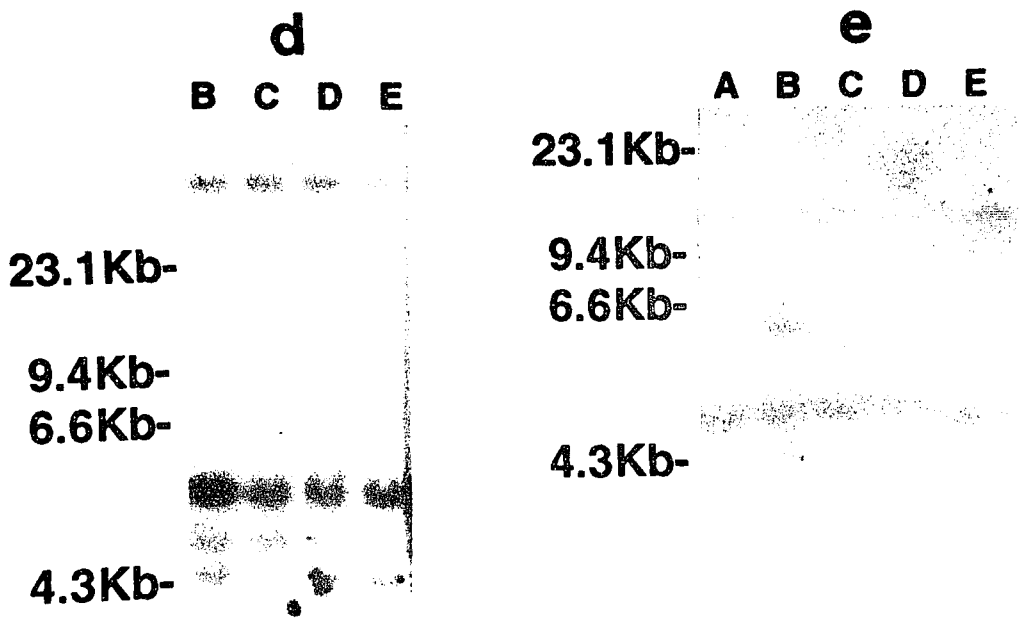


Figure 29.

Southern blot analysis of K-RAS-2 gene in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for K-RAS-2 gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

d: Restriction enzyme digestion with Hind III endonuclease.

e: Restriction enzyme digestion with EcoRI endonuclease.

revealed when the cell lines were initially digested with four restriction endonucleases *(Figure 30, 31).* However, the MCC gene was determined to be rearranged in the immortal cell line AR-5, when additional restriction enzymes were employed *(Figure 32).* Northern analysis showed a 10 Kb band corresponding to the MCC gene product as expected *(Figure 33/a).* Quantitative RNA dot blot analysis demonstrated that the RNA levels of MCC were 100% elevated in AR-5 cells over those in preimmortal cells *(Figure 33/b,c,d).* The rearrangement of MCC (on 5q21) could be possibly correlated either with the translocation t(5;18)(q21;q21) or with the integration of SV40 in 5q21-22. However, it is possible that the translocation itself was a product of the integration event (Henderson, 1982). No other preimmortal (tsA-0) and immortal cell lines (tsA-A, HAL) (containing the same SV40 integration side) had the MCC rearrangement, nor an increase in mRNA levels. The rearrangement and the altered expression of the MCC tumor suppressor gene, regardless of its possible correlation with the translocation t(5;18) or SV40 integration site, suggests that it could be involved in the progression of the immortal phenotype of the cell line AR-5. The mechanism by which MCC could be involved in immortalization is by cooperation with the putative senescence related gene(s) on chromosome 6. This hypothesis is supported by the findings that MCC gene has mainly an indirect role in tumorigenesis. The mutations observed in MCC in some colon carcinomas may follow upon altered activity of another gene acting earlier in the pathway of progression to colon cancer (Joslyn *et al.*, 1991). The data strongly suggest that a combination of events is necessary

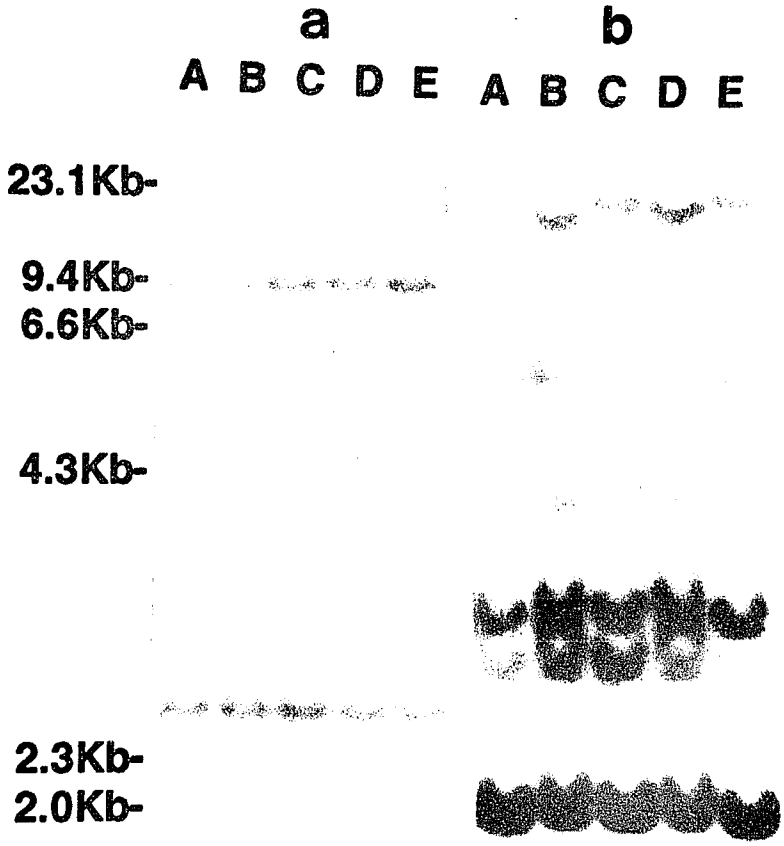


Figure 30.
Southern blot analysis of MCC gene in cellular DNA from all cell lines.
High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for MCC gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.
A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).
a: Restriction enzyme digestion with Hind III endonuclease.
b: Restriction enzyme digestion with EcoRI endonuclease.

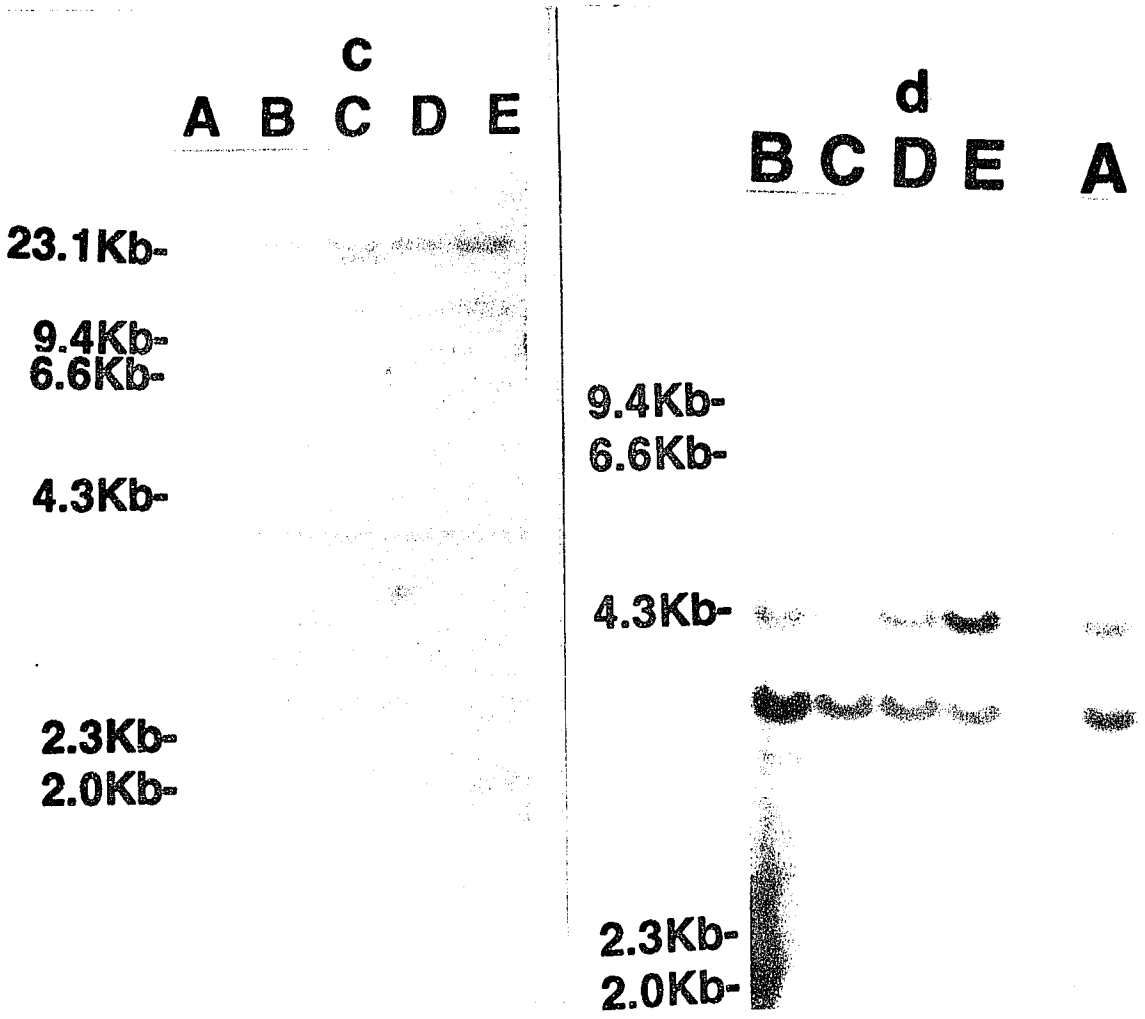


Figure 31.

Southern blot analysis of MCC gene in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for MCC gene was hybridized to the blot. All cell lines show the same hybridization pattern (Germ line), demonstrating no rearrangements of this gene in any of the cell lines.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

c: Restriction enzyme digestion with Bgl II endonuclease.

d: Restriction enzyme digestion with Taq I endonuclease.

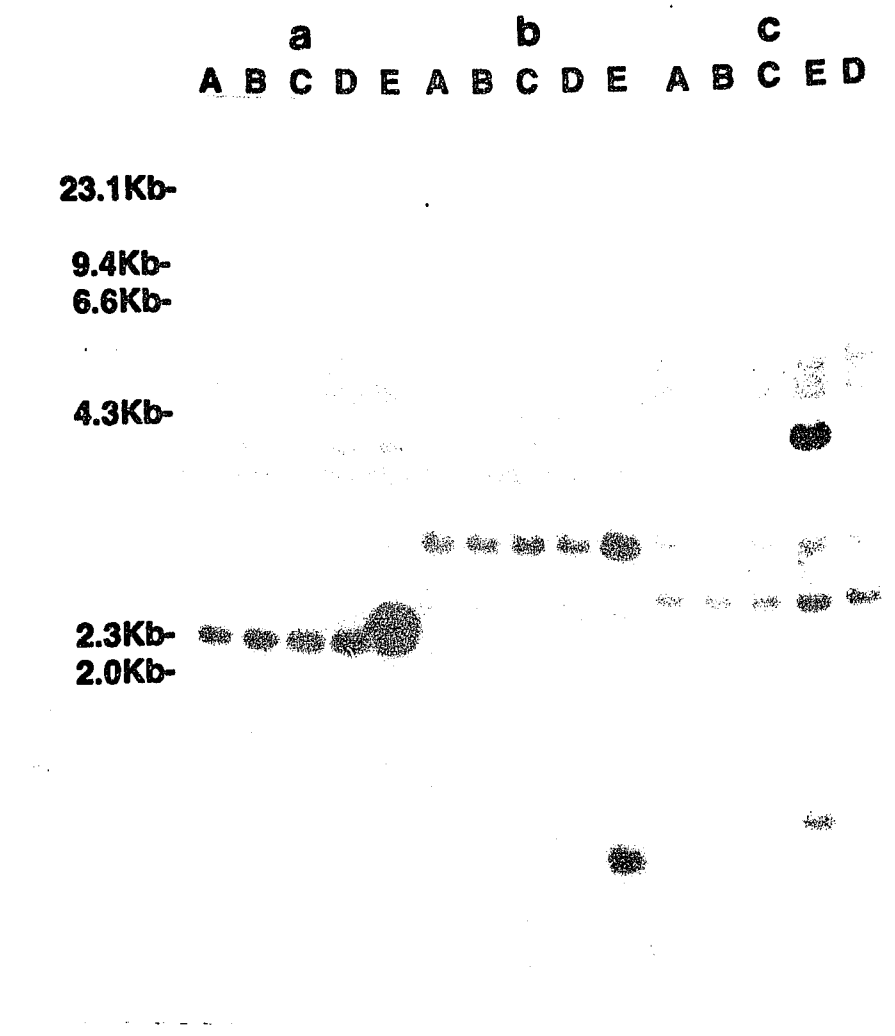


Figure 32.

Southern blot analysis of MCC gene in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for MCC gene was hybridized to the blot. The AR-5 cell line shows different hybridization pattern than all the other cell lines, demonstrating rearrangement of this gene. The intenced band on cell line AR-5 demonstates also DNA amplification.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

a: Restriction enzyme digestion with Pvu II endonuclease.

b: Restriction enzyme digestion with Pst I endonuclease.

c: Restriction enzyme digestion with BamHI endonuclease.

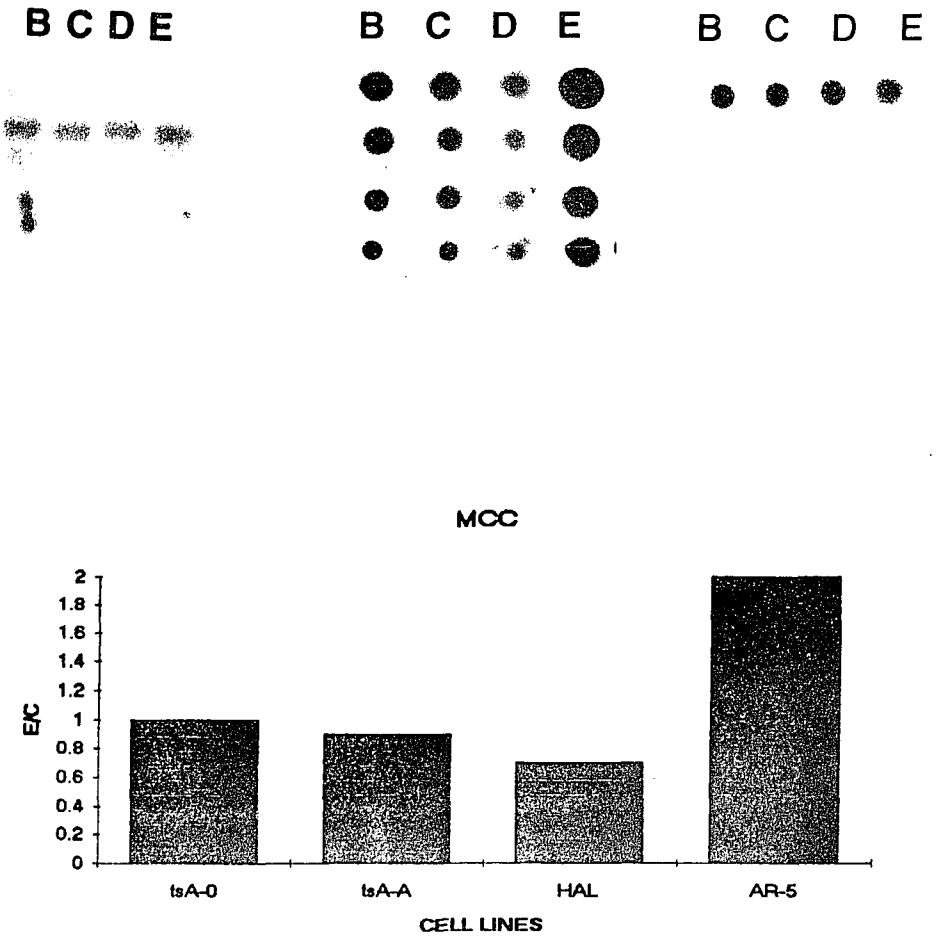


Figure 33.

Northern blot and quantitative RNA dot blot analysis of the MCC gene.

a: Total RNA (10 ug) was separated by electrophoresis through a denaturing gel and transfer to nylon membranes. The RNA of the northern blot was then hybridized with cDNA MCC probe (same as used with Southern blot) and showed the 10 kb RNA of MCC.

b and c: The same preparation of RNA was used for the preparation of two dot blots. One of them was hybridized with cDNA MCC probe (b:experimental) and the other was hybridized with GAPDH probe (c:control)

d: The level of MCC gene expression is showed on a graph after quantitation of the experimental and normalization with the control.

B:(Preimmortal tsA-0/p8); C:(Immortal tsA-A/p31); D:(Immortal HAL/p33); E:(Immortal AR-5/p35)

for a progression in the immortalization state. Another known gene in the area, APC, was also deregulated. APC is located only 100 kb distal to MCC (Kinzler *et al.*, 1991). The DNA of APC tumor suppressor gene was found to be in normal configuration. Further, Northern analysis showed a 9 Kb band corresponding to the APC gene product as expected *(Figure 34/a)*. The level of APC gene expression was, however, reduced *(Figure 34/b,c,d)*. The APC deregulation is important because this tumor suppressor gene has been found to have altered level of gene expression in Familial polyposis coli and colorectal cancer (Stanbridge, 1990). Therefore, APC deregulation could also be associated with the progression of the immortal phenotype.

