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Studies on chromatin, topology, and DNA replication of simian virus 40

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Ann Arbor, MI 48106

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STUDIES ON CHROMATIN, TOPOLOGY, AND DNA REPLICATION OF SIMIAN VIRUS 40

by

Yi Chu

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the the requirements for the Degree of Doctor of Philosophy The City University of New York

1992

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ABSTRACT

Studies on Chromatin, DNA Topology, and DNA Replication of Simian Virus 40

by

Yi Chu

Advisor: Prof. Ming-Ta Hsu

Simian virus 40 (SV40), a member of the small DNA tumor virus family *Papovaviridae*, has provided an excellent experimental system to study the molecular biology of gene expression and DNA replication in mammalian cells. Encoding only a few proteins, the virus depends extensively on the host machinery for its replication. In this thesis, three original findings concerning the SV40 chromatin, DNA topology, and DNA replication are described.

Chapter 2 describes the establishment of P1 nuclease as a probe for active SV40 chromatin. SV40 exists in infected cells in the minichromosome configuration that is virtually indistinguishable from that of cellular chromatin. P1 nuclease was found to cut a small subpopulation of intracellular SV40 DNA only once under the exhaustive digestion condition. The cleavage site has been mapped to either the origin of DNA replication or transcription enhancers. The P1-sensitive fraction was found to have higher superhelical density than the bulk of intracellular SV40 DNA. Newly synthesized viral chromatin as well as replication intermediates were preferentially cleaved by P1 nuclease.

Correlation between P1-sensitivity and the biological activity of SV40 origin of replication was established using SV40 variants containing two origins of replications. In *in*(Or)1411 and *in*(Or)1412 DNA which contain two functional origins, either of the two origins was sensitive to P1 cleavage. On the other hand, the mutated and nonfunctional origin in the virus *in*(Or)1415 was not cleaved by P1 nuclease. These results indicate that P1 nuclease can be used to probe the structure of active chromatin. A major advantage of this method over the conventional DNase I method is that P1 only makes a single cleavage and then stops, whereas DNase I further degrades chromatin to small fragments. P1 nuclease, therefore, is useful to define the active chromatin fraction with altered chromatin conformation.

Chapter 3 points to another aspect of the structure of newly synthesized SV40 chromatin. It was found that superhelical density of DNA in the newly synthesized SV40 chromatin is greatly increased by ellipticine, an intercalating agent and a topoisomerase II inhibitor. Several types of evidence indicate that the increase of superhelical density is due to the intercalation of ellipticine followed by fixation of DNA topology by a topoisomerase. The density of intercalation, estimated to be one ellipticine per 10-20 base pairs, suggests that ellipticine can intercalate within the nucleosome core of newly synthesized SV40 chromatin. This result is consistent with the interpretation that newly synthesized chromatin contains a loosened nucleosome structure which upon maturation becomes inaccessible to ellipticine intercalation.

Chapter 4 of the thesis describes the study on the regulation of SV40 DNA replication in SV40 variant viruses containing two functional origins of replication (*ori*). Using an *in vitro* nuclei replication assay we observed that the two *ori*s in the SV40 variants *in*(Or)1412 and *in*(Or)1411 are not used with equal efficiency. Based on the locations of the two origins in the DNA we investigated the following possibilities for their differential usage: 1) Sequences immediately surrounding the two *ori*s affect their usage. This possibility was eliminated by comparing the efficiency of replication of recombinant clones containing either *ori* and its surrounding sequences in a transient DNA replication assay; 2) Association with an intact transcription unit activates the *ori*. This possibility was eliminated using the similar assay; 3) Transcription passing through the second, inserted *ori* interferes with the initiation from this origin. Attempts of using human cytomegalovirus (HCMV) major immediate early gene promoter/enhancer (P/E) to study transcription interference of replication unexpectedly revealed a strong orientation- and position-dependent suppressing effect of the HCMV P/E on SV40 *ori*. Deletion analyses indicate that it is the enhancer elements that exert the negative effect on SV40 DNA replication. The transcription-interference hypothesis was further tested using a variant of *in*(Or)1412 with the two *ori*s located at the 3' end of a transcription unit. The results of transfection experiments are consistent with this model.

ACKNOWLEDGEMENTS

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CHAPTER 1. INTRODUCTION

I. General biology of simian virus 40

Simian virus 40 (SV40) is a small, icosahedral virus isolated from the rhesus monkey (Sweet and Hilleman, 1960; Liddington *et al.*, 1991). It is non-oncogenic in its natural host, but it induces tumors in newborn rodents (Eddy *et al.*, 1962; Girardi *et al.*, 1962) and transforms cultured cells from a variety of species (Todaro and Green, 1964; Black, 1966). SV40, mouse polyoma virus, human JC and BK viruses, together with papillomaviruses constitute the small DNA tumor virus family, *Papovaviridiae* (Melnick *et al.*, 1974; Tooze, 1980; Eckhart, 1990; Shah, 1990).

The genome of SV40 is a circular double-stranded DNA of 5,243 base pairs (Fig. 1A). It encodes six proteins: two early gene products, large T and small t antigens, and four late gene products, VP1, VP2, VP3 and agnoproteins. The large T antigen is required both for SV40 DNA replication and late gene expression, and is responsible for the transforming ability of SV40. The function of small t antigen appears not to be essential during lytic infection in tissue culture but its role in cell transformation and *in vivo* replication is still not clear. VP1 is the major capsid protein and is responsible for the attachment of SV40 to cells. VP2 and VP3 are minor capsid proteins. The agnoprotein is a small and basic protein. Its function is believed to facilitate the packaging and spreading of SV40 virus particles (Carswell and

Alwine, 1988).

Expression of both early and late proteins and initiation of SV40 DNA replication are regulated by a common regulatory region depicted in Fig. 1B. This region contains the origin of DNA replication, promoters and an enhancer for both early and late transcription. Late in SV40 infection, this regulatory region is found to be devoid of nucleosome organization in about 25% of the intracellular viral chromatin (Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980).

The outcome of SV40-cell interactions depends on host cells. In African green monkey kidney cells (*e.g.*, CV-1 cells), SV40 undergoes productive infection resulting in the production of new infectious virus and cell lysis. In mouse cells, however, SV40 infection is abortive. Only the early genes are expressed and no progeny virus is produced. Human and hamster cells are semipermissive for SV40: virus production is slower than in permissive monkey cells and only a small amount of progeny viruses is produced. One of the factors that determine the permissiveness of host cells to SV40 virus infection is the successful interactions between SV40 origin of DNA replication (*ori*), large T antigen and cellular factors. For instance, cellular DNA polymerase α-primase (Murakami *et al.*, 1986a; 1986b) and p53 proteins (Wang *et al.*, 1989; Friedman *et al.*, 1990) have been shown to contribute to host permissiveness.

As in all non-enveloped viruses, the entry of SV40 into host cells is through receptor-mediated endocytosis (Marsh and Helenius, 1989). While VP-1 has been shown to be responsible for the attachment of the virus to cells

(Shah, 1990), little is known about the cellular receptor for SV40. Pharmacological studies suggested that the cellular receptor for SV40 contains protein and O-linked carbohydrates (Clayson and Compans, 1989). Interestingly, binding competition experiments showed that SV40 and its close relative polyomavirus do not share the same receptor (Clayson and Compans, 1989). With polarized epithelial cells, SV40 binds only on the apical but not on the basolateral surface (Clayson and Compans, 1988).

Following entry of SV40 into cells, virus uncoating occurs in the cell nucleus. Subsequently, "early" viral RNA encoding T/t antigens appears prior to and independent of the onset of viral DNA replication. Following the expression of the early genes, cell DNA synthesis and the activities of cellular enzymes related to DNA synthesis are stimulated.

In the lytic infection, SV40 DNA replication starts around 12 hours post infection (hpi), peaks around 36 hpi, and continues until cell death. After DNA replication commences, the "late" viral RNA encoding VP-1, VP-2, VP-3 and agnoproteins are synthesized. The late structural proteins, together with the amplified viral DNA, are then assembled to form mature virus particles (Eckhart, 1990; Tooze, 1980).

Virus assembly occurs in the nucleus. The 75 S viral minichromosomes are initially condensed into a 180 S previrion, then further condensed into young virions, which mature into stable 220 S virions (Coca-Prados and Hsu, 1979; Fanning and Baumgartner, 1980; Garber *et al.*, 1979). Following virus

assembly cell lysis occurs with the release of 10^4-10^5 infectious progeny. This ends the infection cycle.

One of the most attractive features of SV40 biology is its ability to transform cells and induce tumor in animals. The recent demonstration of the interactions of T antigen with a number of tumor suppressor genes (*e.g.*, Paul and Schmidt, 1989; Zhu *et al.*, 1991 and references therein) makes SV40 an excellent model for studying the mechanism of tumorigenesis. Due to the space limitation, this subject is not further reviewed in this thesis.

II. Chromatin organization and nuclease hypersensitivity

Historically, chromatin was a cytologic entity readily stainable with basic dyes (Flemming, 1880). It became meaningful only after the nucleosome structure was elucidated in the 1970s (Finch *et al.*, 1977; Richmond *et al.*, 1984). The nucleosome constitutes the basic unit of the eukaryotic chromatin. A nucleosome core is composed of an octomer of histones (two molecules each of H2A, H2B, H3 and H4 histones) wrapped left-handedly about 1.75 turns by a double stranded DNA segment of 146 base pairs (bp) in length. In the chromatin fiber, the basic nucleosome cores are linked by a DNA segment of varying lengths (8-114 bp), depending on the cells. Histones H1 or H5 histone are specifically associated with the linker DNA (Lewin, 1990).

Cellular chromatin is packaged into higher-order structures in the cell nucleus. The basic level of organization is the 10 nm fiber consisting of a

string of nucleosomes organized edge to edge, stacked along the fiber or tilted less than 20^0 to the axis (Thoma *et al.*, 1979). The resulting packing ratio is 6. This fiber is then coiled into a 30 nm fiber resulting in another packing ratio of 40. The 30 nm fiber may have a helical coil of 6 nucleosomes per turn, organized radially (Finch and Klug, 1976). To make a mitotic chromosome from the 30 nm fiber, two further orders of magnitude of compaction are generated by specific non-histone proteins which form the skeleton or scaffold of the chromosome (Paulson and Laemmli, 1977). Electron microscopy of histone-depleted metaphase chromosomes suggests that cellular DNA is organized in superhelical loop-domains. Each loop-domain of the chromatin is thought to be attached to the scaffold at its base (Benyajati and Worcel, 1976). Interestingly, subsequent studies have shown that one of the scaffold proteins is topoisomerase II (Lewis and Laemmli, 1982; Earnshaw and Laemmli, 1983).

Considering the fact that in higher eukaryotes only 5-10% of the genome is transcribed (Davidson, 1976), it is not surprising to find that this fraction of the "active" chromatin exhibits structures different from that of the bulk chromatin. In general, active chromatin shows both an increased overall sensitivity to endonucleases like DNase I and micrococcal nuclease and the presence of specific nuclease hypersensitive sites. Frequently, the nuclease hypersensitive sites are present immediately upstream of the transcription initiation site in the sequences that are crucial for the promoter function (Elgin, 1981; Eissenberg *et al.*, 1985; Pederson *et al.*, 1986; Gross and Garrard,

1988).

SV40 has been used as a model system for studying the relationship between chromatin organization and gene expression. It was in the SV40 minichromosome that the existence of nuclease hypersensitivity sites in the regulatory region was first demonstrated. Taking advantage of the SV40 variant *in*(Or)1411 containing an additional DNase I hypersensitive segment (Shenk, 1978; for our result, see Appendix of Chapter 2), Scott's group made a series of deletions within the 273 bp insert and tested for their DNase I hypersensitivity (Gerard *et al.*, 1982; 1985). Since the ratio between the DNase I cleavage of the two DNA segments is about 1:1 in the parent *in*(Or)1411, they were able to evaluate contributions of various sequences within the 273 bp region to DNase I hypersensitivity. Overall, no single short deletion resulted in complete loss of nuclease sensitivity, suggesting that more than one genetic element is responsible for the DNase I hypersensitive chromatin structure (Scott, 1988).

How nuclease hypersensitivity is formed and maintained in a genome is still not well understood (Weintraub, 1985; Yaniv and Cereghini, 1986). Several possibilities have been suggested: (1) the specific sequence of the active genes may disfavor nucleosome formation; (2) modifications of the histone proteins associated with these active genes, such as acetylation and ubiquitination, may cause change in nucleosome organization; and (3) preferential association of non-histone proteins, such as transcription factors

or HMG14 and 17, with active genes (for review, see van Holde, 1989; Lewin, 1990).

Notwithstanding our poor knowledge of its mechanism, DNase I hypersensitivity has been used as an efficient method to probe active genes. In this thesis, I shall describe a new method using P1 nuclease to probe active chromatin. The advantage of the P1 method is discussed in Chapter 2.

III. DNA topology and biological function

When the ends of a base-paired linear double-stranded DNA molecule are joined, the two strands of the DNA become topologically linked. The parameter characteristic of this physical condition is called linking number. The linking number is the summation of the twists (the number of crossovers between two single strands in the duplex) and the writhing number (the number of crossovers between the duplex axes in space) (for review, see Cozzarelli *et al.*, 1990). When the writhing is created due to underwinding of the duplex, it is of negative value (negatively supercoiled). When the writhing is due to overwinding of the duplex, it is of positive value (positively supercoiled). To change DNA linking number, the action of topoisomerases is required. By transiently breaking a DNA strand and passing another strand through the transient break (topoisomerase I, topoI for short) or by transiently breaking a pair of complementary strands and passing another double-stranded segment (topoisomerase II, topoII for short) followed by resealing the

breaks, these enzymes can catalyze many types of topological changes of DNA (for review, see Gellert, 1981; Wang, 1985; Vosberg, 1985).

In prokaryotes, topoI acts to relax negative supercoils, and gyrase (a topoII) acts to add negative supercoils. These two enzymes are regulated homeostatically through the degree of DNA supercoiling. The net result is a negatively-supercoiled genome. In eukaryotes, the corresponding gyrase activity has not been found. The negative supercoiling of the extracted DNA, as clearly demonstrated by some eukaryotic viruses, is the net result of nucleosome organization prior to DNA extraction (Germond *et al.*, 1975). The eukaryotic topoI relaxes both negative and positive supercoils. TopoII also relaxes, but does not add, negative or positive supercoils to DNA.

Mechanistically, eukaryotic topoI covalently links to the 3' phosphoryl group of DNA in the transient cleavage reaction (Champoux, 1977; Hsiang *et al.*, 1985), while prokaryotic topoI covalently links to the 5' phosphoryl group (Depew *et al.*, 1978). On the other hand, both eukaryotic and prokaryotic topoII link to the 5' phosphoryl group in the cleavage intermediate (Hsieh, 1990). The transient cleavage intermediates of DNA by topoI or topoII can be stabilized by topoisomerase inhibitors in the presence of protein denaturants (Drlica and Franco, 1988; Liu, 1990). Hence, topoisomerase inhibitors are useful in mapping the location of topoisomerase cleavage sites.

Camptothecin is the only well-characterized topoI inhibitor. At a concentration of 25 uM, camptothecin traps more than 85% of cellular topoI

(10^5-10^6/cell) on chromosomal DNA (Hsiang and Liu, 1988). These breakage sites have been mapped within the transcribed region of active genes (Gilmour and Elgin, 1987; Rowe *et al.*, 1987; Stewart and Schutz, 1987; Zhang *et al.*, 1988). These mapping results are consistent with immunological studies that show a strong association of topoI with actively transcribed genes (Gilmour *et al.*, 1986). In addition to its functional involvement in transcription, topoI has also been shown to be required in the elongation step of DNA replication (Snapka, 1986; Yang *et al.*, 1987).

Many topoII inhibitors have been identified. They can be generally categorized into two groups: DNA intercalators and nonintercalators (Liu, 1990). Some nonintercalators such as etoposide (VP-16) and teniposide (VM-26) induce significant double-stranded DNA cleavage. Among intercalators are strong inducers of DNA cleavage, including daunomycin and *m*-AMSA, whereas others such as ellipticine and actinomycin are weak inducers. TopoII has been found to be associated with nucleosome-free regions, but not in actively transcribed regions (Yang *et al.*, 1985; Rowe *et al.*, 1986). Active transcription alters the distribution of the topoII cleavage site on *Drosophila hsp70* genes (Rowe *et al.*, 1986). Although the role of topoII in transcription is not established, it is certain that topoII is essential in late stages of DNA replication and in segregation of daughter chromosomes (Yanagida and Sternglanz, 1990).

The biological importance of negative superhelicity, chosen by nature,

is easily understood when one envisions that the very first step in both DNA replication and transcription is the unwinding of the DNA duplex at the origin of replication and the promoter, respectively. Negative supercoiling is the topological equivalent of unwinding. Thus, with an *in vitro* replication system, the efficiency of DNA replication is at least one magnitude higher by using negatively supercoiled DNA as substrate than by using the linear form (Kelly, 1990).

Since DNA superhelicity is closely related to the three-dimensional winding of DNA in chromatin, monitoring DNA topology in a simple molecule like SV40 may help understand the structural organization of different chromatin fractions. Chapter 3 describes experiments using this approach.

IV. SV40 DNA replication: biochemical mechanism

SV40 is the most extensively characterized replicon in eukaryotic cells (for review, see DePamphilis and Bradley, 1986; Challberg and Kelly, 1989; Stillman, 1989). Several advantages of using SV40 for studying DNA replication contribute to its popularity: (1) It contains a small genome (5,243 bp); (2) Its genome is complexed with histones to form a nucleosomal structure similar to cellular chromatin; and (3) Only one viral protein, T antigen, participates in DNA replication; the other components of the replication machinery must be derived from host cells. This makes the study highly relevant to the replication processes in the cells.

The minimal *cis* requirement for SV40 DNA replication is a 65 bp core origin of DNA replication (*ori*, #5210-#31), identified by deletion and point mutation analyses (Deb *et al.*, 1986; Tegtmeyer *et al.*, 1988, and references therein). The core origin is situated inside the regulatory region of the SV40 genome. This region includes a transcription enhancer (two copies of 72 bp repeat), bi-directional promoters (21 bp repeats), and T-antigen binding sites, one of which overlaps with the core origin. The regulatory region is organized in a nucleosome-free conformation in about 25% of the total SV40 chromatin at late times of viral infection (Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980), and is hypersensitive to nuclease cleavages (for review, see Lewin, 1990). The special chromatin conformation at the origin may be crucial for its function since a mutation that inactivates replication also abolishes sensitivity to P1 nuclease digestion (see Chapter 2). The biological activity of the core origin is also influenced by the flanking sequences (see the section below).

Genetic analysis shows that large T antigen is the only viral gene product required for viral DNA replication (Tegtmeyer, 1972). T antigen is a multifunctional phosphoprotein of 708 amino acids. Phosphorylation of T antigen at threonine-124 by CDC2 kinase was recently found to be required for its activity in SV40 DNA replication (McVoy *et al.*, 1989; for review, see Stahl and Knippers, 1987). On the other hand, dephosphorylation by protein phosphatase 2A of T antigen at serine-120 and serine-123 increases *in vitro* DNA replication by 5-fold (Kelly, 1990). T antigen is involved both at the

initiation and the elongation steps of DNA replication.

Studies of SV40 DNA replication in the cell-free system established by Li and Kelly (1984) have elucidated the biochemical steps and host factors involved in SV40 DNA replication. The first step of initiation is the binding of T antigen to the GAGGC motifs at the core origin to initiate DNA replication (Tjian, 1978; DePamphilis and Bradley, 1986). T antigen binding (eventually forming a double hexamer) causes conformational changes in the early palindrome and AT-tract in the core origin. The bound T antigens unwind the origin in the presence of cellular topoisomerase I, single-stranded DNA binding protein (SSB) and ATP (Wobbe *et al.*, 1987; Stillman, 1989). The unwound origin is now poised for DNA synthesis by cellular DNA polymerases. (Borowiec *et al.*, 1990 and references therein).

It is generally agreed that two, instead of only one (as originally thought), types of DNA polymerase participate in SV40 DNA replication. DNA polymerase α-primase serves first to generate an RNA primer (12-16 bp) and subsequently to initiate DNA synthesis in the leading strand. As soon as replication forks are mobilized, DNA polymerase δ replaces polymerase α-primase in directing leading strand synthesis, while the latter starts to serve in lagging strand synthesis (Downey *et al.*, 1988; Tsurimoto *et al.*, 1990). The processivity of polymerase δ is significantly promoted by its accessory factor, proliferating cell nuclear antigen (PCNA) (Prelich and Stillman, 1988). In the elongation step, T antigen which acts as a helicase, cellular SSB and

topoisomerases are continually required along with polymerase activities. The final step in SV40 DNA replication is the separation of the replicated daughter molecules (Sundin and Varshavsky, 1980; 1981); this requires cellular topoisomerase II activity, which was also demonstrated by Yang *et al.* (1987) in *in vitro* experiments.

V. SV40 DNA replication: modulation of origin activity by sequence microenvironment

It has been keenly observed that in both prokaryotic and eukaryotic cells the core origin of replication is closely associated with transcription promoters and enhancers (see for example, DePamphilis, 1988). The role of the transcription regulatory elements in DNA replication is still not well understood. The interactions seem to be rather complex because both positive and negative effects of transcription elements on DNA replication have been reported.

In SV40, the core origin is also flanked by transcription regulatory sequences. The transcriptional control sequences of the SV40 genome are organized as two sets of direct repeats. The GC-rich 21-bp repeat present in three copies is a major component of the early promoter (Everett *et al.*, 1983; Fromm and Berg, 1982; Hansen and Sharp, 1983). The 72 bp repeat present in two copies serves as a position and orientation-independent enhancer of transcription (Banerji *et al.*, 1981; Moreau *et al.*, 1981). The role of these

elements in SV40 DNA replication has been investigated by several laboratories. Deletion analysis has revealed two flanking sequences that can influence the activity of SV40 origin. Auxiliary region 1 (*aux-1*) is located on the early side of the core origin. Deletion of this sequence reduces SV40 DNA replication by six fold. Auxiliary region 2 (*aux-2*) located on the late side of the core origin includes the 21-bp and 72-bp repeats. The presence of the 21-bp repeat adjacent to the core origin increases the efficiency of replication by 10 to 20 fold. The 72-bp repeat is also capable of a similar enhancement in replication if it is juxtaposed to the core origin by the deletion of the intervening 21-bp repeat. However, in the presence of the 21-bp repeat, the 72-bp repeat does not enhance replication any further (Bergsma *et al.*, 1982; Chandrasekharappa and Subramanian, 1987; Fromm and Berg, 1983; Hertz and Mertz, 1986; Lee-Chen and Woodworth-Gutai, 1986). A similar dependence of DNA replication on auxiliary sequences has also been reported for polyoma virus (Fried and Prives, 1986).

Further studies suggest a complex relationship between transcription elements and SV40 DNA replication. For example, reiteration of 72-bp repeats more than ten times inhibits rather than enhances SV40 DNA replication (Kumar *et al.*, 1988). A second origin placed 2 kb away from the repeats, however, is not affected. In another dramatic example, translocation of the 21-bp repeats from the late side to the early side of the core origin results in the inhibition of replication (Lee-Chen and Woodworth-Gutai, 1986). It is not

known why the manipulations of the transcription elements described above cause such opposite effects on SV40 DNA replication.

The mechanism of replication enhancement by the neighboring auxiliary elements has been studied recently using the cell-free *in vitro* system (Guo *et al.*, 1989). Employing extracts from SV40-infected monkey cells that can reproduce the *in vivo* effect, Gutierrez *et al.* (1990) examined the ability of the auxiliary sequences to facilitate binding of replication factors and to promote DNA unwinding. Deletion of auxiliary sequences reduces the affinity of *ori*-core for active T antigen by only 1.6-fold, consistent with the fact that saturating concentrations of T antigen in the cell extract did not reduce the stimulatory effect of the auxiliary (*aux*) sequences in replication. In contrast, deletion of *aux* sequences reduces the efficiency of *ori*-specific, T-antigen-dependent DNA unwinding in cell extracts at least 15-fold. In the presence of purified T antigen and topoisomerase I, *aux* sequences strongly facilitate T-antigen-dependent DNA conformational changes consistent with melting the first 50 base pairs, while exerting little effect on the binding of T antigen to DNA. Thus, this study reveals that a primary role of *aux* sequences in DNA replication is to facilitate T-antigen-dependent DNA unwinding after the T-antigen pre-initiation complex is bound to *ori*-core.

SV40 origin activity has also been found to be influenced by foreign regulatory sequences. SV40 origin activity is inhibited when it is connected with the replicon of bovine papillomavirus (BPV) (Roberts and Weintraub,

1986; 1988), with adeno-associated virus (AAV) genome (Berns *et al.*, 1988) or with Rous sarcoma virus LTR enhancer (Binniger *et al.*, 1989). In the cases of BPV and AAV, both *cis* and *trans*-elements are necessary for the inhibitory effect. On the other hand, placing NF-1 binding site next to the SV40 core origin augments SV40 replication in the presence of NF-1 factor (Cheng and Kelly, 1989). Similarly, polyomavirus DNA replication could be stimulated by yeast GAL4 *cis* and *trans*-elements (Bennett-Cook and Hassell, 1991). Our study showed that SV40 origin activity can be suppressed by the human cytomegalovirus major immediate early gene enhancer in a distance- and position-dependent manner (see Chapter 3).

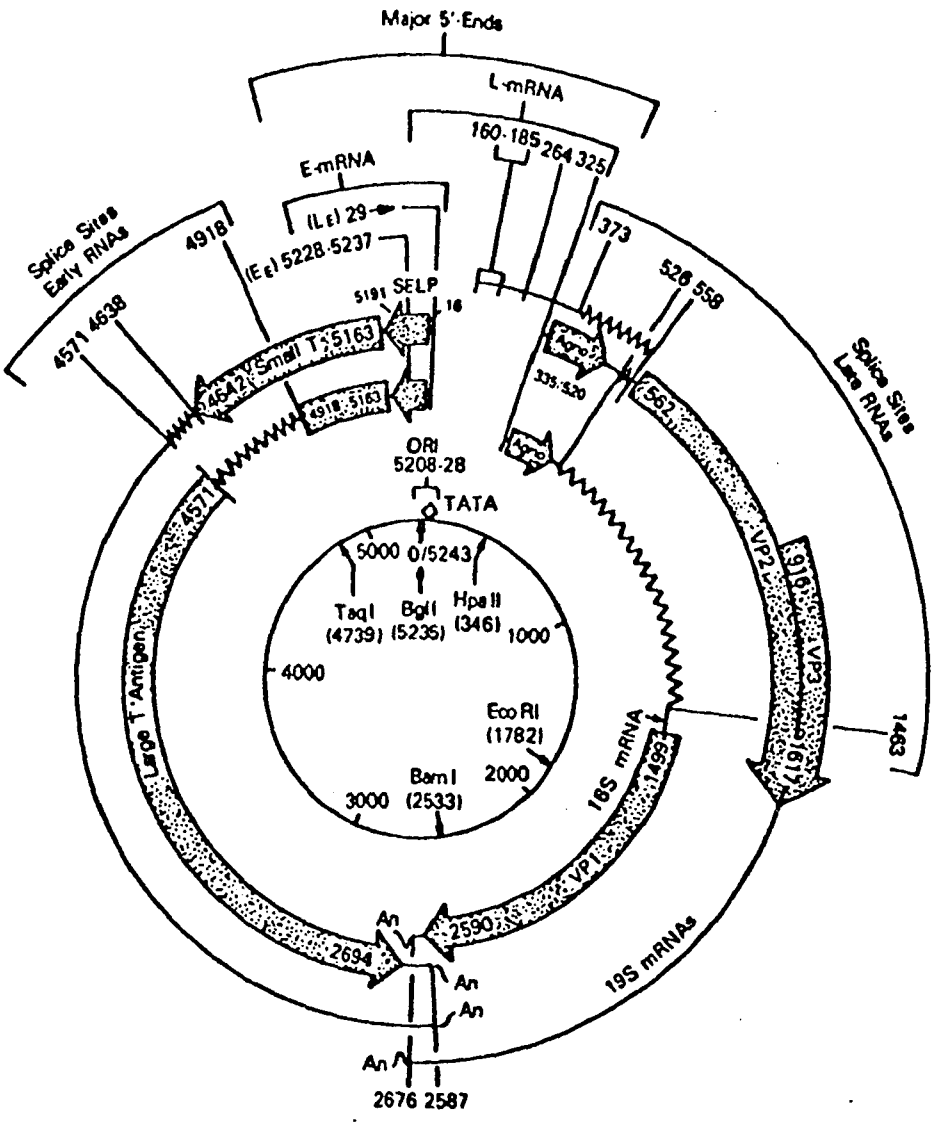
The studies described above show that SV40 origin activity can be modulated by adjacent sequences. The most obvious reason for modulation of origin activity is to control DNA copy number. Viral DNA in a lytically infected cells is designed to be a run-away replicon so that maximum number of progeny can be produced. However, under certain circumstances, viral DNA replication can also exhibit tight control of its DNA copy number. A well-known example is BPV. In the nonpermissive mouse cells it maintains more or less constant copy number of viral genome (about 200 copies per cell) and viral DNA replicates in the episome form only once per cell cycle (Law *et al.*, 1981; Broker and Botchan, 1986; Galloway *et al.*, 1986; Hayward, 1986). The sequence of 69% genome containing the replication origin is responsible for this plasmid maintenance (Rabson *et al.*, 1986).

In summary, studies of modulation of the activity of SV40 origin by adjacent sequences may provide important information about how replicons in mammalian cells are regulated. Chapter 4 of this dissertation describes some findings on sequence microenvironment contributions to SV40 *ori*-mediated DNA replication in a transient transfection system.

VI. Figure legend

Fig. 1A. Genetic map of simian virus 40 (strain 776). The inner circle represents the 5,243 bp-long genome with the origin of DNA replication, the TATA box and several restriction enzyme sites indicated. The outer counterclockwise and clockwise lines show early and late transcripts (E-mRNA and L-mRNA), respectively. The starts and ends of mature transcripts are indicated with numbers. E_E and L_E indicate the initiation site of the early transcripts during early and late infection, respectively. The zigzagged lines represent introns. The stippled boxes indicate protein-encoding regions. (The figure is taken from Remenick and Brady, 1990.)

