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INTEGRATION OF SV40 PROMOTER/ENHANCER SEQUENCES IN AN
ANCHORAGE INDEPENDENT CLONAL SUBLINE OF SV40-INFECTED HUMAN
EPIDERMAL KERATINOCYTES AND ALTERATIONS IN THE VIRAL
SEQUENCES INDUCED BY N-METHYL-N'-NITRO-N-NITROSOGUANIDINE
(MNNG)

By

Guangtian Chen

A dissertation submitted to the Graduate Faculty in
Biochemistry in partial fulfillment of the requirements for
the degree of Doctor of philosophy, The City University of
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This manuscript has been read and accepted for the Graduate Faculty
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the degree of Doctor of Philosophy.

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Abstract

INTEGRATION OF SV40 PROMOTER/ENHANCER SEQUENCES IN AN ANCHORAGE INDEPENDENT CLONAL SUBLINE OF SV40-INFECTED HUMAN EPIDERMAL KERATINOCYTES AND ALTERATIONS IN THE VIRAL SEQUENCES INDUCED BY N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (MNNG)

by

Guangtian Chen

Advisor: Professor Mark L. Steinberg

The work described in the following pages was initiated with the goal of using SV40-immortalized human keratinocytes as a system to study molecular alterations in the DNA of cells exposed to the alkylating carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). For this purpose changes in the endogenous viral sequences in clonal sublines of SV40 transformed human keratinocytes which were exposed to MNNG at sublethal concentrations ranging up to 10 µg/ml for a period of 15 minutes were examined by Southern blot hybridization using full length SV40 DNA as a probe. Of the clonal sublines tested, one (AG34) was found to exhibit certain consistent, dose dependent changes at 4 days post

treatment: a) loss of at least two *EcoRI* fragments of about 4.4 and 3 kb, with the concomitant appearance of two bands migrating between 1.8 and 2.3 kb and, b) loss of a 1.5 kb fragment and the appearance of 2, 3.8 and 5 kb fragments in *Kpn I* digests. Minor variable changes in restriction patterns were seen at 24 hours post treatment but consistent and pronounced effects were observed only at 4 days post treatment. The altered restriction fragment patterns apparently indicate that MNNG causes highly specific rearrangements in some subset of the DNA sequences associated with the integrated SV40 sequences in human epithelial cells. This differs from what has been found for SV40 sequences in other cell lines where amplification has been reported. These results suggest that carcinogen treatment can result in the induction of a common genetic event throughout the entire population of exposed cells.

Since the Southern blot analyses had shown that the viral sequences in AG34 were completely contained on two *BamHI* fragments of approximately 5 and 7 kb in length, genomic libraries were created from *BamHI*-digested genomic DNA in lambda phage vectors in order to clone and then

sequence fragments containing SV40 sequences from MNNG-treated and untreated AG34 cells.

Sequence analysis revealed the larger fragment to be 6.93 kb in length and contained two subgenomic viral integrants: a) viral sequences 5051-1645 and 570 bp of 5' flanking human sequence were located at the right hand end of the fragment (nt 5056 - 6881) and, b) a central integrant containing a portion of this segment containing SV40 bases 5051-468 along with the leftward flanking human sequence was repeated with slight alterations in the same orientation at a location 934 bp upstream from the right hand integrant. Both integrants included the origin/promoter of the virus which contains the viral origin of replication, the early and late gene promoters and short segments of the early and late gene transcripts. A number of mutations within this region were observed: the central integrant contained base substitutions at sequences within T antigen binding sites 1 and 2 and base substitutions falling within the 72 bp repeat viral enhancer elements. Northern blot hybridization followed RT-PCR demonstrated that the early promoter in the central integrant but not the late promoter was functionally active and gave rise to a fusion transcript containing a

short segment of the viral early genes and the adjacent human sequences flanking the integration site. The 5.2 kb fragment was found to consist of full length *Bam*HI-linearized SV40 genomic DNA with numerous point mutations throughout the early gene region. There was a nine nucleotide insertion which results in an insertion of the tripeptide Pro-Ser-Gln at residue 702 of the T antigen polypeptide. A number of other nucleotide substitutions were also present in the late gene sequences, but none of those were predicted to result in alterations in the VP1, VP2 or VP3 polypeptides.

Sequence analysis of the corresponding fragments from the MNNG-treated cells revealed that of 22 nucleotide MNNG-induced alterations in a region of about 10 kb, eight were all located within a 36 bp region within the viral origin of replication; seven of these were G/C base pair substitutions or insertions and one was an A/T insertion. Interestingly, four of the eight alterations were clustered within the segment comprising the second T antigen binding site. The twelve alterations not found in the viral origin were single nucleotide substitutions, deletions or insertions scattered at random mainly throughout the flanking human sequences.

Dedicated to my parents for their sacrifices to provide me the best education as possible. Their love and encouragement will continue to inspire me in the future.

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INTRODUCTION

I. Simian Virus 40 and its roles in transformation

Simian Virus 40 is one of the members of the PaPoVa virus family. It was first discovered in the fluids of rhesus monkey kidney cultures (1) and was a contaminant in the Salk and Sabin poliovirus vaccines. It was found that the virus did not produce cytopathic effects in rhesus monkey kidney cells but produced cytopathic effects in kidney cell cultures derived from the African green monkey. Later, it was shown that the injection of the extract obtained from the rhesus monkey kidney cultures into newborn hamsters induced tumors (2). It was further confirmed that the tumor induction was due to the presence of SV40 virus (3). These discoveries stimulated the extensive studies on the virus since then (4).

1. Structural features of SV40 virus and viral genome

SV40 virus is an icosahedral shaped virus with a diameter of about 45 nm and a sedimentation coefficient of

240 S (3-7). The molecular weight of the SV40 virion is approximately 27×10^6 (8). Molecular characterization of the SV40 genome reveals that the genome consists of a covalently closed circular duplex DNA molecule of 5243 base pairs in length.

The SV40 genomic DNA contains two regions of approximately equal size that are functionally distinct, which are referred to as the "early region" and "late region" (Fig. 1). They are defined in terms of the order of being expressed either before or after the onset of viral DNA replication. The "early region" encodes two nonstructural proteins which are designated T-antigen and t-antigen. The "late region" encodes three structural proteins, VP1, VP2 and VP3 which comprise the viral capsid. The other late protein which is involved in virion assembly, LP-1, is encoded by an open reading frame overlapping with the 5' region of VP1 (9, 10).

2. Structural features and biochemical properties of SV40 T antigen

T-antigen is a nuclear phosphoprotein containing 708 amino acids. The antigen, serving as a multifunctional

regulatory protein determines the course of events in the productive viral infection of permissive cells and abortive infection of nonpermissive cells by controlling its own transcription and DNA synthesis, and the transcription of other viral mRNAs (Table 1).

(1) DNA binding/unwinding

SV40 T antigen binds to specific SV40 binding sites within the control region of the SV40 genome with high affinity (Fig. 2). The control region includes the promoter/enhancer elements and the origin of viral DNA replication with three T antigen binding sites: site I, site II and site III. Site I contains two important GAGGC elements separated by a seven bp AT-rich spacer. SV40 T antigen binds to this 17-bp sequence forming a complex covering 5-7 bp on either side of this core element. The binding of SV40 T antigen to Site I appears to repress early gene transcription (11, 12, 13).

Site II contains four GAGGC elements which are organized as two pairs in opposite orientations, forming a perfect palindrome of 27 bp. Site II, with an imperfect

palindrome on its upstream side and a 17 bp AT-rich stretch on its downstream side forms a 64 bp "minimal" origin of viral replication. Mutations within this region lead to the loss of viral replication (14-17). It has been shown that binding of T antigen to the GAGGC pentanucleotide in site II results in proper location and orientation of T antigen on the origin. It has also been shown that binding of ATP to T antigen increases the binding affinity of T antigen to the origin region. It seems that ATP induces the conformational change within T antigen that stabilize its binding to the binding sites. However, hydrolysis of ATP is not required in this process. Studies of the protein-DNA complex at the origin showed that T antigen assembles into a two-lobed structure, each lobe containing a T antigen hexamer, and the double strands at the origin region are partially separated (18-20). The hydrolysis of bound ATP by the intrinsic ATPase activity within T antigen causes further separation of the double strands at the origin region. Driven by the DNA helicase activity of T antigen, T antigen moves in the 3' to 5' direction on the DNA strands which results in the separation of double strands (21-22).

The exact function of site III has not been determined. In vivo, the binding of T antigen to site III may involve in the interaction of host proteins either with T antigen or with site III (23).

(2) Functional domains within T antigen

There are several domains within T antigen associating with its various regulatory functions. There are DNA binding sites which bind to double stranded DNA to initiate viral DNA synthesis; intrinsic ATPase activity which hydrolyzes ATP during DNA replication; a nuclear localization signal which enables T antigen to translocate from cytoplasm to the nucleus; The Zine-finger motif responsible for protein-protein interaction; a DNA polymerase α -primase binding site which positions the enzyme at the origin region with a correct orientation for DNA replication; a binding site for the tumor suppressor protein p53; and a binding site for the retinoblastoma suppressor protein Rb. These functions are needed for its regulatory functions in viral infection of permissive cells as well as in transformation of nonpermissive cells (Table 2):

Surprisingly, the biochemical activities associated with its replication function are not involved in its ability to transform cells. Studies using deletion and mutation analysis within the early gene region showed that the functions of replication and transformation of T-antigen are genetically separate(24-27). Biochemical analysis of these mutants demonstrated that the ability to bind to the SV40 origin of replication, the ability to hydrolyze ATP and the helicase activity of SV40 T-antigen were necessary for the viral genome replication, but not for T-antigen mediated transformation (28-30).

3. T antigen in viral DNA replication

T antigen is the only viral protein required for SV40 viral DNA replication in combination with the host replication machinery. Similar to the mammalian DNA replication, the viral genome replication starts at a well defined origin, and proceeds in a bidirectional and semidiscontinuous manner (31-34). The initiation of viral replication requires the formation of a double hexamer of T antigen which complexes with the specific sequences within

the origin region. The subsequent binding of the DNA single strand binding protein (SSB), binding of ATP to the ATP binding domain on T antigen and the activities of intrinsic ATPase within T antigen are also required for further unwinding of the strands at the origin region by the T antigen. Binding of DNA polymerase α -primase to T antigen finally forms an active initiation complex for DNA replication (35-37).

4. Regulatory activities of T antigen by phosphorylation

The transcription of T-antigen and other viral mRNAs as well as replication of viral DNA are regulated by the dynamic balance between unphosphorylated and phosphorylated forms of certain amino residues at the N and C termini of the T-antigen.

Dephosphorylation of Ser-120, Ser-123, Ser-677 and Ser-679 is a process of rapid turnover of phosphate in infected cells, which increases T antigen's ability to activate the initiation of SV40 replication (38). The activation seems to be the result of cooperation of protein-protein interaction between the double hexamers of T antigen at the origin. At

least one study has shown that highly phosphorylated T antigen was unable to bind to the origin and therefore was incapable of unwinding DNA at the replication origin(39). Studies in vitro and vivo showed that PP2A, the host origin protein phosphatase, may play a role in dephosphorylation of Ser residues (38, 40). It was also revealed that PP2A may be involved in activation of SV40 DNA replication in the S phase since the combination of extracts obtained from cells in the G1 phase with PP2A stimulated SV40 replication to a level close to that observed with extract from S phase cells (40).

Phosphorylation of Thr-124 of T antigen, however regulates the activity of T antigen in a positive manner. Substitution of Ala for Thr-124 of T antigen weakens its ability to bind to Site II in the SV40 origin and creates a defect in the protein's ability to initiate the SV40 replication (41). Moreover, T antigen expressed in bacteria which was not modified at Thr-124 was unable to induce initiation of SV40 DNA replication until it was incubated with a cell cycle-related protein kinase cdc2 (42), which has been shown to exhibit an ability to phosphorylate Thr-124 (42, 43). It was also shown that the phosphorylation of

Thr-124 on T antigen occurred shortly after it was synthesized in the infected cell (44, 45). Furthermore, the turnover of phosphate on Thr-124 is much slower than that for Ser residues (46). It was concluded from the above evidence that phosphorylation of Thr-124 is required for T antigen to activate SV40 DNA replication. The phosphorylation may be responsible for the S phase specific replication of SV40 DNA in monkey cells(42, 47).

A model has been proposed for the mechanism of phosphorylation of T antigen in activation of DNA replication. In cells newly infected by SV40 virus, a number of serine residues on T-antigen are phosphorylated, but not at Thr-124 which renders the T antigen defective for initiating viral DNA synthesis, but partially active in autoregulation of early gene transcription. A small fraction of the T-antigen binds to p53 and Rb which is a tumor suppressor protein, leading to alteration of their functions so that the cell switches from the resting G1 phase to the growing S phase and the host cell then progresses through the cell cycle; the active forms of *cdc2* kinase and PP2A are generated, which can add the activating phosphate at Thr-124 in T-antigen and remove the suppressing serine

phosphate(s), respectively. This altered form of T-antigen is then competent to initiate DNA replication (47, 48).

The ATP binding and helicase activities of T antigen are not affected by the phosphorylation and dephosphorylation (41, 49, 50, 51).

5. T antigen and transcriptional regulation

It has been shown that large T antigen not only participates in viral DNA synthesis but modulates both SV40 early and late gene transcription, and trans-activates other viral and cellular genes (52-63).

The SV40 early promoter/enhancer region is composed of three key elements: a TATA box-like sequence ATAAAT, two identical 21-bp repeats and two identical 72-bp repeats (Fig. 3). The sequence ATAAAT is involved in the control of SV40 DNA replication and early transcription. It is located at nucleotides 15-20 and is about 30 nucleotides upstream from the early RNA transcription initiation site (64). This TATA box of the early SV40 promoter seems to play a role in fixing the RNA initiation sites to a narrow region (65). Two

other sequence domains also function to regulate early transcription as transcriptional enhancers.

The two identical 21-bp repeats with one homologous 21-bp repeat are located at nucleotides 40-60, 62-82 and 83-103, respectively. All three repeats contain six GC-rich motifs (GGCGGG). The 21-bp repeats may regulate early RNA synthesis by serving as binding sites for the general transcription factor, SP1, of which binding facilitates the binding of RNA polymerase to the early gene promoter sequences, in an orientation-independent fashion (66, 67, 68-71). However, the transcription of early RNA is affected by the distances between these elements and the TATA sequence (72).

The 72-bp repeats are located at nucleotides 107-178 and 179-250 , and are necessary for both early and late transcription. They also confer the cell-type specificity to SV40 transforming activities. Although the enhancer is active in a wide range of cell types, it exhibits maximum activity in its normal host cells or closely related cells. For example, the enhancer functions with higher activity in primate cells than in murine, rat or human cells (73-77).

Previous studies showed that the enhancer sequences serve as the entry site(s) for RNA polymerase and /or transcription factors (78-80). Enhancers control transcription by interacting with cellular factors and this interaction facilitates the binding of RNA polymerase to the promoter which is linked to the enhancer. The interaction of transcription factors with the enhancers ultimately facilitates the formation of the transcriptional complex (81-83).

T antigen at low levels seems to activate the transcription of SV40 early genes (84, 85), while at high levels it suppresses transcription. The binding of T antigen to the promoter which interferes with RNA polymerase binding or progression is partially responsible for the suppression function(12, 13, 86). An additional mechanism required in order for T antigen to repress early gene transcription is the specific interaction between the antigen and the enhancer-binding protein, AP2. The binding of AP2 to the early promoter/enhancer region is required for the full expression of the early genes. T antigen inhibits early transcription by forming T antigen-AP2 complex which

prevents AP2 from being available to the promoter/enhancer region (87).

A shift from early gene transcription to late gene transcription occurs when SV40 DNA replication starts in permissive cells. This shift is accomplished when viral DNA replication changes the structure of the late gene promoter/enhancer region which thereby increases the accessibility to the proteins required for the late gene transcription.

T antigen not only can activate viral early and late transcription but trans-activates other cellular proteins which makes it function as an oncogene in a wider range of host cells. It activates some cellular genes by direct interaction with the promoter/enhancer region of these genes (61, 62). It has also been shown that T antigen activates some genes through interaction with transcription factors which in turn activates the genes which these factors are specific for (57, 65).

6. T antigen in transformation

There are three regions which have been shown to be the transforming domains required for immortalization and transformation of human cells, [A, B and D as listed in Table 2 & Fig. 4 (85)]. Region A is the N-terminal segment containing amino acid residues 1-82 and which binds to cellular protein, p300; Region B is also in the N-terminal segment covering amino acid residues 101-118 and which binds to pRb; Region D is in the central portion of T antigen covering as amino acid residues 351-450 and 533-626 which binds p53, another tumor suppressor protein. The T antigen binding of P53, pRb and p300 play an important role in transformation of SV40 virus infected cells.

(1) T antigen interacting with p53

The p53 tumor suppresser gene was first discovered as a component of complexes containing the oncoproteins of DNA viruses (89, 90); the formation of complexes was found to bring about a dramatic increase in the half life of p53, normally only several minutes long (91, 92). Because cloned p53 sequences were also found to produce immortalization and other properties of transformation including tumorigenicity

when introduced into rodent cells either alone or in concert with known oncogenes such as ras (93-96), p53 was widely presumed to be an oncogene. However, subsequent studies showed that the p53 sequences exhibiting oncogenic properties were actually mutant genes and that wild type p53 actually suppressed growth in the G1 phase of the cell cycle when introduced into transformed cells (97-101). p53 is known to form specific complexes with the proteins of at least four oncogenic DNA viruses: SV40 (SV40 large T antigen), adenovirus (Ad5 E1B), Epstein-Barr virus (EBNA-5) and human papillomaviruses 16 and 18 (E6 early proteins). The oncoproteins of these viruses are thought to abrogate growth control in the cells they infect at least in part by interfering with p53-mediated cell cycle control thereby allowing DNA synthesis and cell division to ensue.

More recent studies have shown that p53 may play a role in a wide variety of cellular functions including transcriptional activation. Wild type p53 has been shown to bind to regions at, or near the TATA box motif either directly or as part of the TATA transcriptional complex where it might function either to activate or repress transcription (102-105). However, p53 does function as a

transcriptional activator in genes whose regulatory sequences contain the p53 consensus binding motif (106). In fact, p53 has been found in association with a number of factors involved in both transcription (CCAAT binding factor, Sp1 transcription factor, TATA binding factor) and DNA replication (replication protein A) and other functions (heat shock protein 70), (summarized in 107). Thus, unlike other genes whose function(s) remain obscure, p53 presents researchers with an overwhelming array of possible functions.

Whatever the critical function(s) of p53 in cellular transformation turn out to be, it cannot be doubted that alterations of these functions are pervasive in cancer. Mutations in p53 are more commonly associated with human cancers than is true for almost any other oncogene (108). A survey of the recent literature shows that p53 mutations are implicated in a vast array of cancers including those of skin and other epithelia (109-112), ovary and cervix (113-115), thyroid (116), breast (117-118), pancreas (120), stomach (121, 122) and prostate (123-125). The nature of the mutations found in cancers from different tissues or even different cancers of the same tissue varies widely.

However, it has been observed that most mutations in human cancers are found within one of four conserved regions of p53 (126) suggesting that neoplastic transformation does indeed result from disruption of a critical p53 function.

The association between p53 and SV40 large T antigen is one of the earliest observations to implicate p53 in transformation. Both p53 and SV40 T antigen are phosphoproteins and phosphorylation of both proteins at critical serine residues appears to be necessary for complex formation which occurs shortly after transport into the nucleus (reviewed in 47, 127). The T antigen region necessary for p53 binding has been mapped by various types of directed mutation procedures to a large region covering amino acid residues 337-672 (the coding regions within the segment spanning SV40 bases 1442-2462:47,127). Since this region also contains the T antigen binding sites for DNA polymerase alpha and ATP, it has been suggested that complex formation with wild type p53 blocks T antigen functions associated with DNA replication. On the other hand, it is possible that T antigen may, instead, modulate one or more of the various p53 functions described above. Since definitive experimental evidence is so far lacking, the

question remains as to which of the two molecules is the effector in the complex and what function(s) of the target molecule might be modulated as a result of complex formation. In a separate line of research, Bargonetti et al. (128), using purified p53 in DNase I footprinting experiments showed that wild-type, but not mutant, p53 formed tight complexes with SV40 DNA at a region around the SV40 origin region. The protected region spanned SV40 bases 37-151 which includes both the 21 and 72 base pair enhancers. It may be concluded that T antigen, interacting with p53 can offset the suppressing activity of p53 imposed on the proliferative potential of cells.

(2) T antigen interacting with pRb

pRb is a nuclear protein with a negative regulatory effect on cell growth. The mutations of this protein such as deletion and rearrangement are associated with human retinoblastoma (126-131). pRb has been shown to be the target of several DNA transforming viruses and is bound by SV40 T-antigen (132), polyoma T-antigen (133), adenovirus E1A protein (134), and human papilloma virus E7 protein (135).

The expression of pRb in a variety of transformed cell lines results in growth suppression and decreases in tumorigenicity (136-139). Both retinoblastoma and osteosarcoma cell lines in cell culture do not readily tolerate wild-type pRb protein expression (136) while bladder and prostate cell lines do (137-139). However, all of these cell lines show reduced tumorigenicity.

Mutant pRb proteins share some properties with p53 in that the mutants do not bind to SV40 T-antigen (140-142), and are not phosphorylated in a cell-cycle dependent manner as wild-type pRb is. It was reported that SV40 T-antigen bound only to the hypophosphorylated form of pRb (143) and that pRb is phosphorylated in a cell cycle-dependent manner (144-148). Since only the hypophosphorylated form of pRb is targeted by both SV40 T-antigen and human papillomavirus E7 protein (143, 149), it has been suggested that the hypophosphorylated form of pRb is the active form, which has a negative growth effect in the G₁ phase of cell cycle. The phosphorylation of pRb can result in the progression of cells into S phase. Microinjection of pRb into the cells which have been synchronized in the G₁ phase will arrest these cells prior to the onset of S phase (150).

Microinjection of c-myc can reverse the cell arrest induced by pRb (151).

Biochemical studies indicate that expression of pRb represses or activates different promoter elements (152, 153). pRb complexes with the cellular transcriptional factors DRTF1 and E2F and the complex formation may play a role in the transcriptional regulatory activity of pRb (154-159). TGF- β downregulates c-myc transcription and this effect can be overcome by expression of E1A and T-antigen which can bind pRb (160). TGF- β arrests cells in the G1 phase and prevents the phosphorylation of pRb. Expression of SV40 T-antigen overcomes this arrest without affecting the phosphorylation state of pRb (161).

The binding site on SV40 T-antigen for pRb has been identified as a motif (LXCXE) and found to be an Rb-binding motif also present in adenovirus E1A and the human papillomavirus E7 protein (135). E2F also binds to pRb but not on this motif. It has been shown that SV40 T-antigen, E1A and E7 protein inhibit the binding of E2F to pRb. Apparently, SV40 T-antigen, E1A and E7 interfere with the E2F-pRb interaction not by direct competition for the same binding site but by some steric effect(s) (162, 163).

It was shown that pRb can inhibit replication of SV40 origin-containing DNA in cells (164) and inhibit SV40 T-antigen from stimulating both the polymerase and primase activities of the DNA polymerase/primase complex (165). In SV40-infected cells, T-antigen can associate with p53 and pRb into a trimolecular complex (141).

It may be concluded from the above evidence that binding of pRb with transcription factors results in growth arrest; and hyperphosphorylation of pRB and complex formation with T antigen can disassociate pRB from the bound transcription factor(s) which releases cells from growth arrest.

(3) T antigen interacting with p300

Little is known about p300. It is reported that p300 is an adenovirus E1A binding protein (166). p300 has been shown to co-immunoprecipitate with TATA-binding protein TBP (167); a monoclonal antibody study implies that p300 shares sequence homology with both pRb and p107 since monoclonal antibodies cross-react with all three proteins (168); p300 is thought to possibly play a role in the regulation of

cellular DNA synthesis (169). the role of p300 in binding to SV40 T antigen is not yet clear.

7. Transformation of human keratinocytes by SV40

More than 80% of all human tumors are of epithelial origin. Early studies of transformation in vitro have largely relied on rodent fibroblasts for which a limited number of transformation markers are available. Our laboratory was the first to demonstrate and characterize the transformation of human epithelial cells (keratinocytes) by SV40 (170). In this system the virus initiates a process of transformation which progresses through distinct stages over a period of many months following infection (Fig. 5; 171-174). These stages can conveniently be divided into precrisis, crisis and post crisis stages(175). These stages are well defined in terms of a series of events displaying certain cytologic and histochemical features.

Precrisis - In this early stage, breakdown of junctional communication is detected by transfer of microinjected fluorescent dye (175, 176), and clonal growth in the absence of feeder layers (170-172) and loss of

histochemical and cornified cell envelope markers of terminal differentiation (170-172) are other 'early' events in the transformation scheme. 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 period when cells enter crisis (60-90 cell generations post infection; 177). Redistribution of fibronectin cables over the cell surface occurs at about the same time (171, 178). Growth dependence on high serum concentrations and anchorage to solid substrates are indicators commonly used as markers of transformation in fibroblasts. In epithelial cells these markers are temporally dissociated from one another. Growth independence from high serum concentrations is manifest quite early i.e. within the first four passages post infection (about 40 cell generations) but anchorage independence is never seen until permanent cell lines are established in the period following crisis (>10 serial passages; (170-172).

Crisis - The initial period of viral-induced growth stimulation of keratinocytes is almost invariably followed

by a period of growth crisis in which growth slows and cells exhibit a characteristic type of cytopathic effect (CPE) involving gross enlargement, vacuolization and detachment of the affected cells from the culture substrate. Human fibroblasts infected by SV40 have also been shown to go through a period of crisis prior to the establishment of cell lines (179). The severity of the crisis has been found to vary widely from one cell line to the next. In its most severe form, the cells are progressively, and completely, destroyed within a period of about ten days following the first signs of CPE. Interestingly, when the number of cell divisions is carefully counted, the onset of the crisis period appears to occur after approximately the same number of cell generations (Fig. 5) as would precede senescence in uninfected cultures suggesting that crisis may be the manifestation of normal senescence in an abortively transformed subpopulation of cells. The crisis period represents an important demarcation between the early and late phases of the transformation process; a number of properties associated with the transformed phenotype including clonal growth appear only after crisis (Fig. 5).

