

Simian Virus 40 Early- and Late-Region Promoter Functions Are Enhanced by the 72-Base-Pair Repeat Inserted at Distant Locations and Inverted Orientations

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Tandemly repeated 72-base-pair (bp) segments located between nucleotides 107 and 250 of the simian virus 40 genome are essential for early region transcription. The functional requirement for the 72-bp repeat was supplied even when that segment was translocated to several locations distant from, and in different orientation, relative to, the promoter. Regardless of the position of the 72-bp enhancer segment, transcription was initiated at the same locations as with the normal promoter. Translocation of the 72-bp repeat segment to other sites in the genome resulted in the appearance of DNase I hypersensitivity at that site in the intranuclear viral minichromosomes. One of the translocations which did not produce enhancement of early- and late-region expression also failed to create a DNase I-hypersensitive site at the translocated 72-bp segment.

The DNA sequences of eucaryotic promoters that are recognized *in vivo* by RNA polymerase II have been investigated extensively, and some of their essential features are known (29). Most but not all promoters contain a TATA box (M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978), which directs transcription initiation 20 to 30 nucleotides downstream. Elimination or alteration of the TATA box causes transcription initiation to occur at many sites (3, 4, 10, 13, 14, 16, 18, 19, 23) and, in some instances, at lower efficiency (14, 16, 19, 23). RNA polymerase II promoters also require nucleotides in the -30 to -110 region; their removal or substantial modification abolishes or greatly reduces transcription, e.g., the herpes simplex virus thymidine kinase promoter (18, 19), the human α -globin (20) and rabbit β -globin promoters (16), and the simian virus 40 (SV40) early-region promoter (10). Two others, the sea urchin histone H2A and SV40 early-region promoters, have absolute requirements for a sequence located between nucleotides -184 and -524 and nucleotides -112 and -255, respectively (4, 10, 15). By contrast, the human α -globin (20) and the herpes simplex virus thymidine kinase (18) promoters show no dependence on the nucleotide sequence in this region. Of particular interest is the finding that the essential sequence in the -184 to -524 region of the sea urchin histone H2A promoter and the -112 to -255 region of the SV40 early promoter can function even when they are inverted relative to

the other promoter components (10, 15, 22). The nucleotide sequence between nucleotides -112 and -255 that is needed for SV40 early-region promoter function contains a tandemly repeated 72-base-pair (bp) sequence; this sequence can also augment correct transcription initiation from the rabbit (2) and human (J. Sklar and P. Berg, unpublished data) β -globin promoters, the herpes simplex virus thymidine kinase promoter (7; M. Fromm, Ph.D. thesis, Stanford University, Stanford, Calif., 1983), the conalbumin and ovalbumin promoters (22), and the adenovirus 2 late promoter (22). The mechanism of this enhancement or augmentation of transcription by sequences acting at a considerable distance and in inverted orientation is not known.

SV40 minichromosomes are known to have a region that is depleted of nucleosomes (27) and especially sensitive to endonuclease digestion (8, 11, 26, 28, 35, 38). This hypersensitive sequence is located between the *Bgl*II (nucleotide 5,243/0) and *Hpa*II (nucleotide 346) restriction sites (for SV40, the SV numbering system [6] is used throughout), a region which contains the SV40 origin of DNA replication (*ori*) and the early- and late-region promoters. The reason for the sparse nucleosome content and increased endonuclease sensitivity is not known, although it has been suggested that this region serves as an entry site for proteins needed in transcription and replication (9).

We have already reported (10) that inverting the DNA segment containing the tandem 72-bp

repeats (nucleotides 95 and 272) does not impair SV40 early- or late-region transcription. In the present study, the same DNA segment was translocated to a site within the intron of the large T antigen gene or to nucleotide 2,666, a position at the 3' end of the early and late region. Early- and late-region transcription were virtually normal in both types of recombinants; furthermore, the early-region mRNAs were initiated at the proper sites and the late-region mRNAs had the characteristic sizes.

Our present studies indicate that the *ori* region of the SV40 minichromosome was hypersensitive to endonuclease cleavage even when it lacked the 27-bp tandemly repeated sequence. Nevertheless, the 72-bp repeat sequence induced endonuclease hypersensitivity when it was translocated to other regions of the SV40 genome. Whether the enhancer activity of the 72-bp repeat segment requires the structural change detected by the endonuclease hypersensitivity was not resolved. It is apparent, however, that the minichromosome structural feature associated with endonuclease hypersensitivity, in the absence of the 72-bp sequence, is not sufficient to ensure SV40 early- or late-region promoter activity.