Fig. 1B. The regulatory region of SV40. Core *ori* is necessary and sufficient to initiate large T antigen (Tag) mediated DNA replication. Tag binding sites (TBS), when bound, trigger the onset of viral DNA replication, the autoregulation of early transcription and the expression of SV40 late genes. T/A box is the equivalent of the TATA box for early transcription. The 21 bp repeats are highly GC-rich, containing six Sp1 binding motifs essential for maximal transcription. The 72 bp repeats are a transcription enhancer. The boundaries of each element are shown with numbers.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E-mRNA
(L ε) 29
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Splice Sites Early RNAs
4918
4571 4638
5191 SELP
16
Small T 5163
4642
4918 5163
373
526 558
Splice Sites Late RNAs
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TATA
4571
5000 0/5243
TaqI (4739)
BglI (5235)
HpaII (346)
1000
4000
EcoRI (1782)
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1613
1463
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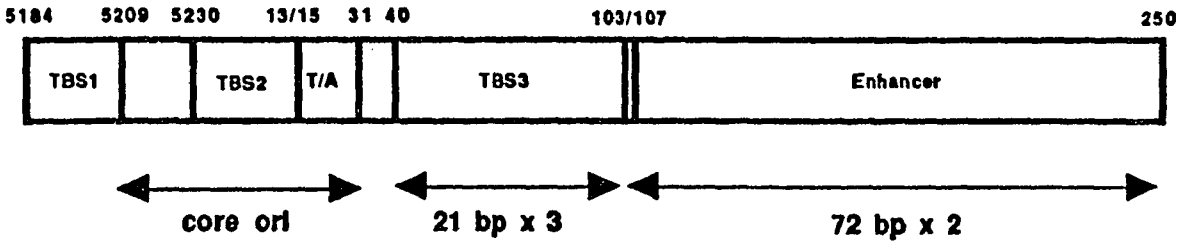
A


5184
5209
5230
13/15
31 40
103/107
250
TBS1
TBS2
T/A
TBS3
Enhancer
core ori
21 bp x 3
72 bp x 2

B

Chapter 2

P1 nuclease defines a subpopulation of active SV40 chromatin ---- a new nuclease hypersensitivity assay

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A. ABSTRACT

Under exhaustive digestion conditions P1 nuclease was found to cleave a subpopulation of intracellular SV40 chromatin only once. The major P1 cleavage site in SV40 DNA was mapped at the origin of DNA replication, and the two minor sites at the SV40 enhancers. The P1-sensitive SV40 chromatin subpopulation was found to have higher superhelical density than the bulk of the intracellular SV40 chromatin. Furthermore, pulse labeled SV40 DNA which had higher superhelical density than that of the steady state viral DNA (S.S. Chen and M. T. Hsu, J. Virol. 51:14-19, 1984) was also found to be preferentially cleaved by P1 nuclease. These results are consistent with a supercoil-dependent alteration of chromatin conformation near the regulatory region of the viral genome that can be recognized by P1 nuclease. Since P1 nuclease cleaves the subpopulation of SV40 chromatin only once without further degradation, this nuclease can be used as a general tool to define viral or cellular chromatin fraction with altered chromatin conformation and to map nuclease hypersensitive sites. Preliminary studies indicate that P1 makes limited double stranded cleavages in cellular chromatin to generate large DNA fragments.

B. INTRODUCTION

The regulatory regions of cellular and viral chromatin have been shown to exhibit altered structures as demonstrated by their hypersensitivity to nucleases (Weintraub and Groudine, 1976, for a review, see Elgin, 1981; Gross and Garrard, 1988). In a subpopulation of the simian virus 40 chromatin, a segment of DNA about 400 base pairs long that encompasses the replication origin, the transcriptional enhancer, and both the late and early SV40 promoters is found to be free of nucleosome organization and hypersensitive to restriction endonuclease and DNase I digestion (Varshavsky *et al.*, 1978; Scott and Wigmore, 1978; Cremisi, 1981; Jongstra *et al.*, 1984). The SV40 chromatin subpopulation has been estimated by electron microscopy to comprise about 25% of the total SV40 chromatin at late times of infection (Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980). However, by blocking the virion assembly, Ambrose *et al.* have shown that the regulatory region of almost all the intracellular late SV40 chromatin becomes nucleosome-free and accessible to restriction enzyme digestion (Ambrose *et al.*, 1986). This result, together with the nuclease digestion studies, indicates that the SV40 chromatin involved in virion assembly does not have the nucleosome-free organization in the regulatory region of the viral genome. On the other hand, accumulating evidence has suggested that the transcriptionally active SV40 minichromosomes have a nuclease-hypersensitive region within the

nucleosome-free domain (Choder *et al.*, 1984; Weiss *et al.*, 1986). Therefore, nucleases such as DNase I can be justifiably used as a means of monitoring alterations in chromatin conformation in active chromatin.

Although DNase I has been shown to preferentially cleave DNA in the regulatory regions of chromatin, it has an avidity for digesting all DNA --- a shortcoming necessitating a careful titration of the enzyme activity to obtain the optimum assay conditions for detecting nuclease hypersensitive sites. In this communication, we report a new method for studying the nuclease hypersensitive sites in SV40 chromatin using P1 nuclease derived from the fungus *Penicillium citrinum* (Fujimoto *et al.*, 1969). P1 nuclease has both phosphodiesterase and phosphomonoesterase activities with no sequence specificity. It has been used to determine the base composition of nucleic acids and to identify the cap structure of eukaryotic mRNAs (for a review, see Shishido and Ando, 1985). In contrast to the other nucleases, we observed that even when used in excess, it cut a subpopulation of SV40 chromatin only once *in vivo* at the regulatory region without further degradation of SV40 DNA. It also cut the cellular chromatin a limited number of times. Therefore, P1 nuclease is a potentially useful reagent for defining chromatin populations with altered chromatin conformation as well as for mapping nuclease-hypersensitive sites in the genome.

C. MATERIALS AND METHODS

Virus infection of CV-1 cells

CV-1 African green monkey kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 ug/ml gentamicin (Gibco). Cells were infected with simian virus 40 at 10 pfu/cell.

Preparation of cell nuclear ghost layer and digestion of nuclei with P1 nuclease

Nuclear ghost layer was prepared from CV-1 cells infected with SV40 virus by lysis with the buffer containing 0.5% Nondet P40, 10 mM Tris-Hcl, pH7.4, 0.2 mM $ZnCl_2$ (TZN). The nuclei obtained by this lysis procedure were firmly attached to the plastic plate allowing later reaction with the nuclei to occur efficiently. The nuclear ghost layer was digested with 50 ug/ml P1 nuclease (Bohringer-Mannheim) in TZN buffer at 37°C for the period indicated in the text. After digestion, the nuclei were lysed by adding SDS and tRNA (Sigma) to a final concentration of 1% and 100 ug/ml respectively. Transfer RNA was used as a competitive substrate to prevent degradation by P1 nuclease of the naked DNA that would result from the detergent lysis. The lysate was extracted immediately with phenol saturated with 0.2% SDS and then with chloroform-isoamyl alcohol (24:1), and was precipitated with ethanol in a ratio of 2 parts ethanol to 1 part lysate.

Gel electrophoresis

Two-dimensional gel electrophoresis of SV40 topoisomers was described previously (Chen and Hsu, 1984). Fluorography of the gels containing ^{3}H-thymidine labelled samples was carried out by immersing the dried gels in 0.7 M sodium salicylate for one hour. The gels were then dried again before exposing to X-ray film at -135°C.

D. RESULTS

P1 nuclease makes a single double-stranded cleavage on a fraction of intracellular SV40 DNA at the site of either the replication origin or transcription enhancers

We examined the P1 nuclease digestion pattern of intracellular SV40 DNA in infected CV-1 nuclei at 48 hrs post infection. As shown in Fig. 1, about 10% of intracellular SV40 DNA was found to have been cleaved once by the P1 into full-length linear molecules, whereas very little or no cleavage was found in the control samples. The percentage of the P1-sensitive fraction remained essentially unchanged after 60 minutes of digestion. This resistance of SV40 chromatin to further cleavage is not due to a rapid decline of the P1 activity. Even after three hours of digestion of SV40 infected nuclei, exogenously added free DNA molecules were still degraded by the P1 in the digestion mix. The kinetic analysis thus indicated that only a subpopulation

of the intracellular SV40 DNA is sensitive to P1 (See below for the nature of this sensitive fraction). The percentage of the P1-sensitive subpopulation depends on the time of infection. At 24 hrs post infection, the P1-sensitive fraction would increase to about 20%. This is because the P1-resistant, mature virus particles accumulate as the infection progresses.

The P1 nuclease cleavage sites in SV40 chromatin were mapped against the BclI, EcoRI and EcoRV endonuclease cleavage sites at nucleotide 2770, 1782, and 768, respectively. The full length linear SV40 DNA generated by P1 cleavage of SV40 chromatin as shown in Fig.1 was purified from the gel and then digested with the restriction endonucleases just described. As shown in Fig. 2, all three enzyme digestions generated specific pairs of subgenomic bands. Two major subgenomic DNA fragments of 2.4 kb and 2.3 kb were found after BclI digestion. Since BclI is 2.7 kb from the origin of DNA replication, this result suggestively placed the P1 cleavage sites near the regulatory region containing the origin of replication and promoters of SV40 DNA. This interpretation was confirmed by the result of EcoR1 digestion which produced two major fragments of 1.7 kb and 1.6 kb. To map the positions of the cleavage sites more precisely, we cleaved the P1-generated linear SV40 DNA with EcoRV that cleaves SV40 DNA at nucleotide 768. As shown in Fig. 2, EcoRV digestion produced a major band of 730 bp and two minor bands of 600 bp and 545 bp in the lower molecular weight region. This result mapped the major P1 cleavage site at nucleotide 38 and two minor sites at nucleotide 168

and 223 respectively. The major cleavage site at nucleotide 38 is located within the minimum origin of SV40 DNA replication (Deb *et al.*, 1986) whereas the minor cleavage sites are located in the SV40 enhancers (Khoury, 1981; Moreau *et al.*, 1981). These sites are comparable with the DNase I hypersensitive sites mapped by Jongstra *et al.* (1984). We found that the major cleavage at nucleotide 38 resides in their region I (which includes the replication origin and the early promoter), and that the minor cleavages at nucleotide 168 and 223 reside in their region II (which includes the enhancer and the late promoter).

A repeated sequence of the known AP-1 binding site, $^{5'}$AATTAGTCAGC$^{3'}$, was found to be located at or near the P1 cleavage sites of SV40 chromatin at nucleotides 27-37, 112-122 and 184-194 respectively. Although P1 nuclease is not known to have sequence specificity, we could not exclude the possibility that, under limiting digestion conditions, P1 might prefer specific sequences. To exclude this possibility, we mapped the P1 cleavage sites of purified SV40 DNA under limiting digestion conditions to generate full length linear SV40 DNA. The conditions that generate full length linear SV40 DNA were first determined by a titration experiment by varying the ratio of P1 to SV40 DNA. At as low as the enzyme to DNA ratio of 0.2 ng P1 to 1 ug DNA, (Fig.3a, lane 4), the supercoiled form I DNA was the preferential target of P1; as the ratio increased, both the form I and form II were first converted to the form III linear DNA and then further degraded. Fig. 3b shows the result of the

restriction endonuclease mapping of P1 cleavage sites of the P1-generated full length linear SV40 DNA isolated from gels (its interpretation is illustrated in Fig. 7.). Some specific cleavages were observed, two being located in the AT rich region near nucleotide 4200 and 1310 respectively (the other two minor cleavages being observed around nucleotide 2260 and 2700 respectively). These cleavages were not at the sites found in the intracellular SV40 DNA. This result indicated that the P1 nuclease cleavage sites in SV40 chromatin were not due to sequence specificity of P1 nuclease. Therefore, the specific cleavage of SV40 chromatin in the regulatory region by P1 nuclease must be the result of altered nucleoprotein structure in this chromatin region. The cleavage of purified SV40 DNA at the AT rich region by P1 nuclease is most likely due to the presence of single stranded region induced by the topological tension of the supercoiled SV40 DNA. Similar cleavage of supercoiled SV40 DNA by S1 nuclease and other nucleases has been previously demonstrated (Beard *et al.*, 1973, and references therein).

We investigated whether the P1-sensitive chromatin could be differentially associated with the nuclear matrix. This was done because P1 was found to cut intracellular SV40 DNA at the DNA replication origin or at the enhancers and also because the replication-and transcription-active template molecules were reported to exist in tight association with the nuclear matrix (Ben-Ze'ev *et al.*, 1982; Watson and Gralla, 1987). Previous studies have shown that active SV40 chromatin in infected cell nuclei is resistant to

extraction with a buffer solution containing 0.2% Triton and 0.2 M NaCl (Green and Brooks, 1976). Using this extraction procedure we observed that viral chromatin extracted from this buffer was resistant to P1 nuclease digestion whereas the chromatin remaining in the nucleus after extraction was highly sensitive to P1 digestion (Fig. 4). This result suggests that the P1-sensitive SV40 chromatin is more tightly associated with the nuclear structure than the bulk of intracellular viral chromatin.

The chromatin with higher superhelical density, the replication intermediates and the newly-synthesized SV40 chromatin are preferentially cleaved by P1 nuclease

Since the P1 nuclease prefers single-stranded DNA or negatively supercoiled DNA as substrate, we surmised that the intracellular SV40 DNA cleaved by P1 might have superhelical tension and thus most likely have higher superhelical density. To test this possibility, we analyzed the superhelical density of the DNA that had been purified from those intracellular chromatin that were resistant to the P1 digestion in infected cell nuclei. The DNA topoisomers of both the control and the P1 digestion samples were resolved in two-dimensional chloroquine gels (first dimension without chloroquine and the second dimension with 4 ug/ml chloroquine). To eliminate variation in experimental conditions the control and P1 digested samples were electrophoresed in the same electrophoretic container. The distribution of

SV40 DNA topoisomers is shown in Fig. 5. When chloroquine at 4 ug/ml was included in the electrophoresis buffer in the second dimension, the supercoiling of the SV40 DNA was still in the negative sense and the topoisomers with higher mobility in the second dimension had the higher negative superhelical density. As shown in Fig. 5, the faster migrating topoisomers that are more negatively supercoiled were absent in the P1 digestion sample. This result is consistent with the interpretation that SV40 chromatin containing DNA with higher superhelical density (those with more than 28 negative superhelical turns) was preferentially digested by P1 nuclease. The curve indicated as RI in Fig. 5 has been shown previously to be SV40 replication intermediates (Chen and Hsu, 1984). The absence of this curve in the P1 digested sample indicates that SV40 replication intermediates were also sensitive to P1 digestion. This could be due to altered chromatin structures of the replication intermediates or to the presence of single stranded region at the replication forks.

Since our laboratory had previously reported that SV40 chromatin with higher superhelical density are employed for DNA replication (Chen and Hsu, 1984), the preferential cleavages of these chromatin by P1 shown in Fig. 5 immediately suggested that the newly synthesized DNA should also be preferentially cleaved. The results shown in Fig. 6 met this expectation. SV40 DNA pulse-labeled with ^{3}H-thymidine for one hour was found to be

preferentially converted by P1 nuclease to full length linear DNA (Fig.6, RFII).

E. DISCUSSION

In the present report, we have demonstrated that P1 nuclease specifically cleaves a subpopulation of intracellular SV40 DNA only once at the regulatory region. The resistance of the SV40 chromatin to further P1 digestion suggests that the cleavage is mediated by DNA topological tension, since cleavage would release the tension and therefore remove the cause for the sensitivity to P1 cleavage. The depletion of the SV40 DNA with the higher superhelical density in the P1 digested sample supports this interpretation. Furthermore, the newly synthesized SV40 DNA with the higher superhelical density is also preferentially converted into full length linear DNA.

Supercoiled DNA with unrestrained topological tension contains free energy. This free energy can be stored in metastable DNA conformations such as Z-DNA, cruciform, single stranded regions, or triple-stranded structure (for a review, see Wells and Harvey, 1988). Since these unusual DNA conformations depend on DNA sequence arrangements (*e.g.*, cruciform requires inverted repeat sequences), the topological free energy becomes manifested at these sites. It is possible that the presence of the unrestrained topological tension in the SV40 DNA with higher superhelical density causes alterations in chromatin structure near the regulatory region of SV40 DNA in such a way

as to render the chromatin sensitive to P1 digestion. In contrast, the topological tension in free supercoiled SV40 DNA in solution is manifested by denaturation of the AT rich region as shown by S1 nuclease (Beard *et al.*, 1973) and P1 digestion (Fig. 3b). The reason for the difference between SV40 DNA in chromatin and in solution in the conversion of topological free energy is not known. It is possible that the AT rich region in chromatin is prevented from denaturation by the binding to histone cores and therefore the extra free energy is forced to be stored as the altered chromatin conformation in the regulatory region. However, we do not know why chromatin conformation in the regulatory region is altered in particular. The enrichment of inverted repeats and direct repeats in the regulatory region of SV40 DNA may play an important role in the formation of the altered chromatin conformation.

The presence of the altered chromatin conformation in the regulatory region of SV40 chromatin has been demonstrated by sensitivity to DNase I and restriction endonuclease digestion and by the absence of nucleosomal structures (Varshavsky *et al.*, 1978; Scott and Wigmore, 1978; Cremisi, 1981; Jongstra *et al.*, 1984; Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980). The results from this investigation further support these analyses. Furthermore, P1 nuclease used in this work has a major advantage over the DNase I and restriction endonuclease digestion techniques in detecting active chromatin with altered chromatin conformation. In contrast to DNase I, which continues to digest DNA in chromatin to oligonucleotides, P1 does not cleave DNA

further after the initial cleavage. This property allows the preservation and quantitation of the chromatin fraction sensitive to P1 digestion. It also avoids the need to determine the optimum enzyme concentration for digestion. The restriction endonuclease digestion technique requires the *a priori* knowledge of restriction enzyme cleavage sites in the chromatin to be investigated. On the other hand, P1 nuclease by the virtue of its low sequence specificity, can be used to investigate the structure of any chromatin. These considerations indicate that P1 should be a useful tool for the analysis of active chromatin which is usually associated with altered chromatin conformations. Because P1 does not cleave chromatin further after the initial cleavages, it would also be possible to clone active genes that can be selectively released by P1 digestion.

Our results that a subpopulation of SV40 chromatin is cleaved by P1 varies with the results of Fujimoto *et al.* (1979). Their results indicated that SV40 minichromosome extracted from the infected cells by a buffer containing Triton X-100 was highly resistant to P1 digestion and only a small amount of full length linear DNA was observed. They interpreted the formation of linear DNA as the result of P1 cleavage through nicks of the nicked circles. Our investigation showed that the P1-sensitive SV40 chromatin was not extractable by the buffer used by Fujimoto *et al.*. As shown in Fig. 4, essentially all the P1-sensitive fraction remained in the nuclei after extraction with the Triton buffer and the extracted chromatin was resistant to P1 digestion. Thus the

discrepancy between their results and the results presented here reflects the differences in the methodology used.

The mechanism of nuclease hypersensitivity of the active chromatin is still not clear (for a review, see Gross and Garrard, 1988). The deletion experiments of a partial duplication mutant have mapped some *cis*-sequences that are crucial for nuclease hypersensitivity of the SV40 regulatory region (Gerard *et al.*, 1982; 1985). These *cis*-sequences may disfavor nucleosome formation and therefore may expose the region to nuclease attacks. Another proposition involves the *trans*-acting protein factors. The binding of these factors to the corresponding DNA sequence may induce some perturbations in the DNA organization that in turn lead to nuclease hypersensitivity. Although both of these propositions could contribute to the results shown in this communication, we tend to favor the DNA conformation, more specifically, the supercoiling hypothesis of nuclease hypersensitivity. Luchnik *et al.* presented the first evidence that the torsionally-strained SV40 minichromosomes were transcriptionally active because they were enriched with endogenous RNA polymerase activity (Luchnik *et al.*, 1982). Later the same authors proved that the same fraction of SV40 minichromosomes was hypersensitive to DNase I and was relaxable by topoisomerase I. Furthermore, they showed that this fraction completely lost its hypersensitivity to DNase I upon relaxation (Luchnik *et al.*, 1985). By studying 5S rRNA plasmid microinjection into Xenopus oocytes (Ryoji and Worcel, 1984), and by comparing ß-globin hypersen-

sitivity to DNase I in chicken red blood cells in the presence and absence of the relaxation agent novobiocin (Villeponteau *et al.*, 1984), Ryoji and Worcel and Villeponteau *et al.* respectively established that a torsional stress is the prerequisite to DNase I hypersensitivity. Our results fit this proposition best, since P1 makes only one double-stranded cleavage without further degradation, and the intracellular SV40 DNAs with higher superhelical density are preferentially cut by P1.

The preliminary data that P1 nuclease in saturation makes only a limited number of cleavages on cellular chromatin (not shown) seems to correlate well with the loop-domain organization of cellular chromatin (Newport and Forbes, 1987). Further research will test the P1 sensitivity of tissue-specific genes in different cell types. The observations that 1) P1 nuclease cuts the SV40 chromatin with higher superhelical density at regulatory regions and that 2) those same chromatin with higher superhelical density are the ones employed in DNA replication (Chen and Hsu, 1984) lead us to believe that P1 nuclease can be used as a general assay to detect active chromatin.

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F. APPENDIX

I. The "preserving" property of P1 nuclease toward SV40 chromatin:

As discussed in the above paper, the most attractive feature of using P1 nuclease to detect active chromatin is that the nuclease makes meaningful cleavage only toward a small subpopulation of SV40 chromatin which is consistent with the chromatin active in DNA replication and/or transcription. P1 nuclease does not make further cleavage once a cleavage has been made either at the replication origin or the transcription enhancer. This "preserving" property of P1 nuclease is further demonstrated as follows.

In the presence of cycloheximide, a protein-synthesis inhibitor used here to inhibit histone synthesis and thus SV40 chromatin maturation, the majority of the newly synthesized SV40 DNA is cleaved once to yield full-length linear molecules (Fig. 8a). In contrast, the ethidium bromide staining shows that the majority of supercoiled SV40 DNA remains resistant to P1 digestion (Fig. 8b). In the control lane, a ladder of topoisomers is resolved in the regular agarose gel (containing no DNA intercalator), confirming deficiency of nucleosome in the SV40 chromatin due to the inhibition of histone synthesis. Under this condition, P1 still makes only a single cleavage in most of the newly synthesized SV40 DNA. This result, then, is consistent with our conjecture

that topological tension is necessary for P1 cleavage. Once the tension is removed (*e.g.*, by a single cut of P1), P1 cleavage is stopped or slowed.

II. A 15 bp sequence at replication origin is responsible for P1 hypersensitivity

One of the tests for the usefulness of the P1 assay would be to observe the P1 sensitivity in the SV40 variants with an additional regulatory segment (containing one 72 bp rather than two) inserted almost 180^0 to the corresponding authentic segment (also containing one 72 bp) (Shenk, 1978). In *in*(Or)1411 and *in*(Or)1412, both the segments are functional in initiating DNA replication. In *in*(Or)1415, however, the inserted segment contains a deletion (by nuclease Bal31) at the center of origin of DNA replication and therefore is nonfunctional as replication origin (Shenk, 1978).

Figure 9 shows the P1 cleavage patterns of these SV40 variants. In *in*(Or)1411 and *in*(Or)1412, P1 cuts only once within either the authentic or the inserted regulatory segment with approximately equal molarity. In contrast, P1 again cuts once within the authentic, but not the inserted, regulatory segment in *in*(Or)1415. This result then establishes the correlation between P1 sensitivity and the functionality of DNA replication. Whether P1 also cuts at the enhancer (now only one copy of the 72 bp repeat rather than two in the wild type SV40) of both the authentic and the insert regulatory segments cannot be concluded from this gel because of resolution. Even if the

cleavage at the enhancer could be resolved, it would still be a minor portion compared with the cleavage at the replication origin.

The complete sequence of these SV40 variants has not been published since their construction (Shenk, 1978). By knowing that the additional regulatory segment was inserted at the HpaI (#2666) site, we synthesized oligonucleotide of #2610-#2631 and used it as the primer to sequence the inserted segment. The results are as follows. In both *in*(Or)1412 and *in*(Or)1415, the additional regulatory segment spans from #5186 through #5243/0 to #286 containing a deletion of the 72 bp unit (#107-#178). This segment was inserted at the HpaI site of the parental *dl*1410 between #2668 and #2669. There is an additional deletion of 15 bp from #5234 through #5243/0 to #5 in *in*(Or)1415. Since the P1 cleavage within the insert region was insignificant in *in*(Or)1415 compared with those in *in*(Or)1411 and *in*(Or)1412, it can be concluded that this 15 bp sequence is directly or indirectly responsible for the P1 sensitivity of the inserted regulatory segment.

Previously, by constructing a series of deletion mutants of *in*(Or)1411 within the inserted region, Scott's group found that there was no single deletion that could completely remove the DNase I sensitivity within the inserted region (Gerard *et al.*, 1982; 1985). It appeared that nuclease sensitivity is determined by a multi-factorial genetics. Our results are consistent with this conclusion, because in *in*(Or)1415, the bands due to the cleavage within the inserted region can still be faintly visualized.

In conclusion, the above results clearly indicate that P1 nuclease, like DNase I, recognizes an altered chromatin conformation that is characterisic of active chromatin.

G. FIGURE LEGENDS

Fig. 1. P1 nuclease makes a single double-stranded cleavage on intracellular SV40 DNA. CV-1 cells were infected with SV40 for 48 hrs and digested with P1 nuclease as described in Materials and Methods. Lane 1: mol. wt. marker; lane 2: Hirt extract, no digestion; lane 3: mock digestion; lanes 4-7: digestion with P1 for 0.5, 1, 2, and 3 hrs, respectively. I, II, and III are supercoiled, nicked, and linear SV40 DNA, respectively.

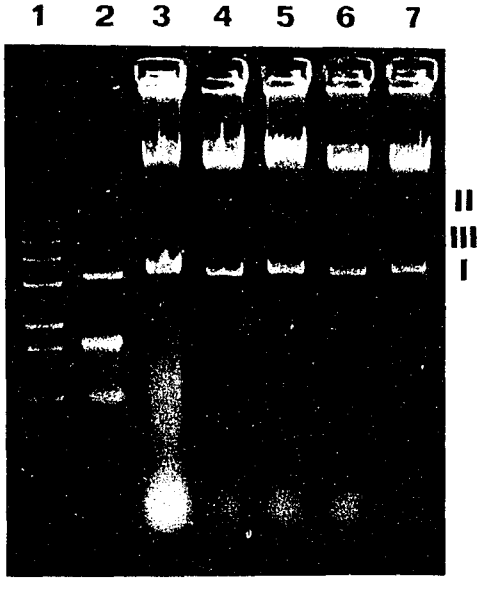

1 2 3 4 5 6 7
II
III
I

Fig. 2. P1 cleavage sites are mapped to the regulatory region on the SV40 genome. The full-length linear SV40 DNA produced by P1 digestion was purified from gel after electrophoresis and then digested with the restriction enzyme BclI, EcoRI or EcoRV. After purification DNA was electrophoresed in 1.2% agarose gel and blotted. The blot was probed with the ^{32}P-labeled total SV40 DNA. Lanes 1-4 are DNA samples digested with P1 nuclease for 0.5, 1, 2, and 3 hrs, respectively. The major and the two minor P1 cleaved DNA bands in the EcoRV digest are marked by a large arrow and two small arrows respectively. The inset panel is a longer exposure of these three bands.

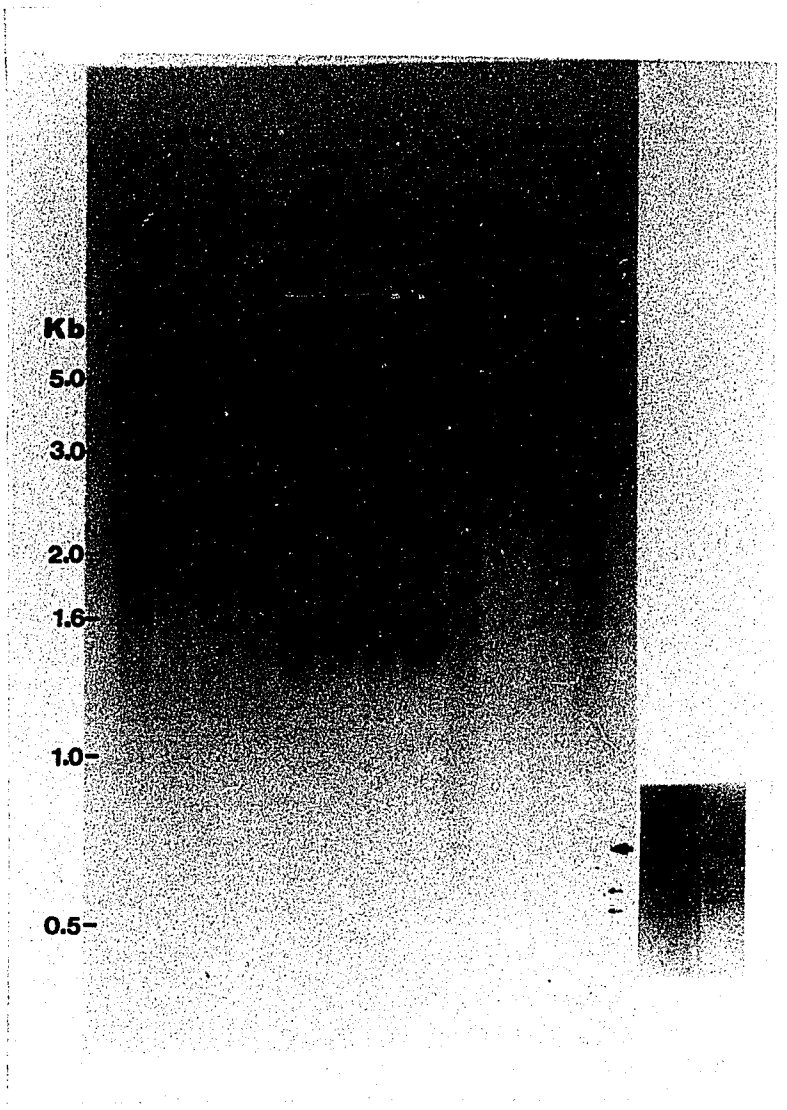

Kb
5.0
3.0
2.0
1.6
1.0
0.5

Fig. 3. Mapping of P1 nuclease cleavage sites on naked SV40 DNA. Panel A: Purified SV40 DNA was digested with P1 at 37°C for 30 min with an increasing enzyme to substrate ratio. Lane 1: mol. wt. marker; lane 2: SV40 DNA substrate; lane 3: mock digestion; lanes 4-7: P1 digestion with the enzyme to SV40 DNA ratio of 0.2, 2, 4, 10 ng/ug, respectively. Panel B: Mapping of P1 cleavage sites using EcoR1 and EcoRV restriction endonucleases. After digestion of naked SV40 DNA with P1 nuclease, full length linear DNA was eluted from agarose gel and redigested with restriction endonucleases EcoR1 and EcoRV. The Southern blot was hybridized with the ^{32}P-labeled total SV40 DNA probe.