The surviving cells are then immortalized and go on to form continuous cell lines.

Post crisis - Upon entering the postcrisis stage, viral infected keratinocytes acquire the capability to form colonies from single cells on soft agar. In addition to anchorage independent growth, the markers of the late phase of the transformation process include altered actin and intermediate filament cytoskeletal elements (171, 173) and formation of undifferentiated carcinoma-like tumors in nude mice (174). It has been shown that the expression of the keratin intermediate filament genes as molecular markers of differentiation is dramatically altered by viral infection (173, 174, 177, 180, 181). Viral-dependent alteration of transcription of the keratins including ectopic expression of genes coding for simple epithelial/fetal keratins and induction of one uncatalogued keratin or keratin-associated protein have also been demonstrated (173, 181). The significance of these findings stems from the clinical observation that the expression of fetal keratins accompanies malignant transformation of epithelial cells and is now widely used as a tool in tumor diagnosis (182-186).

The fact that the transformed phenotype emerges in discernable stages over a long period of time following infection indicates that oncogenic events take place substantially subsequent to viral infection. It has been observed in our laboratory that the viral sequences in the infected cells, while initially present as full length unintegrated viral genomes at high copy number, are ultimately retained as integrated viral sequences in low copy number. Because the cell population is constantly undergoing growth selection in culture, we consider the acquired mutations as having been selected for on the basis of conferring a growth advantage(s) to the cells which contain them. On the other hand, the cells never become cured of the viral sequences so it appears that continued growth in vitro remains dependent on the viral genes.

Based on the paradigm of multistage carcinogenesis, the initiating event in the transformation process is not enough to transform cells. Besides the function of T antigen, the integration of SV40 viral sequences may indicate that necessary genetic alterations in the viral oncogens or other events are the subsequent events responsible for complete transformation of the cells. Therefore, it would be

worthwhile to look into the structural features of the integrated viral sequences as well as their flanking human fragments as the site(s) of such alteration.

In the work presented here, the integrated viral sequences with the human flanking fragments were cloned and sequenced from anchorage independent clonal subline Ag34, which was derived from an immortalized keratinocyte cell line. The sequence structure of the integrated viral fragments and the alterations in viral sequences which are selectively retained in the transformed cells over long term culture were analyzed.

II. Chemical carcinogens

Many chemicals have been identified as carcinogens. They are classified into several groups such as polycyclic aromatic hydrocarbons, aromatic amines, halohydrocarbons, nitrosamines, plant products and microbial products based on their structures or origins. The common property of all the different types of chemical carcinogens is that they can form directly, or be metabolized to reactive electrophiles. Most of them are chemically unreactive and need metabolic

activation to ultimately become carcinogens. When these procarcinogens go through the metabolic process, they can end up to be either in the active form as ultimate carcinogens or as inactive detoxification products. The balance between the metabolic activation and inactivation will determine the potency of a chemical carcinogen (187). However, the direct-acting carcinogens are intrinsically electrophilic and need not to be metabolized. These electrophiles can attack the electron-rich species such as oxygen, nitrogen, or sulfur atoms in DNA, RNA or proteins. The attacks are non-enzymatic reactions, and therefore, they are not specific. Once the DNA is attacked, the damage is irreversible and heritable. The cells then become initiated.

Chemical carcinogens interact with DNA in target cells to form adducts by covalent binding. The adducts can interfere with the hydrogen-bonding between the base pairs of DNA strands, which causes miscoding. The presence of carcinogens in the other sites on DNA can also cause miscoding by distorting DNA which affects the fidelity of DNA replication. The damage in DNA is inherited if the damaged DNA replicates before the repair occurs. The cells carrying the damage become initiated cells. Many tumors

produced in animals after being treated with chemical carcinogens are activated by point mutations, deletions or rearrangements in DNA (188). A well characterized example is the activation of the *ras* family of oncogenes (189). In this study, rats treated with NMU developed carcinomas at high frequency. Analysis of DNA extracted from the tumors revealed that the normal guanine base in the 12th codon was replaced by adenine in all NMU-induced tumors.

1. MNNG

Nitrosamines are indirect-acting carcinogens which are transformed directly into electrophilic alkylating agents by chemical reactions at physiological pH with nucleophiles such as water or thiols (190). One of the representative agents in this group is *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). MNNG results in the formation of a structurally diverse population of alkyl-DNA adducts (191, 192). During DNA replication in mammalian cells, the major lesion, *O*⁶-methyl guanine (*O*⁶MeG), mispairs with thymine and gives rise to a G-C to A-T transition (Fig. 6) and the less frequent lesion, *O*⁴-methylthymine (*O*⁴Met), can mispair with guanine

and results in an A-T to G-C transition (193). In *Escherichia coli*, the same transitions have been observed (194). The molecular mutagenic specificity of MNNG has been studied by several groups in bacteria. MNNG was found to specifically induce G-C to A-T transitions (>97%) in the *lacI* gene of *E. coli* (195, 196) as well as in the *SUP4-o* gene of *Saccharomyces cerevisiae* (197). In one study using human cells, the treatment of cells with MNNG produced a wide spectrum of mutation. The results showed that in both high dose and low dose groups one or more exons were lacking. In the high dose group, the substitutions were G-C to A-T transitions and all of them were located on the non-transcribed strand implying that the formation of O⁶MeG was the intermediate leading to mutagenesis. On the other hand, the majority of base substitutions in the low dose group were A-T to G-C transitions and showed no strand specificity indicating that the intermediate was O⁴MeT (198).

2. The effects of MNNG on integrated SV40 viral sequences in SV40 transformed cells

The mechanisms by which mutagenic chemical and physical agents interact with specific intracellular targets to induce expression of the transformed phenotype have been studied by a wide variety of experimental approaches. Selective amplification of DNA in response to genotoxic stimuli is thought to represent an important consequence of such interactions which can be studied at the molecular level (199, 200). Amplification of integrated SV40 sequences in Chinese hamster cells exposed to mutagenic and carcinogenic agents has been studied with a view towards utilizing the viral sequences as an endogenous genetic probe to elucidate these mechanisms (201-204). In these previous experiments, MNNG caused amplification of integrated SV40 sequences and the adjacent host sequences as well as the host sequences not associated with viral inserts in Chinese hamster embryo cells. These studies suggested that carcinogen-mediated activation of viral replicons was the event that controlled chemical-viral carcinogenesis (205).

In our laboratory, a model experimental system using SV40 transformed human epithelial cells was used to extend

the results of these earlier studies because this system is more relevant to human cancer. For this purpose we have employed lines of SV40-transformed human epidermal keratinocytes which have been extensively characterized in our laboratory over the recent years (170-172, 174, 181, 206). As an additional advantage we have been able to isolate a number of stable clonal sublines containing both integrated and nonintegrated viral sequences in various configurations (171, 206). It was our expectation that different sublines might respond differently to exposure to a given mutagenic agent with respect to amplification of the viral sequences and that these differences might then be related to the integrated state of the viral DNA.

MATERIALS AND METHODS

1. Cell Culture

SV40 transformed human epidermal keratinocytes (SVEK; line 425 at the 12th passage post infection) were cultured in Dulbecco's minimal essential medium plus 10% fetal calf serum and 0.5 $\mu\text{g/ml}$ hydrocortisone as previously described (170-172, 181). Normal human epidermal keratinocytes (NHEK) were grown from newborn foreskin explant cultures and maintained in Dulbecco's minimal essential medium plus 10% fetal calf serum, 0.5 $\mu\text{g/ml}$ hydrocortisone, 30 $\mu\text{g/ml}$ epidermal growth factor and 50 $\mu\text{g/ml}$ cholera toxin.

2. MNNG Treatment

MNNG was dissolved in dimethyl sulfoxide (DMSO) and cells were exposed to MNNG at the indicated concentrations in serum free culture medium for a period of 15 minutes at 37°C after which cell monolayers were rinsed with serum free medium and then incubated in standard medium until cells were harvested for DNA extraction. DMSO was always present

at concentrations of less than 0.1%; controls were incubated in the presence of DMSO alone.

3. Cell Growth measurements

Cells were seeded into small plastic tissue culture flasks (Falcon Plastics; 25 cm²) in triplicate and then subjected to MNNG treatment on the following day as described above. Cell numbers on days subsequent to plating were determined from randomly chosen microscope fields as described previously (170-172, 181).

4. Plating Efficiency/Clonal growth

For plating efficiency replicate cultures of AG34 cells grown in 1 cm diameter wells were treated with MNNG as described above and then immediately trypsinized until cells were completely dispersed. Cells recovered by centrifugation were replated into 60 mm plastic culture dishes (20 cm² growth area) in triplicate at a seeding density of 10⁴ cells/plate. Seven days later colonies were counted in randomly chosen microscope fields and the total

number of colonies/plate was determined from the microscope field area as described (176).

5. 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 Diamine Tetra acetate]; and 0.5% SDS [Sodium Dodecyl Sulfate]) with 500 μ g of proteinase K was added to each plate and the slurry was incubated at 37°C overnight. The plates were scraped gently and the DNA suspension was placed in a 50 ml centrifuge tube. An equal volume of phenol/chloroform-isoamyl alcohol (24:1) in a ratio of 1:1 was then added to each tube. The 50 ml tube was then placed on a rotator and mixed gently for 10 minutes and then centrifuged at 3k rpm for 10 minutes. The supernatant was recovered and the phenol/chloroform-isoamyl alcohol extraction was repeated. DNA was precipitated with two volumes of ethanol, the precipitate was 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

the DNA was dissolved at 37°C overnight. RNase A was added to a final concentration of 10 µg/ml and incubated for an additional 30 minutes. 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 of the genomic DNAs was digested with restriction enzymes according to the supplier's recommendations, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose by passive blotting. Filters were hybridized to a nick translated, ³²P-labeled probe made from full length SV40 DNA. Filters were prehybridized for 1 hr at 42°C in 50% formamide, 0.1% PVP (Polyvinylpyrrolidone), 0.1% Ficoll, 5x SSPE (Saline, Sodium Phosphate, EDTA), 0.1% BSA (Bovine Serum Albumin), and sheared denatured salmon sperm DNA at 100 µg/ml. Hybridization was carried out overnight at 42°C in 50% formamide, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, 5x SSPE, salmon sperm nonspecific DNA at 100 µg/ml and ³²P-labeled probe at a specific activity of approximately 10⁸ cpm/µg of DNA. Filters were washed for 20 minutes twice at 50°C in 0.1x SSC (sodium chloride, sodium

citrate)/0.1% SDS (sodium dodecyl sulfate), and then in 0.1x SSC for another 20 min.

6. Isolation of Total RNA and Northern Blot Analyses

For the isolation of cytoplasmic RNA, 10^8 cultured cells were washed twice with 1x cold PBS (phosphate-buffered saline) and then scraped into a centrifuge tube containing 5 ml of 1x PBS using a rubber policeman. The cell suspensions were pooled in 50 ml Falcon tubes and spun at $2,000 \times g$ for 5 min. Cell pellets were suspended 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 μ l of 2M sodium acetate buffer (pH 4.2) and 0.5 ml of water-saturated phenol was added and mixed. 100 μ l of chloroform was added and the mixture was vortexed. The aqueous and organic phases were separated by centrifugation at $12,000 \times g$ for 20 min. at 4°C . RNA was ethanol precipitated from the aqueous phase, the pellet was collected by centrifugation and the pellet was washed with 70% ethanol, dried and then dissolved in water. For

northern blot analysis, 30 μ g each of cytoplasmic RNAs were dissolved in 20 μ l of 1x running buffer (50% Glycerol; 1 mM EDTA (pH 8.0); 0.25% bromophenol blue and 0.25% xylene cyanol). The RNAs were heated for 5 min. at 65°C and electrophoresed in 1% agarose gels containing 20 mM morpholinopropanesulphonic acid (MOPS), 1 mM EDTA, 5 mM NaAc and 2.2M formaldehyde. The RNAs were then transferred to nitrocellulose filter in 10x SSC. The filter was air dried and baked for 2 h in a vacuum oven at 80°C. Prehybridization was for 2 hr at 42°C in 2x PIPES buffer, 50% deionized formamide, 0.5% SDS, and 100 μ g/ml denatured salmon sperm DNA. Hybridization was carried out in the same solution overnight with a ³²P-labeled probe at 42°C. The final wash was as described for Southern hybridization.

7. Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT - PCR was carried out using total RNA isolated from

AG34 cells as described above. Primer 74L (5' CTCTGCCTGATTTTCATT 3') which covers nucleotides 667->651 bp upstream from the virus/host junction of the central integrant on the 7 kilobase *Bam*HI fragment was used for

first strand cDNA synthesis using avian myeloblastosis virus (AMV) reverse transcriptase under conditions recommended by the supplier (Boehringer-Mannheim Biochemicals). The polymerase chain reaction (PCR) was carried out using Taq polymerase (Boehringer-Mannheim Biochemicals) under conditions recommended by the supplier. Amplification was carried out for 38 rounds (denaturation at 94°C for 1 min., annealing at 50°C for 1 min., primer extension at 68°C for 2 min.) using 74L and 97D-2 (5' ACAGAGAGGAATCTTTGCAGC 3') which covers SV40 map positions 5147-5127 (nucleotides 2966-2946 on the *Bam*HI fragment) as amplimers.

8. Construction of genomic libraries in EMBL 4 and isolation of SV40-containing recombinants by plaque hybridization

To clone the SV40-containing *Bam*HI restriction fragments, genomic DNA libraries were constructed using the lambda bacteriophage variant, EMBL4 (Promega Biotec). Ten micrograms of *Bam*HI digested AG34 DNA was purified by phenol-chloroform extraction and then ligated into *Bam*HI-digested EMBL4 bacteriophage vector DNA in a molar ratio of 3:1 (insert to vector). The ligation reaction was carried out with T4 ligase at 15°C for 24 hours. Following ligation

the mixture was removed and examined for completeness of ligation by gel electrophoresis. For ligation mixtures in which the accumulation of high molecular weight ligated material was observed, 4 μ l of the ligation mixture was used for packaging in vitro using the Gigapac Gold in vitro packaging extract system (Stratagene). The packaged recombinant bacteriophage was suspended in 500 μ l of sterile bacteriophage suspension buffer (SM; 100 mM NaCl; 17 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1 M Tris, (pH 7.5) and 2% Gelatin) followed by the addition of 30 μ l of chloroform. In order to screen the recombinant library for recombinant bacteriophage with inserts containing SV40 sequences, libraries were first plated onto E. Coli host strain NM539 (Promega Biotec), selective for growth of the recombinant. 200 μ l of a bacterial suspension in 10 mM MgSO_4 ($\text{OD}_{600} = 2.3$) was placed in a 15 ml centrifuge tube and mixed with 10-20 μ l of the packaged recombinants. The amount of packaged phage used for screening depends upon the titer (plaque-forming units; PFU) of the library as determined by plaque assay. Usually 10^4 plaques would be plated separately in a 150 mm diameter plate. The mixture of phage and bacteria was allowed to

incubate for 15 min. at 37°C before adding 3 ml of 0.75% top agar in NZYM medium (Sambrook et al.). The agar suspended bacteria with adsorbed phage was then spread evenly over a 1.5% NZYM bottom agar plate and incubated at 37°C overnight. For plaque hybridization, a nitrocellulose filter was placed on the plaque-containing agar plate. The filter's position was marked by making three asymmetric holes using an 18 gauge needle; the holes position were marked on the back of bottom plates with a felt-tipped marker. After the filter was completely wet, it was peeled off and was transferred to a strip of chromatography paper (Whatman 3MM) saturated with denaturing solution (0.5 M NaOH and 1.5 M NaCl), for 4 min. The filter was then transferred to a second 3 MM paper strip saturated with a neutralizing solution (1.5 M NaCl and 0.5 M Tris-Cl, pH 8.0), and left for 7 min. Two strips of 3MM chromatography paper were then used to sandwich the filter and this was baked for 2 hours at 80°C in a vacuum oven.

Approximately 5×10^5 recombinant phages, propagated on host NM539, were screened by plaque hybridization using a nick-translated ³²p-labeled probes. The solutions and conditions of hybridization and filter washes were the same as described for Southern blot hybridization. Regions of

plates identified as containing the putative recombinants were excised from the plate and phage was eluted from the excised agar plugs in SM and rescreened at low plaque density (~100 PFU/plate). After 2-3 rounds of screening, SV40-containing isolates were suspended in 1 ml of SM and grown up in large scale culture. For this purpose, 30 μ l of recombinant phage was mixed with 200 μ l NM539 and $\sim 10^3$ bacteriophage were plated per 100 mm plate in order to achieve complete lysis. Five milliliters of SM buffer was added to the plate the next day, and bacteriophage was eluted with light agitation on a rocker table for 2 hours at room temperature. The bacteriophage stock solution (10^{10} pfu/ml) was collected into a 15 ml centrifuge tube and stored at 4°C. For large scale isolation of cloned DNA, 200 μ l of the recombinant phage stock suspension was incubated with 5 ml of NM539 for 10 min. at 37°C. The mixture was then added to 500 ml of fresh NZYM medium and placed in a shaker overnight at 37°C.

Bacteriophage DNA was isolated by CsCl gradient centrifugation at 55,000 RPM in a Sorval TST 60.4 rotor for 16 hours at 22°C. The next day, the bacteriophage-

containing band was collected and dialyzed against 1000 ml of NTM buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8.0), and 10 mM MgCl₂) for 1 hour with one change of buffer. Then a mixture (20 mM EDTA, 50 mg/ml proteinase K, and 5% SDS) was added to the phage suspension, and then incubated for one hour at 68°C followed by extraction with an equal volume of phenol-chloroform. In order to confirm the presence of SV40 sequences in the recombinant, cloned insert DNAs were released from the recombinant isolate by *Bam*HI digestion, and then electrophoresed on a 1% agarose gel and hybridized to a nick translated ³²P-labeled SV40 probe.

9. Subcloning and Sequence Analysis

Ligation - 7 kb SV40 containing *Bam*HI fragment cloned in EMBL 4 were released by *Bam*HI digestion and purified by electrophoresis through low melting point agarose gels followed by phenol extraction. The fragments were then digested with *Eco*RI completely and subjected to heat treatment at 65°C to denature the restriction enzymes. Then the *Eco*RI digested subfragments were ligated into *Eco*RI and *Eco*RI/*Bam*HI digested M13 sequencing vector variants mp19 and

mp18 DNA in a molar ratio of 2:1 (insert to vector). The ligation reaction was carried out with T4 ligase at 15 °C for 24 hours. Following ligation the mixture was used to transform the competent cells of E. coli strain JM 101.

Preparation of competent cells for transformation - The competent cells were prepared by inoculating a single colony of this bacterial strain carrying an F' episome from minimal (M9) agar plate into 2 ml LB medium for 24 hours; at least 200 µl of the overnight culture was saved for the lawn cell culture and the rest of the overnight culture was diluted in 100 times into a prewarmed LB medium. The culture was incubated for 2-2.5 hours (OD 0.4-0.4 at 590 nm). The cells were harvested by centrifuged at 3k x g for 5 minutes at 4 °C and were gently resuspended in 1/2 x original volume of ice-cold 50 mM CaCl₂. The resuspended culture was incubated on ice for 40 minutes and centrifuged at 3k x g for 5 minutes at 4 °C. The cell pellet was very gently resuspended in 0.1 x original volume of ice cold 50 mM CaCl₂. 300 µl of the cell suspension was Aliquoted into sterile test tubes and maintain on ice (the cells may be stored on ice for up to 5 days although the best results are obtained when used immediately).

Transformation and plating - 5, 10, 50 and 100 ng of the DNA ligation reaction mixture was added to each 300 µl competent cell aliquots, respectively. The mixtures were incubated on ice for 30-40 minutes. During the waiting period, lawn cells were prepared by adding the 200 µl of overnight culture kept to 10 ml of prewarmed LB medium and incubating with shaking at 37 °C. The mixtures were heated shocked at 42 °C for 2 minutes. Each mixture was mixed with 200 µl lawn cells and 3 ml top agar (0.75% agar in LB medium prewarmed at 50 °C), and plated on B-broth plates. After sitting for 5 min., the plates were inverted and incubated at 37 °C for 16-18 hours.

Rapid Screening of recombinants - Single well-isolated plaques with smaller size (recombinants usually grow slower than the wild type) were picked and kept in 1 ml LB medium at 37 °C for 1 hour which allowed phages to diffuse into the medium. 100 µl of suspension was incubated with 2 ml of overnight culture 1:100 diluted in prewarmed LB medium at 37 °C with vigorous shaking for 5-8 hours.

The cells were pelleted and double stranded DNA was extracted by using alkaline method. 1 - 1.5 ml of the infected culture was transferred to a microfuge tube, and

centrifuged at 12,000 g for 5 minutes at room temperature in a microfuge. The supernatant with bacteriophage particles in each tube was transferred to a fresh tube and centrifuged again to remove bacterial pellet left. The supernatant was kept at 4°C for later isolation of single-stranded phage for sequencing. The pellets in each tube was centrifuged again and residual supernatant was removed without disturbing the pellet. The pellet was resuspended by vigorous vortexing in 100 µl of ice-cold Solution I [50 mM glucose, 10 mM EDTA (pH 8.0) and 25 mM Tris·Cl (pH 8.0)] in each tube and stored on ice for 5 minutes. 200 µl of freshly prepared Solution II (0.2N NaOH (freshly diluted from a 10 N stock) was added to each tube and the contents were mixed by inverting the tube rapidly five times. The tubes were stored on ice for 5 minutes. 150 µl of ice-cold Solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml H₂O) was added to each tube. The content in each tube was mixed by vortexing in an inverted position for 10 seconds. The tubes were then stored on ice for 5 minutes. The tubes were centrifuged at 12,000g for 5 minutes at 4 °C in a microfuge. The supernatant in each tube was transferred to a fresh tube. The pellet left were discarded. To each tube of

supernatant, an equal volume of phenol:chloroform was added and the content in each tube was mixed by vortexing. After the tubes were centrifuged at 12,000g for 2 minutes at room temperature, the supernatant in each tube was removed to a fresh tube. Two volume of 99% ethanol was added to the supernatant in each tube and mixed by vortexing. The double-stranded DNA in each tube was precipitated by standing the tube in room temperature (if RNase A was not to be used in the later stage, it would be better not to precipitate the DNA at -78 °C since substantial amount of RNA would be precipitated with the double-stranded DNA). The tubes were centrifuged at 12,000g for 5 minutes at 4 °C in a microfuge. The supernatant in each tube was removed by gentle aspiration and any drops of fluid adhering to the walls of tubes were removed. The pellet of double-stranded DNA in each tube was rinsed with 1 ml of 70% ethanol at 4 °C. the supernatant in each tube was removed completely. the pellet in each tube was dried in air for 10 minutes. 20 µl of H₂O was added to each tube the pellets were resuspended at 37 °C. The yield of DNA in each tube was about 10 µg by using this method.

4 μ l of DNA solution was used for each digestion with *EcoRI* or for double digestion with *EcoRI/BamHI*. The digests were run on 0.8% agarose gels stained with ethidium bromide. Restriction digests of DNA which produced appropriate sizes of inserts were run on agarose gel then subjected to Southern blot analysis to confirm these subfragments containing SV40 sequences.

Sequencing - 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.

10. 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.

RESULTS

1. Alterations in the viral sequences induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)

(1) Effect of MNNG on Cell Growth

To test the effects of MNNG on the growth of AG34 clonal subline after exposure to MNNG, cultures were treated with MNNG at 1 and 2 $\mu\text{g/ml}$ dissolved in 0.1% DMSO and cell counts were taken at 1, 2 and 4 days post-treatment. The results showed cultures treated only with 0.1% DMSO increased in cell number about 9 fold over the four day period while the corresponding increases for cultures treated with MNNG at 1 and 2 $\mu\text{g/ml}$ was 6.5 and 3 fold respectively (Fig. 7). To address the problem of possible selection, the toxicity of MNNG was measured by clonal survival and the results are shown in table 3. In this experiment, cultures were treated with MNNG as described in Materials and methods and immediately trypsinized and replated in triplicate at cell densities where colonies derived from single cells could be observed (clonal growth). It is shown that colony forming

efficiency of the cells from cultures treated with MNNG at 1 $\mu\text{g/ml}$ was still 69- 75% of that seen in control cultures. Exposure of AG34 cells to 2 $\mu\text{g/ml}$ MNNG substantially reduced the fraction of cells able to form colonies under stringent growth conditions. However, even under these conditions the subpopulation of colony forming cells was found to represent a minimum of 25-27% of control cultures.

(2) Amplification of SV40 Sequences

Several clonal sublines of SV40 transformed keratinocytes were examined for amplification of viral sequences following exposure of the cells to MNNG as had been reported earlier for SV40 transformed rodent fibroblasts (201-204). The lines used in these experiments were all derivatives of line 98 transformants obtained via repeated rounds of selection in soft agar. Patterns of restriction fragments containing integrated and unintegrated viral sequences in these lines have been described in previous reports (171, 206). Neither the unselected parental line nor any of four other clonal sublines tested showed significant amplification (2-3 fold) when cellular

DNAs from the treated cells were hybridized to an SV40 probe in dot blot assays. Only one subline (AG06) has been found to show 7 fold amplification at 2 µg/ml MNNG in response to MNNG treatment.

(3) Rearrangements Associated with SV40 Sequences

Altered patterns of SV40-containing restriction fragments were also analyzed in Southern blots following MNNG treatment. These experiments revealed that, in at least one subline (AG34), which had been found to contain integrated subgenomic viral fragments (171, 206) in low copy number (<10 copies/cell), treatment with MNNG resulted in the disappearance of certain restriction fragments concomitant with the appearance of novel fragments detected by the SV40 probe (Fig. 8: 207). Figure 8 shows that *EcoRI* digests of DNA isolated from Ag34 at 4 days after exposure of the cells to DMSO contained at least four SV40 containing restriction fragments of about 5.5, 5, 4.3 and 3 kb. Since there is only a single *EcoRI* restriction site on SV40 most, if not all, of these fragments would be expected to represent truncated or rearranged SV40 sequences. A

detailed description of the viral fragments present in AG34 has been published previously (171, 206). Treatment with MNNG caused the loss of the 4.3 and 3 kb fragments and the appearance of two bands migrating between 1.8 and 2.3 kb. The altered patterns produced by MNNG at 2 µg/ml in the *EcoRI* digests in Figure 7 were not further altered at concentrations up to 10 µg/ml. In contrast, several other clonal sublines (AG62, AG64, AG72, AG07) showed no changes in restriction pattern when examined in Southern blots after MNNG treatment. For example, AG07, a clonal subline containing primarily unintegrated viral DNA, showed no changes in restriction pattern when tested at MNNG concentrations of 1-5 µg/ml and using a variety of restriction enzymes including *EcoRI*, *BglI*, *BamHI* (Fig. 9A), *SalI*, *BglIII* and *KpnI* (not shown). Figure 9B shows similar findings in AG62, a clonal subline containing supragenomic forms of the viral DNA presumed to represent integrated SV40 sequences (171, 206).