The region of chromosome 5q21 which includes MCC and APC tumor suppressor genes was of interest in that it could be associated with SV40 integration and the possibly, resultant translocation t(5;18)in the cell line AR-5. This translocation was one of the few rearrangements noted among the cell lines analyzed. Similar translocations which involved the same region on chromosome 18q21 were observed in the immortal cell line SV/HF-39 (*Figure 15*), and have also been reported in other SV40-transformants (Ray *et al.*, 1992). The expression of bcl-2 gene located at the translocation region on chromosome 18q21 was examined for changes in the level of gene expression (*Figure 35/a).* The bcl-2 gene expression was elevated (about 50%) in tsA-A and AR-5 cell lines. *(Figure 35/b,c,d).* A known oncogenic function for bcl-2 is in blocking programmed cell death (Hockenbery *et al.*, 1991). Over-expression of this protein is thus an antidote

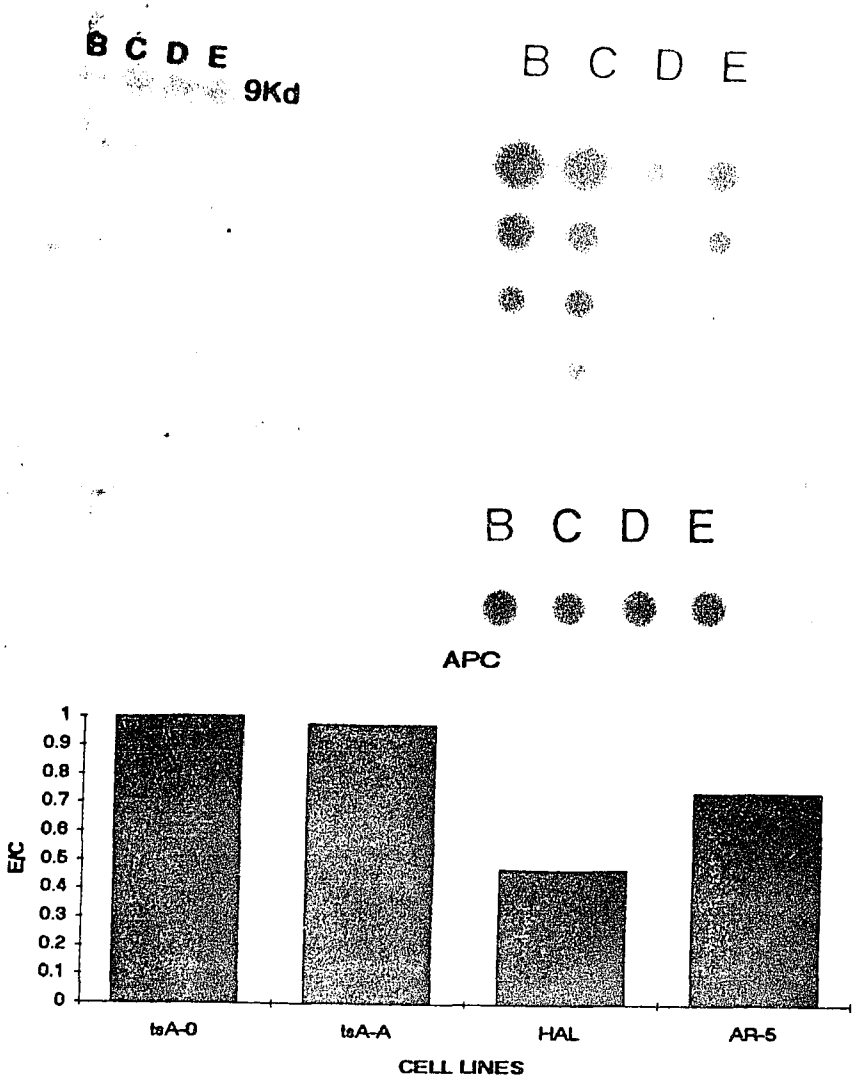


Figure 34.

Northern and quantitative RNA dot blots of the APC gene.

a: Total RNA (10 ug) was separated by electrophoresis through a denaturing gel and transfer to nylon membranes. The RNA of the northern blot was then hybridized with cDNA APC probe (same as used with Southern blot) and showed the 9 kb RNA of APC.

b and c: The same preparation of RNA was used for the preparation of two dot blots. One of them was hybridized with cDNA APC probe (b:experimental) and the other was hybridized with GAPDH probe (c:control)

d: The level of APC gene expression is showed on a graph after quantitation of the experimental and normalization with the control.

B:(Preimmortal tsA-0/p8); C:(Immortal tsA-A/p31); D:(Immortal HAL/p33); E:(Immortal AR-5/p35).

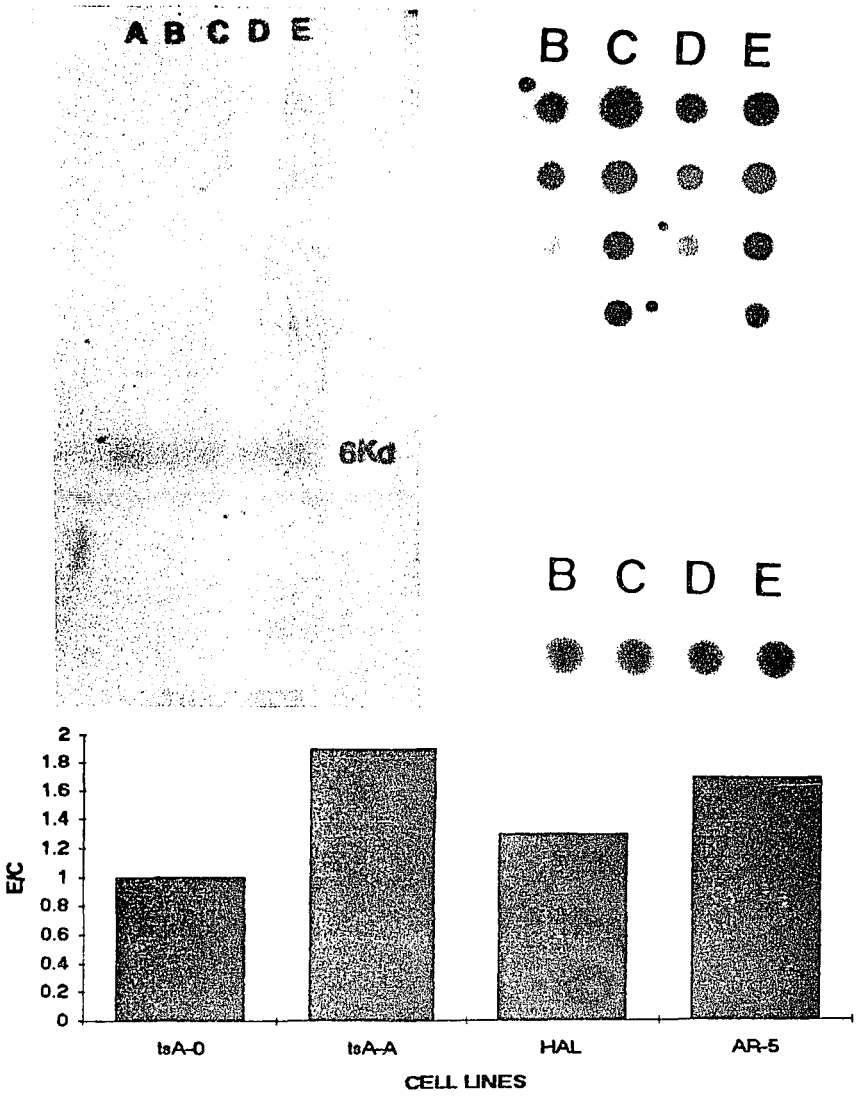


Figure 35.

Northern and quantitative RNA dot blots of the bcl-2 gene.

a: Total RNA (10 ug) was separated by electrophoresis through a denaturing gel and transfer to nylon membranes. The RNA of the northern blot was then hybridized with cDNA bcl-2 probe (same as used with Southern blot) and showed the 6 kb RNA of bcl-2.

b and c: The same preparation of RNA was used for the preparation of two dot blots. One of them was hybridized with cDNA bcl-2 probe (b:experimental) and the other was hybridized with GAPDH probe (c:control)

d: The level of bcl-2 gene expression is showed on a graph after quantitation of the experimental and normalization with the control.

B:(Preimmortal tsA-0/p8); C:(Immortal tsA-A/p31); D:(Immortal HAL/p33); E:(Immortal AR-5/p35).

to this process. Therefore, over-production of bcl-2 RNA and protein could result cell accumulation and tumor development, as well as a progression in the cell phenotype related to immortalization. An example of an involvement of bcl-2 protein overexpression in neoplasia is the human follicular lymphoma [t(8;14)] (Nunez *et al.*, 1989).

Another tumor suppressor gene on chromosome 18q21 is DCC (Fearon, 1990). Northern analysis showed a 10-12 Kb band which corresponded to the known DCC gene product *(Figure 36a).* The level of DCC gene expression was also determined *(Figure 36b,c,d).* The DCC gene expression was elevated about 50% in AR-5 cells *(Figure 35d, 36d).* DCC is involved in carcinogenesis and it is possible that it is also involved in immortalization. The DCC protein shows significant homology to the neural cell adhesion molecules (CAMs) and other related cell surface glycoproteins (Stanbridge, 1990). The protein is related to genes involved in cell surface interactions, and disruption of this protein could result the loss of cell-cell contact inhibition.

The conclusion from the analysis of chromosomal aberrations was that a progression in the immortalization phenotype involved activation or inactivation of multiple cooperating genes. The data also suggests that other gene(s) disrupted by chromosomal translocations and/or the SV40 integration site, are involved in the progression of the immortal phenotype of the cell line AR-5.

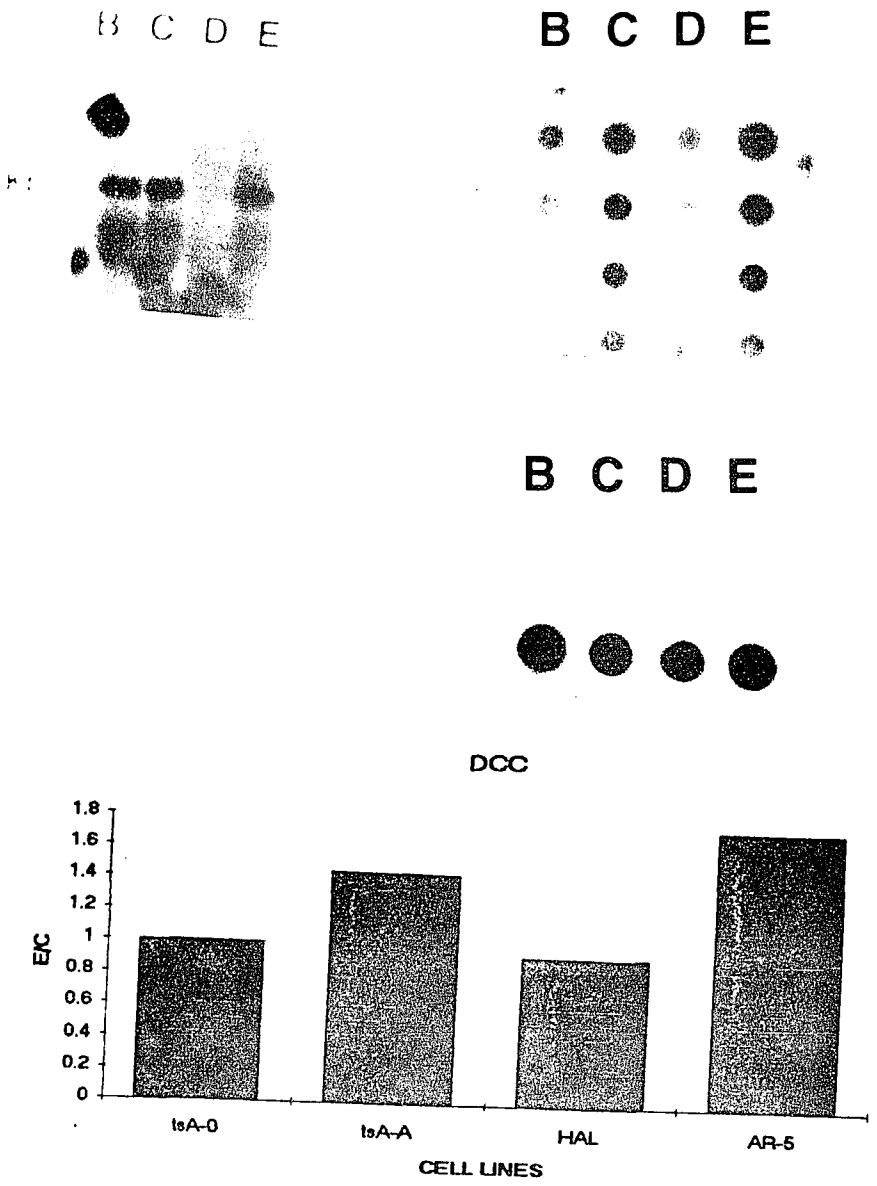


Figure 36.

Northern and quantitative RNA dot blots of the DCC gene.

a: Total RNA (10 ug) was separated by electrophoresis through a denaturing gel and transfer to nylon membranes. The RNA of the northern blot was then hybridized with cDNA DCC probe (same as used with Southern blot) and showed the 10-12 kb RNA of DCC.

b and c: The same preparation of RNA was used for the preparation of two dot blots. One of them was hybridized with cDNA DCC probe (b:experimental) and the other was hybridized with GAPDH probe (c:control)

d: The level of DCC gene expression is showed on a graph after quantitation of the experimental and normalization with the control.

B:(Preimmortal tsA-0/p8); C:(Immortal tsA-A/p31); D:(Immortal HAL/p33); E:(Immortal AR-5/p35).

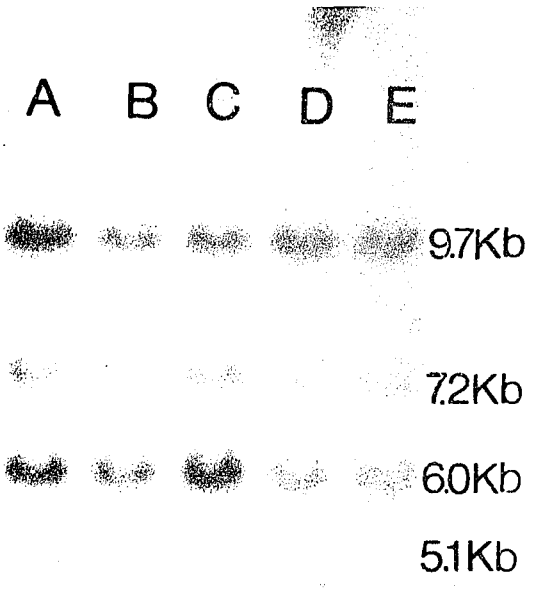
PART THREE: MOLECULAR ANALYSIS OF CHROMOSOME 6

A. Loss of copy number distal to chromosome 6q21; Molecular analysis.

Cytogenetic analysis identified non-random loss distal to chromosome 6q21 (6q21->ter) as the most common aberration among immortal cell lines of the lineage SVtsA/HF-A. More precisely, cell line tsA-A had a deleted chromosome 6 (6q21) and cell lines HAL and AR-5 have a missing chromosome 6. Non-random loss of the chromosome 6q was also found among the immortal cell lines of the SV/HF-5 lineage. The cell lines of this lineage (SV/HF-5) demonstrated very complex karyotypes with many unidentify chromosomes, thus most of the studies were done on the SVtsA/HF-A lineage which showed very few non-random chromosomal aberrations. These cytogenetic findings were confirmed by further analysis of the DNA.

The first experimental series demonstrated the presence of the 6p region in the 5 cell lines of the SVtsA/HF-A lineage. These experiment used a single-copy DNA probe homologous to the small arm (p) of chromosome 6, D6S10 (White *et al.*, 1987) with oligo-A synthetase (chromosome 11) as a quantitative control. The parental HS74 cells, early preimmortal tsA-0 cells and the non-clonal immortal cell line tsA-A, were shown to have two copies of the 6p arm as compared to one copy in HAL and AR-5. This was consistent with the cytogenetic analysis *(Figure 37).*

In a second analysis, the quantitative amount of the protooncogene c-*mas*-1 DNA (single copy on 6q24-27, Ravin *et al.*, 1987) was determined *(Figure 38).* Hybridization analysis showed that HS74 and tsA-0 cells had two copies of the 6q



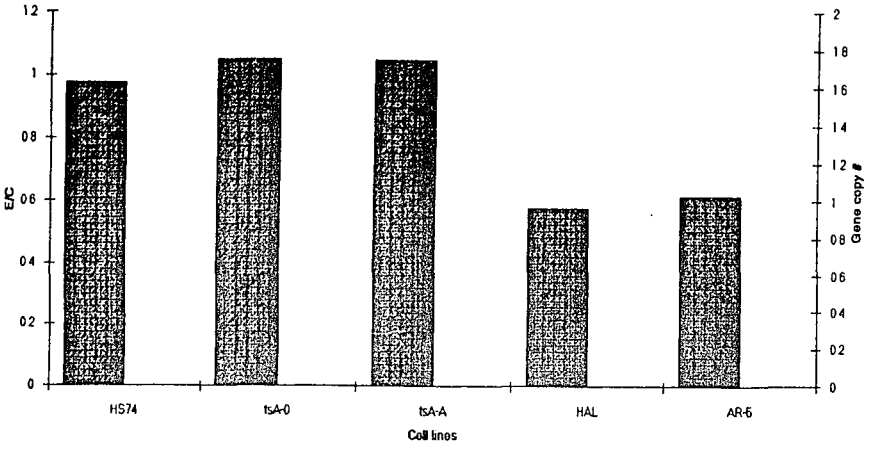


Figure 37.

