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Ha-ras polymorphisms in SV40-transformed human keratinocytes: Cloning and sequence analysis of putative regulatory elements adjacent to the human Ha-ras protooncogene

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City University of New York, 1993

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BY

WEIYI CHEN

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

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Abstract

HA-RAS POLYMORPHISMS IN SV40-TRANSFORMED HUMAN KERATINOCYTES: CLONING AND SEQUENCE ANALYSIS OF PUTATIVE REGULATORY ELEMENTS ADJACENT TO THE HUMAN HA-RAS PROTOONCOGENE

BY

WEIYI CHEN

Advisor: Professor Mark L. Steinberg

Whereas much is known about the process of transformation by retroviruses and their transduced oncogenes, the mechanism of transformation by DNA viruses is still obscure. It is postulated that DNA viruses may cause oncogenic transformation by interacting with host cellular genes including oncogenes, resulting in a cooperative interaction leading to altered patterns of growth. However, very little research has been done. In this study, we used human cultured keratinocytes transformed by SV40 virus as an <u>in vitro</u> system to study structural changes in the cellular proto-oncogenes Ha-ras and myc resulting from infection by SV40. Southern blot analysis of ras sequences in the genomic DNAs of transformed cells using PTB-1, a probe which covers most 5′ region of the Ha-ras, revealed a polymorphic pattern of large restriction

fragments of about 4-5 kb which were present in the normal keratinocytes but which were absent in both PstI and Sau3A digests of transformed cells. To determine the nature of the restriction fragment length polymorphisms (RFLP), we cloned and sequenced the 1.8 kilobases of the region upstream from the 6.4 kilobases BamHI fragment containing Ha-ras. In a further study, ras gene expression by northern blot analyses also indicated that ras proto-oncogenes in SV40 transformed cell lines were expressed at a level of at least five times higher than those from normal keratinocytes. Sequence homology analysis of the 1.8 kilobase upstream region by computer search of the Genbank DNA sequence database revealed: 1) two segments with homology to enhancer regions of previously studied genes and, 2) that the cloned segment contained a novel, previously uncatalogued nucleotide sequence which has been subsequently submitted to Genbank with the accession number L11526. These segments were also found to contain binding motifs for transcriptional activating proteins including T-ag, AP-1 and SP1. Nuclear proteins prepared from EP, HeLa and line 98 were found to bind specifically to subfragments of this region in gel mobility shift assays and it appears that the binding activity in 98 is slightly stronger than in EP. DNase I foot-printing experiments confirmed the existence of nuclease resistant nucleotide segments within the putative enhancer region of the upstream sequences (Fig. 31 and 32).

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I. Introduction and Background

1) Retroviral Oncogene and RAS Oncogene

The idea of oncogenes was proposed by Huebner and Todaro in 1969 in the context of tumorigenesis induced by tumor viruses. They were first described as retrovirus-encoded genes that produced tumors in birds and rodents. In fact, retrovirus oncogenes have arisen by transduction of cellular genes, because every retrovirus oncogene has its own counterpart in cellular genes. It has been shown that a specific oncogene carried by a particular retrovirus shows a high degree of homology with a specific cellular gene. This group of cellular genes was usually referred to as proto-oncogenes, since they can become oncogenes by recombination into a retrovirus or by other means that cause them to be expressed in an abnormal manner.

Since the discovery of oncogenes there has been a rapid proliferation of experimental work aimed at the elucidation of biochemical pathways by which aberrant oncogene expression brings about the transformation of cells from a normal to a malignant phenotype. The importance of this body of research stems from its ability to provide molecular-genetic models of transformation which account for all three major classes of transforming stimuli: chemical, viral and physical.

At the present time, our understanding of oncogenesis is

based largely on studies of the sequences transduced from host genomes by infecting retroviruses. Most proto-oncogenes are thought to encode proteins that are involved in the control of cellular growth and differentiation.

What is the mechanism of activation of proto-oncogenes? Genomic damage or mutations have been found in most cancer cells, and transformed cells. These events may include point mutation, deletions, chromosomal translocation and amplification (reviewed in Bishop, 1987). Point mutation has been discovered to cause activation of the ras oncogene family in a large number and a variety tumor cell lines. In these cases, activating point mutations occurred by G -> T transversions mostly at either the 12th or 61st codon and the mutated genes were about 1000-fold more efficient in inducing foci in the 3T3 cell assay than their normal counterparts (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Yuasa et al 1983). Deletions seem to play little part in the conversion of proto-oncogenes to oncogenes, but they are a relatively common type of alteration in tumor suppressor genes, such as p53 and RB (Sager, 1989; Scrable et al., 1990). Chromosomal translocation apparently contributes to tumorigenesis by activating proto-oncogenes to become oncogenes (Haluska et al., 1987). For example, it was found that specific chromosomal translocations were often associated with plasmacytomas in the mouse and with Burkitt lymphomas in man. These tumors arise from abnormal B lymphocytes. The

common feature in human Burkitt lymphomas is that the c-myc proto-oncogene on human chromosome 8 is translocated to the proximity of an Ig locus on chromosome 14 or other chromosomes. When c-myc is translocated to the Ig locus, it becomes activated (Haluska et al., 1987). Its activation is one of the events associated with converting the cell into a tumorigenic state. The c-myc gene might be activated either by transcription from an immunoglobulin promoter, or by the release of a negative regulator located upstream from its usual location during translocation. Oncogene amplification occurs rarely in normal cells, but appears to become more common as cells progress towards the malignant phenotype (Tlsty et al., 1989; Wright et al., 1990). Many tumors carry abnormally amplified domains of DNA that can include proto-oncogenes and magnify their expression. Amplification of the myc and ki-ras and abl oncogenes have been reported in several tumor cell lines (Reviewed in Bishop, 1991).

At present, genetic damage found in cancer cells not only derives from dominant mutation from which they gain new functions, but also from recessive mutants which lose tumor suppressor genes. The general functions of these suppressor genes may serve as negalative regulators of cellular proliferation (reviewed in Marshall, 1991).

The existence of antioncogenes was postulated on the basis of evidence from cell fusion experiments using transformed and nontransformed mouse (Evans et al., 1982) and

human (Geiser et al., 1986) cells in which suppression of transformation acts as a dominant trait assignable to mouse chromosome 4 and human chromosome 11. These experiments showed that the hybrids were less tumorigenic when normal cells were fused with malignant cells. Loss of chromosomal regions was thought to be the main reason for tumor induction.

The loss of tumor suppressor genes (anti-oncogenes or recessive oncogenes) is found in many human malignancies, such as retinoblastoma, Wilms' tumor, neuroblastoma, small cell carcinoma, acoustic neuromas, colon carcinoma, breast cancer, bladder cancer, neurofibromatosis and chronic myelogenous leukemia (reviewed in Marshall, 1991). One well characterized tumor suppressor gene is the one in the retinoblastoma (RB) locus. Familial retinoblastoma is a disease in which young children develop multifocal tumors in the retina. Non-familial retinoblastoma develops in slightly older children and is characterized by unifocal, unilateral tumors. It has been presumed that both allelic mutations would have to be acquired somatically for the development of human retinoblastoma (Knudson et al, 1971; Comings et al, 1973). Cytogenetic and molecular analyses have supported the prediction that retinoblastoma cells often have two altered allelic copies of a single gene RB-1 located on chromosome 13q14 (Dryja et al., 1986; Lee et al., 1987; McGee et al., 1989).

It is believed that the RB protein may be involved in the negative regulation of cell proliferation (Huang et al,

1988). RB protein is a 110 KD nuclear protein with DNA binding activity. The mechanism of action of this protein was suggested by the finding that RB could form a specific complex with at least 3 major viral transforming products: SV40 large T, adenovirus E1A and human papilloma virus E7 (Dyson et al., 1989; DeCaprio et al., 1988; Whyte et al., 1988). These findings suggest a link between the ability to complex with RB and the ability to acquire transformation.

The strongest current candidate for a second tumor suppressor gene is provided by a gene designated p53, which was first recognized because it encodes a protein that binds SV40 T antigen. Although it was initially thought that p53 was an oncogene, several lines of evidence currently favor the hypothesis that loss of p53 function plays a role in neoplastic development. First, p53 genes are inactivated in several neoplasms. These include murine leukemia cell lines, where p53 locus had undergone insertions and deletions and resulted in complete loss of its expression (Mowat et al 1985). Rearrangements of p53 have also been observed in human osteosarcomas (Miller et al., 1990)). In addition, the p53 gene is frequently lost or mutated in human colon, breast, liver and lung tumors (reviewed in Marshall, 1991). Finally, recent gene transfer experiments have demonstrated that high level expression of the normal p53 gene product can suppress transformation of cultured cells (Finlay et al., 1988; Eliyahu et al., 1989), but p53 cDNA clones isolated from tumor cell

lines showed the cooperation of the p53 protein with ras in transformation (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984). These studies strongly implicate p53 as a tumor suppressor gene whose loss is involved in the pathogenesis of human neoplasms.

The potential of proto-oncogenes to participate in tumorgenesis arises from the fact that their products appear to involve mechanisms of growth control related to the action(s) of growth factors and protein kinase activity. Their products fall into five classes (Table 1):

1) Secreted proteins, that are growth factors e.g., sis (platelet derived growth factor [PDGF] B-chain). There is good evidence that this oncoprotein stimulates cell growth and transform cells
(Carpenter, 1987).

2) Mutant forms of cell surface growth factor receptors. Many of these are derived from receptor protein-tyrosine kinase genes, for example erbB (EGF receptor), fms (M-CSF receptor), and ros. These oncoproteins transform normal cells by delivering a continuous ligand independent mitogenic signal (reviewed in Bishop, 1991).

3) Mutant proteins associated with the inner face of the cytoplasmic membrane. These oncoproteins are of two main

types:

A) Protein kinase, such as: src, yes, fps/fes, fgr and ros are membrane bound proteins which have tyrosine kinase activity and show structural homology to one another in domains that are thought to be responsible for their kinase activity (Carpenter et al., 1987; Heldin et al., 1984; Bishop et al., 1985; Bishop et al., 1987). In cells transformed by these oncogenes, there is a dramatic tenfold increase in the formation of phosphotyosine, and a continuous rather than a ligand regulated signal is delivered (Cantley et al., 1991).

B) Another type is a G-protein like signal transducer, such as ras. The ras oncogene codes for a membrane bound GTP binding phosphoprotein (p21) homologous to the alpha subunit of G proteins. p21 appears to mediate a transmembrane signalling process which brings about altered gene expression via the formation of cAMP (Bishop et al., 1985; Bishop et al., 1987; McCormick et al., 1989).

4) Cytoplasmic oncoproteins such as mos, raf and cot. This group of oncogenes has received a good deal of attention recently because these oncogenes bear a close relationship to signal transduction (Cantley et al., 1991). The protein-serine/threonine kinase encoded by raf plays an important role in intracellular signaling pathways and thus it is important in control the cellular proliferation. The enzymatic activity of this protein can be induced by the direct or indirect

action of diverse cell surface receptors, cytoplasmic PTKs (Phosphotyrosine kinases), and ras (Morrison et al., 1988, 1989, Anne et al., 1993). The Raf kinase may then transfer signals to the nucleus, perhaps by phosphorylating transcription factors, such as jun and fos.

5) Nuclear proteins. This class of oncoproteins has a nuclear location and is believed to cause alternation of gene expression through direct interaction with chromosomal DNA. It has been shown that one such oncogene, jun, is equivalent to a previously identified transcriptional enhancer activating factor, Ap1 (Bohamann et al., 1987). More recently, complex formation between the Ap1/jun and fos oncogene products has been implicated in fos-induced transcriptional activation (Kouzarides et al., 1988). Myb and myc also fall into this class of oncogenes but less is known about how these oncogenes may act to activate transcription. Information regarding a number of other retroviral oncogenes including dbl and bcl-2 is still insufficient to permit classification.

The functions of oncogenes indicate that these proteins play important roles in intracellular signaling. The normal counterparts of these oncoproteins are involved in the generation of a cascade of events which ultimately cause the DNA in the nucleus to start replicating. Signal transduction through a growth factor receptor is viewed in the following way. A growth factor binds to its receptor, the receptor

becomes transiently activated. This in turn activates other proteins involved in the growth-stimulatory pathway. It also activates the production of a variety of small regulatory molecules called second messengers. These signals are ultimately transmitted to the nucleus, where the expression of specific genes is induced, resulting in cell division (Fig. 1).

There are many types of signal transduction mechanism as shown in table 2. One well known mechanism signal transduction is through receptors coupled to G proteins. Ligand binding leads to activation of specific G proteins. Activated G protein alpha-subunits modulate activity of specific targets (e.g., adenyl cyclase, phospholipases C and A_2, ion channels, cGMP phosphodiesterase).

G-proteins are a guanyl-nucleotide-binding proteins, which serve as the information-carrying intermediate between hormone receptors and their specific targets. The hormone-receptor complex binds to the G protein, and induces the exchange of GTP for GDP. The GTP bound G protein is an active form which in turn, activates the adenylate cyclase or other targets depending upon the species, and increases the level of cyclic AMP in the cytosol. G protein also possesses another property- an intrinsic GTPase activity, which hydrolyzes the bound GTP into GDP and switches off the adenylate cyclase or other specific targets. Scolnick (1979) recognized a similarity between the ras proteins and those of the G protein

family.

The acronym of ras is derived from the words rat sarcoma because these genes were first identified as the transforming genes of the Harvey and Kirsten strains of rat sarcoma viruses (Harvey, 1964; Kirsten et al., 1967). There are at least three classes of mammalian ras genes (Bishop, 1983). Two of these c-Ha-ras and c-Ki-ras first appeared the transduced oncogenes of the Harvey and kirstein rat sarcoma viruses (v-Ha-ras and v-Ki-ras). The third (N-ras) was found as a transforming gene in human tumor cells (Shimisu et al., 1983). Ras oncogenes have been isolated with the help of DNA transfection techniques.

Ras genes have been the focus of intense research since 1982 when their transforming alleles were first identified in human tumors. Activated ras oncogenes are thought to account for up to third of all human cancers. In some cases like tumors of pancreas and colon, 50 -90 % result from mutations of ras genes, and 95% of these mutations are point mutations in certain domains such as codon 12, 13 and 61 or alteration in the ras gene expression (reviewed in Barbacid, 1987). The biological significance of these mutations are being studied. By using gene-targeting method to disrupt the activated Ki-ras gene in two colon carcinoma cell lines, DLD-1 and HCT 116, Dr. Shirasawa and his colleges found that cells disrupted at the activated k-ras gene were morphologically altered, lost the capacity for anchorage independent growth, grew more slowly both in vitro and in nude mice, and showed reduced expression

of c-myc. They suggested that Ki-ras gene activated by point mutation plays a key role in colorectal tumorigenesis through altered cell differentiation and cell growth.

The ras pathway has been intensely studied recently. There is more known now about the ras pathway by far than about any other pathway in the cell -Dr. Michael Wigler. Revealing the ras pathway in cells should greatly help our understanding of the regulation of cell growth and human cancer. The ras gene encoded proteins, p21, have similar biochemical properties as the G protein, for example, they bind guanine (GTP and GDP), possess intrinsic GTPase activity, and are associated with the plasma membrane (reviewed in Barbacid 1987). In fact, certain domains of ras proteins exhibit significant sequence homology with the alpha subunit of G proteins which activates adenylate cyclase (Gilman, 1984). These findings suggest that ras proteins are signal transducers for cell surface growth factor receptors. The ras oncogenes have been shown to sustain mutations that render them constitutively active by keeping the proteins in the active GTP-bound state. Recent evidence suggests that the GTPase activity of ras is under the regulation of GAP (GTPase activating protein), a likely candidate for a ras effector protein. A model for the role of GAP has been proposed and is shown in figure 2. The GAP is putatively responsible for the conversion of p21-GTP to p21-GDP, but mutated ras escapes this regulation and remains in the active GTP-bound state, thus a

continuous rather than a ligand regulatory signal is delivered (McCormick et al., 1989). A second class of regulators of ras were also reported recently. They are made up of guanine nucleotide exchange factors that stimulate the slow basal rate at which guanine nucleotide exchange on and off ras after binding to an activated receptor. Since GTP is a predominant guanine nucleotide in the cytosol of mammalian cells, the nucleotide exchange factors act to increase the amount of GTP bound to ras relative to GDP and hence are activators of ras (Downward, 1992). Recently mammalian homology of the yeast Ras exchange factor genes (CDC25, SDC25, and ste6) have been cloned (Martegain et al., 1992; Shou et al., 1992; Wei et al., 1992). In addition, the two murine homologous of the Drosophila Son of Sevenless (SOS) gene have been cloned by low stringency hybridization (Bowtell et al., 1992), and one (hSOS) gene from human has been cloned (Chardin et al., 1993). New details of ras pathway through which a signal is delivered from cell surface to the nucleus has been recently put forward. The current model of the ras pathway is outlined in figure 3. The EGF (Epidermal growth factor) receptor is turned on and binds to two linked proteins, GRB-2 (Growth factor receptor-bound protein 2) and SOS (Son of sevenless). SOS then binds to the membrane-bound ras protein followed by ras binding to the raf-1 proteins which begins activation of a sequence of enzymes. Eventually, genes which encode transcription factors are activated, and thus turn on gene

expression. The latest work focuses on the ras pathway in cancer therapies. Drugs are being devised that deactivate the ras proteins by disabling their anchor to the cell membrane, a positioning essential if ras is to act as a transducer in signal pathway (James 1993).

2) Biological Properties of SV40

Simian Virus 40 (SV40) is an oncogenic member of the papova group of DNA viruses. SV40 was originally isolated from cultures of monkey kidney cells. SV40 virus is an ideal subject to study as it grows easily in tissue culture; it is easily purified and manipulated; and its genome is less than 6 kb. This simple model systems have provided information on transcription, RNA processing, DNA replication, gene regulation, DNA structure, DNA-protein interactions and oncogenic transformation. Fig. 4 shows that the genome of SV40 consists of a single molecule of double stranded, covalently closed circular DNA, just 5243 nucleotide pairs in length. The genome is divided into two functionally distinct genetic regions of about equal size. Each half of the genome encodes a different set of viral functions. The early region codes for two proteins, large tumor antigen (T-ag) and small tumor antigen (t-ag). The late region codes for three proteins, VP1, VP2, and VP3, which are structural components of the

icosahedral capsid and are synthesized in appreciable amounts only at late times during infection. The stretch of DNA between the ends of the early and late mRNAs contains the controlling elements for both DNA replication and transcription in SV40. The origin of viral DNA replication contains a TATA box governing the starting points for early mRNA synthesis, as well as three sets of nucleotide sequences to which the large T antigen binds tightly, and specifically. The control region also provides two tandem repeated 72-bp enhancers sequences just to the left of the origin sequences. The formation of this complex appears to be the critical step in regulation of both expression of the viral genes and viral DNA synthesis (DeLucia et al., 1983).

Lytic growth of SV40 can be divided into two stages. The early stage includes the induction of early transcription of T antigen and cellular DNA synthesis. The SV40 early transcription unit is transcribed into two mRNAs with identical 5′ and 3′ termini but different splice junctions. The smaller 2.3 kb (16S) mRNA encodes large T antigen, a protein of 94,000 daltons apparent molecular weight which is found predominantly in the nuclei of infected cells and is essential for replication and transformation. The larger 2.6 kb (19S) mRNA encodes small t antigen, a protein of 17,000 daltons apparent molecular weight. As the level of large T antigen rises, early transcription is down regulated by T-antigen-mediated autoregulation. Late stage infection includes

the synthesis of late viral mRNA which codes for the structural proteins of the virion.

SV40 can adopt two different life styles, either productive infection or nonproductive infection, depending upon the nature of the host cells. Monkey cells are fully permissive; in such permissive cells the genome of the virus is completely expressed: the viral DNA is replicated to high copy number, progeny virus particles are released, and the cell is killed via a vacuolating process. If however, the viruses are used to infect cultured cells such as mouse, rat, or human, there occurs an abortive or nonproductive response during which very little or no progeny virus is produced and the cells survive. In a fraction of the cells undergoing nonproductive infection, segments of viral DNA become integrated into the cellular genome. The expression of this integrated DNA causes the cells to change their pattern of growth and to acquire properties similar to those of tumor cells. Studies also found there is no specificity apparent in the sites of integration within either the host or the viral DNA although some regions are preferred (Salzman, 1986). Transformation does not appear to be the primary function of SV40 in its natural hosts. This virus is dependent on cellular encoded proteins for the replication of its genomic DNA, and it will induce cellular division and synthesis in a resting population. This is achieved by the action of a viral encoded protein such as T antigen which causes the cell to ignore its

own regulatory signals and enter into its cell cycle.

3) T-antigen and Transformation

Not all viral genes are required for transformation. Several lines of evidence show that only the early region that code for the first 147 amino acid of large T antigen is actually required for transformation (Sompayrac et al., 1984; Sompayrac et al., 1988). T antigen is a heat labile, largely nuclear phosphoprotein of 708 amino acids. T-antigen has multiple biochemical activities. These include: a) the initiation of SV40 DNA replication, which is partly dependent on specific binding to the origin of SV40 replication, b) autoregulation of viral early transcription, c) stimulation of host cellular DNA and ribosomal RNA synthesis, d) induction cellular enzyme synthesis, e) transactivation of selected RNA Polymerase II promoters, including the SV40 late promoter, f) complex formation with cellular protein P53, g) adenine nucleotide binding, h) ATPase and helicase activities (for a review, see Livingston and Bradley, 1987). The T antigen is necessary and sufficient in the viral transforming process and can fully transform cultured cells and induce rodent tumors when present in sufficiently high concentration (for a review, see Tooze, 1981; Fried and Prives, 1986). Since T antigen also binds to sites in mammalian DNA (Martin, 1981), it is widely presumed that transformation by SV40 is based on aberrant host DNA synthesis and/or altered gene expression caused by the

formation of complexes between T antigen and host DNA. The recent discovery of complex formation between T antigen and the retinoblastoma (Rb) gene product (Ludlow et al., 1989) suggests another mechanism of transformation - the modulation of antioncogene activity. The RB binding sites on T antigen have been mapped by deletion mutagenesis to residues 105-114 on the SV40 genome. This region is required both for Rb binding and for transformation (Cherington et al., 1988). This transforming domain is structurally similar to domain 2 of the adenovirus EIA protein (Figge et al., 1988). The binding of Rb to T antigen is regulated in part by the cell cycle-dependent phosphorylation and dephosphorylation. T antigen only binds to the dephosphorylated RB which was only detected during the G0/G1 phase of cell cycle (Ludlow et el., 1989). It has been found that only the dephosphorylated form of RB is active in suppressing cell proliferation. Binding of T antigen may modulate or inactivate this protein so as to obliterate the control of cell proliferation.

The binding of T antigen to retinoblastoma may act to release the infected cells from their G1 block and allow progression through the cell cycle. This will then activate cdc2 kinase and PP2A which will act to convert the T antigen into a form able to initiate DNA replication. A quiescent cell is pushed into cycle, so that the viral DNA can be replicated to a high copy number. This will occur in S phase in concert with the cellular DNA (Reviewed in Christopher, 1991). In a

transformed cell, where cell lysis in not the outcome, the viral DNA continues to generate this signal giving rise to a cell which can divide under conditions where normal cell division is inhibited.

However, one shortcoming of these antioncogen-modulation models is that they fail to account for the progressive nature of the transformation process over long periods of time following the initial infection. This was also noted at a meeting on 'The Role of DNA Viruses in Human Tumors' (Marx, 1989). It was suggested that viral infection may, over the long term, lead to mutations in critical host genes, e.g. oncogenes and that full blown malignancy would require cooperation between one or more activated oncogenes and the persisting transforming genes of the virus. It was also suggested that such mutations might come about as a consequence of repeated rounds of abnormal cell division initiated by viral infection. In this connection it should be noted that: 1) SV40 T antigen has been shown to have mutagenic activity in assays of mutations at the HGPRT locus (Gorbunova et al., 1982; Geissler et al., 1983; Zannis et al., 1983; Shapiro et al., 1984) and, 2) chromosomal breaks and aberrations are a general feature of transformed cells. Activation of oncogenes via chromosomal translocation presumably resulting from chromosomal breakage at certain 'fragile sites' is a frequent observation in some types of tumors (LeBeau, 1986). Insertion of viral DNA sequences leads

to a rearrangement of cellular sequences at the integration site (Wolf et al., 1984; Westaway et al., 1984). The requirement for cooperation between the oncogenic sequences of DNA viruses and the retroviral oncogenes to achieve the malignant phenotype has been well established by transfection experiments (Land et al., 1983; Ruley, 1983; Thompson et al., 1989). The significance of oncoprotein cooperation might be in line with the fact that the normal cell has multiple independent mechanisms for cellular growth control, and that several separate events are needed to override these systems, as well as to induce other aspects of the transformed phenotype (reviewed by Hunter 1991).

Transformation by Herpes Simplex virus provides some of the strongest evidence for activation of host oncogenic processes by a DNA virus (Galloway et al., 1983; Galloway et al., 1984). Rodent cells transformed by Herpes DNA retain different fragments of the transfected DNA depending upon method of selection used (i.e. growth in semisolid medium/focus formation vs. immortalization). Sequences identified as necessary and sufficient for transformation on the basis of transfection of various restriction subfragments of HSV-2 DNA into hamster embryo fibroblasts are not the sequences retained by these cells when whole HSV DNA instead of individual restriction fragments are used; in some cases none of the viral DNA is retained by the transformants.

4) Biological and SV40 Transforming Properties of Cultured Human Keratinocytes

This laboratory was the first to demonstrate and characterize the transformation of human keratinocytes by SV40 (Steinberg et al., 1979). Epithelial cells were chosen as a model system because most human tumors are of epithelia origin and because improved culture systems are now available to study them in vitro. Recent advances in epithelial cell culture have made it easy to follow or observe the oncogenic processes in vitro since the developmental process of epidermal differentiation is maintained in culture.

When dissociated epidermal cells are inoculated together with supporting 3T3 cells, single epidermal keratinocytes can grow into colonies of stratified squamous epithelium (Rheinwald and Green, 1975). Mature colonies of keratinocytes, like a stratified squamous epithelium in vivo, contain basal layers of dividing cells and upper layers of differentiating cells. The cells of the upper surface of colonies appear very flattened and have a much greater surface area, also they have the ultrastructural appearance of keratinocytes such as particles like keratohyhaline granules and cross-linked envelopes and other cytological features characteristic of the cells of the stratum corneum.

In this system the virus initiates a process of transformation which progresses through distinct stages over

a period of many months following infection (Defendi et al., 1982; Steinberg et al., 1983; Steinberg et al., 1985; Steinberg et al., 1986). The transformation process can be divided into three distinct stages (Fig. 5) (Defendi et al., 1982). During the first stage, (precrisis about 10 passage) cells have a higher colony-forming efficiency and higher growth rate (cell multiplication in normally nondividing differentiated central regions), as well as an indefinite life span as compared with uninfected cells which undergo senescence within five to seven serial passages. As more cells become increasingly SV40 T antigen positive, the loss of growth dependence on high serum concentration, breakdown of junctional communication as detected by transfer of microinjected fluorescent dye (Steinberg et al., 1981; Steinberg et al., 1982), clonal growth in the absence of feeder layers (Steinberg et al., 1979; Defendi et al., 1982; Steinberg et al., 1983) and loss of histochemical and cornified cell envelope markers of terminal differentiation (Steinberg et al., 1979; Defendi et al., 1982; Steinberg et al., 1983) are appeared in this early stage. Some of the phenotypic properties whose expression is blocked by SV40 infection are inducible by treatment with 5-azacytidine (5-aza) during this early phase of transformation but response to 5-aza is lost just prior to the crisis period (Okada et al., 1984). The second stage of transformation (crisis 10-15 serial passages) involves a decrease in growth potential and the

appearance of large, bizarre, vacuolated cells. The end of the crisis period represents the onset of a third stage (postcrisis- after passage 15). These cells which survive past the crisis period are established as cell lines with indefinite growth potential (Steinberg et al., 1983). The cultures emerging from crisis acquire the capability to grow as attachment-independent colonies in semi-solid agar. There is also an increase in total synthesis and a redistribution of fibronectin over the cell surface (Edelman et al., 1985).