MATERIALS AND METHODS

Animal cells and viruses. The history, care, and use of CV1 and CV-1P monkey cells for the propagation and assay of SV40 and its mutants are described elsewhere (21).

Enzymes and reagents. Restriction endonuclease *EcoRI* was a gift from M. Thomas, Stanford University, and other restriction endonucleases were purchased from Bethesda Research Laboratories. *Escherichia coli* DNA polymerase I and T4 DNA ligase were gifts from S. Scherer, Stanford University. S1 nuclease was obtained from Boehringer Mannheim, polynucleotide kinase from PL Biochemicals, and the octanucleotide linkers containing an *XhoI* restriction site were purchased from Collaborative Research. [³⁵S]methionine and [γ -³²P]ATP were from New England Nuclear Corp.

Construction of XSLR mutants. Plasmid pXS10 is a recombinant of pBR322 and a mutant strain of SV40 that lacks nucleotides 73 to 272, the region that contains the augmentor or enhancer sequence of the early-region promoter (10). pXS10 DNA was the starting material into which the DNA segment spanning nucleotides 95 to 272 was inserted at various locations in the SV40 genome (see Fig. 1). The general procedures for construction were described previously (10), but the more specific steps were as follows. After 5 μ g of pXS10 DNA was partially digested with *HpaI* endonuclease, 0.25 μ g of *XhoI* octanucleotide linkers were ligated to the blunt ends, and the linear DNA was isolated by electrophoresis in a 0.7% agarose gel. After digestion with *XhoI*, the linear DNA was circularized with T4 DNA ligase (in 10 mM Tris [pH 7.5], 1 mM ATP, 10 mM dithiothreitol, and 10 mM MgCl₂) and transfected into *E. coli* HB101 cells. The plasmid

DNA recovered from ampicillin-resistant colonies were analyzed by restriction enzyme digestions to identify the derivative with the *XhoI* linker inserted at the *HpaI* restriction site at nucleotide 2,666 [pXS10-*XhoI*(2666)].

pXS10-*XhoI*(2666) DNA (5 μ g) was partially digested with *TaqI* endonuclease and incubated with 4 U of calf alkaline phosphatase, and the linear DNA was isolated by electrophoresis in a 0.7% agarose gel. *TaqI* cleaves pXS10-*XhoI*(2666) DNA at two locations in the pBR322 sequence and three locations in the SV40 sequence. A DNA segment containing the 72-bp tandem repeats (nucleotides 95 to 272) was obtained from a derivative of pX-100 DNA (10) by cleavage with *TaqI* at an *XhoI* restriction site at nucleotide 95 and an *Sall* site at nucleotide 272 (*TaqI* cleaves both *Sall* and *XhoI* restriction sites). The resulting 185-bp fragment, which spanned the sequence between nucleotides 95 and 272 and contained *TaqI*-cohesive ends, was ligated to the linear pXS10-*XhoI*(2666) DNA, and the products were transfected into *E. coli* HB101 cells. Insertions at either of the two *TaqI* restriction sites in the 2.3-kb pBR322 portion of pXS10 probably destroy ampicillin resistance or the origin of plasmid DNA replication, since clones with such insertions were not recovered. Insertion of the 72-bp repeat segment into any of the three *TaqI* restriction sites in the SV40 portion of pXS10-*XhoI*(2666) provided the XSLR derivatives. Restriction enzyme analysis confirmed that the 72-bp repeat segment was inserted into the positions and orientations shown for the six XSLR plasmid recombinants in Fig. 1.

pBR322 DNA sequences were subsequently removed by *EcoRI* endonuclease digestion, and the SV40 portion of each mutant was recircularized with T4 DNA ligase and transfected into CV-1P monkey cells to form virus plaques. Virus stocks were prepared from the plaques, and the viral DNA structures were confirmed by restriction enzyme analysis.

Isolation and characterization of early and late RNAs. Cytoplasmic RNA was isolated (37) from virus-infected CV1 cells, treated with DNase I, and absorbed to polydeoxythymidylate-cellulose to recover the polyadenylated [poly(A)⁺] fraction (1). The 5' ends of the SV40 early mRNAs were located by the Weaver and Weissmann modification (36) of the Berk and Sharp (5) procedure, using a single-strand DNA probe obtained from the wild-type genome that extended from nucleotide 272 to the ³²P-labeled end at nucleotide 5,118 (10). The mixture of cell RNA and DNA probe was precipitated with ethanol, the pellet was dissolved in buffer containing 50% formamide, 400 mM NaCl, 40 mM PIPES [piperazine-N,N'-bis(Z-ethanesulfonic acid)] (pH 6.4), and 1 mM EDTA, and the solution was heated at 80°C for 5 min and kept at 37°C for 12 h. The hybridized samples were diluted 10-fold with a buffer containing 250 mM NaCl, 30 mM sodium acetate, 1 mM zinc acetate, 5% glycerol, 20 μ g of heat-denatured salmon sperm DNA per ml, and 3,000 U of S1 nuclease per ml. After incubation at 37°C for 30 min, 10 μ g of *E. coli* tRNA was added, and samples were precipitated with ethanol, dissolved in loading buffer containing 80% formamide, and, after denaturation at 90°C for 3 min, electrophoresed in 8 M urea-8% polyacrylamide gels. Dried gels were autoradiographed as described previously (10).