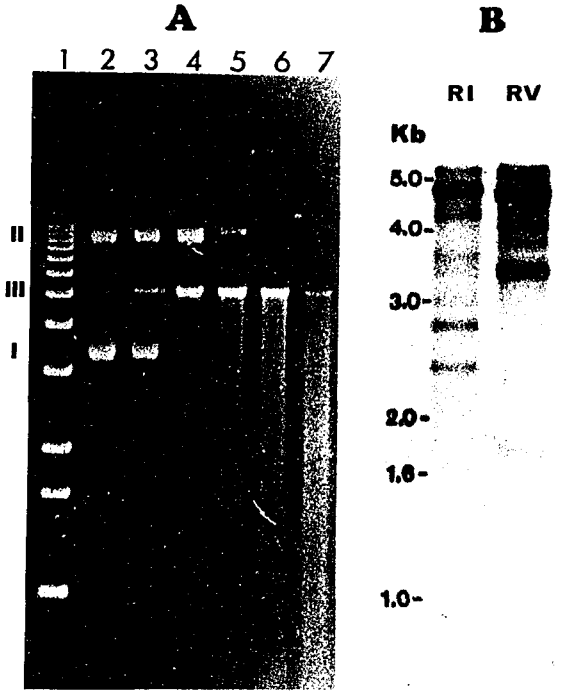

A
1 2 3 4 5 6 7
II
III
I
B
RI RV
Kb
5.0-
4.0-
3.0-
2.0-
1.6-
1.0-

Fig. 4. Resistance of P1-sensitive SV40 DNA to the extraction by Triton buffer. At 48 hrs post infection the CV-1 cells were extracted with a buffer containing 0.2% Triton-X100, 0.2 M NaCl, 1 mM EDTA and 40 mM Tris, pH7.4. After two rounds of extraction, the extract and the nuclei were digested with P1. SV40 DNA purified from the digested Triton extracts and from the remaining nuclei are shown in lane 3 and 5 respectively; lanes 2 and 4 are the corresponding mock digestion.

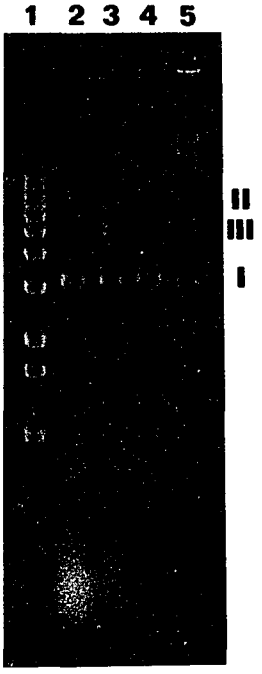

1 2 3 4 5
II
III
I

Fig. 5. SV40 DNA topoisomers with higher superhelical density and SV40 replication intermediates are sensitive to P1 cleavage. SV40 DNA in the infected cell nuclei digested or mock digested with P1 nuclease for 3 hrs at 37°C was analyzed by 2D gel electrophoresis. 1st D: left to right, 1% agarose in TEA buffer; 2nd D: top to bottom, 1.2% agarose gel in TEA buffer supplemented with 4 ug/ml chloroquine. The gel was Southern-blotted and the blot was probed with the ^{32}P-labelled total SV40 DNA. The curve labeled as RI represents SV40 replication intermediates. RF I, II, and III are supercoiled, nicked, and linear SV40 DNA, respectively.

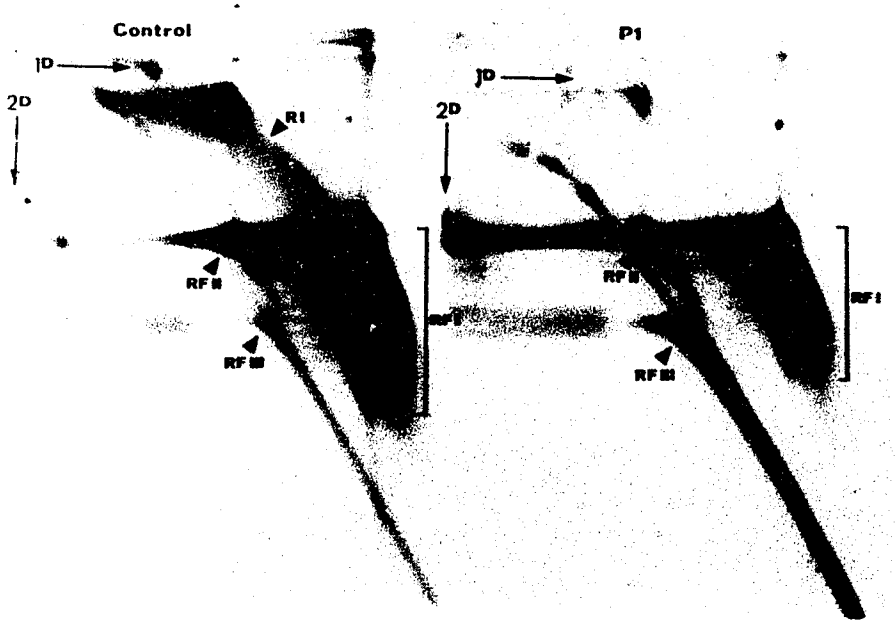

Control
P1
1D
2D
RI
RF II
RF III
RF I

Fig. 6. Newly-synthesized SV40 chromatin is preferentially digested by P1 nuclease. At 47 hrs post infection, the cells were pulsed with ^{3}H-thymidine at 0.1 mCi/ml for 1 hr, then digested with P1 for 3 hrs, against a mock digestion. Both the samples were analyzed by the 2D gel electrophoresis as described in Fig. 5. The direction of the 1st D for the P1 digested sample was opposite to that of the control in order to compare more clearly the distribution of DNA topoisomers in the two samples.

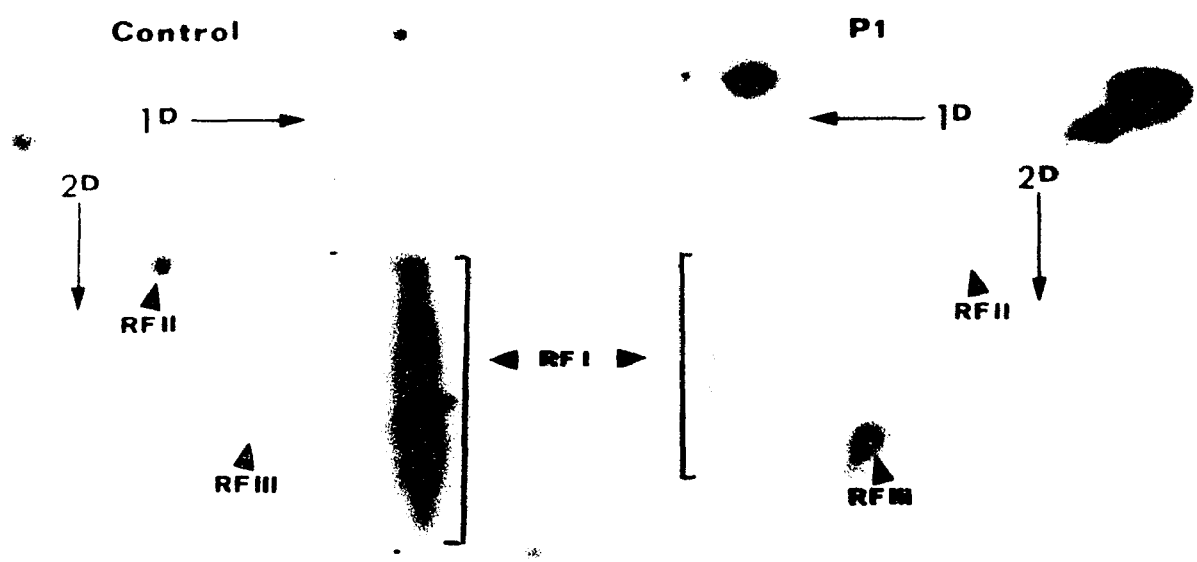

Control
P1
1D
1D
2D
2D
RF II
RF II
RF I
RF III
RF III

Fig. 7. Summary of P1 nuclease cleavage sites on intracellular SV40 DNA and on naked SV40 DNA. The dots on the circle show the two major P1 cleavage sites (nucleotide 1310 and 4200) and two minor sites (nucleotide 2260 and 2700) on the naked SV40 DNA. The origin-enhancer region of SV40 DNA is expanded in the upper part of the figure to show the locations of the three P1 cleavage sites on SV40 chromatin. The shaded box shows the A/T rich region, and the bar above the expanded region shows the minimum origin of DNA replication. The boxes with darker diagonal lines are the three 21 bp direct repeats, and the boxes with lighter diagonal lines are the two 72 bp direct repeats (the enhancer). The arrow on the left indicates the major P1 cleavage site at the replication origin, and the two arrows on the right indicate the two minor P1 cleavage sites at the enhancer.

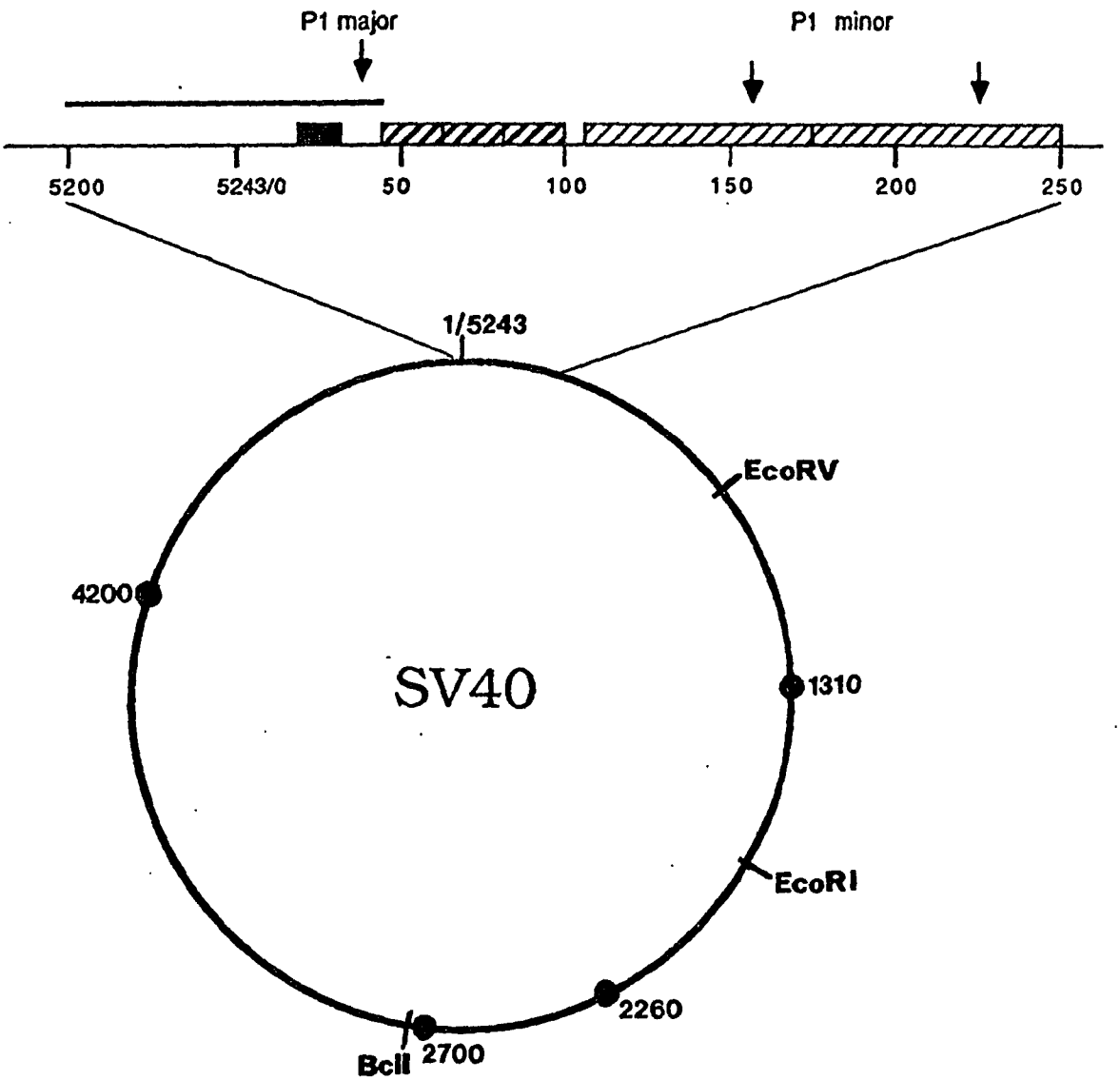

P1 major
P1 minor
5200
5243/0
50
100
150
200
250
1/5243
EcoRV
4200
SV40
1310
EcoRI
2260
BclI
2700

Fig. 8. SV40 chromatin synthesized in the presence of protein-synthesis inhibitor cycloheximide is cut by P1 nuclease to full-length linear DNA. Thirty four hrs after infection with wild type SV40, the cells were labeled with 0.1 mCi ^{3}H-thymidine for 3 hrs in the presence of 25 ug/ml cycloheximide. The cells were digested with P1 nuclease. The DNA were Hirt extracted and resolved in gel. Ethidium bromide staining of the gel is shown in panel B. The gel was subsequently soaked in 0.7 M Na Salicylate for 0.5 hour, dried and exposed to XAR film for 2 days. Panel A shows the fluorograph. Lane 1 shows control without P1 digestion; lane 2 P1 (100 ug/ml) digested for 1 hr; and lane 3 P1 digested for 3 hrs. III indicates the full-length linear SV40 DNA; I and II indicate closed and nick circular DNA, respectively.

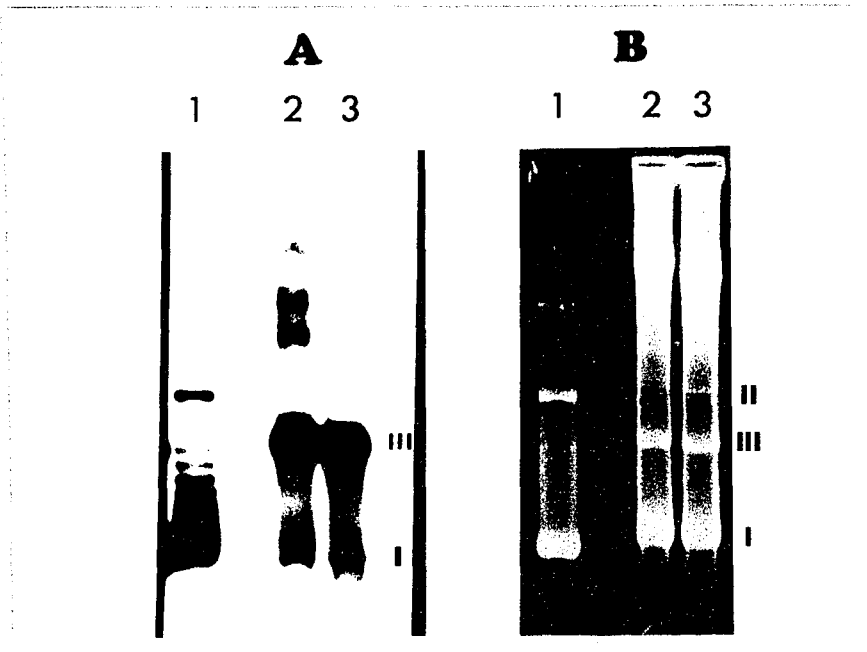
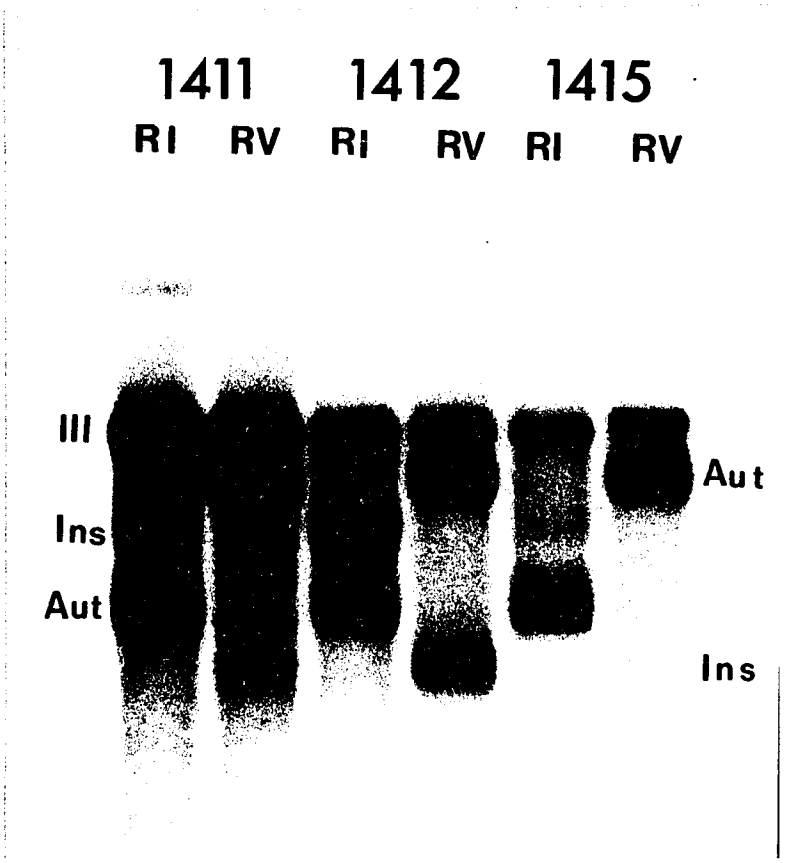

A
1
2
3
III
I
B
1
2
3
II
III
I

Fig. 9. P1 nuclease cuts at both functional replication origins in *in*(Or)1411 and *in*(Or)1412; it cuts at the functional authentic origin but does not cut at the inactivated insert origin in *in*(Or)1415. Forty eight hrs after infection, cells infected with each virus were digested with P1 nuclease. The full-length linear DNA were purified from gel and the P1 cleavage sites were mapped against EcoRI and EcoRV cleavage sites, respectively. The Southern blot was probed with total SV40. The bands derived from P1 cleavages at authentic and insert origins are indicated as aut and ins respectively. III indicates the position of the full-length DNA.


1411
1412
1415
RI
RV
RI
RV
RI
RV
III
Ins
Aut
Aut
Ins

Chapter 3

Induction of highly supercoiled SV40 DNA by intercalation of ellipticine within nucleosome core

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A. ABSTRACT

We investigated the *in vivo* effect of ellipticine, a mammalian topoisomerase II (topoII) inhibitor, on SV40 DNA topology. In contrast to epipodophyllotoxins, ellipticine does not cause significant double stranded cleavage of intracellular SV40 DNA. Furthermore, ellipticine reduces cleavages induced by epipodophyllotoxins, VP16 and VM26. Unexpectedly, ellipticine was found to increase dramatically the superhelical density of a fraction of intracellular SV40 DNA, from -26 to about -47 turns. Newly synthesized viral DNA is preferentially converted to the highly supercoiled form by ellipticine. Several lines of evidence suggest that the formation of this highly supercoiled DNA species (Ih form DNA) is not due to the inhibition of topoII *per se*, but the result of intercalation by ellipticine into the DNA of the newly synthesized SV40 chromatin followed by the fixation of DNA linking number by a topoisomerase activity. Based on the linking number change and the known unwinding angle of ellipticine, the intercalation density can be calculated as one ellipticine molecule per 10-20 bp in the Ih DNA. This result suggests the newly synthesized SV40 nucleoprotein complexes contain altered nucleosomal organization that allows the intercalation within nucleosome core.

B. INTRODUCTION

Topoisomerases are enzymes required for resolving the topological constraints of DNA imposed by DNA replication, transcription and packaging (Gellert, 1981; Wang, 1985; Vosberg, 1985). Because of their importance in cell proliferation and gene expression, these enzymes have been targeted for chemotherapy of malignancy and of microbial infections (for review, see Potmesil and Silber, 1990). Only one major inhibitor, camptothecin with its derivatives, has been found for eukaryotic topoisomerase I (Liu, 1990). On the other hand, many inhibitors are known for eukaryotic topoisomerase II (Liu, 1990). Studies of the mechanisms of topoII inhibitors have revealed two groups among these inhibitors. Those in the first group, referred to as "cleavage complex" type inhibitors, induce double stranded cleavages of the substrate DNA with the enzyme being joined covalently to the cleavage sites. This group of inhibitors is exemplified by epipodophyllotoxins and *m*-AMSA (Liu, 1990). Those in the second group of eukaryotic topoII inhibitors such as merbarone (Drake *et al.*, 1989) and amilorides (Besterman *et al.*,1989) do not induce a significant amount of double stranded cleavages of the substrate DNA; rather, they prevent DNA cleavages induced by the "cleavage complex" inhibitors. The reason for the difference of these two groups of inhibitors in inducing double stranded DNA cleavages is not understood. Apparently, the ability to induce DNA cleavage does not correlate with anti-tumor activity of

these topoII inhibitors.

We showed previously that, in contrast to epipodophyllotoxins, ellipticine did not induce double stranded cleavages in intracellular viral DNA in infected HeLa cells even though it inhibited adenovirus transcription and packaging (Wong and Hsu, 1988). Since ellipticine has been shown previously to be a "cleavage complex" type topoII inhibitor (Filipski and Kohn, 1982; Multon *et al.*, 1989; Ross and Bradley, 1981; Zwelling *et al.*, 1982), we asked whether the inability of ellipticine to cleave intracellular adenovirus DNA is specific only to adenovirus. In this report, we show that ellipticine also fails to induce cleavage of the intracellular SV40 genome, which is organized into nucleosome structures. Furthermore, we show that, like the second group of topoII inhibitors, ellipticine reduces cleavages of SV40 and adenovirus DNA by VP16 and VM26.

Since DNA cleavage by inhibitors such as epipodophyllotoxins could preclude the examination of the very DNA on which topoII acts, we used ellipticine to investigate the role of topoII in SV40 DNA replication and in the maintenance of SV40 DNA topology *in vivo*. Unexpectedly we found that treatment of SV40 infected CV-1 cells with ellipticine dramatically increased the superhelical density of a fraction of intracellular SV40 DNA. We present evidence showing that the induction of the highly supercoiled SV40 DNA is because ellipticine is capable of intercalating into the nucleosome core in a subfraction of intracellular SV40 nucleoprotein complexes.

C. MATERIALS AND METHODS

Cell culture and isolation of SV40 chromatin and DNA

CV-1 cells were cultured in Dulbecco modified minimum essential medium (Sigma) supplemented with 10% fetal calf serum and 50 ug/ml of gentamicin. Cells were infected with SV40 virus at 10 pfu/cell in the medium containing 2% fetal calf serum. At 24 or 48 hours postinfection, fresh media containing topoisomerase inhibitors were added to the cells. Lengths of the drug treatment and the drug concentration used are described in the text. The stock solutions of topoisomerase inhibitors are the following: camptothecin (Sigma), 7.2 mM in dimethyl sulfoxide; ellipticine (Sigma), 10 mM in 10 mM HCl; VP16 (Bristol-Meyers Laboratories), 34 mM as in the *VePesid Injection* vial; and VM26 (a gift from Bristol-Meyers), 50 mM in dimethyl sulfoxide. SV40 chromatin was isolated from infected cells at 48 hours postinfection by extraction with 0.2% Triton-X100, 0.4 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4. SV40 DNA was isolated from infected cells using a filtration technique (Wong and Hsu, 1988).

Gel electrophoresis of SV40 DNA topoisomers

One per cent agarose gel containing 4 ug/ml of chloroquine was used for the analysis of SV40 DNA topoisomer distribution in a one-dimensional (1-D) analysis as previously described (Chen and Hsu, 1984). For two-dimensional (2-D) gel analysis, SV40 DNA was first electrophoresed in a 1% agarose gel

containing 4 ug/ml chloroquine and then electrophoresed in the second dimension in the presence of 50 ug/ml chloroquine. Fluorography of gels was performed by soaking gels in 0.7 M of sodium salicylate as described previously (Chen and Hsu, 1984).

Electron microscopy

SV40 DNA was spread from 60% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5, as described by Davis *et al.* (1971). Grids were shadowed by platinum-palladium (80:20) using an Edwards E306A coating system and analyzed in a Zeiss 10CA electron microscope.

D. RESULTS

Ellipticine does not induce significant double stranded cleavage in SV40 DNA *in vivo*

The topoII inhibitors, VM26 and VP16, are known to induce double stranded DNA breaks (Chen *et al.*, 1984). When added to SV40 infected cells, these agents induced double stranded cleavages of SV40 DNA. As shown in Fig. 1B, the full length linear SV40 DNA, due to the drug-induced topoII cleavage of covalently closed circular SV40 DNA, appeared when VM26 (lane 4) or VP16 (lane 5) was added at 100 uM. In contrast, we found that ellipticine, another topoII inhibitor, did not induce double stranded cleavage of intracellular SV40 at concentrations up to 100 uM (lanes 3 to 5 in Fig. 1A

and lane 3 in Fig. 1B). Similar results were obtained for human adenovirus DNA in HeLa cells (data not shown).

Since ellipticine has been shown to induce DNA cleavage of mammalian cell DNA *in vivo* (Filipski and Kohn, 1982; Multon *et al.*, 1989; Ross and Bradley, 1981; Zwelling *et al.*, 1982) and of SV40 chromatin *in vitro* (Tewey *et al.*, 1984), the above results could be due to the poor uptake of ellipticine by the virus infected cells. To exclude this possibility, we examined the structure of SV40 DNA extracted from the ellipticine treated cells in the electron microscope. It has been shown that inhibition of topoII activity results in the formation of SV40 DNA catenanes with highly intertwined newly replicated DNA (Snapka, 1986). This highly intertwined SV40 DNA was indeed found in the viral DNA extracted from ellipticine-treated cells (Fig. 2). This observation indicates that topoII activity in SV40 infected cells was indeed inhibited by ellipticine. Therefore, the absence of cleavage of SV40 DNA was not due to the failure of ellipticine to inhibit topoII in our experimental protocol.

If ellipticine indeed could inhibit topoII activity without causing significant cleavage of intracellular SV40 or Ad5 DNA, then one would predict that pretreatment of cells with ellipticine should inactivate topoII, thereby reducing, if not blocking, the subsequent induction of DNA cleavage by VM26 or VP16. This is indeed the case. As shown in lanes 6 and 7 in Fig. 1B, pretreatment with 100 uM of ellipticine for 30 minutes significantly reduced subsequent cleavage of intracellular SV40 DNA induced by VM26 or VP16. In

VM26 and VP16 treated samples, fragmented cell DNA that could be extracted by the Hirt procedure was observed near the top of the gel in Fig. 1B (lanes 4, and 5). The amount of this fragmented cell DNA was also reduced by the pretreatment with ellipticine (compare lanes 4 and 6 as well as lanes 5 and 7). Similarly, ellipticine pretreatment was found to inhibit the induction of cleavage of intracellular Ad5 DNA in HeLa cells infected by Ad5 (data not shown). These results indicate that ellipticine was taken up by the cells and could compete with VM26 or VP16 to reduce the induction of double stranded DNA cleavages induced by these two drugs.

Ellipticine induces the formation of a highly supercoiled SV40 DNA species (form Ih DNA)

Since ellipticine did not induce significant cleavage of intracellular SV40 DNA, we used this drug to investigate the effect of its inhibition of topoII on the DNA topology of SV40 DNA *in vivo*. Treatment of SV40 infected cells with 50-100 uM ellipticine from 24 to 26 hours post infection dramatically increased the superhelical density of SV40 DNA as shown by the analysis using two-dimensional chloroquine gel to resolve SV40 DNA topoisomers. We found that this effect was ellipticine specific, regardless whether other topoisomerase inhibitors were incubated together with ellipticine (also see below). Figure 3a shows a comparison of distribution of SV40 DNA topoisomers between the untreated sample and the ellipticine-camptothecin treated sample. The

superhelical density of this ellipticine-induced highly supercoiled SV40 DNA species (Ih DNA for convenience) is about 1.5-1.7 times that of the control SV40 DNA, corresponding to about 38-47 superhelical turns in each SV40 DNA molecule. When DNA was pulse labeled with ^{3}H-thymidine for one hour before the addition of ellipticine, the majority of the labeled SV40 DNA was found in the highly supercoiled Ih DNA form (Fig. 3a, compare panel D with C).

This effect is shown more clearly at 48 hours postinfection when the proportion of DNA that is newly replicated is smaller than at 24 hours post infection (Fig. 3b). Comparison between the pattern of ethidium bromide-stained bulk DNA with that of the fluorographed pulse-labeled DNA in a 1-D chloroquine gel clearly demonstrates that newly labeled SV40 DNA is preferentially converted to the highly supercoiled Ih DNA in the ellipticine-treated sample (Fig. 3b, compare lanes c and c').

In vivo and *in vitro* evidence that alteration of SV40 DNA linking number by ellipticine is due to its intercalation within nucleosome cores of SV40 chromatin followed by linking number fixation

We considered two possible mechanisms for the formation of the highly supercoiled Ih DNA by ellipticine treatment. Since ellipticine is a topoII inhibitor, it is possible that Ih DNA in the infected cells normally is rapidly relaxed by topoII, and that its existence is revealed only when topoII relaxation activity has been inhibited with no concomitant DNA cleavage. The other

possibility, since ellipticine is known to be an intercalating agent (Kohn *et al.*, 1975), is that the linking number change of SV40 DNA induced by ellipticine is due to its intercalation into SV40 chromatin followed by the fixation of linking number by a topoisomerase activity.