Southern blot analysis of the single-copy DNA D6S10 (6p15) in cellular DNA from all cell lines.

DNA was prepared from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines. DNA was digested with Bgl II for analysis by Southern procedure and hybridized simultaneously with ^{32}P-labeled probes for the single-copy DNA D6S10 and oligo-A synthetase which were radiolabeled seperately. The 9.7 Kb band for oligo-A synthetase provides an internal standard for quantitation. A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

Densitometry measurements shows the level of hybridization to the D6S10 probe after normalization to the internal control (oligo-A synthetase).

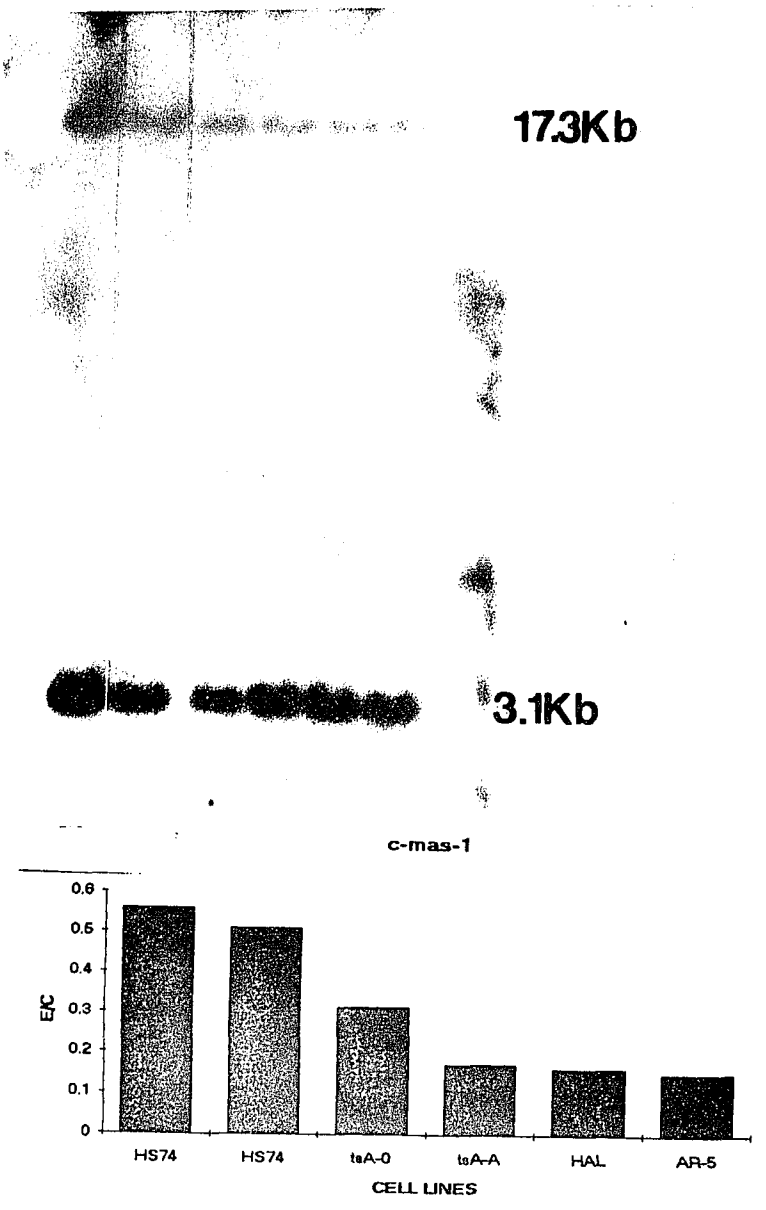


Figure 38.

Southern blot analysis of the c-mas-1 ongogene (6q24-27) in cellular DNA from all cell lines.

DNA was prepared from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines. DNA was digested with Hind III for Southern blot analysis and hybridized simultaneously with ^{32}P-labeled probes for c-mas-1 and oligo-A synthetase which had been radiolabeled seperately. A 3.1 Kb band for oligo-A synthetase provides an internal control for quantitation.

1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25); 4:(Immortal HAL/p24); 5:(Immortal AR-5/p27).

Densitometry measurements shows the level of hybridization to the c-mas-1 probe after normalization to the internal control (oligo-A synthetase).

arm as expected on the basis of karyotype. This should be compared to the one copy observed in tsA-A, HAL and AR-5 cells. These two experiments confirmed the previous cytogenetic findings in that the non-clonal immortal cell line tsA-A had a deletion 6q21 and HAL and AR-5 had a missing chromosome 6. This was important for further determination of the breakpoint.

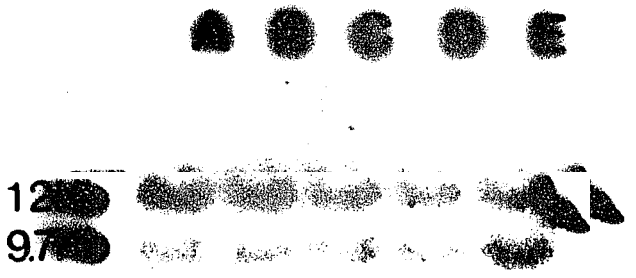
The non-random deletion of chromosome 6q21->ter among immortal cell lines was also confirmed by hybridization analyses using the protooncogenes c-*ros*-1 (6q21-22) (Sharma *et al.*, 1989) and c-*myb*-1 (6q22-23) (Frannchini *et al.*, 1983; Hubbard-Smith *et al.*, 1992). These experiments showed that DNA sequences distal to 6q21 are missing in the immortal cell lines. PCR assays also demonstrated a lkss of heterozygosity. Several microsatellite polymorphic dinucleotide repeats [$(CA)_n$] are localized distal to chromosome 6q21 *(Ozer, unpublished data).* Whereas HS74 and preimmortal cell line tsA-0 are heterozygous for each of these microsatellite polymorphic dinucleotide repeats [$(CA)_n$], the immortal cell lines are missing one of the DNA repeats. Interestingly, the two immortal lineages, AR-5 and HAL, have retained different $(CA)_n$ repeats. This indicates that the two immortal lineages, AR-5 and HAL, (cytogenetically characterized by missing one whole chromosome 6) have retained a different parental chromosome 6. The AR-5 line was derived from a clonal isolation immediately after transfection. HAL was derived from a non-clonal line established many generations after transfection. The results suggests that regions of either of the parental chromosomes 6 could be responsible for immortalization. This finding

also supports an immortalization hypothesis of "reduced dosage" of a gene (discussed later) directly associated with immortalization.

B. Flanking the exact breakpoint on chromosome 6 with single-copy DNA sequences.

The aims of this portion of the study were to flank the breakpoint on chromosome 6q, and determine more precisely the region of interest. This study was made on the basis of determining non-deleted single-copy DNA (two copies) and deleted single-copy DNA (one copy) in proximity to the putative breakage point on chromosome 6. The single-copy DNA probes were previously localized by hybridization *in situ* to this general region 6q11->22 (Golubic *et al.*, 1991).

DNA from the parental cells, HS74, the preimmortal cells, tsA-0, and immortalized derivatives, tsA-A, HAL and AR-5, were probed for the single-copy D6S116 (6q15) *(Figure 39).* The immortal cell lines had one copy of D6S116 DNA, as compared with two copies for normal HS74 and preimmortal cells. This finding proved that D6S116 DNA was located distal to the chromosome 6 breakpoint. Identical results were obtained when all normal, "preimmortal" and immortal cell lines were digested and probed for the single-copy D6S122 DNA (6q14), which is located closer to the centromere and breakpoint *(Figure 40).* Further analysis localized another single-copy DNA, D6S125, to 6q12 (Golubic *et al.*, 1991) *(Figure 41).* D6S125 DNA was not deleted in the immortal cell line tsA-A (which had a deleted chromosome 6q21); it is therefore distal to the breakpoint. This DNA was



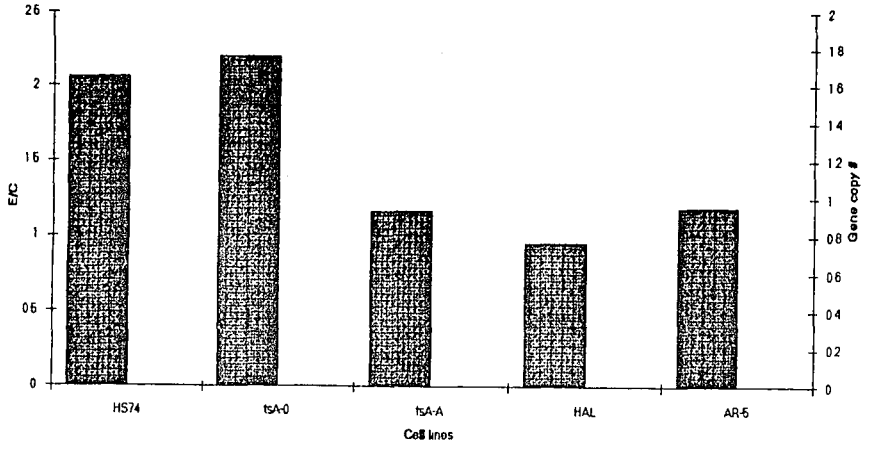


Figure 39.

Southern blot analysis of the single-copy DNA D6S116 (6q15) in cellular DNA from all cell lines.

DNA was prepared from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines. DNA was digested with bgl II for Southern blot analysis and hybridized with ^{32}P-labeled probes for D6S116 and oligo-A synthetase. A 9.7 Kb band for oligo-A synthetase provides an internal control for quantitation.

1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25); 4:(Immortal HAL/p24); 5:(Immortal AR-5/p27).

Densitometry measurements shows the level of hybridization to the D6S116 probe after normalization to the internal control (oligo-A synthetase).

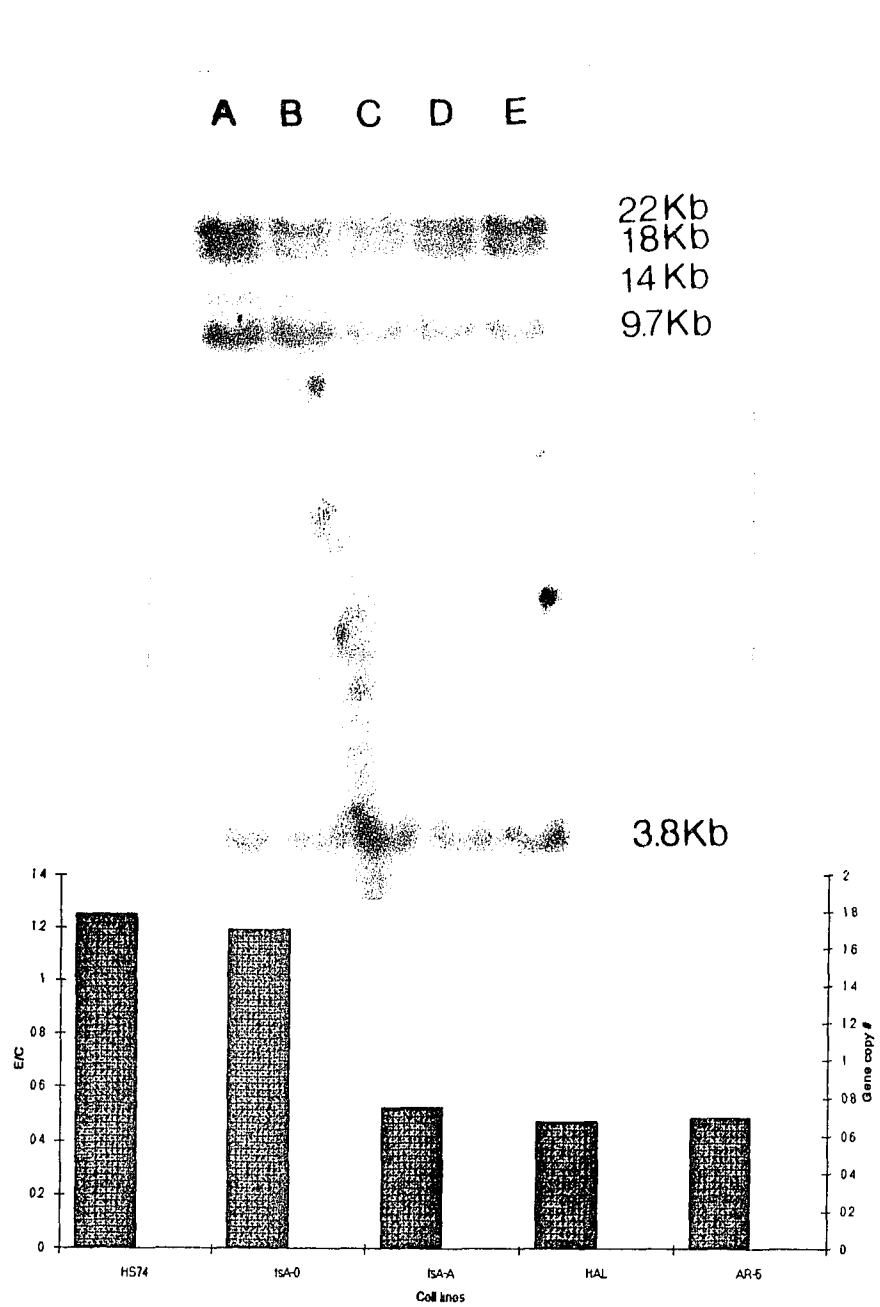


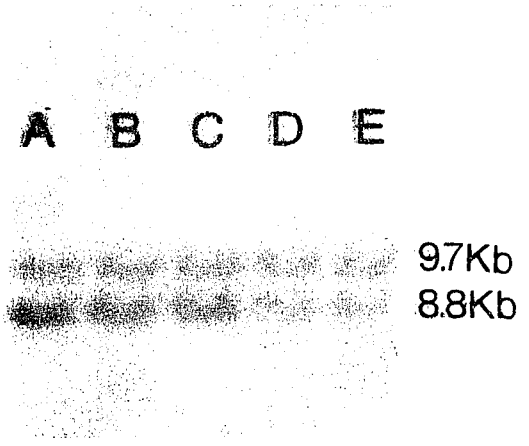
Figure 40.

Southern blot analysis of the single-copy DNA D6S122 (6q14) in cellular DNA from all cell lines.

DNA was prepared from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines. DNA was digested with bgl II for Southern blot analysis and hybridized simultaneously with ^{32}P-labeled probes for D6S122 and D15S16 (internal control). A 9.7 Kb band shows the hybridization signal from D6S122.

1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25); 4:(Immortal HAL/p24); 5:(Immortal AR-5/p27).

Densitometry measurements shows the level of hybridization to the D6S122 probe after normalization to the internal control (D15S16).



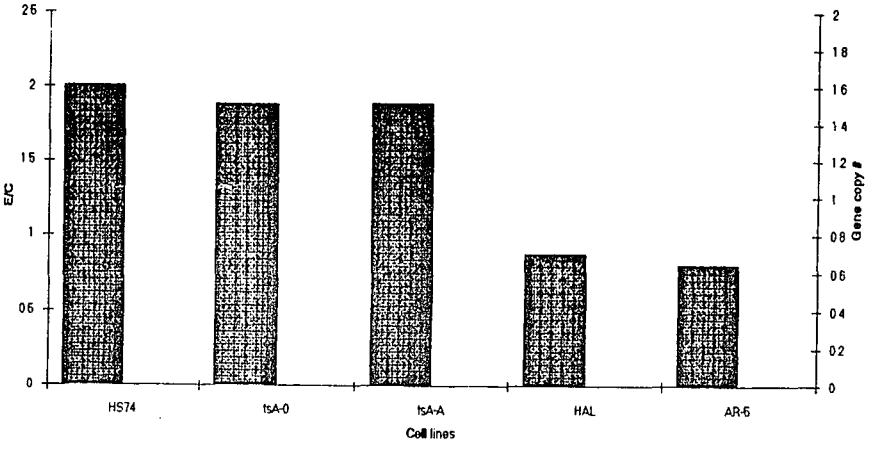


Figure 41.

Southern blot analysis of the single-copy DNA D6S125 (6q12) in cellular DNA from all cell lines.

DNA was prepared from normal HS74BM, preimmortal tsA-0 and the other immortal cell lines. DNA was digested with bgl II for Southern blot analysis and hybridized with ^{32}P-labeled probes for D6S125 and oligo-A synthetase (internal control). A 9.7 Kb band for oligo-A synthetase provides an internal control for quantitation.

1:(HS74BM/p7); 2:(Preimmortal tsA-0/p9); 3:(Immortal tsA-A/p25); 4:(Immortal HAL/p24); 5:(Immortal AR-5/p27).

Densitometry measurements shows the level of hybridization to the D6S125 probe after normalization to the internal control (oligo-A synthetase).

deleted in the immortal cell lines HAL and AR-5 and the results were consistent with a missing chromosome 6.