The size of the viral mRNAs was determined by

electrophoresing glyoxylated poly(A)⁺ RNA on a 1.5% agarose gel, transferring it to nitrocellulose (34), and hybridizing it to SV40 DNA labeled with ³²P by nick translation (24). This probe hybridizes to all SV40 RNAs.

DNase I digestion of SV40 minichromosomes. Nuclei were isolated (39) 36 to 40 h after CV1 cells were infected with 10 to 20 PFU of either SV40 or mutant virus per cell and incubated at 37°C for 30 min with various amounts of DNase I (0.04 to 1.0 µg in 0.10 ml). After the DNase I reaction was stopped by the addition of sodium dodecyl sulfate and proteinase K, DNA was isolated by phenol extraction and ethanol precipitation. Those DNase I-digested samples which produced distinctive bands in Southern blots (32) after cleavage with *Eco*RI endonuclease were analyzed by Southern blotting after digestion with other restriction enzymes.

RESULTS

The mutant SV40 strain XS10 lacks the 72-bp repeats and three adjacent short guanine-plus-cytosine (G+C)-rich repeats (nucleotides 73 to 272) of the SV40 early promoter (10). The mutant XS10 strain is defective for early- and late-region expression but competent for replication (10). The 72-bp repeat region (nucleotides 95 to 272) is essential for early-region expression; late-region expression is defective if nucleotides 72 to 114 are also absent. We showed previously (10) that insertion of the 72-bp repeat segment (nucleotides 95 to 272) into a deletion mutant resembling XS10 restored early- and late-region expression. Therefore, strain XS10 was used to

test whether insertion of the 72-bp repeats at other sites in the SV40 genome and different orientations relative to the promoter sites could restore the defects in early- and late-region transcription.

The SV40 genome contains only three regions which do not code for essential SV40 proteins (30); therefore, these regions could accommodate a translocated 72-bp repeat segment without affecting viability. One of these is the region surrounding the 72-bp repeats; the second is within the large T antigen intron; and the third is between the 3' ends of the early and late regions. Consequently, the segment between nucleotides 95 and 272 was inserted at its original site (XSLR20 and XSLR21), within the large T antigen intron (XSLR24 and XSLR25), and at the *Hpa*I restriction site at nucleotide 2,666 (XSLR22 and XSLR23), in both possible orientations at each of these sites. The genomic structures of these mutants and wild-type SV40 are shown in Fig. 1.

Viability of mutants containing translocated 72-bp repeat segments. The parental and XSLR mutant genomes were transfected into CV-1P monkey cells, and plaques were counted after 2 weeks. All but the parent, XS10, and XSLR23 strains yielded plaques; therefore insertion of the 72-bp repeat segment at the locations and orientations used in mutants XSLR20, XSLR21, XSLR22, XSLR24, and XSLR25 can complement the defects in both early- and late-region expression of mutant strain XS10.