To test whether ellipticine can intercalate into SV40 chromatin *in vivo*, we examined the effect of ellipticine on the intercalation of SV40 DNA by psoralen. Intercalation of psoralen into double stranded DNA is a prerequisite for its photocrosslinking the DNA (Shen and Hearst, 1977). If ellipticine indeed intercalates into SV40 DNA *in vivo*, one would expect that pretreatment with ellipticine would inhibit the photocrosslinking of SV40 DNA by psoralen due to competition of intercalation. To test this possibility, SV40 infected cells were pretreated with different concentrations of ellipticine for two hours before photocrosslinking by aminotrioxsalen (AMT). AMT-crosslinked SV40 DNA was extracted, linearized with EcoRI and analyzed in an alkaline agarose gel. In the alkaline gel, non-crosslinked DNA is denatured into single strands. The single stranded DNA has faster electrophoretic mobility than the crosslinked DNA which contains two single strands linked at the crosslinked sites under the denaturing condition. The gel mobility of the crosslinked DNA also varies according to the degree of crosslinking. The more highly crosslinked DNA migrates at faster rate because the single stranded bubbles between the crosslinks are smaller.

As shown in Figure 4, pretreatment of cells with ellipticine before the

AMT addition inhibited AMT-induced photocrosslinking of SV40 DNA *in vivo*. This is evidenced by the appearance of the fast migrating, uncrosslinked single stranded DNA in the ellipticine-pretreated sample (Fig. 4, lanes 3-5, arrow). Furthermore, the gel mobility of the crosslinked DNA in ellipticine-pretreated sample is lower than that of the control without ellipticine pretreatment (compare lanes 3, 4 and 5 with lane 2, bracket) suggesting that they have lower crosslinking density than that of control. These results are consistent with the interpretation that ellipticine can block photocrosslinking of DNA by psoralen, presumably by competing against psoralen intercalation into DNA. This conclusion is supported by the positive control which uses another intercalating agent, ethidium bromide, to interfere with psoralen crosslinking (lanes 6 and 7). However, ethidium bromide is much less efficient in blocking psoralen crosslinking, probably because of the poorer uptake by the cells.

On the other hand, if form Ih DNA were formed by the inhibition of topoII without intercalation by ellipticine, one would expect highly supercoiled DNA to pick up more psoralen. This is opposite to what we have observed. Therefore, the data described above are consistent with the generation of Ih form by intercalation and not by inhibition of topoII. This conclusion is further supported by the observation that treatment of SV40 infected cells with known intercalating agents, *e.g.*, actinomycin D and acridine orange, also causes an increase in the superhelical density of intracellular SV40 DNA (data not shown).

To determine whether ellipticine can induce alteration of DNA linking number in SV40 chromatin by intercalation, we incubated SV40 chromatin with 100 uM ellipticine *in vitro*. SV40 chromatin preparation is known to contain an endogenous topoI activity as described by Keller *et al.* (1977). This activity was present in our SV40 chromatin preparation as evidenced by the relaxation of the indicator plasmid DNA added to the chromatin preparation (Fig. 5, lane 2). Addition of ellipticine to SV40 chromatin resulted in the formation of the highly negatively supercoiled species (Ih) of both SV40 DNA and of the added plasmid DNA (Fig. 5, lane 3). The one hour ellipticine treatment following a half hour pretreatment with camptothecin (a topoI inhibitor), VP16, or VM26 gave rise to a similar pattern as that of the ellipticine treatment alone (Fig. 5, lanes 4-9). These results are again consistent with intercalation as the mechanism for the alteration of SV40 chromatin DNA linking number by ellipticine. Furthermore, the alteration of SV40 DNA linking number in the presence of camptothecin, VP16, or VM26 suggests that the topoisomerase involved in the fixation of SV40 DNA linking number subsequent to the ellipticine intercalation is insensitive to these topoisomerase inhibitors.

On the other hand, if the failure of observing the Ih form in the absence of ellipticine were due to the relaxation of Ih by topoII, then this DNA species should also be found when the cells are treated with other topoII inhibitors that do not intercalate into DNA. Treatment of SV40 infected cells with

novobiocin, VP16 or VM26 at various concentrations failed to induce Ih DNA (data not shown). Considering the fact that these drugs cause DNA cleavage, SV40 DNA extraction from the drug-treated cells was preceded by a treatment of the cell nuclei with 0.5 M NaCl and 20 mM EDTA which has been shown to reverse topoII cleavage (Tewey *et al.*, 1984). These treatments still failed to reveal the Ih DNA.

Furthermore, the majority of ^{3}H-thymidine pulse-labeled SV40 DNA remained as supercoiled DNA and was not cleaved by treatment with VP16 or VM26 (data not shown). As ellipticine converted the majority of newly synthesized DNA to form Ih DNA (Fig. 2), the absence of the Ih DNA in VP16- or VM26- treated samples could not be simply due to the cleavage of *all* the Ih DNA into the linear form by the treatment with VP16 or VM26. As shown in Fig. 5, pretreatment of cells with VM26 or VP16 for thirty minutes before the addition of ellipticine failed to prevent the induction of Ih DNA. If the failure of VP16 or VM26 to induce the Ih DNA were due to the cleavage of Ih DNA by topoII, then the pretreatment should have prevented the formation of Ih DNA in the presence of ellipticine.

In summary, the data presented above argue strongly for intercalation as the mechanism of increasing *in vivo* SV40 DNA superhelical density by ellipticine. Thus, it becomes possible to calculate the intercalation density of ellipticine in SV40 chromatin by the known unwinding angle (17^{0}) of ellipticine intercalation (calculated from the data in Kohn *et al.*, 1975, using the

unwinding angle of ethidium bromide of 26^0 in Keller *et al.*, 1977 as standard). For the highly supercoiled Ih DNA with 38 to 47 superhelical turns, the intercalation density is about one ellipticine molecule per 10-20 bp of SV40 DNA. Since SV40 chromatin is organized into nucleosome structures with a 146 bp nucleosome core and 40-50 bp linker DNA, this intercalation density indicates that ellipticine can intercalate within the nucleosome cores of the newly synthesized SV40 chromatin.

E. DISCUSSION

Induction of double stranded DNA cleavage by ellipticine

Our observation that ellipticine did not cause significant double stranded cleavage in intracellular SV40 and Ad5 DNA seems to differ from the results of previous studies which conclude that ellipticine can cause double stranded DNA breaks in several mammalian cells *in vivo* (Filipski and Kohn, 1982; Multon *et al.*, 1989; Ross and Bradley, 1981; Zwelling *et al.*, 1982) and *in vitro* (Tewey *et al.*, 1984). The concentration of ellipticine used in the present study is similar to that used by these other studies. Therefore, it is unlikely that the absence of DNA cleavage observed in the present study is due to the self-inhibitory phenomenon of the intercalating drugs at the concentration higher than optimum (Tewey et al., 1984; Pommier *et al.*, 1985). The trivial possible explanation of poor uptake of ellipticine by CV-1 or HeLa cells has been

excluded by our results which indicate that ellipticine treatment inhibits SV40 and Ad5 DNA replication, induces the formation of highly intertwisted SV40 DNA, and interferes with VP16 and VM26 in their inducing DNA cleavage.

Therefore, the discrepancy appears due to the difference of experimental protocols used to detect DNA cleavages. Gel electrophoresis has been used in the present study to assess double stranded DNA breaks whereas alkaline elution has been used in the others. The alkaline elution examines the overall DNA breaks in the cells, whereas gel electrophoresis and Southern blotting detect DNA breaks in a specified gene. For example, double stranded cleavage of the *c-myc* gene is not detected in ellipticine treated cells even though double stranded breaks are found in total cell DNA (Zwelling *et al.*, 1982).

Induction of the DNA-topoII cleavable complex by ellipticine derivatives has been shown to depend on the presence of 9-OH group (Auclair, 1987). Since ellipticine lacks this functional group, it is not surprising that much fewer cleavage of viral DNA was induced by ellipticine than by VP16 and VM26.

Recently, several topoII inhibitors have been shown to produce few topoII-associated double stranded break, including the antitumor drugs merbarone (Drake *et al.*, 1989), amilorides (Besterman *et al.*, 1989), 3'-N-alkyl derivatives of doxorubicin and daunorubicin (Bodley *et al.*, 1989), gossypol (Adlakha *et al.*, 1989) and ethidium bromide (Tewey *et al.*, 1984). Merbarone, amilorides, gossypol and ethidium bromide have also been shown to interfere

with epipodophyllotoxins in inducing double stranded breaks. The behavior of ellipticine in our study resembles that of these drugs. The molecular basis for the drug interactions remains to be elucidated.

Intercalating agents as probes for studying chromatin structure and DNA topology *in vivo*

The observation that ellipticine induces a highly supercoiled SV40 DNA with superhelical density corresponding to about 1.5-1.7 superhelical turns per nucleosome was unexpected. The untreated SV40 DNA has the superhelical density corresponding to about one superhelical turn per nucleosome. Since DNA wraps around histone cores about 1.75 turns, theoretically, each nucleosome should contribute to 1.75 negative supercoils. The discrepancy between the observed superhelical density of intracellular SV40 DNA and that expected from the nucleosome structure has been termed the "linking number problem" (Finch *et al.*, 1977). The deficiency of supercoil numbers in SV40 DNA has been thought to arise either from the difference between the double helical twist in SV40 chromatin (Hayes *et al.*, 1990) and that in solution (Wang, 1979) or from the reduction of writhing number by the relative orientation of nucleosomes (Worcel *et al.*, 1981; Butler,1983; Woodcock *et al.*, 1984; Zivanovic *et al.*, 1988; Grigoryev and Ioffe, 1981). It has been shown that a random orientation of nucleosomes in SV40 chromatin could result in the observed reduction in superhelical density (Grigoryev and Ioffe, 1981), as well

as in the broad distribution of SV40 DNA topoisomers (Shure *et al.*, 1977).

Our observation of highly supercoiled Ih DNA with superhelical density corresponding to that with no deficiency in supercoil numbers was thought originally to provide a possible solution to the linking number problem. Thus, the nucleosome in the newly synthesized chromatin would be organized in such a way that there would be no reduction of writhing number. Subsequently, a topoII activity would be responsible for the randomization of the orientation of nucleosomes to achieve a reduction of writhing number from 1.75 per nucleosome to 1.0 per nucleosome.

However, our analysis indicates that the formation of this highly supercoiled SV40 DNA species is caused not by the inhibition of topoII by ellipticine *per se* but but the intercalation of ellipticine into the nucleosome cores of SV40 chromatin. This conclusion is based on several types of evidence: (1) ellipticine induces Ih formation in the SV40 chromatin extract *in vitro*; (2) ellipticine inhibits the crosslinking of SV40 chromatin by psoralen which is also an intercalating agent; (3) other intercalating agents such as actinomycin D and acridine orange also increase supercoiling density of SV40 DNA *in vivo*; and (4) non-intercalating topoII inhibitors such as VP16, VM26 and novobiocin fail to induce Ih formation of intracellular SV40 DNA, and also to prevent the induction of Ih formation by ellipticine.

Alteration of DNA linking number in SV40 chromatin by chloroquine, another intercalating agent, has been reported by Esposito and Sinden (1987).

However, the *in vivo* effect of chloroquine which is not a known topoII inhibitor on SV40 DNA linking number is small even at the extremely high concentration of 10 mg/ml. The difference in effect between chloroquine and ellipticine on SV40 DNA linking number appears due to a stronger binding of ellipticine to DNA than chloroquine. In this respect, ellipticine and other strong intercalating agents are better probes for studying DNA topology *in vivo*.

From the change in the linking number of Ih DNA and the known unwinding angle of ellipticine intercalation (Kohn *et al.*, 1975), the intercalation density is estimated to be about one ellipticine molecule per 10-20 bp. This value implies that ellipticine is able to intercalate into the DNA within nucleosome cores in the newly synthesized SV40 chromatin. This result suggests that newly synthesized SV40 chromatin has more loosely organized nucleosomes than the bulk of SV40 chromatin. Therefore, intercalating agents such as ellipticine may provide a useful tool to probe the structures of nucleoprotein complexes *in vivo*.

Previous studies also have shown that several intercalating agents intercalate within nucleosomes. Chen *et al.* (1990) showed that actinomycin D preferentially intercalates into the nucleosomes of the transcriptionally active chromatin. The carcinogen, benzo[a]pyrene diol epoxide, has also been shown to intercalate within nucleosomes (MacLeod *et al.*, 1989). Altered organization of nucleosome structure either in the active nucleolar gene of

Dictyostelium or in chromatin at high pH has been shown to allow psoralen to bind and crosslink DNA within nucleosomes (Sogo *et al.*, 1984). These results suggest that subtle differences in chromatin organization can be probed with intercalating agents.

ACKNOWLEDGEMENTS

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F. FIGURE LEGENDS

Fig. 1. Agarose gel electrophoretic analysis of SV40 DNA extracted from cells treated with topoII inhibitors. Panel A shows the result of treatment with ellipticine. Lane 1: kb mol. wt. marker (BRL); 2: control without ellipticine treatment; 3: ellipticine 10 uM; 4: ellipticine 33.3 uM; 5: ellipticine 100 uM. Panel B shows the effect of ellipticine pretreatment on VP16 or VM26 induced cleavages. Lane 1: kb mol. wt. marker; 2: control without drug treatment; 3: 100 uM ellipticine for 90 minutes; 4: 100 uM VM26 for 60 min.; 5: 100 uM VP16 for 60 min.; 6: 100 uM ellipticine for 30 min., then 100 uM VM26 for 60 min. in the presence of ellipticine; and lane 7: 100 uM ellipticine for 30 min., then 100 uM VP16 for 60 min. in the presence of ellipticine. I, II, and III label the positions of supercoiled, nicked and linear SV40 DNA, respectively. The DNA above II is cellular DNA.

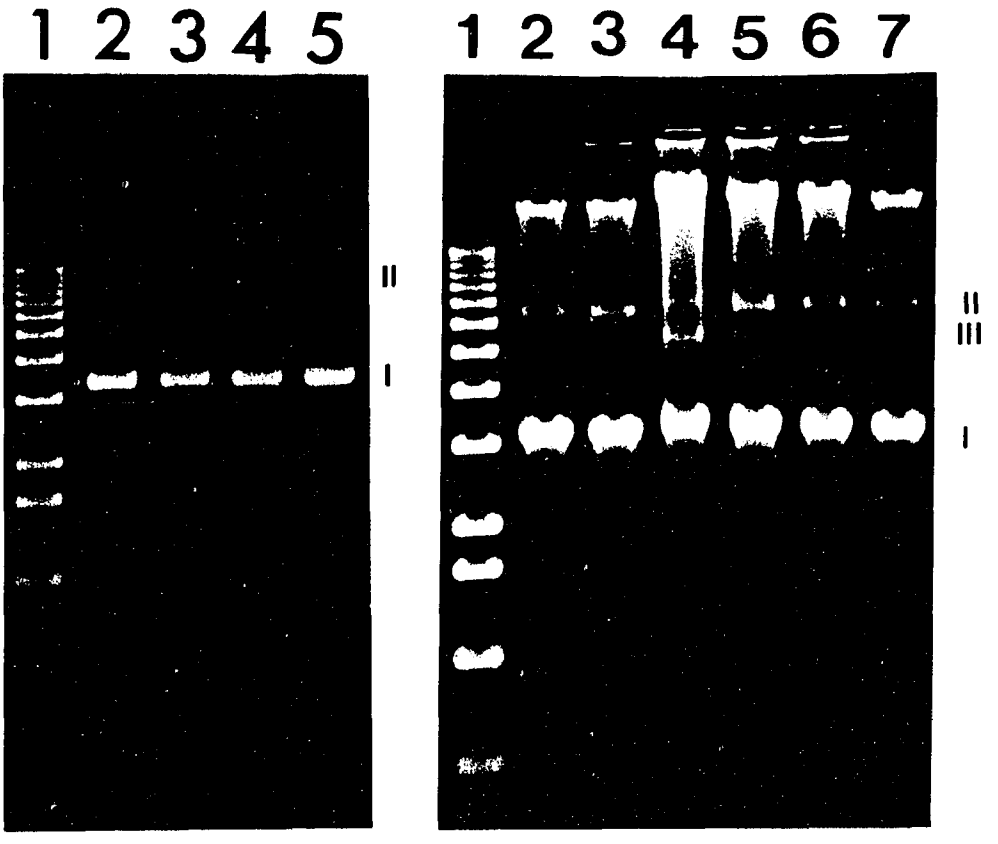

1 2 3 4 5
II
I
1 2 3 4 5 6 7
II
III
I

Fig. 2. Electron micrographs of highly intertwisted SV40 DNA induced by ellipticine treatment. Panels a and b show two SV40 DNA molecules highly intertwisted, a result of inhibition of topoisomerase II during DNA replication. Panels a' and b' are hand-drawn interpretations of panels a and b, respectively. Thick and thin lines indicate two intertwisted daughter molecules.

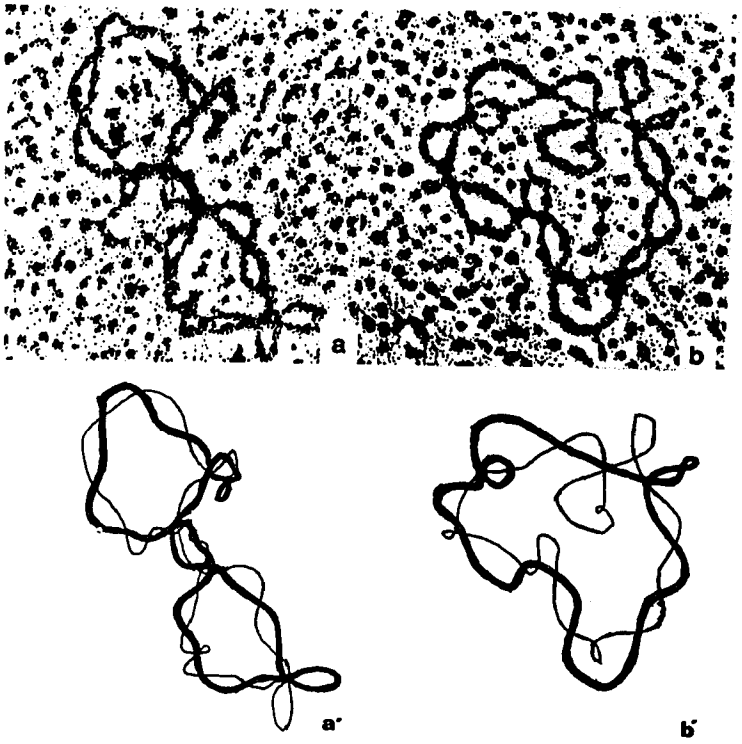
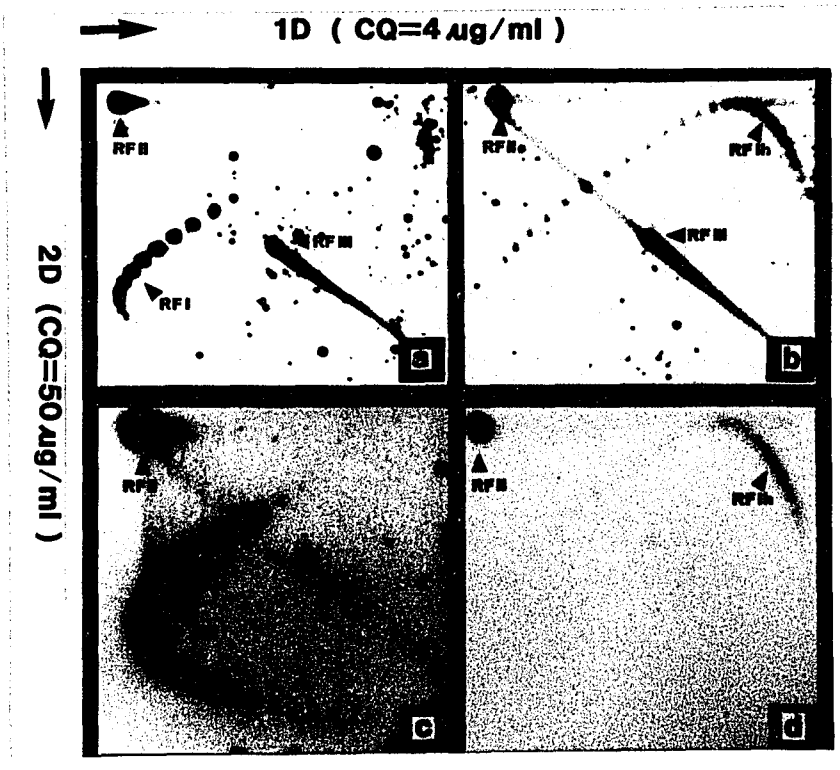

a
b
a'
б'

Fig. 3. Chloroquine agarose gel electrophoresis of SV40 DNA topoisomers in ellipticine-treated and control samples. Fig. 3a: Two-dimensional gel electrophoresis (1st D, 4 ug/ml chloroquine; 2nd D, 50 ug/ml chloroquine) of SV40 DNA extracted from cells without (panels a and c) or with (panels b and d) 100 uM ellipticine treatment from 24 to 26 hr. p.i. (100 uM camptothecin was included in the treatment but similar results were obtained with ellipticine alone). Panels a and b are Southern blots whereas panels c and d are fluorographs of the DNA labeled with ^{3}H-thymidine for one hour before drug treatment. Highly supercoiled topoisomers in ellipticine-treated samples are indicated as "Ih" DNA. II and III indicate the position of nicked and linear SV40 DNA. Fig. 3b: 1-D chloroquine gel of SV40 DNA extracted from cells at 48 hr. p.i. Lanes a and a': mock treated; b and b': 100 uM camptothecin for 2 hours; c and c': 100 uM ellipticine for 2 hours. The left panel shows the ethidium bromide staining picture and the right panel the fluorograph of the same gel.


1D (CQ=4 μg/ml)
2D (CQ=50 μg/ml)
RFII
RFIII
RFI
a
b
c
d


EtBr
3H
a b c
a' b' c'

Fig. 4. Alkaline agarose gel electrophoretic analysis of EcoRI linearized SV40 DNA extracted from control cells (lane 1), cells crosslinked with psoralen (lane 2), cells pretreated with ellipticine followed by psoralen crosslinking (lanes 3, 10 uM ellipticine, lane 4, 50 uM, lane 5, 100 uM) or cells pretreated with ethidium bromide followed by psoralen crosslinking (lane 6, 0.5 ug/ml, lane 7, 5 ug/ml ethidium bromide). Crosslinking was performed by incubating cells with 6 ug/ml AMT followed by irradiation with a 302 UV light for 20 minutes. Arrow points to the position of denatured single strand SV40 linear DNA and the bracket indicates the region where crosslinked SV40 DNA resides.

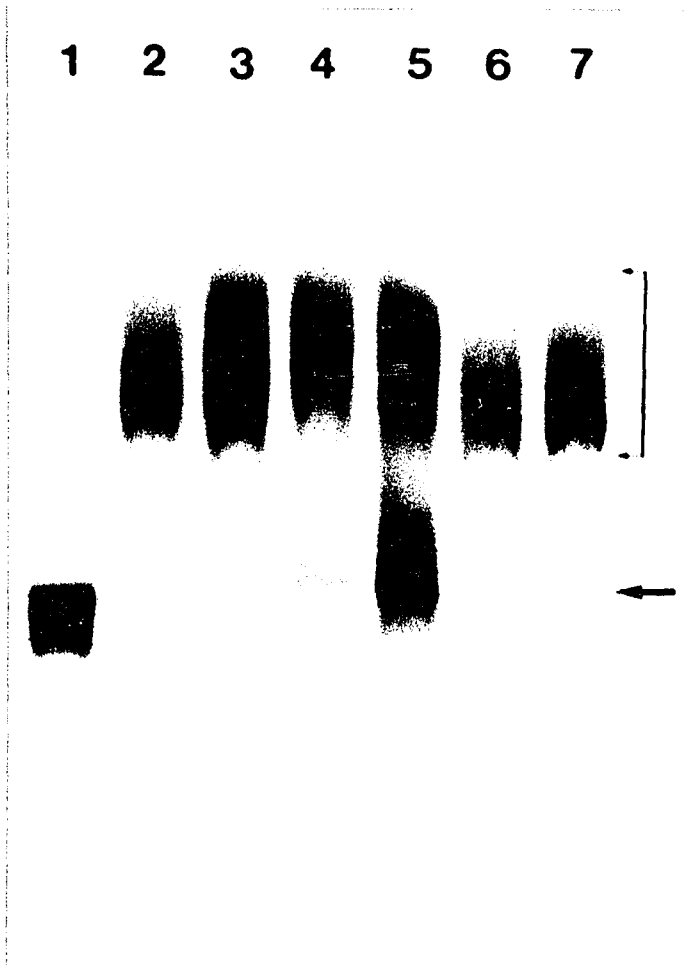
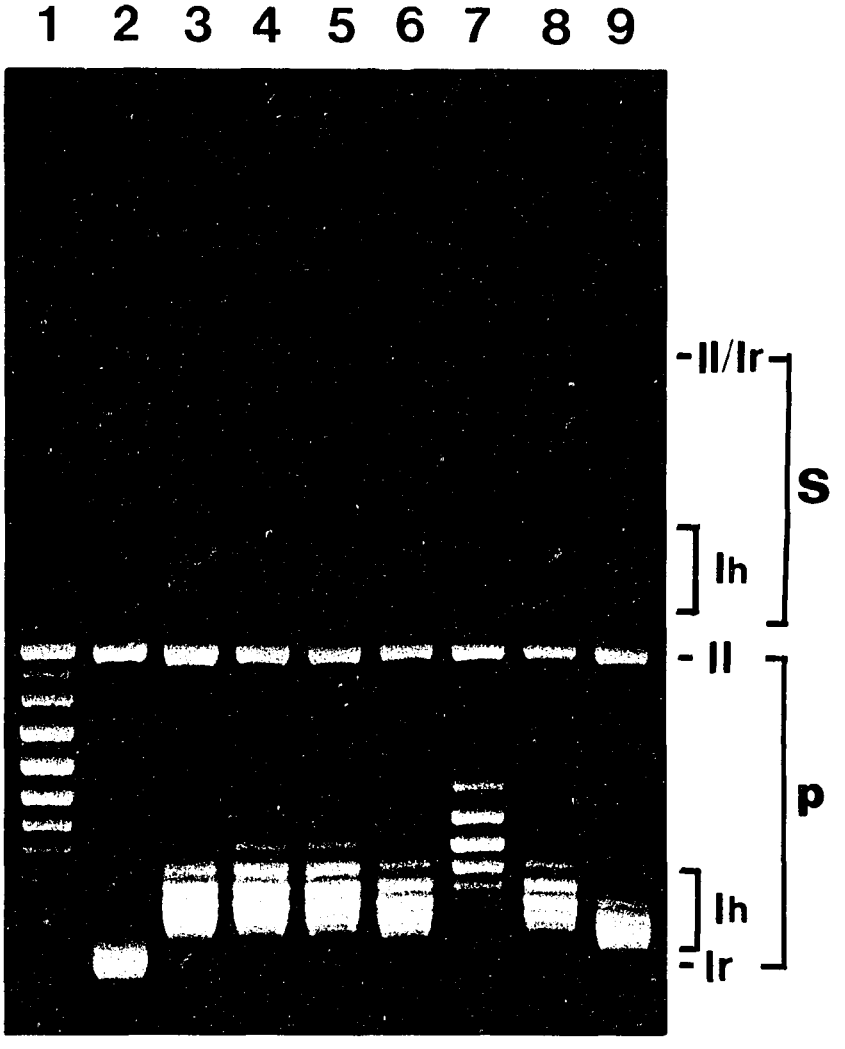

1 2 3 4 5 6 7

Fig. 5. Agarose gel electrophoretic analysis of DNA topoisomers of indicator plasmid (pBluescriptKS$^+$, 3 kb; Strategene) and SV40 chromatin. Lane 1, plasmid DNA alone. Lane 2, incubation of plasmid DNA with SV40 chromatin for 1.5 hrs. Lane 3, same as lane 2, except in the presence of 100 uM ellipticine. Lane 4, chromatin plus plasmid DNA in the presence of 10 uM camptothecin for 0.5 hr, followed by 1 hr incubation in the presence of 100 uM ellipticine. Lane 5, same as lane 4, except for camptothecin being 100 uM. Lane 6, same as lane 4, except for camptothecin being replaced by 10 uM VP16. Lane 7, same as lane 5, except for camptothecin being replaced by 100 uM VP16. Lane 8, same as lane 4, except for camptothecin being replaced by 10 uM VM26. Lane 9, same as lane 5, except for replacement of camptothecin by 100 uM VM26. Ih indicates highly negatively supercoiled DNA species; Ir relaxed DNA species, and II nick circle DNA. Bracket marked S indicates the region where SV40 DNA resides; bracket marked p the region where the indicator plasmid DNA resides.


1 2 3 4 5 6 7 8 9
-II/Ir
S
Ih
-II
p
Ih
-Ir

Chapter 4

Effects of sequence microenvironment on the biological activity of the SV40 origin of DNA replication

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A. ABSTRACT

The effects of sequence microenvironment on the biological activity of SV40 origin of replication were investigated. Using SV40 virus variants that contain two identical origins, *in*(Or)1411 and *in*(Or)1412 (*in*1411 and *in*1412), we observed that the authentic origin is used at higher frequency than the second, inserted origin. We examined the following hypotheses that might explain differential usage of the two origins: 1) sequences adjacent to the origin can affect its activity; 2) association of an origin with an active transcription unit facilitates origin activity; 3) transcription passing through an origin interferes with that origin's activity; 4) different chromatin organization of the two origins can affect their activities. Results of cloning and transient DNA-replication assay were not consistent with the first two hypotheses. Experiments using P1 nuclease to examine altered chromatin conformation at the two origins indicated a similar chromatin organization in the two functional origins. The transcription interference model was tested by using a transcription unit driven by the major immediate early gene promoter/enhancer (P/E) of the human cytomegalovirus (HCMV). Unexpectedly, the analysis showed that the HCMV enhancer sequence strongly suppresses SV40 origin activity. The suppression was found to be independent of transcription, and to be distance- and position-dependent. To test further the transcription-interference hypothesis, we constructed a variant of *in*1412

by inverting the sequence between the two origins. The resultant DNA, *in*(Or)1412v (*in*1412v), contains both origins located at the 3' end of a transcription unit. Transfection experiments indicated that *in*1412v replicated significantly less efficiently than the parental *in*1412. This result is consistent with the hypothesis that transcription passing through the origin interferes with the initiation of replication from that origin.