Keratins are a group of water-insoluble proteins which are the major differentiation-specific proteins of epithelial cells. Since their expression is very tightly linked to the cell type and the stage of differentiation, the keratin proteins serve as a useful biochemical marker of epithelial differentiation. Extensive analysis of keratin gene expression in cultured keratinocytes has shown that SV40-transformed human keratinocytes express an altered set of keratin proteins as compared with normal cultured keratinocytes. The transformed cells have lost the 58, 56, 50, 48, and 46kD keratins that are expressed by in normal cultured keratinocytes. Instead, these cells express mainly 52, 45 and 40KD keratins, a set highly characteristic of simple epithelial cells which are not found in their untransformed counterparts (Steinberg et al., 1985). The changes in keratin expression indicate the differentiation program of keratinocytes may have been changed to reflect that of a

simple epithelium.

In addition to the expression of simple epithelial keratins in SV40 transformed keratinocytes, a group of novel proteins has also been detected in keratin immunoprecipitates from SV40- transformed keratinocytes such as 48, 54.5 and 43 KD proteins with PI 5.8, 5.0 and 5.7 respectively (Morris and Steinberg, 1985). cDNA libraries have been created from a line of SV40 transformants. From these libraries we have now isolated at least one cDNA (pG1) which corresponds to an unknown cytoskeletal protein marker of transformation in both viral and spontaneous (i.e. squamous cell carcinoma derived) transformants (Genbank name: HUMPG1., Morris and Steinberg, 1989). These findings strongly suggest that some cellular genes have been activated in SV40 transformed human keratinocytes and that the expression of these genes might be closely related to the emergence of the transformed phenotype (Morris and Steinberg, 1989)

The findings of new proteins in human keratinocytes induced by SV40 infection and the progressive nature of the transformation process over long periods of time following the initial infection further suggest that transformation of human epithelial cells by SV40 may interfere with the functioning of other critical cellular genes in a process involving multiple stages. This multistep process parallels features of multistep carcinogenesis seen in malignant transformation in vivo. Studies on the genetic basis of

coloretal tumor development by Fearon's group demonstrated that at least four genetic alterations had been found, including mutational activation of the ras oncogene and deletions of chromosomes 5q, 17p, and 18q which include loci of tumor suppressor genes (p53 and DCC). In addition, they found that these genetic alterations often occurred according to a preferred sequence (Fearon et al., 1990). These findings suggest that the development of malignancy would require cooperation between one or more active oncogenes.

Based on the knowledge we have now, we hypothesize that the appearance of transformed properties which occur in a progressive way results from activation of host oncogenic sequences subsequent to SV40 infection.

From previous work done in this lab, we already know that myc becomes amplified and undergoes changes in methylation as a result of SV40 infection. Evidence is accumulating that structural changes in myc and ras are the most common oncogene alterations found in human tumors (Barbacid, 1987). Also the view that tumorigenesis involves direct mutation of ras has been implicated in several forms of experimental carcinogenesis tests (Sukumar, 1989). In this study, we studied oncogene-related alterations in human epithelial cells which result from infection of human epithelial cells by SV40. We have examined restriction fragment length polymorphisms in the ras oncogene from both normal and SV40 transformed keratinocytes in Southern blot analyses. We have also examined

the levels of ras expression in normal and SV40 transformed cells by northern blot analyses. A 1.8 kb ras upstream region was cloned and sequenced. Computer analyses, DNase I footprinting of this region indicated the presence of DNA-binding motifs for the factors such as T-ag, Ap-1 and SP1 in the upstream region of the Ha-ras protooncogene. Possibility that SV40 and other transforming agents may activate ras by transcriptional enhancement of gene expression acting within the upstream region.

II. MATERIALS AND METHODS

1) Isolation of Genomic DNA and Southern Blot Analyses

For isolation of genomic DNA, 10 x 100 mm plates containing 10^8 cells were rinsed with 1 x PBS twice. 5 ml of extraction buffer (100 mM Tris, pH 7.9; 10 mM NaCl; 10 mM EDTA [Ethylene D? Tetraacetic Acid]; and 0.5% SDS [Sodium Dodecyl Sulfate]) with 500 μg of proteinase K was added to each plate and this was allowed to incubated at 37°C overnight. The plates were scraped gently and the DNA mixture was placed in a 50 ml centrifuge tube. Equal volume of phenol/chloroform-isoamyl alcohol in a ratio of 1:1 was then added to each tube. The ratio of chloroform to isoamyl alcohol is 24:1. The 50 ml tube was then placed on a gentle mixer for 10 minutes. This was then centrifuged at 3k rpm for 10 minutes. The supernatant was saved and phenol/chloroform-alcohol extraction was repeated. DNA was precipitated with two volumes of ethanol, washed with 70% of ethanol, and dried for 15 minutes under vacuum. To each tube 2 ml of TE (10 mM Tris, pH7.4 and 0.5 mM EDTA) was added and allowed to incubated at 37°C overnight. RNase A was added to a final concentration of 10 μg/ml. The phenol-chloroform extraction and the ethanol precipitation were then repeated. DNA was resuspended in 0.5 ml of TE (pH 7.5) and dialyzed for two days against 1000 ml of TE (pH 7.5). For Southern blot analysis, ten micrograms each of the genomic

DNAs from normal cultured keratinocytes (EP), a line of squamous carcinoma cells (SCC), and two lines of SV40 transformed keratinocytes (425 and 98) were digested with a series of restriction enzymes which can generate appropriate numbers and size of restriction fragments, electrophoresed in 1% agarose gel, and transferred to nitrocellulose. To examine the alterations of ras containing fragments, filters were hybridized to a nick translated, ^{32}P-labelled probe made from the plasmid, pTB-1, which covers a 4.8 kb exons-containing segment of the T24 human ras oncogene starting from the left BamH I site (Goldfarb et al., 1982) (Fig. 6). To examine length polymorphisms in the myc containing restriction fragment, blots were hybridized to a ^{32}P-labelled probe made from plasmid, pMC413RC, which contains the third exon of the myc oncogene (Dalla et al., 1984) (Fig. 7). Prehybridizations were for 1 hr at 42^{0}C in 50% formamide, 0.1% PVP (Polyvinypyrlidone), 0.1% Ficoll, 5x SSPE (Saline, Sodium Phosphate, EDTA), 0.1% BSA (Bovine Serum Albumin), and sheared salmon sperm DNA at 100 μg/ml. Hybridizations were for overnight at 42^{0}C in 50% formamide, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, 5x SSPE, salmon sperm nonspecific DNA at 100 μg/ml and probes with specific activity 10^8 cpm/μg of DNA. Final washes were for 20 min twice at 55^{0}C in 0.1x SSC (Sodium Chloride, Sodium Citrate)/0.1% SDS (Sodium Dodecyl Sulfate), then in 0.1x SSC for another 20 min. The hybridization patterns of ras or myc containing restriction fragments were

compared between normal and transformed cells to show the polymorphisms.

2) Isolation of Total RNA and Northern Blot Analyses

Cells from uninfected keratinocytes (EP), and two lines of SV40 transformed keratinocytes (425 and 98) were grown in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal calf serum. For the isolation of cytoplasmic RNA, 10^8 cells were washed twice with 1x cold PBS (phosphate-buffered saline), and cells were scraped into 5 ml of 1x PBS with a rubber policeman. Cell suspensions were pooled in 50 ml Falcon tubes and spun at 2,000 rpm for 5 min. Cell pellets were dissolved in 0.05 M Sodium Citrate buffer (Containing 4M Guanidinium isothiocyanate, 0.1M Beta-mercaptoethanol and 0.5% Sodium Sarcosyl) at 0.5 ml for each flask. The mixture was then transferred to an Eppendorf tube, and 50 μl of 2M Sodium acetate buffer (PH 4.2) and 0.5 ml of water-saturated phenol were added. The solution was mixed and 100 μl of chloroform was added followed by vortexing. Aqueous and organic phases were separated by centrifugation at 10,000 rpm for 20 min at 4^0C. RNA was precipitated from the aqueous phase by ethanol, the pellet was collected by centrifugation and the pellet was washed, dried and dissolved in water. For northern blot analysis, 30 μg each of cytoplasmic RNAs derived from normal keratinocytes and SV40 transformed cell lines were dissolved

in 20 μl of 1x running buffer (50% Glycerol; 1 mM EDTA (pH 8.0); 0.25% Bromphenol blue and 0.25% Xylene cyanol). The RNAs were heated for 5 min at 65^{0}C and electrophoresed in 1% agarose gels containing 20 mM morpholinopropanesulphonic acid, 1 mM EDTA, 5 mM NaAc and 2.2 M formaldehyde. The RNAs were then transferred to nitrocellulose paper in 10x SSC. The filter was air dried and baked for 2 h in a vacuum oven at 80^{0}C. Prehybridization was for 2 hr at 42^{0}C in 2x PIPES buffer, 50% deionized formamide, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA. Hybridization was in the same solution for overnight at 42^{0}C with a probe of ^{32}P-labelled EJ fragment (Shih et al., 1982), kindly provided by Dr. Seymour Garte of NYU (Fig. 8). The final wash was in the same solution and conditions as in Southern hybridization. The hybridization intensity and mobility of the hybridizing species were compared between the normal and transformed cells to examine the alteration of expression of ras. The same blot was washed again and rehybridized to a myc probe (pMC413RC) (Fig. 7) to examine the expression of myc gene in the same samples. To confirm the quantity and quality of the RNAs we isolated previously, the blot which had been hybridized to the ras probe was washed in 0.1x SSC for 30 min at 90^{0}C and rehybridized to a probe which containing a cloned actin gene.

3) Cloning of Ras and Myc-Containing Fragments

Previous Southern hybridization analysis had shown ras and myc containing fragments generated by BamHI digested were 6.6 Kb and 15 Kb in size respectively. To clone ras and myc containing fragments, genomic DNA libraries were constructed using BamHI-digested DNA from normal and SV40 transformed human keratinocytes. 10 μg resulting BamHI digested DNA fragments were purified by phenol-chloroform extraction and then ligated into EMBL4 bacteriophage vector DNA which has been previously digested by BamHI (Promega Biotec) (Fig. 9) in a molar ratio of 3:1 (insert to vector). The ligation reaction was carried out by using T4 ligase in 15^0C water bath for 24 hours. Once the ligation mixture was removed, it was tested by gel electrophoresis. After the ligation procedure was finished, 4 μl of the ligation mixture was then used for packaging in vitro by using Gigapac packaging system (Stratagene), followed by the addition of 30 μl of chloroform and 500 μl of sterile phage buffer SM (100 mM NaCl; 17 mM $MgSO_4 7H_2O$; 1 M Tris-Cl, (PH 7.5); and 2% Gelatin).

To screen the human genomic library constructed by BamHI digestion, first, EMBL4 bacterial phage carrying the insert was used to infect the E.Coli host NM539 (Promega Biotec) by placing 200 μl of bacterial in a 15 ml test tube in the presence of 10-20 μl of packaged mixture. The amount of packaging mixture used depends on the titer of plaque- forming

unit (PFU), usually 10^4 plaques would be plated separately in a 150 mm plate. The mixture of phage and bacteria was allowed to incubated for 15 min at 37^0C before adding 3 ml of 0.75% NZYM top agar. It was then spread evenly on the 15% of NZYM bottom agar plate and was placed in a 37^0C incubation overnight.

Second, nitrocellulose filter was placed on the top of the agar plate. The filter's position was marked by making three asymmetric holes using a 18 gauge needle, and marked on the back of bottom plates with a permanent pen. After the filter was completely wet, it was peeled off and was transferred to the 3 MM paper which was saturated with denaturing solution (0.5 M NaOH and 1.5 M NaCl), and was allowed to stand for 4 min. The filter was then transferred to a second 3 MM paper that has been saturated with neutralizing solution (1.5 M NaCl and 0.5 M Tris-Cl, PH 8.0), and was left for 7 min. Two pieces of 3 MM whatman papers were then used to sandwich the filter and this was baked for 2 hours at 80^0C in a vacuum oven.

Approximately 5 x 10^5 recombinant phages, propagated in the E.Coli host NM539 (Promega Biotec), were screened by plaque hybridization using nick-translated 32p-labelled probes: a 1.9 kb BamHI\XbaI fragment isolated from plasmid pEJ, which contains 5′ end of 6.6 kb Ha-ras fragment from bladder carcinoma cell line (Shih et al., 1982) (Fig. 8), and a 0.86 kb PvuII fragment isolated from plasmid pMC41 (Dalla et

al., 1984), which contains the first exon of myc (Fig. 7). The solution and conditions of hybridizations and final washes were the same as in Southern hybridization. After 2-3 round screen, ras and myc containing positive clones were isolated and allowed to diffused in 1 ml SM buffer. 30 μl of recombinant phage was mixed with 200 μl NM539 and placed on the plate to get a complete lysate. 5 ml of SM buffer was added to the plate the next day, and was allowed to shake for 2 hours in a rocker at room temperature. The phage stock solution (10^{10} pfu/ml) was collected into a 15 ml tube and stored in 4^0C. For large scale isolating ras or myc cloned DNA, 200 μl of phage cloned stock solution was incubated in 5 ml of NM539 for 10 min at 37^0C. The mixture was then added to 500 ml fresh NZYM medium and placed in a shaker overnight at 37^0C.

Phage DNA was isolated by CsCl gradient centrifugation at 55,000 rpm for 16 hours at 22^0C. The next day, phage band was collected and dialyzed against 1000 ml of dialysis solution (10 mM NaCl, 50 mM Tris-Cl (pH 8.0), and 10 mM $MgCl_2$) for 1 hour twice. Then a mixture (20 mM EDTA, 50 mg/ml proteinase K, and 5% SDS) was added to the phage solution, and allowed to incubated for one hour at 68^0C followed by extraction with an equal volume of phenol-chloroform. For confirming the ras and myc clones, cloned insert DNAs were released from recombinant phage by BamHI digestion, then electrophoresesed on 1% agarose gel and hybridized to a ras or myc containing probe.

4) Subcloning and Sequencing Analysis of ras Containing Fragments

For sequence analysis of the 5′ region of the Ha-ras, 1.9 Kb BamHI\XbaI fragments from transformed and nontransformed cell lines which contain the promoter\enhancer and the first exon Ha-ras were released from the 6.6 Kb ras containing clones by BamHI and XbaI digestion, and purified by electrophoresis through low melting point agarose gels and phenol extraction. These 1.9 Kb B/X fragments were then subcloned into M13 sequencing vector variants MP19 and MP18 (Promega Biotec) (Fig. 10). The method of isolation double strands DNA of MP13 was as described (Promega Biotec). A cyclone system was used to generate a set of overlapping subclones about 250 base pairs in size for sequencing (IBI) (Fig 11). First, the single stranded of MP19 recombinant was annealed to a EcoRI oligomer and the annealed hybrid was then digested with EcoRI to generate a nick. Then the linearized fragment was digested by T4 DNA polymerase at 3 min intervals to generate an overlapping set of subclones. In the final step, T4 DNA ligase was added to seal the remaining nick. The sequences of each of the subcloned fragments were determined by the dideoxynucleotide method of Sanger (1977) using Sequenase (United States Biochemical) and the M13 universal primer. All the sequencing was carried out on 6% polyacrylamide gel containing 7 M urea.

5) Cloning and Sequencing Analysis of 5′ Upstream Region of Ras

To further study the 5′ adjacent region of Ha-ras, new genomic libraries were constructed in the lambda bacteriophage vectors GEM-11 (Promega Biotec Fig. 12) using the same method as described in part 3 above. Previous Southern blot analysis had indicated that XbaI digestion would generate a 3.7 Kb fragment when using 1.9 Kb B/X ras containing fragment as a probe. For cloning, vector DNA was digested with XbaI. Genomic DNAs derived from normal keratinocytes and SV40 transformed cell lines (425 and 98) were digested with XbaI and ligated into the XbaI sites of the vector, and the phage DNAs were packaged in vitro using the Gigapac packaging system (Stratagene). LE392 cells (Promega Biotec) were infected, yielding 2x 10^5 recombinants as determined by plaque assay. The libraries were then screened with a 0.3 kb RsaI c-ras fragment (Fig. 8) derived from the pEJ fragment labeled by nick-translation. The solution and conditions of hybridization and final washes were the same as for Southern hybridization.

For sequencing the ras adjacent region, the cloned 3.7 kb XbaI fragments which cover 1.9 kb of the EJ ras fragment and 1.8 kb 5′ upstream of EJ were isolated as described in part 3 above, and further subcloned into the XbaI site of pGEM-3, a vector which contains T7 and SP6 promoter primers for sequencing inserts from both ends (Promega Biotec) (Fig. 13).

To determine the orientation of the XbaI fragments cloned in pGEM-3 with respect to EJ, sequencing was started from two ends using primers SP6 and T7. After the orientation was determined, a primer flanking the 5′ end of 3.7 Kb fragment was used as a first primer in sequencing. Sequencing was continued by primer walking with the second primer. Second primer was designed to continue the sequencing based on the data from the end of the first sequencing run, and so on until sequencing to the end of the adjacent region was completed. DNA sequencing was performed by the dideoxynucleotide termination technique of Sanger (1977) on double stranded plasmid DNA using a sequenase kit (USB).

6) Preparation of Nuclear Extracts

Cells from HeLa and line 98, SV40-transformed human keratinocytes, were cultured in DMEM medium supplemented with 10% fetal calf serum as described by Rheinwald and Green (1975). Cells were allowed to attain near confluence at the time of harvesting.

Method of isolation of nuclear extracts was as described by Dignam et al. (1983). Nuclear extracts were prepared from a total at 10^8 cells. Cells were washed twice in mololayer with 5 ml cold 1x PBS and then scraped into 5 ml of PBS with a rubber policeman, and pooled in a 50 ml Falcon tube and pelleted at 2,000 rpm for 5 min. All steps below were

performed at 4^0C. Cells were resuspended in 4 ml cold buffer A (10 mM Tris, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT [dithiothreitol] and 0.5 mM PMSF [Phenylmethylsulfonyl fluoride]) and allowed to swell on ice for 10 min. lysed cells by 10-20 strokes Dounce homogenizer and spun at 2,000 rpm for 10 min. The resulting pellet was resuspended in 200 μl buffer C (20 mM Tris, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM PMSF and 0.5 mM DTT), transferred to an Eppendorf tube and centrifuged for 5 min. In a microfuge KCl was added to a final concentration of 0.3M to the resulting suspension and rocked for 30 min to extract nuclear proteins. The suspension was centrifuged for 15 min in a microfuge at 12,000x g to pellet nuclei and chromatin. The supernatant was dialyzed for 2-4 hr against 250 ml BC100 buffer (20 mM Tris, pH 7.9; 20% Glycerol; 0.1M KCl; 0.2 mM EDTA; 0.5 mM DTT and 0.5 mM PMSF) with one change of buffer. Aliquot were frozen and stored in liquid nitrogen rapidly or stored at - 70^0C.

7) Gel Retardation Assays

For DNA protein-binding assays, four subfragments (F1, F2, F3 and F4) were generated from the 1.8 Kb XbaI/BamHI fragment containing the 5′ adjacent region of Ha-ras isolated from a 3.7 Kb XbaI clone derived from the SV40 transformed cell line, 98, and subcloned into the PGEM-3 vector. The 1.8 Kb DNA fragment derived from this subclone was first released

by XbaI and BamHI (X/B) digestion, and eluted from a low melting point agarose gel. The X/B fragment was then digested by StyI which has two cut sites within the 1.8 kb fragment thereby generating three fragments, fragment A (0.39 kb), fragment B (0.8 kb) and fragment C (0.6 kb). The three fragments were purified from low melting point agarose gels. Fragment A was designated F1, fragment B and C were further digested by SfaNI and BbvI respectively yielding F2, F3, and F4 (Fig. 14). These four fragments were eluted from low melting point agarose gels, and labelled with appropriate ^{32}P-nucleotide by Klenow enzyme.

Each gel shift reaction mixture contained 4 μl of 5x Gel shift buffer (25% glycerol; 50 mM Tris, pH 7.5; and 250 mM KCl; 1 μl 25 mM DTT) 1 μl 1% Nonidet P-40, 2 μl sperm DNA (hsDNA) (1mg/ml), 1 μl Normal Mouse Serum (NMS), and 1 μg nuclear extracts, probe containing 5 ng DNA of about 200-300 bp fragment 10^5 (CPM) and H_2O up to 20 μl. The mixture was incubated at room temperature for 30 to 40 min and 10 μl of each reaction mixture was loaded into a pre-made 4% polyacrylamide gel with 2 μl tracking dye in the first lane. The gel was run at 150 V until the dye reached approximately 2/3 the length of the gel. The dried gel was put on x-ray film overnight for autoradiography (Varshavshy, 1987).

8) DNAase I Foot-Printing

The F2 and F3 fragments which were used in gel shift assays were amplified by PCR with two pairs of primers. The first amplimers were primer L6 (5'-ACTTAGGCCAGGCACGG) corresponding to the nucleotide 1420 and primer T7 (Promega Biotec). The second amplimers were L5 (5'-CCAGGCTGTCTCAGACT) corresponding to nucleotide position 1180 and primer T7 (Promega Biotec). Primers L6 and L5 were end labeled with gamma-^{32}P-ATP and T4 polynucleotide kinase before PCR amplification. The PCR products were then run on, and isolated from a 1% agarose gel and extracted with phenol/chloroform. The end labeled DNAs (10^4 cpm) (2 to 5 ng) were incubated with various amounts of nuclear extract isolated either from line 98 (98NE) or Hela (HNE), and 1 μg of sonicated calf thymus DNA in 50 μl for 30 to 40 min at 30^{0}C. The 50 μl of reaction mixture contained, in addition to the probe, nuclear extract and nonspecific DNA, 50 mM KCl, 32.5 mM Hepes (pH 7.8), 6.25 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 5% glycerol, 100 μg/ml BSA and 0.025% NP-40. After the incubation, 50 μl of cold 10 mM $MgCl_2$/ 5 mM $CaCl_2$ was added to the binding reaction, followed by 1 μl of freshly diluted DNase I at a final concentration of 10 μg/ml. DNase I digestion was done for 1-2 min on ice. The reaction was stopped by the addition of 90 μl of 1% sodium dodecyl sulfate (SDS), 20 mM EDTA, 200 mM KCl, 250 μg of yeast tRNA per ml. The DNA was phenol-chloroform (1:1) extracted

once and ethanol precipitated before running on a 6% polyacrylamide, 6 M urea, sequencing gel. In order to define the position of the protected regions, a G+A sequence ladder was prepared (Maxam and Gilbert, 1977) and run along side the reaction products in the gel. The complementary strands of F2 DNA containing fragment was tested also by using the same method as described above, but the primer to be labelled was T7 instead of L6 used previously.

9) Computer Analyses

The LFASTA and PCGENE programs were used to align the ras sequences between normal keratinocytes and SV40 transformed cell lines. The PC GENE program was also applied to nucleotide analysis, such as restriction mapping, ORF (open reading frame), head-loop structure and repeat sequence. Another program GENBNANK was used to search genes homologized to our 5′ adjacent region of Ha-ras, and also to find ALU, enhancer like fragments. One more program SSCN was used to search the DNA binding motifs.

III. RESULTS

1) Ras-Associated Polymorphism in SV40 Transformed Keratinocytes

Figures 15 and 16 show Southern blot analyses of the ras containing restriction fragments from normal cultured keratinocytes, a line of human squamous carcinoma cells and two lines of SV40 transformed human keratinocytes. The probe used was pTB-1, a Harvey ras probe covering a 4.8 kb exon-containing segment of the EJ oncogene region starting from the left BamHI site (Goldfarb et al., 1982). Pst I digestion of DNA from both normal and transformed keratinocytes produced three fragments of about 0.8, 1.1 and 1.9 kb consistent with the sizes of restriction fragments generated from within the region covered by pTB-1. However, Pst I digestion of DNA from normal keratinocytes and squamous carcinoma cells also produced an additional fragment of about 3.5 kb which was presumed to represent a segment extending leftward from the first Pst I site and including sequences flanking the EJ region on the 5′ side since, based on the published sequence of EJ, there are no fragments of this size within the probe region itself. In contrast, this fragment was not observed in either of two lines of SV40 transformed keratinocytes although a larger fragment of about 4.4 kb was seen in one of them (line 425). Similar results were obtained with Sau3A; DNA

from normal keratinocytes and squamous carcinoma cells generated a fragment of about 3.8 kb which was absent from the SV40 transformants. There was an additional prominent band of about 1.3 kb generated from line 425 DNA. There were no obvious alterations seen in Pvu II digests of the same DNAs. Absence of the large (3.5-4.0 kb) Pst I and Sau3A fragments has also been observed in another line of SV40 transformants (line 22; data not shown).

2) Myc-Associated Polymorphism in SV40 Transformed Keratinocytes

Restriction polymorphisms associated with the myc oncogene have also been found. Figure 17 shows a Southern blot analysis of the myc containing fragments from normal cultured epidermal keratinocytes (EP), two SV40 transformed cell lines (425 and 98), and a line of squamous carcinoma cells (SCC). The probe used was pMC413RC, a restriction subfragment of pMC41, which contains the third exon of the myc oncogene. Pst I digests of DNA from line 98 SV40 transformants do not display the 6.6, and 3.0 kb restriction fragments found in uninfected keratinocytes, but shows highly amplified sequences of about 4.0 kb not seen in normal cells. Also the 6.6 and 3.0 kb fragments found in uninfected keratinocytes were absent in line 425 transformants as well. There were no obvious

alterations found in Pvu II digests of DNAs from both uninfected and infected keratinocytes. These results suggest the alterations associated with SV40 transformed might occur in the 3′ end of the pMC413 covering region.

3) Isolation and Restriction Mapping of ras Containing Fragments Derived from Normal and SV40 Transformed Keratinocytes

Both the 6.6 kb BamHI ras and the 15 kb BamHI myc containing fragments derived from normal and SV40 transformed keratinocytes have been cloned from six EMBL4 genomic DNA libraries as described in Materials and Methods. About 2-3 ras or myc containing clones out of approximately 6 x 10^4 recombinant phage were isolated. To confirm the clones which contain ras or myc proto-oncogene, all cloned DNAs were digested by BamHI and run on 1% agarose gel along with a DNA molecular weight marker. Figure 18 shows the 6.6 kb ras containing fragments derived from the clones of EP, 425 and 98. Restriction mapping analyses of these ras cloned inserts were conducted to identify regions which contain polymorphisms when cloned inserts from viral transformed keratinocytes were compared to the normal keratinocytes. The 6.6 kb BamHI ras containing fragments from individual clones were isolated on

low melting agarose gels and digested with a series of restriction enzymes, such as AvaI, PstI, PvuII, SacI, Sau3A, SmaI and XbaI. These results revealed no indication of restriction polymorphisms in cloned fragments from the transformed cell lines. However, these results do not exclude some subtle changes, such as point mutation, which may not be present within the restriction enzyme recognition sites. In fact, there is much evidence that a point mutation is mostly responsible for activation of the ras oncogene (Reddy, 1982).