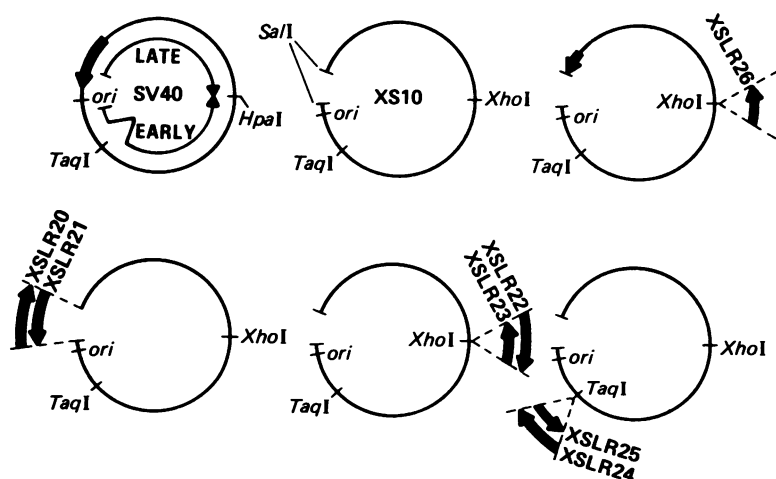


FIG. 1. Structure of SV40, XS10, and XSLR mutant DNAs. Circles represent the SV40 or mutant DNAs, and the discontinuous portion of the circle near *ori* indicates the region from which the 72-bp repeat segment was deleted. Short arrows indicate the location and orientation of the inserted 72-bp repeat segment; the arrow on each inserted 72-bp segment points from nucleotide 272 to 95. Arrowheads within the circle showing wild-type SV40 DNA indicate the direction and extent of the late region and large T antigen transcripts; *Hpa*I and *Taq*I mark the insertion sites of the 72-bp segment in strains XSLR22 and XSLR23 and in strains XSLR24 and XSLR25, respectively.

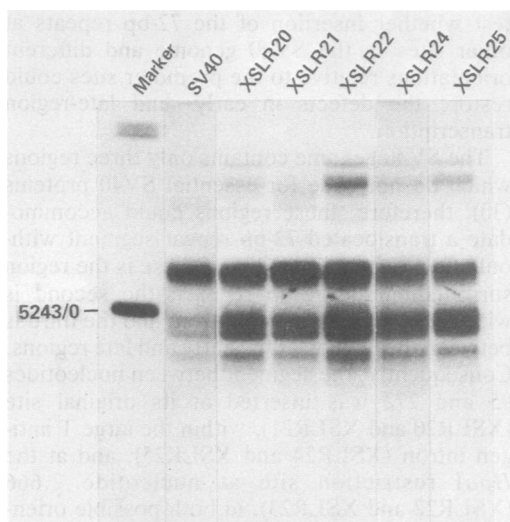


FIG. 2. Mapping the 5' ends of early region mRNAs produced by SV40 and the XSLR mutants during lytic infection. Poly(A)⁺ RNA was isolated 48 h after CV1 cells were infected. After hybridization of the RNA to an SV40 DNA fragment labeled with ³²P at the 5' end of the *Ava*II restriction site (nucleotide 5,118), the hybrids were digested with S1 nuclease, and the protected DNA fragments were electrophoresed in 8% polyacrylamide–8 M urea gels and autoradiographed. The marker was the DNA probe cut with *Bgl*I.

Translocation mutants make normal early- and late-region mRNAs. Virus stocks were prepared from plaques of the five viable XSLR mutants, and their DNA structures were confirmed by electrophoretic analysis of restriction enzyme digests. To compare transcripts from the viable mutants and the wild type, we isolated RNA 48 h after infection of CV1 cells with 10 to 20 PFU of SV40 or mutant virus per cell. The 5'-end positions of the early mRNAs of each virus were mapped by annealing the poly(A)⁺ mRNA to single-stranded SV40 DNA containing a ³²P-labeled 5' end at nucleotide 5,118, digesting this with S1 endonuclease, and electrophoresing the resultant products on an 8% polyacrylamide–urea gel. Each of the viable mutants produced the wild-type pattern of upstream- and downstream-initiated early mRNAs (10, 12, 17) (Fig. 2).

The sizes of the SV40-related RNAs made by the five viable mutant genomes were determined by electrophoresing the glyoxylated poly(A)⁺ RNA on a 1.5% agarose gel and hybridizing the blots (34) with nick-translated (24), ³²P-labeled SV40 DNA (Fig. 3). The patterns of 19S mRNAs encoding the T antigens VP2 and VP3 and the 16S mRNA encoding VP1 were virtually indistinguishable from those obtained with wild-type

SV40. Thus, it appears that the five viable mutants produce early- and late-region transcripts that are the same as those made by wild-type SV40, even though the 72-bp repeat segment occurs at a remote location or in an inverted orientation relative to the promoter sites.