B. INTRODUCTION

The double-stranded circular genome of simian virus 40 (SV40) represents a simple model system for studying mechanisms of mammalian DNA replication. With only one encoded viral protein T antigen involved, the virus depends extensively on cellular machinery for its DNA replication (Kelly and Challberg, 1989; Stillman, 1989). Replication starts at a unique site, and the elongation of nascent DNA chains proceeds bidirectionally at equal rates (Danna and Nathans, 1972; Fareed *et al.*, 1972). Analyses of naturally arising evolutionary variants and deletion mutants have narrowed the limits of the minimum *cis*-element for the initiation of SV40 DNA replication (core *ori*) to a segment of 65 bp (#5210-#31 in the SV40 sequence map) including a 27-bp palindrome (#5230-#13, Tag binding site II) and an AT-rich sequence (#15-#31) (Bergsma *et al.*, 1982; DiMaio and Nathans, 1980; 1982; Gutai and Nathans, 1978; Learned *et al.*, 1981; Myers and Tjian, 1980; Subramanian and Shenk, 1978). Contribution of each nucleotide in this segment has been further determined by point mutation analysis (Tegtmeyer *et al.*, 1986 and references therein).

A common theme for DNA replication is that the origin of DNA replication is neighbored by sequences regulating transcription (DePamphilis, 1988). The interactions between replication and transcription regulatory elements seem rather complex. In SV40 DNA, the core *ori* is neighbored by

Tag binding site I (#5184-#5209) on the early transcription side, and three 21-bp GC-rich tandem repeats (Tag binding site III, #40-#103) and two 72-bp tandem repeats (#107-#250) on the late transcription side. The 21-bp repeat is a bidirectional promoter for SV40 transcription while the 72-bp repeat is a transcription enhancer (Everett *et al.*, 1983; Banerji *et al.*, 1981). In the presence of Tag binding site I and 21-bp repeats the *ori*-core activity is increased by 100-fold (Guo *et al.*, 1990 and references therein). In the absence of the 21-bp repeats, the 72-bp repeats substitute for the 21 bp repeats. However, when the 72-bp repeats are reiterated more than ten times, these sequences exert an inhibitory effect on *ori*-core activity (Kumar *et al.*, 1988 and references therein). Translocation of the 21 bp repeats to the early side of the origin also inhibit the origin activity (Lee-Chen and Woodworth-Gutai, 1896).

The molecular basis by which these transcription regulatory sequences activate or inhibit *ori*-core activity is not well understood. One critical requirement for the study is to establish *in vitro* replication systems that could reproduce these mostly *in vivo* phenomena. Studies of SV40 DNA replication in the cell-free system have revealed similar enhancing activity of the auxiliary sequence on SV40 *ori*-core (Guo *et al.*, 1989). *In vitro* replication analysis also points to chromatin assembly (Cheng and Kelly, 1989), participation of transcription activator containing activation domain (Bennett-Cook and Hassell, 1991), and facilitation of DNA unwinding (Gutierrez *et al.*, 1990) as possible mechanisms for replication activation by transcription sequences.

How two identical origins containing transcription sequences are used in a single SV40 molecule is a more complex question. It may have some bearing on eukaryotic cell DNA replication which is initiated from many sites in the genome. Initiation of DNA replication from multiple sites must be tightly controlled and coordinated so that the genome is replicated only once per cell cycle. How this process is regulated is still poorly understood.

In this chapter, we describe our approach to this problem by using SV40 variants containing two copies of replication origin as a simple model system. We have obtained evidence that, despite the presence of all the sequences needed for the initiation of SV40 DNA replication, the two origins in the virus variants differ significantly in their biological activity during lytic infection in CV-1 cells. The authentic *ori* is used significantly more frequently than the inserted one in an *in vitro* nuclei replication assay.

Examination of the two *ori*s in the SV40 variants reveals several differences between them: (1) The DNA sequences surrounding the two *ori*s are different; (2) The more active *ori* is associated with two active transcription units whereas the less active *ori* is not; and (3) The less active *ori* is located at the 3' ends of the two SV40 transcription units. Based on these differences we designed experiments to test which difference is responsible for the observed different replication efficiency.

Using a transient DNA replication assay in Cos-1 cells we eliminated the first two possibilities. Our data are consistent with, but do not directly prove,

the third possibility. During the analysis of the third possibility we accidentally discovered an interesting phenomenon that the early promoter/enhancer of human cytomegalovirus (HCMV E/P) could suppress the activity of SV40 origin in a distance-dependent manner. Deletion and competition analyses suggest that the CMV enhancer sequence acts as a *cis*-element that suppresses the function of SV40 origin.

C. MATERIALS AND METHODS

Cells and parental plasmids

CV-1 and Cos-1 African green monkey kidney cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 50 ug/ml gentamicin (Gibco).

Plasmid pSV2neo contains SV40 *ori* sequences followed immediately by an intact transcription unit coding for the G418-resistance *neo* gene (Southern and Berg, 1982). Plasmid pUC19 was kindly provided by Jiaojiao Zhang in Dr. Irwin Gelman's lab. Plasmid pBluescriptKS$^+$ was kindly provided by Jianmin Chen in Dr. Lu-Hai Wang's lab. Plasmid pCMV.poly (pCMV) was kindly provided by Dr. Eseng Lai of the Memorial Sloan-Kettering Cancer Center. All four plasmids contain a bacterial replication origin and the gene for ampicillin resistance, which allow the plasmid transformants to grow under ampicillin selection. The simplified restriction maps of pSV2neo and pCMV are

illustrated in Fig. 1. The detailed maps of pUC19 and pBluescriptKS$^+$ can be found in Biolab and Stratagene catalogs, respectively.

Analysis of *ori* usage in SV40 variants using *in vitro* nuclei replication and neutral/alkaline 2D gel electrophoresis

CV-1 cells, when 90-95% confluent, were infected with wild type SV40 or the variant *in*1411 or *in*1412 at a MOI of 20 PFU/cell. At 48 hours postinfection (hpi), infected cells were lysed with the lysis buffer (0.2% NP-40, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM HEPES, pH7.4). After washing with the reaction buffer (50 mM HEPES, pH7.6, 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$), *in vitro* nuclei replication reaction was processed in 1 ml of the reaction buffer containing 2.5 uM each of dATP, dTTP and dGTP, and 50 uCi of α-^{32}P-dCTP at 37^{0}C for 20 min. The reaction was terminated by adding SDS to 1% and EDTA to 20 mM. Proteinase K was added to 100 ug/ml and the mixture was incubated at 37^{0}C for additional 2 hours. Viral DNA was subsequently separated from cell DNA by a modified Hirt extraction method (Wong and Hsu, 1988). Free α-^{32}P-dCTP in the DNA sample was eliminated by passing the sample through a Sephadex G-50 spin-column. The free counts were trapped in the column and DNA with incorporated isotope was ethanol precipitated from the eluate.

Since replication intermediates (RIs) contain single-stranded regions, RIs were enriched by passing through a Benzoylated-Naphthoylated DEAE

cellulose (BND-DEAE, Sigma) column. The procedure was as follows: a BND-DEAE column was packed in a Pasteur pipet (1 ml bed vol.) and equilibrated with 6 ml of Buffer A: 0.3 M NaCl in TE (10 mM Tris, 1 mM EDTA, pH8.0), at 200 ul/min. DNA sample was loaded in 200 ul of Buffer A. The column was washed with 3.0 ml of Buffer A and then with 3.0 ml of Buffer B (1 M NaCl in TE), and the high salt (HS) fractions were collected. (These fractions are enriched with double-stranded DNA). The column was further washed with 2.0 ml of Buffer C (1.8% caffeine in Buffer B) and the eluted fractions were collected. (These fractions are enriched with RIs and RNA). Both HS and caffeine fractions were precipitated with ethanol.

The above caffeine fractions were pooled and digested with appropriate restriction enzymes (*e.g.*, EcoRV or KpnI+PstI). The digested DNA was first resolved in a neutral 1.0% agarose gel (first-dimension). After staining with ethidium bromide the lanes were cut out. The lanes then were laid horizontally on the top of a new gel plate onto which a 1.2% alkaline agarose gel was to be made. The alkaline gel was made by first melting agarose in 0.9 volume of H_2O and then adding 0.1 volume of 10x alkaline buffer (400 mM NaOH, 10 mM EDTA) when the temperature of the melted gel had decreased to 65^{0}C. This second-dimension gel was run at 200 mA per 30 cm for 24-48 hours in a cold room. The gel after electrophoresis was vacuum-dried and exposed to XAR film (Kodak).

Construction of recombinant plasmids

I. General preparations and reactions

(1) Plasmid DNA preparation: After appropriate transformation and identification of the desired plasmid, a single bacterial colony was propagated in 100-150 ml of TB medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72mM K_2HPO_4; Sambrook *et al.*, 1989) containing 50 ug/ml ampicillin for 12-16 hours in a 37°C shaker. The bacteria were collected by centrifugation and subjected to alkaline lysis (Maniatis *et al.*, 1982). The plasmid-containing supernatant was collected after centrifugation and precipitated with ethanol. The pellet was resuspended in 10 ml of TE containing 10 ug/ml of RNase, incubated at 37°C for 30 minutes and precipitated again with ethanol. The pellet was resuspended in 6.0 ml of TE. After the addition of 6.0 g of CsCl and 300 ul of ethidium bromide (500 mg/ml), the solution was distributed into two 4 ml Quick-Seal ultracentrifuge tubes (Beckman). The tubes were balanced and sealed, placed in the TLN100 rotor and centrifuged in a TL-100 table ultracentrifuge (Beckman) at 100,000 rpm for 4 hours. The supercoiled plasmid DNA band was collected, repeatedly precipitated with ethanol to remove CsCl, extracted with butanol to remove ethidium bromide, and precipitated with ethanol. The purified DNA was resuspended in 300 ml of TE. The yield was generally 1-5 mg by OD_{260nm} reading. The quality of DNA was evaluated by agarose gel electrophoresis. Usually 90% was supercoiled and the remaining 10% was nicked plasmid

monomer DNA.

(2) Preparation of plasmid vector and insert DNA for subsequent ligation: Appropriate amounts of plasmid DNA were digested with restriction enzymes (Boehringer-Mannheim, Promega, or Biolab). The vector DNA was further subjected to phosphatase treatment (CIP, Promega). The DNA fragments were resolved in a 0.8% low-melting-point agarose (Bethesda Research Lab.) gel. The desired bands were cut out, collected in Eppendorf tubes, and incubated at 65°C for 3 min. NaCl was added to the melted gel to a final concentration of 0.25 M and the tubes were further incubated at 65°C for 3 min. The DNAs were purified by extraction twice with phenol and once with chloroform/iso-amyl alcohol (24:1). The purified DNAs (the vector DNAs being dephosphorylated and the insert DNAs being phosphorylated) were quantitated by gel electrophoresis and were ready for ligation reactions.

(3) Ligation reaction: The molar ratio between the vector and the insert DNA was usually chosen as 1:5 and the amount of vector DNA used was usually 20 ng. The ligation reaction was processed in a volume of 10, 15, or 20 ul using T4 DNA ligase (Promega or Biolab), and incubated at 14°C for 8 hours or room temperature for 2 hours.

(4) Transformation and screening for the desired clones: The ligated DNA was diluted five-fold with TE and 1.5 ul was used to transform 20 ul of competent bacteria (DH5α from BRL or INV1αF' from Invitrogen) in an Eppendorf tube placed on ice. Thirty minutes after incubation on ice, the tube was transferred

to a 42°C incubator and heat-shocked for 45 seconds. The tube was added with 80 ul of SOC (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose; Sambrook *et al.*, 1989) and shaken at 250 rpm in a 37°C shaker for 1 hour. The entire 100 ul of the transformed bacteria were subsequently plated in a LB/Ampicillin plate (Maniatis *et al.*, 1982) and the plate incubated in a 37°C warm room. Twelve to 24 hours after incubation, Amp^r colonies were picked and propagated in 1.5 ml of TB/Amp liquid in a snap-capped tube in a 37°C shaker. Plasmid mini-preparation was made by the alkaline-lysis method and the DNA was analyzed by digestion with an appropriate set of restriction enzymes. After the desired plasmid was identified, the DNA was prepared in large scale as described in the section above.

II. Construction of plasmids

The maps of all the plasmids to be described are summarized in Fig. 2.

(1) Cloning of the two origins of replication and their surrounding sequences in *in*1412 virus into pUC19 vector: pSVOR1, pSVOR2 and pSVOR3 (Fig. 2a): DNA of the SV40 variant *in*1412 was prepared by a modified Hirt extraction method from the virus-infected CV-1 cells (Wong and Hsu, 1988). The purified DNA was digested with HindIII or EarI plus DrdI. The digested DNA was electrophoresed in a low-melting-point agarose gel. The 1 kb HindIII B fragment containing the authentic origin of replication (*ori*), the 2 kb HindIII A fragment containing the inserted, second *ori*, or the 1.5 kb EarI-DrdI

fragment containing the authentic *ori* was purified as described above. The locations of these fragments in *in*1412 map is shown in Fig. 13. The HindIIIA and HindIIIB fragments were ligated to HindIII-linearized pUC19 as described above. The EarI-DrdI fragment was treated with T4 DNA polymerase (Biolab) to fill in the ends, and blunt-end-ligated to SmaI-linearized pUC19. The presence of the correct insert in the plasmids was checked by restriction enzyme digestion. The plasmids containing the HindIIIA and HindIIIB fragments are designated as pSVOR1 and pSVOR2 respectively (Fig. 2a). Digestion of these plasmids with HindIII enzyme released the corresponding insert fragments as predicted. The plasmid containing the 1.5 kb EarI-DrdI fragment is designated as pSVOR3 (Fig. 2a). The presence of the correct insert was confirmed by digestion with EcoRV which generated a single 4.2 kb band, with PstI plus EcoRI which generated 1.5 kb (EarI-DrdI insert) and 2.7 kb (pUC19) bands, and with BglI nuclease which generated 2.3, 1.1 and 0.8 kb bands.

(2) Construction of a plasmid from pSV2neo by replacing the *neo* transcription unit with a nonspecific lambda phage DNA. plasmid pSV2L (Fig. 2b): The *neo* transcription unit (excluding the promoter) in the plasmid pSV2neo DNA (Southern and Berg, 1982) was excised out of the plasmid by digestion with HindIII and EcoRI (see Fig. 1). The 2.6 kb DNA fragment containing SV40 *ori* and the vector sequences was isolated from low-melting-point agarose gel. After phosphatase treatment it was ligated with the 1.9 kb HindIII-EcoR1

bacteriophage lambda DNA fragment (nucleotide 21,226-23,130 in the lambda phage map). The presence of the proper insert in the plasmid was confirmed by a single HindIII or EcoRI digestion to generate linear full-length (4.5 kb) DNA, and by the double digestion to generate equimolar bands of 2.6 kb and 1.9 kb.

(3) Construction and cloning of variants of *in*1412: *in*1412v, *in*1412neo and their parental plasmids p1412, p1412v and p1412neo (Fig. 2c): DNA of *in*1412 was purified using the micrococcal nuclease digestion method (Lai and Chu, 1991) to achieve better purity than the Hirt method. To construct an orientation variant of *in*1412, the viral DNA was cleaved at the two *ori*s with BglI to generate 2.8 and 2.4 kb fragments (see Fig. 14). The fragments were separately purified and ligated in an equimolar amount. Because the two BglI cleavage sites have identical sequences, ligation of the two fragments should give equimolar amount of two circular DNA containing the 2.8 and 2.4 kb fragments in two different orientations. One of the circles would be the parental *in*1412 whereas the other circle (the *in*1412v desired) would contain the 2.4 kb (or the 2.8 kb) fragment in the inverted orientation relative to that in *in*1412 (see Fig. 13). Analysis of the ligation products by restriction enzyme digestion indicated that this was indeed the case. As shown in Fig. 14, digestion with BclI plus EcoRI produced a pair of 3.9 and 1.3 kb fragments for *in*1412, and a pair of 3.3 and 1.9 kb fragments for *in*1412v. These two pairs are approximately equimolar. Subsequently, the ligation product was digested

with EcoRI and the resulting full-length linear DNA was gel-purified as described above. The DNA then was ligated to EcoRI-linearized pUC19 to generate p1412 and p1412v plasmids (see Fig. 2c). After transformation, single colonies were screened for p1412 and p1412V, respectively, by PstI analysis.

In order to construct p1412neo, pBluescriptKS$^+$ was chosen as the plasmid vector due to the convenience of its polylinker. The plasmid was cut with HindIII+BamHI, phosphatase-treated, and the 3 kb fragment was purified as described above. DNA of *in*1412 was cut with HindIII+EcoRV and the resultant 0.7 kb fragment containing the authentic *ori* was purified, or cut with EcoRV+BglI and the resultant 2.1 kb fragment containing partial inserted *ori* was purified. DNA of pSV2neo was cut with BglI+BamHI and the resultant 2.4 kb fragment containing partial SV40 *ori* followed by *neo* and SV40 splicing/polyadenylation signal sequences was purified. Then the 4 pieces were ligated by a forced-ligation strategy. After an arduous screening following transformation, one correct colony with the plasmid consisting of the 4 pieces was identified by various sets of restriction digestion. This plasmid was first linearized with SacI (whose location is on the other side of the polylinker relative to the XhoI site in the polylinker, separated by the insert), then blunt-ended with T4 DNA polymerase and ligated to XhoI linker. After transformation, the colonies containing two XhoI sites flanking the *in*1412neo piece were identified and the plasmid DNA was prepared in large scale. This plasmid is designated p1412neo.

To obtain the corresponding recombinant viruses, p1412 and p1412v were digested with EcoRI to release the 5.2 kb SV40 DNA, and self-ligated to circularize the DNA. The circularized DNA was transfected into Cos-1 cells, and progeny viruses were produced during a period of 2-4 weeks. The viruses are designated as *in*1412 and *in*1412v, respectively. For the production of *in*1412neo virus, p1412neo DNA was digested with XhoI to retrieve the 5.2 Kb *in*1412neo. The 5.2 kb DNA was circularized by self-ligation and transfected into Cos-1 cells to generate the recombinant virus *in*1412neo.

(4) Construction of plasmids containing insertion of alpha satellite DNA into the HindIII site of pSV2neo: pM and pT (Fig. 2d): Digestion of cell DNA extracted from CV-1 cells by HindIII restriction endonuclease generated a 172 bp DNA ladder (Fig. 3). This ladder has been shown previously to be the alpha satellite DNA of monkey cells. The monomer, dimer, trimer, tetramer and pentamer DNA bands were excised from the gel, purified and cloned into the HindIII site of pSV2neo. Plasmid DNA isolated from Amp^r colonies were screened by HindIII digestion to determine the size of the insert which should correspond to the multiples of 172 bp. Southern blotting analysis using gel-purified 172 bp monomer DNA as a probe was used to confirm the insert as alpha satellite DNA. The plasmids containing a monomer or a trimer are designated pM and pT, respectively.

To construct length-control plasmids, lambda DNA was cut with HindIII, and the resulting 125 bp (control for monomer) and 564 bp (control for trimer)

were gel-purified and ligated to HindIII-linearized pSV2neo. The resultant plasmids are designated pNL1 and pNL2, respectively (see Fig. 2b).

(5) Construction of plasmids with HCMV P/E inserted at various positions of pSV2neo: pSCM1, pSCM2, pSCM3(+) and pSCM3(-) (Fig. 2e):

a) pSCM1 with HCMV P/E inserted at the HindIII site of pSV2neo: HCMV P/E was excised from the plasmid pCMV as follows: pCMV was first linearized with ClaI, the HCMV P/E start site in pCMV. Then the DNA was treated with Klenow enzyme to fill the 5'-protruding ends, and ligated to phosphorylated HindIII linker (d(pCAAGCTTG), Biolab) in a molar ratio of 1:100. Following digestion of HindIII, a 0.7 Kb fragment containing the HCMV promoter/enhancer was obtained (one end was the original ClaI site and the other end was the HindIII site located at the 3'-end of the pGem-1 polylinker connected downstream to the HCMV sequence in pCMV). The fragment was purified and ligated to HindIII-linearized pSV2neo. Only one positive clone was identified when screened by PstI digestion. The expected SV40*ori*-HindIII-to-HindIII(ClaI) ligation did not work properly, therefore this HindIII site was destroyed in the plasmid. The reason for this was that the HindIII linker had not been ligated to the ClaI end, suggested by subsequent DNA sequencing. DNA sequencing analysis also confirmed that the overall configuration was correctly arranged as designed: the SV40 *ori* sequence ended at #5176 and the HCMV P/E started at #-598, connected by a 5 bp junction (CGATG). Furthermore, DNA sequencing from the other orientation confirmed that SV40

ori was intact and was not mutated during the cloning procedure.

b) pSCM2 with SV40 *ori* in pSCM1 moved to the EcoRI site: The construction scheme for this plasmid is depicted in Fig. 4.

c) pSCM3, with HCMV P/E inserted at the 3' end of the *neo* transcription unit (BamH1 site): pCMV plasmid was first linearized with ClaI. The DNA was then treated with Klenow enzyme and ligated with a BamHI linker (d(pCGGGATCCCG), Biolab). Following BamHI cleavage, a 0.7 kb BamHI (linker)-BamHI (internal polylinker) fragment was purified and ligated to BamHI-linearized pSV2neo. In the positive clones, this 0.7 kb fragment was retrievable with BamHI. The clones with SV40 early promoter and the HCMV promoter in the same direction were designated as pSCM3(+), whereas those with the two promoters in the opposite direction were designated as pSCM3(-).

(6) Construction of mutants of pSCM1 with deletions in the HCMV P/E sequences (Fig. 2f): Deletion in the HCMV promoter region in pSCM1 was achieved by Bal31 digestion from the SacI site (-16) near the TATA box of the HCMV promoter. Because there is also a SacI site in the polylinker, digestion of pSCM1 with SacI would remove 78 bp of DNA containing the transcription start site driven by the HCMV promoter (Boshart *et al.*, 1985). Following ethanol precipitation, the DNA was resuspended in 89 ul of H_2O plus 10 ul of 10x Bal31 buffer (6 M NaCl, 120 mM $CaCl_2$, 120 mM $MgCl_2$, 200 mM Tris, pH 8.0, 2 mM EDTA) and digested with 0.5 unit of Bal31 at 30°C. Ten microliter aliquots were removed from the reaction every 30 seconds and placed in an ice-

cold Eppendorf tube containing 1 ul of 0.5 M EGTA. To the tubes then were added 400 ul of TE and 20 ul of 5 M NaCl. Following phenol and chloroform extractions, the DNA was ethanol-precipitated and 1/100 for each reaction time point was self-ligated at 14°C overnight. Taking advantage of the presence of restriction sites in the polylinker, deletion mutants of pSCM1 could be identified by digestion with SacI (which shouldn't cut), XbaI (whose cleavage site at the polylinker is 16 bp away from SacI) and HindIII (37 bp away from SacI). Both XbaI and HindIII are unique-cutting enzymes in pSCM1. Hence, assuming that Bal31 proceeds from both ends at the same speed, a set of deletion mutants were identified with deletion of less than 32 bp, 32-74 bp and more than 74 bp. Four clones were randomly chosen for subsequent transfection experiments, and they were all sequenced to identify the sequences deleted.

Transfection and transient replication assays

Cos-1 cells were passaged 1-2 days prior to transfection experiment. The cells were used for transfection when they reached 50-60% confluency. Cells were first washed with PBS, then 400 ul of the transfection mixture (approximately 50 ng DNA and 0.25 mg/ml DEAE-Dextran in PBS) was gently loaded onto the cells. After an incubation at room temperature (in a culture hood) for 20 min., the transfection mixture was removed by suction and 5 ml of DMEM medium containing 5% FCS and 100 uM chloroquine was added to

the dishes. Following a 3-hour incubation in a CO_2 incubator the medium was replaced with fresh DMEM medium containing 10% FCS. This time point was counted as zero for the transient replication assay. Plasmid DNA was harvested using a modified Hirt procedure at different times after transfection. The cells were lysed with Hirt buffer (1% SDS, 10 mM Tris, pH7.4, 1 mM EDTA, 0.1 M NaCl) and proteinase K added to 100 ug/ml. The lysate was incubated at 37°C for 2-4 hours and then filtered through a 1 ml syringe plugged with glass wool. The filtrate was phenol and chloroform extracted and the plasmid DNA was purified by ethanol precipitation. The purified DNAs were cut with appropriate restriction enzymes in order to differentiate cotransfected plasmids. For 0-hour samples, a half amount of the digests was saved as plasmid input; the other half was further subjected to DpnI digestion. For the remaining-time-point samples, the digests were further subjected to DpnI digestion to degrade the input methylated plasmid DNA. Finally 1/4 or 1/2 of each sample was resolved by 1% agarose gel electrophoresis. The gel was blotted to Nytran membrane by capillary action in 10xSSC (1xSSC: 0.15 M NaCL, 0.015 M Na Citrate). After baking at 80°C in a vacuum oven for 2 hours, the membrane was first prehybridized in a sealed bag containing 0.25% non-fat milk in 6xSSC at 42°C for 3 hours, and then hybridized to ^{32}P-labeled pUC19 probe in the hybridization buffer (50% formamide, 0.25% non-fat milk, 6xSSC) in the sealed bag at 42°C for 6 or more hours. The membrane was washed at 65°C first in 2xSSC and 1% SDS, and then in 0.2xSSC and 1% SDS.

Appropriate washing was monitored by Geiger counter. Finally the membrane was exposed to XAR film (Kodak).

D. RESULTS

(1) Differential usage of origins of DNA replication in SV40 viable variants *in*(Or)1412 and *in*(Or)1411 which contain two functional origins.

Differential initiation at the two origins of DNA replication in the SV40 variants *in*1412 and *in*1411 was analyzed by *in vitro* nuclei replication. This technique was chosen because there is no re-initiation of DNA replication in the isolated nuclei and the elongation reaction detected reflects the initiation events that have occurred prior to the isolation of nuclei.

Replication intermediates (RI) were labeled by the incorporation of α-^{32}P-dCTP. RI were first separated from the mature DNA using BND-DEAE chromatography. After restriction enzyme digestion, they were analyzed by a neutral/alkaline two-dimensional (2D) gel electrophoresis. Figure 5a shows the 2D autoradiography result of EcoRV digested samples; Fig. 5b shows that of KpnI+PstI digested samples. The results indicate that (1) by looking at where the replication intermediates curve approached its upper limit (Figs. 5a and 5b), the majority of replication initiation started from the authentic *ori* while few started from the insert *ori*; and (2) by looking at which restriction

fragment the ladder of gapped fragments (derived from the lagging strand synthesis) appeared under, only the restriction fragment corresponding to the termination site from the authentic *ori* (the smallest PstI-PstI fragment in fig. 5b) was found. Under the largest KpnI-PstI fragment where the termination would be expected if the insert *ori* is utilized, several faint spots were noticeable, suggesting the insert *ori* had also been used but with low frequency.

(2) Test of First Hypothesis: neighboring sequences around *ori* affect *ori* usage in *in*1412. The two separated *ori*s of *in*1412 replicated with equal efficiency in a transient replication assay

The above results show that the authentic *ori* is used significantly more frequently in initiating SV40 DNA replication than the inserted *ori*. To determine whether this is due to the effect of the neighboring sequences surrounding the two *ori*s, the two HindIII fragments containing either the authentic or the insert *ori* in *in*1412 (see Fig. 13) were separately cloned into the pUC19 plasmid as shown in Fig. 2a. The plasmids containing the authentic and insert *ori*s are designated as pSVOR1 and pSVOR2, respectively.

The efficiency of DNA replication of the two plasmids was tested by their transient DNA replication in Cos-1 cells which contain the SV40 T antigen necessary for the initiation of DNA replication from SV40 *ori* (Gluzman, 1981). As shown in Fig. 6a, the two plasmid DNA accumulated to the same extent during 40 hour period after co-transfection into Cos-1 cells. This result

indicates that there is no negative sequence elements surrounding the insert *ori* to account for its lower initiation efficiency used in *in*1412 *in vivo*.

The construction of pSVOR1 has removed the early gene sequence upstream from the authentic *ori*. This upstream sequence is absent in the insert *ori* in both *in*1412 and *in*1411. Thus, it remains possible that the early gene upstream sequence may exert an enhancing effect on the authentic but not on the insert *ori* usage in *in*1412. The absence of this enhancing element in the plasmid constructs would then account for their equal efficiency of replication in Cos-1 cells.

To test this possibility, a new plasmid, pSVOR3, was constructed to contain the authentic *ori* with the upstream sequence. The EarI-DrdI fragment of *in*1412 containing the authentic *ori* together with the upstream early sequence (see Fig. 13) was purified, treated with T4 DNA polymerase to become blunt-ended, and cloned into the SmaI site of pUC19. Co-transfection into Cos-1 cells of pSVOR3 with either pSVOR1 or pSVOR2 showed that the three plasmids had similar efficiency in replication (Fig. 6b).

In conclusion, this series of experiments indicates that when separated in a single molecule, both *ori*s of the variant *in*1412 function equally efficiently in a transient replication assay. The neighboring sequence of the insert *ori* does not inhibit the *ori* usage, and the neighboring sequence of the authentic *ori* does not stimulate the *ori* usage.

(3) Test of Second Hypothesis: Association of *ori* with an intact transcription unit increases the efficiency of replication initiated from that *ori*. No effect of linkage of *ori* to a functional transcription unit in transient replication assay.

In *in*1412, the authentic *ori* serves simultaneously as the promoters for both early and late transcription units, while the insert *ori* is not followed by a functional transcription unit. To test whether the linkage of SV40 *ori* to a transcription unit may increase its usage, pSV2neo (in which *ori* is followed by an intact transcription unit) and pSV2L (in which *ori* is followed by a piece of nonspecific lambda phage DNA without an intact transcription unit) were co-transfected into Cos-1 cells. As shown in Fig. 7, the replication efficiency is similar between the two plasmids. Thus, in the transient replication system, linkage of SV40 *ori* to a transcription unit does not appear to affect the *ori* usage.

(4) Test of Third Hypothesis: Location of the insert *ori* at the 3' ends of transcription units in *in*1412 or *in*1411 inhibits its function. *Cis*-suppressing effect of HCMV E/P on SV40 *ori* usage

The third difference between the insert and the authentic *ori* in *in*1411 and *in*1412 is that the insert *ori* is located at the 3' ends of both the early and late gene transcription units. The localization of the insert *ori* is such that RNA polymerase has to pass through the *ori* sequence to produce both the

early and late mRNA. The transcription process, therefore, may interfere with the binding of protein factors for the initiation of DNA replication from the insert *ori* as demonstrated in prokaryotic cells and in yeast (see Discussions). This problem does not exist for the authentic *ori* because transcription initiated at the authentic *ori* terminates well before reaching the authentic *ori* (Ford and Hsu, 1978).