The molecular analysis confirms the cytogenetic findings of a deletion distal to chromosome 6q21 in cell line tsA-A and the absence of chromosome 6 in the cell lines, HAL and AR-5. Based on the DNA analysis, the breakage on chromosome 6 was determined to be at 6q13 since it was flanked by the two single-copy DNAs, D6S122 (6q14) and D6S125 (6q12). However, cytogenetic analysis showed a 6q21 deletion. It is possible that in the deleted chromosome 6, the 6q21 is not a terminal deletion, thus the chromosome 6's breakpoint is more proximal to the centromear and the rest of the telomeric region belongs to other chromosome. Another possibility is that the assigments for D6S122 and D6S125 with hybridization *in-situ* are not precisely correct by Gobulin *ed al.*, since this method is not perfect relative to the site of location. Further studies were designed to determine the discrepancy between the molecular and cytogenetic findings (see section E). These studies finally determined that 6q13 was the region where 6 "ends", therefore the location of D6S122 (6q14) and D6S125 (6q12) was also precisely confirmed.

C. Screening a YAC library with D6S116 and D6S122.

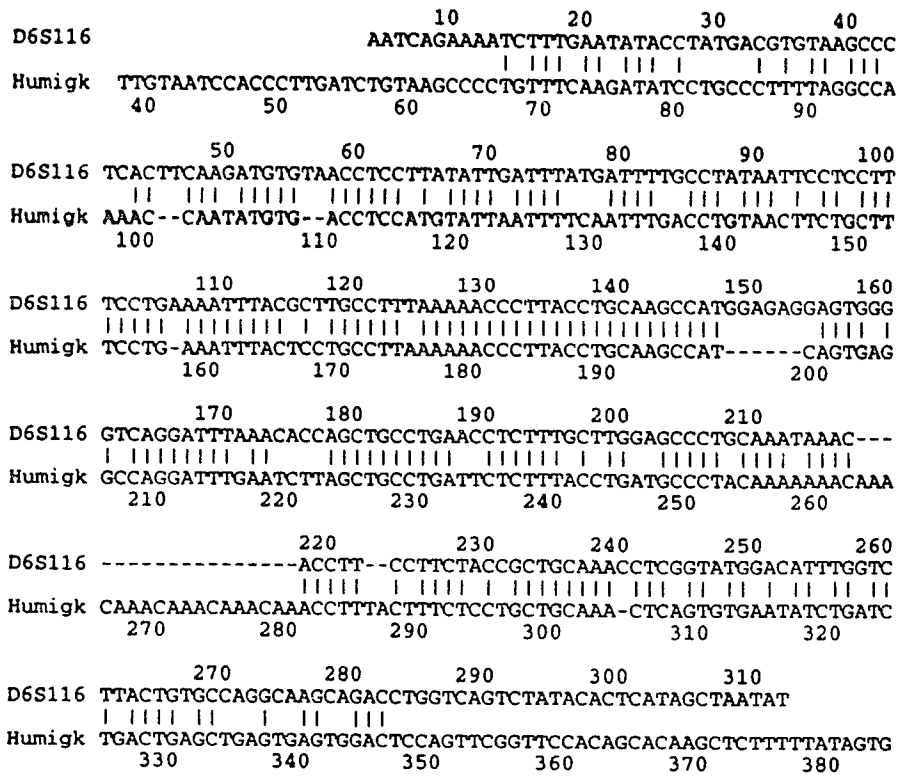
YAC library screening were done in an attempt to isolate as many DNA sequences as possible in close proximity to the chromosome 6 breakpoint. The YAC library (obtained from CEPH Institute in France) contains seven haploid

genome equivalents and average 450 kb of human DNA per YAC clone (Albertsen *et al.*, 1990). Many problems developed during this analysis, and therefore, the results were inconclusive.

The YAC library as provided from CEPH institute in France is designed to be screened by PCR. The only information obtained from the principal investigator (Gobulin *et al.*, 1991 and personal communication) about D6S116 and D6S122 DNAs was that they were unique and localized at 6q15 and 6q14, respectively. These DNAs do not overlap in homology and are assumed not to be contiguous. In order to screen the YAC library with the PCR, primers were made. DNA sequences of D6S116 and D6S122 were identified for this purpose.

The first 350 bp of both D6S116 and D6S122 DNAs was sequenced. Comparison of these sequences with all known human sequences found in Genbank revealed that D6S116 (6q15) had 68.8% homology with the human Igk variable immunoglobulin region. The D6S122 (6q14) DNA, (closest to the breakpoint) had no homology with any other human sequences. For this reason, only D6S122 DNA was used for the YAC library screening. Three sets of primers (24 bp) were designed to amplify D6S122 DNA *(Figure 42)*. A PCR reaction determined the efficiency and specificity of the primers by amplifying the expected size of DNA, from plasmid D6S122 an' genomic DNA *(Figure 43)*. The primers showed no amplification with pBR322, SV40, D6S116, lambda DNAs, demonstrating specificity and purity.

The YAC library contained approximately 100,000 YAC clones (Albertsen

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 51  TCAAGAATGG CCCACAGGGG GGGTGCTATT AAAAAATGGG AAGGAAAGCT
101  CATCATCACA TCGTCATCTT AGTTTCGCGG ATCATGATAG GCCCCTGCTT
151  ACCAGCCATT CATACGTTTC CTAGTAACTT ACATAGGAAG CTGTGCTTAC
201  CAAAGGTCCC TGCTGATTTG GCCCTTTCTA ATTCTCCAAG CCCATCTCAT
251  TCCACTTTTT CTCCTTTCCT CCGACTAAGC TCTAGCTCAC TGGCCTCATC
301  ACAGTTCCTT GAACACAGTG AATTTC
Product 211bp.

  1  CCACTCTACC TTTTCTCAGG TCCAGATCTA AGGAAATTAT TTTAAGTTAC
 51  TCAAGAATGG CCCACAGGGG GGGTGCTATT AAAAAATGGG AAGGAAAGCT
101  CATCATCACA TCGTCATCTT AGTTTCGCGG ATCATGATAG GCCCCTGCTT
151  ACCAGCCATT CATACGTTTC CTAGTAACTT ACATAGGAAG CTGTGCTTAC
201  CAAAGGTCCC TGCTGATTTG GCCCTTTCTA ATTCTCCAAG CCCATCTCAT
251  TCCACTTTTT CTCCTTTCCT CCGACTAAGC TCTAGCTCAC TGGCCTCATC
301  ACAGTTCCTT GAACACAGTG AATTTC
Product 286bp.