Mutant XSLR23 early- and late-region functions. The sixth mutant, XSLR23, produced a few tiny plaques after 3 weeks; consequently, we judged it to be defective. Although none of the other XSLR mutants produced in the primary cloning operation gave rise to defectives, three independent isolates of strain XSLR23 could not be propagated as a virus. It is therefore unlikely that the defect in strain XSLR23 was caused by a defective 72-bp repeat segment. Mutant XSLR23 could not be complemented with either SV40 mutant strain *tsA58* or *tsB201*, which supply late and early functions, respectively (33); hence, it appears to be defective for expression of both early and late genes. Alternatively, strain XSLR23 may be unable to replicate its DNA normally, or perhaps the particular orientation of the insert at that site has some other deleterious effects. To explore this point, XSLR23 was modified by inserting a portion of the 72-bp repeat region (nucleotides 228 to 272) at its normal location, producing XSLR26 (Fig. 1). At its normal location, this particular segment of the 72-bp repeat region is sufficient to

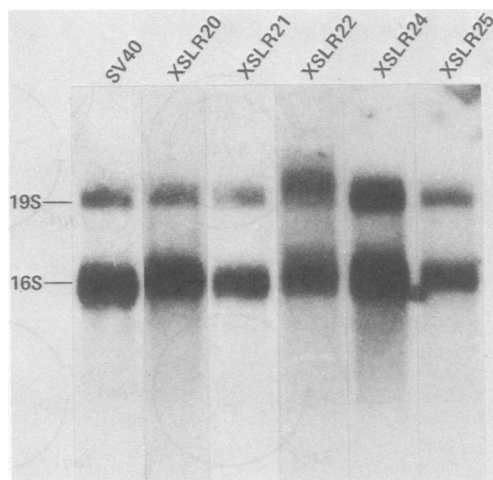


FIG. 3. Analysis of XSLR mutant RNAs made during lytic infection of CV1 cells. Glyoxylated poly(A)⁺ RNAs from wild-type and mutant SV40 strains were electrophoresed in 1.5% agarose gels, transferred to nitrocellulose, hybridized to ³²P-labeled, nick-translated SV40 DNA, and autoradiographed. The positions of the 19S and 16S RNAs are indicated.

permit early and late function (see mutant XS12 in reference 10). Thus, XSLR26 could be used to test whether inserting the 72-bp repeat segment between the ends of the early and late regions, as it exists in XSLR23, is in itself lethal. However, strain XSLR26 was viable and had the same growth characteristics as the parent strain XS12 after transfecting CV-1P cells in the standard plaque assay. Therefore, the 72-bp repeat fragment located at nucleotide 2,666, as it exists in mutant XSLR23, does not prevent multiplication of the virus; it seems to be more plausible that the 72-bp repeat sequence in mutant XSLR23 fails to enhance SV40 early and late transcription.

DNase I hypersensitivity of translocation mutant DNA. Intranuclear SV40 minichromosomes of infected cells contain DNase I-hypersensitive sites in the region between the *Bgl*I and *Hpa*II endonuclease cleavage sites (8, 11, 26, 28, 35, 38), the region which contains *ori* and the early- and late-region promoters. We examined the DNase I sensitivity of the intranuclear viral genomes in infections with the various XSLR derivatives to determine whether the 72-bp repeat segment produces DNase I hypersensitivity at the translocated sites. Nuclei obtained 36 to 40 h after infection of CV1 cells with mutant and wild-type genomes (20 PFU/cell) were incubated at 37°C for 30 min with increasing concentrations of DNase I, after which the DNA was isolated (see above). Samples that had been optimally digested with DNase I were cleaved with various restriction enzymes, electrophoresed, blotted, and hybridized with ³²P-labeled SV40 DNA probe. Figure 4 shows the autoradiograph of *Eco*RI-digested DNAs from SV40 and a mutant strain (XS12) lacking part (nucleotides 115 to 227) and from one strain (XS13) lacking all (nucleotides 115 to 272) of the 72-bp repeat region (10). In each case, the DNase I-*Eco*RI-digested DNA yielded fragments about 3.5 and 1.4 to 1.7 kilobases (kb) long, the expected sizes if the DNA is cleaved near the *ori* region and at the *Eco*RI restriction site. Comparable digestions of purified SV40 DNA yielded a smear and no comparable fragments, indicating that there was no preferential cleavage of free DNA by DNase I. This experiment showed, as did those referred to above, that the intranuclear SV40 DNA is preferentially cleaved by DNase I at or near *ori*; moreover, removal of part or all of the 72-bp repeat region does not eliminate the DNase I hypersensitivity of the *ori* region.