4) Sequence Analyses of Cloned Ras Fragments

Based on the result of Southern Blot analyses, we presume that the ras restriction fragment polymorphisms in our SV40 transformed cell lines might be located in the 5′ end of ras containing fragment. Therefore we performed sequence analysis on the 5′ end of the Ha-ras containing region. From previous work we knew that the promoter, enhancer and the first exon which include the codons 12 and 13 are located in this 5′ end region of the ras (Fig. 19). Point mutation in the codons 12 and 13 have been reported to result in the acquisition of oncogenic potential by ras (Barbacid, 1987).

The 5′ end of 1.9 kb BamHI/XbaI ras fragment isolated from the 6.6 kb ras clone was subcloned into M13 sequencing vector variants M19 and M18 (Promega Biotec). Once we had the BamHI/XbaI inserts derived from normal and two lines of SV40

transformed keratinocytes subcloned, we employed a cyclone system to generate a set of overlapping subclones of about 250 base pairs in size as described in Materials and Methods. Complete sequence analysis has been done on three subcloned fragments derived from normal cell (Ep) and transformed cell lines (425 and 98) by the dideoxnucleotide chain termination technique. Sequence data were analyzed by comparing the sequences to one another using computer sequence analysis software (PC GENE; Intelligenetics). These results indicated that there were no SV40 related sequence polymorphisms in these 1.9 Kb BamHI/XbaI ras fragments when the sequences from SV40 transformed cell lines (425, 98) were compared to the sequence derived from normal keratinocyte. This finding suggests that the polymorphisms observed in the Southern blot might be located in a much further region from that covered by our cloned sequences. However, polymorphisms in position 1782, within the first intron of ras, were found in all of our samples including normal keratinocytes and two SV40 transformed cell lines compared with the EJ sequence from published data; G was changed into A in all three of our samples (Fig. 20). The single base difference in all our samples compared with the EJ, a bladder carcinoma ras oncogene might represent the tissue polymorphisms.

5) Cloning and Sequencing Analysis of 5′ Upstream Region of Ha-ras

Our previous sequence analysis on the 1.9 kb B/X 5′ region of the Ha-ras revealed no sequence prolymorphisms there and suggested that the prolymorphisms might occur in the further upstream sequences of the ras. We therefore carried out restriction mapping and sequence analysis of our cloned fragments covering this region. Based on literature and GENBANK searches this region has not been characterized despite the possibility that upstream regulatory elements within this segment could affect the expression of the ras gene 2-4 kb downstream.

A 3.7 kb XbaI fragment, which covers the 1.9 kb 5′ end of the ras EJ containing fragment and its 1.8 kb 5′ end adjacent region (Fig. 21), was cloned from libraries created in lambda GEM-11 (Promega Biotec) using, as a probe, a 0.3 kb RsaI fragment isolated from pEJ (Fig. 8). We were able to obtain clones derived from both normal keratinocytes and SV40 transformed cell lines. In all, three positive clones from normal keratinocytes, two from line 425 and three from line 98 viral transformants were isolated from libraries. To confirm the identify of the clones, XbaI digestion, agarose gel electrophoresis and hybridization were performed. Fig. 22 shows the 3.7 kb XbaI fragments which hybridized to the 1.9 kb BamHI\XbaI ras probe. Restriction mapping was conducted in the

following way: the 3.7 Kb XbaI inserts derived from normal and transformed cell lines were isolated and digested by a series of restriction enzymes, such as Pst1, PvuII and SacI, then run on gels and hybridized to a probe containing the 3.7 kb clone. No difference was found in the restriction pattern of fragments derived from normal and transformed cell lines.

For sequence analysis, the 3.7 kb XbaI cloned inserts were further subcloned into the plasmid vector, pGEM-3 (Promega Biotec) which contains T7 and SP6 promoter-primers for sequencing double stranded inserts from both ends. The 1.8 Kb fragments containing the upstream region 5′ to the Ha-ras protooncogene derived from normal keratinocytes and SV40 transformed cell lines have been sequenced by using following oligonucleotide primers: T7 (promega Biotec), L1 (5′-TTGACTGTGCTTATGTGTAT) corresponding to the nucleotide at position 210, T2 (5′-ACGAGACAAATAGAAATAAT) at position 450, L3 (5′-CAGAGACAAATAGAAATAAT) at position 720, L4 (5′-GCCTGGAGTGCAGTGGCATG) at 1000, L5 (5′-CCAGGCTGTCTCAGACT) at 1180, L6 (5′-ACTTAGGCCAGGCACGG) at 1420, L7 (5′-GCCGAGATGGTGCCACT) at 1660. We found no sequence alteration in this 1.8 kb 5′ adjacent region of the human Ha-ras gene specifically attributable to SV40 transformation.

6) Elevated Expression of Human Ras Gene in SV40 Transformed Keratinocytes

To determine whether cellular Ha-ras transcripts are elevated in SV40 transformed cultured human keratinocyte, northern blot analyses on total cellular RNA were performed. Total RNA samples were prepared from both normal and SV40 transformed cell lines, 98 and 425, and hybridized on northern blots with a pEJ probe as described in Materials and Methods. Figure 23 showed the presence of major bands near the 28S band of ribosomal RNA which (about 5-kb), and in several cases a much less intense bands of about 2-kb near the 18S band of ribosomal RNA were seen also. Densitometric scanning of the 5-kb bands using a Hewleh-Packard Scanjet Plus Flatbed scanner and Image Star software (Microtek, Inc) showed the ras related transcripts to be at least 5 times higher amounts in two SV40 transformed cell lines (425 and 98) then in normal cultured keratinocytes (EP). The same blot was also rewashed and rehybridized with a probe created from a 0.86 kb PvuII restriction fragment containing the first exon of the myc protooncogene. Myc transcripts of about 1.8 kb were seen. The results showed no change in the expression of myc in both SV40 transformed cell lines as compared with normal keratinocytes (Fig. 24). To confirm the amount and integrity of RNA samples, the blot shown in Figure 23 was washed and rehybridized with a cloned probe which includes a ß-actin gene. This

demonstrated that approximately equivalent amounts of undegraded RNA were present in each lane (Fig. 25). We conclude that the ras transcripts are significantly elevated in SV40 transformed keratinocytes. The heightened transcription might be due to the amplification of the gene or to altered regulation of ras transcription. To address the question of gene amplification, we also conducted a DNA dot blot hybridization analysis. A total of 20 μg of DNAs from normal and two SV40 transformed cell lines, 425 and 98 were applied to a hybridization membrane, and probed with the entire 6.6 kb EJ fragment. Densitometric quantification of the intensities of the autoradiographic spots were carried out on the dot blots. The results of the DNA dot blots hybridization analysis indicated no significant amplification of ras sequences were seen in SV40 transformed cell lines as compared to the normal keratinocytes. This result indicates that the increase in the level of ras transcripts in both SV40 transformed cell line is due to the increased transcriptional activity instead of gene amplification.

7) Defining Putative Ras Upstream Regulatory Elements

From the sequence data of the 1.8 kb XbaI\BamHI fragment derived from pGEM-3 clone (Appendix I), we determined that this fragment is bounded by the 5′ XbaI site of the fragment on the left and a 43 bp overlap with the 5′ end BamHI site of

the human EJ Ha-ras sequence. An extensive homology search of the 1.8 kb sequence against known sequences catalogued on the Genbank sequence databank revealed the following features (summarized in Fig. 26)

I). There are two Alu-like repeat sequences at positions 940 to 1240 and positions 1510 to 1655. These Alu-like sequences have been found in a number of human genes (table 3). Alu sequences have been reported as function as origins of DNA replication in mammalian cells (Huberman et al., 1968; Tamm et al., 1979) and as a reducer to decrease the expression of genes (Saffer et al., 1989).

II). There are multiple homopolymeric T and A stretches. These are found in association with many virus genes (table 4), although their functions are as yet, still unclear. In addition, some long direct repeat sequences and inverted repeat sequences were also found. A pair of 16-bp(AAAGCAGAACCCCAGA) long direct repeat sequences were found at position 667 and 721, and another pair of 12-bp (CTCAGGTCAGTC) long direct repeats were found at positions 902 and 917. Two pairs of inverted repeat sequences (CTCCCAAAGTGCTGGGATTACAGGCAT) at (TGTCACCCAGGCTGGAGTGCAGTGGCA) were found at positions 1216, 1443, 989 and 1665 respectively. Long repeat sequences are commonly associated with enhancer activity.

III). The 1.8 kb region adjacent to C-Ha-ras does not contain any large continuous coding sequences in any of the three possible open reading frames.

IV). There are some consensus sequences of DNA-binding motifs in this region as shown in table 5. Further analysis by gel retardation and DNAase I footprint have been conducted.

V). There are two putative enhancer like consensus sequences: positions 989-1012, and 1414-1562. These sequences were found in at least two other genes: the human prothrombin gene Genbank name (HUMPROTH) and the human papillomavirus Genbank name (PPHPA18E).

A restriction analysis of the 1.8 kb 5′ upstream region of the Ha-ras is shown in Appendix II.

8) Interaction of Keratinocyte Nuclear Proteins with 5′ Upstream Sequences of Human Ha-ras

The computer analyses above indicated the existence of putative DNA binding motifs and enhancer-like sequences within the 5′ adjacent region of Ha-ras. If so, it may be expected that there may be a specific interaction between these elements and trans-acting proteins resulting in the regulation of ras gene expression downstream. We therefore carried out a series of gel shift assays to determine if nuclear proteins

from both normal keratinocytes (EP) and an SV40 transformed cell line, (line 98) would interact with sequences on the 1.8 kb fragment. Nuclear extract from HeLa and serum response factor binding fragment (FT) as well as its competitor XGL and XGLM (a gift given by Ha Zhu in Columbia University) were used as a positive control in the gel retardation assay. We have generated four subfragments: F1, 395 bp generated by XbaI/StyI (bases 1-395); F2, 206 bp generated by BbvI/BamHI (1581-1787); F3, 371 bp generated by StyI/BbvI (1210-1581); F4, 452 bp generated by StyI/SfaN (395-847) and 363 bp generated by SfaN/StyI (847-1210) (Fig. 14). The four fragments were end labeled by end-filling with the klenow enzyme. Fragments were incubated with nuclear extracts made from culture HeLa cell and line 98, SV40 transformed keratinocytes. No migration change was found for F1 and F4 DNAs when those fragments were incubated with nuclear extracts from both HeLa and line 98. DNA-protein complexes migrating more slowly than free DNAs were observed with F2 and F3 which are just adjacent to the 5′ BamHI site of the ras fragment when incubated with an extract from HeLa (Fig. 27). But no binding activity was seen when F2 and F3 DNAs were incubated with nuclear extracts from line 98. Possibly the concentration of critical factors in nuclear extract from line 98 may be low or be of low binding affinity in vitro, or may be absent in line 98. To clear up these questions, we have conducted another series of gel shift assays. A control experiment was set up to test the technique

that we used and the activity of the nuclear extracts that we isolated. The test DNA we used in this experiment was a 180 bp c-fos oncogene containing fragment (a gift from Dr. Ha Zhu of Columbia University) covered a binding site of SRF (serum response factor). Slow migrating bands were observed when incubated with nuclear extracts both from HeLa and 98 (Fig. 28), but weaker bands were seen when the nuclear extract from line 98 was used as compared with Hela nuclear extracts. This finding further suggests that the nuclear extract from 98 may lack critical factors which are more abundant in HeLa cells. The results also showed binding specificity. When a 52 bp double stranded oligonucleotide, XGL, which binds SRF with high affinity (Prywes et al., 1988) was added, this oligonucleotide bound to SRF such that SRF could not bind to its target DNA, and the complex bands disappeared. However, when XGLM which has two nucleotide mutations compared to the XGL sequence (Manak et al., 1990) was added, binding activity was recovered, slow bands appear again since the mutations abolish the binding of SRF to XGLM so that the SRF is released. These results suggest that the binding of SRF is specific. From the test experiment we knew that the assay system worked well.

In the experiment shown in Figure 28, twice the amount of nuclear extract from 98 and same amount from HeLa were used as compared with the experiment shown in Figure 27. Both F2 and F3 fragments exhibited a retarded gel migration after

incubation with nuclear extracts from both HeLa and 98. In addition, two binding complexes were seen with F2 DNA; these might represent two or more proteins with different molecular weights binding to this F2 region. The lower band in the control lane of free F3 DNA indicates some minor contamination of the preparation with F2. Taken together these results suggest that there are specific protein binding activities within F2 and F3 fragment regions which bind to factors present in HeLa and 98 cell nuclear extracts.

To confirm that the slowly migrating bands in F2 and F3 represent specific complexes, we carried out a binding reaction in the presence of a 10 fold excess of nonradioactive competitor fragments. The results shown in Figure 29 indicate that the complex bands in F2 and F3 were competitively displaced after the addition of unlabeled F2 and F3 DNA respectively when tested with nuclear extracts from both HeLa and 98.

We have also compared the DNA-binding activity using equal amount of crude nuclear extracts from normal keratinocytes (EP) and line 98. Figure 30 shows DNA-binding activity appear in both EP and line 98. Slightly stronger binding activity was seen in line 98. This observation suggests that the increasing binding in 98 may result from the the SV40 transformation.

9) Defining the Nuclear Protein Binding Domains within the Fragment 2 and 3

To further locate the regions to which the proteins bind, DNAase I foot-printing was performed using radiolabeled F2 and F3 fragments. For this purpose F2 DNA was amplified by PCR with the L6 and T7 primers. Before PCR amplification, primer L6 was 5′ end-labeled by T4 polynucleotide kinase and gamma-^{32}p-ATP. Digestion of the labeled F2 fragment with DNase I in the presence of nuclear proteins produced three protected regions spanning nucleotides 5′-GAGGTC at position 1492-1497; 5′-AAGACCAGCCT at position 1506-1516 and 5′-GAAACCCC at position 1528-1536 (Fig. 31). A replicate DNA foot-printing assay shown in fig. 30 indicates that the DNase I protected region spanning nucleotides 1506-1563 was repeatable (Fig. 32). An examination of the nucleotide sequence revealed that the protected region was located within the second putative enhancer region (position 1453-1609). This finding suggests that this putative enhancer region may play a role in regulating the expression of ras gene by interacting with trans-acting factors.

IV. DISCUSSION

1) The Ras Polymorphisms in SV40 Transformed Human Keratinocyte

This project was based on previous work from this laboratory which demonstrated that transformation of human epidermal keratinocytes by the oncogenic virus, SV40 is a progressive process which is only fully manifest over many cell generations; long after expression of the viral oncogenes (i.e. T antigens) themselves. For this reason it was proposed that viral infection may only represent the initial event in transformation and that other events such as the activation of host protooncogenes by a process either directly or indirectly set in motion by the virus might be responsible for the appearance of some transformed properties many weeks subsequent to infection. Southern blot hybridizations using myc and ras probes did appear to indicate the presence of oncogene polymorphisms in the DNA from both viral transformed and naturally transformed cells.

Southern blot analyses of ras sequences in the genomic DNAs of transformed cells using pTB1, a probe which covers the left portion of the 6.4 kb ras EJ region revealed a polymorphic pattern (Figs. 15 and 16) which is only partially accounted for by the known sequence data (Table 6). For example, complete digestion of c-Ha-ras by Pst I would account

for the three fragments of about 1.9, 1.1 and 0.8 kb but not for the larger fragments of between 4-5 kb observed in digests of EP, SCC and 425 DNAs. Similarly, the Sau 3A fragments of about 0.8, 1.1 and 1.9 kb would be anticipated in the blots shown in figures 15 and 16 but not the fragments of 2 kb or larger. Interestingly, the majority of the polymorphic transformation-related changes are manifest in these larger fragments. The Sau 3A digests shown in figure 16 demonstrate not only a polymorphism resulting in the appearance of a 2 kb fragment in all three transformed lines but also in the loss of the normal 1.1 and 1.8 kb fragments which are present in digests of normal keratinocyte (EP) DNA. On the basis of the available ras sequence data we predicted that the larger fragments might be generated from a region upstream from ras including about 2 kb of unsequenced DNA. DNAs presumed to contain the polymorphisms were cloned as a 1.8 kb BamHI/XbaI fragment(s) from lambda phage libraries created from the genomic DNAs from normal cultured keratinocytes and two lines of SV40 transformed keratinocytes. However, sequence analysis of the cloned DNAs proved that no fragments of a length larger than about 2 kb, or less, could result from PstI, and Sau3A digestion even after taking into account the additional 1.8 kb of DNA.

One possibility for the appearance of the polymorphic large bands is that they may represent cross hybridization between pTB-1 and other members of the ras family, N- and K-

ras. However, this is unlikely under the stringent hybridization conditions used since the N and K ras sequences do not show sufficient homology with c-Ha-ras to hybridize to the probe under stringent conditions (> 95% sequence homology). In fact, N-ras was originally cloned by taking advantage of the fact that N-ras is detected only at low hybridization stringency with an Ha-ras probe (Shimizu et al., 1983). On the other hand it may be that the ras sequences may be substantially more methylated in normal keratinocytes and less methylated in SV40 transformed cells. The reasons we consider the possibility of methylation are, first, the fragment pattern cannot be explained without some consistent alteration in restriction sites. The most possible methylated sites that result in the unexpected 4 kb Pst I and Sua3A fragments appeared in EP and SCC are the Pst I cut sites at position 1061 or 6741; Sau3A cut sites at 1839, 2447, 3625 and 4295. In addition, the appearances of both normal restriction pattern and the extra 4 kb fragments in EP and SCC could be due to partial methylation of sequences at these cut sites; A restriction map which includes the 1.8 kb upstream sequences and the EJ ras region is shown in fig. 21. Demethylation in those restriction sites may account for the loss of the 4 kb Pst I and Sua3A fragments in the SV40 transformed cell lines. Secondly, the enzymes we used are all methylation sensitive suggesting this as the most likely possibility. Third, repeatable restriction fragment length polymorphisms were only

found in genomic DNA but not in the cloned DNA. This difference reflects the different natures of methylation in prokaryotic and eukaryotic systems. Fourth, ras expression levels are dramatically increased in transformed cells. Demethylation as a means of activating gene expression is consistent with this observation.

It is widely believed that methylation of promoter and enhancer segments in both prokaryotic and eukaryotic organisms is a mechanism of control of gene expression with DNA methylation generally associated with lowered levels of gene expression. Genes, such as HLA-E and mts1 have been shown to be inactive when methylated and expressed when demethylated (Boucraut et al., 1993; Tulchinsky., 1992). When enzyme-deficient rodent cell lines were treated with 5-azacytidine, a potent demethylating agent, they were strongly reactivated to wild type (Holliday, 1987). Several studies have also shown that methylation at the promoter region is sufficient to repress gene activity (Ohtani-Fujita et al., 1993; Razin et al., 1991; Doerfler et al., 1989). Changes of DNA methylation in protooncogenes in the process of radiation-induced transformation of mouse m5S/1M cells in vitro have been reported also (Yasuzawa et al., 1992). Therefore, it is possible that demethylaion of the upstream region in Ha-ras in our SV40 transformed cell lines may be responsible for the greatly enhanaced expression of the ras gene.

However, The most majority of methylations involved in

regulation of gene expression in eukaryotic organisms appear to be CpG-dependent methylation. That means the methylation usually occurs in the area of a CG repeated cluster. No such CpG islands were found in our possible methylation sites. Our data seems to rule out the possibility of CpG-dependent methylation suggesting another type of methylation may involve in our system. One way this might be tested is by the method of Maxam-Gilbert (1979).

2) Elevated Levels of Ha-ras Gene Expression Associated with SV40 Transformation

The expression of ras gene was markedly increased after SV40 transformation. Our northern hybridization using the EJ probe demonstrated that significantly higher levels of ras RNA were expressed in two SV40 transformed cell lines as compared to non transformed keratinocytes. This result suggests that the elevated levels of Ha-ras expression are linked to transformation induced by SV40. This finding provides further evidence that SV40 may transform normal cells by interacting with other cellular genes and that proto-oncogene may be a target of this process. Altered expression of cellular genes might directly or indirectly mediate the process of transformation. Altered gene expression following infection by SV40 is not a new observation. Several groups have isolated cDNA clones which correspond to mRNAs which are present at

higher levels in SV40-transformed cells than in the normal parental untransformed cell lines (Schutzvank et al., 1982; Scott et al., 1983). Among these mRNAs, some may occur at elevated levels in many transformed cell lines, irrespective of the nature of the transforming agent. But other mRNAs are apparently more specific to SV40 transformation as exemplified by the mRNA encoding a 58K mitochondrial protein (Zuckerman et al., 1984). Another example is the reactivation of silent rRNA genes in somatic cell hybrids after SV40 infection (Soprano et al., 1980). The reactivation of rRNA genes is controlled by large T, because it is abolished at the restrictive temperature when tsA mutants of the virus are used, it is not clear whether the stimulation of rRNA synthesis plays any role in cell transformation. In addition, it has been reported that SV40 transformation could affect the expression of some cellular proto-oncogenes in other systems. An SV40 induced increase in Ha-ras expression in SV40 transformed human epidermal keratinocytes has not, to our knowledge, been previously reported. The observation of increasing ras expression in SV40 transformed cell lines is consistent with our methylation hypothesis. Demethylation may activate the ras expression. In addition, our computer analyses also indicated the presence of an SV40 T-ag binding motif in our sequence suggesting the SV40 T-ag might also be involved in the regulation of ras expression.

Elevated Ha-ras transcription has also been reported in

a number of naturally occurring tumors including those of bladder (Viola et al., 1985), colon (Gallick et al., 1985), and prostate (Viola et al., 1986). Increased expression of the normal ras proto-oncogene by linkage of this gene to retroviral regulatory elements (LTR) resulting in the malignant transformation of NIH3T3 cells has also been demonstrated (Chang et al., 1982). All of these observations suggest that a constitutive change in the expression of ras could represent an important mechanism in the oncogenic transformation or the pathogenesis of some cancers. In the present case, it is still unclear whether the induction of ras expression in SV40 transformed cells is the only event involved in the appearance of "late-stage" transformation. Interestingly, SV40 transformed human tracheal epithelial cells which could not form tumors in nude mice became tumorigenic if they were also transfected with ras, whereas neither oncogene (i.e. ras or SV40) could induce tumorigenesis acting alone (Rhim et al., 1985). To address the question of whether other oncogenes may be activated by SV40 infection, DNA transfection in a 3T3 cell focus-forming assay might be utilized.

The molecular mechanism by which overexpression of ras transforms normal cells into the malignant state is not yet clear. More recently, the discovery of the GTPase activating protein, GAP (Trahey and McCormick, 1987) has shed light on understanding the ras regulation events. Evidence now

indicates that GAP interacts with ras-GTP and stimulates its intrinsic GTPase activity dramatically, suggesting that the role of GAP is to downregulate ras-GTP (fig. 2) (Zhang et al., 1990; Downward et al., 1990). However, other evidence suggests that GAP may also act in some systems at least, as a positive effector of p21 ras (Yatani et al., 1990). In the context of Zhang's negative theory, there is one current model which suggests that the abundance of the GAP is limited and consequently, ras expression must be tightly controlled to avoid saturation of its regulator (Cohen et al., 1989). From this point of view, overexpression of the ras gene could result in escape from the GAP down regulation. Ras p21 could accumulate in the active GTP-bound state in these cells and thus contribute to abnormal cellular proliferation. If this is true, we believe that introducing extra exogenous GAP could reduce the overexpression of ras and thus rescue the abnormal cells.

The fact that our SV40 transformed cell lines exhibit overexpression of ras raises the question of when this abnormal expression starts; in early or late stage of SV40 transformation? The cell lines are apparently still T-Ag dependent after the alteration of ras expression since we have never observed that any of our long term SV40 transformants ever become "cured" of SV40 sequences.

This question might be addressed by northern blot hybridization analyses using different stages of SV40

transformed cell lines infected with a tsA (temperature sensitive) mutant cell line. Stage-specific activation of ras has been reported in a number of carcinogen studies. Ras mutations may be associated with the late phases of transformation, although conflicting evidence has been reported (Barbacid 1987). Activated ras has been reported unnecessary for the early immortalization of mouse liver epithelial cells derived from normal C3H mice but ras transfection was able to convert the immortalized but nonmalignant cells into hepatocellular carcinoma cells by transfection (Lee et al., 1990). This is direct evidence for an activated ras role as a "progressor" in mouse hepatocarcinogenesis. Further support for the hypothesis that ras is necessary in late stage is provided by the finding of a higher incidence of ras activation in late rather than in early stage dimethylnitrosamine-induced lesions (Stowers et al., 1988). However, the available data do not exclude the possibility that ras activation may also occur during the initiation phase as has been stated in many carcinogen studies (Wiseman et al., 1986; 1987).

The availability of thermosensitive mutants of SV40 which produce thermolabile large T proteins, enables determination of whether an active T-Ag gene product is continuously required to maintain part or all of the transformed phenotype. A number of early reports concluded that cells, which have been transformed at the permissive temperature with SV40 tsA

mutants, lost their transformed phenotype at the restrictive temperature (Osborn and Weber, 1975; Kimura and Itagaki, 1975; Tegtmeyer, 1975; Brugge and Butel, 1975; Martin and Chou, 1975).

Actually, it has been reported that tsA mutants can produce two types of transformants. Some transformants, designated as N transformants by Seif and Cuzin (1977), display a thermosensitive transformation phenotype, whereas type A transformants keep their transformed phenotype at the restrictive temperature. Both types can be obtained with SV40 tsA mutants (Rassoulzadegan et al., 1978). The outcome of a transformation experiment in terms of N or A phenotype appears to depend on the experimental conditions. When the infected cells are maintained in active growth for a critical number of cell divisions before selection of the transformants, N transformants are more frequent than A transformants (Roassoulzadegan et al., 1978; Brockman, 1978; Seif and Martin, 1979). N or A phenotype also depends on the multiplicity of infection and type of cells which are infected. Current studies are more in favor of a requirement for the continuous expression of SV40 large T in the maintenance of the transformed phenotype. This conclusion is also supported by the observation that nontransformed revertants selected from the SV40-transformed rat 14B cell line, no longer express large T (Steinberg et al., 1979). As stated above we have never observed this in our SV40

transformed cell lines; T-Ag is continuously expressed.

3) Characterization of Putative Regulatory Element Adjacent to the 5′ End of the Human Ha-ras Proto-oncogene

A 1.8 kb fragment adjacent to the 5′ end of human Ha-ras gene derived from both normal keratinocytes and SV40 transformed keratinocytes has been cloned and sequenced in this project. Computer analysis of the sequence has revealed that there are two putative enhancer-like fragments and multiple repeat sequences within this region as well as many DNA-binding motifs including sites binding by SV40 T-ag. The binding of T-ag within the 1.8 kb ras-upstream may implicate the possible interaction between T-ag and ras expression. It is widely believed that T-ag can regulate the early and late transcription in SV40 by binding to the three consensus sequences within the region containing the origin of replication. The same principle can also be applied to the regulation of cellular genes. Activation of silent rRNA gene after transfected T-ag is one example (Soprano et al., 1980). Our finding of dramatically increased ras expression in the SV40 transformed cell lines is in line with the function of T-ag in transcriptional regulation. Therefore, we have reason to believe that SV40 may cause transformation by interacting with cellular genes, specially cellular oncogenes. The specific

binding activity of T-ag in the upstream region of the ras gene could be further tested by another gel shift assay using purified T-ag.