To test whether placing SV40 *ori* at the 3' end of a transcription unit interferes with its function, we inserted SV40 *ori* at the 3' end of the *neo* transcription controlled by HCMV major immediate early gene promoter/enhancer (P/E). As a control we also placed the *ori* at the 5' end of the HCMV P/E controlled transcription unit. For convenience's sake we started out by constructing the control plasmid with SV40 *ori* at the 5' end of HCMV P/E. This was achieved by inserting HCMV P/E into the HindIII site of pSV2neo between SV40 *ori* and the *neo* transcription unit. One plasmid with proper orientation of HCMV promoter relative to the *neo* transcription unit was obtained. This plasmid is designated pSCM1 (Fig. 2e). The SV40 *ori* in this plasmid was subsequently moved to the EcoR1 site at the 3' end of the now HCMV P/E controlled transcription unit (see Fig. 4 for its construction). This plasmid is designated pSCM2. We also constructed two pSV2neo variants in which the relative position of HCMV P/E and SV40 *ori* was inverted relative to that in pSCM2. The HCMV P/E was inserted at the BamH1 site which is at the 3' end of the SV40 *ori* controlled transcription unit. These plasmids are

designated pSCM3(+) and pSCM3(-) depending on the relative orientation of HCMV and SV40 early promoters (Fig. 2e).

The results of the transfection experiments using these plasmids were unexpected. The amount of pSCM1 DNA accumulated at 40 hours post-transfection was reduced at least 100 fold relative to that of pSV2neo or pSV2L (Fig. 8). The replication of the other plasmids, pSCM2, pSCM3(+) and pSCM3(-), was more efficient than that of pSCM1 but was still reduced relative to that of pSV2L or pSV2neo. Among the three, the level of reduction was consistently higher in pSCM2 than in pSCM3(+) and pSCM3(-), whereas the latter two showed the similar level of reduction. As controls, the HCMV sequence in pSCM1 was substituted by a 564 bp HindIII lambda phage DNA fragment or by the α-satellite DNA sequence of CV-1 DNA. These constructs did not differ in replication efficiency from the parental plasmid, pSV2neo (Fig. 9 and data not shown). Thus the vast reduction of pSCM1 replication could not be simply due to the insertion of a foreign sequence between SV40 *ori* and the *neo* transcription unit. These results suggest that HCMV P/E can inhibit SV40 *ori* activity by a distance-dependent mechanism. Further characterization of this unexpected phenomenon is described in the section below.

(5) Analyses of the mechanisms of suppression of SV40 *ori* activity by HCMV P/E

We consider the possibility that protein factors binding to the enhancer region of HCMV P/E may affect the assembly of initiation complex on SV40 origin. If this is the case then competition of protein binding by an excess of competing HCMV P/E sequence should alleviate the inhibitory effect of HCMV P/E on SV40 origin. Competition was carried out using PCMV, the plasmid from which HCMV P/E was derived. The result showed that with 50 fold molar excess, PCMV failed to stimulate the replication of pSCM1 in a co-transfection experiment (data not shown). Since PCMV lacks SV40 origin and therefore cannot replicate in Cos-1 cells, the failure of competition may be due to the rapid degradation of PCMV in the cells. To provide a better competing DNA, we co-transfect pSCM1 with 10 or 100 fold molar excess of pSCM2. As shown above, pSCM2 can replicate much more efficiently than that of pSCM1. The amplified pSCM2 would then provide the templates for competing for the binding of protein factors. This competition experiment again failed to show stimulation of pSCM1 replication by pSCM2 (Fig. 10). These results therefore are not consistent with the requirement of *trans*-acting factors for the inhibition of SV40 origin by HCMV P/E.

Deletion analysis was used to delineate the sequence elements in HCMV P/E important for the suppression of SV40 *ori* activity in pSCM1. The HCMV P/E sequences were deleted from the unique SacI site located near the

transcription initiation site by Bal31 digestion (see Fig. 2f). As shown in Fig. 11, deletion of the sequence up to -91 nucleotide from the transcription start site did not relieve the inhibitory effect on SV40 *ori* activity. In pSCM1dl1, pSCM1dl2 and pSCM1dl4, the deletion removes both the CAAT and the TATA elements of HCMV promoter but does not affect the enhancer sequences. This result suggests that it is the enhancer element in HCMV P/E that is responsible for the suppression of SV40 origin.

In the transfection system that we have used, DNA replication saturates at about 40 hours posttransfection. To eliminate the possibility that the lower replication of pSCM1 is due to the slower replication kinetics, we compared the replication level of all plasmids in the pSCM series at 72 hours posttransfection. As shown in Fig. 12, pSCM1 and its HCMV promoter deletion variants consistently exhibit a significantly lower level of DNA replication than the other pSCM constructs. This result suggests that the suppression of pSCM1 DNA replication occurs independent of the time at which the DNA replication is measured.

(6) Second attempt to assess the effect of placing *ori* at the 3' end of a transcription unit: Construction and biological activities of an *in*1412 orientation variant and *in*1412neo with each *ori* located at the 3' end of a transcription unit (Fig. 13).

Because the HCMV enhancer unexpectedly affects the activity of SV40

origin, the use of foreign promoter/enhancer to test the transcription-interference model may be complicated by the effect of the foreign regulatory sequence on SV40 origin. We were forced, therefore, to design a scheme using only SV40 P/E driven transcription units to test this model.

We took advantage of the fact that *in*1412 DNA contains two copies of SV40 *ori* in the inverted orientation. Cutting the two origins with BglI restriction endonuclease will generate two fragments of DNA, 2.8 kb (A) and 2.4 kb (B), each bounded by the origin sequence. Re-ligation of the two fragments should in principle generate equimolar amounts of two orientation isomers of circular DNA: one represents regenerated *in*1412 and the other consists of A and B in the inverted orientation relative to that in *in*1412 (see Fig. 13). The latter DNA is named *in*1412v for *in*1412 variant. In contrast to *in*1412, the two *ori*s in *in*1412v are now both at the 3' end of a transcription unit. The transcription of *in*1412v should occur only on one strand and in one direction as compared to *in*1412 which contains two transcription units in the opposite polarity. If placing *ori* at the 3' end of a transcription interferes with its replication activity, then the replication efficiency of *in*1412v should be substantially reduced relative to that of *in*1412.

Ligation of BglI-cleaved *in*1412 DNA indeed produced an equimolar amount of the two products. Digestion of the ligation mixture by BclI and EcoR1 restriction endonucleases generated four equimolar fragments (Fig. 14, panel A). One pair, 4.0 and 1.2 kb, is derived from *in*1412 whereas the other

pair, 3.4 and 1.8 kb, is derived from *in*1412v. However, the progeny viral DNA derived from transfection of the ligation mixture in Cos-1 cells produced predominantly the pair of 4.0 and 1.2 kb bands characteristic of *in*1412 (Fig. 14, panel B). This result suggests that *in*1412 DNA replicates significantly more efficiently than *in*1412v.

We have also cloned *in*1412 and *in*1412v DNA separately into the EcoRI site of PUC19 plasmid. The two viral DNA were then released from the vector by digestion with EcoRI and transfected into Cos-1 cells. Preliminary experiments indicated again that *in*1412v DNA is less efficiently replicated than that of *in*1412.

(7) Effect of chromatin structure on SV40 origin usage

Since the SV40 regulatory region including *ori* has been found to exhibit altered chromatin structure (Chapter 2), we also considered the possibility whether chromatin organization may account for the differential usage of the two *ori* in *in*1411 and *in*1412. We used P1 nuclease to probe the altered chromatin structure at the two origins in *in*1412 and *in*1411. As shown in the Appendix of Chapter 2, both origins are equally sensitive to P1 digestion. On the other hand, the non-functional origin in *in*1415 is resistant to P1 cleavage. The analysis of chromatin organization using P1 nuclease, therefore, indicates that the two origins in *in*1411 and *in*1412 are both organized in altered P1 sensitive conformation.

E. Discussion

Differential usage of the origins in SV40 variants, *in*1411 and *in*1412.

Using *in vitro* nuclei replication assay to determine the relative initiation rates from the two origins (*ori*), we observed that DNA replication in *in*1411 and *in*1412 *in vivo* was initiated significantly more frequently from the authentic *ori* than the inserted *ori*. Since the two origins have identical sequences and both contain all the *cis* elements (the minimum origin plus the surrounding auxiliary sequences, *ori*, for short) necessary for SV40 DNA replication, the difference in their biological activity must be somehow determined by their locations in the DNA molecule.

Examination of the *in*1411 and *in*1412 molecules reveals a number of differences in microenvironment surrounding the two *ori*s: 1) neighboring nucleotide sequences; 2) association with active transcription units; and 3) locations relative to the transcription units. Experiments described in the text tend to eliminate the first two factors as the causes for the differential activity of the two *ori*s. However, it remains possible that the transient replication assay used to assess the replication efficiency of the cloned origin may not represent the same situation during lytic infection in which the difference in the usage of origins was originally found. For example, the late gene products (capsid proteins) could somehow differentially affect the two origins. We have attempted to test this possibility by studying the replication of the plasmids

in cells infected by SV40 virus. These experiments were not successful because SV40 virus unexpectedly suppressed the replication of plasmids containing SV40 origin. The reasons for the suppression are not known but they could be due to competition by the excess amount of wild type virus DNA present in the infected cells.

We have also considered the possibility whether the difference of the activity of the two origins in *in*1411 and *in*1412 results from chromatin organization. An example of influence of chromatin structure on DNA replication was published recently (Simpson, 1990). It was found that the active yeast ARS sequence is located in the linker region between nucleosome in the plasmid TRP1ARS1. When the phase of nucleosome organization was altered by deletion or insertion such that ARS is located inside nucleosome, the ARS activity is markedly decreased. In SV40 chromatin, the origin of replication is known to adopt a "nucleosome-free" conformation which is hypersensitive to nuclease digestion (see Chapter 2 for review). The active promoter activity of the authentic *ori* in *in*1412 may be preferentially organized into "active" chromatin structure as defined by nuclease hypersensitivity. We tested this possibility by comparing the cleavage frequency of the two origins by P1 nuclease *in vivo*. The result showed no significant difference between the two *ori*s in sensitivity to P1 digestion.

To further test the effect of chromatin organization on origin activity we inserted an α-satellite DNA fragment next to SV40 origin. Previous studies

have shown that a dominant nucleosome phase is formed on α-satellite DNA (Zhang *et al.*, 1983; Neubauer *et al.*, 1986). It has also been shown that the presence of α-satellite DNA can suppress yeast *PHO5* promoter activity by interfering with the organization of active chromatin structure in the nearby promoter (Straka and Hörz, 1991). We inserted the HindIII monomer, dimer, trimer, tetramer and pentamer of the CV-1 α-satellite repeat at the HindIII site of pSV2neo adjacent to SV40 origin. The results of transient replication assay, however, showed that the insertion of the α-satellite sequence next to SV40 origin did not produce any significant effect on SV40 origin activity (Fig. 9). Because the distance between the boundary of the satellite sequence to that of SV40 origin is only about 70 bp, formation of nucleosome on the satellite DNA would be expected to affect the nucleoprotein organization at the origin. The negative result observed suggests either that the satellite sequence does not dominate nucleosome formation in the transfected DNA or that the nucleoprotein organization at the origin is dominant over that at the satellite sequence. Further analyses of the nucleosome phasing in the satellite containing plasmids would be needed to resolve these possibilities.

Our analyses so far are most consistent with the interpretation that placing origin at the 3' ends of transcription units in SV40 genome interferes with the origin activity. This is suggested by the greatly reduced amount of *in*1412v DNA accumulated in Cos-1 cells despite the equal input of *in*1412 and *in*1412v DNA for transfection (Fig. 14). However, it is also possible that the

inversion of the late transcription in *in*1412v abolishes this transcription unit and thus indirectly affects the replication efficiency of this DNA. We think this is unlikely because 1) the late gene expression in *in*1412 would provide a complementation in the co-transfection and subsequently the co-infection; and 2) eventually *in*1412v could grow out as virus in Cos-1 cells. Thus despite the inversion of late transcription unit so that early and late transcription are now in the same polarity, late gene products apparently can be produced to form virus particles. The slow growth of *in*1412v in Cos-1 cells, therefore, more likely result from inefficient DNA replication. However, kinetic analysis of DNA replication of *in*1412 and *in*1412v DNA would be needed to confirm this conclusion.

The result of *in*1412v and the differential usage of the two *ori*s in *in*1411 and *in*1412 are consistent with the hypothesis that active transcription passing through origin would inhibit the origin activity. Interference of *CEN* (centromere) and *ARS* (autonomous replication sequence) by transcription into them has indeed been observed in yeast (Kipling and Kearsey, 1989; Snyder *et al.*, 1988 and references therein). That active transcription through the ARS is necessary for interference is shown by elimination of the effect by placing a transcription termination signal between the *ARS* and the promoter (Snyder *et al.*, 1988). Examples of transcription interference of origin activity has also been reported in prokaryotic cells (Lambert and Reznikoff, 1985; Seiki *et al.*, 1982; Tanaka and Hiraga, 1985; Brewer, 1988). The mechanism of inhibition

is presumably due to the interference of assembly of replication initiation complexes at the origin by the transcription complexes.

If the differential usage of the *ori*s in *in*1411 and *in*1412 is indeed due to transcription interference, then stopping transcription by using transcription inhibitors such as α-amanitin or by placing a transcription termination signal between the promoter and the inserted origin should increase the relative frequency of replication initiated from the inserted origin. These predictions remain to be tested in the future.

Effect of HCMV P/E on SV40 origin activity

The unexpected result of the analysis of SV40 replication is that HCMV P/E sequence inhibits transient replication of plasmids containing SV40 origin. Deletion of the HCMV promoter sequence did not alleviate the inhibitory effect, suggesting that the enhancer sequence is responsible for the effect observed. This result also indicates that transcription from the HCMV promoter is not responsible for the inhibition. However, further mutational analysis would be required to define the sequence elements within the enhancer responsible for the suppression of SV40 origin activity. Toward this goal, we have cloned fragments of HCMV P/E into pSV2neo.

Studies of prokaryotic and eukaryotic DNA replication origin have consistently shown that replication origin is intimately associated with regulatory sequence of transcription (Kornberg and Baker, 1992). In SV40

DNA the enhancer/promoter elements near the origin positively regulate the origin activity (Bergsma *et al.*, 1982; DeLucia *et al.*, 1986; Hertz and Mertz, 1986; Lee-Chen and Woodworth-Gutai, 1986; Li *et al.*, 1986). However, transient DNA replication experiments using recombinant constructs with transcription regulatory elements placed at various locations and orientations relative to SV40 core origin reveal a complex relationship between transcription elements and SV40 origin activity. For example, repeats of SV40 enhancer sequence cause inhibition of SV40 replication instead of activation (Kumar *et al.*, 1988). The inhibition effect is found to be distance-dependent and orientation-independent. Translocating the 21 bp repeat of SV40 promoter from the late to the early side of origin also results in inhibition rather than activation of SV40 origin (Lee-Chen and Woodworth-Gutai, 1986). In addition, SV40 origin activity is suppressed when it is associated with adenovirus major late promoter (DeLucia *et al.*, 1986), Rous sarcoma virus LTR enhancer (Binninger *et al.*, 1989), adeno-associated genome (Berns *et al.*, 1988), or with bovine papillomavirus genome (Roberts and Weintraub, 1986; 1988). The latter two cases require virus-coded *trans*-acting factors in addition to the *cis*-elements for the suppression.

Our analysis showed that HCMV P/E caused inhibition of SV40 origin function in the distance-dependent manner. When placed immediately at the early side of SV40 origin (in pSCM1), it greatly suppressed the plasmid replication. When the distance between HCMV P/E and SV40 origin was

farthest (in pSCM3), the suppression was the smallest. When the distance was closer (in pSCM2), the suppression became more severe. In a recent construct which contains HCMV sequence immediately at the late side of origin, however, the suppression was greatly diminished (data not shown). Therefore, in addition to the distance, the exact position of the HCMV P/E insertion determines the activity of suppression by HCMV P/E. These effects of HCMV P/E toward SV40 origin resembles that of the 21 bp repeat of SV40 promoter (see discussion above). It is interesting to note that both HCMV enhancer and 21 bp repeat contain multiple binding sites for the transcription factor Sp1. In fact, a homology of 9 bp sequence covering a Sp1 binding site was found between the two sequences. It would be interesting to determine whether mutations in the Sp1 binding sites in HCMV enhancer can alter or abolish its inhibitory effect on SV40 origin.

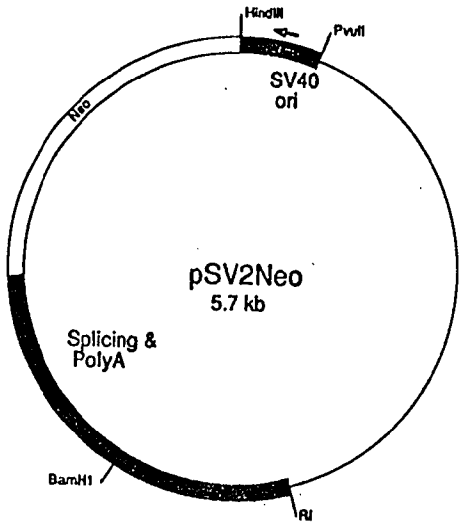
How various transcription regulatory elements affect SV40 origin is an intriguing question that remains to be analyzed. The tight association between replication origin and transcription elements suggests that the transcription process may modulate the activity of replication origin. However, since our results and those obtained by others indicate that enhancer alone can modulate origin activity, it appears that transcription near the origin *per se* is not required to regulate origin activity. Enhancer sequences have been shown to contain multiple binding sites for *trans*-acting factors. The binding of these factors may cause allosteric effect on the structure of the neighboring DNA

sequences. The opposite effect of the 21 bp repeat on SV40 origin upon translocation from the late to the early side of origin suggests that either a polarity of the 21 bp repeat or that the two ends of the replication origin respond differently to the regulatory sequence. Since inversion of 21 bp repeat on the late side of origin only reduces the enhancement of replication two fold (Lee-Chen and Woodward-Gutai, 1986), the latter mechanism seems more likely. Our data are consistent with this interpretation.

The modulation of SV40 origin activity by HCMV enhancer and a number of viral regulatory sequences cited above suggests that initiation of DNA replication can be fine tuned by its neighboring sequences in mammalian cells. So far all the known replication origins are shown to be associated with transcription regulatory elements. The initiation of DNA replication therefore would be tightly linked to gene expression and cellular physiology. The well-characterized SV40 replicon can provide a very useful model for understanding the sequence elements and mechanisms involved in the modulation of origin activity.

F. FIGURE LEGENDS

Fig. 1. Maps of the starting plasmids pSV2neo and pCMV. Thin line represents pBR322 sequence containing prokaryotic origin of DNA replication and the coding region for the ampicillin resistance gene. The stippled boxes represent SV40 sequences. In pSV2neo, the arrow by one stippled box indicates the direction of transcription driven by SV40 early promoter (PvuII→ HindIII); the open box represents the sequence encoding *neo* (G418 resistance) gene. In pCMV, the open box represents human cytomegalovirus early promoter/enhancer (HCMV P/E) sequence (Boshart *et al.*, 1985); the arrow inside the box indicates the direction of transcription driven by the promoter (ClaI→HindIII). The region starting from RI to HindIII is the polylinker derived from pGem-1 (Promega), which facilitates subcloning of HCMV P/E. The restriction enzyme sites shown occur only once in the plasmid.


HindIII
PvuII
SV40
ori
Neo
pSV2Neo
5.7 kb
Splicing &
PolyA
BamH1
RI

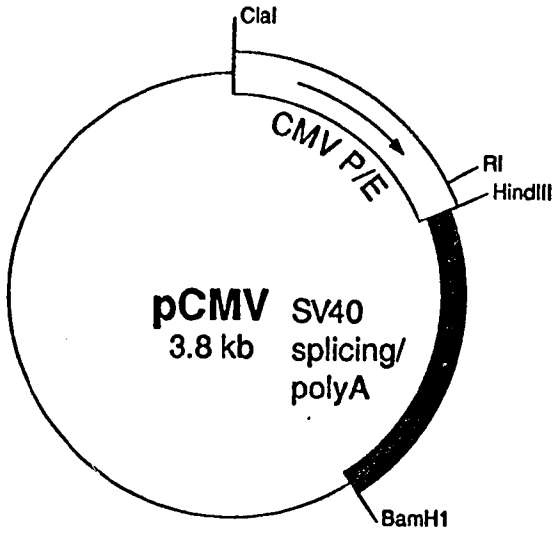

ClaI
CMV P/E
RI
HindIII
pCMV
3.8 kb
SV40
splicing/
polyA
BamH1

Fig. 2a. Plasmids containing the authentic or inserted origin of replication of *in*1412 DNA. The HindIIIB, HindIIIA and EarI to DrdI fragments of *in*1412 were cloned into pUC19 vector to yield pSVOR1, 2, and 3, respectively. The open bars represent SV40 sequences. The location of the authentic or the inserted origin is indicated.

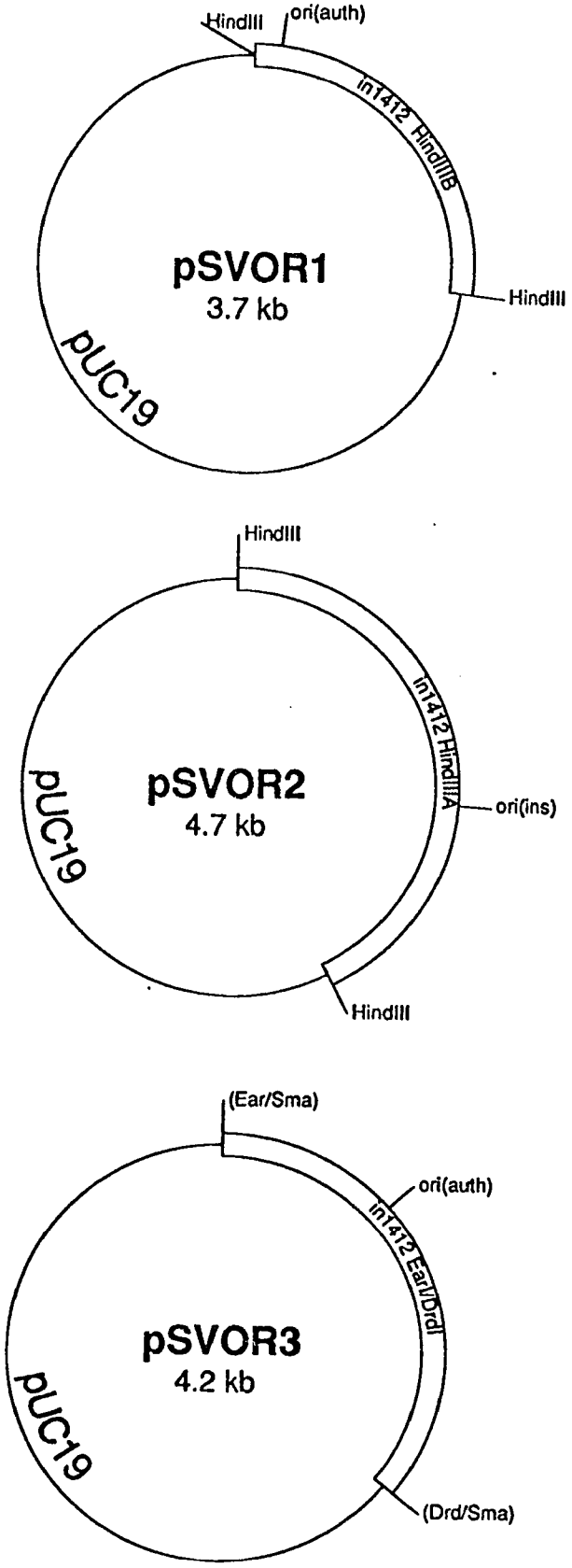
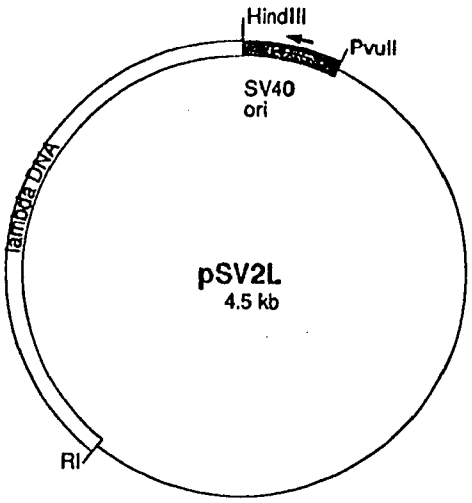
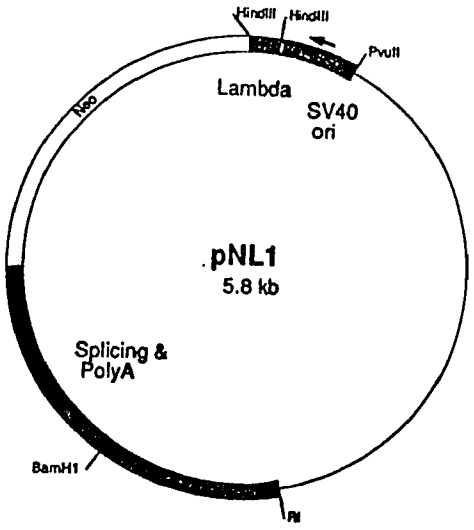

HindIII
ori(auth)
in1412 HindIIIB
pSVOR1
3.7 kb
pUC19
HindIII
HindIII
in1412 HindIIIA
pSVOR2
4.7 kb
pUC19
ori(ins)
HindIII
(Ear/Sma)
ori(auth)
in1412 EarI/DrdI
pSVOR3
4.2 kb
pUC19
(Drd/Sma)

Fig. 2b. Plasmids containing no intact transcription unit (pSV2L) or phage lambda/HindIII fragments inserted in the HindIII site of pSV2neo (pNL1 and pNL2).


HindIII
PvuII
SV40
ori
lambda DNA
pSV2L
4.5 kb
RI


HindIII
HindIII
PvuII
Lambda
SV40
ori
Neo
pNL1
5.8 kb
Splicing &
PolyA
BamH1
RI

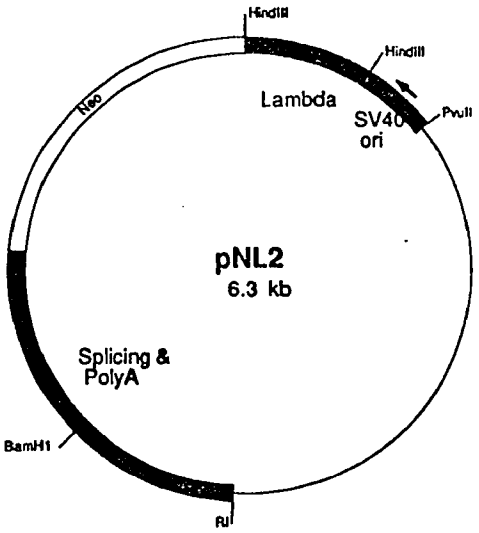
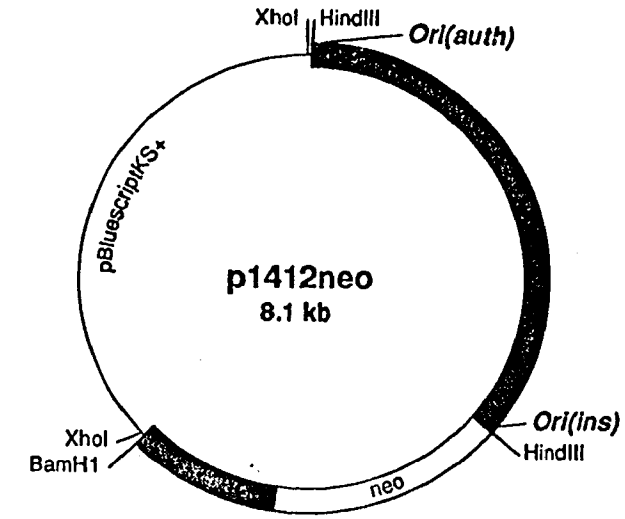
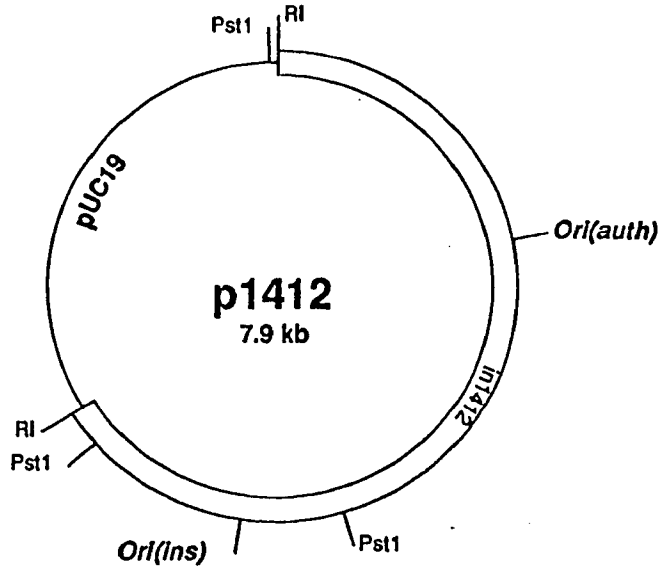

HindIII
HindIII
Lambda
SV40
ori
PvuII
Neo
pNL2
6.3 kb
Splicing &
PolyA
BamH1
RI

Fig. 2c. Plasmids containing variants of *in*1412 virus DNA. In p1412neo plasmid, the early region of *in*1412 DNA was replaced by a transcription containing neo-resistance gene and the variant DNA cloned into pBluescriptKS+ plasmid. The two origins relative to the *neo* transcription unit are indicated. XhoI sites adjacent to the boundaries of vector-SV40 sequences were used to retrieve the 1412neo DNA for transfection experiments. Plasmid p1412 contains *in*1412 DNA cloned into the R1 site of pUC19. In p1412v plasmid, the late transcription of *in*1412 DNA was inverted relative to the early transcription unit. This results in the change of the locations of origins and the PstI sites. PstI restriction endonuclease was used to distinguish p1412 from p1412v during screening of recombinant clones and in co-transfection experiments.