Analysis with the strains XSLR20 and XSLR21 pair yielded the same fragments as were obtained with wild-type SV40 (Fig. 5). However, the strains XSLR24 and XSLR25 pair yielded additional fragments diagnostic of preferential DNase I cleavages at or near the 72-bp

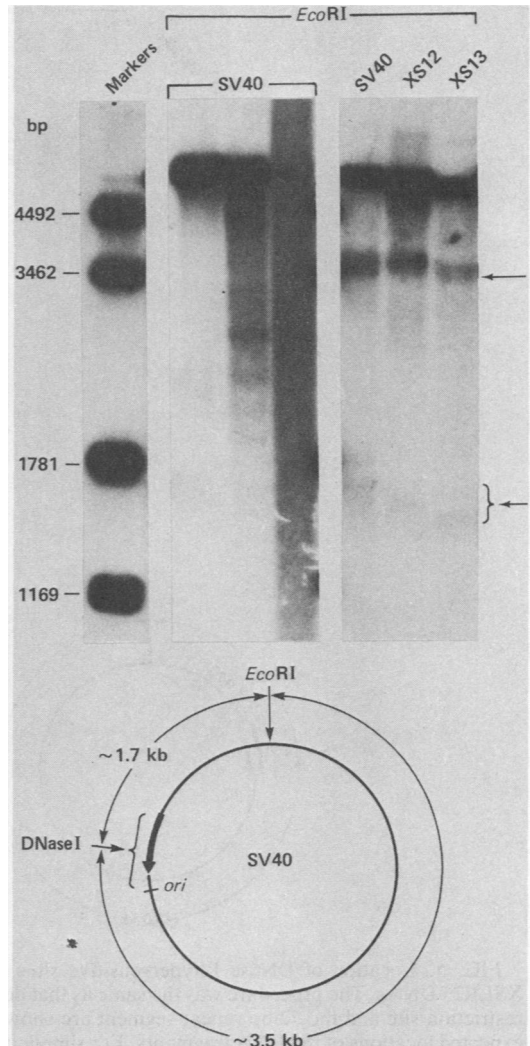


FIG. 4. Location of DNase I-hypersensitive sites in intranuclear SV40, XS12, and XS13 DNAs. DNAs isolated from DNase I-digested nuclei were cleaved with *Eco*RI, electrophoresed in 0.8% agarose gels, blotted, hybridized, and visualized by autoradiography. The DNA fragment sizes expected from DNase I cleavage at the SV40 *ori* region (26, 38) are shown at the bottom, and the expected positions of these fragments are indicated by arrows at the right of the gel. The pattern of fragments generated by DNase I digestion of purified SV40 DNA indicated no preferential cleavage at comparable sites.

repeat segment in the large T antigen gene (Fig. 5). The existence of preferential DNase I cleavage at or near the 72-bp repeat segments in XSLR24 and XSLR25 was also supported by the appearance of fragments of the expected size after *Bcl*II endonuclease digestion (Fig. 5).