To characterize the 5′ upstream of ras gene, we have also detected interaction of DNA-binding proteins within this 5′ adjacent region of ras using a gel mobility shift assay. Figs. 27-29 show DNA-binding activity in the F2 and F3 fragments when used with nuclear extracts from both HeLa and line 98. Our DNase I foot-printing assays also revealed three regions extending from position 1506 to position 1563 which are protected by nuclear extracts derived from a SV40 transformed cell line, 98. The protected regions coincide quite well with the second putative enhancer region. The presence of putative enhancer region and the DNA-binding activity suggest the existence of a regulatory element(s) mediated by trans-acting factor(s) in the region flanking the human Ha-ras gene that regulates the distal Ha-ras expression. To further examine the effect of this region on the regulation of expression of the ras gene, CAT assays to demonstrate enhancer activity and <u>in vitro</u> transcriptional assay to examine the length and abundance of ras transcripts have been conducted although the results have been inconclusive.

Recent studies have established that cis-regulatory elements in the 5′ flanking region of eukaryotic genes play an important role in regulation of gene expression. Two cis-regulatory elements, the TATA box and the upstream promoter

element, the CCAAT box, are present in most, but not all mammalian genes. These elements are generally located within 120 bp upstream from the transcription start site (Dynan et al., 1986). Their functions are thought to ensure precise and efficient initiation of transcription. In addition, enhancer or reducer elements have also been found in the 5′ flanking region of many eukaryotic genes at various locations (Walker et al., 1983; Wu et al., 1987; Godbout et al., 1986), although their presence in the intron sequence (Banerji et al., 1983; Gillies et al., 1983) and 3′ flanking sequence have also been well documented (Choi et al., 1986). Enhancers or reducers are required for increasing or decreasing the rate of transcription from a promoter respectively. In addition, recent evidence suggests that enhancers and some upstream regulatory elements of eukaryotic genes play a central role in conferring inducibility, tissue specificity (Steinberg et al., 1987). The precise mechanism concerning how these cis-regulatory elements act to control gene expression, especially in specific cell types or at different stages during development is not fully understood. However, it has been found that most motifs of enhancer and upstream regulatory elements are apparently binding sites for nuclear proteins (Wu et al., 1987; Godbout et al., 1986; Grosschedl et al., 1985; Edlund et al., 1985). This finding suggests that gene expression is probably regulated by the interaction of a variety of trans-acting factors with multiple cis-regulatory

elements.

The distinctive characteristic of enhancers or reducers is that they can act on cis-promoters or hetero-promoters at great distances in an orientation-independent manner and can also function down-stream from the transcription unit (Serfling et al., 1985). Enhancer sequences that can affect the transcription of a gene thousands of base pairs away have been observed. For instance, sequences upstream of the early promoter of the simian virus 40 (SV40) were found to stimulate transcription of a linked beta-globin gene by more than two orders of magnitude over distances of more than 3000 bp in either orientation (Banerji et al., 1981; Moreau et al., 1981; Fromm et al., 1983). How does an enhancer or an upstream sequence affect an event at a distant location on the same molecule? Three classes of mechanisms have been postulated for such distal actions: tracking or translocation of a protein along a DNA, the association of two proteins bound at separate sites to form a DNA loop in between, and distal interactions that are affected by the topology of the DNA (Wang et al., 1988).

Regulation of gene expression is a very complex process. In addition to the consensus promoter and enhancer elements there are sequences governing negative and/or positive regulation. Multiple elements participating in the regulation of gene expression have been reported in many eukaryotic genes. For instance, two genetically separable positive

regulatory domains and an overlapping negative control sequence have been found in the ß-interferon gene which control gene expression under the uninduced or induced condition (Goodbourn et al., 1986).

The 5′ flanking sequence of the human Ha-ras gene has been studied by a few laboratories (Ishii et al., 1986; Jones et al., 1987; Lowndes et al., 1989; Nagase et al., 1990; Lee et al., 1991) using 5′ deletion analysis. The ras promoter region has been identified and shown to lack the TATA box characteristic of many eukaryotic genes. Instead, it appears to possess multiple start sites and contain copies of the hexanucleotide sequences GGGCGG or CCGCCC, which were identified by DNA foot-printing using the purified Sp1 factor (Ishii et al.,1986), and two NF-1 binding sites, one strong and one weak (Jones et al., 1987). Such GC motifs are also contained within other cellular promoters and within several viral promoter such as those of the herpes virus thymidine kinase gene (Mcknight et al., 1984) and the SV40 early transcription unit. In addition, enhancer activity has been found in the 0.8 kb SstI fragment located about 1 kb at the 5′ of the human Ha-ras (Spandidos et al., 1986). However, very little is known about the other regulatory element, particularly those in the 5′ upstream region.

To our knowledge, we are the first laboratory to sequence and characterize the 5′ upstream region adjacent to the human Ha-ras gene. Neither this sequence nor any homolog

was discovered during sequence homology searches of the Genbank nucleotide database and we were able to register the sequence in Genbank under the accession number L11526. Nagase et al (1990), using transient expression assays in African green monkey kidney cells (CV-1) and human A431 epidermoid carcinoma cells, have previously reported that no additional control elements for expression of the Ha-ras exist outside of the promoter region affected the promoter activity. However, their conclusion seems still arguable, because they didn't use equal molar amount of DNAs derived from different deletion mutants in their CAT assays. Therefore, we still think it is necessary to reexamine and reassess the role of putative enhancer elements in the ras flanking region.

The presence of potential regulatory elements and the demonstration that nuclear proteins from line 98 and Hela bound specifically to the F2 and F3 DNA fragments further support the contention that this 5' upstream region might be important in controlling ras expression. It has recently been shown that the 5' upstream region immediately adjacent to the mouse N-ras gene contains a reducer-like negative regulatory element. Studies revealed that this negative regulatory element was active on the N-ras promoter as well as in transient assays, and down regulated the heterologous herpes simplex virus thymidine kinase promoter. Foot-printing analyses and in vivo transfection competition experiments indicated that a trans-acting factor is responsible for the

negative effect on transcription (Paciucci and Pellicer, 1991). However, no sequence homology was found between the human Ha-ras and the mouse N-ras in the 5′ upstream region.

We wondered whether the DNA-binding activity shown above is induced by the infection of SV40, therefore we have also conducted another gel shift assay using nuclear extracts from normal keratinocytes (EP) and a SV40 transformed cell line (98). Compared the results obtained with EP and line (98), Fig. 30 revealed the presences of binding activity in both EP and line 98, but slightly more binding intensity was seen in line 98. We presumed the binding affinity or the amount of crucial binding factors was increased in line 98 after SV40 infection. This finding again suggests that the sequence within the 5′ upstream region of Ha-ras maybe involved in the regulation of ras gene expression which is linked to the transformation induced by SV40. This is consistent with other evidence which supports the hypothesis that cooperation with other cellular events might account for the SV40 transformation.

Finally, it is worth noting that the size of the ras transcript seen in many published northern blot hybridizations strongly suggests that at least some transcripts may begin well into the flanking region although we are unaware of studies specifically addressing this point. Our own Northern blot hybridization analysis using EJ probe revealed the presence of one main band of about 5 kb in all our total

cellular RNA from human keratinocyte. In several cases a much less intense band of about 2 kb were seen too. Actually a similar pair of 5 kb and 2 kb transcripts have been found in EJ, T24 tumor cell lines and their derived transfectants with pEJ or rat c-Ha-ras 1 as probe (Parada et al., 1982). The exact nature of this transcript is still incompletely understood. The mechanisms which explains the varied Ki-ras transcripts have been reported in terms of alternative splicing patterns (Shimizu et al., 1983; McGrath et al., 1983). However, changes in splicing patterns may not be the case in Ha-ras because the size of whole transcript, even including the all the intron, is no larger than 3 kb. This larger transcript might come from another transcription initiation site in the further upstream sequence of the Ha-ras which was cloned in the present study.

V. Conclusion

The work reported herein has provided evidence to support the possible cooperation between SV40 and cellular oncogene in the process of SV40 transformation in human keratinocytes. Our findings include:

1) No SV40 induced sequence polymorphism in the 3.8 kb ras upstream region sequenced was found. Instead ras restriction fragment length polymorphisms associated with SV40 transformation were found in our Southern blot analyses. Methylation may account for such change. Because repeatable RFLP were found only in genomic DNA but not in the cloned DNA. In addition, fragment pattern cannot be explained without some consistent alteration in restriction sites. The enzymes we used are all methylation sensitive. Dramatically increases in ras expression were also observed in SV40 transformed cell lines. Demthylation as a means of activating gene expression is consistent with this observation.

2) Computer analyses indicated the presence of a T-ag binding motif in the 5′ upstream region of the Ha-ras. This finding implicates the possible interaction between T-ag and cellular genes in the SV40 transformed cell. T-ag binding may account for the increase of ras gene expression after infected by SV40 virus.

3) A novel segment with many properties associated with promoter/enhancer has been cloned and characterized. Computer analyses, DNase I foot-printing and gel shift assays show specific DNA-binding activity within this 1.8 kb upstream region of Ha-ras. In addiction, the 5 kb ras transcript suggests the possible existence of another transcription start site within this cloned region.

VI. Figures and Tables

Fig. 1 Signal transduction by a membrane-bound receptor. A schematic depiction of the events which follow the binding of a growth factor (GF) to its membrane growth factor receptor (GFR).

MB assoc denotes membrane associated.

ST denotes signal transducer.

Source: Druker et al. 1989

Figure 1. Signal Transduction by a Membrane-Bound Receptor

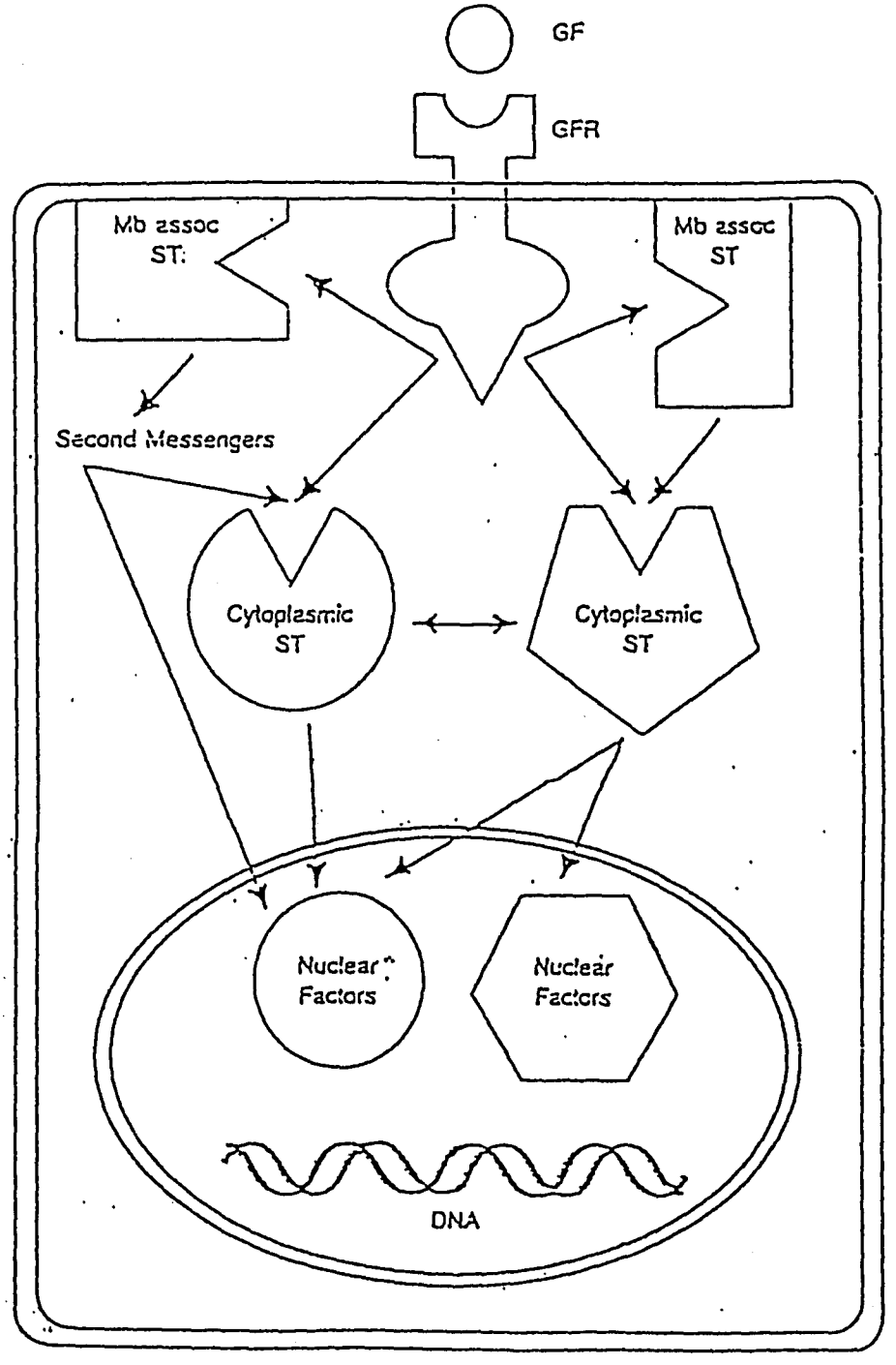


Fig. 2 Model for GAP as downstream effector and regulator of ras activity. The model at left depicts wild-type ras p21. At right an oncogenic position 12, 59, or 61 p21 protein ($p21^{*}$) is shown generating uncontrolled signal output.

Sources: McCormick, 1989.

Figure 2. Model for GAP as Downstream Effector and Regulator of Ras Activity

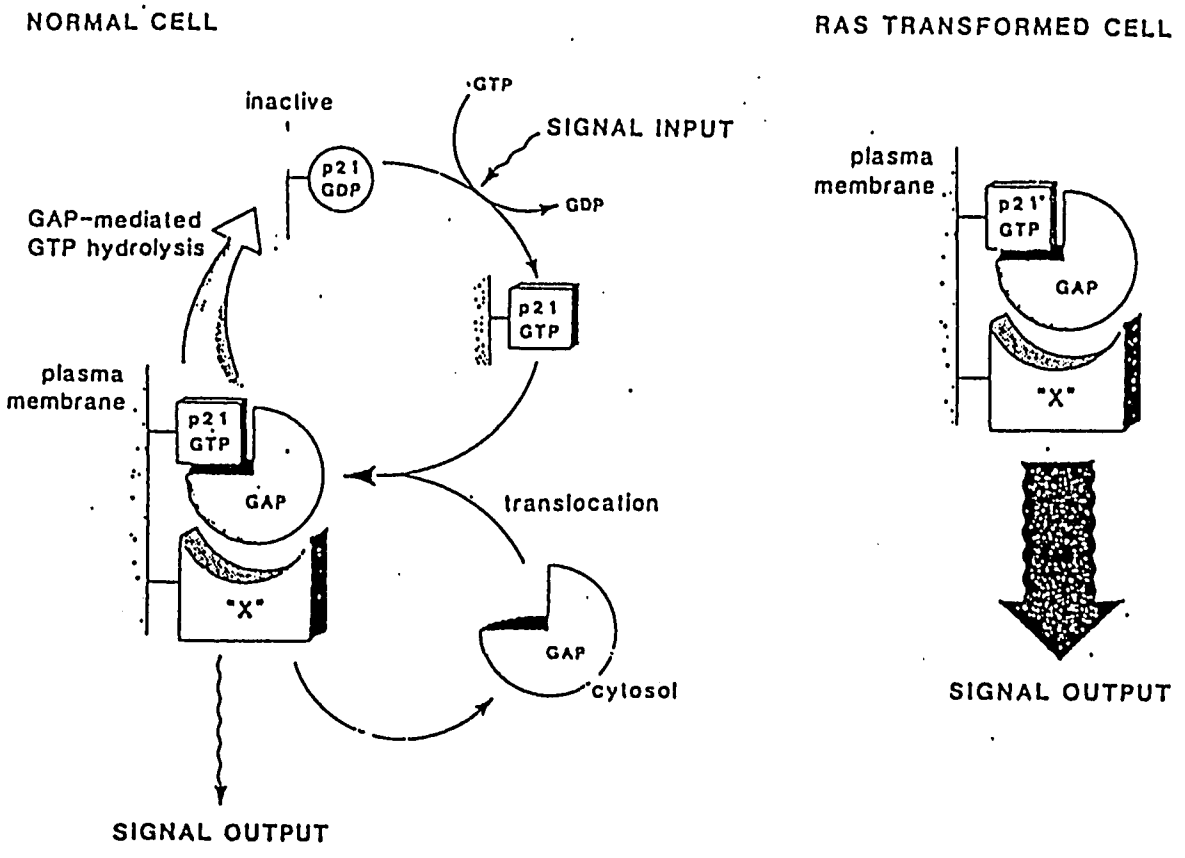


Fig. 3 Ras pathway in signal transduction. The complex of EGF-GRB-2-SOS* binds to the membrane-bound ras protein, and facilitates the ras protein binds to the raf-1 protein. Raf-1 will help to send the signal to the nucleus where genes that regulate gene expression are turned on.

EGF denotes Epidermal Growth Factor.

Grb-2 denotes Growth Factor Receptor-Bound Protein 2 (adapter protein).

SOS denotes Son of Sevenless (nucleotide exchange factor).

Sources: Merck, (Science) 1993.

Figure 3. Ras Pathway in Signal Transduction

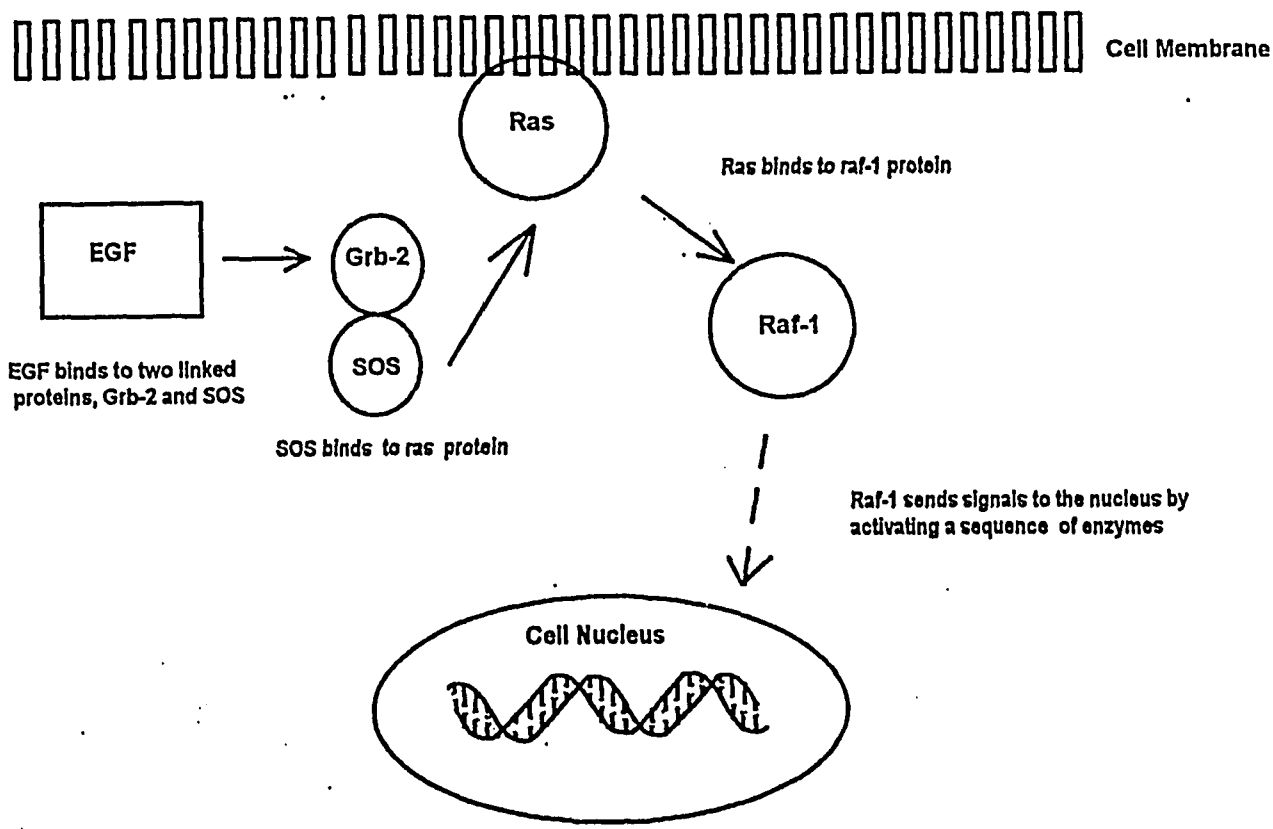


Fig. 4 Genetic organization of the SV40 genome. The major structural features of DNA transcription, RNA processing, and RNA translation are indicated and their locations are given according to the nucleotide numbering system of Buchman et al. (1980). Cleavage sites of five single-site restriction endonuclease are shown for reference. Indicated on the map are the origin of replication (ORI), 5′ and 3′ ends of early (Small t- and large T-antigens) and late (16 S and 19S) mRNAs, an the early gene TATA box. The protein coding regions of mRNAs are designated by open arrows. Within these, the first number locates the A in the AUG codon, and the second number identifies the nucleotide that immediately precedes.

Sources: John N. Brady and Norman P. Salzman (1986)

Figure 4. Genetic Organization of the SV40 Genome

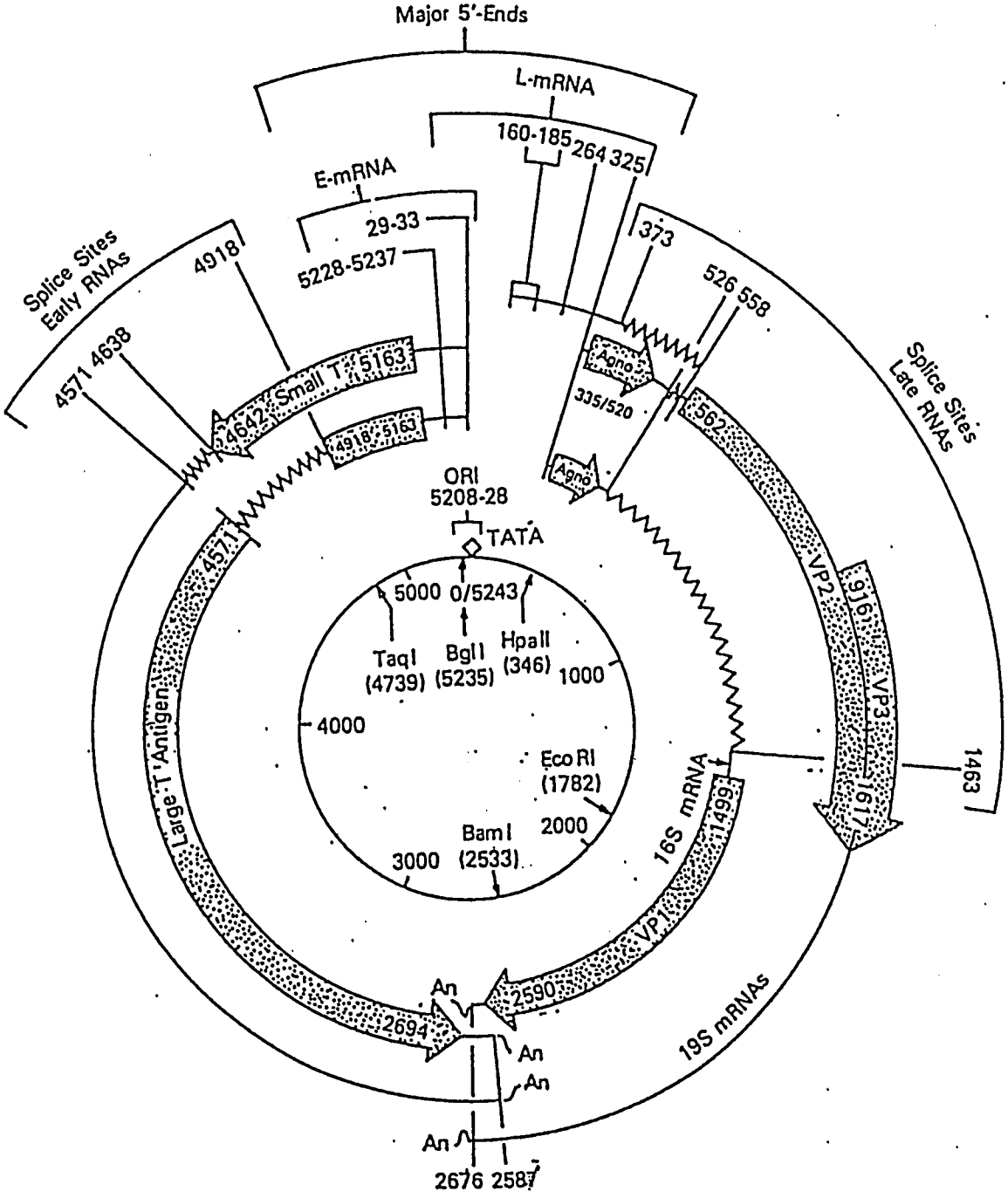


Fig. 5 Diagrammatic representation of the emergence of transformed properties over time following infection of human epidermal keratinocytes by SV40. Infection of the cells and maintenance and growth properties of the culture have been described (Steinber and Deferndi 1979).