  1  CCACTCTACC TTTTCTCAGG TCCAGATCTA AGGAAATTAT TTTAAGTTAC
 51  TCAAGAATGG CCCACAGGGG GGGTGCTATT AAAAAATGGG AAGGAAAGCT
101  CATCATCACA TCGTCATCTT AGTTTCGCGG ATCATGATAG GCCCCTGCTT
151  ACCAGCCATT CATACGTTTC CTAGTAACTT ACATAGGAAG CTGTGCTTAC
201  CAAAGGTCCC TGCTGATTTG GCCCTTTCTA ATTCTCCAAG CCCATCTCAT
251  TCCACTTTTT CTCCTTTCCT CCGACTAAGC TCTAGCTCAC TGGCCTCATC
301  ACAGTTCCTT GAACACAGTG AATTTC
Product 238bp.
```

Figure 42.

DNA sequence analysis of the single-copy DNA D6S122.

A. Comparison of D6S116 DNA sequenses with all known human sequences found in Genbank reveal that the single copy DNA D6S116 (6q15) had 68.8% homology with the human Igk variable immunoglobulin region. B: 350bp sequense of the single-copy DNA D6S122 and three sets of primers (1,2,3) choose for PCR amplification of the locus.

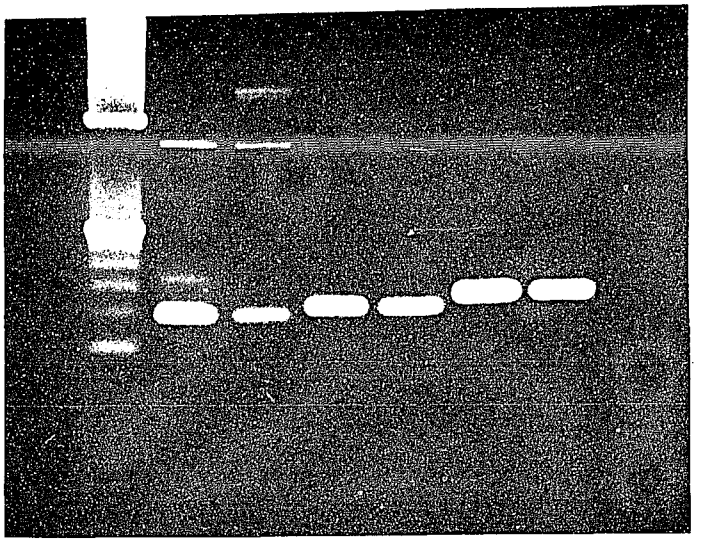


Figure 43.
PCR amplification of the single-copy DNA D6S122.
Three sets of primers from the single copy DNA D6S122 with the plasmid D6S122 and genomic DNA, demonstrated PCR amplification of the expected size.
1:100 bp ladder; 2:D6S122 with primer set #1; 3:Genomic DNA with primer set #1; 4:D6S122 with primer set #2; 5:Genomic DNA with primer set #2; 6:D6S122 with primer set #3; 7:Genomic DNA with primer set #3.

et al., 1990). The screening was divided in two phases: During the first phase, 113 DNA pools were screened with PCR. Each of these pools contained DNA from 8 microtiter plates; each microtiter plate contains 96 YAC clones. In the second phase, the positive DNA pool(s) were reported to the CEPH institute and they provided subpools of DNA from each positive pool. After the second screening, the positive subpools were again reported to the CEPH Institute. Based on the position of the positive subpools, they submitted a number of YAC clones for further processing and screening. Among those, at least one YAC was expected to be positive.

Primers from D6S122 amplifying 211 bp DNA *(Figure 42)* were run in a PCR reaction with genomic and plasmid DNA as positive controls and with no DNA as negative control. All DNA pools were screened with the same way and two positive pools were found, #47 and #60 *(Figure 44).* All 113 pools were screened again with a different set of primers (286 bp product, see figure 42) and again the #47 and #60 pools were positive *(Figure 45/a).* Stringent hybridizations demonstrated that the amplified DNA was homologous to D6S122 locus *(Figure 45/b).* The above result was also repeated with the third set primers which amplify a different size of DNA (238 bp product, see *Figure 42*).

The positive DNA pools (#47, #60) were reported to CEPH and a set of primers for this locus was obtained from CEPH. They provided 28 subpools of DNA from each positive pool found. During the second screening, a problem of non-specific amplification in PCR arose. Almost all subpools showed positive

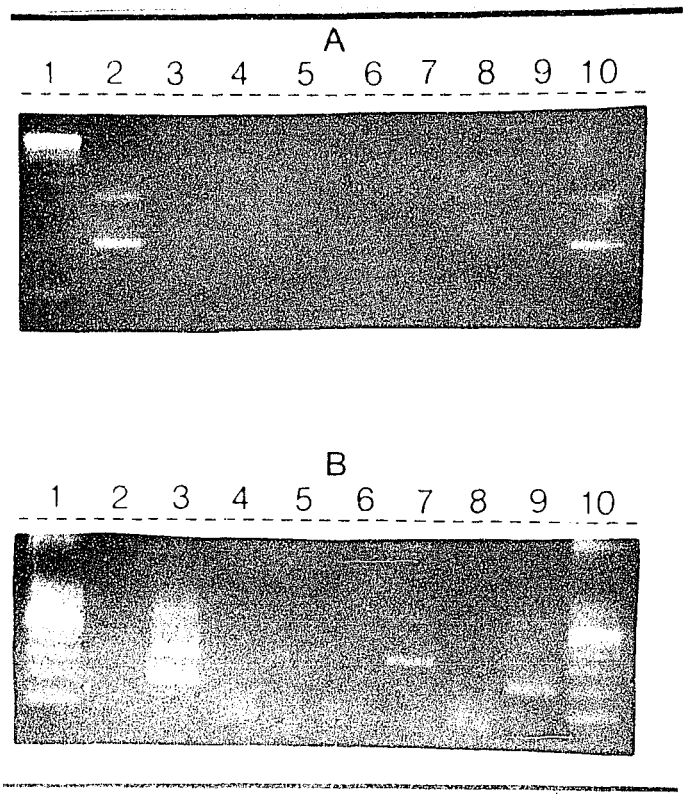
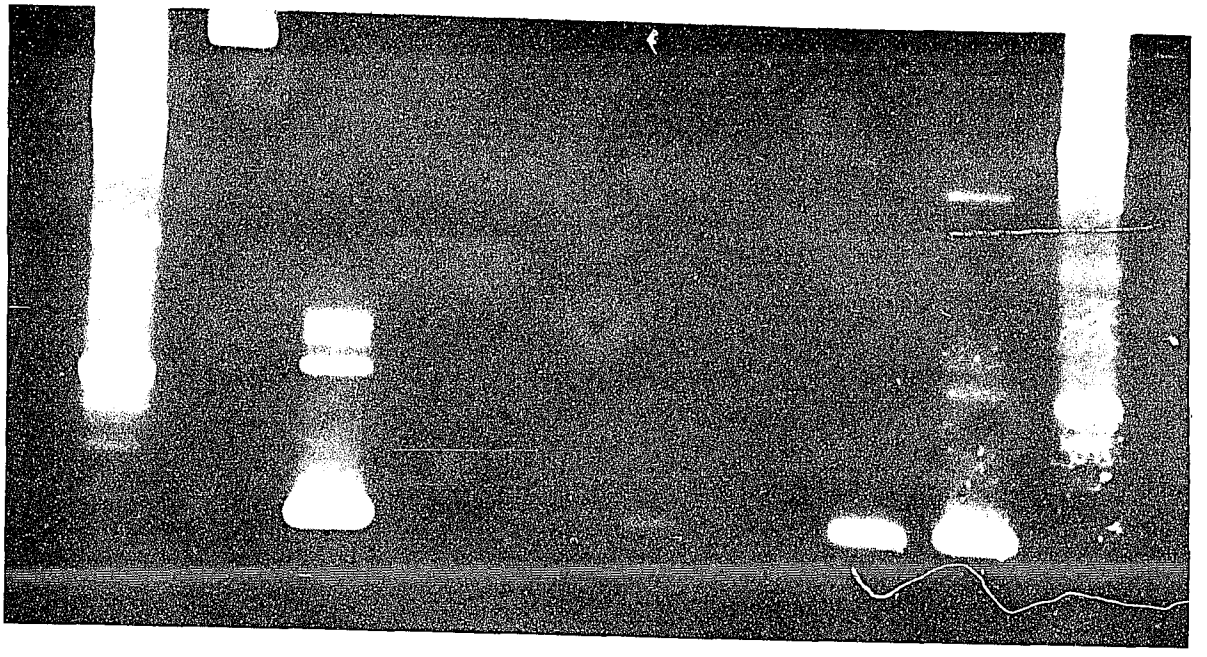


Figure 44.
PCR amplification of DNA YAC library pools.
Primers from D6S122 amplifying 211bp DNA band, were used in a PCR reaction. YAC DNA pool was amplified along with genomic and plasmid DNA as positive controls and with no DNA as negative control. Products of PCR amplification were run in 2% agarose gel and DNA was vizualised with EtBr staining.
A: None of the first six DNA pools showed any amplification (lanes 3-8).
1:100 bp ladder; 2:D6S122 DNA; 3:Pool #1; 4:Pool #2; 5:Pool #3; 6:Pool #4; 7:Pool #5; 8:Pool #6; 9:No DNA; 10: Genomic DNA.
B: DNA pools #47 and #60 showed positive amplification.
1:100 bp ladder; 2:No DNA; 3:D6S122 DNA; 4:Pool #59; 5:Pool #60; 6:Pool #46; 7:Pool #47; 8:Pool #49; 9:Genomic DNA; 10:100 bp ladder.



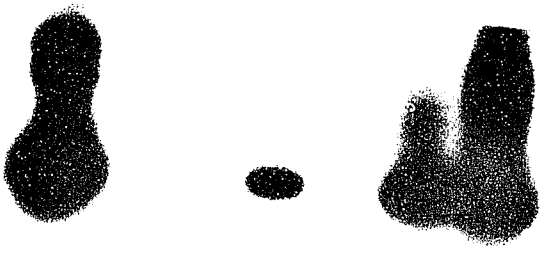


Figure 45.
PCR amplification and Southern analysis of positive YAC pools.
A: Primers from D6S122 amplifying 286bp DNA band, were used in a PCR reaction. YAC DNA pool was amplified along with genomic and plasmid DNA as positive controls and with no DNA as negative control. Products of PCR amplification were run in 2% agarose gel and DNA was vizualised with EtBr staining. DNA pools #47 and #60 showed positive amplification. **B:** Southern hybridization of the above gel with a D6S122 probe.
1:100 bp ladder; 2:No DNA; 3:D6S122 DNA; 4:Pool #43; 5:Pool #68; 6:Pool #60; 7:Pool #35; 8:Pool #47; 9:Genomic DNA; 10:100 bp ladder.

amplification with all sets of primers from D6S122 *(Figure 46)*. The non-specific amplification was also reported from the CEPH Institute, using the same primers. The phenomenon of non-specific amplification has been reported as a common occurrence (Green *et al.*, 1990). To overcome this problem, the following approaches were made. We first addressed the question of whether the primers for D6S122 gave positive amplification with yeast DNA. A PCR reaction with yeast DNA and D6S122 primers was run. Unfortunately, the answer was yes; primers for D6S122 amplified yeast DNA (*Figure 42).* The second approach was to determine if the product of yeast amplification was the same as the product from D6S122 amplification. Southern analysis demonstrated that the product of yeast amplification was not the same with the product from D6S122 amplification *(Figure 47).*

After the problem of non-specific amplification was overcome, the screening of the subpools continued. The 29 DNA subpools that were positive for pool #47 were screened with PCR followed by Southern hybridization. This experiment demonstrated two positive subpools (#16, #22) *(Figure 48).* The other 29 DNA subpools that were received for positive pool #60 were processed in the same way, but positive subpools were found *(Figure 49).* The autoradiographies were sent to CEPH institute in France. Based on which YAC DNA each of the subpools contained, they sent six YAC clones, of which one was expected to be positive.

The six YAC clones were analyzed with PFGE and Southern blots. DNA from the complete DNA D6S122 and purified DNA of the 238 bp amplified product

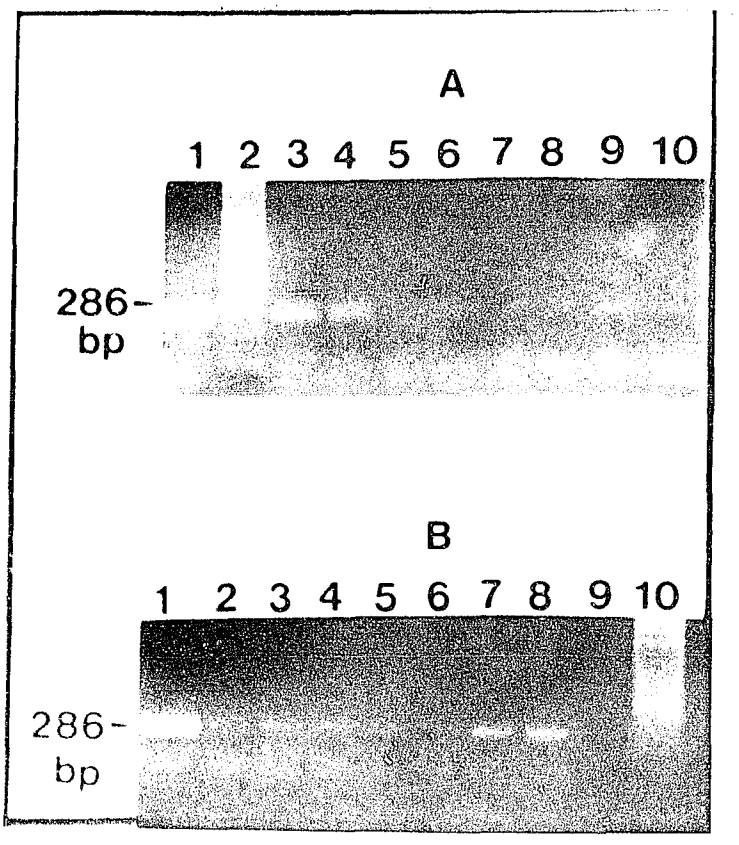


Figure 46.
PCR amplification of DNA YAC library subpools.
Primers from D6S122 amplifying 286bp DNA band, were used in a PCR reaction. YAC DNA subpools (**A:** #47, **B:** #60) were amplified along with genomic and plasmid DNA as positive controls and with no DNA as negative control. Products of PCR amplification were run in 2% agarose gel and DNA was vizualised with EtBr staining. Almost all subpools showed non-specific amplification.
A: 1:D6S122 DNA; 2:100 bp ladder; 3:Subpool #1; 4:Subpool #2; 5:Subpool #3; 6:Subpool #4; 7:Subpool #5; 8:Subpool #6; 9:Subpool #7; 10:Subpool #8.
B: 1:D6S122 DNA; 2:Subpool #1; 3:Subpool #2; 4:Subpool #3; 5:Subpool #4; 6:Subpool #5; 7:Subpool #6; 8:Subpool #7; 9:No DNA; 10:100bp DNA ladder.

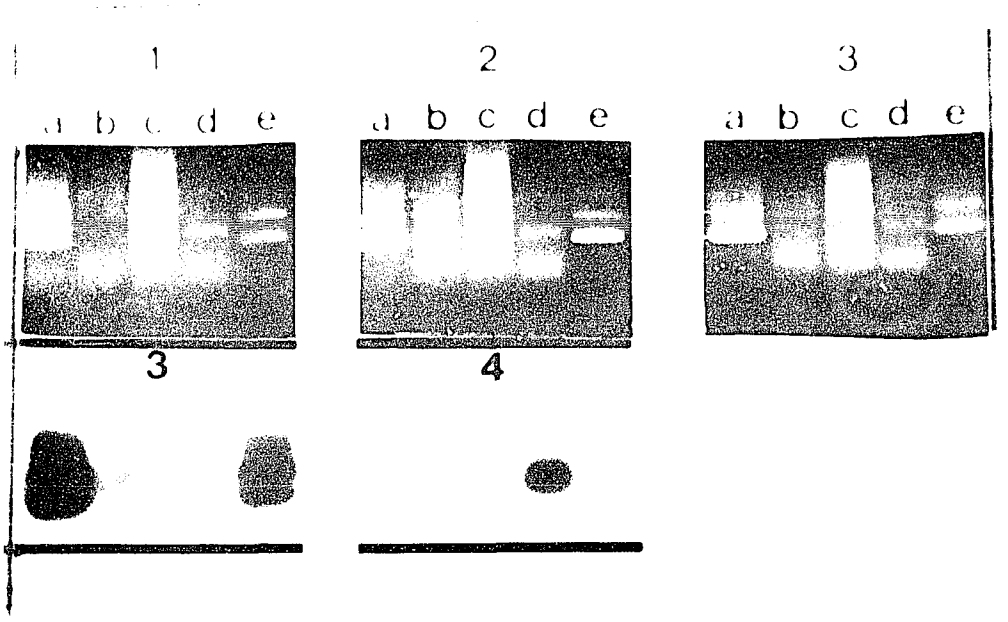


Figure 47.

PCR amplification and Southern analysis of positive YAC pools.

PCR reactions with the 238bp primers were used to amplify plasmid containing D6S122, genomic DNA and yeast DNA, as well as c-fos primers and genomic DNA. The 2% agarose gels, 1, 2 and 3 shows products of PCR amplification with primers which amplify a 238bp band. The gel 3 was used to purify the 238bp amplified band from D6S122 (probe-A) and the approximately 250bp from yeast (probe-B) with gene-clean Kit. The gels 1 and 2 were transferred and hybridized with probe-A (D6S122 product) and probe-B (yeast product) under very stringent contitions. The two autoradiographs, 4 and 5 shows the level of hybridization with probe-A (D6S122 product) and probe-B (yeast product) respectively. The conclusion from the autoradiography is that the product of yeast amplification is not the same with the product from D6S122 amplification.

a: D6S122 DNA; b: Genomic DNA; c: Primers for c-fos gene and Genomic DNA; d: Yeast DNA; e: D6S122 DNA.

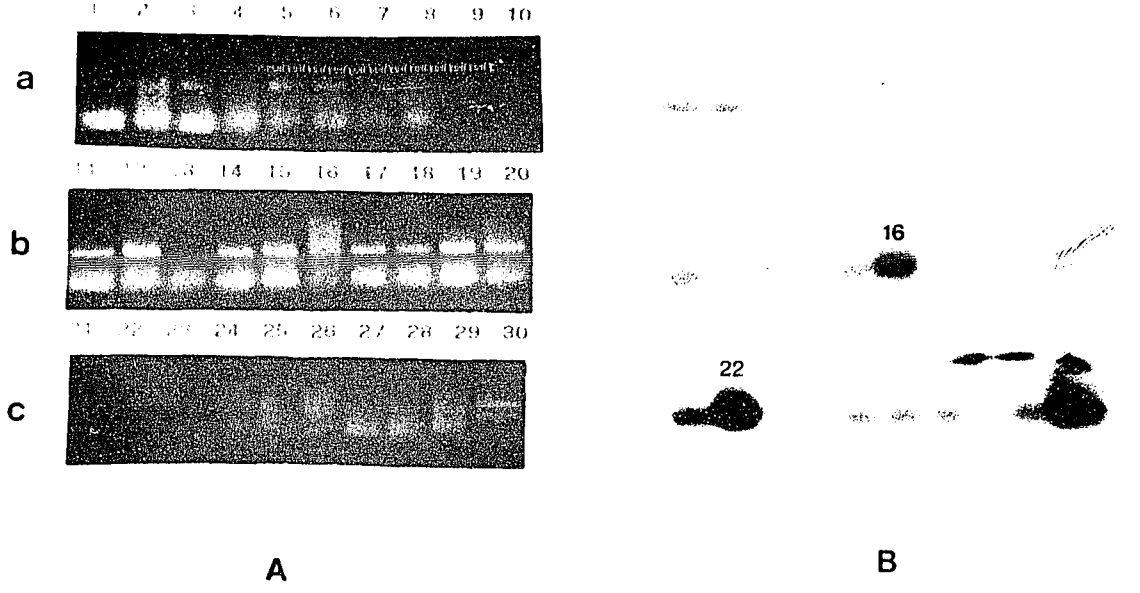


Figure 48.
Screening the subpools from the positive pool #47.
A: Primers from D6S122 amplifying 238bp DNA band, were used in a PCR reaction. All 28 YAC DNA subpool was amplified along with genomic DNA as positive controls. Products of PCR amplification were run in 2% agarose gels and DNA was vizualised with EtBr staining. The 2% agarose gels, a, b and c shows products of PCR amplification with the subpools 1-10, 11-20 and 21-29 respectively. Number 30 is the positive control showing amplification with D6S122.
B: Southern blot of the above gels with a probe-A (D6S122 product) probe under very stringent conditions. The subpools #16 and #22 appeared positive.

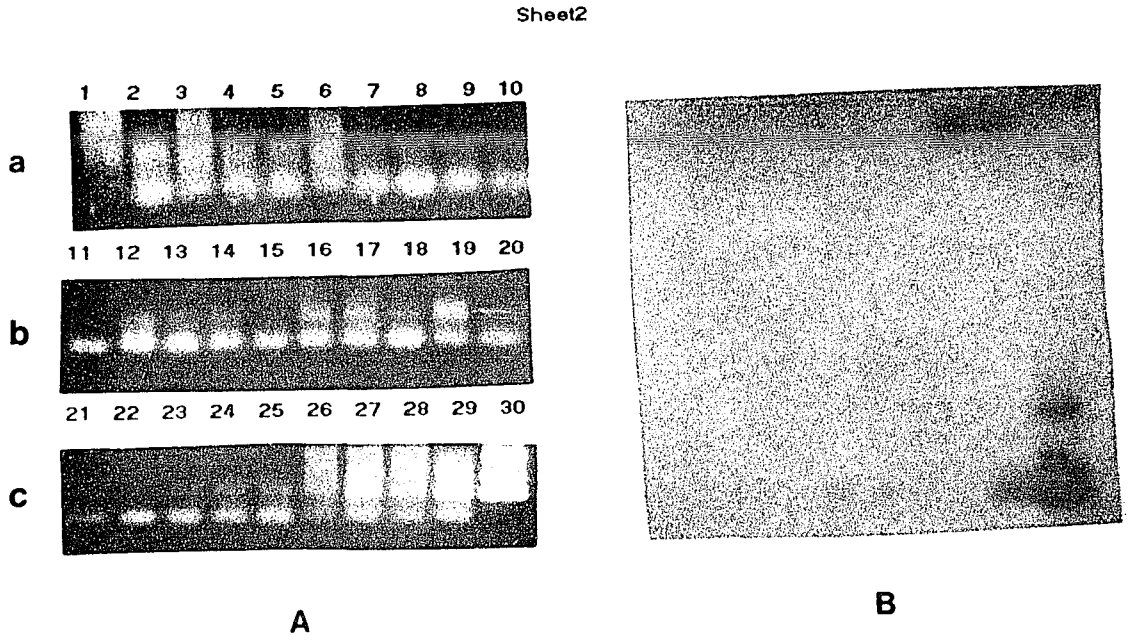


Figure 49.

Screening the subpools from the positive pool #60.

A: Primers from D6S122 amplifying 238bp DNA band, were used in a PCR reaction. All 28 YAC DNA subpool was amplified along with genomic DNA as positive controls. Products of PCR amplification were run in 2% agarose gels and DNA was vizualised with EtBr staining. The 2% agarose gels, a, b and c shows products of PCR amplification with the subpools 1-10, 11-20 and 21-29 respectively. Number 30 is the positive control showing amplification with D6S122.
B: Southern blot of the above gels with a probe-A (D6S122 product) probe under very stringent conditions. No subpools appeared positive.

of D6S122 were used as probes. None of the six clones had any homology to the D6S122 sequence. PFGE and Southern analysis of DNA from the six clones using pBR322 probe (homology with the YAC vector $pYAC_4$), showed the yeast artificial chromosomes which contained the human DNA *(Figure 50).*

The screening at the CEPH Institute YAC library identified no positive YAC clones for the locus D6S122. Several problems could be the cause in the failure to identify positive YAC clones. This include: (i) non-specific amplification of D6S122 with yeast DNA; (ii) the lack of availability (from the CEPH Institute) of filters for screening with hybridization together with PCR screening technique (both methods are available and recommended with other YAC libraries); (iii) the CEPH institute gives adequate DNA for pools and subpools for 2 PCR reactions; there was not enough material for further experiments to solve the problem of the non-specific amplification, and (iv) several laboratories in the United States involved in the human genome project (users of this library), have publicly complained about the inadequate of the library (Anderson *et al.*, 1993).

D. Further analysis of homology of D6S116, D6S122 and D6S125 DNAs.

The following experiments were designed to determine if the available probes known to be homologous to regions near the breakpoint on chromosome 6 were actually involved in the breakpoint, or near enough to be identified on the basis of proximity to the breakpoint. The following questions were addressed: (i) do D6S116 and D6S122 single-copy DNAs have coding sequences; (ii) is either

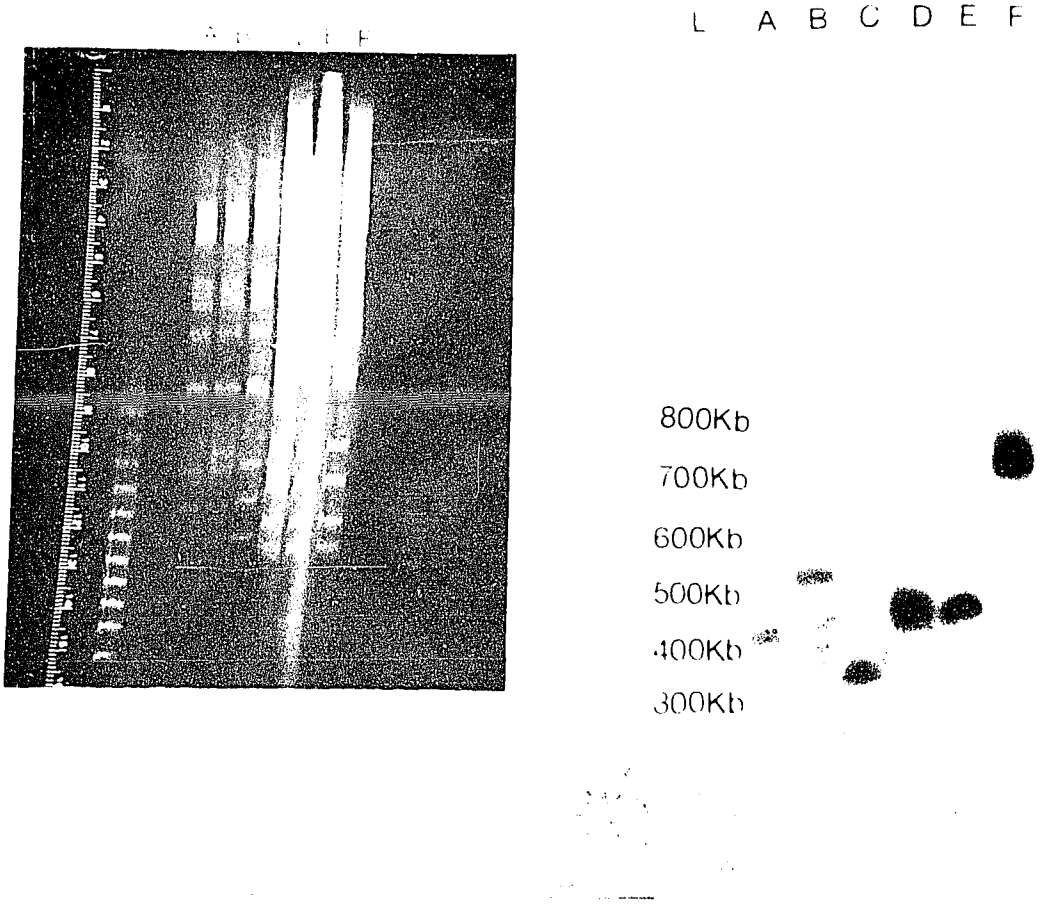


Figure 50.
Southern blot analysis of YAC DNA seperated by PFGE.