*Hpa*II endonuclease cleavage of DNase I-

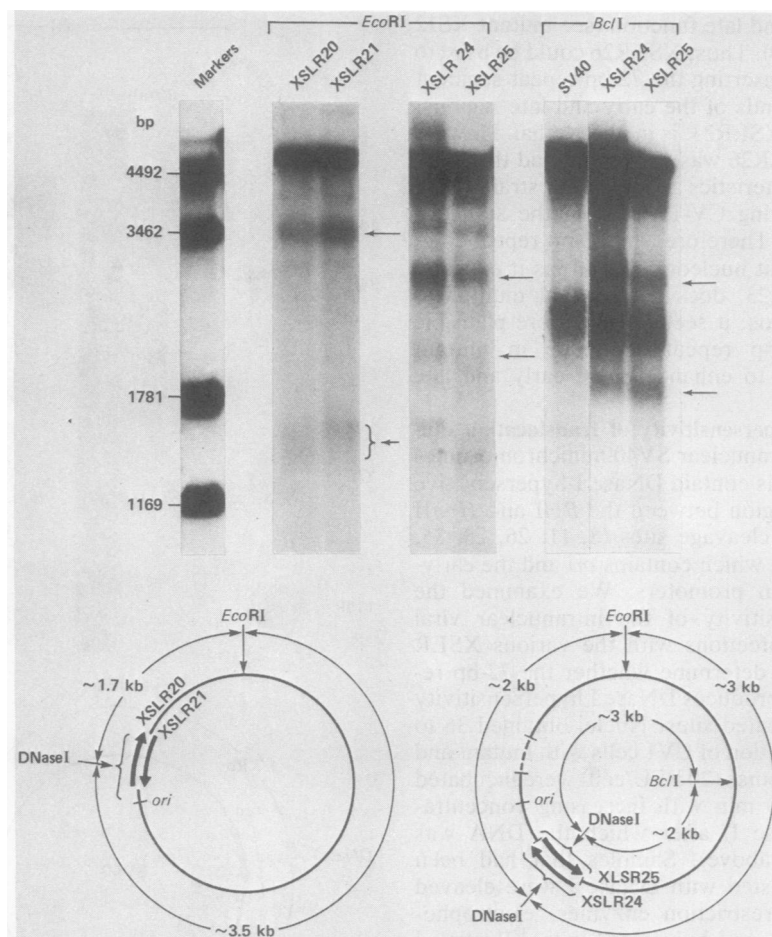


FIG. 5. Location of DNase I-hypersensitive sites in intranuclear strain XSLR20, XSLR21, XSLR24, and XSLR25 DNAs. The procedure was the same as that described in the legend to Fig. 4. The distances between the restriction site and the 72-bp repeat segment are shown at the bottom, and the arrows beside the gel show the expected locations of the DNA fragments. For simplicity, fragments arising from cleavage at the known DNase I-hypersensitive site at *ori* are not indicated; they can be deduced by comparing the pattern obtained for SV40 DNA with the corresponding restriction enzyme. The *EcoRI*- and *BclI*-digested samples were electrophoresed separately but with the same size markers. The markers shown at left are from electrophoresis of *EcoRI* analysis; markers for the *BclI* digestion have been omitted for simplification.

digested nuclei from strain XSLR22-infected cells yielded distinctive fragments of 2.7 and 2.3 kb; the major product of comparable digests with material from SV40-infected cells was about 5 kb, although small amounts of fragments about 3 kb long were also produced (Fig. 6). The result with strain XSLR22 was consistent with the occurrence of an additional DNase I-hypersensitive site at or near the translocated 72-bp repeat segment. Similar digestions with *EcoRI* produced a 4-kb band in addition to the approximately 3.5-kb band expected from cleavages at or near *ori* and the *EcoRI* restriction site (Fig. 4); the 4-kb band indicated that some molecules were initially cleaved by DNase I at or near the

72-bp segment at nucleotide 2,666. This contention was further supported by the finding that digestion with *BglI* at *ori* after DNase I treatment yielded fragments approximately 2.5 to 2.6 kb long (data not shown).

An unexpected finding was that mutant XSLR26, which contains the 72-bp repeat segment at the same location as in mutant XSLR22 but in the opposite orientation, was less hypersensitive to DNase I at the translocated 72-bp repeat segment. This followed from the absence or reduced quantity of the diagnostic 2.7- and 2.3-kb fragments after *HpaII* digestion and the 4-kb fragment after *EcoRI* digestion (Fig. 6). Whether the failure of the 72-bp repeat segment

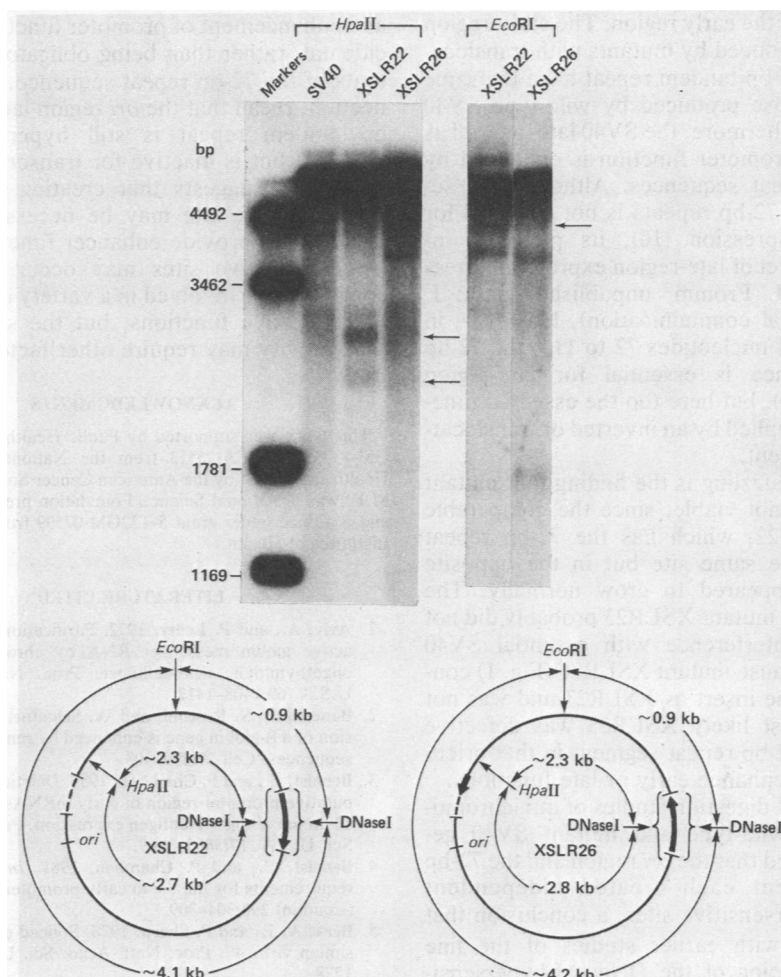


FIG. 6. Location of DNase I-hypersensitive sites in intranuclear strain XSLR22 and XSLR26 DNAs. The experimental procedure and comments are described in the legends to Fig. 4 and 5.

at this location and orientation to induce a distinctive DNase I-hypersensitive site was the cause of its inability to enhance the inactivated SV40 early and late promoters in mutant XSLR23 needs further study.