Source:

Vittorio Defendi, Piotr Naimski, Mark L. Steinberg (1982)

Figure 5. Progressive Nature of SV40 Transformation

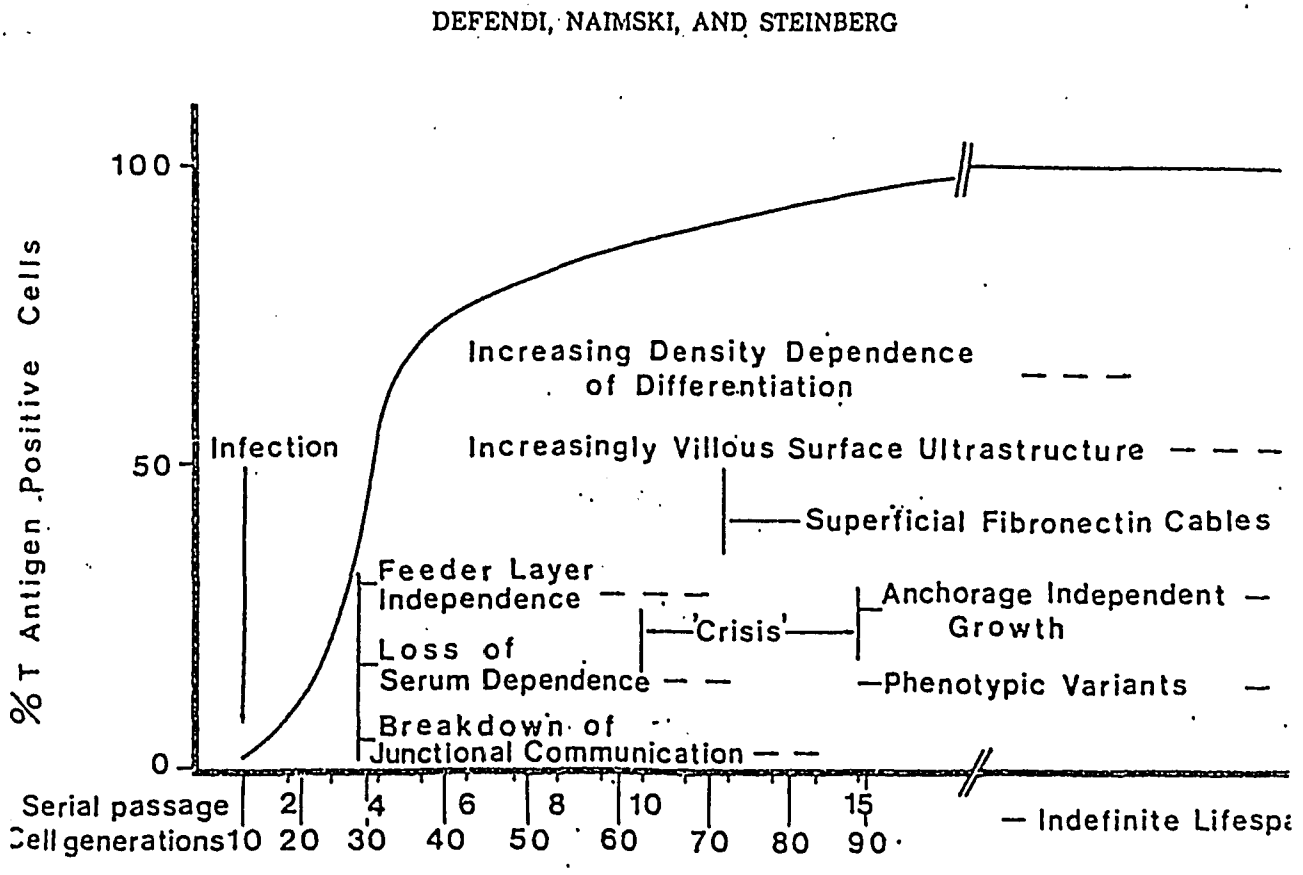


Fig. 6 Circular map of pTB-1 (Goldfarb et al., 1982). The BamHI/HindIII fragment of lambda sup2.9 is cloned into pBR322 (open box). The T24 ras DNA is denoted by a black box. Below is shown a more detailed restriction map of the cloned T24 bladder carcinoma transforming sequences. B, BamHI; Ps, PstI; Pv, PvuII; S, Sau3A; X, XbaI.

Figure 6. Circular Map of PTB-1

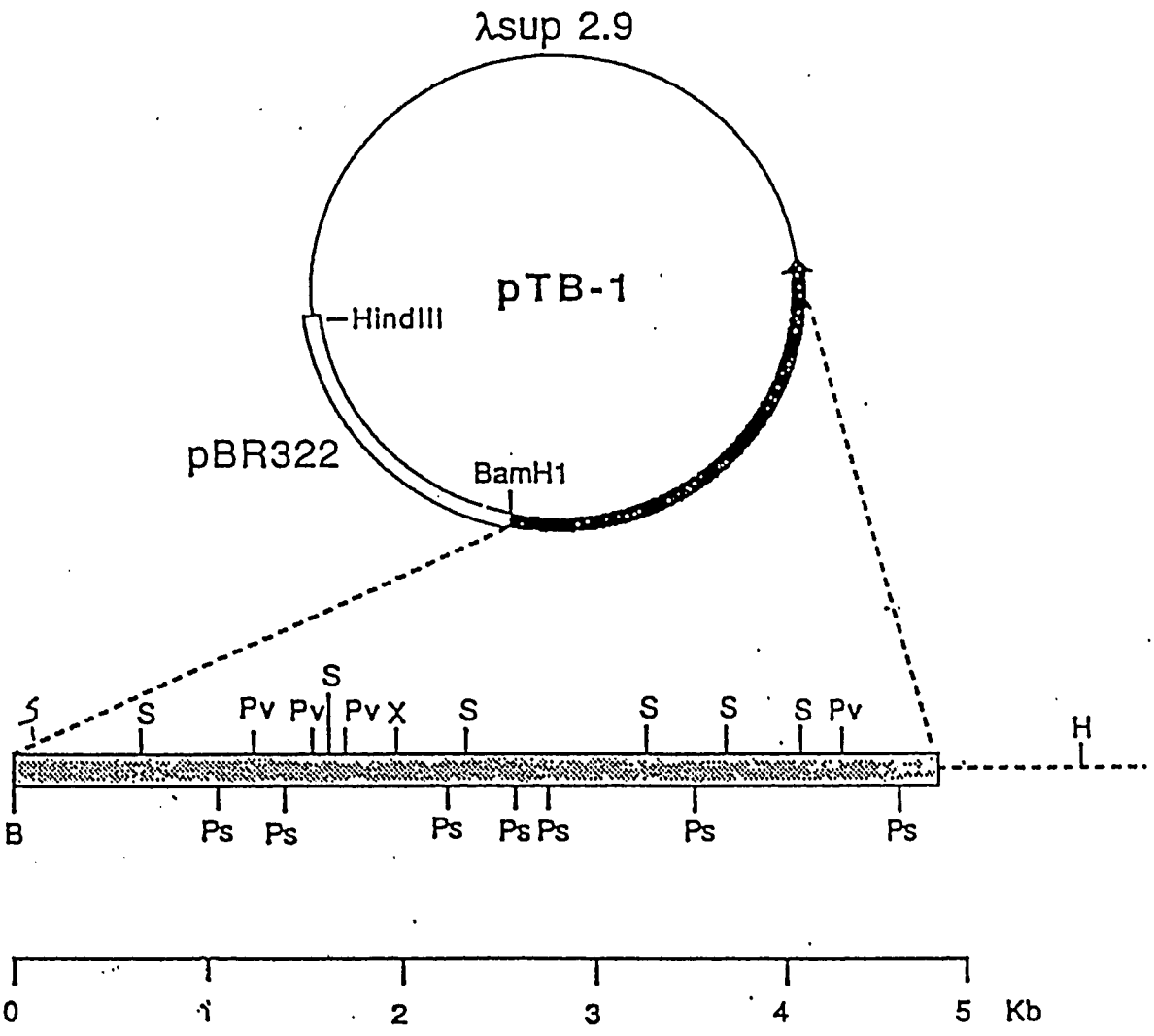


Fig. 7 Circular map of the plasmid, pMC41 (Dalla et al., 1984). The 15 kb myc gene containing insert denoted by a shaded box is cloned into the BamHI sites of pBR322 (denoted by a thin line). The myc region with its three exons and restriction sites for the restriction enzymes ClaI, PstI and PvuII are shown below. The 0.86 kb PvuII fragment containing the first exon and the EcoRI/ClaI fragment (pMC413RC) probes are also indicated.

Figure 7. Circular Map of the Plasmid PMC41

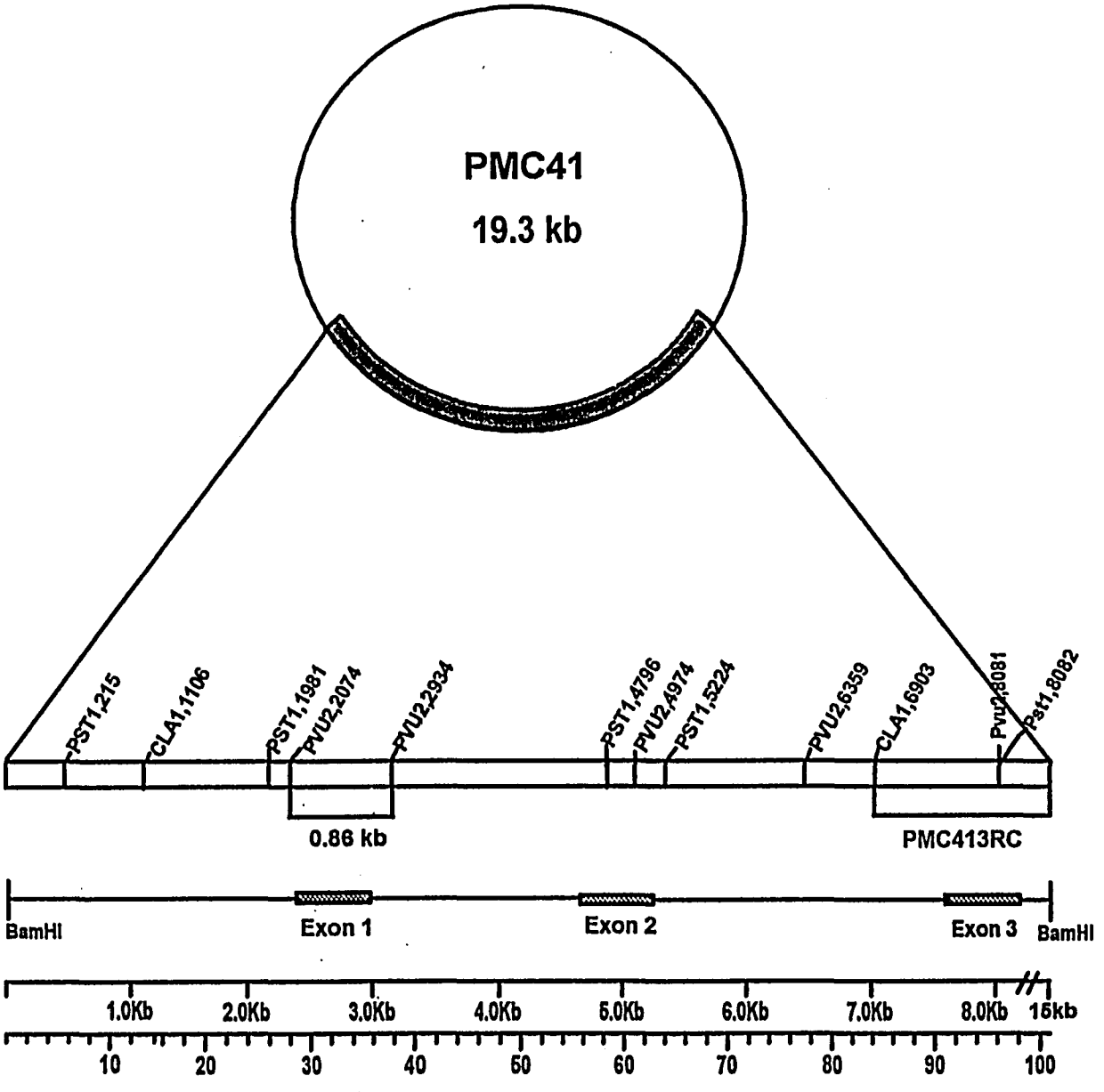


Fig. 8 The 10.3 kb plasmid pEJ, with the 6.4 kb BamHI/BamHI EJ ras containing fragment denoted by shaded box cloned into pBR322 (denoted by the thin line). The EJ region with restriction sites for the enzymes PstI, PvuII and Sau3A is diagrammed below. The map also indicates the 1.9 kb BamHI/XbaI fragment and 0.3 kb RsaI fragment which were used as probes in screening the libraries.

Figure 8. The Circular Map of Plasmid PEJ

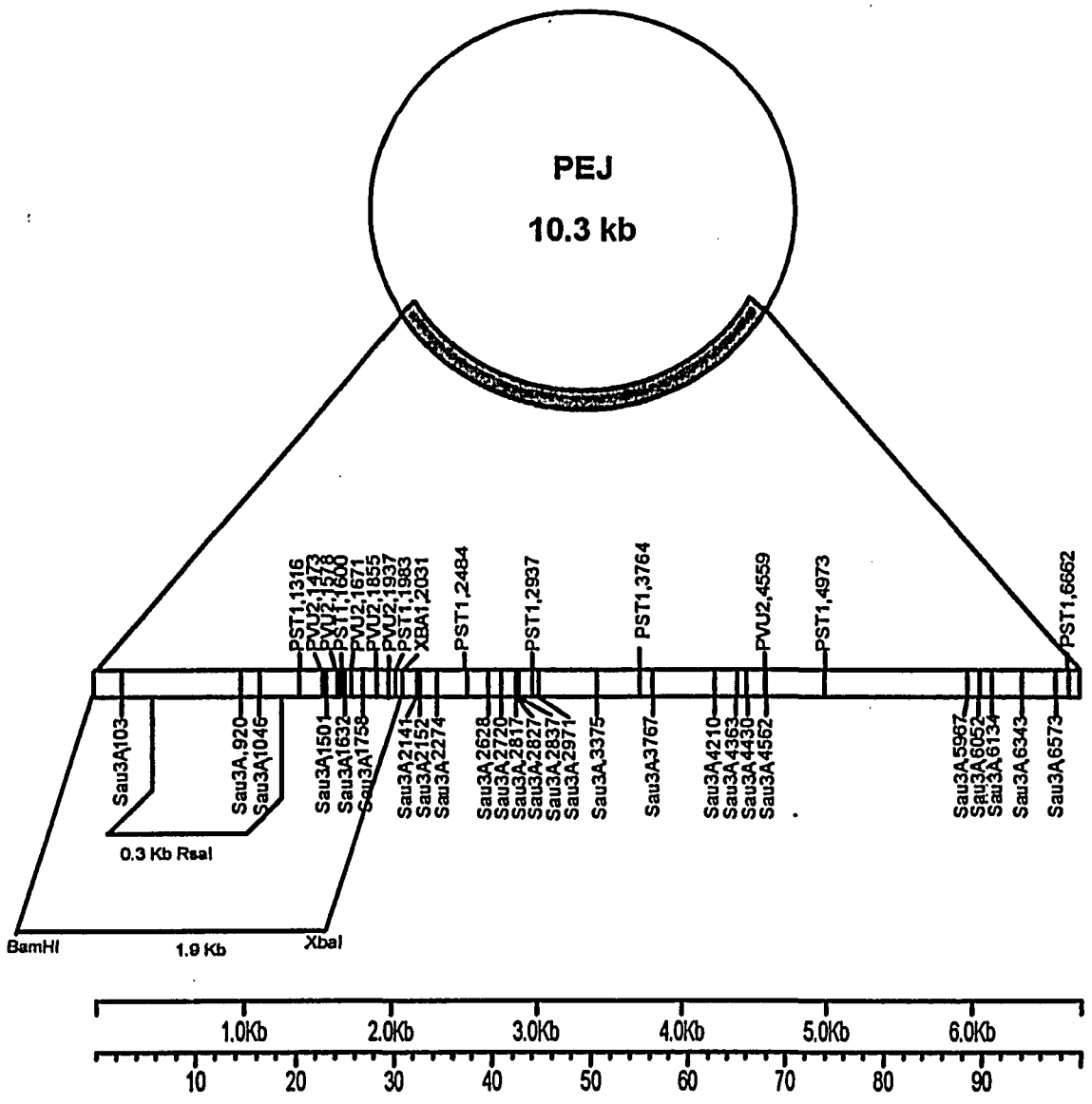


Fig. 9 Schematic diagram of the EMBL4 lambda cloning vector showing the relative lengths of the left arm, right arm and central stuffer of this vector. The multiple cloning sites are also indicated.

Figure 9. Schematic Diagram of the EMBL4 Lambda Cloning Vector

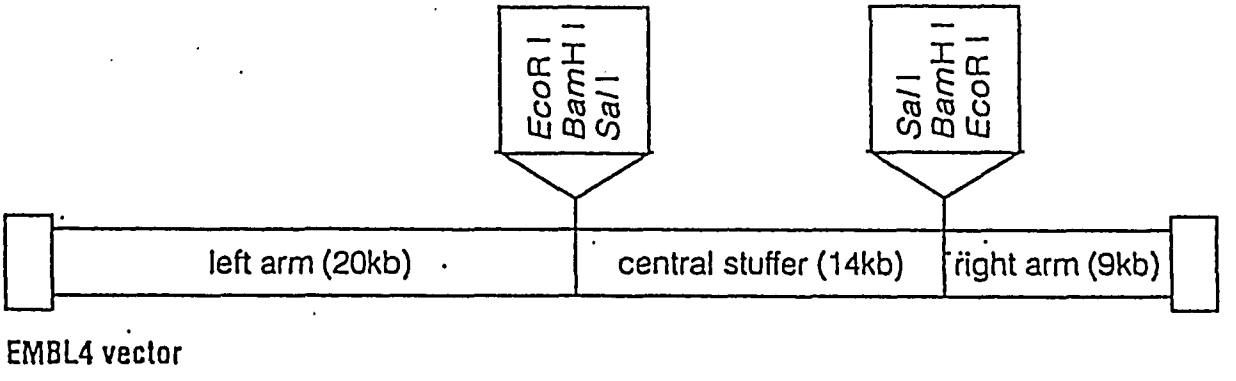


Fig. 10 Circular maps of bacteriophage M13 vectors mp18 and mp19. The figure shows the LacZ′, LacI gene regions and polylinker. The vectors differ from one another only in the orientation of the polycloning sites.

Figure 10. Circular Maps of Bacteriophage M13 Vectors

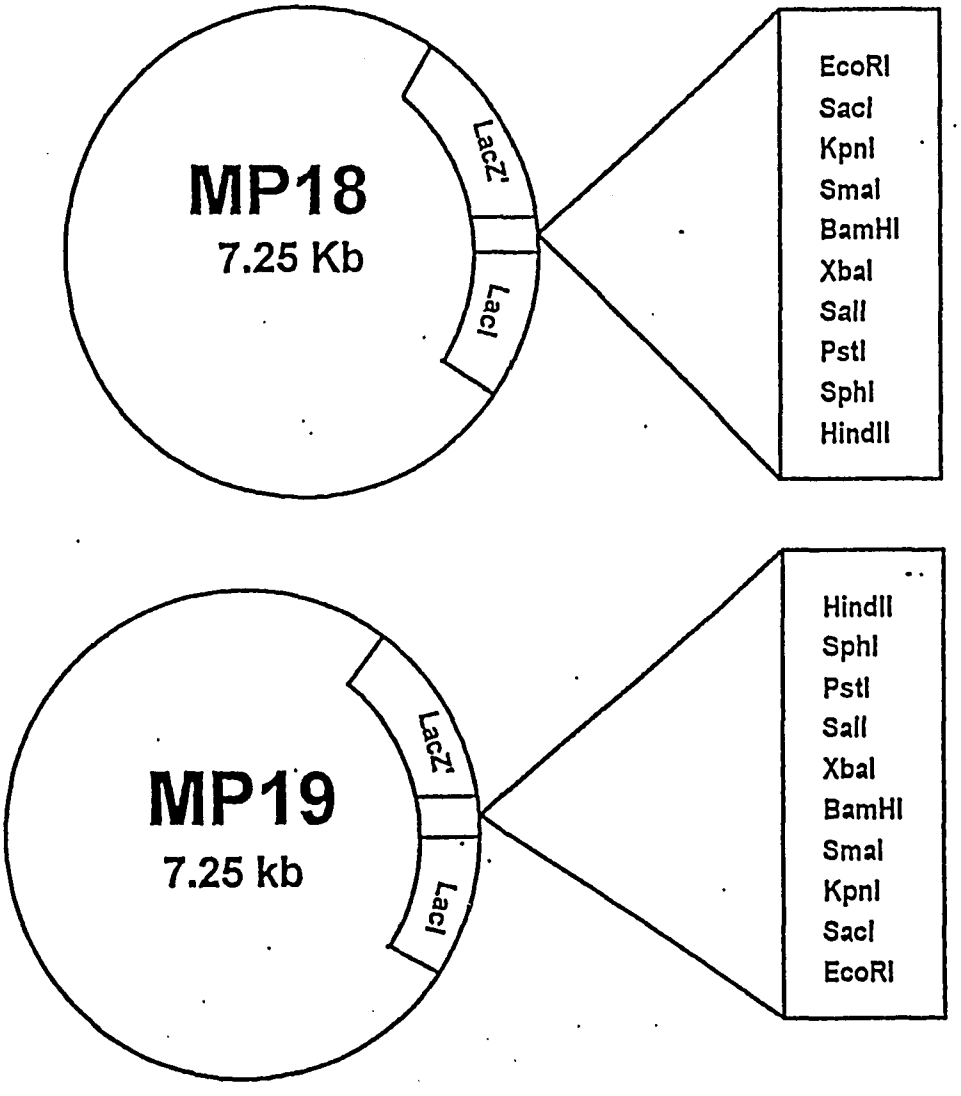


Fig. 11 Outline of the procedure for creating single stranded nested deletions in inserts cloned into mp19. The solid bar represents the insert and the sawtooth line the polylinker region of M13. An oligonucleotide complementary to the polylinker and containing a terminal EcoRI site is annealed to the single stranded template which is then cleaved with EcoRI. The single stranded 3′ end is digested by the 3′ -> 5′ activity of T4 DNA polymerase for various times to create the nested deletions. An oligo dG tail is then added to the 3′ end of the shortened insert with terminal deoxynucleotide transferase (TdT). An oligonucleotide containing (dC) and the EcoRI complementary overhang TTAAG are annealed to glue" the single stranded ends to reform the circle which is then transformed into an appropriate bacterial host such as JM101.

Figure 11. Outline of the Procedure for Cyclone

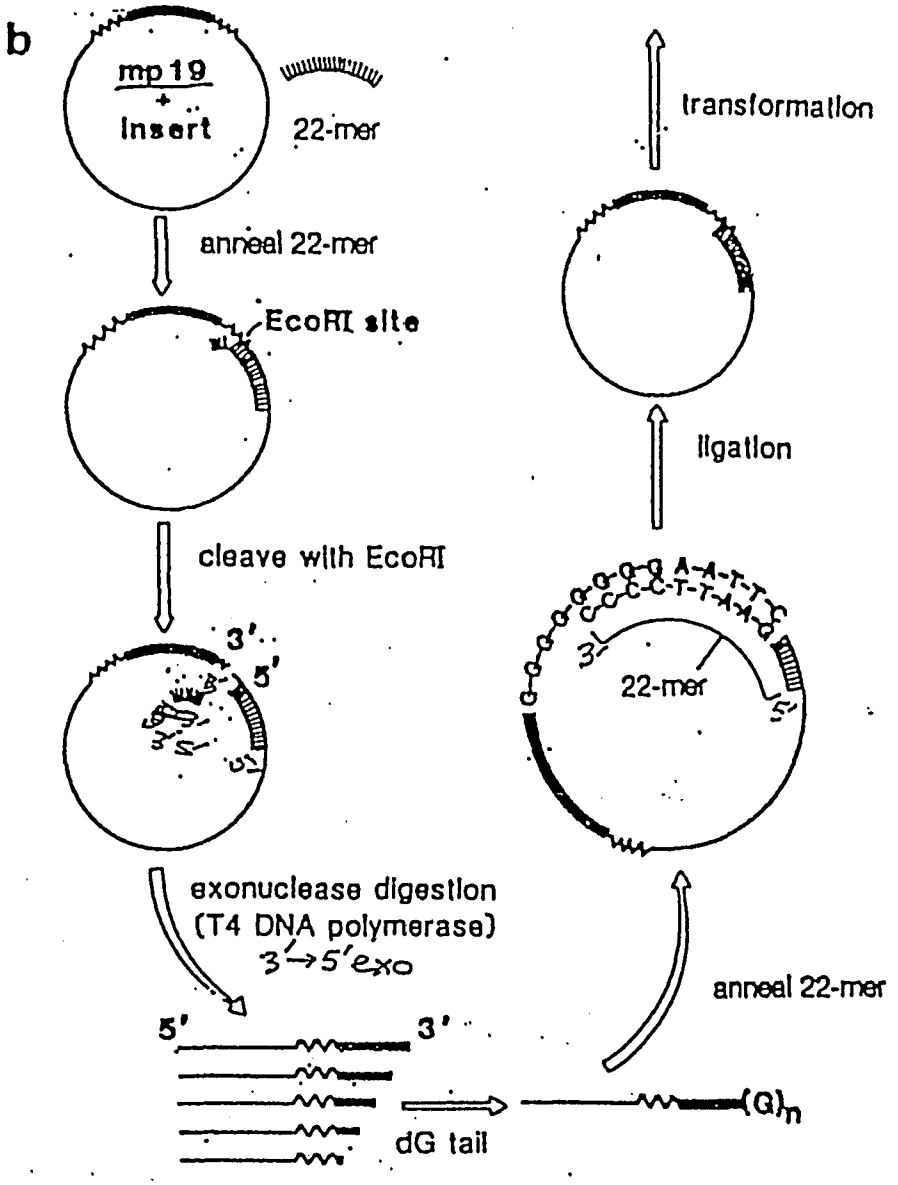
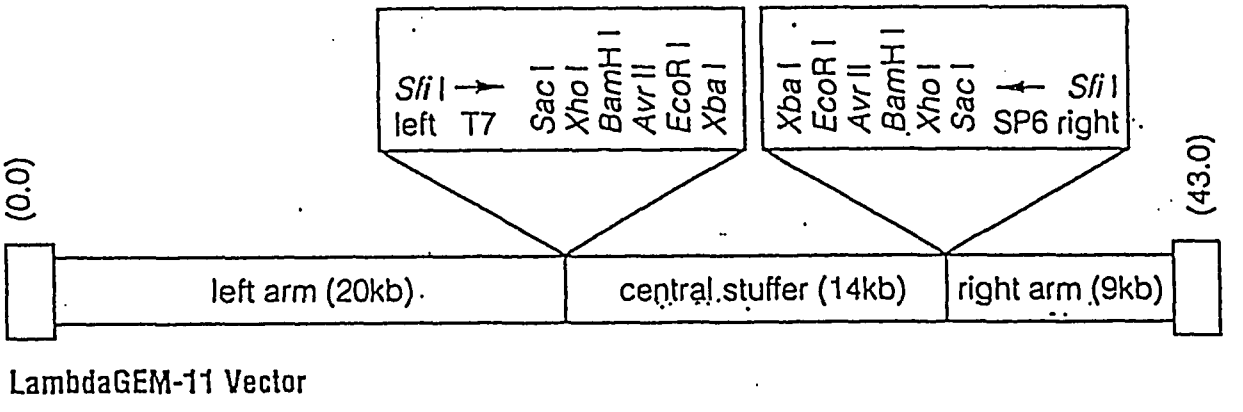


Fig. 12 Structural map of the Lambda GEM-11 vector. The lambda GEM-11 arms are derived from EMBL3 and the stuffer fragment is derived from lambda 2001. The map shows the sizes of arms and multiple cloning sites. Arrows indicate the direction of T7 and SP6 primers which complementary to the T7 and SP6 RNA polymerase promoters.

Figure 12. Structural Map of the LambdaGEM-11 Vector



LambdaGEM-11 Vector

Fig. 13 A map of the plasmid vector pGEM-3 showing the size of the vector and the multiple cloning sites. Arrows indicate the direction of primers complementary to the T7 and SP6 RNA polymerase promoters. The open box indicates the ß-lactamase coding region.