Yeast cells were grown and DNA was prepared in agarose inserts, and seperated in PFGE. Concatamers of 51 Kb lambda DNA were run as size markers and are indicated in left.
A: The six yeast clones 185B8, 186B8, 187B8, 188B8, 185A8, 186A8, were were analyzed with PFGE and the yeast chromosomes including the YAC appeared by staining with EtBr.
B: The gel was transferred on a nylon membrane and hybridization with ^{32}P-labeled probe for pBR322 (homology with the YAC vector $pYAC_4$).

D6S122 (6q14) or D6S125 (6q12) DNAs rearranged in the tsA-A cell line which contains the deleted chromosome 6 and (iii) is there DNA homology to D6S122 (6q14) and D6S125 (6q12) that is close enough to the breakpoint (100-300 kb) to be detectable using PFGE?

(i) Analysis for coding sequences. The experiments used both Northern and dot blot analysis with tha single-copy DNAs, D6S116, D6S122, and D6S125 as probes. No hybridization signal showed on the blots. The same result was obtained when the same experiments were repeated three times with increasing quantities of RNA (Figures are not shown, because they showed no signal). These experiments demonstrated that these DNAs do not code any gene.

(ii) Analysis for D6S122 (6q14) or D6S125 (6q12) rearrangement. Southern blot analysis of all cell lines digested with a group of four different restriction endonucleases and hybridized with D6S122 DNA, showed no rearrangements or deletions *(Figures 51)*. In a similar experiment, D6S125 showed no rearrangement after digestion with five different restriction endonucleases *(Figures 52)*. These two experiments demonstrated that D6S122 and D6S125 DNAs were not directly included in the breakpoint on chromosome 6.

(iii) Pulsed Field Gel Electrophoresis (PFGE) analysis of D6S122 and D6S125. With the Pulsed Field Gel Electrophoresis (PFGE) technique, high-molecular-weight DNA fragments of 50-2000 Kb were separated and further analyzed with the Southern procedure *(Figures 53, 54)*. Hybridization of the membrane with D6S122 and D6S125 DNA probes demonstrated that these

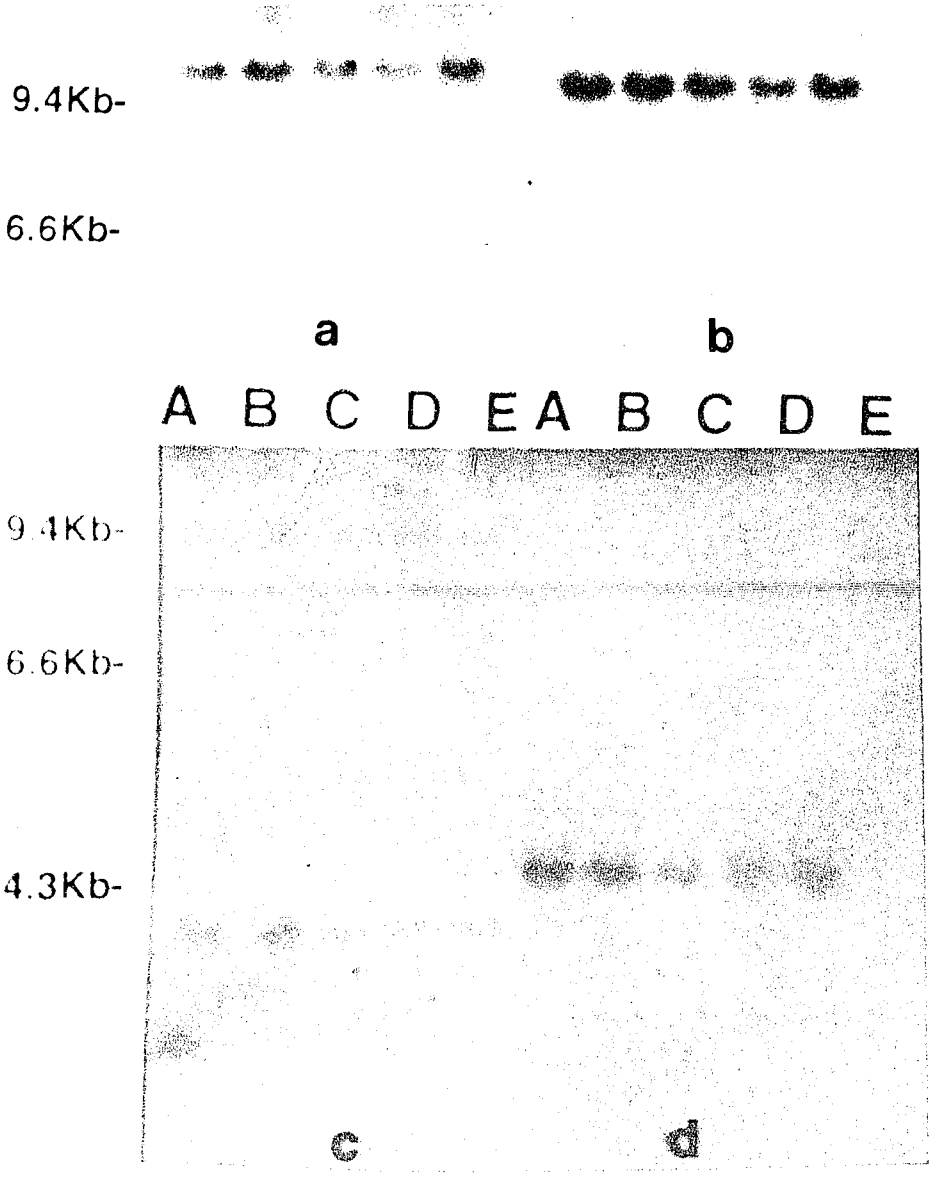


Figure 51.

Southern blot analysis of single-copy DNA D6S122 in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for the single-copy DNA D6S122 was hybridized to the blot. All cell lines show the same hybridization pattern, demonstrating no rearrangements of this locus in any of the cell lines.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

a: Restriction enzyme digestion with EcoRI endonuclease.

b: Restriction enzyme digestion with Bgl II endonuclease.

c: Restriction enzyme digestion with Pvu II endonuclease.

d: Restriction enzyme digestion with Pst I endonuclease.

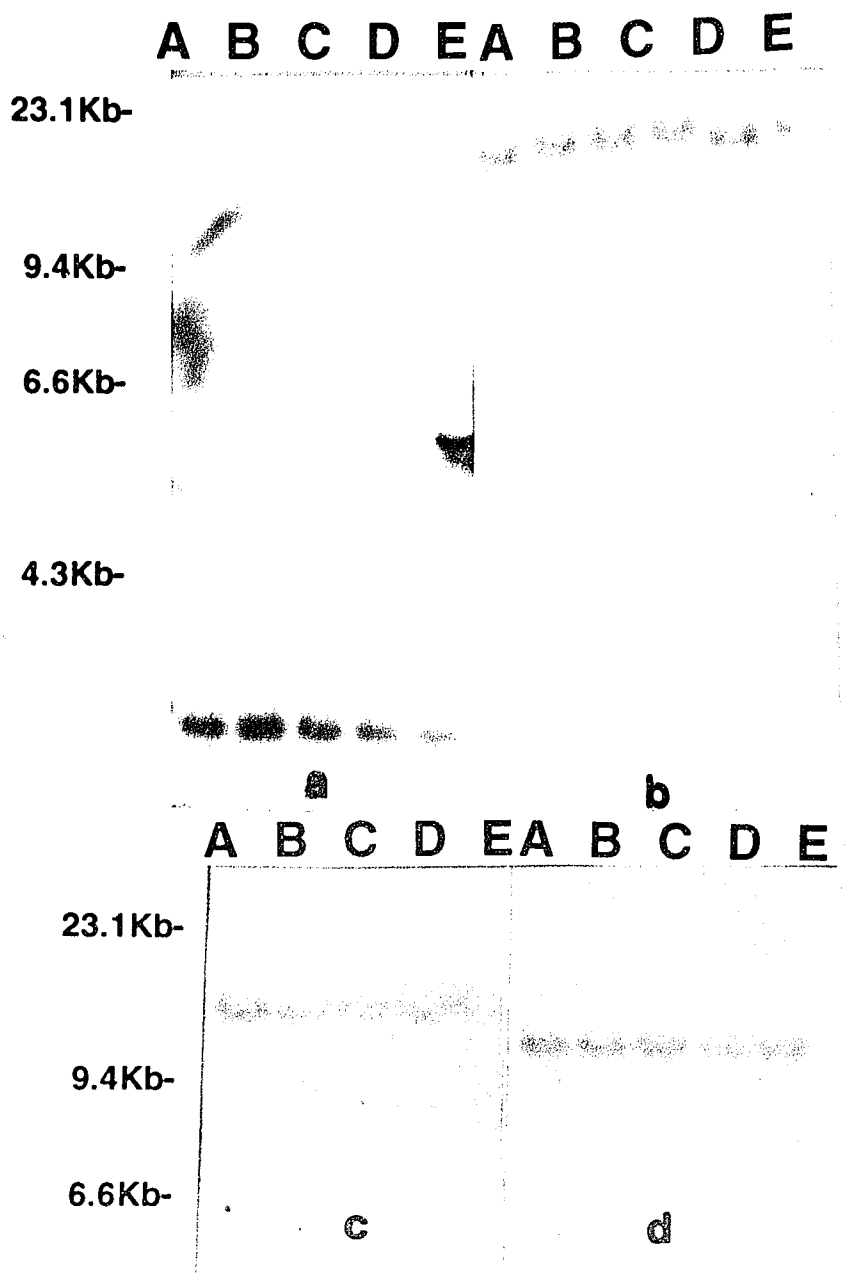


Figure 52.

Southern blot analysis of single-copy DNA D6S125 in cellular DNA from all cell lines.

High-molecular-weight DNA prepared from the normal, preimmiortal and immortal cell lines was digested with restriction enzyme and analysed by Southern blot. ^{32}P-labeled probes for the single-copy DNA D6S125 was hybridized to the blot. All cell line shows the same hybridization pattern, demonstrating no rearrangements of this locus in any of the cell lines.

A:(HS74BM/p7); B:(Preimmortal tsA-0/p9); C:(Immortal tsA-A/p25); D:(Immortal HAL/p24); E:(Immortal AR-5/p27).

a: Restriction enzyme digestion with Hind III endonuclease.

b: Restriction enzyme digestion with EcoRI endonuclease.

c: Restriction enzyme digestion with Pvu II endonuclease.

d: Restriction enzyme digestion with Bgl II endonuclease.

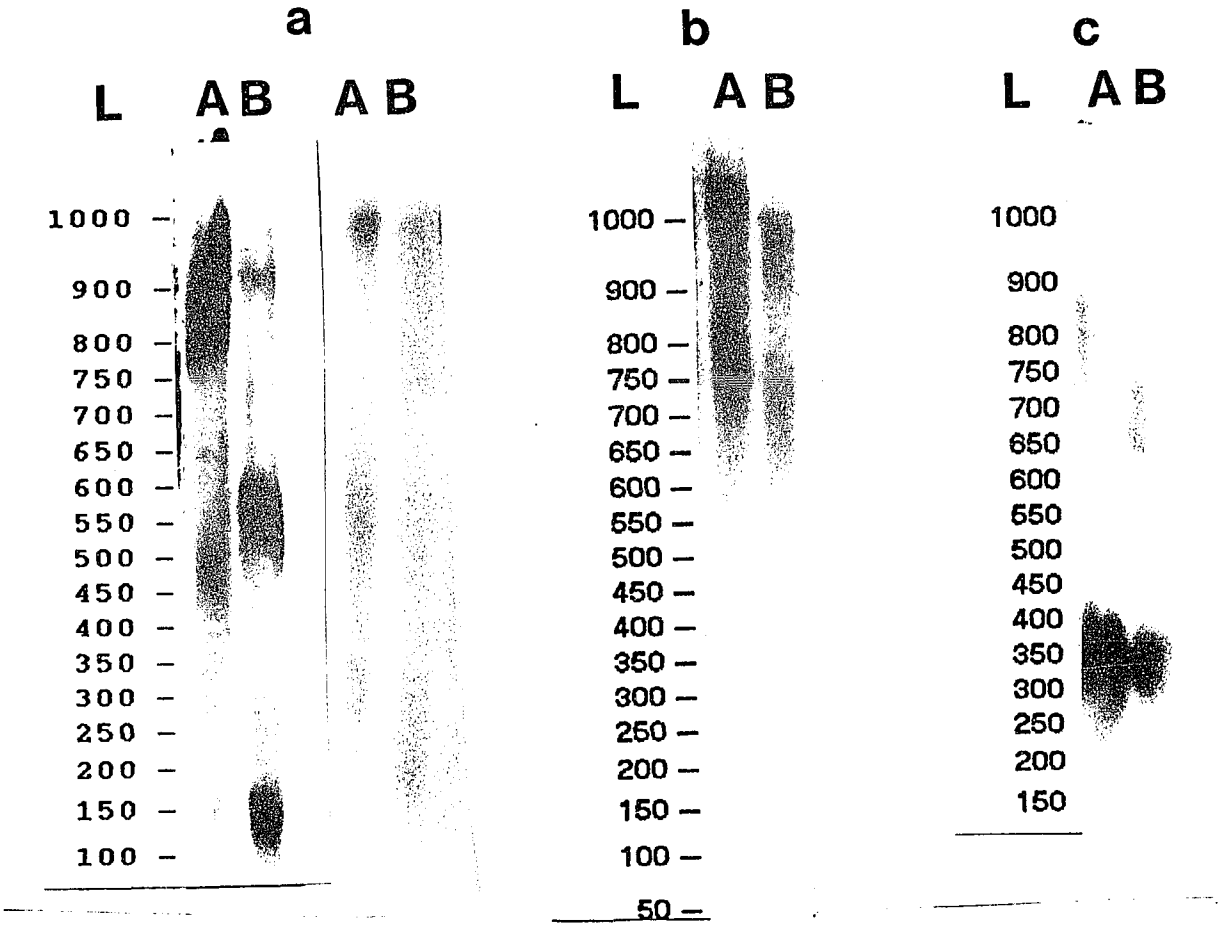


Figure 53.

Southern blot analysis of cellular DNA seperated by PFGE.

DNA was prepared in agarose inserts, digested with rare cutter restriction enzyme and seperated in PFGE. Concatamers of 51 Kb lambda DNA were run as size markers and are indicated in left. ^{32}P-labeled probe for the single-copy DNA D6S122 was hybridized to the blot. The lambda concatamers DNA were vizualized by hybrization with lambda probe. The cell lines tsA-0 and tsA-A (contains the 6q21 deletion) shows the same hybridization pattern, demonstrating no rearrangements of this locus.

L: Lambda ladder (51Kb); A: Preimmortal tsA-0/p9; B:Immortal tsA-A/p25.

a: Restriction enzyme digestion with Xho I endonuclease.

b: Restriction enzyme digestion with Nru I endonuclease.

c: Restriction enzyme digestion with Nae I endonuclease.

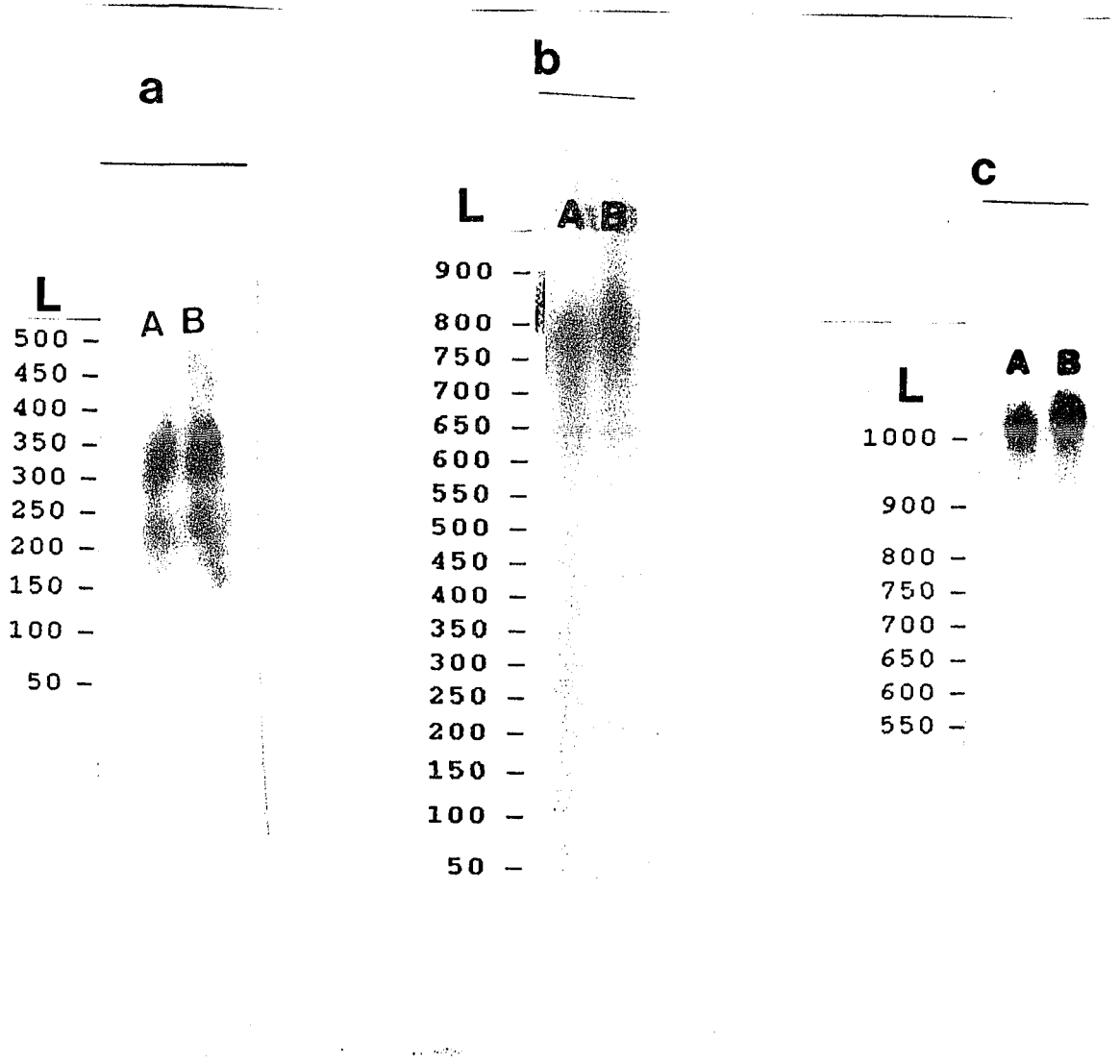


Figure 54.

Southern blot analysis of cellular DNA seperated by PFGE.

DNA was prepared in agarose inserts, digested with rare cutter restriction enzyme and seperated in PFGE. Concatamers of 51 Kb lambda DNA were run as size markers and are indicated in left. ^{32}P-labeled probe for the single-copy DNA D6S125 was hybridized to the blot. The lambda concatamers DNA were vizualized by hybrization with lambda probe. The cell lines tsA-0 and tsA-A (contains the 6q21 deletion) shows the same hybridization pattern, demonstrating no rearrangements of this locus.

L: Lambda ladder (51Kb); A: Preimmortal tsA-0/p9; B:Immortal tsA-A/p25.

a: Restriction enzyme digestion with Xho I endonuclease.

b: Restriction enzyme digestion with Nru I endonuclease.

c: Restriction enzyme digestion with Sal I endonuclease.

sequences were not rearranged. In addition, these two single copy DNAs showed no overlapping homology to the same DNA fragment.

E. FISH of the chromosomal breakage on chromosome 6.

Cytogenetic analysis revealed a 6q21 deletion. The molecular analysis, however, flanked the breakpoint on chromosome 6 at region 6q13 between D6S125 and D6S122. One explanation for the discrepancy regarding the exact location of the breakpoint was that chromosome 6q21 could consist of a broken chromosome 6, at 6q13, plus a telomeric region from chromosome 6 or another chromosome. This would occur as the result of an interstition deletion (for example, breakage on 6q13 and subsequent deletion of 6q13->25) or a translocation. This possibility was tested using the Fluorescent *In Situ* Hybridization (FISH) technique. Specific probes which stain chromosome 6 and differentiate it from other chromosomes were used. Cell line tsA-A (tetraploid) showed two intact and two deleted chromosomes 6 on the basis of differential color. The chromosomes 6 stained green, but all other chromosomes stained red. The deleted chromosomes 6, however, stained green up to 6q12-13 and the rest of the chromosome (6q13->21) stained red *(Figure 55).* This experiment demonstrated that the actual breakage on chromosome 6 is indeed on 6q12-13 (as molecular analysis confirms) and DNA from 6q13->21 belongs to a chromosome other than chromosome 6.

Another FISH experiment on the immortal cell line AR-5 (tetraploid missing

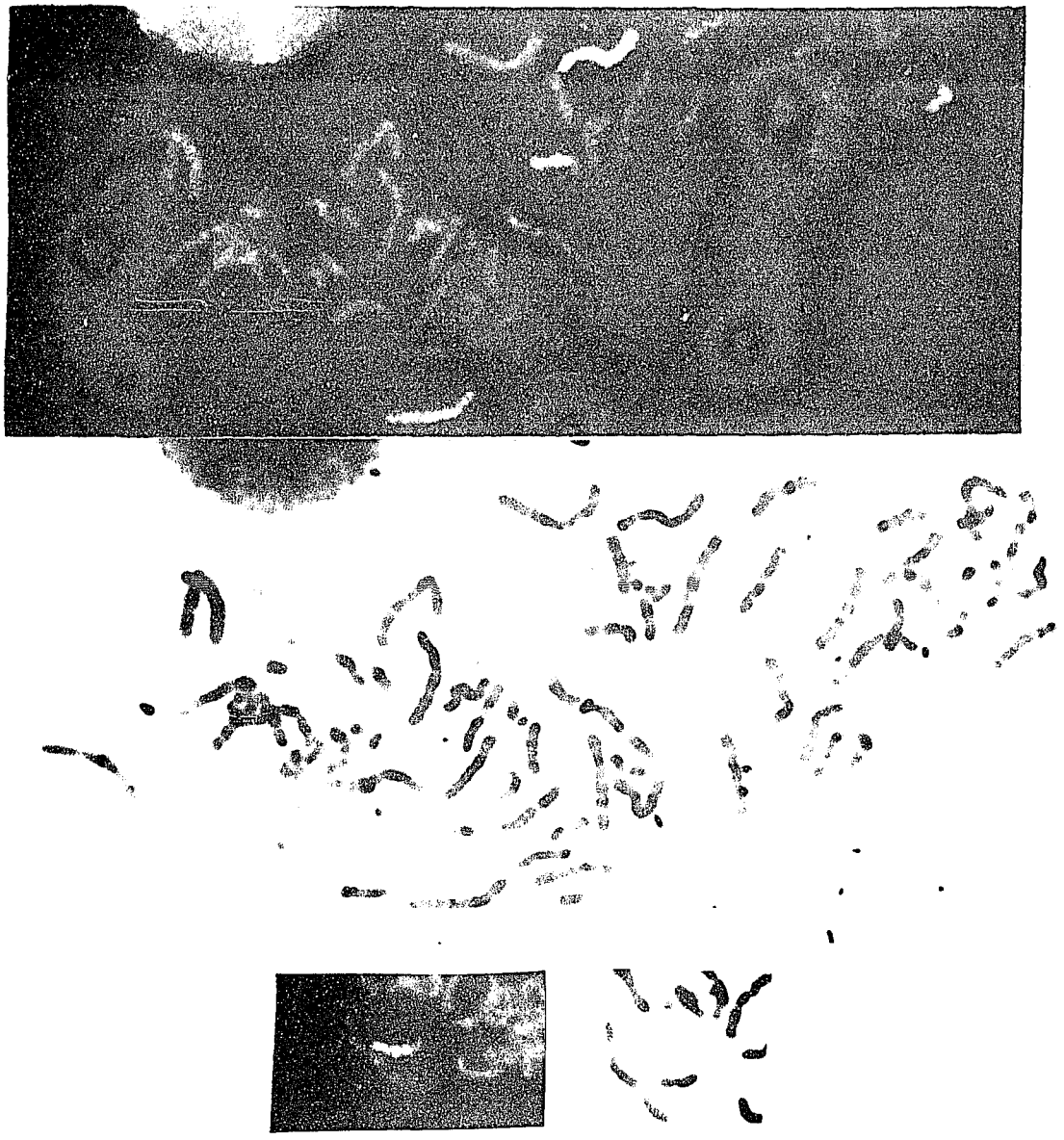


Figure 55.

Fluorescent *In Situ* Hybridization (FISH) using whole chromosome 6 spesific probe with the immortal cell line tsA-A.

A: Specific whole chromosome 6-avidin probe was hybridized to metaphase spread. The fluorescent yellow chromosomes arised from the signal amplified with avidin and anti-avidin antibody (see materials and methods). The metaphase spread is stained with propidium iodine and the backgrount chromosomes appear red. The tetraploid cells of the cell line tsA-A show two intact and two deleted chromosomes 6 stained green, with all other chromosomes stained red. The deleted chromosomes 6, stained green up to 6q13 and the rest of the chromosome (6q13->21) stained red.

B: Partial karyotypes of another metaphase plate showing chromosome 6 in the same FISH experiment and after staining with G-banding.

two chromosomes 6), confirms the cytogenetic data by demonstrating only two intact chromosomes 6 *(Figure 56).*

A FISH experiment was also done on the cell line SV/HF-5/39 which is an immortalized derivative from the lineage SV/HF-5, transformed with the wild type SV40(ori-). This experiment was designed to determine if the lineage (SV/HF-5) has an identical breakpoint on chromosome 6. The cytogenetic analysis of this immortal tetraploid cell line showed a deleted 6q21 chromosome. The FISH analysis shows that the actual breakage point of chromosome 6 is not on 6q21 but more proximal, close to the region 6q15 *(Figure 57), i.e.,* the chromosomal region from 6q15->21 does not belong to chromosome 6. Based on this finding several hypotheses can be made. It is possible that there are more than one sites of breakage on chromosome 6q. This hypothesis is supported by the many different deletions on chromosome 6 reported in neoplasm ranging from 6q12 until the telomere. It is also possible that the consistent region on chromosome 6 which is involved in immortalization is not lying on the breakpoint, but distal to 6q15.

The FISH analysis confirmed both cytogenetic and molecular findings. In addition, these experiments demonstrated that deleted chromosome 6q21 is not a terminal deletion on chromosome 6. The chromosome 6 breakage is more proximal to the centromere and the abnormal chromosome 6q21 contains a telomeric region from another chromosome. Different deletions of chromosome 6, from 6q12 to the telomere are consistently found in human neoplasia, like neuroectodermal tumors (Third international workshop, 1981), acute lymphoblastic

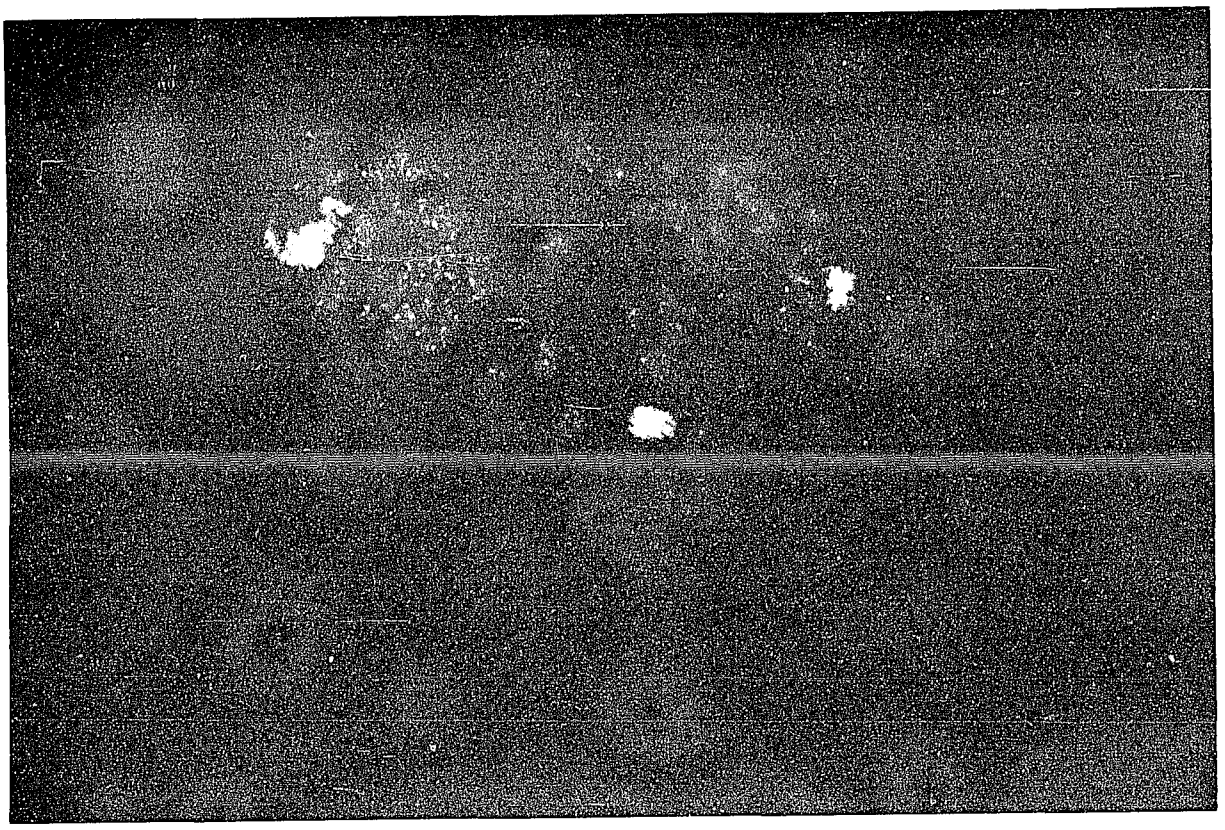


Figure 56.
Fluorescent *In Situ* Hybridization (FISH) using whole chromosome 6 spesific probe with the immortal cell line AR-5.
Specific whole chromosome 6-avidin probe was hybridized to metaphase spread. The fluorescent yellow chromosomes arised from the signal amplified with avidin and anti-avidin antibody (see materials and methods). The metaphase spread is stained with propidium iodine and the backgrount chromosomes appear red. The tetraploid cells of the cell line AR-5 show two intact chromosome 6 only (Cytogenetic analysis showed two chromosome 6 and the other chromosome 6 are missing). Chromosome 6 stained green, and the all other chromosomes besides 6 stained red.

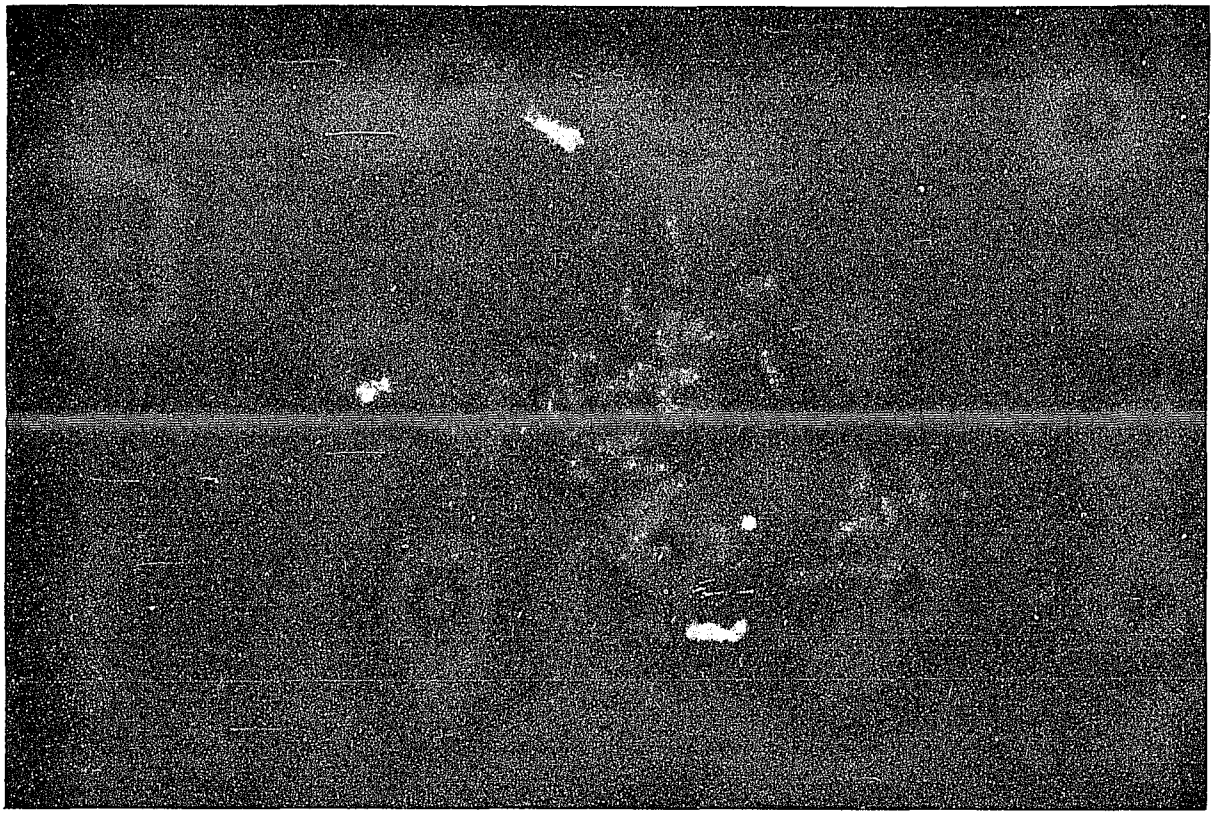


Figure 57.
Fluorescent *In Situ* Hybridization (FISH) using whole chromosome 6 spesific probe with the immortal cell line SV/HF-5/39.
Specific whole chromosome 6-avidin probe was hybridized to metaphase spread. The fluorescent yellow chromosomes arised from the signal amplified with avidin and anti-avidin antibody (see materials and methods). The metaphase spread is stained with propidium iodine and the backgrount chromosomes appear red. The tetraploid cells of the cell line SV/HF-5/39 show two intact and one deleted chromosome 6, and a translocated chromosome 6 stained green, and the all other chromosomes besides 6 stained red. The deleted chromosomes 6, stained green up to 6q13 and the rest of the chromosome (6q13->21) stained red.