DISCUSSION

All eucaryotic promoters recognized by RNA polymerase II contain an essential region located between nucleotides +1 and -110; that region is analogous to the +1 to -35 region of procaryotic promoters (25, 31) in its involvement in RNA polymerase binding and transcription initiation (10, 15, 16, 19, 20, 23). However, some eucaryotic promoters also require a sequence upstream of the -110 region for efficient transcription (4, 10, 15). The sea urchin histone H2A gene promoter, for example, requires a sequence that occurs 184 to 524 nucleotides

upstream of the site of transcription initiation, and significantly this region functions normally even if it is inverted (15). The SV40 early-region promoter also requires a sequence that occurs between 112 and 255 nucleotides upstream of the initiation sites of the early mRNAs (4, 10). This region contains a tandemly repeated 72-bp sequence which, if inverted at its usual genomic location, can also enhance early-region transcription (10, 22).

The present experiments establish that the enhancing function of the 72-bp repeat segment is expressed even when it is inserted at locations distant from the site of transcription initiation, e.g., within the large T antigen intron or even at the 3' end of the early region; furthermore, the 72-bp segment functions in either orientation at its normal location or within the large T antigen intron, but in only one orientation when inserted

at the 3' end of the early region. The early-region transcripts produced by mutants with translocations of the 72-bp tandem repeat have the same 5' ends as those produced by wild-type SV40 genomes. Furthermore, the SV40 late- as well as early-region promoter function is enhanced by the 72-bp repeat sequences. Although the sequence in the 72-bp repeats is not essential for late-region expression (10), its presence increases the level of late-region expression three- to fivefold (M. Fromm, unpublished data; J. Mertz, personal communication). However, in the absence of nucleotides 72 to 114, the 72-bp repeat sequence is essential for late-region expression (10), but here too the essential function can be supplied by an inverted or translocated 72-bp segment.

Somewhat puzzling is the finding that mutant XSLR23 was not viable, since the comparable mutant XSLR22, which has the 72-bp repeat segment at the same site but in the opposite orientation, appeared to grow normally. The nonviability of mutant XSLR23 probably did not result from interference with essential SV40 functions, because mutant XSLR26 (Fig. 1) contained the same insert as XSLR23 and was not defective. Most likely XSLR23 was defective because the 72-bp repeat segment in that orientation cannot enhance early or late functions.

The DNase I digestion studies of minichromosomes from wild-type and mutant SV40 genomes indicated that the *ori* region and the 72-bp repeat segment each created independent DNase I-hypersensitive sites, a conclusion that is consistent with earlier studies of the fine structure mapping of the DNase I-hypersensitive sites of SV40 (8, 11, 26, 28, 35, 38). Except for the inference that DNA at these sites is more accessible to DNase I and presumably to intracellular proteins as well (9), very little can be said about the nature or significance of these regions in regulating gene expression. Nonetheless, it is evident that the sequence within the 72-bp repeat that enhances homologous as well as heterologous promoters from distances up to several kb and in opposite sequence orientations relative to the promoter (2, 10, 15, 22) also creates localized DNase I hypersensitivity. Particularly intriguing was the inability of the transplanted 72-bp repeat segment in one of the two possible orientations at nucleotide 2,666 to enhance early- and late-region expression. This site also did not induce the distinctive DNase I hypersensitivity. Quite possibly the enhancer function is mediated by an alteration of minichromosome structure, which in turn facilitates the entry of RNA polymerase to the promoter site or increases the efficiency of transcription initiation per se. Of course, it is entirely possible that the induction of DNase I hypersensitivity

and enhancement of promoter function are coincidental, rather than being obligatory concomitants of the 72-bp repeat sequence. In this connection, recall that the *ori* region lacking the 72-bp tandem repeat is still hypersensitive to DNase I but is inactive for transcription initiation. This suggests that creating a DNase I-hypersensitive site may be necessary but not sufficient to provide enhancer function; DNase I-hypersensitive sites may occur at chromosomal regions involved in a variety of regulatory or replicative functions, but the specificity of that activity may require other factors.

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