Figure 13. A Map of the Plasmid Vector PGEM-3

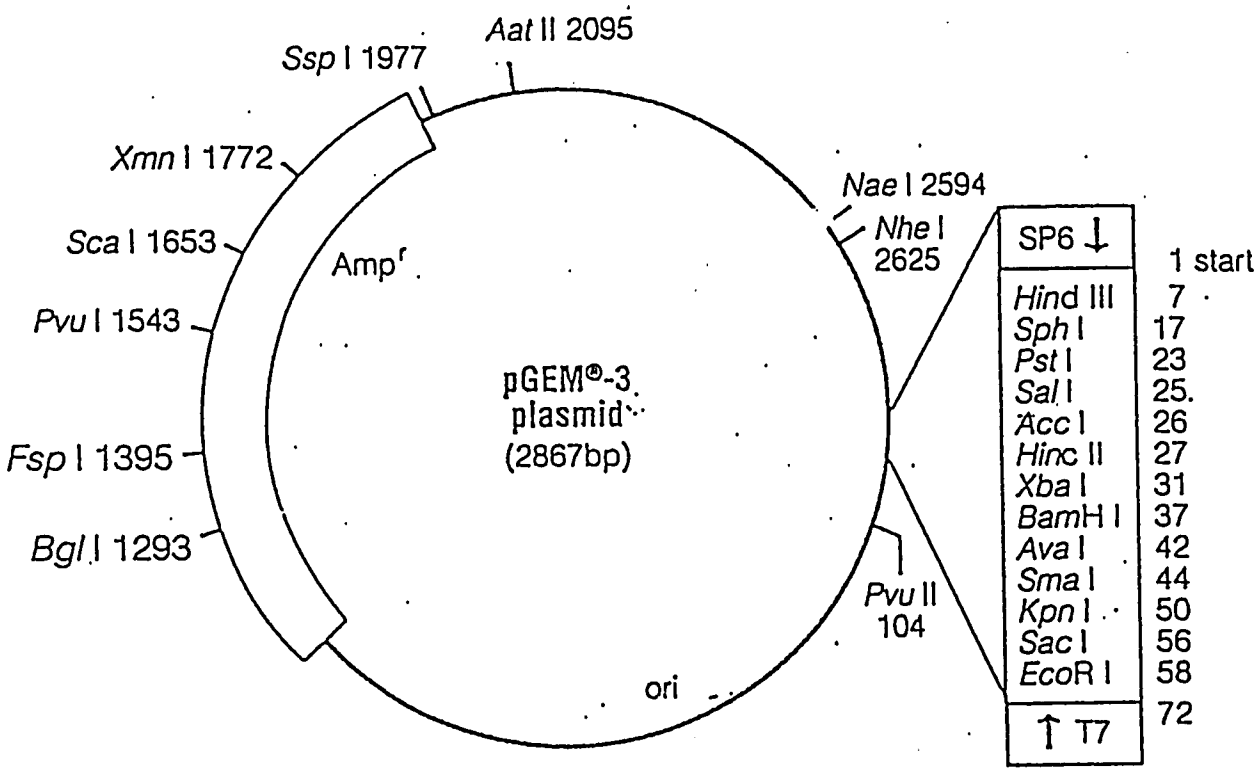


Fig. 14 A map of the DNA probes used for gel retardation assays. The 1.8 kb XbaI/BamHI fragment just adjacent to the 5'end of the 6.4 kb ras containing fragment was isolated from the subclcone pGEM-3. Four fragments (F1, F2, F3 and F4) were generated by restriction enzyme digestion and fractionated by gel electrophoresis. The restriction enzymes used to generate the fragments were XbaI, StyI, SfaNI and BamHI.

Figure 14. A Map of the DNA Probes Used for Gel Retardation Assays

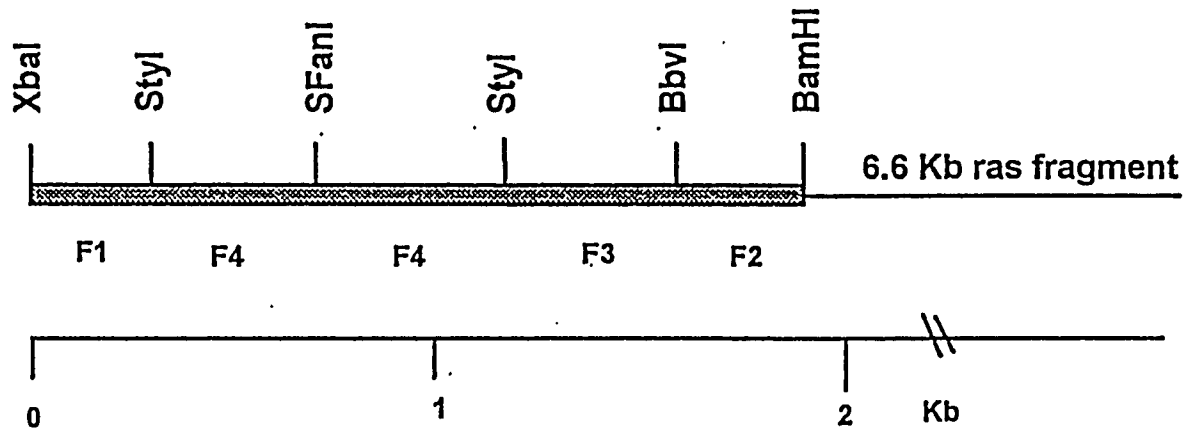


Fig. 15 Restriction polymorphisms in the c-Ha-ras sequences of SV40 transformed keratinocytes. Genomic DNAs derived from normal cultured keratinocytes (EP), line 324 squamous carcinoma cells derived from facial epidermis and two lines of SV40 transformed keratinocytes (lines 98 and 425 at the 55th and 46th serial passages respectively) were digested to completion with the indicated restriction enzymes, run on an agarose gel, blotted onto nitrocellulose and hybridized to ^{32}P-labeled pTB-1, a 4.8 kb fragment containing the four Ha-ras exons plus about one kilobase of both 3′ and 5′ flanking DNA. The migration of Hind III digested lambda phage fragments as markers is indicated.

Figure 15. Restriction Polymorphisms in the C-Ha-Ras Sequences

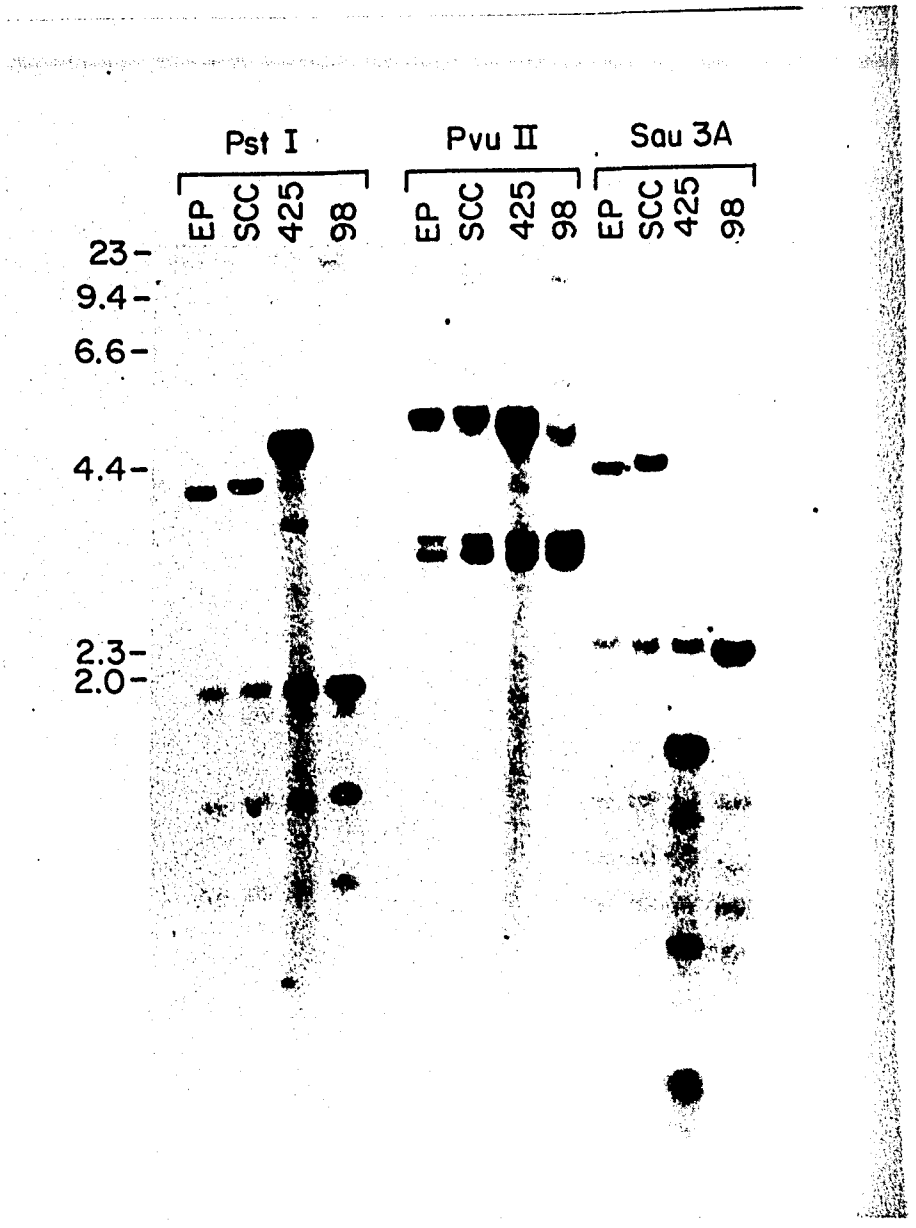


Figure 16. A replicate Southern blot of the restriction polymorphisms as shown in figure 15.

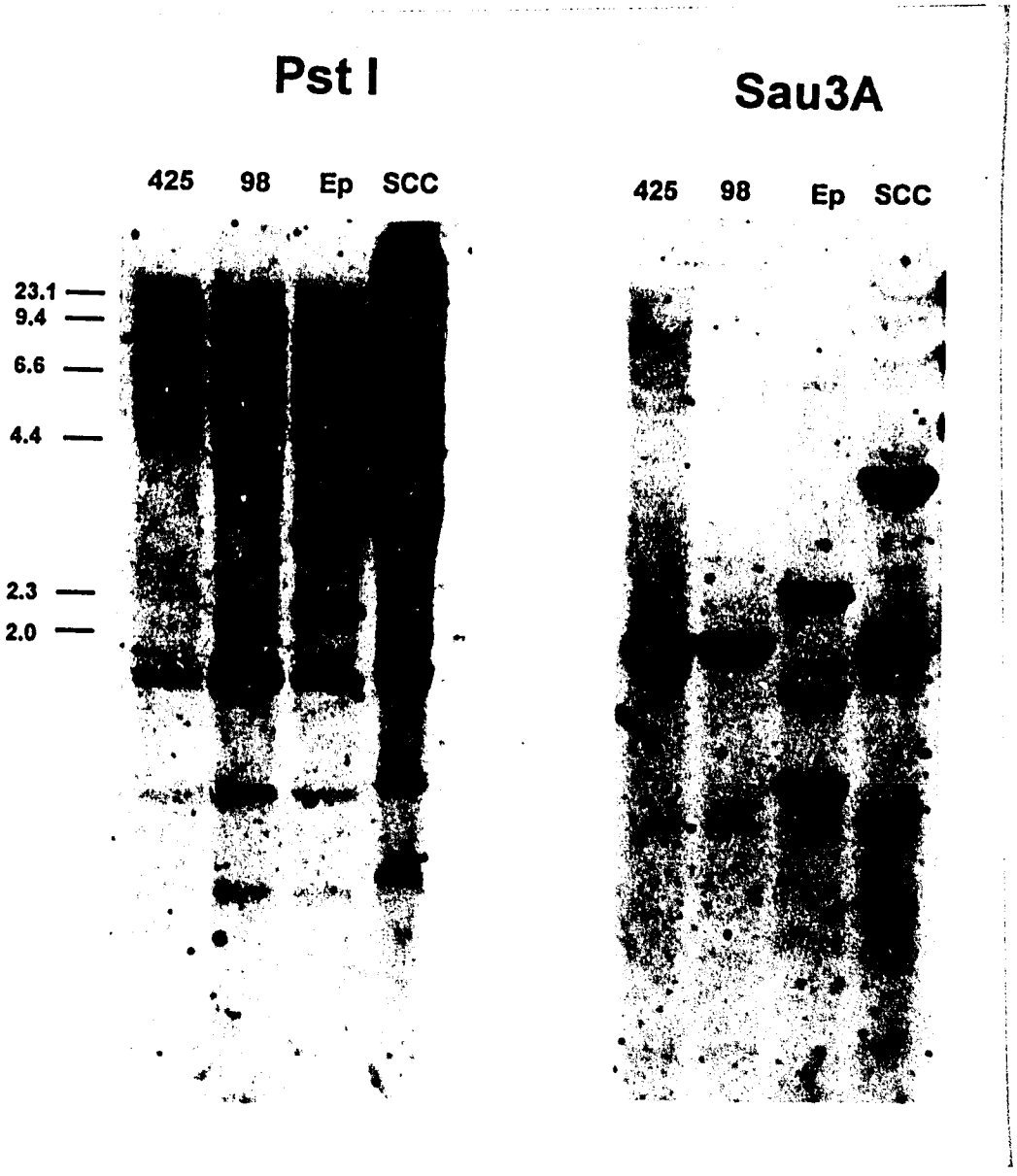


Fig. 17 Restriction polymorphisms in the c-myc sequences of SV40 transformed keratinocytes. 10 μg of each DNA were digested with endonuclease PstI and PvuII. The digested DNAs were electrophoresed on a 1% agarose gel, blotted onto nitrocellulose, and hybridized to ^{32}P-labelled pMC413RC, an EcoRI/ClaI restriction fragment containing the 3d exon of the myc protooncogene. Genomic DNAs were from normal cultured keratinocytes (EP), line 324 squamous carcinoma cells and lines 98 and 425 SV40-transformed keratinocytes at the 51st and 45th passage respectively. The migration of lambda phage Hind III fragment markers is indicated.

Figure 17. Restriction Polymorphisms in the C-Myc Sequences

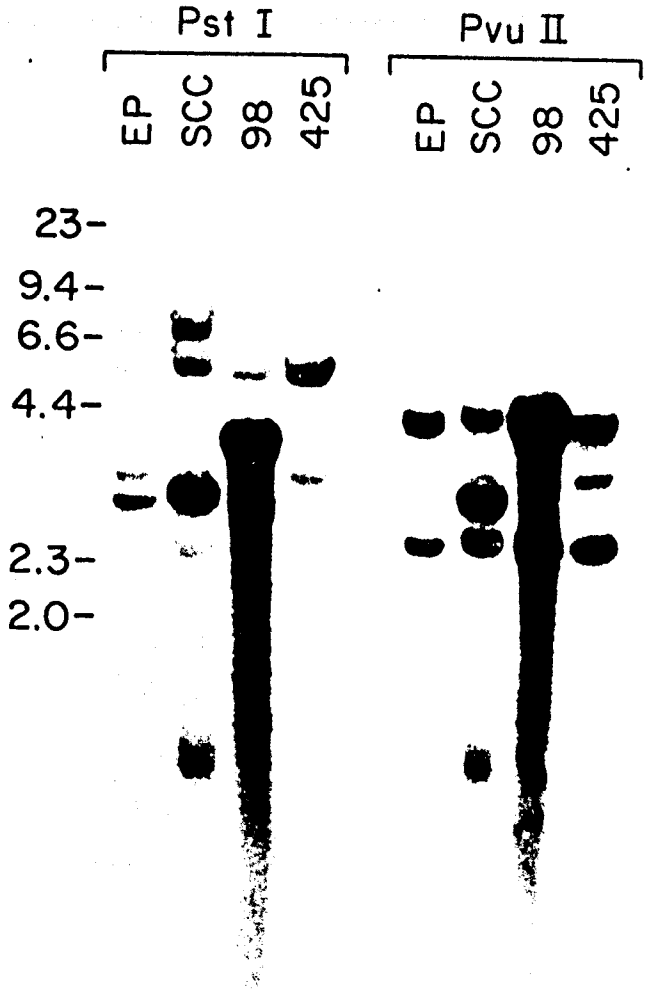


Fig. 18 Ethidium bromide stained agarose gel of cloned 6.4 kb ras-containing fragments derived from EP, lines 425 and 98 clones were purified from agarose gels and run on a 1% agarose gel. The migration of lambda phage Hind III fragment markers is indicated.

Figure 18. Ethidium Bromide Stained Agarose Gel of Cloned 6.4 kb Ras-Containing Fragments

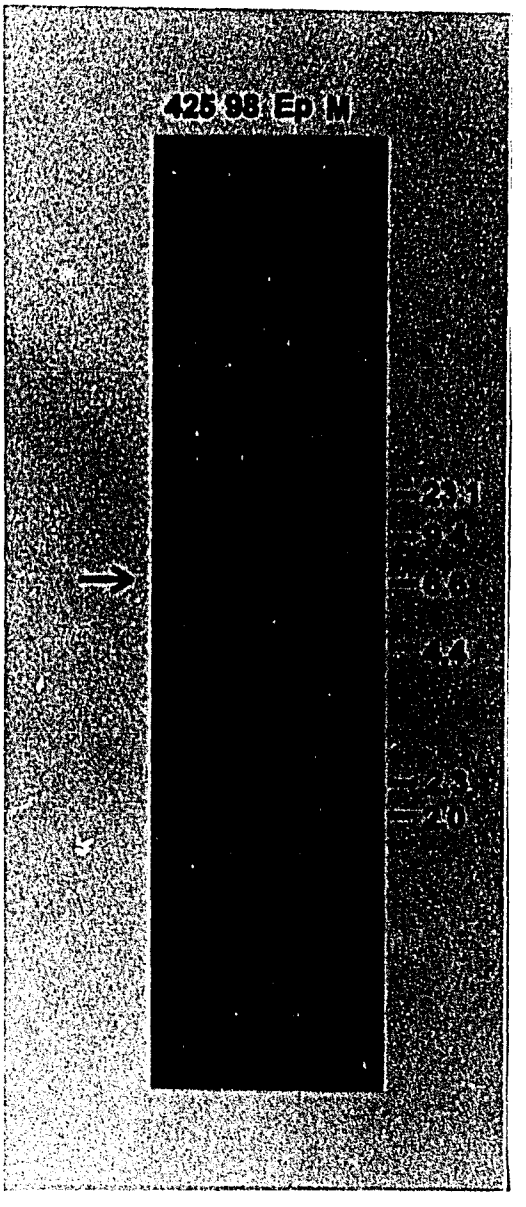
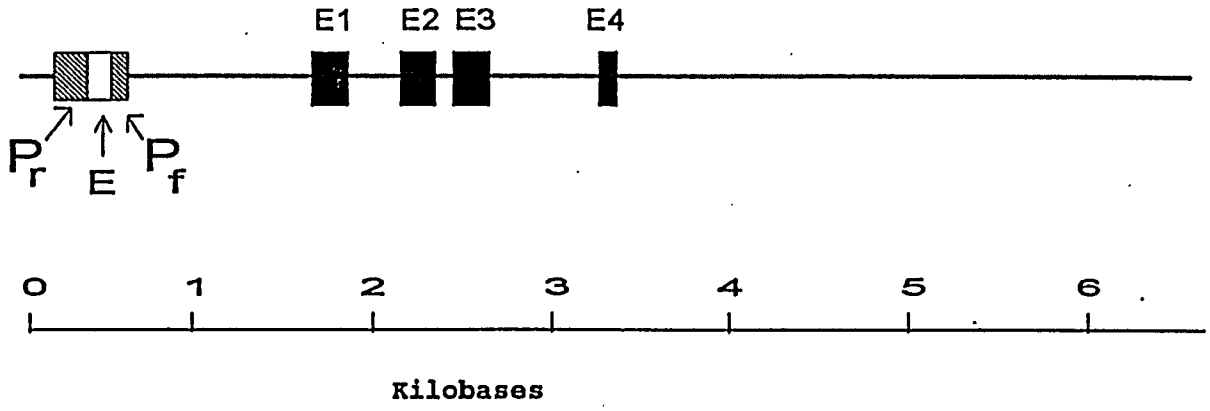


Fig. 19 Summary of regulatory sequences in the 5′ region of Ha-ras. Four exons are indicated by black boxes. Promoters including forward promoter and reverse promoter are indicated by shaded boxes. Enhancer is indicated by open box. Blow shows the positions of promoters, enhancer, exons, and splice sites.

Figure 19. Summary of Regulatory Sequences in the 5' Region of Ha-Ras



	base no.
Forward promoter	380 --> 560
Reverse promoter	298 --> 328
Transcriptional enhancer	333 --> 380
Splice donor site	576
Splice acceptor site	1516
Exon 1	1664 --> 1774
Exon 2	2042 --> 2220
Exon 3	2374 --> 2533
Exon 4	3231 --> 3350

Fig. 20 Polymorphism in the ras gene. The EJ ras sequence from base numbers 1769 to 1792 is shown. Guanidine (G) has been changed to Adenine (A) in all three corresponding sequences from EP (normal keratinocytes), lines 425 and 98 (SV40 transformed keratinocyte cell lines) as compared with the bladder carcinoma Ha-ras oncogene EJ.

Figure 20. Polymorphism in Ras Gene

```
EJ ras   TAGAGGTGAGCCTAGCGCCGCCG 1792
Ep ras   ----------G---------
425 ras  ----------G---------
98 ras   ----------G---------
```

Fig. 21 Restriction map of 3.8 kb XbaI fragment including the 5′ end of Ha-ras and 5′ adjacent region of Ha-ras. This diagram shows the restriction cut sites of pstI MboI (isoschizomer of Sau3A), and PvuII. The ruler below shows the length of the fragment.

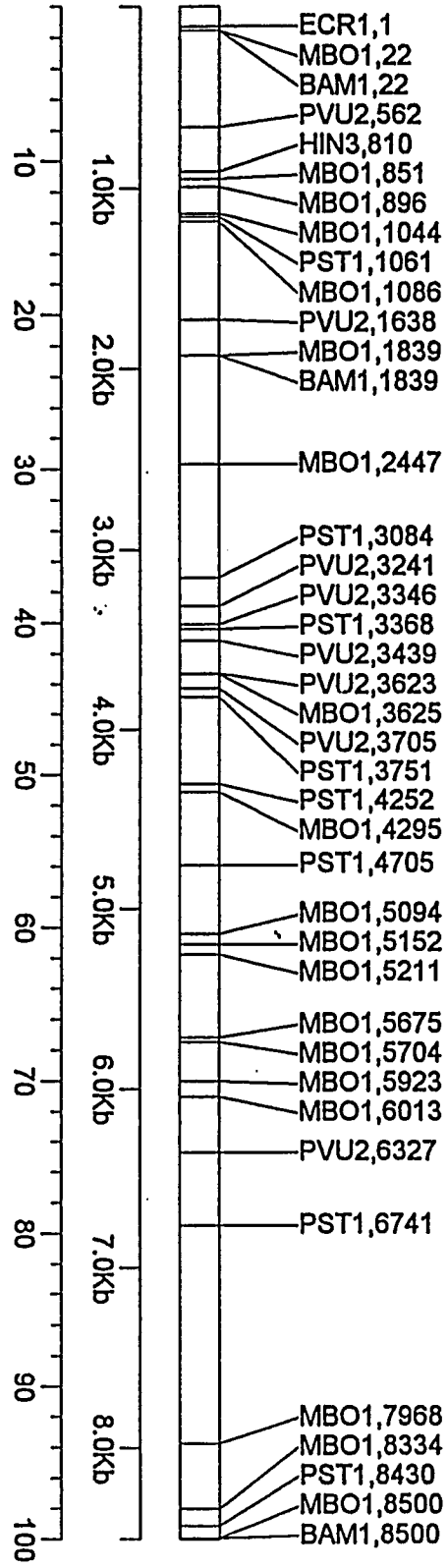


Figure 21. Restriction Map of 3.8 Kb XbaI Fragment

Fig. 22 Ethidium bromide stained agarose gel showing the comigration of the XbaI cloned DNAs derived from normal cultured keratinocytes (EP) and SV40 transformed lines 425 and 98. The identity of clones was confirmed by Southern blot hybridization using the 0.3 kb RsaI fragment probe which was used to screen the XbaI libraries. The migration of lambda phage Hind III fragment markers is indicated.

Figure 22. Ethidium Bromide Stained Agarose Gel of Cloned 3.8 kb XbaI Fragments

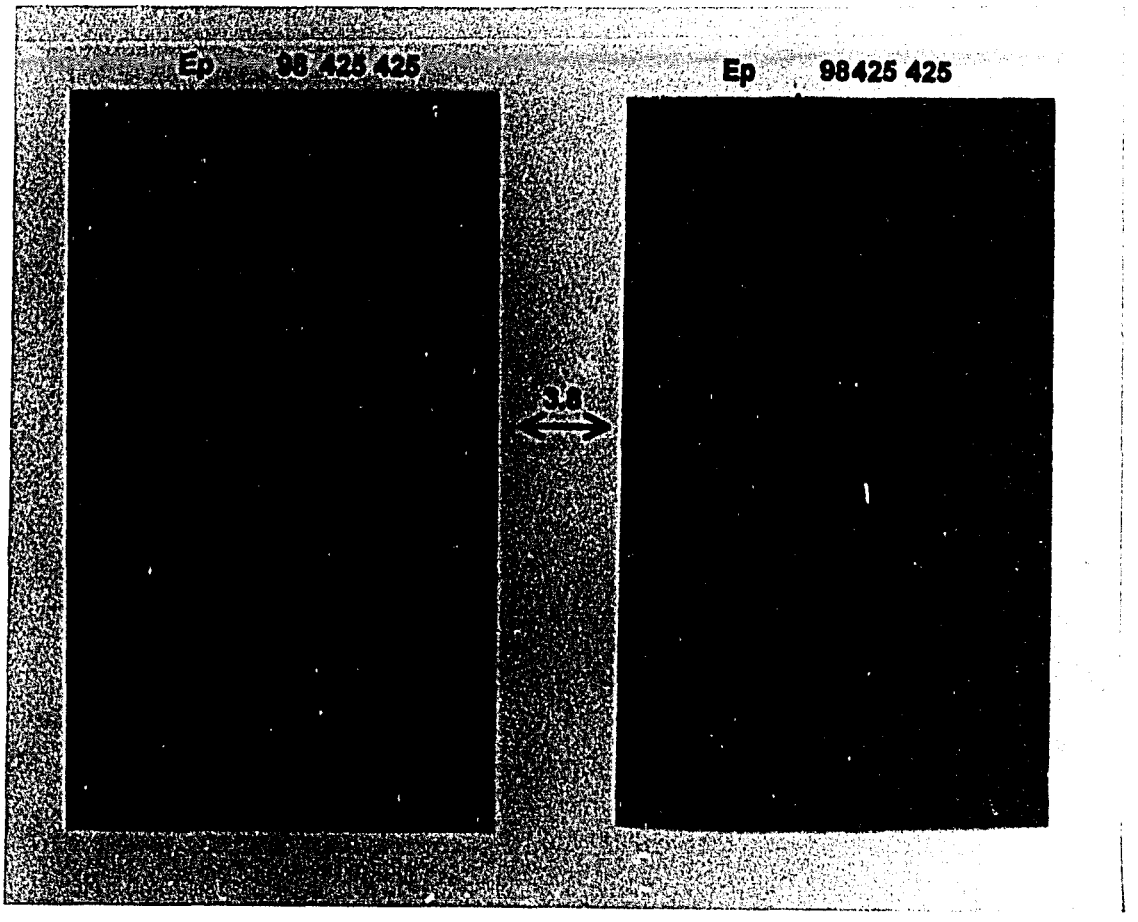


Fig. 23 Northern blot hybridization of ras transcripts in normal human keratinocytes (EP) and lines 425 and 98 SV40 transformed cell lines. Total cellular RNAs (30 μg per lane) were electrophoresed through denaturing formaldehyde gels, blotted onto nitrocellulose and hybridized to the full length 6.4 kb EJ ras containing fragment (fig. 8) labelled with 32 by nick translation. RNAs from normal human keratinocytes (EP), line 425 and line 98 SV40 transformed cell lines were used. The migration of the 28S and 18S rRNAs are indicated at the left.