leukemia and lymphomas (Hayashi *et al.*, 1990), ovarian carcinomas (Ehlen *et al.*, 1990) and malignant melanoma (Trent *et al.*, 1983). The consistency of the breakpoint we found on 6q13, with the breakpoint on the deleted chromosomes 6 found in human tumors, is presently unknown. It is likely that deletions previously described on chromosome 6 are not terminal (Trent *et al.*, 1993).

Conclusion

This study, using genetically matched SV40-transformed preimmortal and immortal cell lines, demonstrated that specific changes in the cellular genome are required for immortalization. Cytogenetic and molecular analysis showed that a consistent loss of sequences of chromosome 6q was involved. FISH and molecular analysis determined the deleted chromosome 6 breakpoint at 6q13; two single copy DNA probes were determined to flank the region of the breakage. This is the first definitive demonstration that a chromosome region(s) distal to 6q13 is critical to the immortalization process. This genomic deletion, along with the presence of T-antigen, is adequate for immortalization to occur.

The rearrangement of the gene MCC, and deregulation of MCC, APC, bcl-2, DCC genes, all located on the breakpoints of the non-random translocation t(5;18) or the integration of SV40, show that stabilization or progression of immortalization could be the result of a multiple cooperation of many genes. It is possible that other gene(s) disrupted by chromosomal aberrations and/or the SV40 integration site, are involved in the progression of the immortal phenotype.

DISCUSSION

A. Overview

SV40-mediated transformation of normal human cells HS74 is an excellent experimental system to study the basis of immortalization in HDF cells. Several hypothesis have been advanced to explain the mechanism of immortalization. The two step model of immortalization, proposes at least two classes of cellular functions as requirements for transformation and immortalization. In the first class, the cells express the SV40 large T antigen and bypass the first stage, which is characterized by the loss in growth arrest in G_1 stage, and proliferate until the second stage is initiated. Inactivation of the second step could result in immortalization. The second event is hypothesized to involve specific genetic change(s) ij the genome.

This hypothesis is supported by the following observations. SV40 T antigen, as well as viral proteins for other DNA tumor viruses, have been shown to form complexes with human proteins (Pereira-Smith *et al.*, 1983; Resnick-Silverman *et al.*, 1991). The interaction has been interpreted to result in the inactivation of their growth inhibitory functions. Such complex formation might explain aspects of the transformed phenopype in cells expressing an SV40 genome, but they are insufficient to explain all aspects of the transformed phenotype in immortalized human fibroblasts. The conclusion is most simply based on the fact that the preimmortal and immortal cell lines of both SVtsA/HF-A and SV/HF-5 lineages

failed to show significant change in the viral gene function (Hubbard-Smith *et al.*, 1992; Neufeld *et al.*, 1987). In addition, immortal cell lines cease to proliferate (reach senescence) when large T antigen is removed or inactivated (Radna *et al.*, 1987). Thus, large T antigen is required for the maintenance of the immortalization phenotype in human fibroblasts, but since immortalization with SV40 is a rare event, it is insufficient for immortalization *per se*.

The conclusion is that an additional cellular event is required for immortalization. The specific change(s) in the cellular genome required for immortalization during the second stage have common genetic bases. This is supported by complementation analysis. Twenty one cell lines were assigned to four complementation groups relative to genes required for indefinite division. All immortal SV40-transformed cell lines studied were assigned to the same complementation group A (Pereira-Smith *et al.*, 1988). This experiment indicates that the genetic changes existing in different SV40 immortal lines cannot complement each other, and therefore, immortalization of human cells by SV40 occurs by the same processes. This finding supports the hypothesis that cellular senescence is a result of genetic program and that there is a limited number of gene products which are key factors involved in senescence and immortalization processes.

B. Critical role of chromosome 6q in immortalization

A second class of function is required for indefinite cell growth

(immortalization). The second event is specific change(s) in the cellular genome. Some examples could be the inactivation of growth suppressor gene(s) and/or changes in gene regulation of a proto-oncogene(s) or growth factor(s) or growth factor receptor(s). The inactivation of a single or multiple genes product is common in the development of cancer cells. Paradigms for these mechanisms are the inactivation of Rb gene in Retinoblastoma (Levine *et al.*, 1990), and the series of inactivation of c-Kras-1, APC, p53 and DCC genes in Sporadic Colorectal Cancer (Stanbridge, 1990). Chromosomal analysis of human tumors reveals non-random chromosomal aberrations which facilitated the identification of chromosomal regions containing oncogenes and tumor suppressor genes (Motocura *et al.*, 1991; Lee *et al.*, 1987).

The breakpoints of non-random chromosomal abnormalities in human tumors host oncogenes or tumor suppressor genes (Joslyn *et al.*, 1991; Cleary *et al.*, 1986). In point, studies of breakpoints of these non-random aberrations lead to the identification of many oncogenes (Kinzler *et al.*, 1992; Lee *et al.*, 1987). In the present study, cytogenetic analyses allowed a determination of differences between genetically matched normal, preimmortal and immortal SV40-transformed cell lines. Analysis of the two independent SV40-transformants, SVtsA/HF-A and SV/HF-5, demonstrated a non-random deletion of the distal portion of chromosome 6q21 among all clonal immortal cell lines. Since the chromosomal alterations in the SVtsA/HF-A set of SV40 transformants are few, the non-random abnormalities in chromosome 6 become more significant.

During the course of our research, a critical role of chromosome 6q in immortalization was supported by cytogenetic data from Ray and Kraemer (Ray and Kramear, 1992). They found non-random breaks at 6q21 in 6 out of 9 immortal SV40-transformed HF. Another immortal cell line showed a translocation of 6q21 to chromosome 1. Other immortal cell lines showed various chromosomal aberrations commonly seen after transformation with SV40 occurring at chromosomal regions 1p13, 11q13, 12p11 and 18q21 (Ray *et al.*, 1992).

A senescence-related gene could be involved in immortalization using the same mechanisms that tumor suppressor genes use in their involvement in carcinogenesis. A loss of heterozygosity and/or non-random chromosomal aberrations has been noted in leukemias, lymphomas and many human tumors (Hayashi *et al.*, 1990; Lee *et al.*, 1990; Becker *et al.*, 1983). A loss of heterozygosity and/or non-random chromosomal aberrations has been also been noted in neuroectodermal tumors (Third international workshop, 1981), acute lymphoblastic leukemia (Hayashi *et al.*, 1990; Barletta *et al.*,1986), ovarian carcinomas (Ehlen *et al.*, 1990; Lee *et al.*, 1990; Whang-Peng *et al.*, 1984), non-Hodgkin's lymphoma (Bloomfield *et al.*, 1983; Fifth international workshop, 1987; Schouten *et al.*, 1990), renal cell carcinoma (Morita *et al.*, 1991), and malignant melanoma (Balaban *et al.*, 1984; Becker *et al.*, 1983; Pathak *et al.*, 1983; Trent *et al.*, 1983; Trent *et al.*, 1989).

A viable hypothesis, then, is that a tumor suppressor gene(s) is located in the long arm (q) of chromosome 6, and it is involved both immortalization and

tumorigenesis (Barletta *et al.*, 1986; Schouten *et al.*, 1990; Trent *et al.*, 1990; Yamada *et al.*, 1990). The following study has approached the question of whether a senescence-related gene is present on 6q. A chromosome 6q was transfered into an immortalized cell line [sub-line of HAL (HAL/neo)], and suppression of immortalization was observed (Sandhu *et al.*, 1991). This indicated the presence of a senescence related gene on chromosome 6 responsible for immortalization, because the reintroduction of this missing sequence in an immortal cell line enabled cells to reach senescence. In addition, the introduction of a normal chromosome 6 by microcell mediated chromosome transfer into malignant melanoma (Trent *et al.*, 1990) and uterine endometrial carcinoma cells (Yamada *et al.*, 1990) also resulted in suppression of the tumorigenicity (ability of cells to form tumor in mice) of these cell lines. The above experiments support the hypothesis that a tumor suppressor gene is located on chromosome 6. It is consistent and demonstrate the critical role of chromosome 6 in immortalization and carcinogenesis.

There are experimental models as to how a deletion of a gene for "senescence" on chromosome 6 could result in immortality. For example, retinoblastomas arise from two sequential mutational events on the Rb-1 gene, in what is called the "two hit" hypothesis (Lee *et al.*, 1987). Immortalization would then result from a combination of mutation and loss of gene(s) on the deleted chromosome. On the other hand, the APC tumor suppressor gene is known found to cause cancer when its expression is reduced. The loss of one allele is sufficient

to decrease levels of the APC gene product, such that efficient control of cell proliferation is not longer possible (Bodmer *et al.*, 1987). This is called the "reduced dosage" hypothesis. In the present case, immortalization is assumed to result from the reduced dosage of the senescence related gene(s) on 6q, as a result of the absence of one of the chromosomes 6. Thus our findings support the hypothesis that a reduced dosage of gene(s) on 6q, as a result of a missing chromosome 6 (or portions of 6q) in the immortal cell lines, is the event responsible for immortalization.

The non-random deletion of chromosome 6q21->ter noted in our study, was also confirmed by DNA analysis (Hubbard-Smith *et al.*, 1992; present study). All of the genes that were investigated were in a position that could be explained by the 6q13 breakpoint. Further analysis, however, which flanked the chromosome 6 breakpoint with two single copy DNA probes, showed the breakage to be on 6q13. FISH, using specific probes staining chromosome 6 determined the breakage on chromosome 6 on the same position. This experiment demonstrates that the actual breakage on chromosome 6 is at 6q13. Many chromosomal deletions are not terminal deletions (Fifth Inter. Work., 1993). Therefore, the reported deletions on chromosome 6 in immortalized cell lines or human tumors, ranging from 6q12 to 6q27, may not be true terminal deletions.

From the localization of single-copy DNA known to be at/or near chromosome regions 6q12 and 6q14, it can be estimated that the region between them is approximately 10-15000 Kb (Golubic *et al.*, 1991). Conventional