Figure 10A shows a time course experiment in which DNA isolated from AG34 cells at 2 and 4 days following exposure to MNNG at 5 µg/ml was similarly analyzed. In this as well as in other such experiments altered restriction patterns

were consistently observed at 4 days post treatment although some changes were also occasionally observed after 2 days (Figure 10B and C). Interestingly, such alterations were only seen with certain restriction enzymes, for example *KpnI* which showed at least two new fragments at about 3.8 and 5 kb, but not for the *PstI* or *BamHI* digests in figure 9A. The experiments in figure 4 serve to illustrate the repeatable features of the altered restriction patterns which appear following MNNG treatment. The novel 5 kb *KpnI* fragment is apparent in fig. 10B and C; the MNNG induced 3.8 kb fragment which can be seen at 4 days in figure 9A and ref. 219 is weakly visible at 2 days post treatment in figure 10C. In contrast the loss of some of the normal *KpnI* fragments was variable from experiment to experiment; in figure 10 the signal intensity of the normal 3 kb fragment was greatly reduced in figure 10B but not in figure 10C.

From the previous experiments, it was constantly shown that *BamHI* digests of DNAs from Ag34 cells at 4 days after exposure to MNNG or DMSO contained two SV40 containing fragments which were migrated at about 5.2 kb and 6.9 kb. In this work, the experiments were repeated and the repeatable results were obtained. Figure 11A and B clearly showed that

*Bam*HI digests of DNAs from Ag34 cells isolated at day 4 after exposure of the cells to MNNG or DMSO contained two SV40 containing fragments migrating at about 5.2 kb and 6.9 kb. It was our deduction that the alterations caused by MNNG should be located within these two restriction fragments. In order to isolate and identify the alteration induced by MNNG treatment on these fragments, it was reasonable to have these sequences cloned and sequenced.

(4) Cloning of the MNNG-induced polymorphism in EMBL 4

Cellular DNAs were isolated at day 4 after exposure of cells to MNNG or DMSO. The DNAs were completely digested with *Bam*HI and ligated into the EMBL 4 *Bam*HI site, respectively. Four different genomic libraries were created from MNNG-treated AG34 cells and two libraries were constructed from untreated AG34 cells. From screenings, 8 independent phage recombinants constructed from genomic DNA of DMSO treated control AG34 cells (75,000 plaques screened) and 6 recombinants derived from the DNA of MNNG treated cells (85,000 plaques screened) were isolated. The clonal DNAs from these isolates were extracted and digested

completely with *Bam*HI and then run on 0.8% agarose gels. Out of eight isolates derived from control cells examined, four were found to exhibit an SV40-containing *Bam*HI fragment insert corresponding in size to the expected 5 kb fragment while the other four showed inserts corresponding to the second anticipated fragment migrating in the 7 kb size range; among six isolates derived from MNNG treated cells, three showed an SV40-containing *Bam*HI insert with a size of about 5 kb while the other three were in the 7 kb size rang. The results are shown in the Southern blot analysis in Figures 12-14. In figure 12, DNAs from two genomic clones from DMSO treated Ag34 cells and one from 2 µg/ml MNNG treated AG34 cells were digested with *Bam*HI and subjected to Southern blot analysis with a ³²p-labeled SV40 probe. It can be seen that each of these clones contained an SV40-containing fragment migrating at about 6.9 kb. In figure 13 and 14, genomic clones from different isolates were similarly analyzed. The results shown in these figures indicate that inserts isolated from the clones derived from Ag34 cells treated with MNNG or DMSO contained SV40 sequences and that these inserts also migrated at about 5.2 kb. For purpose of sequence analysis, the 5.2 and 6.9 kb

fragments were subjected to restriction digestion and the subfragments were cloned into mp18 or mp19 sequencing vectors.

(5) Characterization of 6.9 kb SV40-containing fragment

The 6.9 kb SV40-containing *Bam*HI fragments cloned in EMBL4 from both MNNG treated and untreated Ag34 cells were digested completely with *Bam*HI and the digests were run on an 0.8% low melting agarose gel. The 6.9 kb *Bam*HI inserts were extracted from a low melting gel and digested completely with *Eco*RI. The digests were run on an 0.8% agarose gel and subjected to Southern blot analysis as shown in Figure 14, C1 and M1 lanes. The results revealed that three subfragments were generated with apparent sizes of 3 kb, 2.5 kb and 1.3 kb. The arrangement of these subfragments within the 6.9 kb fragment was determined as described below.

(6) Subcloning of subfragments from *Eco*RI digests of the 6.9 kb SV40-containing *Bam*HI DNA fragments into the M13/mp19 sequencing vector

The three subfragments generated from *EcoRI* digest of 6.9 kb SV40-containing *BamHI* fragments derived from MNNG treated and untreated Ag34 cells were ligated into *EcoRI* digested sequencing vector M13/mp19 as well as *EcoRI/BamHI* digested M13/mp19. After the ligations were completed, the ligated DNA inserts were transfected into the cells of an *E. coli* strain, JM101 for amplification. The inserts were then isolated for the Southern blot analysis. Figures 15-19 show Southern blot analysis of the inserts from these subclones.

In Figures 14, DNAs from subclones CE and ME were isolated, digested with *EcoRI* completely, and then subjected to Southern blot analysis. "CE" and "ME" represent subclones derived from Ag34 cells following exposure to DMSO and MNNG respectively, and for which have two *EcoRI* cut sites. *EcoRI* digests of inserts from subclones CE1 and ME1 generated subfragments comigrating with the 3.1 kb subfragments derived from *EcoRI* digests of inserts from genomic clones C1 and M1; *BamHI/EcoRI* double digests of inserts from subclones CS and MS generated subfragments comigrating with the 2.5 kb subfragments derived from *EcoRI* digests of inserts from genomic clones C1 and M1. "CS" and "MS" represent subclones

derived from Ag34 cells following exposure to DMSO and MNNG respectively; *Bam*HI/*Eco*RI double digests of inserts from subclones CH1 and MH1 generated subfragments comigrating with the 1.3 kb subfragments derived from *Eco*RI digests of inserts from genomic clones C1 and M1. "CH" and "MH" represent subclones derived from Ag34 cells following exposure to DMSO and MNNG, respectively. These experiments also demonstrated that the 3.1 kb and 2.5 kb DNA subfragments were able to hybridize with ³²p-labeled full length SV40 genome DNA while the 1.3 kb fragments did not.

In figure 15, subclones containing the 3.1 kb inserts in M13/mp19 sequencing phage vector derived from 6.9 kb SV40-containing *Bam*HI fragments from DNA isolated from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml were analyzed. The inserts from these subclones were digested with *Eco*RI and subject to electrophoresis. Subclones CE1 to CE5 and ME1 to ME5 all contain *Eco*RI/*Eco*RI inserts migrating at approximately 3.1 kb which are able to hybridize to ³²p-labeled SV40 probe.

In figure 16, subclones with the 2.5 kb inserts in M13/mp19 sequencing phage vector derived from the 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days

following exposure to MNNG at 2 $\mu\text{g/ml}$ were analyzed. The inserts from these subclones were doubly digested with *Bam*HI and *Eco*RI, and subjected to electrophoresis. "CS" and "MS" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively and which hybridized with the full length SV40 probe. The results show that subclones CS1 to CS5 and MS1 to MS4 contain *Bam*HI/*Eco*RI inserts migrating at approximately 2.5 kb which hybridized to SV40.

In figure 17, 1.3 kb inserts in M13/mp19 sequencing phage vector derived from 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days following exposure to MNNG at 2 $\mu\text{g/ml}$ were analyzed. "CH" and "MH" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively. Figure 17 shows that these subclones contain no SV40 sequences as indicated by hybridization.

In figure 18, a Southern blot analysis of subclones of 3.1 kb and 2.5 kb inserts in M13/mp19 sequencing phage vector derived from the 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days following exposure to MNNG at 2 $\mu\text{g/ml}$ is shown. The inserts from CE and ME subclones were double digested with *Bam*HI and *Eco*RI, and those from CS and MS were digested with *Eco*RI alone and the

digests were subjected to electrophoresis. After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to a ^{32}P -labeled full length SV40 genome DNA. The results shown suggest that the 3.1 kb inserts in *Bam*HI and *Eco*RI double digests contain less SV40 sequences since the signals from hybridizing these inserts with ^{32}P -labeled SV40 probe is weaker than the ones from the 2.5 kb inserts although the same amount of DNA was loaded in each lane.

The fact that the inserts of about 3 kb were generated from the *Eco*RI digest demonstrates that the restriction sites on both ends of this subfragments are *Eco*RI; and that the ones of 2.5 kb and 1.3 kb were generated from the *Bam*HI/*Eco*RI double digests indicates that there is one *Bam*HI and one *Eco*RI sites on each end of these inserts. Figure 19 is the diagrammatic display of 6.9 kb fragment based on the data interpreted from the above Southern blot analysis. The 3 kb *Eco*RI insert(s) is located within the center of the 6.9 kb fragment and is flanked by the 1.3 kb and the 2.5 kb subfragments which have *Eco*RI and *Bam*HI cut sites.

- (7) Subcloning 5.2 kb and 6.9 kb SV40-containing *Bam*HI DNA fragments into PGEM 3 and PGEM3Z sequencing vectors

The 5.2 kb and 6.9 kb SV40-containing *Bam*HI fragments derived from MNNG treated and untreated Ag34 cells were directly subcloned into the sequencing vectors pGEM 3Z and pGEM 3, respectively. The subclones with 6.9 kb fragments as inserts were subjected to partial sequencing to determine the orientations of the three subfragments, 3 kb, 2.5 kb and 1.3 kb within the fragments. Figure 20 shows a Southern blot analysis of subclones containing the 5.2 and 6.9 kb SV40-containing *Bam*HI fragments in pGEM 3 and pGEM 3Z sequencing vectors derived from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. "C" and "M" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively. The inserts of the subclones were digested with *Bam*HI and subjected to electrophoresis in a 0.8% agarose gel and hybridized to ³²p-labeled full length SV40 genome DNA. The results indicated that C1, C2 and M1-M3 carry a 6.9 kb SV40-containing *Bam*HI fragment(s) and C3-C5, M4 and M5 carry a 5.2 kb SV40-containing *Bam*HI fragment(s). The results shown suggest that the 6.9 kb inserts contain less SV40 sequences since the signals from hybridizing these inserts with ³²p-labeled SV40 probe is weaker than the ones from the 5.2 kb

inserts although the same amount of DNA was loaded in each lane.

- (8) Sequencing analysis of 6.9 kb and 5.2 kb SV40-containing *Bam*HI fragments derived from MNNG treated and untreated SV40 transformed Ag34

The sequencing data shows that the large and small SV40-containing fragments are indeed 6930 and 5253 bases long, respectively. The data also shows that the subfragments generated from *Eco*RI digest of 6.93 kb fragments are 3,121, 2,458 and 1,351 bases, respectively.

The 5.2 kb fragments from both the MNNG treated and untreated Ag34 cells were completely sequenced. Comparison of both sequences shows various alterations within the fragment derived from the MNNG treated Ag34 (Fig. 21). There were 8 alterations within in a region spanning 435 bases (from SV40 nucleotides 410 - 5218) and 7 of them were located within the viral origin replication. Among the 7 changes within the viral origin of replication, 6 were tightly clustered within T antigen binding site II. These 6 alterations include deletions of "C" at SV40 nucleotide 11 and 4; insertions of "G" at 8 and 2; G->C substitution at

5237, CC->G substitution at 5234 and 5235; An insertion of A at 5218. The alterations are summarized in Table 4.

The 6.9 kb SV40-containing *Bam*HI fragment from MNNG untreated Ag34 cells was sequenced. The sequence data on the MNNG treated and untreated ones were compared. Comparison of the sequencing data shows alterations caused by MNNG treatment (Table 5). It has revealed that there were 11 nucleotide alterations in a region of about 4.5 kb. of 11 alterations observed, there was a G deletion at SV40 nucleotide 210 within one of the 72 repeats. All the other alterations were located within the flanking human sequences. The alterations are shown diagrammatically in figure 22.

In summary, sequence analysis of the cloned fragments from MNNG-treated cells have revealed that of 20 nucleotide alterations observed in the 12.1 kb sequenced, 2 were of the GC -> AT type base substitutions; 4 were of G/C pair substitutions; 14 others were insertions or deletions. Interestingly, while there was only one change in the short SV40 integrant within the 6.9 kb *Bam*HI fragment, 8 changes were contained within the viral origin in the 5.2 kb SV40 *Bam*HI fragment.

The sequence data obtained indicated that the mutations caused by MNNG could not explain the *EcoRI*, *KpnI* and *PstI* polymorphism on these *BamHI* fragments which as expected from the results as in figure 8 and 10.

2. Characterization of the integrated viral sequences in SV40 transformed human keratinocytes clonal subline Ag34

Both Southern blot and sequence analysis have revealed that there are two SV40-containing *BamHI* fragments in the genomic DNA derived from SV40 transformed human keratinocytes clonal subline Ag34, and that these fragments are in the actual sizes of 5,253 and 6,930 bases, respectively. Further analysis and experiments were conducted to study the structural features of these integrated SV40 sequences, and their flanking human sequences. For these purposes, inserts from four independent recombinants representing the 5.2 kb virus-containing fragments and from four recombinants carrying the 6.9 kb insert were isolated from six different libraries. Partial sequence analysis of the inserts from each clone indicated that there was only one unique fragment of each size class in the EMBL libraries.

(1) Integrated viral sequences on the 6.93 kb *Bam*HI fragment

The 6.93 kb fragment contained two subgenomic viral integrants (fig. 23) with the following features: a) viral nucleotides 5051-1645 and 570 bp of 5' flanking human sequence were located at the right hand end of the fragment (nt 5056 - 6881) with 48 nucleotides of human sequences at the 3' end and, b) a central integrant containing SV40 nucleotides 5051-468, a portion of the right hand integrant along with the leftward flanking human sequence was repeated with slight alterations in the same orientation at a location 934 bp upstream from the right hand integrant. Both integrants included the origin/promoter region of the virus which contains the viral origin of replication, the early and late gene promoters and short segments of the early and late gene transcripts. A number of mutations within this region were observed: the central integrant contained nucleotide substitutions at sequences corresponding to SV40 nucleotides 5209, 5235 and a deletion at 5237, alterations which fall within T antigen binding sites 1 and 2. The base substitutions, C->TG and TGGTTGC-->CTAA represent changes at

SV40 nucleotides 113-114 and 179-185 respectively falling within the 72 bp repeat viral enhancer elements (Table 6). The base substitutions at SV40 nucleotides 5209 and 179-185 were also present in the right hand integrant along with four alterations in the SV40 27 base palindromic enhancer element.

(2) Northern blot analysis of viral promoters in the SV40 integrants within the 6.9 kb *Bam*HI SV40-containing fragment

In order to determine whether the viral promoters were maintained in a functional state, northern blot analysis was used to detect transcripts derived from the human host sequences adjacent to the integrants. For this purpose, two restriction fragments containing the human sequences flanking with the central integrant were isolated from the *Eco*RI subfragment of the 6.9 kb *Bam*HI fragment by double digestion of the subfragment with *Sfa*NI and *Dra*III. The *Eco*RI subfragment was digested with *Sfa*NI and *Dra*III to obtain the leftward and rightward human flanking fragments. Figure 24 shows Southern blot analysis of digests of the *Eco*RI/*Eco*RI subfragment with *Sfa*NI, *Dra*III alone as well as with *Sfa*NI and *Dra*III together. The results show that double

digest of the subfragment with *Saf*NI and *Dra*III produced two fragments migrating with apparent size of 1.5 kb and 0.9 kb which did not hybridize to an SV40 probe. A restriction map of the *Eco*RI/*Eco*RI subfragment is shown in Figure 25, in which there is a *Dra*III cut site and a *Sfa*NI cut site located just adjacent to, and not within the SV40 sequences. These two restriction sites generated two subfragments containing only human sequences, which are migrated at about 1.5 and 0.9 kb. Sequence data indicates that the *Eco*RI/*Dra*III fragment is 1473 bp flanking the 5' end of the central integrant and the *Sfa*NI/*Eco*RI fragment is a 939 bp fragment just adjacent to the 3' end of the same integrant and covering the unique human sequences between the two integrants in the 6.9 kb *Bam*HI fragment. These two fragments which contain only human sequences were used as probes in the northern blot analysis.

RNA isolated from Ag34 was run on denaturing gel and was blotted onto nitrocellulose for northern analysis. In Figure 26, Ag34 RNA showed a single transcript slightly smaller than the 18s ribosomal RNA which hybridized to the ³²P-labeled *Eco*RI/*Dra*III probe while no species hybridizing

to the *Sfa*NI/*Eco*RI probe was detected. The blot was able to hybridize to ³²p-labeled full length SV40 probe (fig. 27).

To test the specificity of transcription derived from the promoter within the central SV40 integrant in the 6.9 kb *Bam*HI fragment, RNAs derived from Ag34 cells together with normal cultured human epidermal keratinocytes (Ep), two squamous carcinoma cell lines (SCC) and three lines of SV40 transformed keratinocytes 130, 425, and 98 which is the parental line from which Ag34 was derived were run together on a denaturing gel and subjected to northern analysis. Figure 28 shows that, except for Ag34, RNAs derived from other cells failed to hybridize to the *Eco*RI/*Dra*III probe.

(3) Confirming the transcript by reverse transcription-PCR assay and sequencing

To further identify the putative transcript shown in figure 26, reverse transcription-PCR (RT-PCR) was carried out. For this purpose total RNA was isolated from Ag34 cells as described previously (181). Figure 29 shows the following primers which were used in Reverse transcription and PCR analysis. Primer 74L (5' CTCTGCCTGATTTTCATT 3') which covers nucleotides 667->651 bp upstream from the

virus/host junction of the central SV40 integrant was used for first strand cDNA synthesis and amplification was carried out using 74L and 97D-2 (5' ACAGAGAGGAATCTTTGCAGC 3') which covers SV40 map positions 5147-5127 (nucleotides 2966-2946 on the *Bam*HI fragment) as amplimers. The polymerase chain reaction (PCR) was carried out using Taq polymerase (Boehringer-Mannheim) under conditions recommended by the supplier. cDNAs generated from the reactions using the above combination of primers were analyzed in electrophoresis as shown in figure 30. Fragments 1, 2, 3 and 4 were made from primers H2, 28, 74L and 97D1, respectively. The fragment lengths met the above expectations. "15" and "20" represent for numbers of cycles performed.

The PCR reaction product was sequenced by direct cycle sequencing using a Taq polymerase system (Boehringer-Mannheim). Sequence analysis confirmed the cDNA to be identical to that of the region bounded by these amplimers. Similar RT-PCR experiments using primers 87G-1 and 87G-2 (Fig. 29) designed to amplify cDNAs of transcripts arising from either DNA strand in the right hand flanking human sequences failed to produce a detectable PCR product.

(4) Viral sequences on the 5.2 kb *Bam*HI fragment

The 5.2 kb fragment was found to consist of full length *Bam*HI-linearized SV40 genomic DNA (fig. 30). There were numerous point mutations throughout the early gene region while comparing with the sequences of input SV40 virus which initially was used to transform the cells, but none of these mutations were predicted to alter the T antigen primary structure (Table 7). However, there was also a nine nucleotide insertion (TGTGAGGGC) following SV40 nucleotide 2793 resulting in the tripeptide insertion Pro-Ser-Gln at residue 702 of the T antigen polypeptide (fig. 31).

A number of other nucleotide substitutions were also present in the late gene sequences, but none of those were predicted to result in alterations in the VP1, VP2 or VP3 polypeptides. The origin/promoter region of the full length virus was found to contain only the base substitutions at SV40 map positions 5209 and 179-185 that were also present in the integrants on the 6.93.kb *Bam*HI fragment.

The results from the study of 6.93 kb and 5.3 kb SV40 integrants are summarized in figure 32.

DISCUSSION

I. Integration of the SV40 promoter/enhancer sequences in Ag34 cells

1. The SV40 integrants in SV40 Transformed Human Keratinocytes

In this study, integrated viral sequences from an anchorage independent subline of SV40 transformed keratinocytes were sequenced with a view towards examining alterations which accompany the acquisition of the transformed phenotype. The viral sequences in this subline consisted of both full length SV40 DNA and two subgenomic fragments containing the viral regulatory region but not the early genes.

(1) 5.2 kb SV40 containing *Bam*HI fragment is an integrated viral fragment

This 5.2 kb full length viral fragment was found to contain an intact origin of replication and therefore could represent full length functional unintegrated virus. However, Southern blot studies of the viral sequences had previously indicated that virtually all the viral sequences

in Ag34 were present in an integrated state (171, 206). In addition, densitometric analyses of Southern blots of the viral sequences in this subline showed that the viral sequences were present in low copy number (about 1-3 genome equivalents/cell; not shown) consistent with the finding that the EMBL4 libraries contained only two unique SV40-containing *Bam*HI fragments. The low copy number is consistent with integrated rather than free, actively replicating virus. Thus, it appears likely that the 5.2 kb *Bam*HI fragment represents a portion of an integrated, tandemly repeated, or partially repeated full length viral genome.

In summary, the evidence showing that the 5.2 kb fragment is an integrated viral sequence is: (1)previous Southern blot analysis; (2)densitometric analysis of Southern blots analysis indicates the viral sequences are in low copy number, 1-3 genome equivalents/cell; and (3)partial sequence analysis on each of individual clones indicates that there is only one virus.

(2) The subgenomic SV40 integrants on the larger *Bam*HI fragment were derived from the full length viral genome

The SV40 viral origin/promoter regions in all three viral fragments exhibited the same two base substitutions at SV40 map positions 5209 and 179-185 but, while these were the only alterations present in the origin/promoter region of the full length virus there were additional alterations in the subgenomic integrants on the larger *Bam*HI fragment. This suggests that the integration of the subgenomic viral fragments on the larger *Bam*HI fragment is an event which occurred subsequent to the integration of the full length virus. Although there were numerous alterations throughout other regions of the virus, the sequences of the minimal origin of replication were retained in an unaltered form in all three viral fragments. Since the origin of replication is apparently maintained in a functional state, the subgenomic integrants may have been derived by recombination following DNA synthesis initiated at the replication origin of the virus. Replication could occur either directly from the integrated sequences resulting in multiple copies of the viral sequences in an 'tandem repeat' arrangement (8, 203, 208), and then excision of the viral sequences followed by reintegration of an origin-containing subsegment of the virus at a distal site. A second round of replication-

integration initiated from the viral origin and extending into the flanking human sequences would result in the tandemly repeated virus-human segments observed in the larger *Bam*HI fragment (Fig. 33).

In summary, three viral integrants were apparently derived from the same virus since they all have the same mutations at bases 5209 and 179-185; the central viral integrant was probably derived from the right hand viral integrant since: (1) the 5' human flanking sequences are the same on both integrants; (2) the right hand integrant contains a larger segment of viral sequences than the central integrant; and (3) some of the mutations on the right hand integrant are different than those contained in the central integrant. These were probably acquired after the integration of the central integrant.

(3) A tripeptide was introduced by an insertion of nine nucleotide in SV40 T antigen

Of the ten separate changes that were found in the nucleotide sequence of the early gene region of the full length virus, only the nine nucleotide insertion after SV40 nucleotide 2793 translates into a change in the primary

structure of the T antigen. This is consistent with the idea that maintenance of the transformed state remains dependent on fully functional T antigen. It has been shown that the C terminal end of T antigen is not required for transformation (88, 209). On the other hand, little is known of the functioning of this region. Mutations affecting the amino acid residues 621-768 alter the viral host range (211) and this segment also appears to be responsible for the helper function provided by SV40 in adenovirus infection of monkey cells (211, 212). It is possible that the tripeptide insertion in this region may also extend the range of host genes whose expression is modulated by T antigen thereby accounting for, at least in part, the expression of 'late' transformed properties observed only many cell generations subsequent to viral infection.

2. Transcriptional Activity of the SV40 integrants

The persistence of integrants containing the viral origin/promoter region in long term culture is intriguing since this suggests that expression of nearby host genes may

be subject to control by the viral regulatory elements. If true, this may be a mechanism for activation of transformation-related host genes similar to 'promoter insertion' gene activation by retroviruses (213). Fusion transcripts such as that emanating from the early promoter of the central integrant in the Ag34 subline might be a mechanism of direct regulatory control of host genes in some SV40 transformed cell lines. Interestingly, no such transcript appeared to be present in line 98 which was the parent line from which AG34 was derived by anchorage independent selection. This would suggest that a rearrangement of the viral sequences in line 98 was specifically selected for under the anchorage independent conditions.

The C->T base substitutions at SV40 map position 5209 fall within T antigen binding site 1, an element responsible for regulation of transcription of the early genes; mutations in this region are known to result in overexpression of the early genes (11) and would, in the present case, also be expected to result in high level expression of the fusion transcript. In this connection, with the exception of a single Alu type repeat spanning

nucleotides 553-->666 no significant homology between the flanking human sequences and any of the currently catalogued sequences in the GenBank (release 90.0) was found. The presence of the nucleotide substitutions at SV40 map positions 179-185 seems to destroy the 72-bp palindromic enhancer. However, the ACTTTCCA has been identified as the critical sequence within the 72-bp repeats (215). From this finding, the 72-bp repeats in the central and the right hand SV40 integrant in the 6.9 kb *Bam*HI fragment and the ones in 5.2 kb *Bam*HI fragment may still be in a functional state.