Figure 23. Northern Blot Hybridization of Ras Transcripts

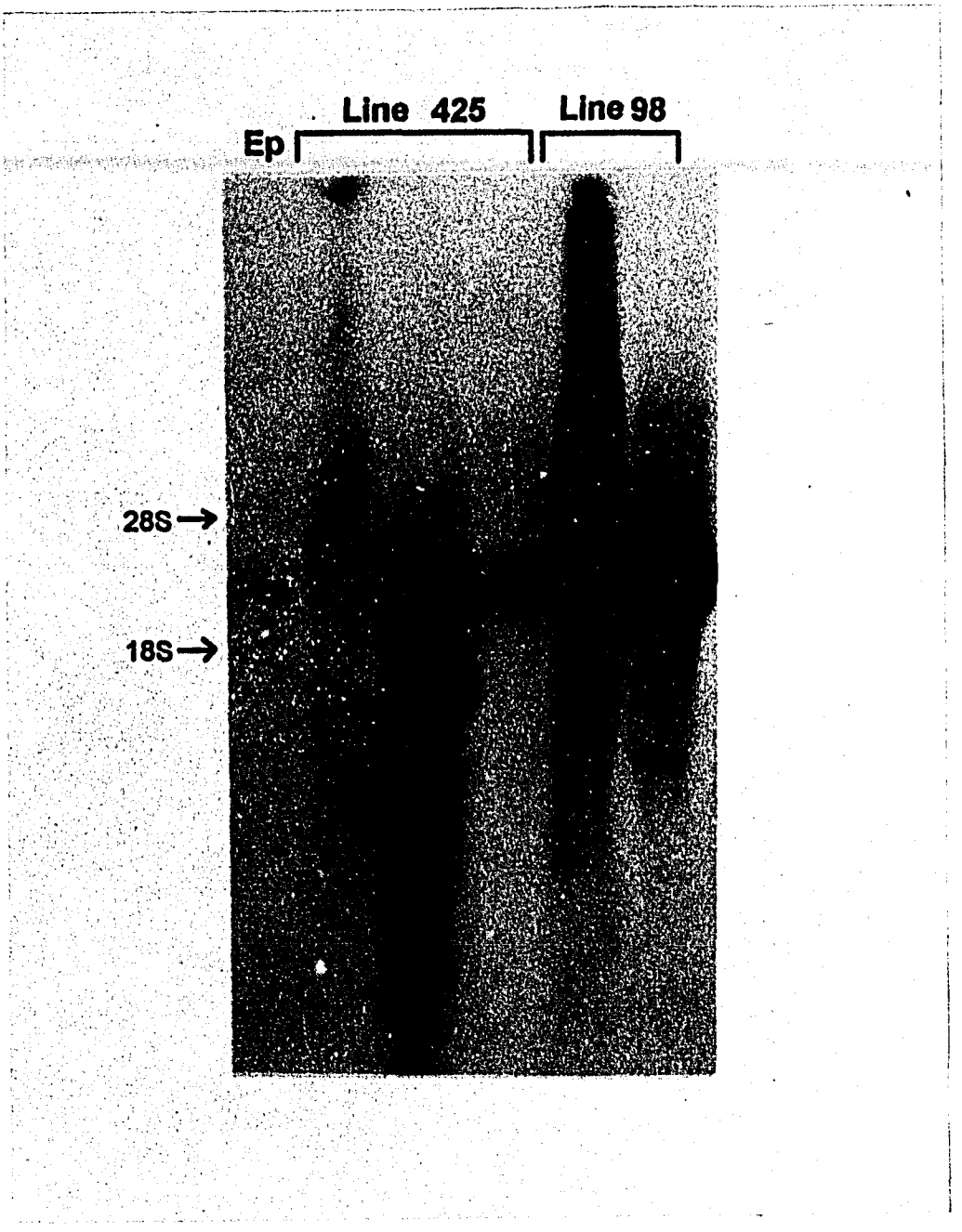


Fig. 24 Rehybridization of the northern blot shown in figure 23 to a ^{32}P labelled pMC413RC myc gene probe. The blot shown in Fig. 23 was stripped of ras probe and rehybridized as described in fig. 23.

Figure 24. Northern Blot Hybridization of Myc Transcripts

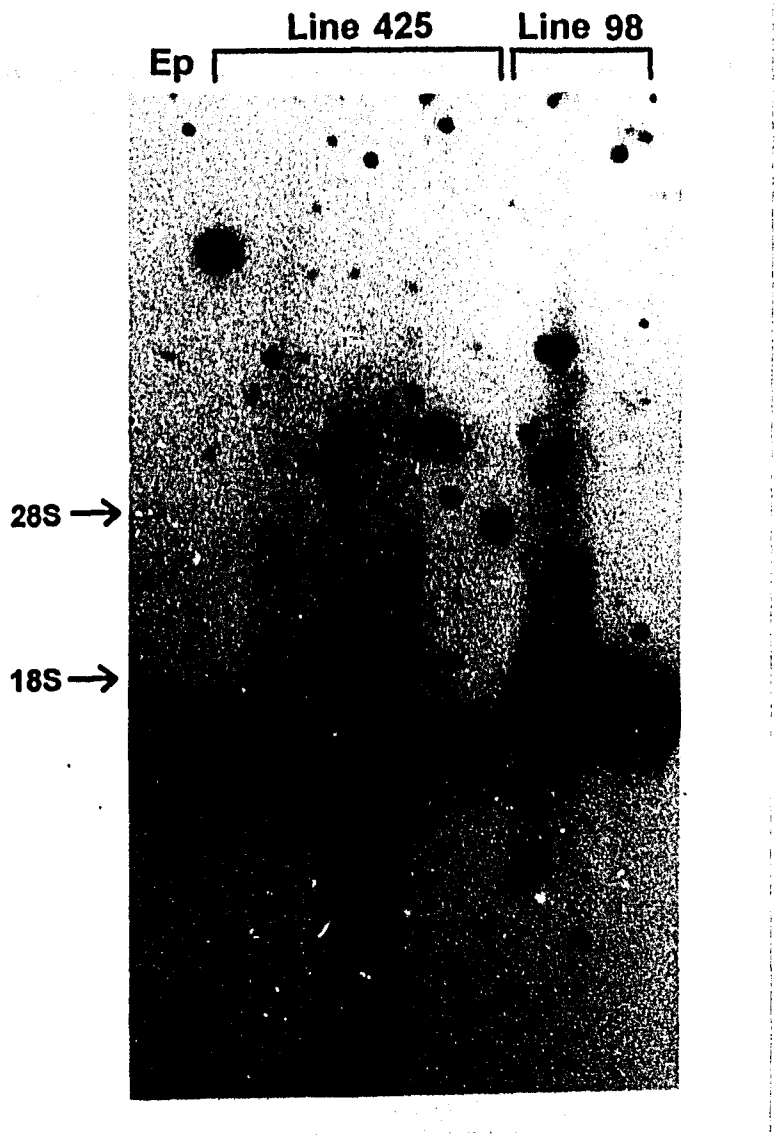


Fig. 25 Rehybridization of the northern blot shown in figures 23 and 24 to a ^{32}P labelled actin gene probe to control for potential errors in loading in interpretation of the myc and ras hybridizations.

Figure 25. Northern Blot Hybridization of Actin Transcripts

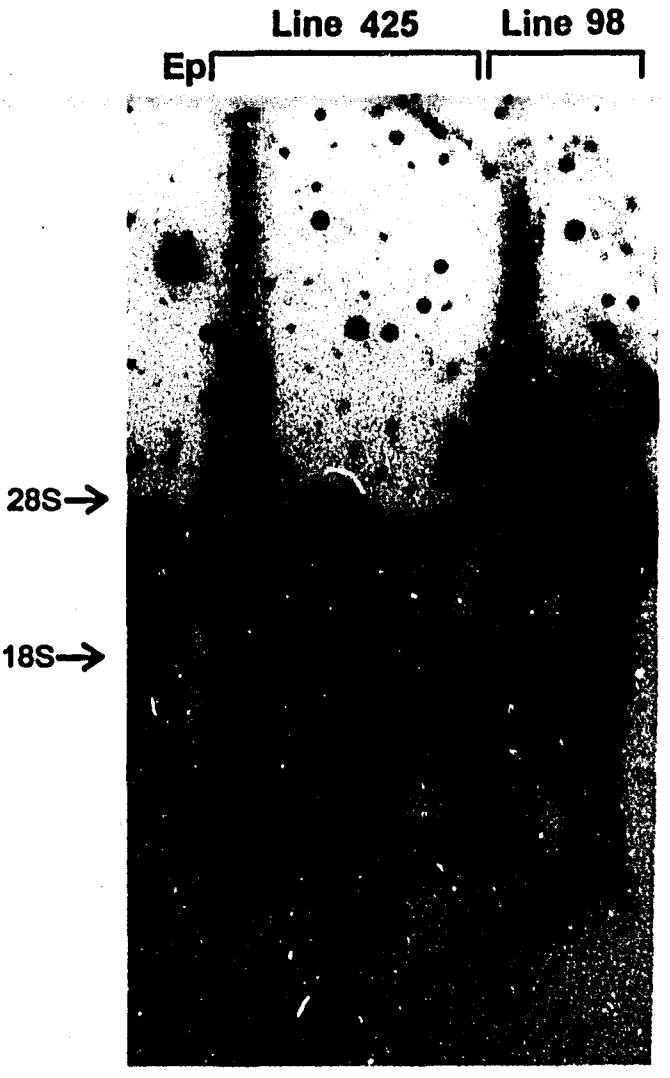


Fig. 26 Summary of sequence analysis of the 1.8 kb region adjacent to the 5′ end of the Ha-ras protooncogene. Long direct repeats are indicated by the open square box. Putative enhancer sequences are indicated by the shadow square box. Alu sequences are indicated in black square box. DNA-binding motifs within the putative enhancer region are also indicated below the map.

Figure 26. Summary of Sequence Analysis of the 1.8 kb Region

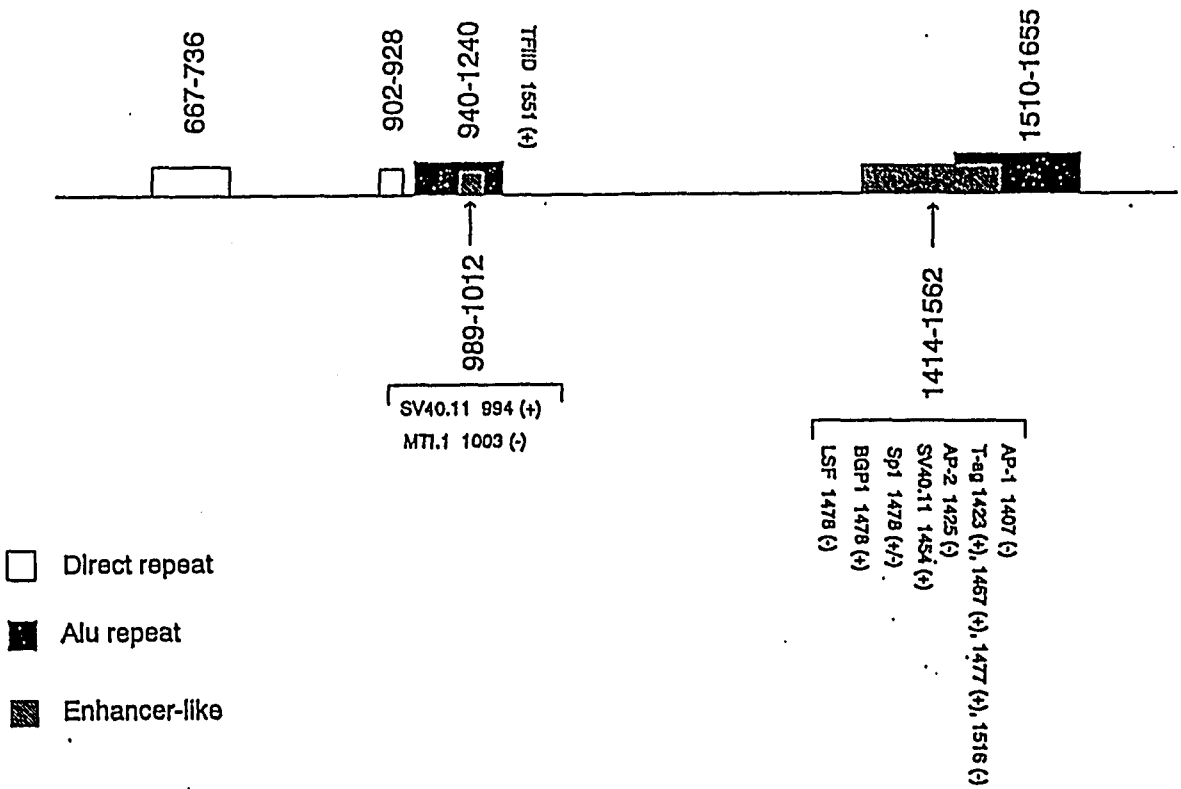


Fig. 27 Assays of binding of nuclear proteins from Hela cells and line 98 SV40 transformed keratinocytes to different subfragments of the ras upstream sequences by gel migration shift. Nuclear extracts prepared from HeLa (HNE; 3.4 μg of protein/reaction) and SV40 transformed cell line 98 (98NE; 1.5 μg of protein/reaction) and 1 μg of hsDNA were incubated with end-labelled probes (5 ng of DNA representing 10^5 cpm). The probes used for the assay were the fragments shown in figure 14: F1 (base nos. 1 to 395); F2 (base nos. 1581 to 1787); F3 (base nos. 1210 to 1581) and F4 (base nos. 395 to 847 and 847 to 1210) (fig 14). Specific DNA-protein complexes were analyzed by the electrophoretic mobility shift assay (see Materials and Methods). Free DNA probes without nuclear extracts were also run as a control. Arrows indicate the bands representing DNA-protein complexes.

Figure 27. DNA-Binding Assay I

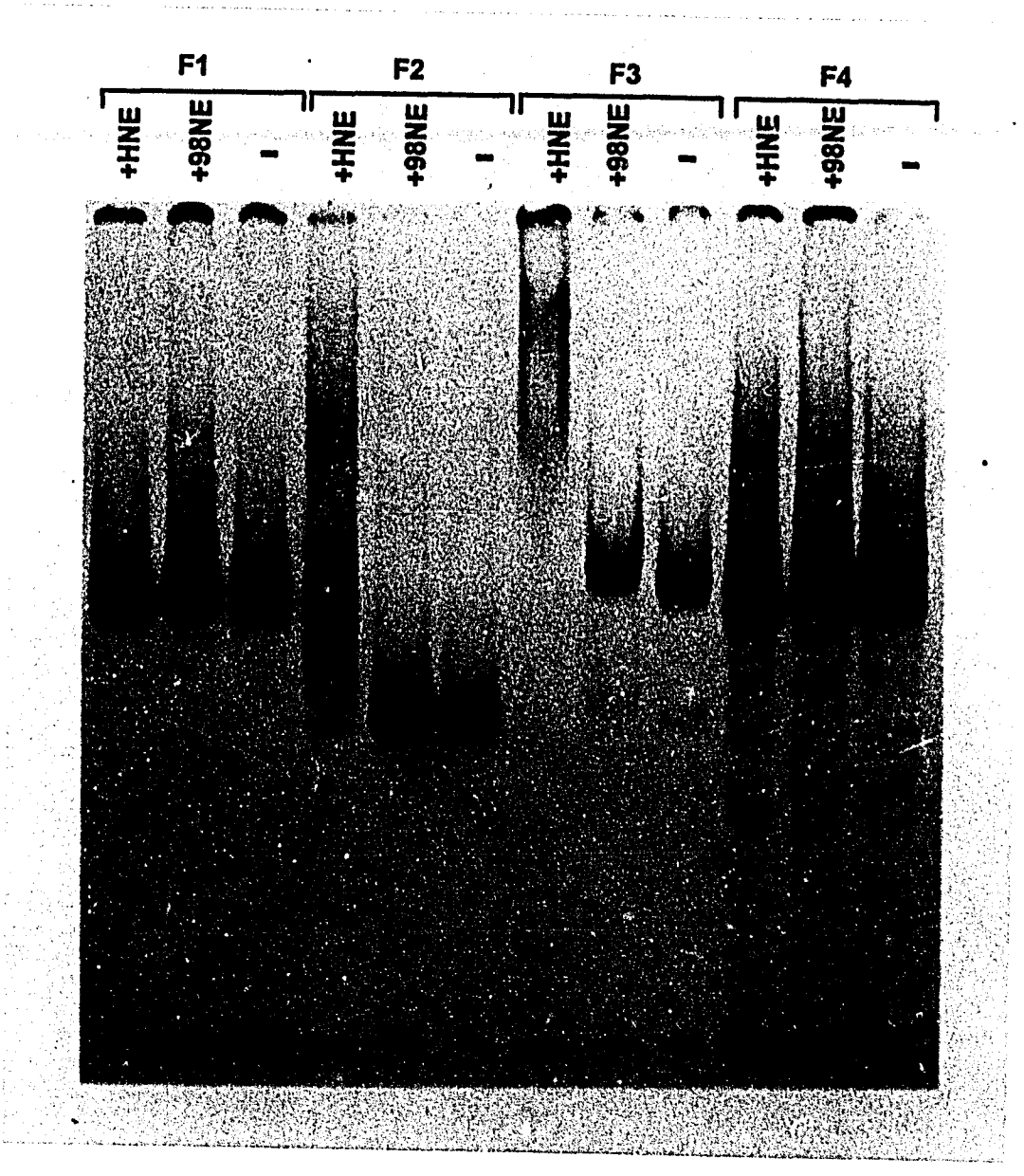


Fig. 28 Gel mobility shift assay showing binding of Hela and line 98 nuclear proteins to the labelled probes F2 (base nos. 1851 to 1787) and F3 (base nos. 1210 to 1581). The assay conditions were the same as described in the legend to fig 27 except that 3 μg of nuclear extract from line 98 were used. The left side of this figure shows the control experiment. Nuclear extracts from Hela (3.8 μg per reaction) and line 98 (3 μg per reaction) were incubated with 5 ng of ^{32}P end-labelled FT fragment DNA, a SRF binding site (serum response factor) in the presence or absence of 30 fold molar excess of XGL, a SRF competitor or XGLM a point mutant of XGL which fails to bind nuclear factors. The DNA-protein complex is indicated by the arrow.

Figure 28. DNA-Binding Assay II

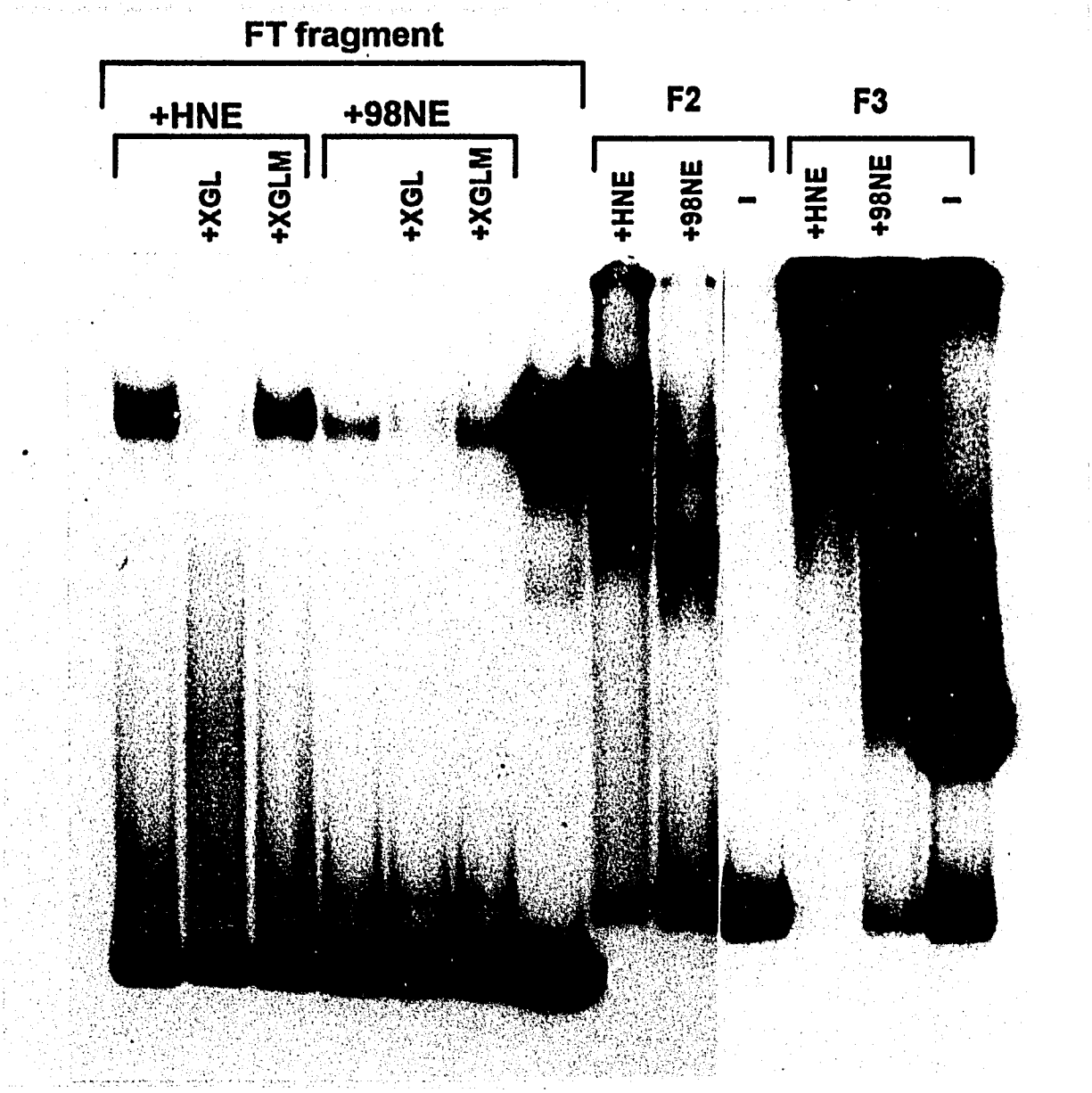


Fig. 29 Gel mobility shift assay demonstrating dose-dependence of binding by nuclear extracts from Hela (HNE) and line 98 (98NE) to the labelled probes F2 (base nos. 1851 to 1787) and F3 (base nos. 1210 to 1581) in the presence of competitor DNA. The assay conditions were as described in the legend to fig. 27 except that a 10 fold molar excess of unlabeled F2 and F3 DNAs were used for competition.

Figure 29. DNA-Binding Assay III

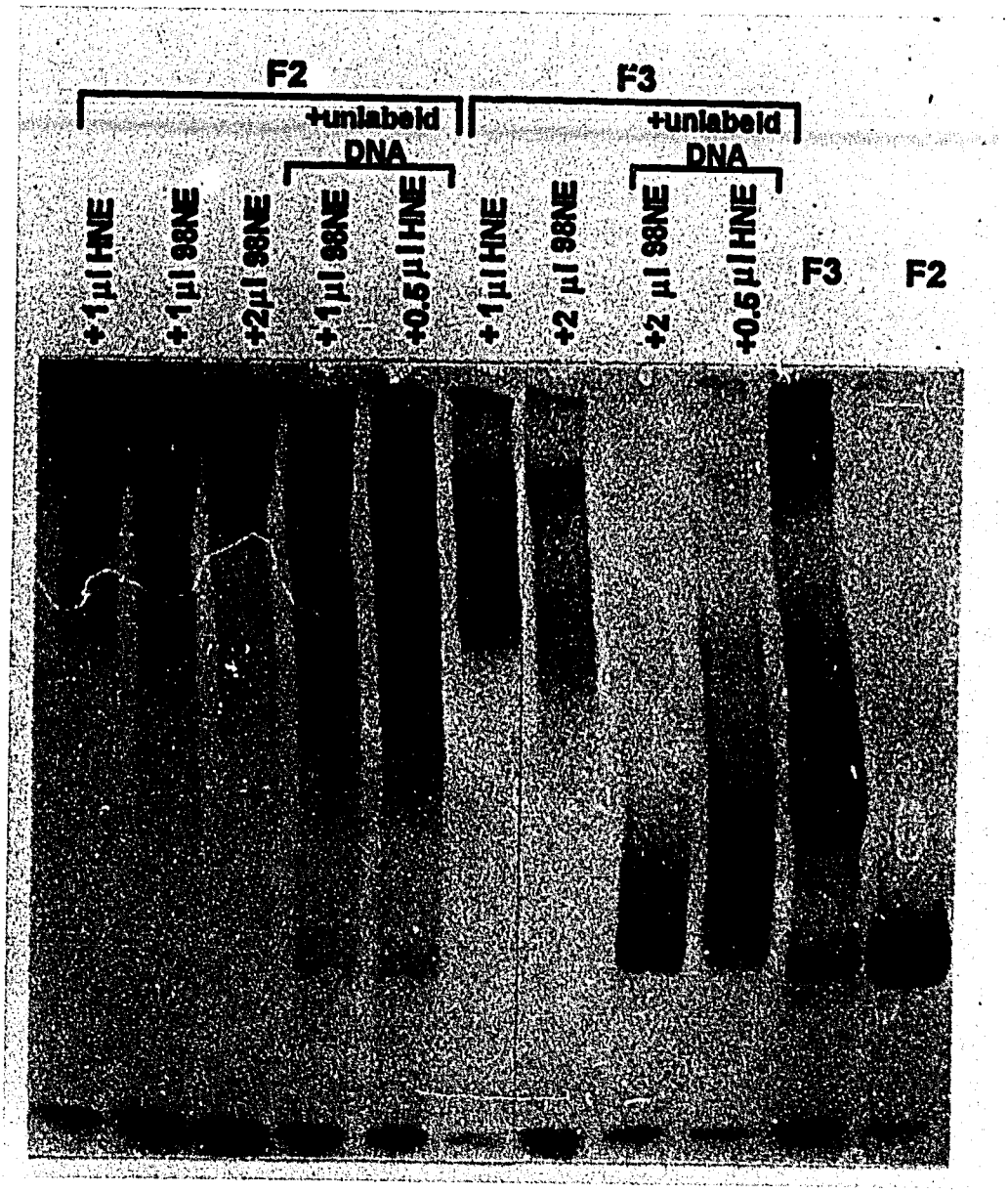


Fig. 30 Gel mobility shift assay demonstrating the binding of nuclear extracts from Normal keratinocytes (EPNE) and line 98 (98NE) to the labelled probe F2 (base nos. 1851 to 1787). The assay conditions were as described in the legend to Fig. 27 except that 0.5, 1, 1.5 and 2 μg of nuclear extracts were used. B represents the longer exposure of A.

Figure 30. DNA-Binding Assay IV

Fig. 31 DNase I foot-printing of the line 98 nuclear proteins-DNA complex. The F2 fragment was amplified by PCR using T7 and L6 as primers. The L6 primer was labeled at the 5′ end by T4 polynucleotide kinases and gamma-^{32}P-ATP prior to PCR. 5 μg of probe DNA was incubated with increasing concentration of nuclear proteins (0, 2 and 4 μg) in the presence of 1 μg of sperm DNA. DNase I digestion was carried out for 2 minutes after which the reaction was terminated and the samples were applied to a 6% polyacrylamide/7M urea sequencing gel as described in "Materials and Methods".