electrophoresis, PFGE, and Southern blot analysis with D6S122 and D6S125 as probes, demonstrated that these two single copy DNA's are not rearranged. Therefore, the breakage point on chromosome 6 is not with these two DNAs or closer to them for 300 Kb. It is unfortunate that the region could not be more closely resolved. The major problem was in the nature of the YAC library at CEPH, which is suspect. Several laboratories in the United States involved in the human genome project (users of this library), have publicly complained about the inadequate of the library (Anderson *et al.*, 1993). Flanking of the breakpoint on chromosome 6 could facilitate the isolation of DNA sequences with microdissection (Fa-Ten *et al.*, 1992). Since the region of interest is precisely determined between these two single copy DNAs on 6q12 and 6q14, microdissection could isolate all the sequences in this area. A similar approach resulted the identification of the gene responsible for the Prader-Willi syndrome (Buiting *et al.*, 1992).

C. Relevance of the integration site of the SV40

Our results are consistent with the model which proposes at least two classes of cellular functions as requirements for transformation and immortalization. It is not yet understood why SV40 T antigen can apparently direct immortalized rodent cells while human cells have to go through crisis before immortalization occurs. Hence, an additional step must be considered in human cell immortalization. In view of the low frequency of immortalization, a combination of events is assumed to occur. The initial event, however, remains the same, that

of integration of the SV40 genome into human DNA and subsequent expression of T-antigen.

In the usual case, integration sites for transfected DNAs appear to be, at least superficially, random (Henderson, 1982). At least twenty four different integration sites have been identified in different SV40 immortalized human cell lines, so a specific site of integration does not appear to play a significant role (Shay *et al.*, 1989). In immortalized cell lines obtained at higher frequency or without crisis, like SVtsA/HF-A, the integration site *per se* in the human genome could effect other gene(s) and further the immortalization process.

Hybridization *in situ* to all SVtsA/HF-A cell lines, shows that the integration site of SV40 is on chromosome 5q21. Two tumor suppressor genes, APC (Adenomatous Polyposis Coli) (Croden *et al.*, 1991) and MCC (Mutated in Colorectal Cancer) (Kinzler *et al.*, 1991), are known to be present in this region. The MCC gene is rearranged in the immortal cell line AR-5, and the RNA levels are elevated. The rearrangement of MCC (on 5q21) could be possibly correlated either with a translocation t(5;18)(q21;q21) or with the integration of SV40 in 5q21. No other preimmortal (tsA-0) and immortal cell lines (tsA-A, HAL) containing the same SV40 integration side had the MCC rearrangement, nor an increase in mRNA levels. Therefore the altered expression of the MCC gene suggests that a role in the progression of the immortal phenotype in the cell line AR-5, rather than being a direct cause of the immortalization event.

Approximately 150 Kb distal to MCC (5q21) is the structurally related APC

gene which is mutated in both sporadic cancer and Familial Adenomatous Polyposis (FAP) (Lindgren *et al.*, 1992). The level of APC in the immortal cell lines is reduced and in the cell line AR-5 was lower (by 100%) that that of the preimmortal cell line tsA-0. This is a very important finding because APC tumor suppressor gene, found to cause cancer when its expression is reduced by 50%(Macdonald *et al.*, 1992). The decrease levels of the APC gene product result the lost of control of cell proliferation (Bodmer *et al.*, 1987)* Both MCC and APC are tumor suppressor genes and they share similarity with the region of the mAChR (m3 muscarinic acetylcholine receptor) known to regulate specificity of G protein coupling (Kinzler *et al.*, 1991). Previous observations have related G protein with neoplasia (Xu *et al.*, 1990; Martin *et al.*, 1990). It will be interesting to see if these two genes interact with G protein, and play a role in immortalization and carcinogenesis.

D. Is immortalization or progression of the immortalization phenotype an activation of a single gene or multiple cooperation of cellular oncogenes?

In human tumors, an activation of single or multiple cooperating cellular oncogenes is in itself sufficient to create a cancerous cell. For example, the retinoblastoma cells lack a functional Rb protein (Lee *et al.*, 1987). When the wild type Rb-1 gene is introduced into cancer cells, their tumor-forming property is suppressed (Huang *et al.*, 1988). On the other hand, tumorigenesis in colorectal cancer proceeds through a series of genetic alterations, starting with K-ras gene

activation and progressing with the tumor suppressor genes on chromosome 5 (APC), then 17 (p53) and 18 then (DCC) (Stanbridge, 1990).

On the basis of our results, primary immortalization can occur in the presence of T-antigen (phase 1) and a subsequent change involving a gene(s) on chromosome 6 (phase 2). There are changes, however, that occur with progression of the immortalization phenotype that are suggestive of additional events that could be involved in immortalization *per se*. For example, could immortalization in SV40 transformed cells be caused, in part, by the activation of a single oncogene (or inactivation of a single anti-oncogene), or multiple cooperation of several oncogenes?

Different passages of the respective sublines showed characteristic chromosomal changes with persistenca of prior rearrangements, suggesting a progression of karyotypic alterations. All SVtsA/HF-A cell lines have a reduced number of chromosome 16, proving a common paternal origin. Since the parental cells have this deletion of chromosome 16, it is assumed to be uninvolved in immortalization. Phis is also supported by the fact that SV40 transformed lines from other studies, do not involve chromosome 16. Cytogenetic data on the lineage SVtsA/HF-A shows non-random chromosomal abnormalities in immortal cell lines, involving non-random alterations on chromosome 1,4,7,13 and translocations involving t(10;12)(p11;q11), t(5;18)(q21;q21) and t(11;22)(q13;p11). The above aberations are among the very few found in the SVtsA/HF-A cell lines, therefore they become even more significant. Since the breakpoints of these

aberrations involve genes critical to neoplastic transformation, it is possible that the translocations are indicative of a previous malfunction of one or more of the genes involved in the chromosomal anomalies. A survey of these genes showed no defects relative to KRAS2, APC, FAD, TCR, PRAD-1, MCC, DCC, bcl-2 and Cyclin D-1 oncogenes (see table 3).

The alternative is that critical genes on the breakpoints of the abnormalities could be involved by acting as additional genomic changes in immortalization, as proposed for changes at the site of SV40 integration. The model of multiple cooperating cellular oncogenes activation in immortalization is further supported by the analysis of the immortal cell lines (SV/HF-37, SV/HF-39) of the SV/HF-5 lineage, which includes the same non-random aberrations on the chromosomal regions 12p11, 11q13 and 18q21. Another analysis of nine different SV40-transformants showed abnormalities, other than 6q21, occurring at chromosomal regions 1p13, 11q13, 12p11 and 18q21 (Ray *et al.*, 1992). The above chromosomal regions were found to be involved also in immortal lines in our studies (SVtsA/HF-A lineage).

The rearrangements of MCC (5q21) possibly developed from the translocation t(5;18)(q21;q21) in the cell line AR-5. The genes DCC (18q21) and bcl-2 gene (18q21) were not rearranged, but they showed elevated levels of gene expression. DNA sequences could be deregulated without showing any obvious genomic changes, when the gene itself is not disrupted, however the transcriptional regulation of the gene is effected; for example juxtaposed next to

strong promoter or effected by transcription factors. The deregulation and overexpression of these two genes becomes very important because both proteins were found to be overexpressed in human tumors. The DCC protein shows significant homology to the neural cell adhesion molecules (CAMs) and other related cell surface glycoproteins (Stanbridge, 1990). The protein is related to genes involved in cell surface interactions, and disruption of cell adhesion can result in a loss of cell-cell contact inhibition. The loss of cell-cell contact inhibition and cell communication are critical events in neoplastic transformation and properties of metastasis and the immortal phenotype. On the other hand, the bcl-2 (18q21) is located in the same chromosomal region with DCC and it could be also affected from the t(5;18)(q21;q21). The bcl-2 gene has the oncogenic function of blocking programmed cell death (Hockenbery *et al.*, 1991). Bcl-2's function is an antidote to apoptosis. Over-expression of bcl-2 RNA and protein, results cells accumulation because they fail to die, demonstrating prolonged survival. An example of this process is the uncontrol proliferation of B cells in human follicular lymphoma. In this lymphoma, the [t(8;14)] juxtaposes the Bcl-2 gene from chromosome 18 with the immunoglobulin heavy chain (IGH) locus on 14. This creates a fusion gene that is deregulated, resulting in overproduction of Bcl-2 protein (Nunez *et al.*, 1989).

The deregulation of the genes MCC, APC, DCC and Bcl-2 on chromosomes 5q21 and 18q21, suggests that immortalization involves other genes in a possible multiple cooperation. These genes either lead to stabilization and/or

the progression of immortalization.

E. Conclusion

Senescence appears to involve repression of proliferation-promoting genes as well as new expression of anti-proliferative genes. Identification of the participating genes and clarification of there mechanism of action will help to elucidate the mechanism involved in biological aging. In addition, it will provide insight into those events that characterize the rare escape of cells from senescence leading to immortalization and carcinogenesis.

This study, using genetically matched SV40-transformed preimmortal an' immortal cell lines, demonstrates that at least one specific change in the cellular genome is required for immortalization. Cytogenetic and molecular analysis indicate that a loss of sequences on chromosome 6 distal to 6q13 is involved. The rearrangement of the gene MCC and the altered expression of the genes MCC, APC, bcl-2, DCC which are all located on the breakpoints of the non-random translocation t(5;18), show that immortalization and/or the progression of the immortalized phenotype could be the result of a multiple cooperation of genes.

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