The basis for the lack of detectable transcriptional activity from the early promoter of the right hand integrant is unclear but is presumably due to one or more of the nucleotide changes at map positions 5243, 3, 8 and 12 (Table 6) which are not present in the transcriptionally active integrant. It could also be resulted from the C->G and G->C substitutions at the position of nucleotide number 4563 and 4564 in the human repeat flanking the 5' end of the right hand integrant which does not occur in the one flanking the central integrant. There are at least two examples showing that the 5' flanking fragment of the promoter does affect the transcription from the promoter (221 and 222).

With the obtained data and current clues, the following experiments could be performed to study the biological significance of the integrated SV40 fragments in human keratinocytes. The SV40 integrants can be cloned from 98, the parental line from which Ag34 was derived as well as other clonal sublines derived from 98; the sequences of the integrants, and their human flanking regions would be compared with the ones from Ag34. Data from the comparison may be used to establish a pattern of SV40 integration which relates to its transforming activities; the origin/promoter region may be cloned and be used to transfect the same host cell to explore its role in transforming human keratinocytes if there is any.

II. MNNG-induced base alterations in the SV40 integrants and their human flanking sequences

1. The sequencing data obtained suggests that MNNG-induced alterations involved in a different mechanism from amplification subsequent to methylation

MNNG is an agent known to methylate nucleic acid base residues resulting in the formation of the corresponding mainly O⁶-methyl derivatives (198). Previous studies on amplification of integrated SV40 sequences in Chinese

hamster cells exposed to mutagenic and carcinogenic agents showed that the integrated viral sequences and its flanking human sequences were amplified in response to the treatments with carcinogenic agents, such as MNNG (201-204). It was our original thought that an anchorage independent SV40 transformed keratinocytes clonal subline Ag34 established in our laboratory was optimum material for studying the mechanism of DNA amplification in response to carcinogens because Ag34 cells contain mainly integrated viral sequences in low copy number (1-3 copies: 171, 206 & 210) and the integrated viral sequences themselves might serve as internal markers for monitoring the amplification and rearrangement if there were any.

At the time, it was our deduction that the difference between our results (high frequency rearrangement) and those described previously by others using SV40 transformed rodent cells (amplification; 201-204) might reflect differences in the sites of integration of the viral sequences. Amplification of transfected CAD (205) and DHFR (206) genes has been shown to be dependent upon the site at which these genes become integrated in the genome of the recipient CHO cells. Amplification of viral sequences is a consequence of

MNNG treatment in another clonal subline (207), indicating that human keratinocytes are capable of gene amplification. In our case, the suggestion that integration site might be a factor in determining the consequence of MNNG treatment derives from the observations that: a) the AG34 cell line shows several viral fragments which appear to represent independent integrants yet only some of them are altered by MNNG and others are not and, b) while this subline shows a number of polymorphism after MNNG treatment this was not found to be true for any of the 3 other sublines which appear to contain primarily unintegrated viral DNA (i.e. AG07, AG72, AG64).

There was at least one example where activation of an oncogene by MNNG appears to have occurred by specific rearrangements of genomic DNA (216). Activation of the met oncogene was analyzed in a human sarcoma cell subline after prolonged periods of MNNG treatment. This suggests that such an activating rearrangement may be derived over long periods from the type of rearrangements observed here.

Because the effects of the agent described in our report (207) are manifest only after several days subsequent to MNNG exposure, our results apparently represent a

separate process subsequent to the formation of adducts. The previous results based on Southern analysis from our laboratory indicated a process requiring a high degree of specificity which cannot be accounted for by adduct formation alone. The specificity of MNNG action is evident from the following observations:

A) At the observed rates of cell growth, it is not possible that selective overgrowth by a small subpopulation of cells which carried the alterations could have occurred over the 4 day post treatment period even if such a subpopulation continued to grow at the rate of the untreated cells. This is also evident in the experiment conducted at 1 $\mu\text{g/ml}$ MNNG which showed only a very minimal effect on cell growth but produced the same DNA alterations as seen at 2 $\mu\text{g/ml}$. Based on growth under clonal growth conditions (table 3), 69-75% of the general cell population maintains colony forming capability after exposure to MNNG at 1 $\mu\text{g/ml}$.

B) The appearance or disappearance of discrete bands on Southern blots means that only relatively short, highly specific sequences must be modified; in this regard it is important to stress the repeatability of the MNNG-induced changes, for example the altered restriction pattern shown

in the *KpnI* digests is essentially the same as reported previously in separate experiments (219). C) The alterations were seen only in one cell subline and only in a small subset of the restriction fragments generated with the restriction enzymes tested; this would not be true if random methylation of bases were the causal mechanism.

Studies of MNNG induced mutations in human cell systems have shown that MNNG produces more O⁶MeG than O⁴MeT on DNA strands when cells are exposed to MNNG (Fig 6). At low doses of MNNG, human methyl-transferase remains active and is able to remove methyl groups from O⁶MeG but not O⁴MeT (198). The unrepaired O⁴MeT could mispair with a guanine due to its structural similarity to cytosine, which causes A · T to G · C transitions (210, 215-216:). The AT → GC changes in this study may be caused by this mechanism. It has been reported that O⁶MeG derived from nucleotide pools could incorporate into DNA strands during replication at a position opposite to the thymidine residue on the template strand due to its ability of pairing with thymidine residues. Then the methyl groups are removed by methyl-transferase before DNA replication leaving the guanines in the strand resulting in the incorporation of C - G pairs

(217, 218). The C--> G changes in this study may be the result of mispairing of thymidine with methylated O⁶MeG which was not repaired.

However, in this study most of the mutations induced by MNNG were not A · T to G · C transitions as expected but consisted of deletions and insertions which might result from base excision. This suggests that they were not the direct results from the methylations of bases in the DNA strands. Also the consistent changes in Southern blot analysis of DNA isolated at day 4 after the exposure of the cells to MNNG would suggest a separate mechanism subsequent to methylation since polymorphism derived directly from methylation would have been appeared at day 1 after exposure of the cells to MNNG and weaken during the post-treatment.

It was very interesting that on the 5.2 kb *Bam*HI fragment, 7 of 20 MNNG-induced alterations observed were located within the 24 base segment comprising T antigen binding site II in the viral origin of replication, to which the DNA polymerase - T antigen complex binds. In contrast, in the two integrants in the 6.9 kb *Bam*HI fragment, both of which contain numerous mutations in the viral origin

sequences, no such localization of base alterations was observed.

From the above findings, a mechanism subsequent to methylation which caused the alterations in DNA induced by MNNG may be proposed, which may be involved in the action of the T antigen-DNA polymerase α interaction. The fact that the MNNG-induced alterations were found to cluster within T-antigen binding site II suggests that the T antigen-DNA polymerase α complex may carry out a novel excision function. The appearance of altered patterns in restriction digests at day 4 following treatment of the cells to MNNG at 2 μ g/ml (Fig. 9 and Fig. 10) are consistent with this replication related mechanism since the cell number doubles between day 2 and 4 (Fig. 5), a time period covering one round of DNA replication. It is of note that no DNA amplification occurred. This mechanism, if can be testified, is different from the one suggested by Lavi (205). In Lavi's model, treatment of the cells with various carcinogens activates cellular replicons, and the repeated activation of replicon sequences then result in a localized amplification of host sequences associated with the replicon and increased the expression of the genes encoded by the amplified

sequences. The process eventually leads to the amplification of certain genes e.g. DHFR and oncogenes (205).

2. The sequence data obtained could not explain the *EcoRI*, *KpnI* and *PstI* polymorphism on these *BamHI* fragments as shown in Southern blot analysis

The MNNG-induced changes seen in the sequence data obtained did not show the *EcoRI*, *KpnI* and *PstI* polymorphisms on these *BamHI* fragments as shown in the Southern blot analysis in figure 8 and 10. This may be explained as the follows: 1) the mutations which caused the altered restriction patterns as seen were possibly located in regions outside of the cloned 5.2 and 6.9 kb *BamHI* fragments; or 2) not every cell in the culture carries exactly the same alterations in DNA sequences so that the DNA fragments containing polymorphism seen might not be cloned in the isolates obtained from the libraries.

Table 1

Properties of SV40 Large-T Antigen

- * Transforms non permissive cells to a malignant phenotype
- * Binds to specific sequences of SV40 DNA in order to initiate each round of viral DNA replication in the infected cell
- * DNA helicase activity that is required for unwinding and DNA chain elongation during DNA replication
- * Negative regulation of SV40 early gene transcription
- * Trans-activation of selected RNA polymerase II promoters including the SV40 late promoter
- * Induction of cellular rRNA synthesis
- * Induction of cellular DNA synthesis
- * ATPase activity
- * Binds to cellular factors including p53, p105-Rb and DNA polymerase α

Table 2. Transforming domains of SV40 large T antigen

<u>Region</u>	<u>Position of Amino Acids</u>	<u>Functions</u>
A	1-82, N-terminus	Binding to cellular protein, p300 DNA polymerase α
B	101-118	Binding to pRb and p107
C	126-132	Nuclear localization
D	351-450	Binding to p53, DNA polymerase α
	533-626	Binding to p53

Table 3. Toxicity of MNNG as measured by clonal survival

Treatment	<u>Survival (% of control)</u>	
	<u>I</u>	<u>II</u>
DMSO control	(100) ^a	(100) ^a
1 µg/ml MNNG	75	69.2
2 µg/ml MNNG	24.6	27.0

Culture were treated with MNNG as described in Materials and Methods and immediately trypsinized and replated in triplicate at cell densities where colonies were derived from single cells (clonal growth), colony forming efficiency of the cells from cultures treated with MNNG at 1 µg/ml was still 69- 75% of that seen in control cultures. Exposure of AG34 cells to 2 µg/ml MNNG substantially reduced the fraction of cells able to form colonies under stringent growth conditions. However, even under these conditions the subpopulation of colony forming cells was found to represent a minimum of 25-27% of control cultures.

^aCloning efficiency of control cells was 31.2% for group I and 24.5% for group II.

Table 4. Alterations on 5.2 kb SV40 containing fragment derived from MNNG-treated human keratinocytes clonal subline Ag34 by comparing with the one derived from MNNG untreated human keratinocytes clonal subline Ag34

<u>5.2 kb fragment nucleotide</u>	<u>SV40 base</u>	<u>nucleotide change</u>
After 2124	410	C insertion
2523 (C)	11	deletion
After 2526	8	G insertion
2530 (C)	4	deletion
After 2532	2	G insertion
2540 (G)	5237	C Substitution
2542, 2543 (CC)	5235, 5234	G substitution /deletion
After 2559	5218	A insertion

Table 5. Alterations on 6.9 kb SV40 containing fragment derived from MNNG-treated human keratinocytes clonal subline Ag34 by comparing with the one derived from MNNG untreated human keratinocytes clonal subline Ag34 cells.

<u>6.9 kb fragment nucleotide</u>	<u>nucleotide change</u>
On the human subfragment with EcoR I/BamH I site	
91 (C)	deletion
92	A insertion
293	T insertion
300	A insertion
439 (T)	G substitution
579	AT insertion

On the EcoR I/EcoR I Subfragment containing SV40 sequences	
3199 (G)	deletion*
3685	T insertion
4059 (A)	deletion
4134 (A)	C substitution

On the EcoR I/BamH I subfragment containing SV40 sequences	
4563 (C)	G substitution
4564 (G)	C substitution

* indicates the deletion at SV40 nucleotide 210 within one of the 72 repeats.

Table 6. SV40 nucleotide alterations occurred in 6.9 kb
SV40 containing fragment

CENTRAL INTEGRANT

<u>SV40 nucleotide</u>	<u>nucleotide change^a</u>	<u>functional element</u>
5209 C	T (substitution)	T-Ag binding site 1
5235 G	C (substitution)	27 bp palindrome minimal origin
5237 C	- (deletion)	27 bp palindrome minimal origin
113	T (insertion)	72 bp palindromic enhancer element
114 C	G (substitution)	72 bp palindromic enhancer element
179 185 TGGTTGC	CTAA (substitution)	72 bp palindromic enhancer element

RIGHT HAND INTEGRANT

<u>SV40 nucleotide</u>	<u>nucleotide change^a</u>	<u>functional element</u>
5209 C	T (substitution)	T-Ag binding site 1
5243 G	- (deletion)	27 bp palindrome minimal origin
3 C	G (substitution)	27 bp palindrome minimal origin
8 C	G (substitution)	27 bp palindrome minimal origin
12	G (insertion)	27 bp palindrome

179	185		minimal origin
TGGTTGC		CTAA(substitution)	72 bp palindromic enhancer

Table 7. SV40 nucleotide alterations occurred in 5.2 kb SV40 containing bamH I fragment

<u>SV40 nucleotide</u>	<u>nucleotide change^a</u>	<u>functional element</u>
179 185 TGGTTGC	CTAA (substitution)	72 bp palindromic enhancer
1756 A	C (substitution)	VP1
1951 T	C (substitution)	VP1
2209 G	A (substitution)	VP1
2239 T	G (substitution)	VP1
2384 C	A (substitution)	VP1
2793	TGTGAGGGC(insertion)	T antigen (C terminus)
3755 A	G (substitution)	T antigen
4071 T	A (substitution)	T antigen
4110 C	T (substitution)	T antigen
4299 C	T (substitution)	T antigen
4642 G	T (substitution)	T antigen intron
4839 C	T (substitution)	T antigen intron
4849 C	T (substitution)	T antigen intron
4879 C	T (substitution)	T antigen intron
5209		

C T (substitution) T-Ag binding site 1

^abase alterations reflect changes as compared with the
sequence of virus from the original virus stocks

Figure 1 - Genetic structure and function map of SV40 genome. Given on the map are structural features of DNA transcription, RNA processing, and RNA translation. Cleavage sites of five single-site restriction endonucleases are indicated for reference. The origin of replication (ORI), 5' and 3' ends of early (small t- and large T-antigens) and late (16S and 19S) mRNAs, and the early gene TATA box are shown on the map. Open arrows designate the protein coding regions of mRNAs. Within the arrows, the first number locates the A in the AUG codon, and the second number identifies the nucleotide that immediately precedes the termination codon. mRNAs splicing sites are indicated by wavy lines. Early and late transcription proceed on the viral minus and plus strands in counterclockwise and clockwise directions, respectively.

Source: John N. Brandy and Norman P. Salzman (1).

Figure 1

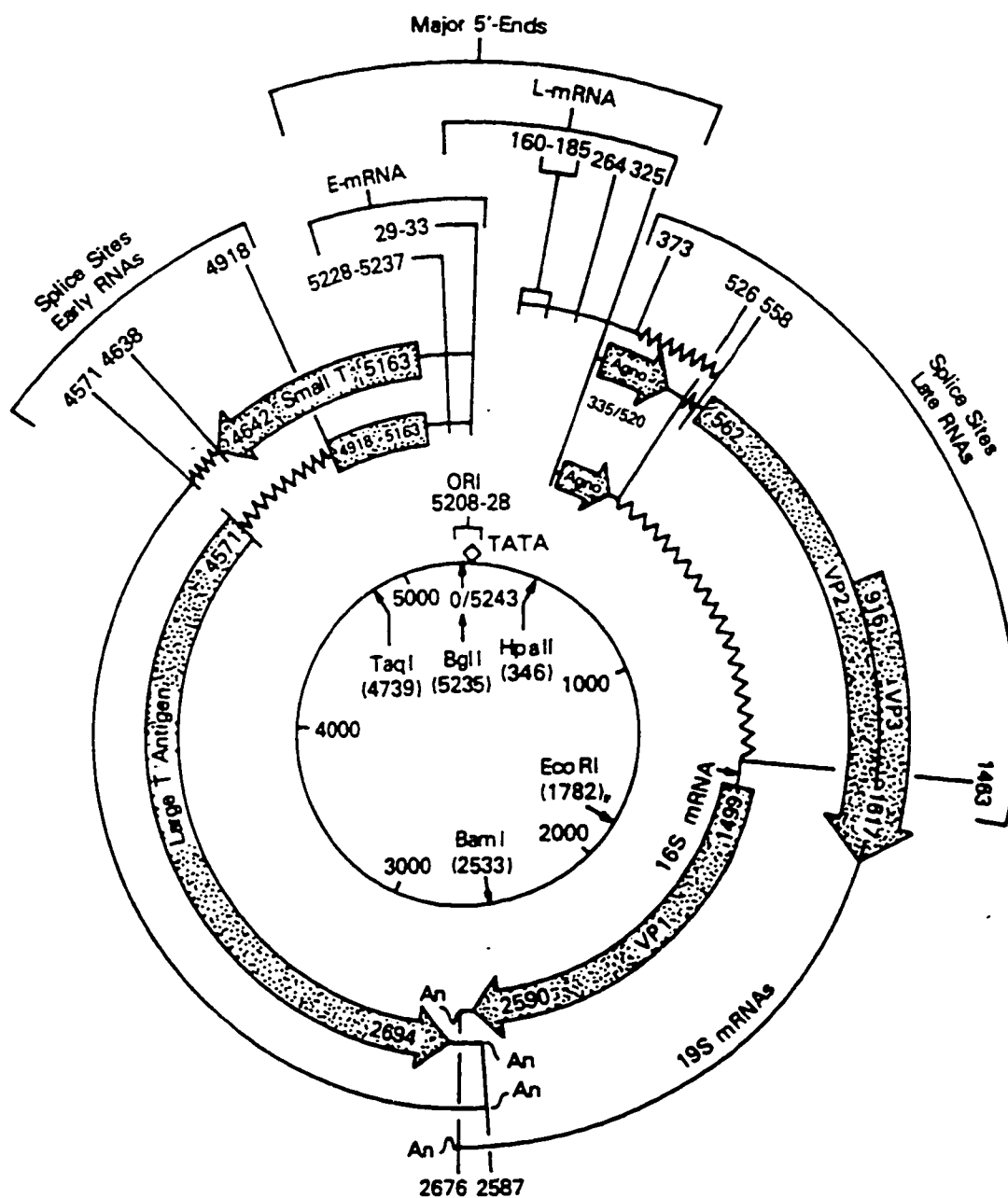


Figure 2 - Diagrammatic illustration of the transcription map and T-antigen binding sites at and near the SV40 replication origin.

Figure 2

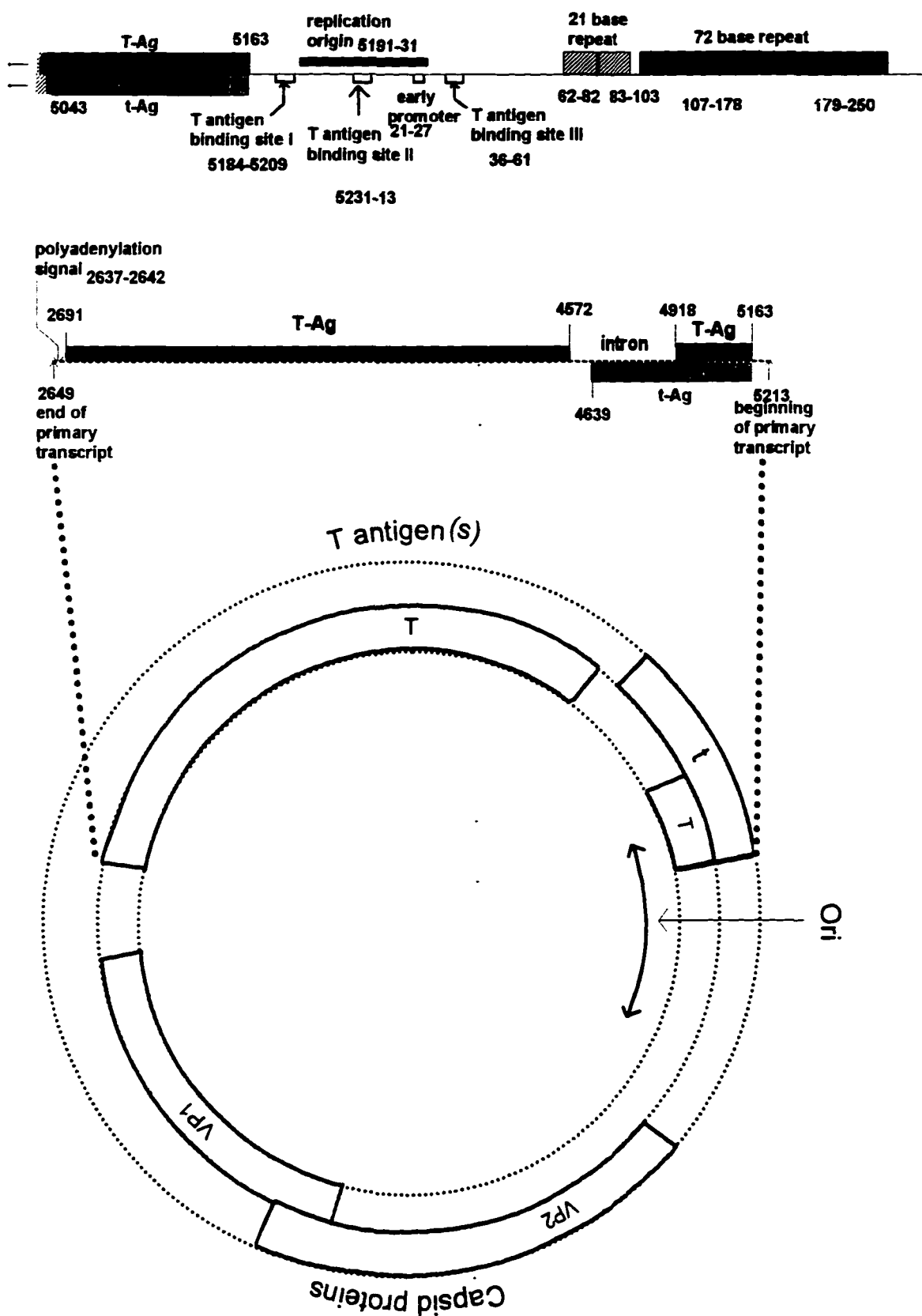


Figure 3 - The SV40 genomic control region showing replication origin, T antigen binding sites and promoter/enhancer region (209)

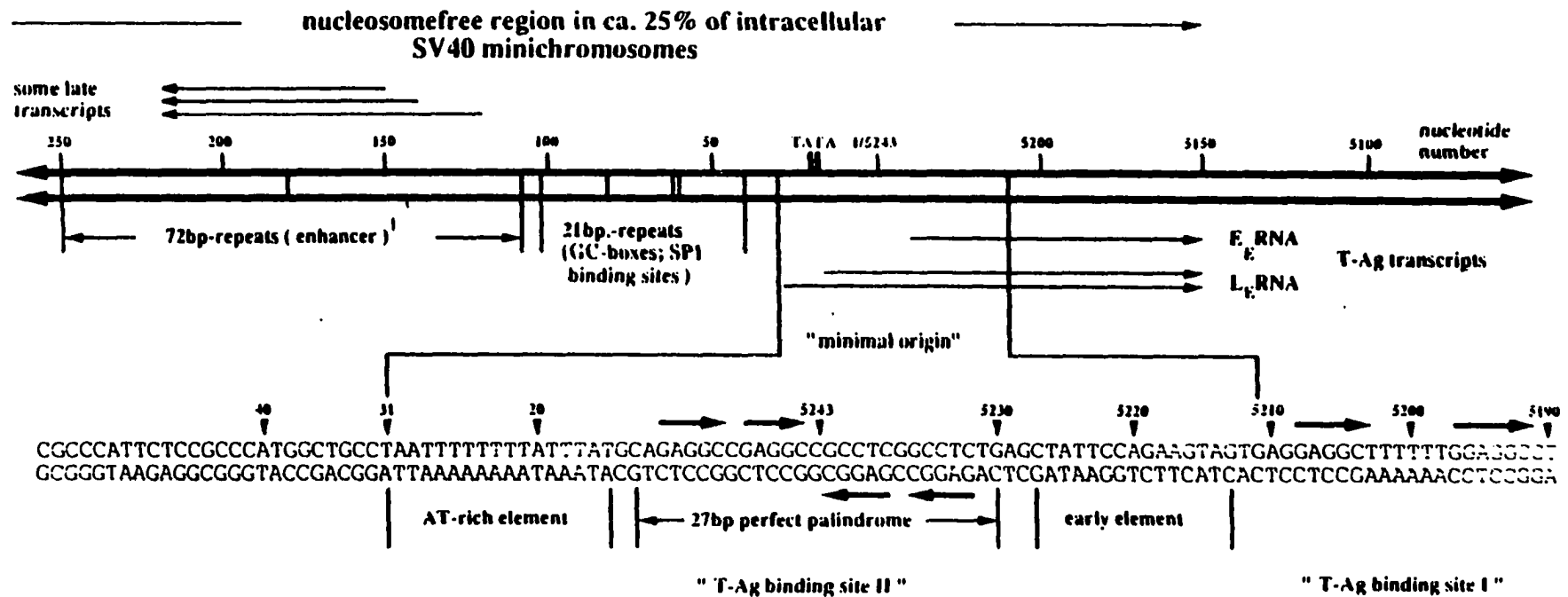


Figure 3

Figure 4 - Regions of SV40 T antigen that are involved in its oncogenic activity. Four regions are designated as A, B, C and D according to their activities. Region A is at the N-terminus covering amino acid residue 1-82. It contains the binding sites for DNA polymerase α , and cellular protein p300. Region B covers amino acid residues 101-118 which is the binding sites to oncogenic protein pRb and p107. Region C extends from amino acid 126 to 132 which includes a nuclear localization signal. Region D includes two sections, one is from amino acid residue 351 to 450 which contains binding sites to oncogenic protein p53 and DNA polymerase α ; the second section covers amino acid residue from 533 to 626 which binds to p53(88).