Figure 31. DNAase Foot-Printing Assay I

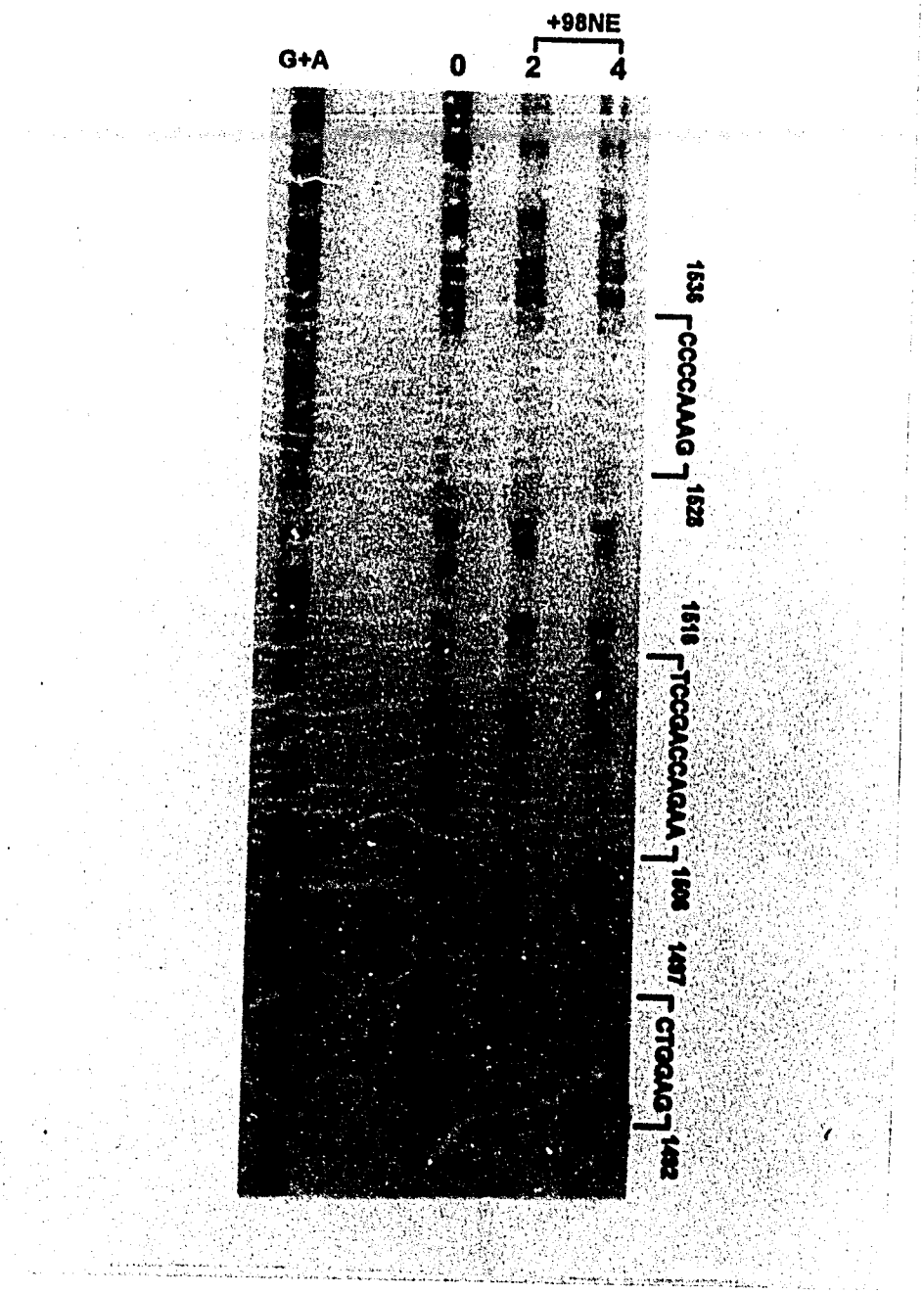


Figure 32. A replicate of the DNAase foot-printing assay shown in Fig 31.

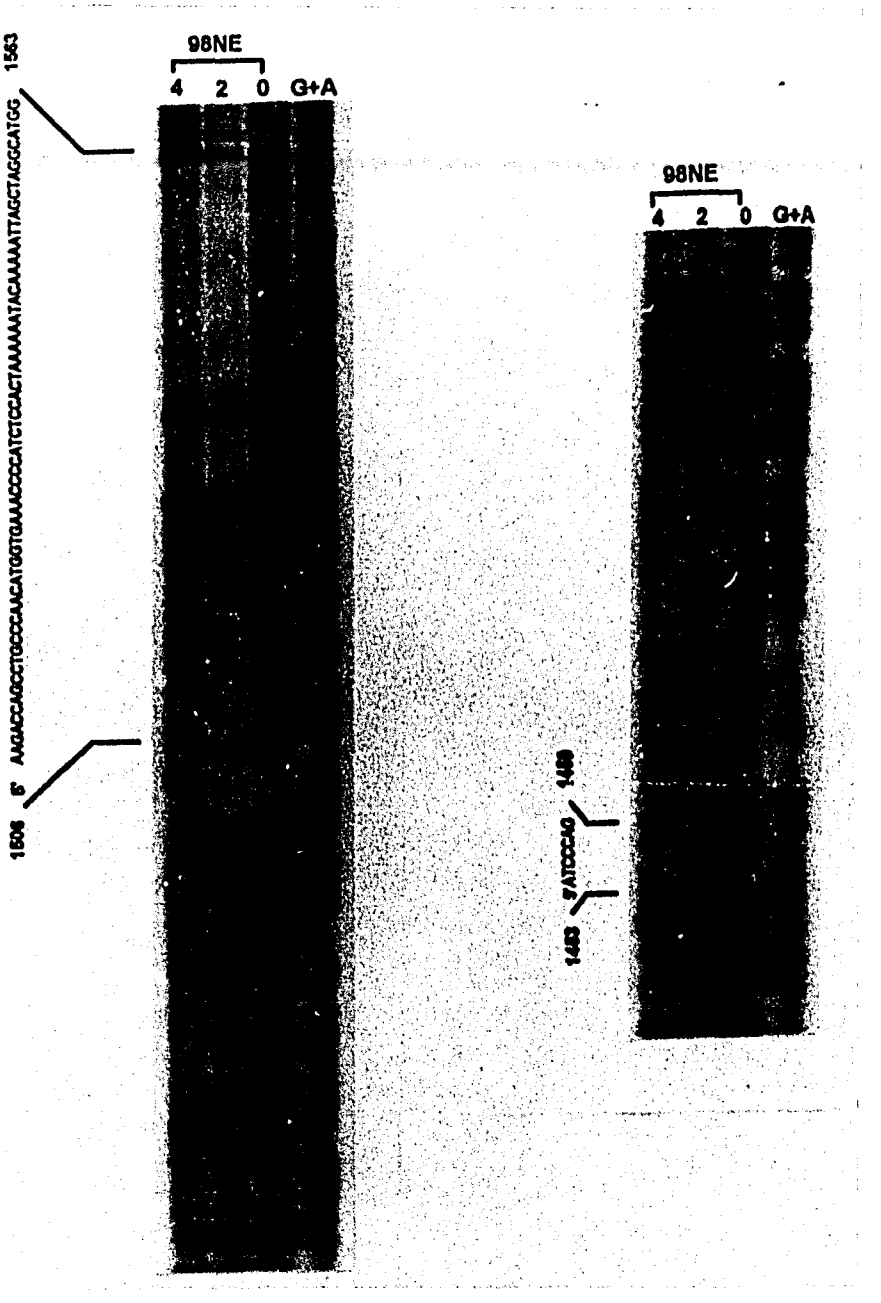


Table 1. Functions of
Cell-Derived Oncogene Products

	Oncogene	Functions
Class 1-Growth Factors		
	sis	PDGF B-chain
	int-2	FGF-related grwoth factor
Class 2-Receptors		
	erB	EGF receptor
	fms	M-CSF receptor
	ros	Receptor like PTK*
Class 3-Membrane-associated		
A. Protein Tyrosine kinase		
	src	Protein tyrosine kinase
	yes	Protein tyrosine kinase
	fps	Protein tyrosine kinase
	fgr	Protein tyrosine kinase
	ros	Protein tyrosine kinase
B. G Proteins	H-ras	GTP-binding/GTPase
	K-ras	GTP-binding/GTPase
	N-ras	GTP-binding/GTPase
4. Cytoplasmic PSK*		
	mos	Protein-serine kinase
	raf	Protein-serine kinase
	cot	Protein-serine kinase
5.Nuclear Proteins		
	jun	Part of AP-1, DNA-binding protein With c-jun ptoduct
	fos	to form AP-1 transcription factor
	myc	DNA-binding protein
	myb	DNA-binding protein

* PTK-Protein Tryrosine Kinase
PSK-Protein Serine Kinasse

Table 2. Primary Signal Transduction Mechanisms

1. Protein kinase receptors
   Ligand binding leads to activation of intrinsic protein kinase catalytic domain. Receptors can be either protein-tyrosine kinases or protein-serine kinases.

2. Receptors coupled to G proteins
   Ligand binding leads to activation of specific G proteins. Activated G protein α subunits modulate activity of specific targets (e.g., adenylyl cyclase, phospholipases C and A_2, ion channels, cGMP phosphodiesterase).

3. Ion channels
   Ligand binding leads to channel opening or closing.

4. ANF receptor
   Ligand binding leads to activation of intrinsic guanylyl cyclase catalytic domain.

5. Phosphotyrosine phosphatase receptors
   Ligand binding may activate or inhibit intrinsic phosphatase catalytic domain.

6. IL-2, IL-3, IL-4, IL-6, GM-CSF, EPO, GH, PL receptors
   These receptors are all related in structure but have an unknown signaling mechanism. The IL-2β, IL-3, IL-4, and EPO receptors form a subfamily of receptors that are related in their cytoplasmic domains as well.

7. Nuclear receptors of steroid gene family
   Ligand binding induces or represses gene expression.

Table 3. Homologies of Alu consensus sequences in the 5' upstream region of Ha-ras with Alu sequences in other genes.

Sequence Homology	Position	Name	
ALU like repeat (940/1240)		Human ADA	950/1310 70%
		Human ENO	960/1130 78%
		BABAPO	990/1270 72%
		Human BMY	960/1250 75%
		Human APO	950/1250 75%
		Human CSF	960/1270 71%
		Human AGG	960/1270 71%
Alu like repeat (1510/1655)		Human G6P	1350/1730 76%
		Hum CRYGBC	1400/1730 77%
		Human CCI	1430/1730 80%
		Human GHC	1420/1730 80%
		Human BCR	1380/1760 79%
		Human GHC	1360/1740 76%
		Human GLA	1400/1720 78%
		Human ACT	1420/1750 70%
		Human ADA	1420/1730 86%
		CHPRSA	1340/1730 76%

Table 4. Homopolymeric T and A stretches in viral genes

Name of viral gene	Position	Homology
BBM3AC	1710-1730	84%
BTV9V	700-730	81%
APHRNA	1710-1740	78%
AAFVMA	1720-1740	65%

Table 5. DNA-binding motifs in the putative regulatory region of Ha-ras

Factor Sequence	DNA-binding site		Consensus
Adh1 US2	18	(-)	CCCCGG
GATA-1	50	(-)	CCAATCT
CTF/CBP	51	(+)	GATTGG
C/EBP	161	(+)	TAAGACTC
GATA-1	350	(-)	GATAAG
GATA-1	351	(+)	TTATCTC
GATA-1	352	(+)	TATCTC
GH-CSE	575	(-)	TAAATTA
ADH US2	1039	(+)	CACCTCCC
AP-1	1046	(-)	TTGAGCCAG
CP1	1197	(+)	AACCAAT
CTF	1199	(-)	GATTGG
GCN4	1360	(-)	TGAGTG
GATA-1	1394	(+)	TATCTT
GCN4	1407	(+)	TTACTC
SP1	1478	(+)	GGGCGG
TFIID	1551	(+)	TACAAA
HC3	1570	(-)	CCACCA
CTF	1783	(+)	GATTGG
CBP	1784	(-)	CCAAT

Table 6. Data of Restriction Analysis of the 3.8 kb XbaI Fragment of Ha-ras

Pst I (CTGCAG) :
Sites: 1033
2992
3268
3639
3826
4124
4561
5388
6531
8166

Sizes: 73, 187, 276, 298, 371, 437, 827, 1033, 1143, 1635, 1959

Pvu II (CAGCTG) :
Sites: 548
3145
3246
3337
3515
3593
6131

Sizes: 78, 91, 101, 178, 548, 2479, 2108, 2538

Sau 3a (GATC) :
Sites: 22
829
872
1016
1056
1787
2377
3517
4165
4938
4994
5051
5501
5528
5741
5827
7720
8074
8234
Sizes: 5, 22, 27, 40, 43, 56, 57, 86, 144, 160, 213, 354, 450, 590, 648, 731, 773, 807, 1140, 1893

VII. Appendixes

Appendix I

```
LOCUS       HUMRAS1A     1830 bp ds-DNA             PRI       22-FEB-1993
DEFINITION  Homo sapiens ras Alu-like and direct repeat regions, enhancer
            elements and c-Ha-ras (EJ fragment) gene, 5'end cds.
ACCESSION   L11526
KEYWORDS    Alu-like repeat; direct repeat; enhancer element;
            regulatory element.
SOURCE      Homo sapiens DNA.
  ORGANISM  Homo sapiens
            Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria;
            Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE   1  (bases 1 to 1830)
  AUTHORS   Chen,W. and Steinberg,M.L.
  JOURNAL   Unpublished (1993)
  STANDARD  full staff_review
FEATURES             Location/Qualifiers
     misc_feature    1..33
                     /standard_name="M13/mp18 polylinker"
     repeat_region   667..736
                     /rpt_type=direct
                     /rpt_unit=667..682
     repeat_region   902..928
                     /rpt_type=direct
                     /rpt_unit=902..913
     repeat_region   940..1240
                     /rpt_family="Alu-like"
     enhancer        989..1012
                     /note="putative"
     enhancer        1414..1562
                     /note="putative"
     repeat_region   1510..1655
                     /rpt_family="Alu-like"
     mRNA            1787..>1830
                     /note="EJ fragment overlap"
                     /product="c-Ha-ras protein"
                     /gene="c-Ha-ras"
BASE COUNT      482 a   448 c    423 g    477 t
ORIGIN
        1 gaattcgagc tcggtacccg gggatcctct agagaaggac tggtgctgaa gattggggga
       61 ggagaagcct ttaaaatgtg cacaatcttt gagaaaataa ttctttaaaa tgttggcacg
      121 ctccacattt ttgttcctgg gcccctggtg agtgtgcctt taagactctg aatgtatatg
      181 tgcatatatg tgtgtatgtg tgtgttgact gtgcttatgt gtatatgaat aggtgttggg
      241 gactaccatg taaattgtga taactaagag cccctgcgag tttggagcca gcagatgtgt
      301 tcctgcactc ctcccctccc actttatgtg ctctgacact cccactatgc ttatctctgt
      361 agctctcatc agaagcagag gctgggatta tctgcctagg gctgagtccc ttcatattaa
      421 caacagactg aagatgtact gactgcaacg agacaaatag aaataatatg gtcacattca
      481 cctccatttc ataaagcaag tggaacctgg gctcactgcg atgaatgagc ttcaataatt
      541 catcccagct gctggtgtgg tattgacata aagctaattt aacactgggg gagccagggg
      601 aagtagggag cagttagcct tcgcttacag catggtcact ctgctagcac tctggtgcta
      661 tgctggaaag cagaacccca gagtagaagc aaaccccaaa cagaacccaa acagaacccc
      721 aaagcagaac cccagacctg gggaggtcag ttgccccctt ccctccttgt agccagttga
      781 ctttctgaag ctttgttagg tatggtattt tcttcttgat gatactcttg atcttttgca
      841 tcaggaatag agacttccca cccctgtctt cagatctgaa cttctcaaca aagagatgtt
      901 tctcaggtca gtcagactca ggtcagtctc tccagggaat caaatcaagg ttgagggttt
      961 ttgtttgttt tgagacaagg tctccctgtg tcacccaggc tggagtgcag tggcatgatc
```

```
1021 tcgtctgact gcagcctcca cctccctggc tcaagcgatc ctgccacctc agcctccaaa
1081 gtagctgaga ctacaggcct gtgccatcac accccgctaa ttgtttctgt tttgttttgt
1141 ttttctaaag aaggagtttc gccacattgc ccaggctgtc tcagactcct gagctcaacc
1201 aatcgcctgc cttggctccc aaagtgctgg gattacaggc ataagccacc atgcacagca
1261 atgttgaggt ttttgctaga gaaaaaggga acctgtggtt catatcttaa atgatattca
1321 gcaacccatt tgatttttt ctttctcaaa aataaaagtc actcattcaa catccaaaac
1381 atgtttgagt tcctatcttt cttcatttac tcaacaaata cttaggccag gcacggtggc
1441 tcatgcctgt aatcccagca ctttgggagg cggaggtggg cggataactt gaggtcagga
1501 gttcaagacc agcctgccca acatggtgaa accccatctc cactaaaaaa tacaaaaatt
1561 agctaggcat ggtggtgggt gcctgtaatc tcagctgctc gggaggctga agcaaggaga
1621 atcgcttgaa cccaggaggc agaagttgca gtaagccgag atggtgccac tgcactccag
1681 cctgggtgac agagcgagac tccatctcac aaacaaaaaa caaaaaacaa aaaaaacccc
1741 acatatttag taagcacttc ctggtactag gtgaaccaga cggattggat cccagccttt
1801 ccccagcccg tagccccggg acctccgcgg
```

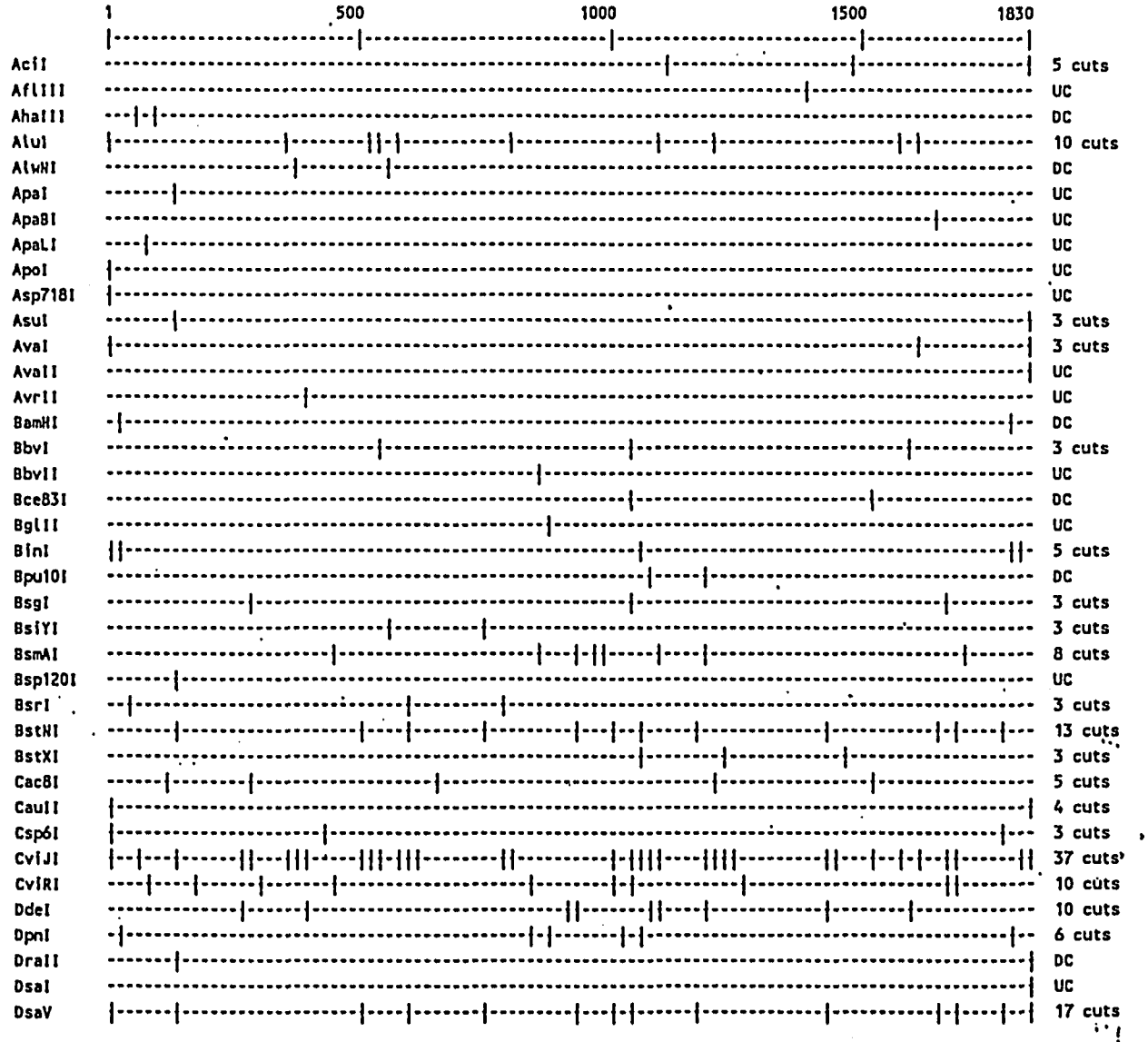
Appendix II

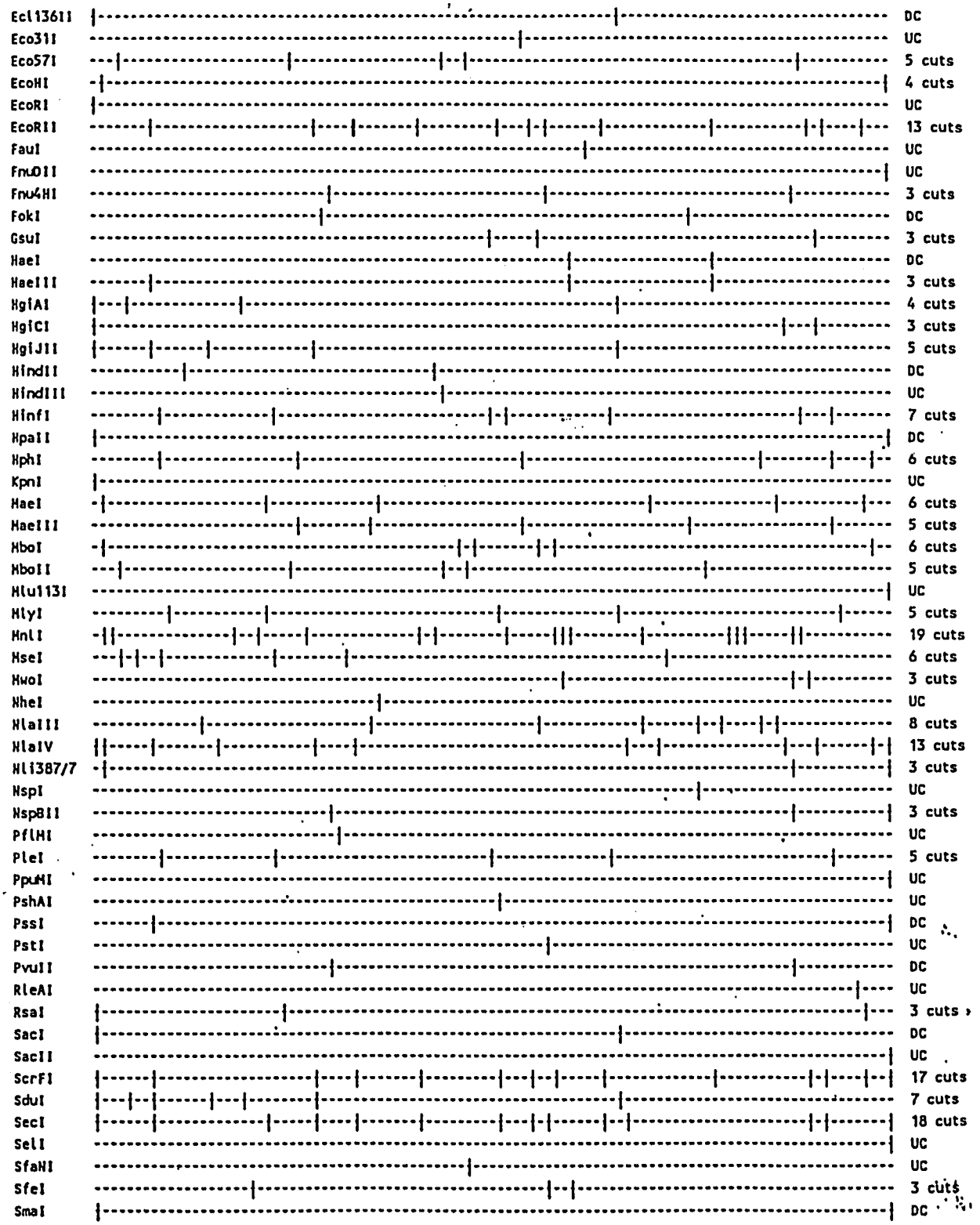
===22-JUN-1993==========PC/GENE===

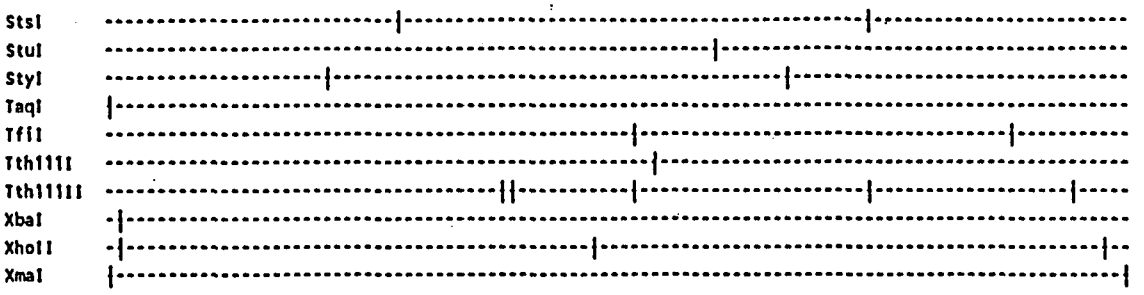
****
* SEMI-GRAPHICAL REPRESENTATION OF THE RESTRICTION MAPS *
****

Map for DNA sequence EP4K.

Analysis done on bases 1 to 1830.




Ecl13611 DC
Eco31I UC
EcoS7I 5 cuts
EcoHI 4 cuts
EcoRI UC
EcoRII 13 cuts
FauI UC
FmuDII UC
Fnu4HI 3 cuts
FokI DC
GsuI 3 cuts
HaeI DC
HaeIII 3 cuts
HgiAI 4 cuts
HgiCI 3 cuts
HgiJII 5 cuts
HindII DC
HindIII UC
HinfI 7 cuts
HpaII DC
HphI 6 cuts
KpnI UC
MaeI 6 cuts
MaeIII 5 cuts
MboI 6 cuts
MboII 5 cuts
Mlu1131 UC
MlyI 5 cuts
MnlI 19 cuts
MseI 6 cuts
MwoI 3 cuts
NheI UC
NlaIII 8 cuts
NlaIV 13 cuts
Nli3877 3 cuts
NspI UC
NspBII 3 cuts
PflMI UC
PleI 5 cuts
PpuMI UC
PshAI UC
PssI DC
PstI UC
PvuII DC
RleAI UC
RsaI 3 cuts
SacI DC
SacII UC
ScrFI 17 cuts
SduI 7 cuts
SecI 18 cuts
SelI UC
SfaNI UC
SfeI 3 cuts
SmaI DC



===22-JUN-1993==========PC/GENE===

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