XhoI
HindIII
Ori(auth)
pBluescriptKS+
p1412neo
8.1 kb
Ori(ins)
HindIII
XhoI
BamH1
neo


Pst1
RI
pUC19
Ori(auth)
p1412
7.9 kb
IN1412
RI
Pst1
Ori(ins)
Pst1

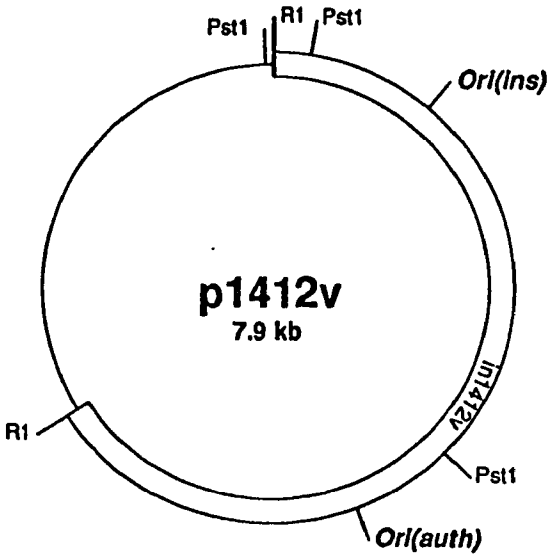
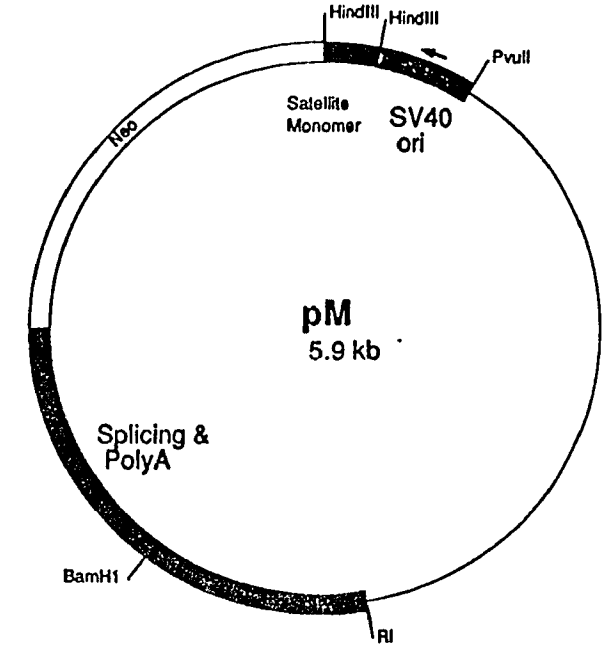

Pst1
R1
Pst1
Ori(ins)
p1412v
7.9 kb
IN1412V
R1
Pst1
Ori(auth)

Fig. 2d. Plasmids containing CV-1 α-satellite DNA sequences (HindIII fragments, Rosenberg *et al.*, 1978) inserted at the HindIII site of pSV2neo. The plasmids, pM and pT contain a 172bp monomer and 516bp trimer of satellite sequence respectively. Similar constructs containing the dimer and the tetramer were also obtained.


HindIII
HindIII
PvuII
Satellite Monomer
SV40 ori
Neo
pM
5.9 kb
Splicing & PolyA
BamH1
RI

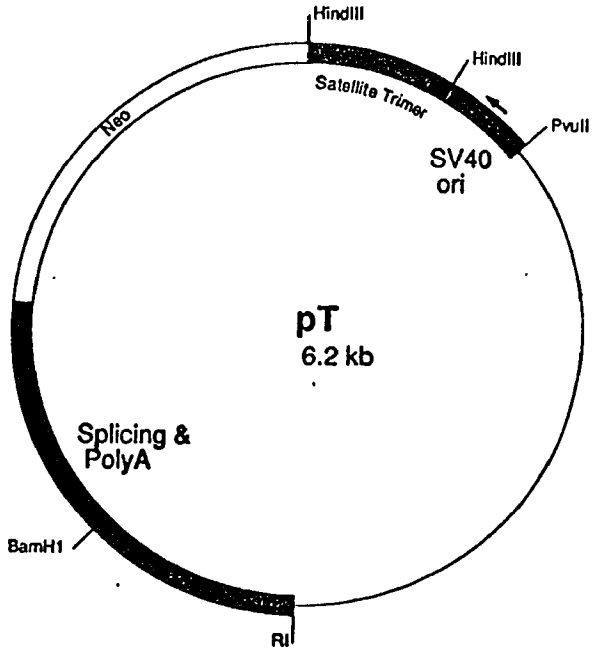
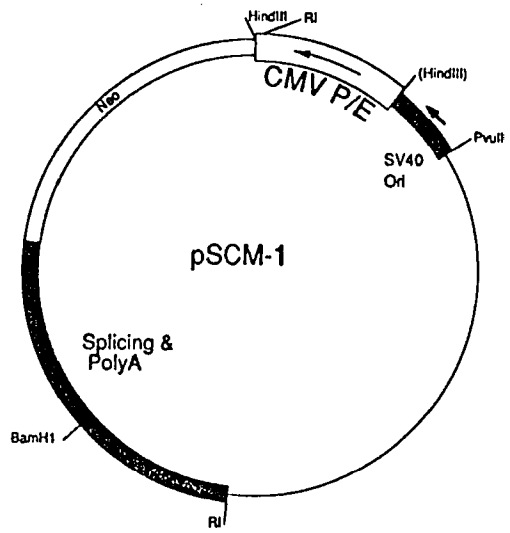
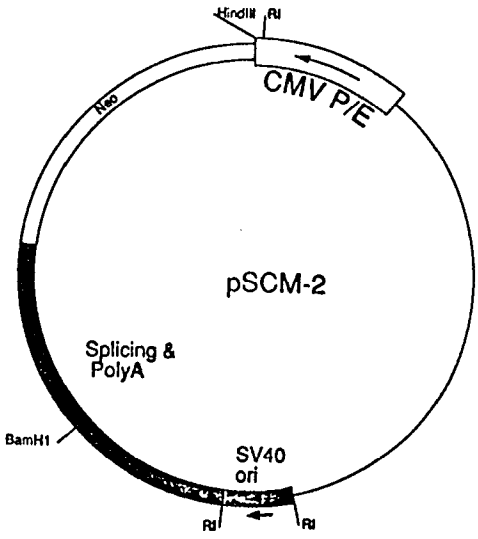
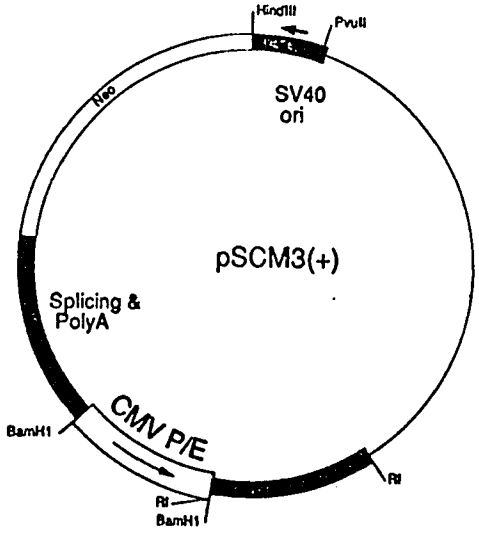

HindIII
HindIII
Satellite Trimer
PvuII
SV40 ori
Neo
pT
6.2 kb
Splicing & PolyA
BamH1
RI

Fig. 2e. Plasmids containing HCMV P/E sequence (Boshart *et al.*, 1985) inserted at different sites of pSV2neo plasmid (pSCM1, pSCM2, pSCM3(+) and pSCM3(-)). (1) Plasmid pSCM1 contains HCMV P/E fragment cloned at the HindIII site of pSV2neo. The directions of transcription from HCMV promoter and SV40 early promoter are shown by arrows. One of the HindIII sites at the border of SV40 origin-HCMV junction was destroyed during cloning by a small deletion of 4 bp. Sequence analysis showed no changes in SV40 origin sequence. (2) Plasmid pSCM2 is a variant of pSCM1 by translocating the origin of SV40 DNA to the RI site. The directions of SV40 early promoter and CMV promoter are shown. (3) Plasmids pSCM3(+) and pSCM3(-) contain HCMV P/E inserted at the BamHI site of pSV2neo. The two plasmids differ in the orientation of the inserted HCMV fragment. The relative directions of HCMV promoter and SV40 early promoter are shown.


HindIII
RI
CMV P/E
(HindIII)
PvuII
SV40
Ori
Neo
pSCM-1
Splicing &
PolyA
BamH1
RI


HindIII
RI
CMV P/E
Neo
pSCM-2
Splicing &
PolyA
BamH1
SV40
ori
RI
RI


HindIII
PvuII
SV40
ori
Neo
pSCM3(+)
Splicing &
PolyA
CMV P/E
BamH1
RI
RI
BamH1

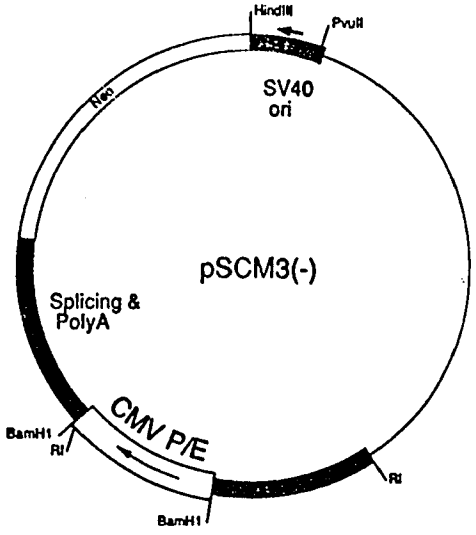
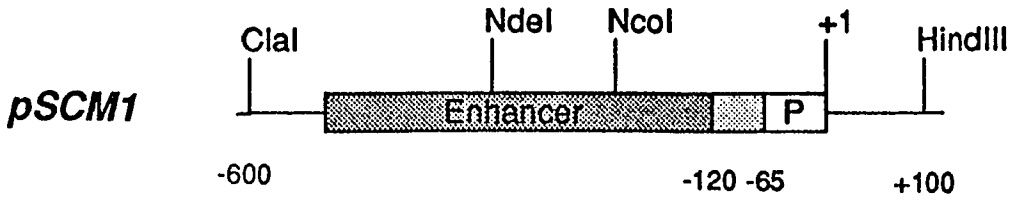

HindIII
PvuII
SV40
ori
Neo
pSCM3(-)
Splicing &
PolyA
CMV P/E
BamH1
RI
RI
BamH1

Fig. 2f. Deletion variants of pSCM1: pSCM1dl1-4. Only the HCMV P/E region (ClaI-HindIII) of the plasmids is shown: +1 indicates the transcription initiation site; P box contains the promoter region including TATA and CAAT boxes; heavily- and lightly- stippled boxes indicate the major and minor enhancer region, respectively (adapted from Stamminger and Fleckenstein, 1990). The open boxes with numbers inside show the deleted regions for each deletion variants. The deletion variants were constructed by Bal31 deletion from the SacI site located at nucleotide +16.


pSCM1
ClaI
NdeI
NcoI
+1
HindIII
Enhancer
P
-600
-120 -65
+100

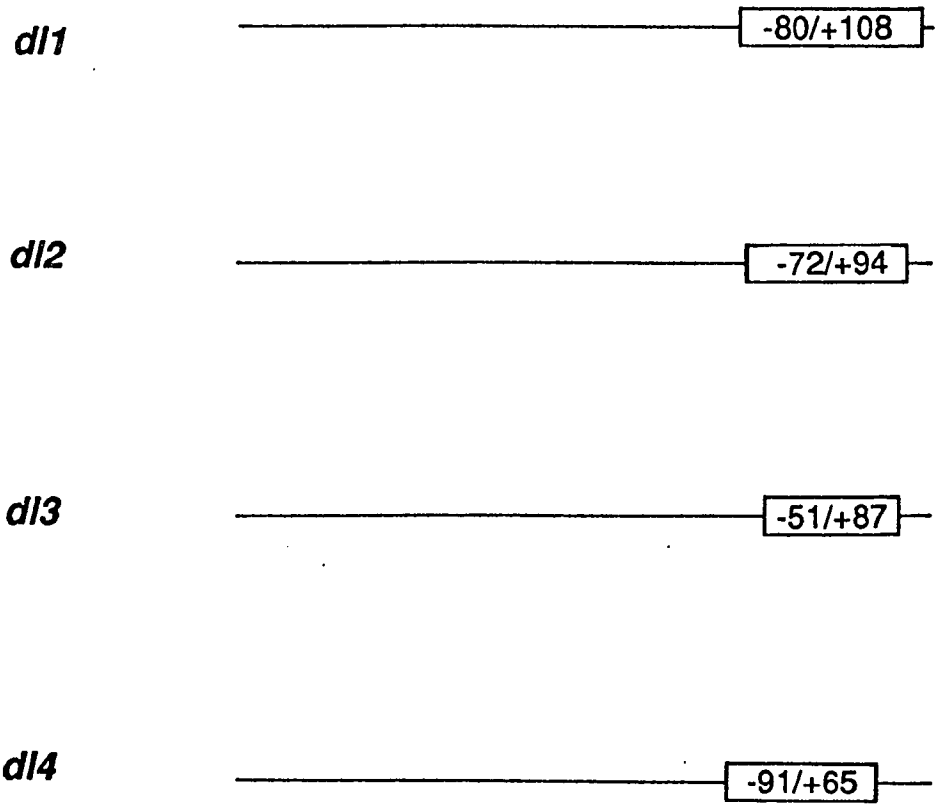

dl1
-80/+108
dl2
-72/+94
dl3
-51/+87
dl4
-91/+65

Fig. 3. CV-1 α-satellite DNA. CV-1 cell DNA was extracted, digested with HindIII to completion, and resolved in a 1.6% agarose gel by electrophoresis. Lane 1: kb mol. wt. marker (BRL); lane 2: CV-1 DNA digested with HindIII: m, monomer; d, dimer; t, trimer. Tetramer and pentamer can also be seen in the picture.

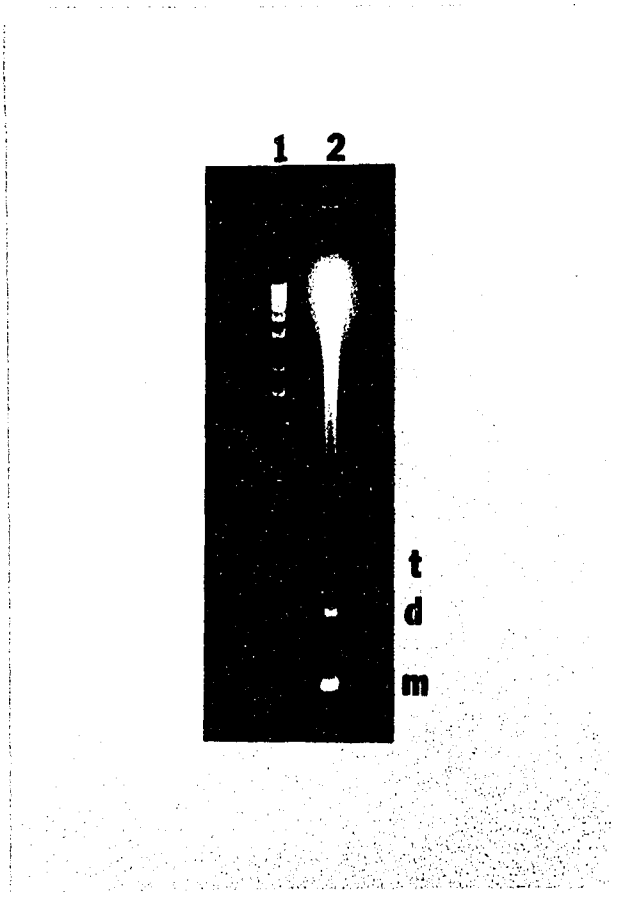

1
2
t
d
m

Fig. 4. Construction scheme of pSCM2. The DNA fragment containing SV40 origin in pSV2neo was first retrieved by digestion with PvuII and HindIII restriction endonucleases. The sticky ends of HindIII was filled by Klenow polymerase and the ends then converted to RI sequence by ligation with R1 linker. This fragment was then inserted at the RI site of pSV2neo. The resulting plasmid containing two SV40 origin of replication was cut with HindIII and BamHI to generate two fragments. One of the origins in the larger fragment was removed by digestion with PvuII leaving the inserted origin in the fragment. This fragment was then ligated with the HindIII-BamHI fragment containing the *neo* gene and with the HCMV P/E fragment retrieved from pCMV plasmid as shown in the left part of the figure. The three-piece ligation reaction yielded the plasmid pSCM2 as shown.

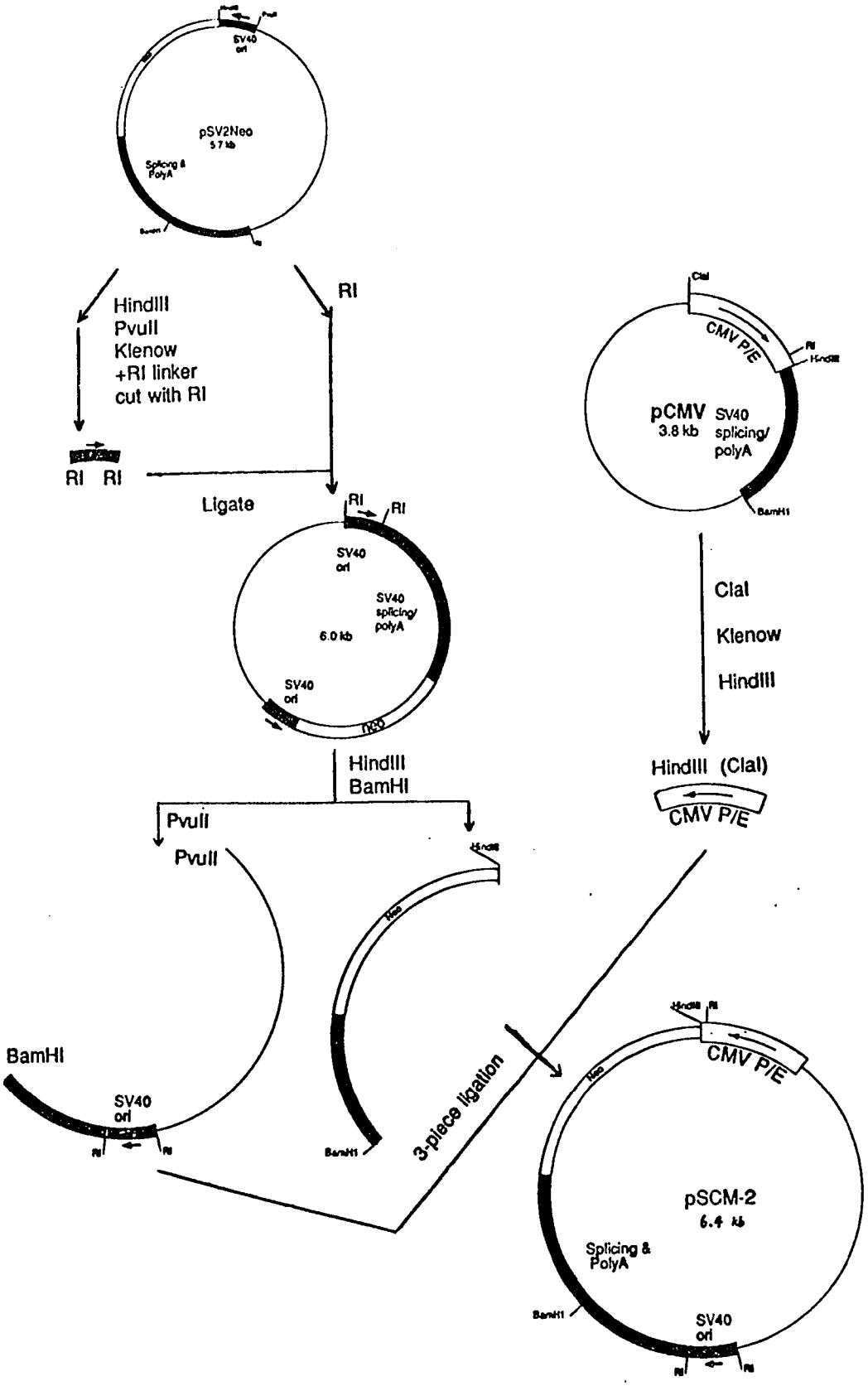

pSV2Neo
5.7 kb
SV40 ori
Splicing & PolyA
HindIII
PvuII
Klenow
+RI linker
cut with RI
RI
RI RI
Ligate
RI RI
SV40 ori
SV40 splicing/ polyA
6.0 kb
SV40 ori
Neo
HindIII
BamHI
PvuII
PvuII
Hindlll
Neo
BamHI
SV40 ori
RI
RI
BamHI
3-piece ligation
ClaI
CMV P/E
RI
HindIII
pCMV
3.8 kb
SV40 splicing/ polyA
BamHI
ClaI
Klenow
HindIII
HindIII (ClaI)
CMV P/E
HindIII RI
CMV P/E
Neo
pSCM-2
6.4 kb
Splicing & PolyA
BamHI
SV40 ori
RI
RI

Fig. 5. The *ori* usage of the insert *ori* is significantly lower than that of the authentic *ori* in SV40 variants *in*1411 and *in*1412. Forty eight hours after infection, nuclei were prepared and assayed for *in vitro* replication. The labeled viral DNA was cut with EcoRV (upper panel, a) or with KpnI+PstI (lower panel, b) and was analyzed by 2D gel electrophoresis (native-denaturing). The autoradiographs are shown. The interpretation of Panel b is shown below. The curves and spots derived from the viral DNA after KpnI+PstI digestion that appeared in the autoradiograph are shown in solid line and spot, respectively; those that were expected but did not appear in the radiograph are shown in dashed line and spot, respectively. The "aut" indicates the curves and spots diagnostic of the authentic *ori* being used; "ins" indicates those diagnostic of the inserted *ori* being used.

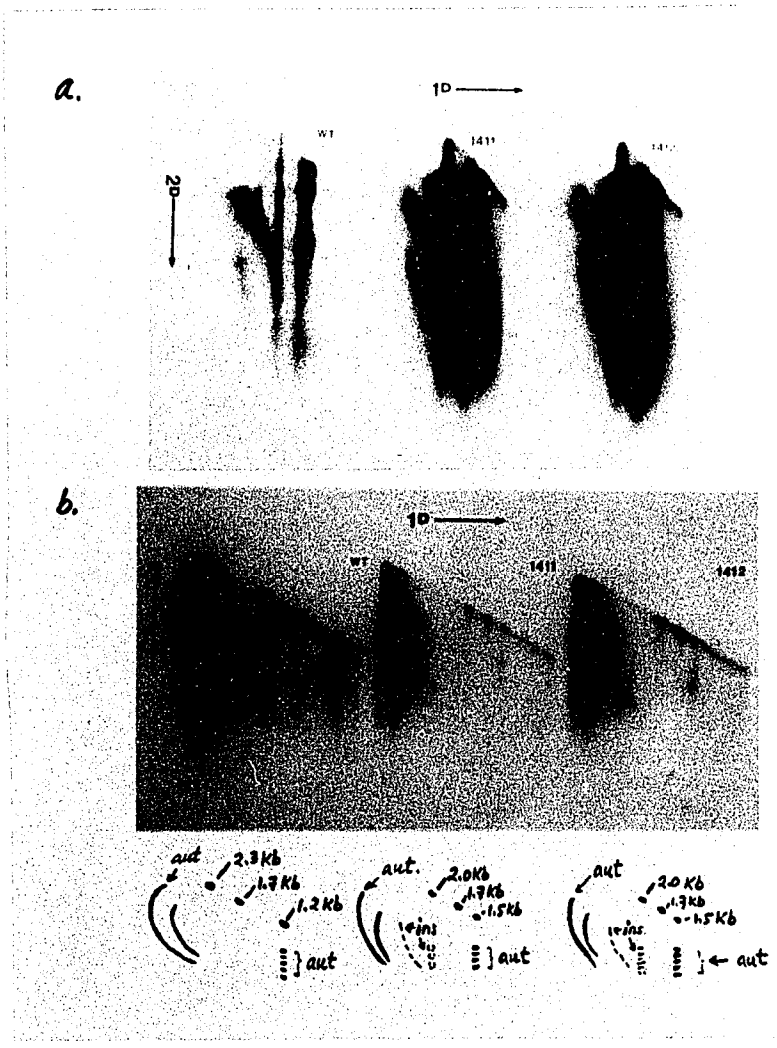

a.
1D
2D
WT
1411
1412
b.
1D
WT
1411
1412
aut
2.3 Kb
1.7 Kb
1.2 Kb
aut
aut.
2.0Kb
1.7Kb
1.5Kb
aut
aut
2.0 Kb
1.7Kb
1.5Kb
aut

Fig. 6. Transient replication of (pSVOR1+pSVOR2), (pSVOR1+pSVOR3) and (pSVOR2+pSVOR3) after co-transfection in Cos-1 cells. Equimolar amounts of the two plasmids were used in co-transfection of Cos-1 cells. Plasmid DNA was harvested at 0, 24, 32, and 40 hours after transfection. DNA was digested with XbaI which cuts each of these plasmids only once. One half of 0 h sample and the total extracted DNA of the other time points were digested with DpnI which cuts methylated (unreplicated) input DNA many times. One fourth amount of each sample was electrophoresed in a 1% agarose gel followed by Southern blotting and hybridization with ^{32}P-labeled pUC19 probe. The autoradiographs are shown. Panel A: co-transfection of (pSVOR1+pSVOR2). Lanes 1 and 2: input pSVOR1 and pSVOR2 (both XbaI-linearized), respectively; lanes 3-6: XbaI and DpnI digestions of DNA obtained at 0, 24, 32, and 40 hours after co-transfection, respectively. Arrows a and b indicate the position of pSVOR1 and pSVOR2, respectively. Panel B: co-transfection of (pSVOR1+pSVOR3) and (pSVOR2+pSVOR3). Lane 1: input DNA (0 h, cut with XbaI but not DpnI); lanes 2-5: XbaI and DpnI digestions of DNA obtained at 0, 24, 32, and 40 hours after co-transfection, respectively. Arrows a, b, and c indicate the position of pSVOR1, 3, and 2, respectively.

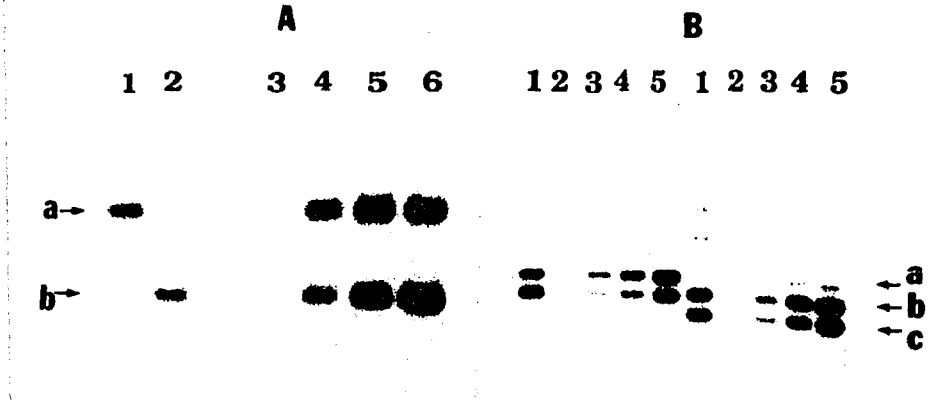

A
B
1 2 3 4 5 6
1 2 3 4 5 1 2 3 4 5
a
b
a
b
c

Fig. 7. Transient replication of (pSV2neo+pSV2L) after co-transfection in Cos-1 cells. Lane 1: input DNA (0 h, cut with HindIII but not DpnI); lanes 2-5: HindIII and DpnI digestions of DNA obtained at 0, 24, 32, and 40 hours after co-transfection, respectively. Arrows a and b indicate the position of the linearized pSV2neo and pSV2L, respectively.

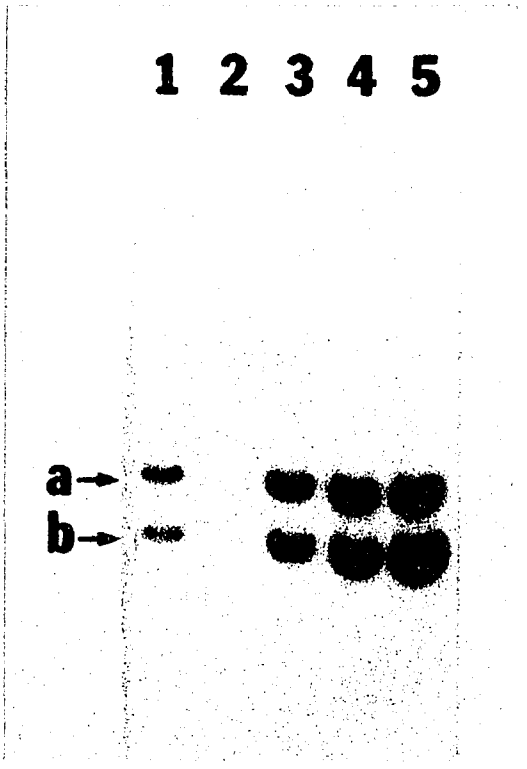

1 2 3 4 5
a
b

Fig. 8. Transient replication of the plasmids containing HCMV P/E inserted at different positions in the parental pSV2neo. Equimolar amounts of the test plasmid and the internal control pSV2L were used in co-transfection of Cos-1 cells. Plasmid DNA was linearized with HindIII. Lane 1: input DNA (0 h, not cut with DpnI); lanes 2-5: DNA further cut with DpnI: 0, 24, 32, and 40 hours after co-transfection, respectively. Panel A: (pSV2neo+pSV2L); B: (pSCM1+pSV2L); C: (pSCM2+pSV2L); D: (pSCM3(+)+pSV2L); and E: (pSCM3(-)+pSV2L). Arrows a, b, and c indicate the position of pSCM, pSV2neo, and pSV2L, respectively.