Figure 4

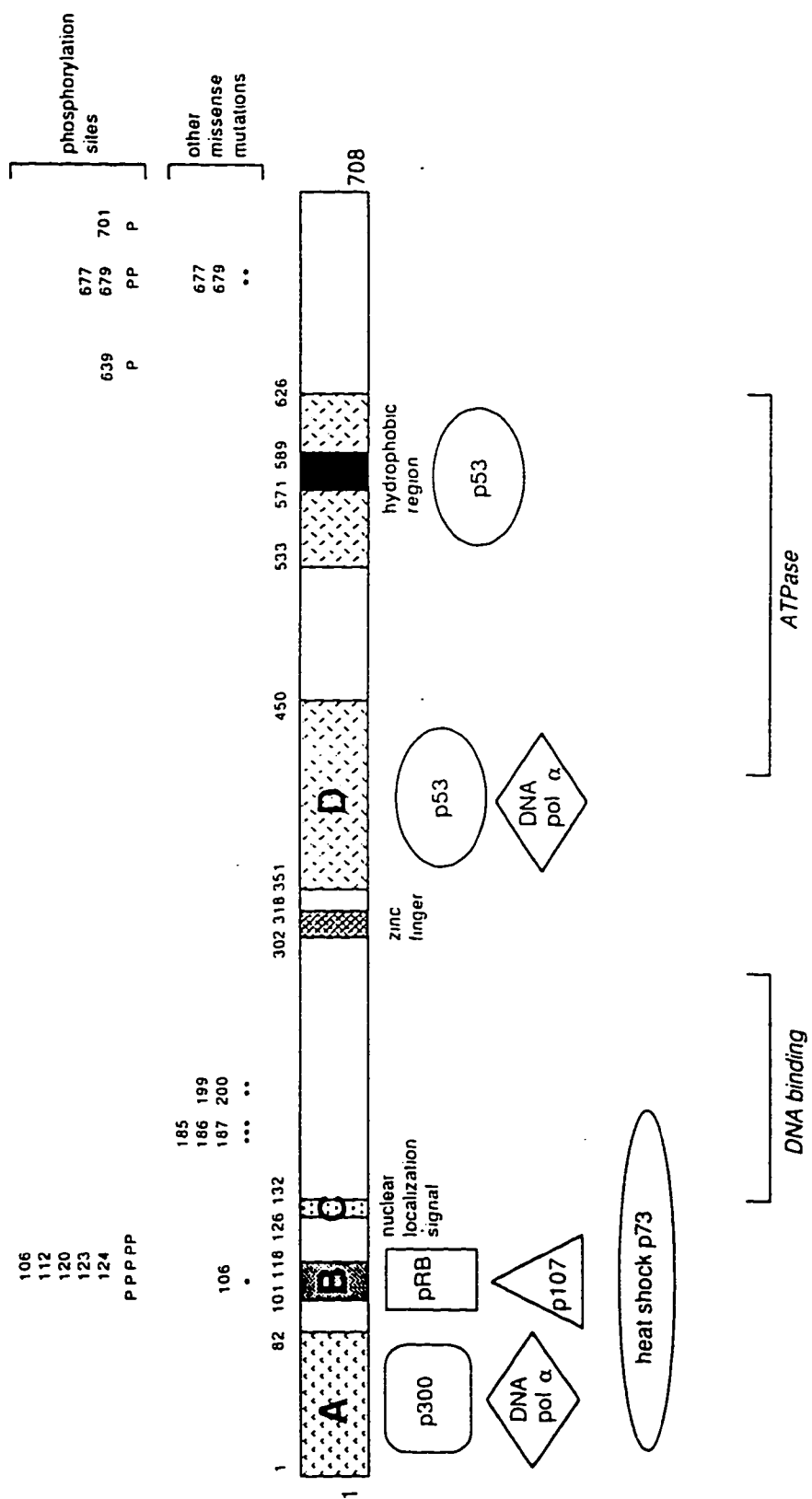


Figure 5 - Diagrammatic representation of the emergence of transformed properties over time following infection of human epidermal keratinocytes by SV40(171). The stage-specific nature of the transformation process is illustrated as delineated by various transformation-related markers. In the "precrisis" stage, feeder layer independence, serum independence and breakdown of junctional communication are the growth properties that infected cells first show; loss of stratified keratin gene expression is the subsequent events; and the induction of simple epithelial keratin gene expression lasts into the "crisis" stage; delocalization of fibronectin to apical cell surface starts in "crisis" and lasts to the late stage, "post crisis". The infected cells growth in the late stage becomes anchorage independent, and organized actin cables forms.

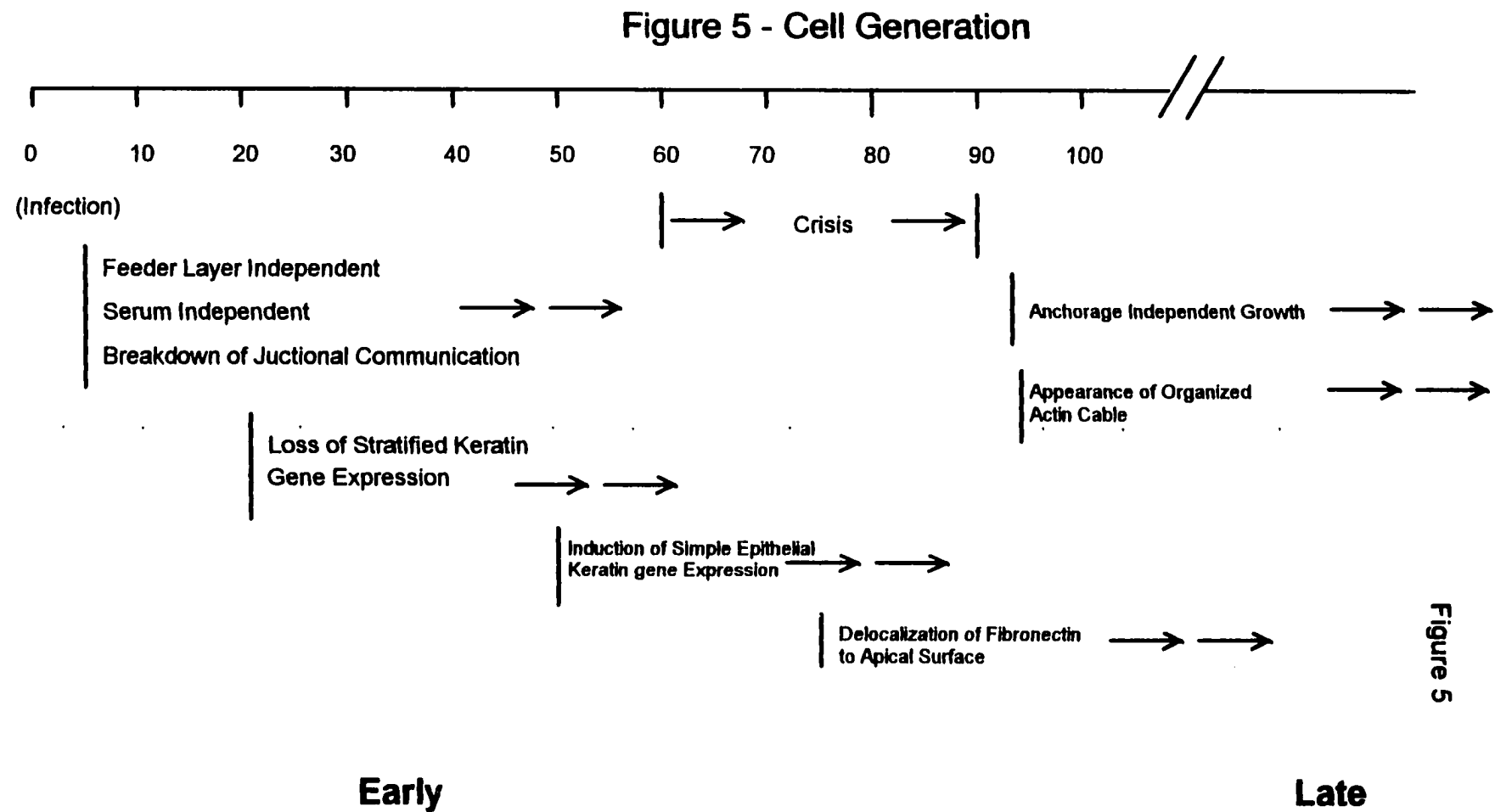


Figure 5

Figure 6 - Methylation of ^6O in guanine by MNNG and the process of mispairing of $^6\text{OMeG}$ with thymine in DNA chain. The mispairing of O^6MeG with thymine in DNA chain results in the transition of G-C to A-T if the adducts formed are not removed before DNA replication starts.

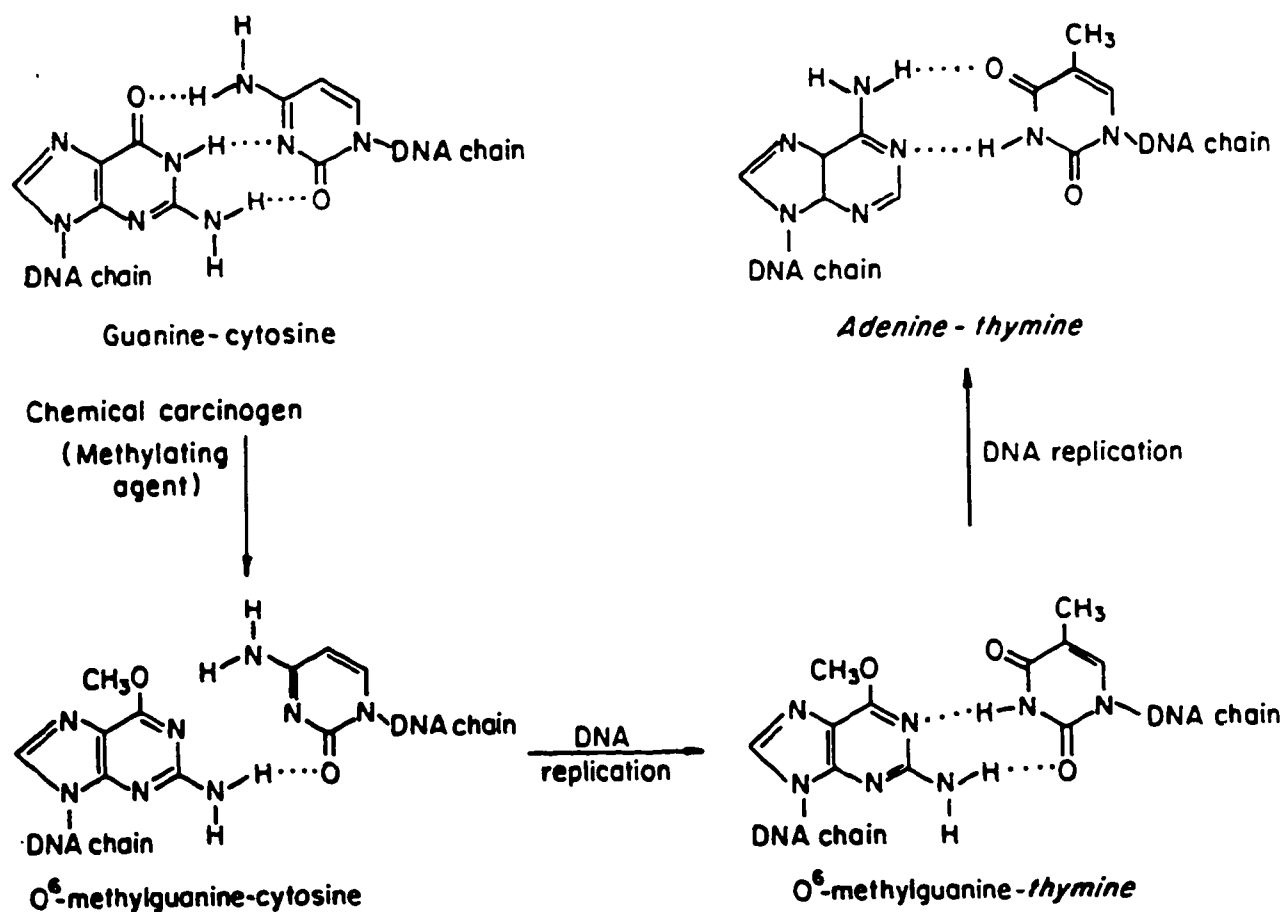


Figure 7 - Growth of AG34 cells following exposure to MNNG. Flasks of AG34 cells were treated with MNNG as described in Materials and Methods and cell counts were taken at 1, 2 and 4 days post treatment. Values represent the average of three flasks. x----x, DMSO controls; o----o 1 $\mu\text{g/ml}$ MNNG; •----•, 2 $\mu\text{g/ml}$ MNNG. The figure shows that Ag34 cells treated only with 0.1% DMSO increased in cell number ~9-fold over the 4-day period; the corresponding increases for cultures treated with 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ MNNG were 6.5- and 3-fold respectively.

Figure 7

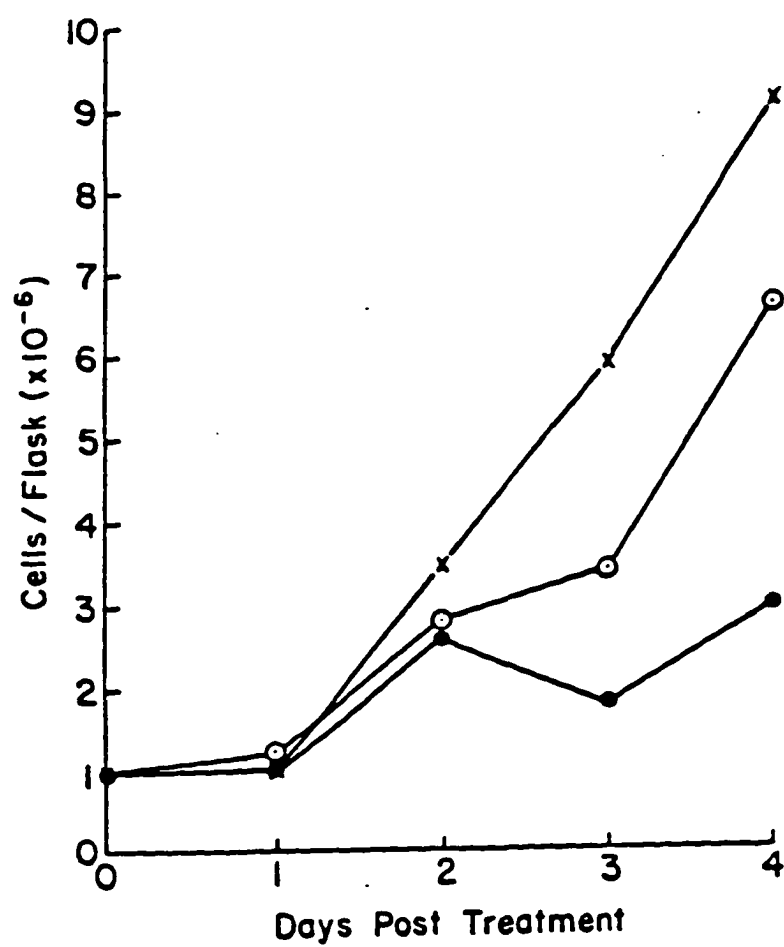


Figure 8 - Altered patterns of SV40-containing restriction fragments following exposure of clonal subline Ag34 to various concentrations of MNNG. Genomic DNAs from Ag34 were isolated from cultures 4 days following exposure of the cells to MNNG at the indicated concentrations. Restriction fragments from *EcoRI* digests were hybridized to an SV40 probe. The migration of lambda (*HindIII* digested) markers in kilobases (kb) is indicated. Note the disappearance of normal fragments migrating at about 3 and 4 kb and the appearance of a novel 2 kb fragment in the lanes representing 2,5, and 10 $\mu\text{g/ml}$.

Figure 8

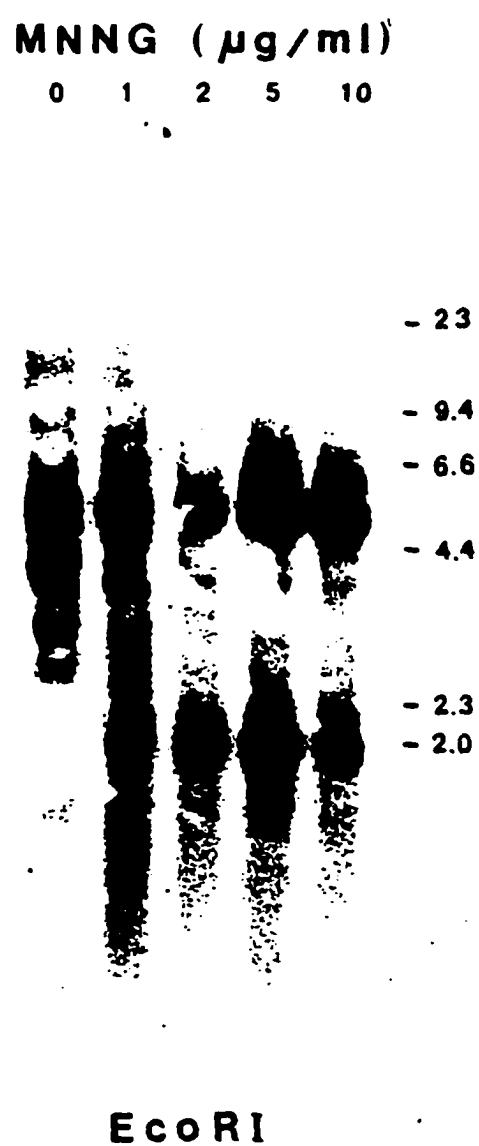


Figure 9 - Cell sublines not responsive to MNNG treatment.

A) SV40 containing restriction fragments from AG07 following MNNG treatment. Cells were treated with MNNG at the indicated concentrations and analyzed as in figure 7. There is no apparent change in patterns produced by *EcoRI*, *BglI* or *BamHI*. B) SV40 containing restriction fragments from AG62 following MNNG treatment. AG62 cells were treated with MNNG at 2 µg/ml and analyzed as in figure 7 using various single cut restriction enzymes for SV40. DNA from treated (+) and untreated (-) cells is indicated. A = *AccI*, BH = *BamHI*, B = *BglI*, E = *EcoRI*, K = *KpnI*.



Figure 10 - Time course of response to MNNG in AG34. A) Genomic DNAs were isolated at 2 or 4 days following exposure to MNNG (5 $\mu\text{g/ml}$) and restriction fragments were hybridized to an SV40 probe. Altered patterns of SV40 containing restriction fragments were observed in Kpn I (but not in BamHI or Pst I) digests at 4 days, but not at 2 days post treatment. B) and C) Separate experiments in which AG34 cell were treated with MNNG ant 2 $\mu\text{g/ml}$. Genomic DNAs were digested with Kpn I; lanes represent DMSO controls and MNNG treated cells are labled 'C' and 'M' respectively. Arrows point to the 5 kb band which is induced after MNNG treatment in A) at 4 days post treatment and in B) and C) at 2 days post treatment.

Figure 10

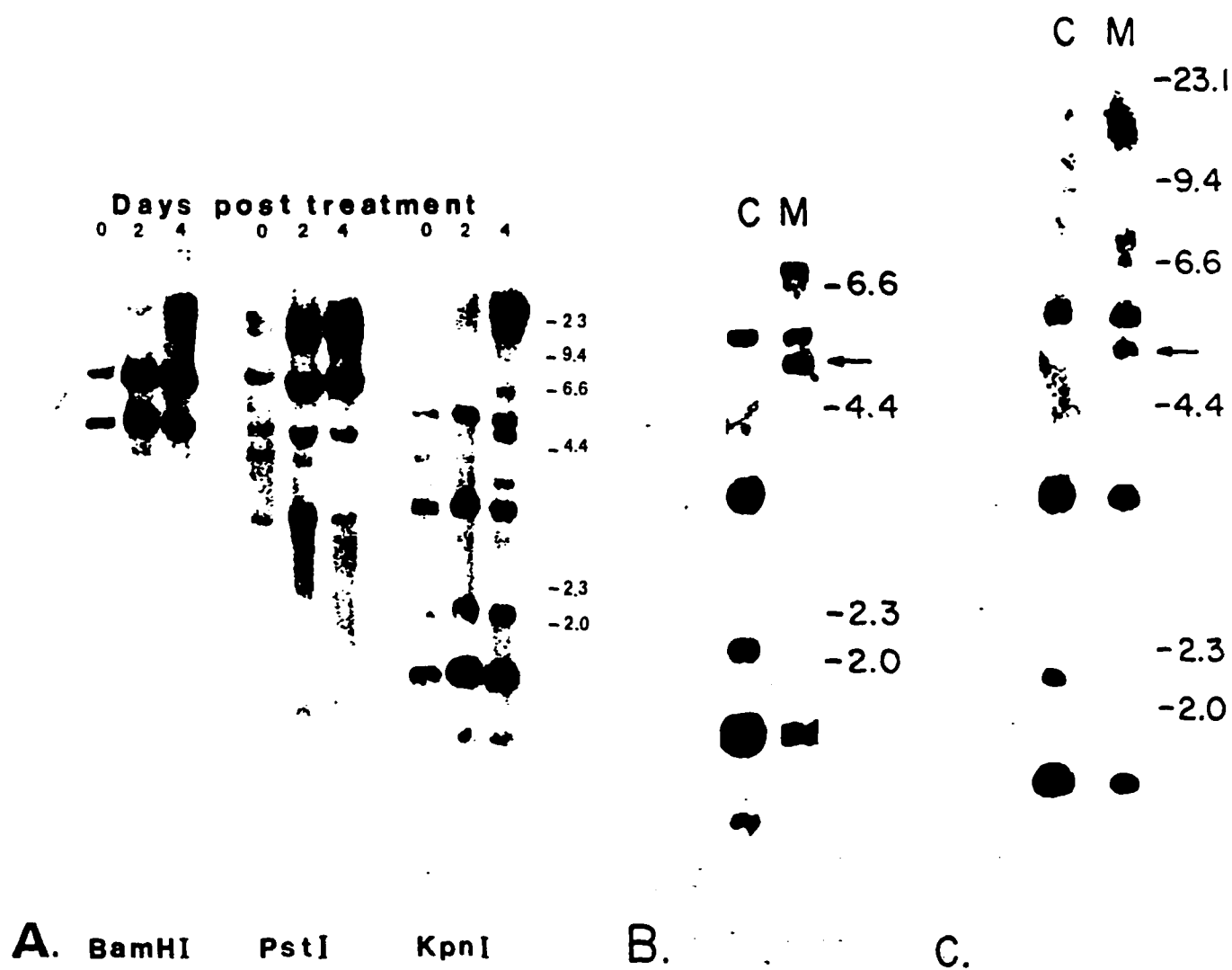


Figure 11 - Southern blot analysis of *Bam*HI digests of genomic DNAs isolated from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. "C" and "M" represent DMSO control and MNNG treatments, respectively. "SV" represents a *Bam*HI digest of full length SV40. "1-3" represent genomic DNAs prepared from Ag34 cells in different MNNG treatments. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated.

A. Left panel: Ethidium bromide stained 0.8% agarose gel. Right panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to a ³²p-labeled full length SV40 genome. Two hybridizing fragments with approximate sizes of 6.9 kb and 5.2 kb, respectively can be seen. A 5.2 kb fragment from *Bam*HI digest of SV40 genomic DNA was observed in ethidium bromide stained gel as well as in Southern blots. No changes in the restriction pattern caused by MNNG was observed.

B. Restriction fragments from *Bam*H I digest of genomic DNA was hybridized to ³²p-labeled SV40 probe. The results shown here comply with the ones obtained in A.

Figure 11

A.

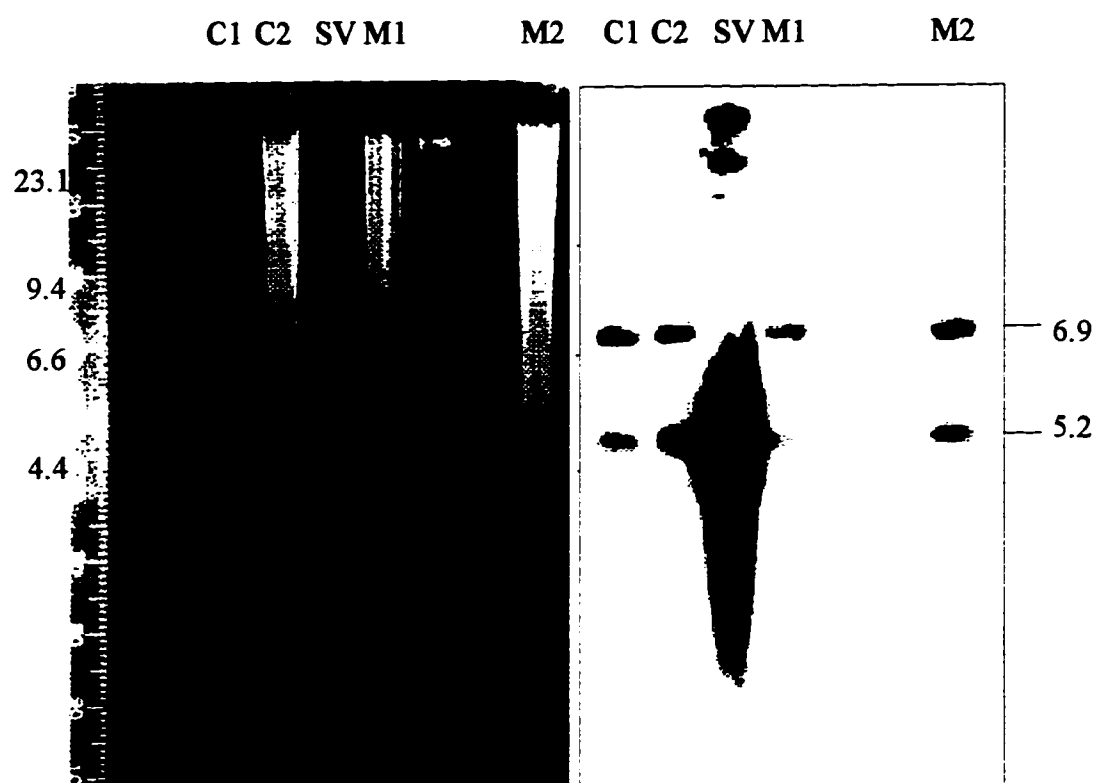


Figure 11

B.

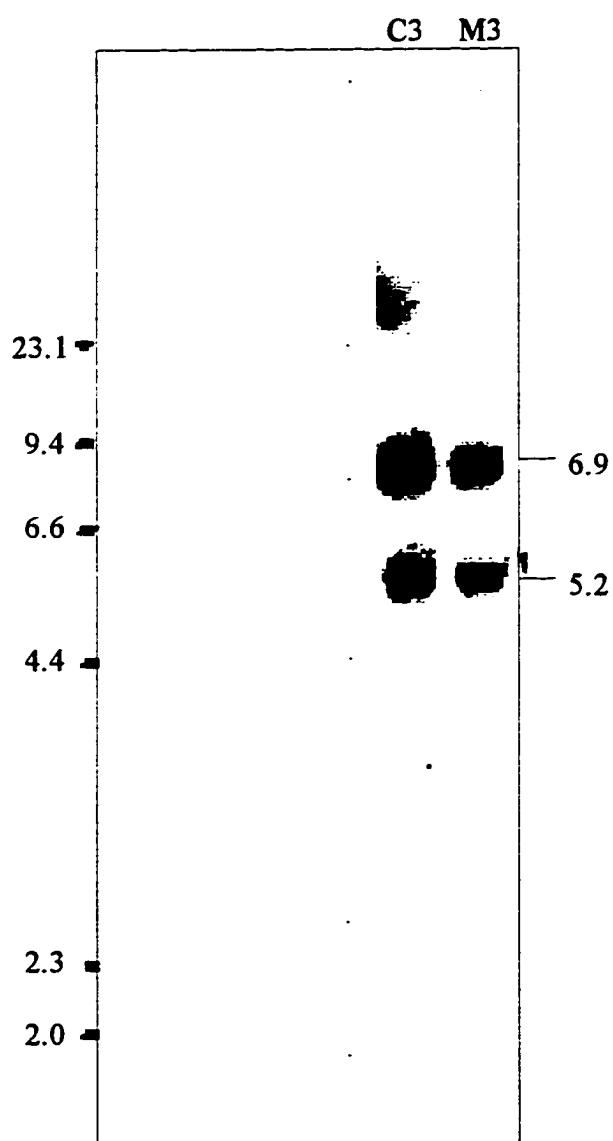


Figure 12 - Southern blot analysis of *Bam*HI digests of the DNAs from genomic clones in EMBL 4 derived from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. "C" and "M" represent DMSO controls and MNNG treatment, respectively. "C1" and "C2" represent clones isolated from different genomic libraries. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated.

Left panel: Ethidium bromide stained 0.8% agarose gel after electrophoresis was completed.

Right panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to ³²p-labeled full length SV40 genome.

*Bam*HI digests from these clones contain fragments migrating at approximately 6.6 kb.

Figure 12

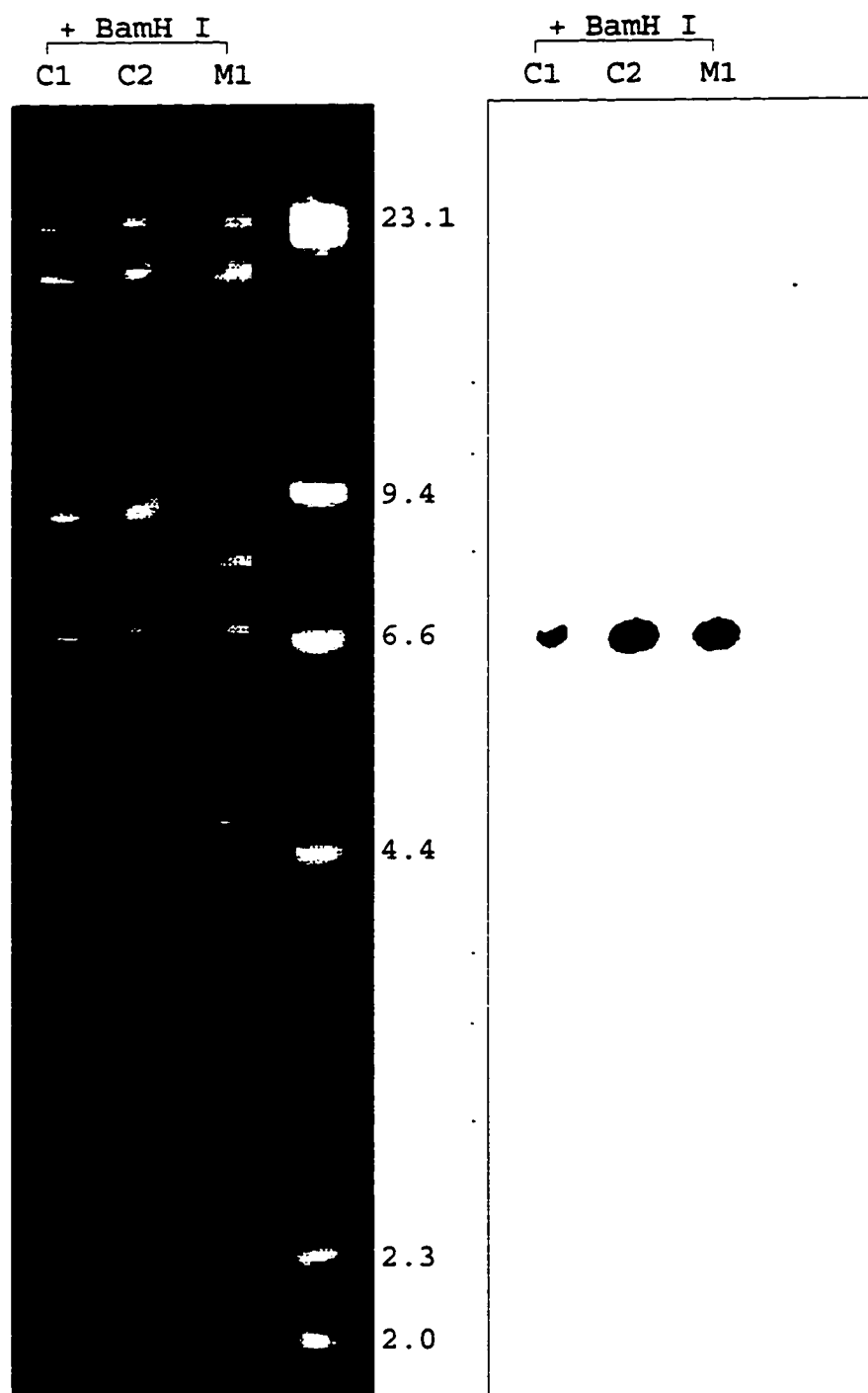


Figure 13 - Southern blot analysis of *Bam*HI digests of the DNAs from genomic clones in EMBL 4 from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. "C" and "M" represent for DMSO controls and MNNG treatment, respectively. "M2" and "C3" represent different clones isolated from genomic libraries. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated.

Left panel: Ethidium bromide stained 0.8 % agarose gel.

Right panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to a ³²p-labeled full length SV40 genome.

*Bam*HI digests from these clones contain fragments migrating at about 5.2 kb which hybridized to the SV40 probe.

Figure 13

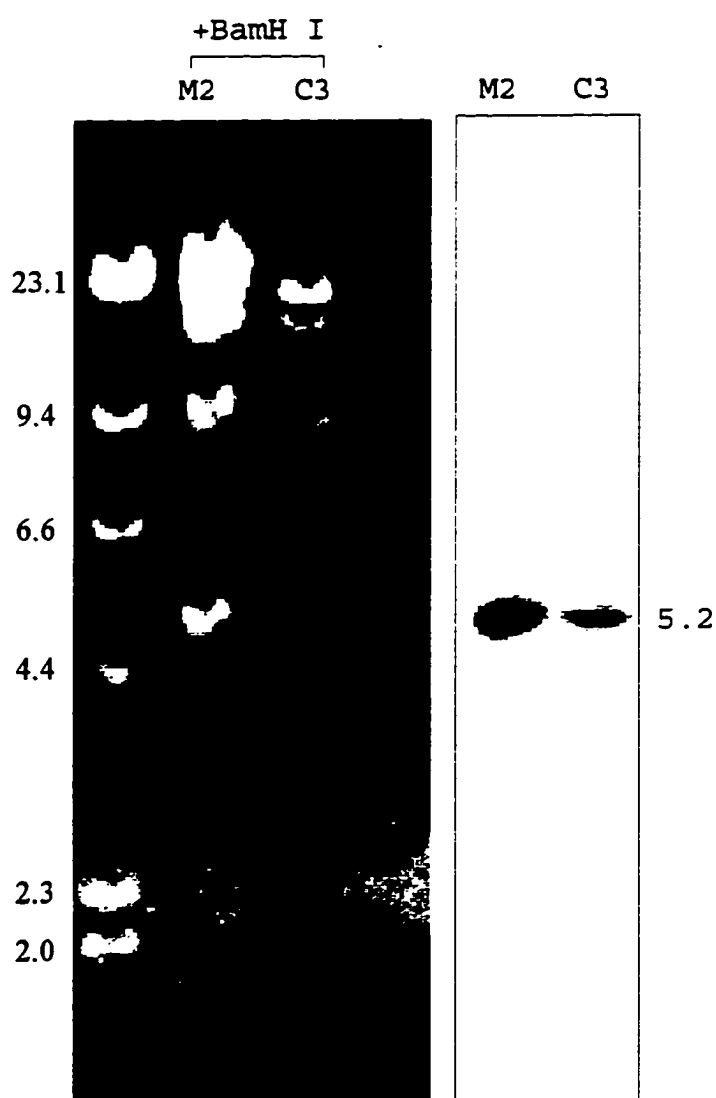


Figure 14 - Characterization of 6.9 kb *Bam*HI DNA fragments from genomic clones and of subfragments in the subclones with phage vector M13/mp19 from *Eco*RI digests of the 6.9 kb *Bam*HI DNA fragments. "C" and "M" represent genomic clones and subclones derived from Ag34 cells at 4 days following exposure to DMSO and MNNG, respectively. "1" represents genomic clones "1" and the subclones derived from them. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated.

Upper panel: Ethidium bromide stained 0.8% agarose gel.

Lower panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to ³²p-labeled full length SV40 genome.

Figure 14

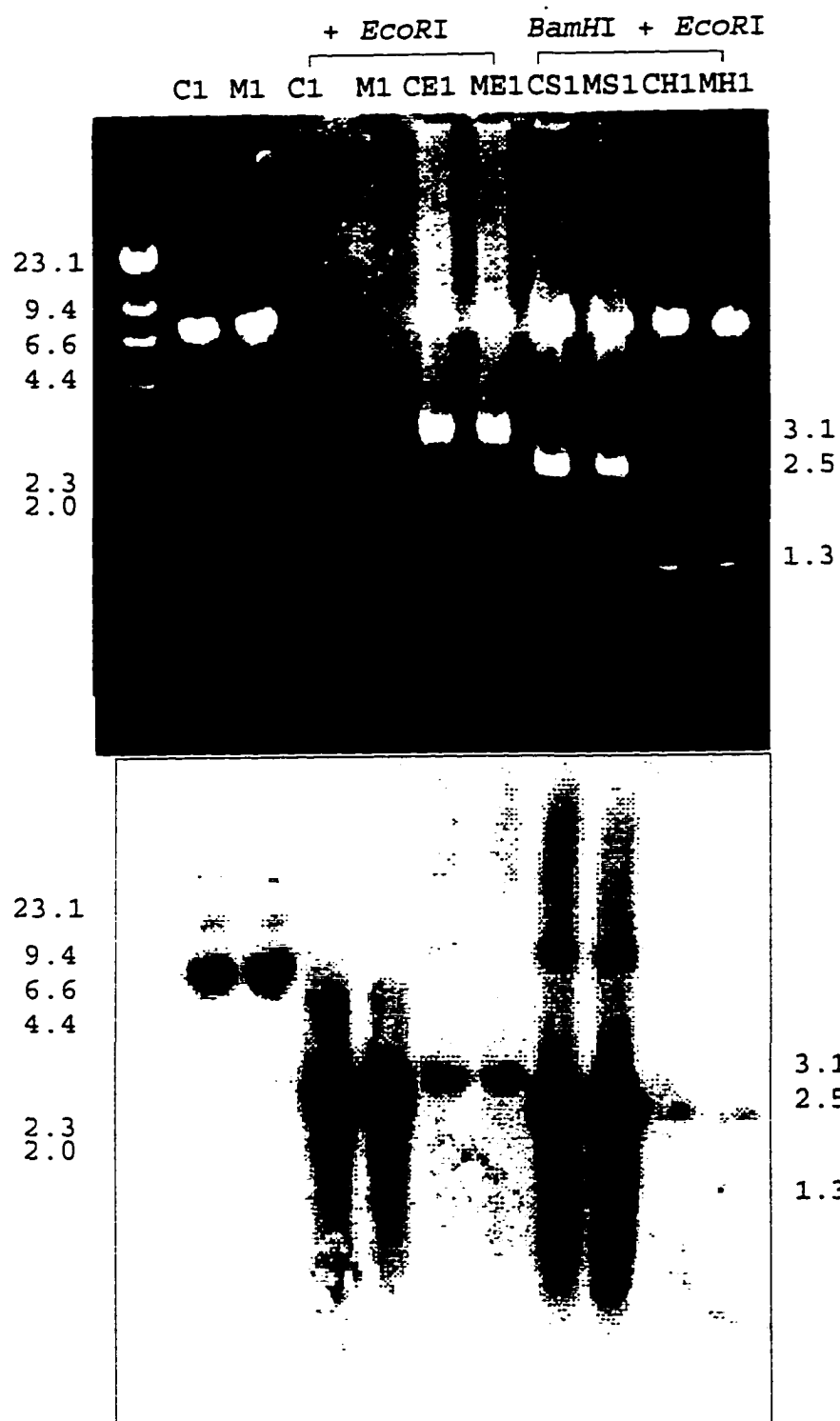


Figure 15 - Southern blot analysis of subclones containing 3.1 kb subfragments in an M13/mp19 sequencing phage vector derived from 6.9 kb SV40-containing *Bam*HI fragments from DNA isolated from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. The DNAs from these subclones were digested with *Eco*RI and subjected to electrophoresis. "CE" and "ME" represent for subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively, which hybridized to SV40 probe. The migration of *Hind*III digested lambda phage fragments is indicated in kb.

Upper panel: Ethidium bromide stained 0.8% agarose gel.

Lower panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to ³²p-labeled full length SV40 probe.

CE1 - CE5 and ME1 - ME5 contain *Eco*RI subfragments migrating at approximately 3.1 kb which hybridized to the SV40 probe.

Figure 15

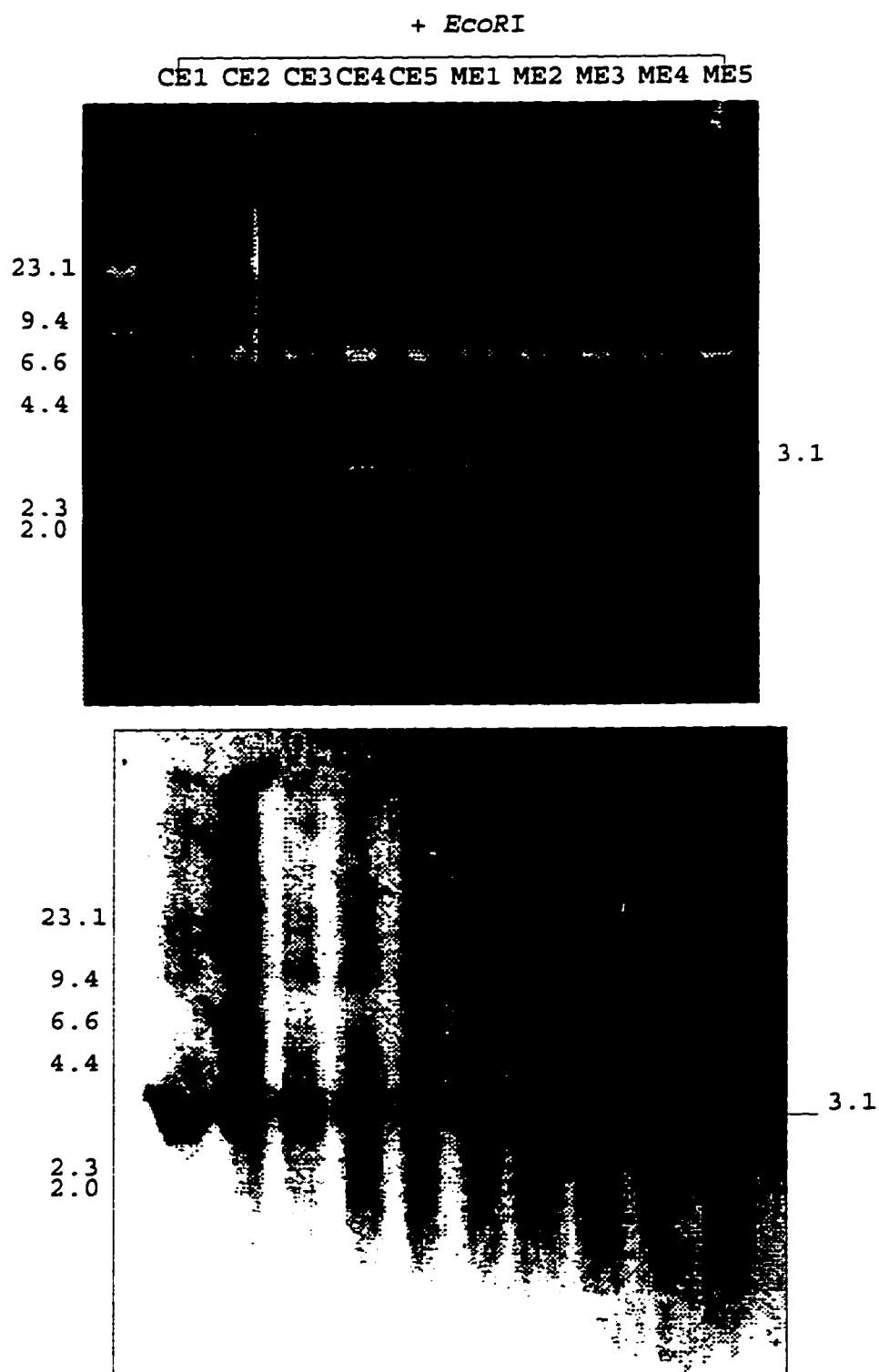


Figure 16 - Southern blot analysis of subclones of 2.5 kb subfragments in an M13/mp19 sequencing phage vector derived from the 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. The DNAs from these subclones were digested with *Bam*HI and *Eco*RI and subjected to electrophoresis. "CS" and "MS" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively, which might contain SV40 sequences. The migration of *Hind*III digested lambda phage markers is indicated in kb.

Upper panel: Ethidium bromide stained 0.8% agarose gel.

Lower panel: After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to ³²p-labeled full length SV40 genome DNA.

Subclones CS1 - CS5 and MS1 - MS4 contain subfragments with *Bam*HI/*Eco*RI ends migrating at approximately 2.5 kb which hybridized to the SV40 probe.

Figure 16

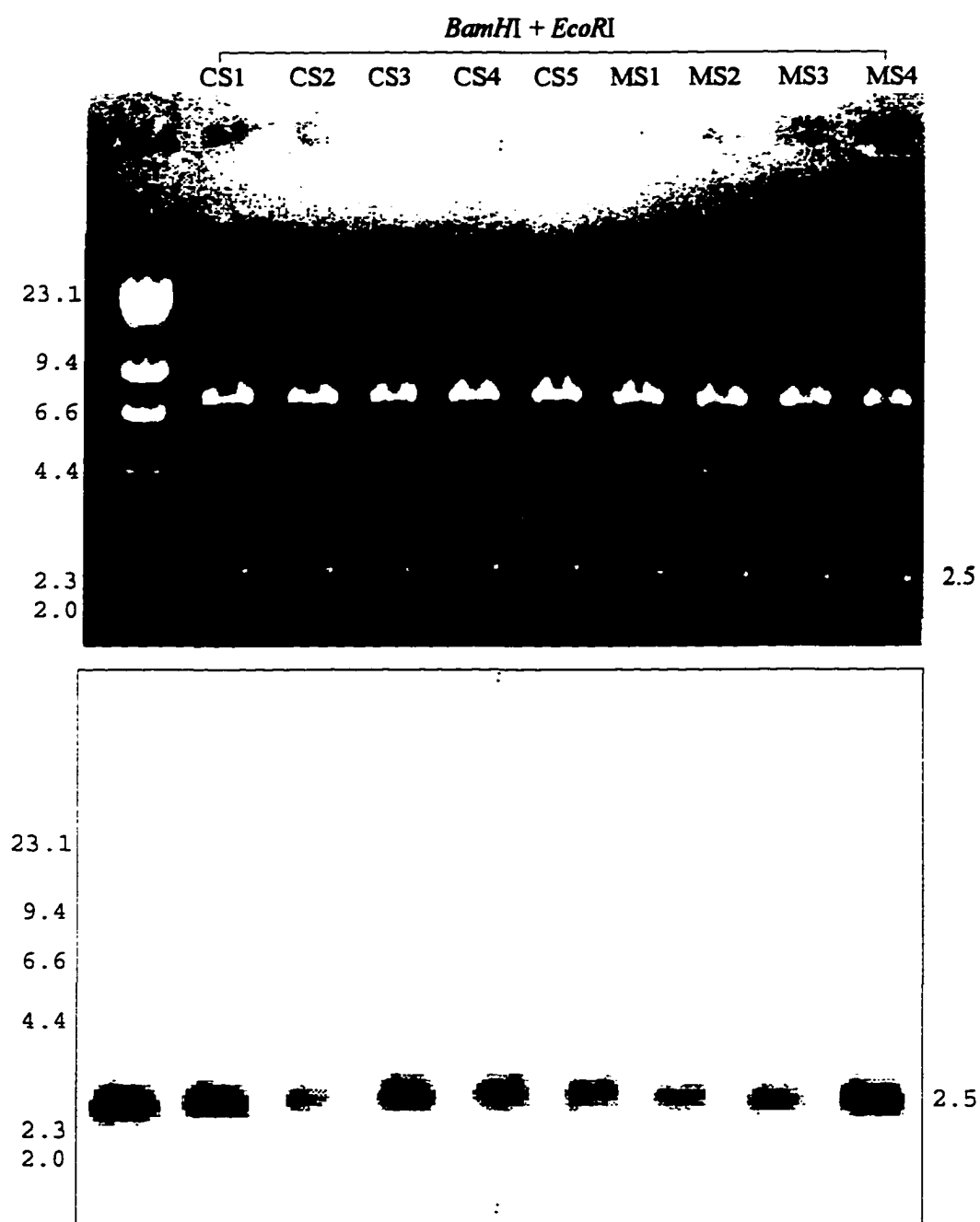


Figure 17 - Restriction analysis of 1.3 kb subfragments cloned in an M13/mp19 sequencing phage vector derived from 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. The DNAs of the subclones were digested with *Bam*HI and *Eco*RI and subjected to electrophoresis in an 0.8% agarose gel. The DNA subfragments in the gel were visualized by staining with ethidium bromide and the inserts migrating at approximately 1.3 kb can be seen. Hybridization of the blot to a ³²p-labeled full length SV40 genomic DNA showed that these subfragments were not able to hybridize to the SV40 probe.

The migration of *Hind*III digested lambda phage fragments is indicated in kb. "CH" and "MH" represent subclones derived from Ag34 cells exposed to DMSO and MNNG respectively.

Figure 17

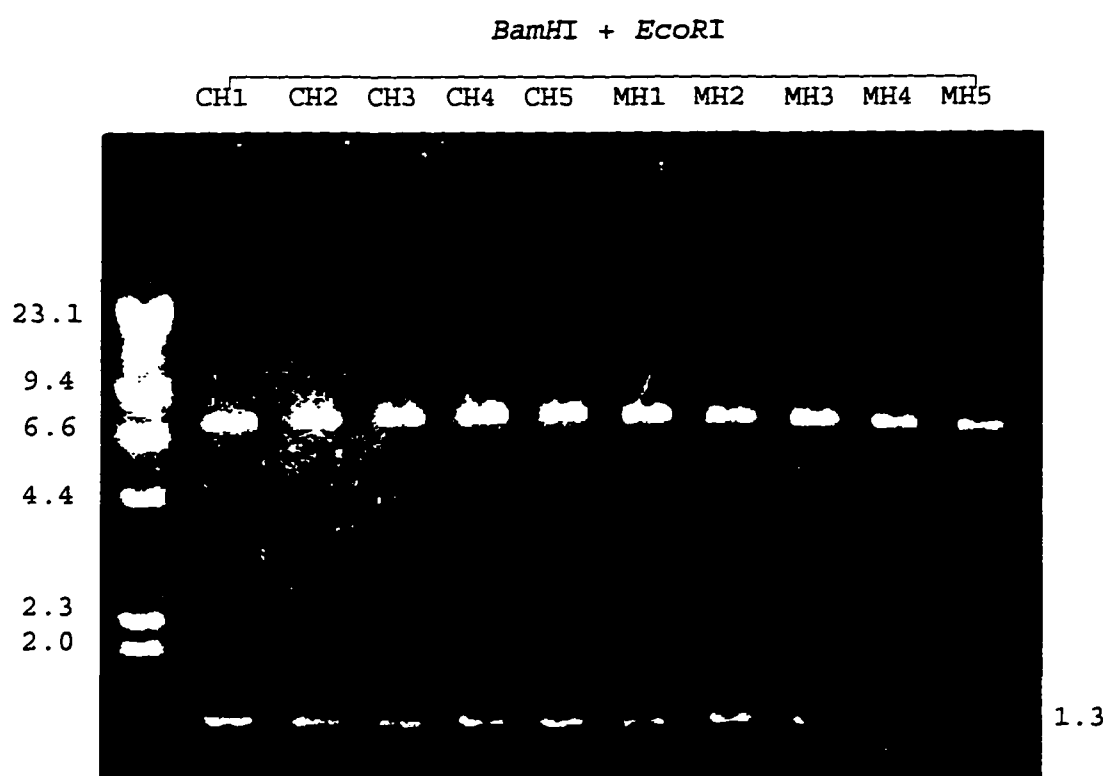


Figure 18 - Southern blot analysis of subclones of the 3.1 kb and 2.5 kb subfragments in an M13/mp19 sequencing phage vector derived from the 6.9 kb SV40-containing *Bam*HI fragments from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. The DNAs from CE and ME subclones were double digested with *Bam*HI and *Eco*RI, and the ones from CS and MS were digested with *Eco*R I alone. The digests were subjected to electrophoresis. "CE" and "ME", and "CS" and "MS" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively. The migration of *Hind*III digested lambda phage markers is indicated in kb. An ethidium bromide stained 0.8% agarose gel after electrophoresis was completed is shown in upper panel. After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to a ³²p-labeled full length SV40 probe and the result is shown in the lower panel.