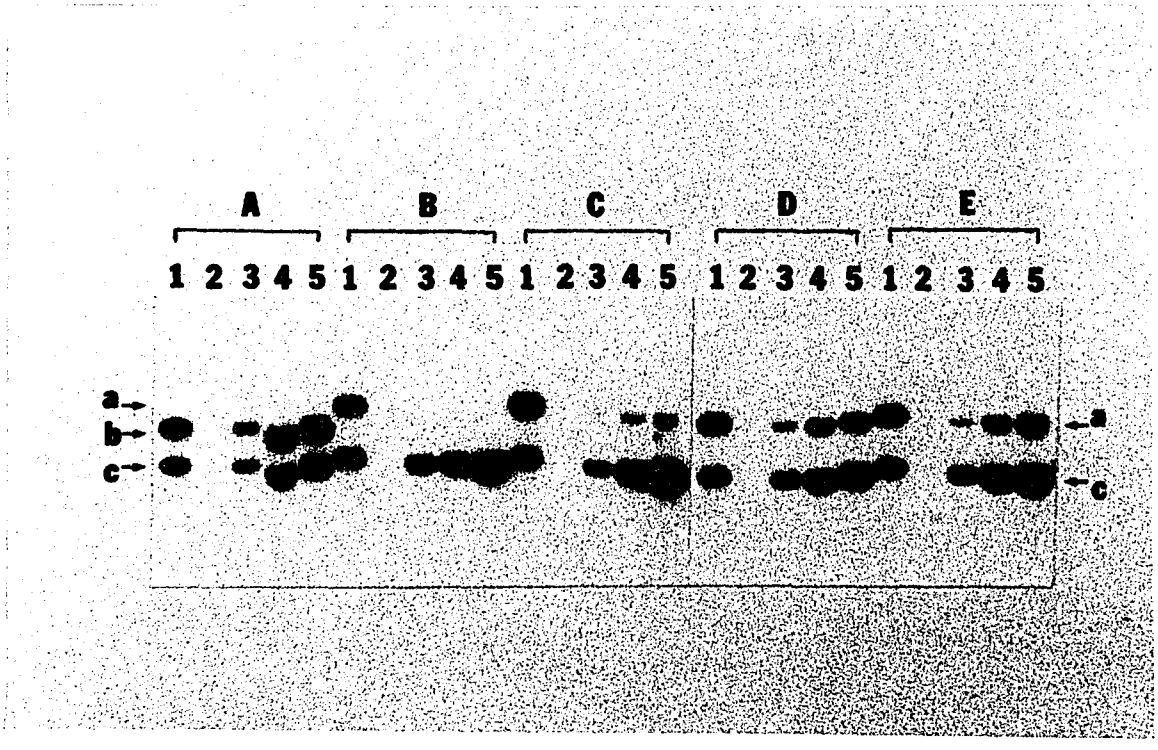

A
B
C
D
E
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
a
b
c
a
c

Fig. 9. Transient replication of the plasmids containing CV-1 α-satellite DNA sequence. Equimolar amounts of the test plasmid and the internal control pSV2L were used in co-transfection of Cos-1 cells. Lane 1: input DNA (0 h, cut with BamHI but not DpnI); lanes 2-5: BamHI and DpnI digestions of DNA obtained at 0, 24, 32, and 40 hours after co-transfection, respectively. Panel A: (pSV2neo+pSV2L); B: (pM+pSV2L); C: (pNL1+pSV2L); D: (pT+pSV2L); and E: (pNL2+pSV2L). Panel F: DNA standard: lanes 1-3, 10^{-3}, 10^{-2}, and 10^{-1} amount of (pSV2neo+pSV2L) used in co-transfection. Arrows a and b indicate the position of each test plasmid and the internal control pSV2L, respectively.

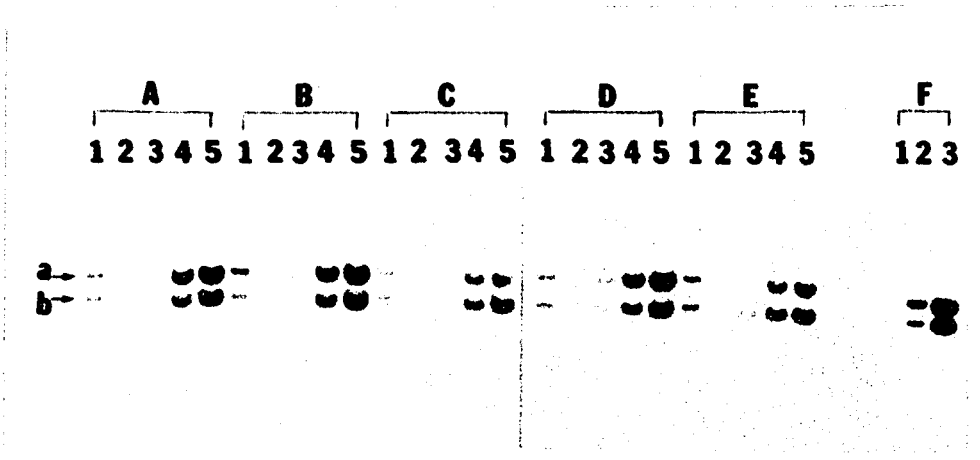

A
B
C
D
E
F
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3
a
b

Fig. 10. Transient replication of pSCM1 in Cos-1 cells: Competition experiment. pSCM1 was co-transfected with equimolar amount of pSV2L (Panel A), pSCM2 (Panel B), or with 10-fold molar excess of pSCM2 (Panel C). Plasmid DNA was digested with EcoRI to differentiate the pairs. Lane 1: input DNA (0 h, not cut with DpnI); lanes 2-6: DNA further cut with DpnI: 0, 24, 32, 40, and 72 hours after co-transfection, respectively. Arrows a, b, and c indicate the position of the linearized 4.5 kb pSV2L, the 2.9 kb band derived from pSCM1, and the 2.6 kb band derived from pSCM2. Bracket d indicates the input DNA fragments resulting from DpnI digestion.

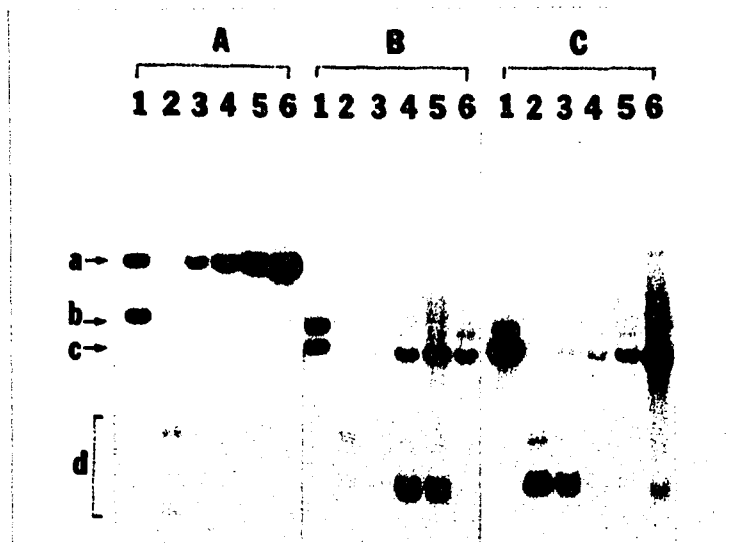

A
B
C
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
a
b
c
d

Fig. 11. Transient replication of pSCM1 and its variants with deletions of the promoter sequence. Equimolar amounts of the test plasmid and the internal control pSV2L were used in co-transfection of Cos-1 cells. Lane 1: input DNA (0 h, with no DpnI digestion); lanes 2-5: DNA further cut with DpnI: 0, 24, 32, and 40 hours after co-transfection, respectively. Panel A: pSCM1; B: pSCM2; C: pSCM1 variant 1 (pSCM1dl1) with a deletion of -80 to +108; D: pSCM1dl2 with a deletion of -72 to +94; E: pSCM1dl3 with a deletion of -51 to +87; F: pSCM1dl4 with a deletion of -91 to +65. Arrow a indicates the position of linearized pSCM1 deletion variants (6.2-6.3 kb); b, the position of linearized pSV2L (4.5 kb); c, the position of the 3.3 kb band derived from EcoRI digestion of pSCM1; d, the position of the 3.0 kb band derived from EcoRI digestion of pSCM2; and e, input plasmid DNA fragments resulting from EcoRI and DpnI digestions.

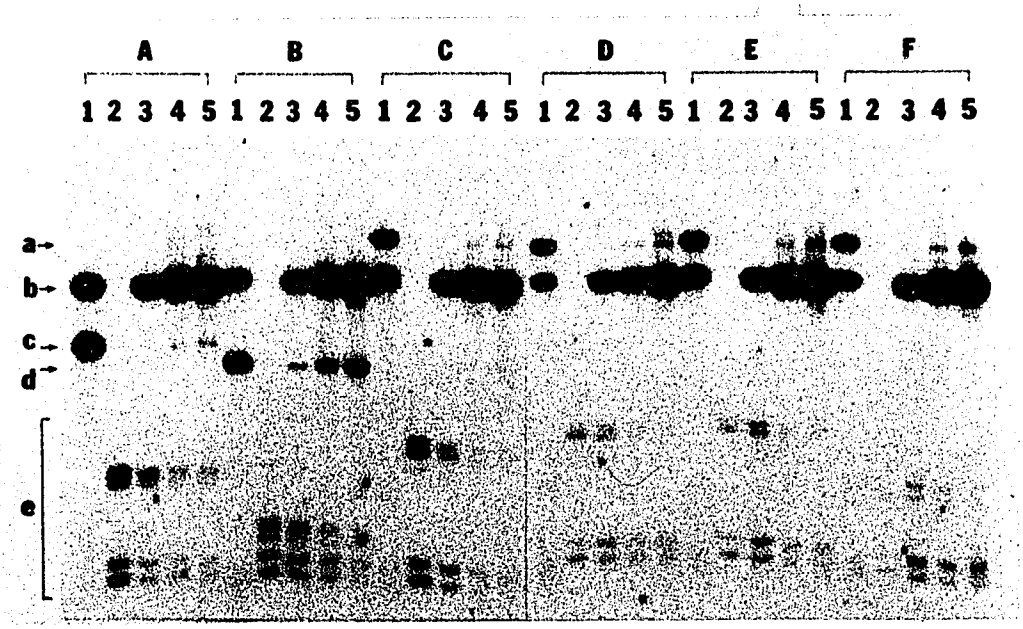

A
B
C
D
E
F
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
a
b
c
d
e

Fig. 12. Transient replication of the pSCM plasmid series at 72 hours after transfection. The pSCM plasmids and the internal control pSV2L in a molar ratio of 5:1 were used in co-transfection of Cos-1 cells. Lane 1. input DNA (0 h, linearized with HindIII or EcoRI, with no DpnI digestion); lane 2: DNA harvested at 72 hours after co-transfection, linearized with HindIII or EcoRI, and further digested with DpnI. Panel A: pSV2neo (linearized with HindIII); B: pSCM1 (HindIII); C: pSCM2 (HindIII); D: pSCM3(+) (HindIII); E: pSCM3(-) (HindIII); F: pSCM1dl1 (EcoRI); G: pSCM1dl2 (EcoRI); H: pSCM1dl3 (EcoRI); and I: pSCM1dl4 (EcoRI). Arrows a, b, and c indicate the position of each pSCM plasmid, pSV2neo, and pSV2L, respectively.

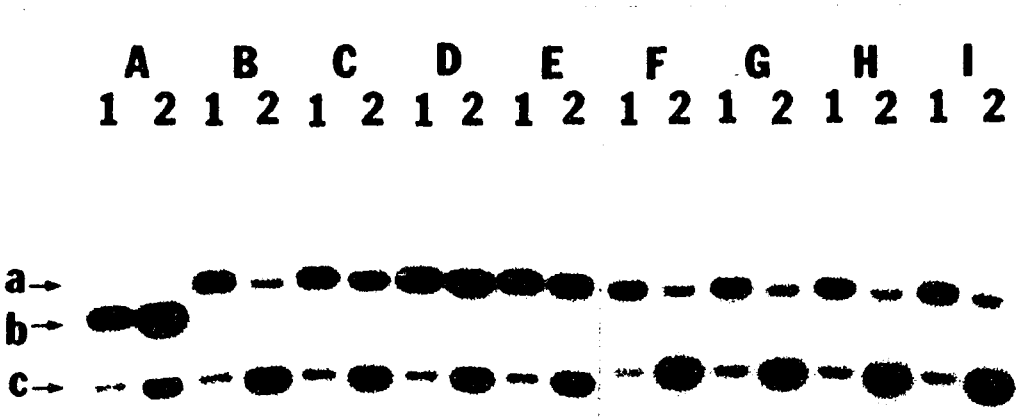
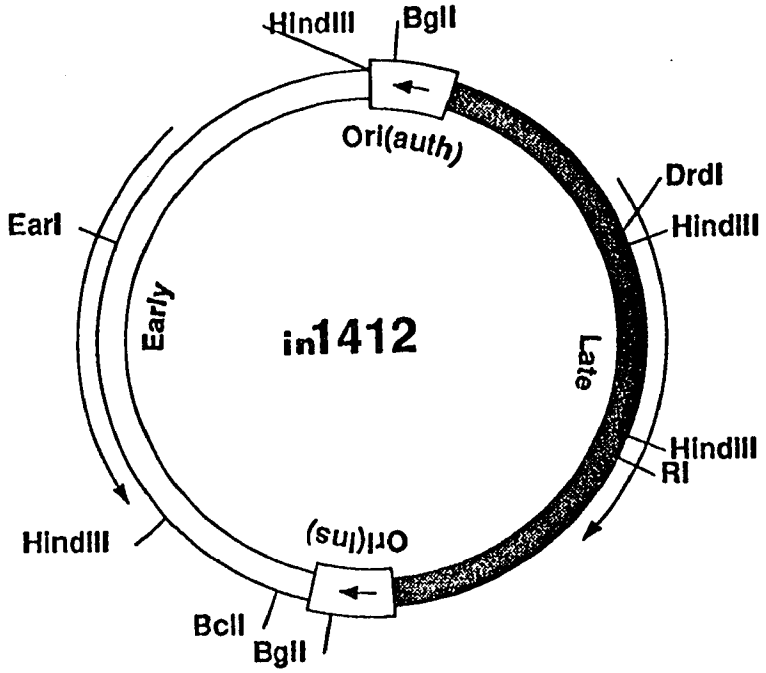

A B C D E F G H I
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
a→
b→
c→

Fig. 13. Genetic maps of SV40 variants *in*1412 and *in*1412v. The SV40 variants containing an additional *ori* were constructed by Shenk (1978). The parental deletion variant *dl*1410 contained two deletions: one within the Tag intron, and the other a 72-bp unit within the enhancer (see the SV40 map in Chap. 1). The HindIII(#5170)-KpnI(#294) segment containing the same deletion of a 72 bp unit was inserted to *dl*1410 at the HpaI site (#2666): the resultant variant containing the insert in the same orientation or in the opposite orientation with the authentic segment was named *in*(Or)1411 and *in*(Or)1412, respectively. The variant derived from *in*(Or)1412 with a small deletion at the BglI site of the inserted segment (thus inactivating the origin function) was named *in*(Or)1415. Here *in*(Or)1412 is shown (*in*1412, for short). Also shown is its variant *in*1412v, which was constructed by BglI digestion and re-ligation. Arrows indicate the direction of early and late transcription, respectively; ori(auth) and ori(ins) indicate the authentic and the inserted replication origin, respectively; and the enzymes shown are those used in this part of work.


HindIII
BglI
Ori(auth)
DrdI
HindIII
EarI
Early
in1412
Late
HindIII
RI
Ori(ins)
HindIII
BclI
BglI

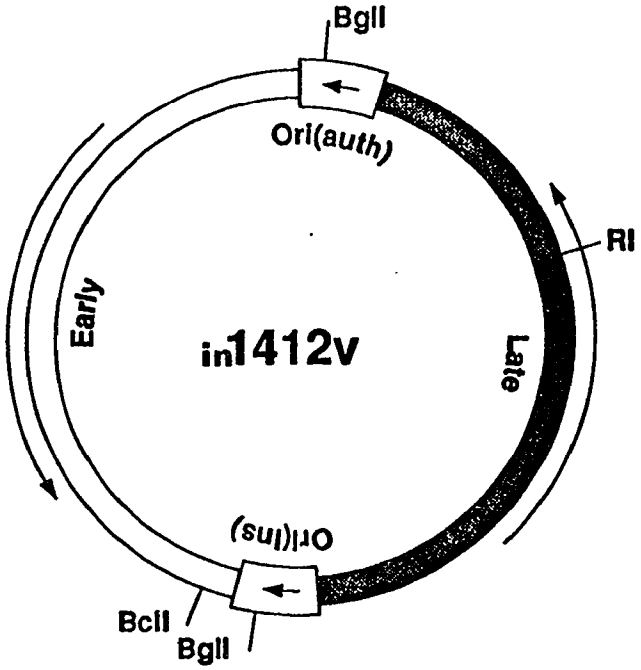
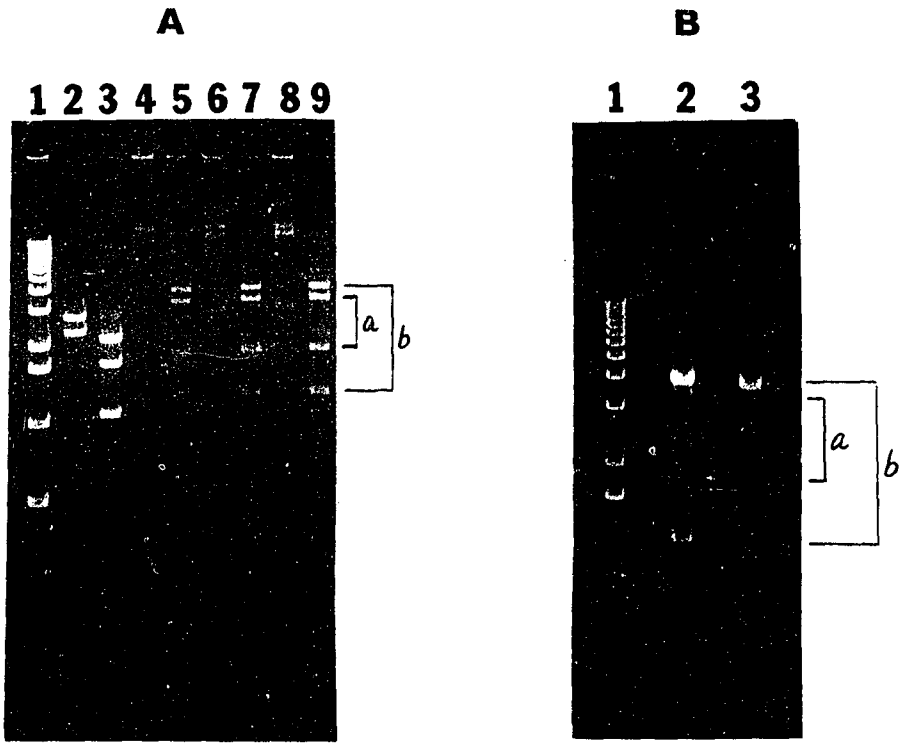

BglI
Ori(auth)
RI
Early
in1412v
Late
Ori(ins)
BclI
BglI

Fig. 14. Growth of *in*1412 and *in*1412v DNA in Cos-1 cells. To generate *in*1412v DNA with inversion of late region of *in*1412, *in*1412 DNA was cleaved with BglI, which cuts at the two origins in *in*1412 DNA generating two DNA fragments of 2.8 and 2.4 kb (panel A, lane 2). Religation of the cleaved DNA generated *in*1412 DNA as well as the variant *in*1412v that contains inverted late region. Panel A: After BglI digestion of *in*1412 DNA, the resultant 2.8 and 2.4 kb fragments were gel-purified and ligated to generate the orientation isomers of *in*1412 and *in*1412v (Fig. 13). The equal molarity of the isomers was confirmed with BclI+EcoRI digestion (see Fig. 13 for the restriction map). Lane 1, kb mol. wt. marker (BRL); lane 2, the purified 2.8 and 2.4 kb fragments in 1:1 ratio prior to ligation; lane 3, the two fragments cut with BclI+EcoRI; lane 4, 6, and 8, ligation products resulting from three reactions with increasing amount of DNA used as substrate; lanes 5, 7, and 9, ligation products from the three reactions characterized with BclI+EcoRI digestion. Pairs a and b indicate the DNA bands resulting from BclI+EcoRI cleavage of *in*1412v and *in*1412 (resulting from the ligation), respectively. Panel B: The ligation products shown in Panel A (1:1 ratio of in1412 and in1412v) were transfected into Cos-1 cells. The resultant virus from these cells was collected and propagated again in Cos-1 cells. The virus lysate then was used to infect CV-1 or Cos-1 cells. DNA of the progeny viruses was purified using the micrococcal nuclease method (Lai and Chu, 1991), and analyzed with BclI+EcoRI digestion. Lane 1, kb mol. wt. marker (BRL); lane 2, viral DNA purified from infected CV-1 cells; lane 3, viral DNA purified from infected Cos-1 cells. Pairs a and b indicate the DNA bands resulting from BclI+EcoRI cleavage of *in*1412v and *in*1412, respectively.


A
1 2 3 4 5 6 7 8 9
a
b
B
1 2 3
a
b

CHAPTER 5: DISCUSSION

A: Structure of active SV40 chromatin

The central theme of this thesis concerns the relationship between the structure and functions of SV40 chromatin. It has been well known that SV40 chromatin active in transcription or replication constitutes only a small fraction of the entire intracellular population (reviewed in DePamphilis and Bradley, 1986). As a runaway replicon, SV40 genome is amplified up to 10^6 copies per cell during infection. Thus, how the small fraction of active SV40 chromatin is chosen from seemingly "equal" progeny molecules resulting from DNA replication is an interesting question.

The first step toward the solution is to find what distinguishes the active SV40 chromatin from the bulk. Studies carried out in Dr. Hsu's laboratory have shown that the SV40 chromatin destined to be packaged into virus particles differs from that remaining in the non-virion pool by the modifications of histones and by the superhelical density of DNA (Coca-Prados *et al.*, 1979). The SV40 chromatin used for the initiation of DNA replication distinguishes itself from the bulk by having the highest superhelical density (Chen and Hsu, 1984). These results suggest that SV40 chromatin fractions destined for different pathways are marked with different properties. The observation that newly synthesized SV40 DNA preferentially re-enters the

DNA replication pool (Green and Brooks, 1976) is consistent with this interpretation.

In this thesis we continued the search for probes to distinguish active chromatin from the bulk chromatin. A characteristic of active chromatin is its hypersensitivity to DNase I. The inherent difficulty of this technique is that the difference in sensitivity to DNase I is only relative. Eventually all the chromatin (whether active or not) is degraded by the nuclease into small fragments. Demonstration of nuclease hypersensitivity, therefore, requires careful titration. To overcome this difficulty we investigated whether P1 nuclease could provide a better tool for probing the active chromatin. P1 nuclease was chosen based on the reasoning that the excess topological tension in the active chromatin may induce local unwinding of the double helix so that the DNA may exhibit a partial single-stranded character. P1 nuclease is known to prefer attacking a single-stranded DNA region. We reasoned that cleavage of the single-stranded region by P1 nuclease would release the topological tension and therefore would render the chromatin resistant to further digestion.

The experiments described in Chapter 2 provided data indicating that the above reasoning was correct. The bulk of SV40 chromatin organized in the nucleosome structure was not cleaved by P1 nuclease after prolonged digestion. In contrast, a small population of SV40 chromatin was cut by P1 and the cleavage occurred only once per molecule. This P1-sensitive fraction had

higher superhelical density as expected. P1 cleavage sites were mapped to the regulatory region of SV40 DNA. These results are consistent with our prediction that P1 responds to the topological tension in active chromatin by making a single cleavage. The observations that only one of the two regulatory sequences in *in*1411 and *in*1412 was cleaved by P1 at one time further support this interpretation. Although both regions in the same molecule should be equally sensitive to digestion, cleavage of one of the sites releases the topological tension and thus prevents the cleavage of the other site. Alternatively, the topological tension only has the energy enough to alter the structure of one of the regulatory regions.

Since the cell genome is organized as higher-order loop-domain structures, the P1 nuclease hypersensitivity has good potential to provide a useful tool to investigate active cellular genes as well as to clone the regulatory regions. Consistent with this conjecture we found that P1 made only a limited number of cleavages in CV-1 cell chromatin. It will be interesting to determine whether any one of the cleavage sites corresponds to the regulatory region of genes active in the cells.

Following the thinking that active chromatin has topological tension and that different viral nucleoprotein population has its characteristic superhelical density, we designed to use topoisomerase inhibitors to investigate the process involved in the establishment of DNA topology in intracellular SV40 DNA. Treatment of SV40 infected CV-1 cells with ellipticine, a topoII inhibitor,

unexpectedly caused a large increase in the superhelical density of a subpopulation of intracellular SV40 DNA. The superhelical density of ellipticine-sensitive DNA was found to correspond to about 1.75 superhelical turns per nucleosome. We originally interpreted this result to be due to the inhibition of topoII-induced relaxation of unconstrained supercoils in active chromatin by ellipticine. Subsequent investigations rejected this interpretation and showed instead that the increase in supercoiling is due to the intercalation of ellipticine within the nucleosome cores of SV40 chromatin. Although unexpected, this study did show that active chromatin has a more loosened nucleosome organization which allows the intercalation of ellipticine. The altered nucleosome organization of active chromatin would be consistent with the observation that active chromatin as a whole is more sensitive to endonuclease digestion.

Taken together, the work described in Chapters 2 and 3 suggests that active SV40 chromatin exhibits altered structures that can be probed with P1 nuclease and DNA intercalators like ellipticine. Although the ultimate question of how active chromatin is formed and maintained remains to be answered, it is believed that our results are helpful toward future investigations.

B. Effects of sequence microenvironment on the biological activity of SV40 origin of replication.

Following up on the finding that the two origins of DNA replication in the SV40 variant *in*1411 and *in*1412 are preferentially used in an *in vitro* nuclei replication assay, we set out to investigate effects of sequence microenvironment on the biological activity of SV40 origin of replication.

Our results described in Chapter 4 are most consistent with the hypothesis accounting for the preferential usage in *in*1411 and *in*1412 that active transcription passing the replication origin inhibits the usage of the origin. Although there have been several precedents indicating such a mechanism of suppression of replication by active transcription, our hypothesis deserves further test. For example, should the pattern of the origin usage be changed following the treatment of α-amanitin to inhibit RNA polymerase B? Or what pattern should arise when the promoter region has been mutated?

Previous studies have revealed several effects of transcription on DNA replication. In prokaryotes, transcriptional activation of DNA replication was initially found in phage lambda by genetic analysis (Dove *et al.*, 1969). Three mechanisms are plausible for this activation of DNA replication by transcription. First, a transcript passing through *ori* may form an RNA-DNA hybrid (R-loop) that, as exemplified by the replication of ColE1, may serve as the primer after its cleavage by RNase H (Itoh and Tomizawa, 1980). Second,

as suggested by biochemical experiments with *oriC* plasmids, RNA-DNA hybrid formation may free a stretch of single-stranded DNA and thus facilitate the assembly of an initiation complex (Baker and Kornberg, 1988). Third, as shown in *in vitro* experiments with both *E. coli* (Ogawa *et al.*, 1985) and lambda DNA replication (Mensa-Wilmot *et al.*, 1989), transcription near *ori* may affect the nucleoprotein structure in that region to facilitate the initiation of DNA replication.

In the SV40 system, "transcriptional activation" has been suggested solely based on the fact that the auxiliary sequences stimulating DNA replication happen to be also responsible for regulating transcription. Unlike the above prokaryotic examples, which require actual RNA polymerase binding and/or transcription, all SV40 studies only show the cis existence of the "transcription sequences" in a strictly position-dependent manner. No actual RNA polymerase binding/transcription has been shown to be needed for the transcriptional activation of DNA replication.

In addition to the transcriptional activation of DNA replication, suppression of DNA replication by several replication/transcription-independent sequences has been reported. Noteworthy with the SV40 origin of DNA replication, it can be converted from a runaway replicon to a controlled replicon by the connected BPV replication origin plus the E2 protein (Roberts and Weintraub, 1985; 1988). When the SV40 regulatory segment (HindIII-PvuII) is inserted into AAV genome, its origin function becomes overridden by

the AAV terminal repeat and one or more *rep*-encoded proteins (Berns *et al.*, 1988). The SV40 origin activity can also be suppressed by the reiteration of the endogenous 72 bp repeats (Kumar *et al.*, 1988). Exogenous LTR enhancer of Rous sarcoma virus, when connected to SV40 origin, abolishes the SV40 origin activity in the distance-independent manner (Binninger *et al.*, 1989).

Our results of the suppression of SV40 origin activity by human cytomegalovirus immediate early gene promoter/enhancer sequence (HCMV P/E) represents another unique example of SV40 origin-exogenous enhancer interaction. First, unlike the examples mentioned above, this suppression is mediated purely by the HCMV P/E sequence, devoid of any other elements that may result in complications. Second, HCMV P/E suppresses the SV40 origin activity strictly in the distance-dependent manner. Third, this suppression is independent of promoter region and thus independent of transcription. Our preliminary result (not shown in this thesis), together with the deletion variants of pSCM1 shown in the thesis, has narrowed the sequence mediating this suppression in the HCMV P/E to -350 to -91. This region includes four copies of 18 bp repeat, two copies of 19 bp repeat and one copy of 21 bp repeat (Boshart *et al.*, 1985). DNase I footprinting assay has shown six protected regions are within this region (Stamminger and Fleckenstein, 1990).

Based on the results presented in Chapter 4, the following experiments may be performed in the future to further characterize the interactions between the HCMV enhancer and SV40 origin: 1) to generate more detailed

deletion and point mutants of HCMV enhancer and study their effects on SV40 origin; 2) to determine the biochemical steps in SV40 replication affected by the HCMV sequence, specifically, the binding of T antigen to origin and the unwinding of origin by T antigen; 3) to isolate mutants of pSCM1 that can replicate efficiently in Cos-1 cells and determine the mutations in either SV40 or HCMV sequences that reverse the inhibitory effect of HCMV enhancer.

It is interesting to note that the HCMV enhancer was originally defined by an "enhancer-trapping" assay using enhancerless SV40 sequence to isolate viable virus that has trapped a functionally substitutable enhancer (Boshart *et al.*, 1985). Thus, our findings, conceptually, can be categorized into the group analogous to the suppression of SV40 origin by reiteration of the 72 bp enhancer elements regardless the difference in their location relative to SV40 origin (Kumar *et al.*, 1988). The uniqueness of the HCMV enhancer is that one copy of it is sufficient to suppress the SV40 origin activity in the presence of SV40's own enhancer; and 2) that although the suppression is distance-dependent, the suppression is much more far-reaching than the suppression exhibited by the 72 bp reiteration. Indeed, the HCMV enhancer is by far the strongest enhancer found, if the strength of the enhancer accounts for the ability of the suppression.

In summary, future studies using *in vitro* systems may find the suppression of SV40 origin activity by HCMV enhancer as an excellent model to study protein-DNA and protein-protein interactions in SV40 DNA

replication.

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