Figure 18

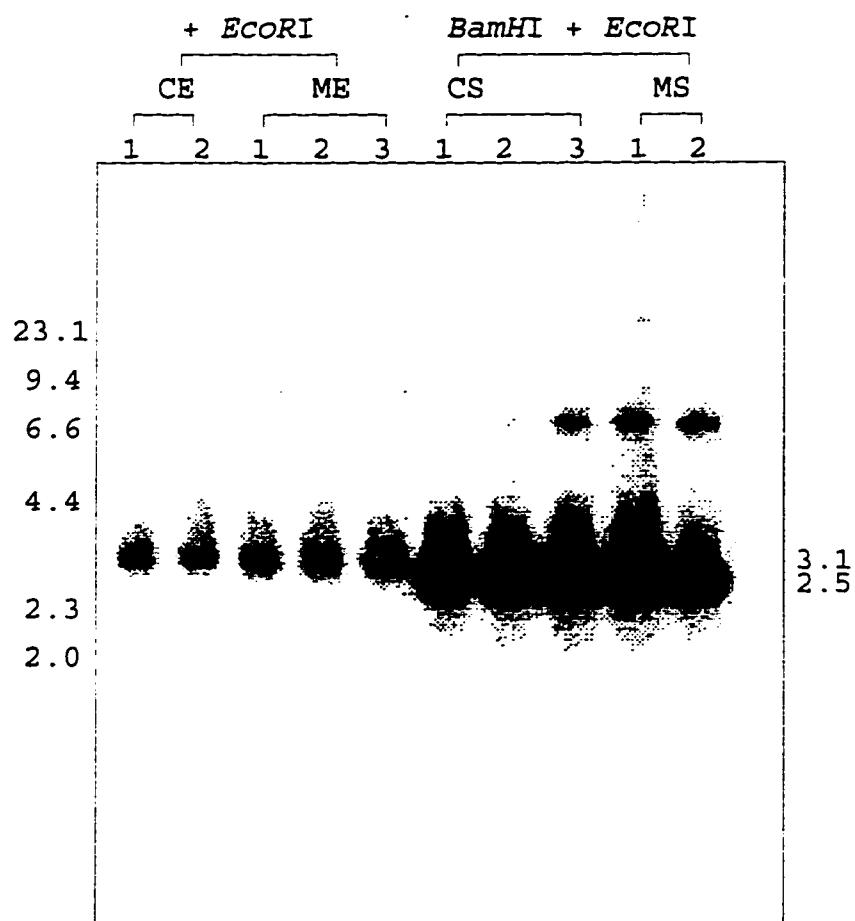


Figure 19 - Diagram of the 6.9 kb SV40-containing *Bam*HI fragment based on the information from restriction digestion of the fragment with *Bam*HI and *Eco*RI. "B" and "E" represent *Bam*HI cut sites and *Eco*RI cut sites, respectively. "CH" or "MH" represent the left handed subfragments which contain no SV40 sequences and have *Eco*RI-*Bam*HI ends; "CE" or "ME" represent the central subfragments which contain SV40 sequences and have *Eco*RI-*Eco*RI ends; "CS" or "MS" represent the right handed subfragments which contain SV40 sequences and have *Eco*RI-*Bam*HI ends.

Figure 19

B	CH/MH	E	CE/ME	E	CS/MS	B
	Human/1.3 kb		SV40 integrant/3.1 kb		SV40 integrant/2.5 kb	

Figure 20 - Southern blot analysis of subclones containing 5.2 kb and 6.9 kb SV40-containing *Bam*HI fragments in pGEM 3 and pGEM 3Z sequencing vectors derived from Ag34 cells at 4 days following exposure to MNNG at 2 µg/ml. "C" and "M" represent subclones derived from Ag34 cells exposed to DMSO and MNNG, respectively. The migration of *Hind*III digested lambda phage marker is indicated in kb.

The DNAs of the subclones were digested with *Bam*HI and subjected to electrophoresis in a 0.8% agarose gel. The upper panel shows the ethidium bromide stained 0.8% agarose gel after electrophoresis. After electrophoresis, the gel was blotted onto nitrocellulose and hybridized to a ³²p-labeled SV40 probe (lower panel).

Figure 20

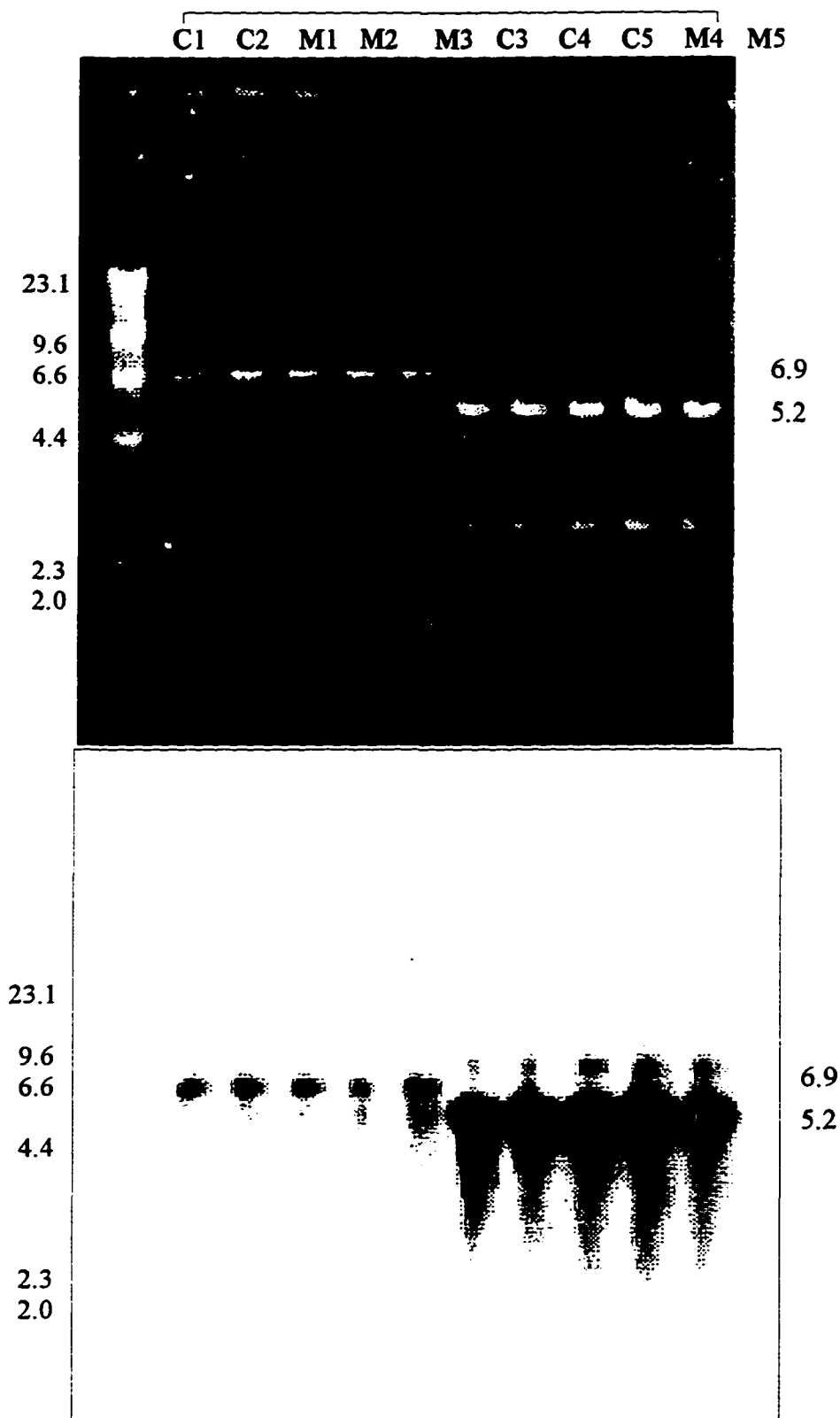
+ *Bam*HI

Figure 21 - Diagramm of the MNNG-induced base alterations on the 5.2 kb SV40-containing *Bam*HI fragment. There were 7 base alterations contained within a 24 base segment comprising T antigen binding site II in the viral origin of replication, which are deletions, insertions and substitutions.

MNNG-Induced base alterations on the 5Kb BamHI Fragment

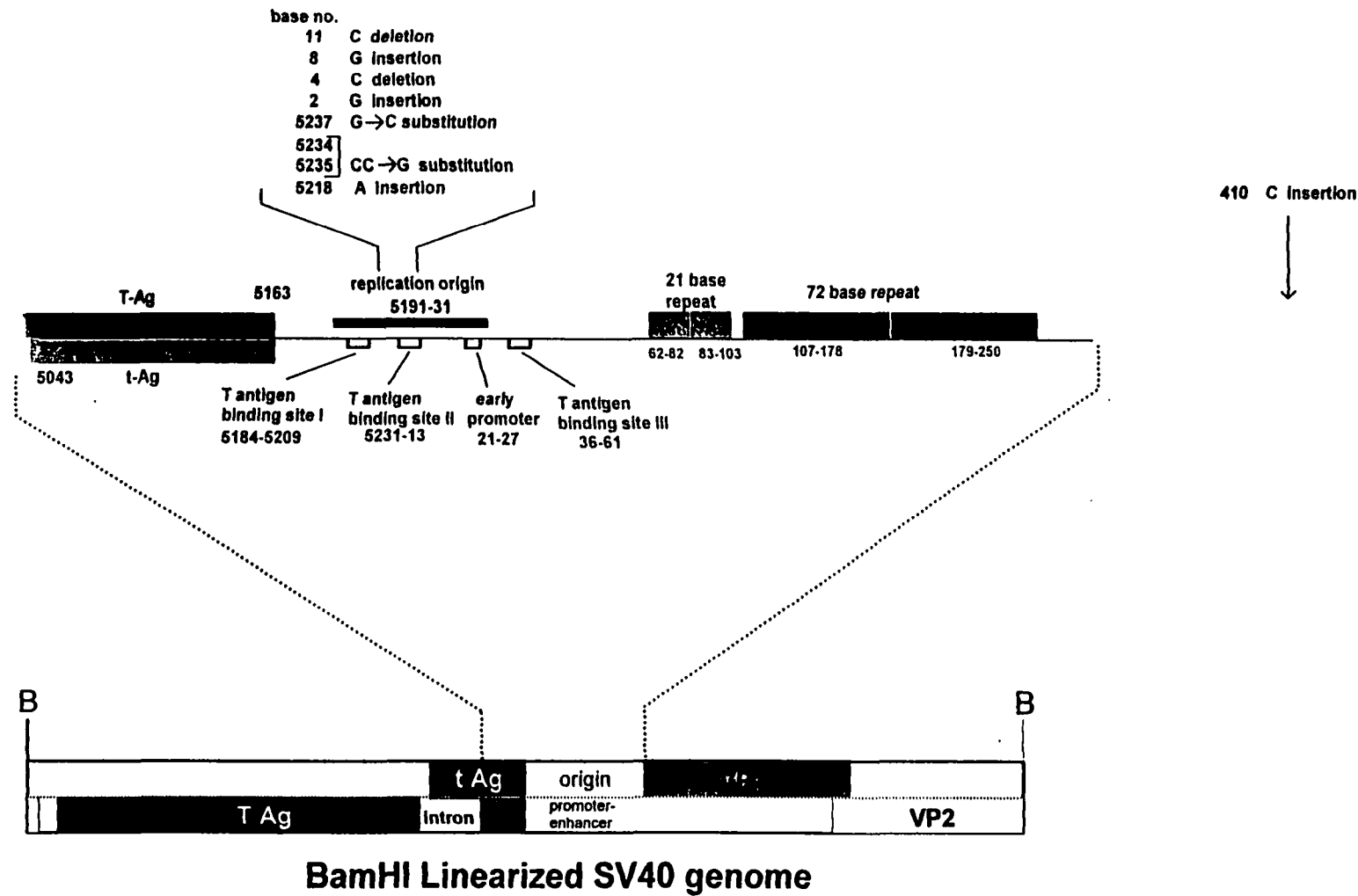


Figure 21

Figure 22 - Diagram showing the MNNG-induced base alterations on the 6.9 kb SV40-containing *Bam*HI fragment. There were 13 base alterations contained within the fragment, which are deletions, insertions and substitutions. However, none of these alterations was located in the viral origin of replication within the two small SV40 integrants on this fragment, which was seen in the 5.2 kb fragment.

MNNG-Induced base alterations on the 7Kb BamHI Fragment*

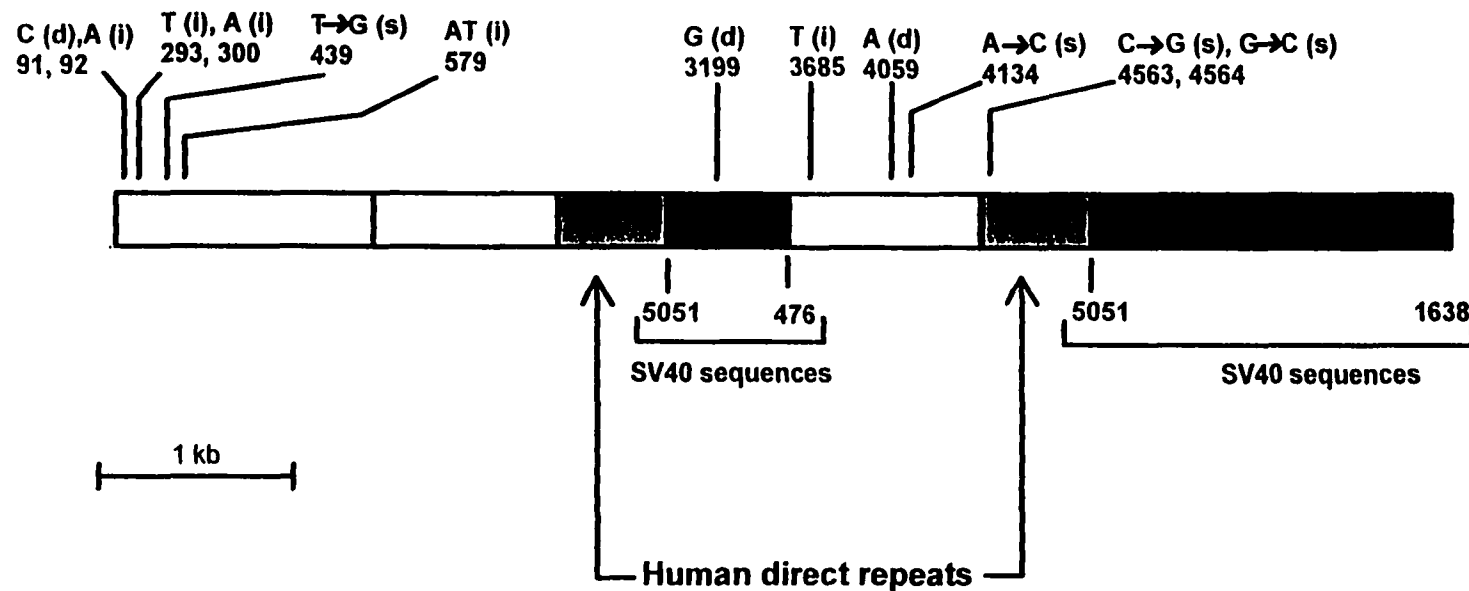


Figure 22

*GenBank accession no. U08313

Figure 23 - Diagram of the 6.9 kilobase SV40-containing *Bam*HI fragment. There are two SV40 integrants flanked by almost identical 594 bp human sequences (HR). The repeated segments are separated by a region containing unique human sequences (fragment nucleotides 3535-4476). The central integrant is contained on a 3121 base *Eco*RI-*Eco*RI subfragment. An Alu family repeat is located at nucleotides 553-666. The right hand integrant is contained on a 2458 base *Eco*RI - *Bam*HI subfragment with a small segment of 48 human sequences. The central SV40 integrant contains a region covering SV40 nucleotide 5051-476 and the right hand integrant contains SV40 nucleotides 5051-1638. Both integrants contain short segments of the early and late regions as well as the replication region (196).

"B" and "E" represent *Bam*HI and *Eco*RI cut sites, respectively.

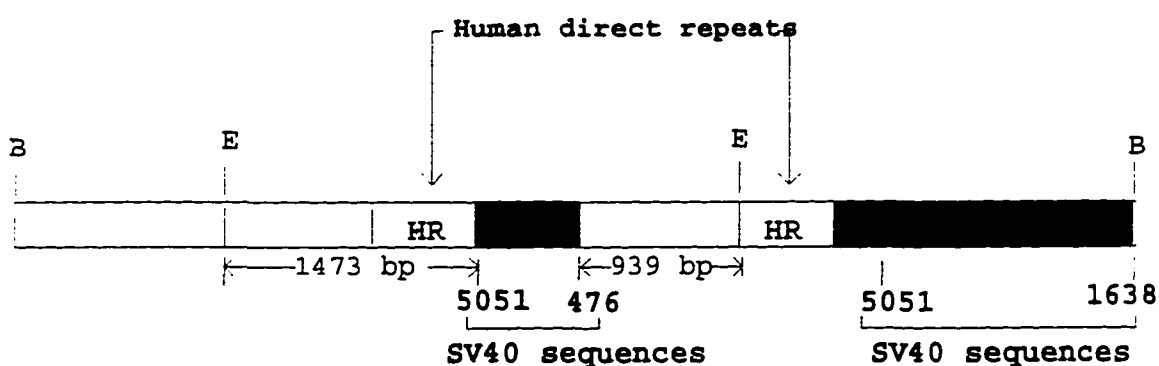


Figure 24 - Southern blot analysis of double digests of *EcoR* I-*EcoR*I subfragment on the 6.9 kb SV40-containing *Bam*HI fragment with *Saf*NI and *Dra*III. The subfragment was digested with *Saf*NI and *Dra*III to obtain the leftward and rightward human fragments flanking the central SV40 integrant. The *EcoR* I subfragment, digests of *EcoR*I-*EcoR*I subfragment with *Saf*NI and *Dra*III alone as well as with *Saf*NI and *Dra*III together were subjected to electrophoresis in 0.8% agarose gel. After the electrophoresis was completed, the gel was blotted onto nitrocellulose and hybridized to a ^{32} P-labeled probe. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated. "Eco-Eco" represents the *EcoR* I subfragment; "SfaN I" represents digest of the subfragment with *Saf*NI; "DraIII" represents the digest of the subfragment with *Dra*III; "SafNI and DraIII" represents the double digest of the subfragment. The left panel shows the ethidium bromide stained agarose gel after electrophoresis was completed. The gel was blotted onto nitrocellulose and hybridized to a ^{32} P-labeled SV40 probe. The right hand panel shows that double digests of the subfragment with *Saf*NI and *Dra*III produced two fragments migrating at approximately 1.47 kb and 0.94 kb which did not hybridized to the SV40 probe.

Figure 24

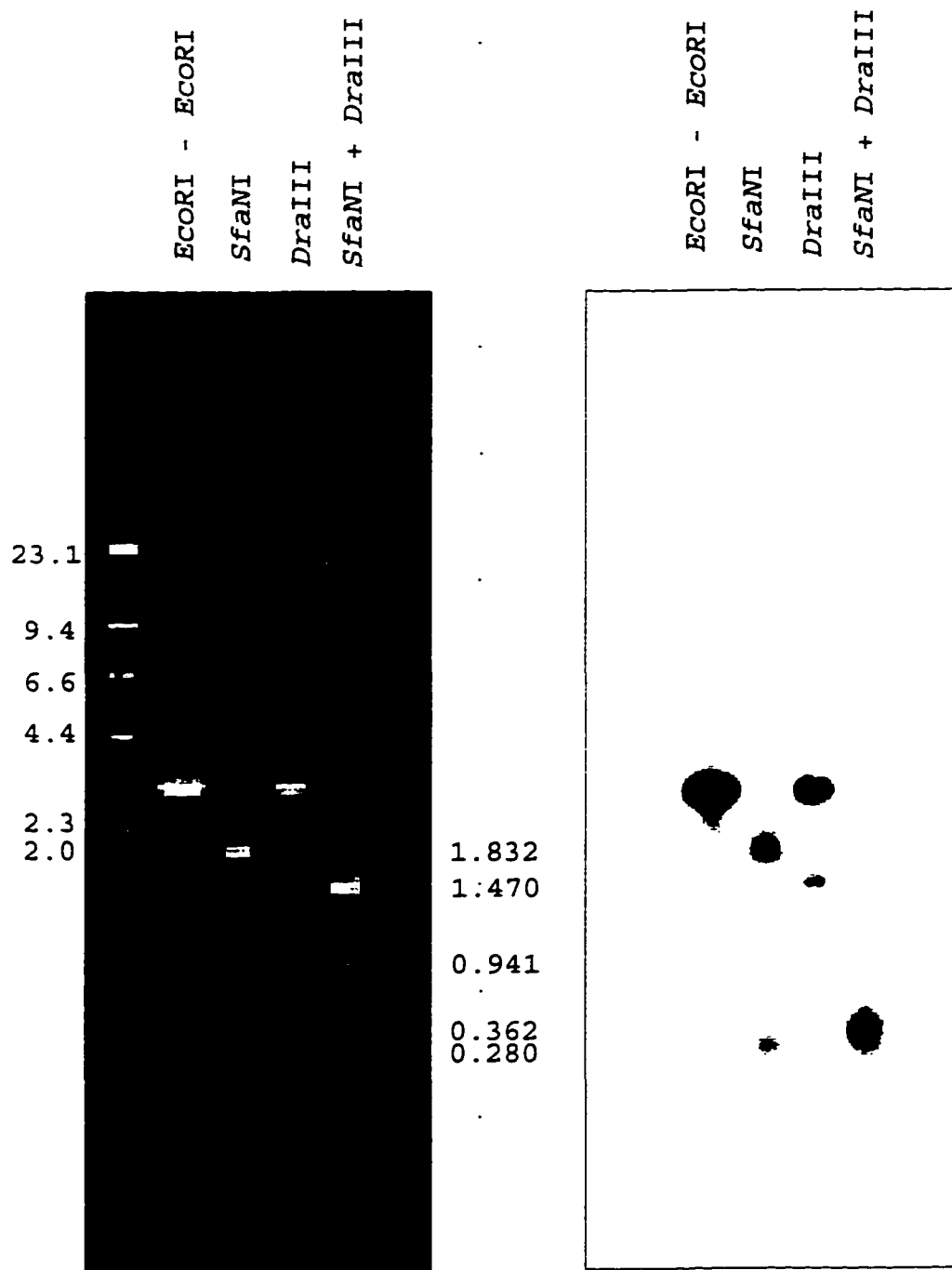


Figure 25 - Diagram of the 3.1 kb *EcoR* I subfragment on the 6.9 kb SV40-containing *Bam*HI fragment with restriction sites for *Sfa*NI and *Dra*III. There are 3 *Sfa*NI sites on the subfragment at nucleotide 1,833, 1,902 and 2,186; there is one *Dra*III site on the subfragment at nucleotide 1,470. Double digests of the subfragment with *Sfa*NI and *Dra*III produced 2 fragments migrating at approximately 1.47 kb and 0.939 kb. "HR" represents human repeat and "E" represents *Eco*RI cut sites. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated.

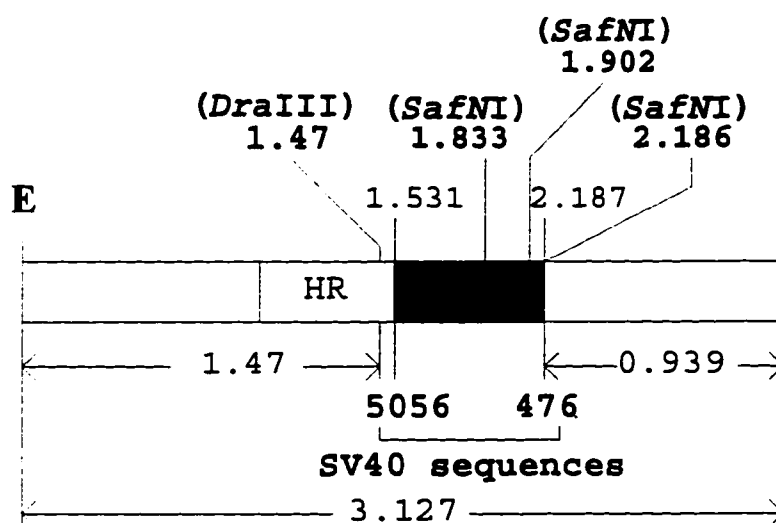


Figure 26 - Northern blot analysis of cytoplasmic RNA from Ag34 cells. Cytoplasmic RNAs from Ag34 were loaded on a 1.0% denaturing agarose gel and subjected to electrophoresis. After electrophoresis was completed, the gel was stained with ethidium bromide and then blotted onto nitrocellulose and hybridized to a ^{32} P-labeled 1.47 kb *Sfa*NI/*Dra*III human fragment flanking the central SV40 integrant within the 3.121 kb subfragment on the 6.9 kb SV40-containing *Bam*HI fragment from Ag34 cells. The migration of lambda (*Hind*III digested) markers in kilobases (kb) is indicated. "28S" and "18S" represent migration of the 28S and 18S ribosomal RNAs. The left panel shows ethidium bromide stained agarose gel after electrophoresis was completed and the right panel shows the hybridization of RNAs on the nitrocellulose.

Figure 26

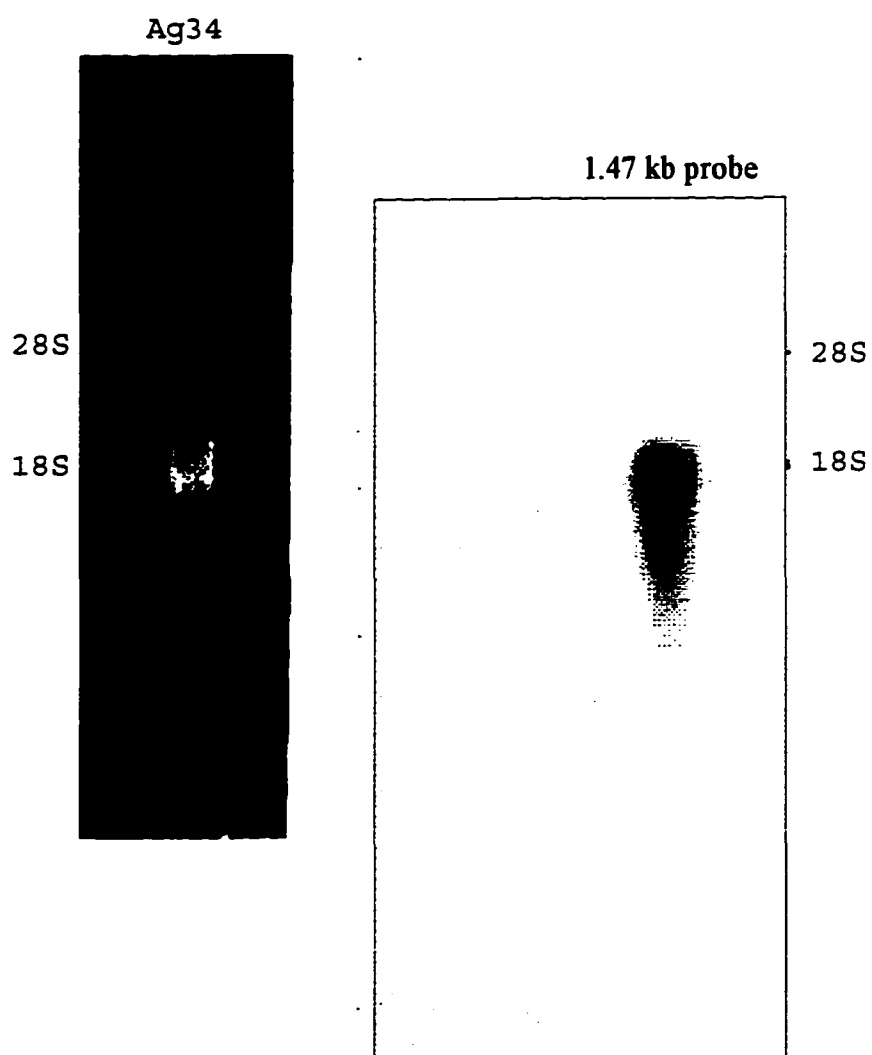


Figure 27 - Comparison of results from northern blot analysis of cytoplasmic RNAs from Ag34 hybridized to the ^{32} P-labeled 1.47 kb fragment and ^{32} P-labeled full length SV40 genomic DNA. The RNA was run on a 1% denaturing agarose gel. After the electrophoresis was completed, the gel was blotted onto nitrocellulose and hybridized to a ^{32} P-labeled 1.47 kb *EcoRI/DraIII* human subfragment and re-hybridized with ^{32} P-labeled full length SV40 genomic DNA after the 1.47 kb fragment was washed off from the blot. The left panel shows the results from northern blot analysis when the RNA was hybridized to a ^{32} P-labeled 1.47 kb *EcoRI/DraIII* human fragment flanking the central SV40 integrant within the 3.121 kb central subfragment. The right panel shows the results of northern blot analysis of the RNA hybridized to a ^{32} P-labeled SV40 probe. Two major bands were hybridized to the SV40 probe. The migration of lambda (*HindIII* digested) markers in kilobases (kb) is indicated.

Figure 27

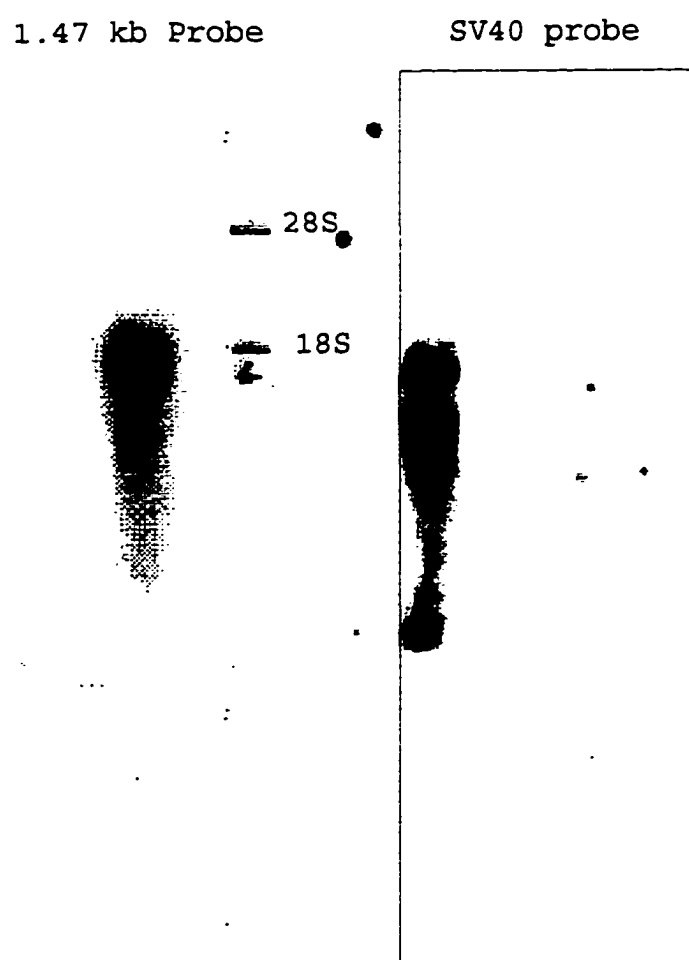


Figure 28 - Northern blot analysis of cytoplasmic RNAs from normal cultured keratinocytes Ep and SV40 transformed keratinocytes cell lines 425, 130, 98 and its clonal subline Ag34 as well as human carcinoma cell lines SCC25 and SCC324. The RNAs were run on a 1% denaturing agarose gel.

Left panel: Ethidium bromide stained 1.0% denaturing gel. 425, Ep, 98, 130, SCC324, SCC25 and Ag34 represent the RNAs from the corresponding cell lines.

Right panel: After the electrophoresis was completed, the gel was blotted onto nitrocellulose. The blot was hybridized with a ^{32} P-labeled 1.47kb *Sfa*NI/*Dra*III human leftward flanking subfragment of the central SV40 integrant on the 6.9 kb *Bam*HI fragment.

Only RNA from Ag34 cells was able to hybridized with 1.47 kb human *Sfa*NI/*Dra*III fragment. The result would imply that the 1.47 kb human fragment was able to hybridize with transcripts expressed from the promoter in the central SV40 integrant.

Figure 28

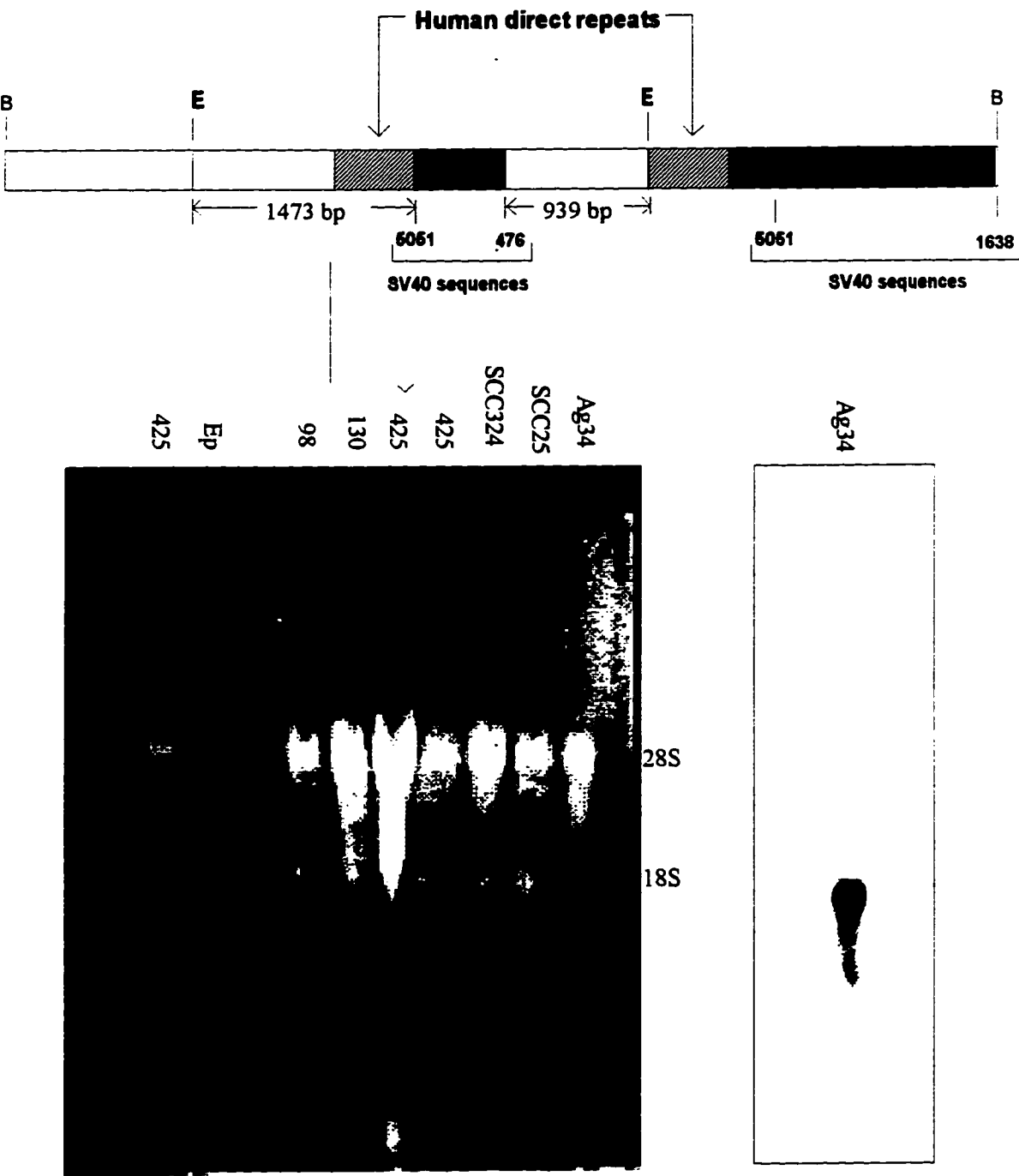
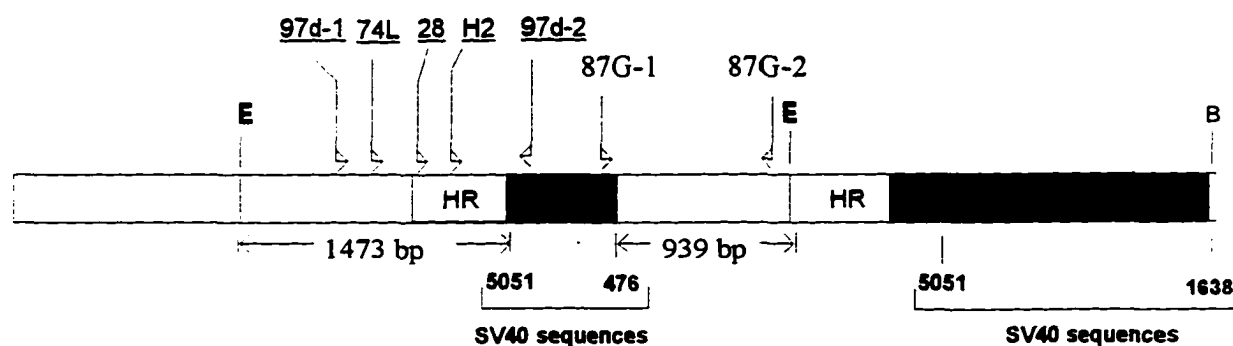


Figure 29 - Reverse transcription and PCR analysis of cytoplasmic RNA derived from SV40 transformed human keratinocytes clonal subline AG34 (196). Reverse transcribed products from cytoplasmic RNA were made using primer 74L and 97d-1, and amplified using 97D2 as amplimer, respectively. The cDNA fragments were amplified coupling with primers, H2, 28, 74L and 97d-1 which located on different positions on the rightward human sequences flanking the central SV40 integrant on *EcoRI* subfragment. The diagram below shows the position of the primers for PCR amplification. 87G-1 and 87G-2 were the primers which were not able to amplify the region surrounded by them. "HR" represents human repeats. "E" and "B" represent *EcoRI* and *BamHI* cut sites, respectively.



Primer	Position of primer on 6.9 kb Fragment	Expected cDNA
74L (for first strand cDNA)	2211 - 2227	
97D2 (amplimer)	2946 - 2966	
H2 (for PCR)	2554 - 2570	412 bp
28 (for PCR)	2398 - 2429	608 bp
74L (for PCR)	2211 - 2277	755 bp
97d-1 (for first strand cDNA)	2056 - 2075	
97d-2 (amplimer)	2946 - 2966	
97d-1 (for PCR)	2056 - 2075	910 bp

cDNA generated from the reactions using the above combination of primers were analyzed in electrophoresis as shown in the following page. Fragments 1, 2, 3 and 4 were made from primers H2, 28, 74L and 97d-1, respectively. The fragment lengths met the above expectations. "15" and "20" represent for numbers of cycles performed.

Figure 29

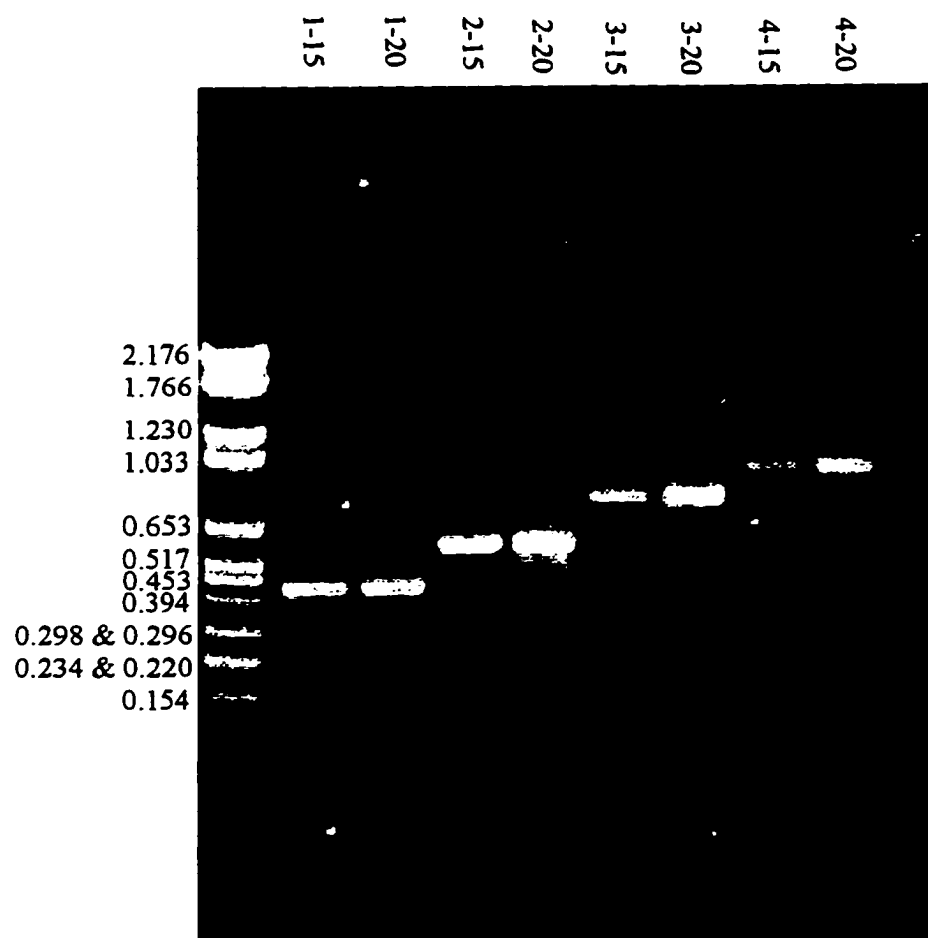


Figure 30 - Diagram of the 5.2 kilobase SV40-containing *Bam*HI fragment. The fragment contains full length *Bam*HI linearized SV40 genomic DNA. There were numerous point mutations throughout the early gene region. There was also a nine nucleotide insertion(TGTGAGGC)following SV40 nucleotide 2793(196) .

Figure 30

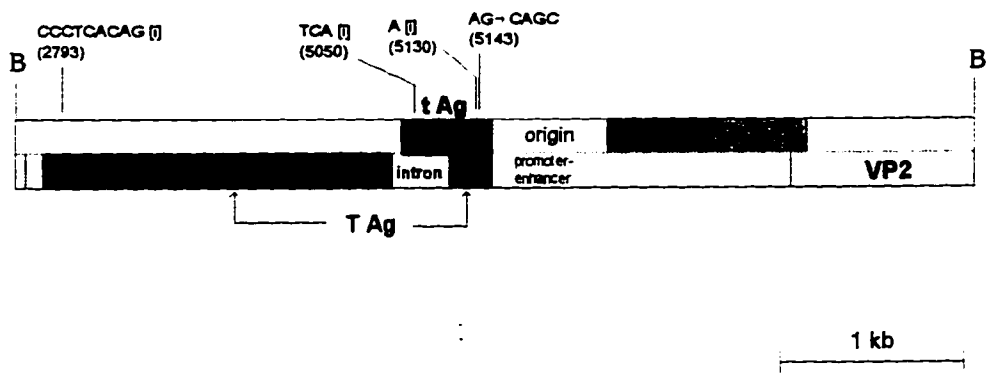


Figure 31 - A nine nucleotide insertion (underlined) near the 3' end of the SV40 early region in the 5.2 kb *Bam*HI fragment. The corresponding change in amino acid sequence is also shown (underline). The arrow indicates the position where the nine nucleotide inserts. Standard numbering of SV40 nucleotides is shown for the normal SV40 sequence (GenBank identifier: SV40CG)

2801		2787
...	GCC CCT CAG TCC TCA...	
...	Ala Pro Gln Ser Ser...	
673		677

...	GCC CCT CAG <u>CCC TCA CAG</u> TCC TCA...
...	Ala Pro Gln <u>Pro Ser Gln</u> Ser Ser...

Figure 32 - Summary of the study on the 6.9 and 5.2 kilobase SV40-containing *Bam*HI fragments. A Southern blot hybridization of an SV40 probe to *Bam*HI-digested AG34 DNA is shown at the left in which two hybridizing fragments with apparent sizes of about 5 and 7 kb can be seen. For reference, a diagram of the SV40 circular genome indicating the early (E) and late (L) gene regions and the origin/promoter region (O/P) is also shown in the bottom right; the SV40 nucleotide numbers at the viral/host junctions are parenthesized. In the diagram of the 6.9 kb fragment shown at the top right, there are two SV40 integrants flanked by almost identical 594 bp human sequences (HR). The repeated segments are separated by a region containing unique human sequences (fragment nucleotides 3535-4476). The central integrant is contained on a 3121 base *Eco*RI subfragment. The rightward integrant is contained on a 2477 base *Eco*RI-*Bam*HI subfragment. An Alu family repeat is located at nucleotides 553-666. The locations of sequences corresponding to the various primer pairs used for RT-PCR (74L/97D-2 and 87G-1/87G-2) are indicated. In the diagram of the 5.2 kb fragment at the bottom, the nine nucleotide insertion at the C terminal end of the early gene region is shown(169).

Figure 32

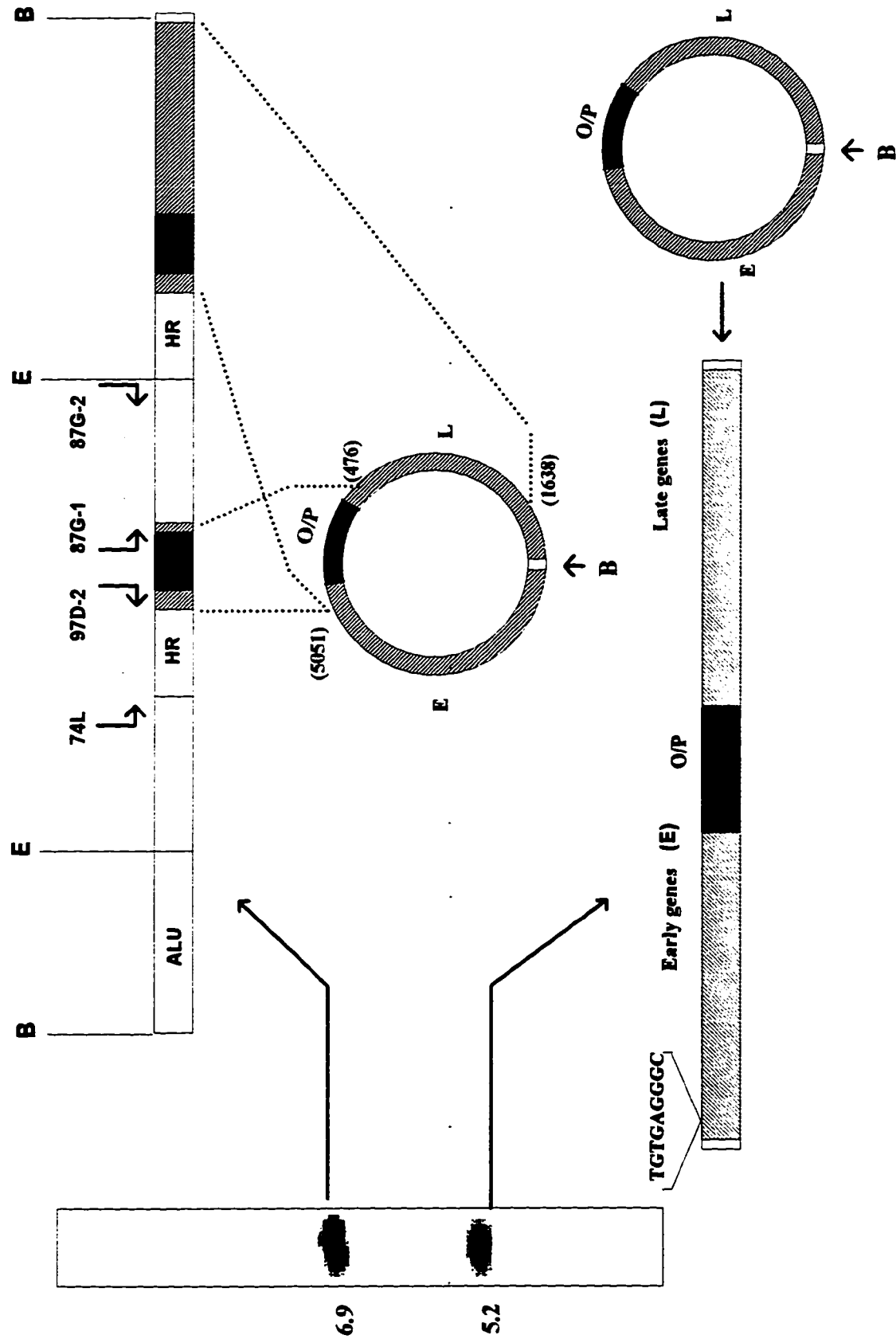
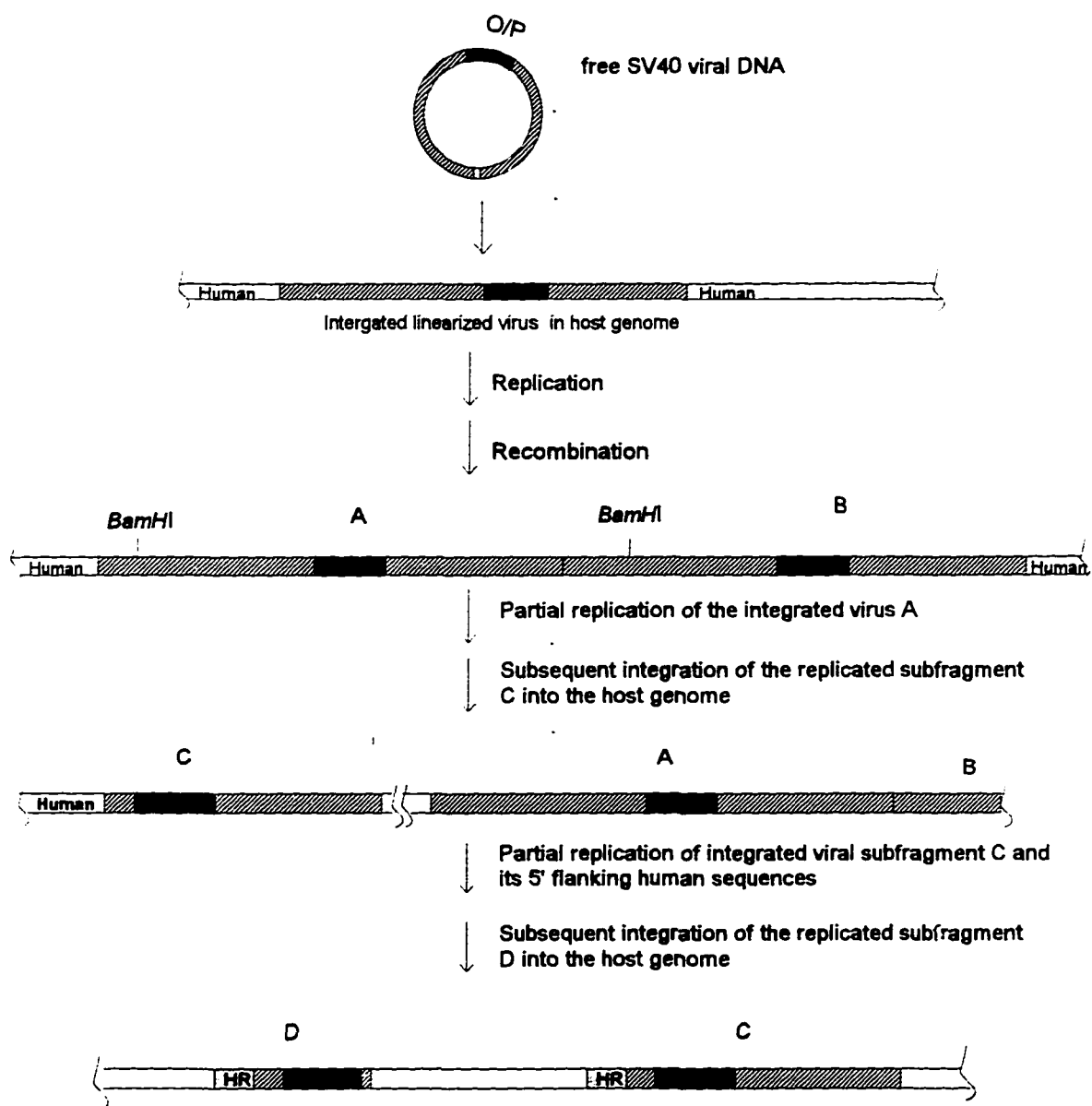


Figure 33 - Diagram of partial replication of free SV40 viral sequences, integration of the replicated subfragments into host genome and the subsequential events.



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