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The Genomic Instability Associated with Integrated Simian Virus 40 DNA Is Dependent on the Origin of Replication and Early Control Region

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DNA rearrangements in the form of deletions and duplications are found within and near integrated simian virus 40 (SV40) DNA in nonpermissive cell lines. We have found that rearrangements also occur frequently with integrated pSV2neo plasmid DNA. pSV2neo contains the entire SV40 control region, including the origin of replication, both promoters, and the enhancer sequences. Linearized plasmid DNA was electroporated into X1, an SV40-transformed mouse cell line that expresses SV40 large T antigen (T Ag) and shows very frequent rearrangements at the SV40 locus, and into LMtk$^-$, a spontaneously transformed mouse cell line that contains no SV40 DNA. Stability was analyzed by subcloning G-418-resistant clones and examining specific DNA fragments for alterations in size. Five independent X1 clones containing pSV2neo DNA were unstable at both the *neo* locus and the T Ag locus. By contrast, four X1 clones containing mutants of pSV2neo with small deletions in the SV40 core origin and three X1 clones containing a different *neo* plasmid lacking SV40 sequences were stable at the *neo* locus, although they were still unstable at the T Ag locus. Surprisingly, five independent LMtk$^-$ clones containing pSV2neo DNA were unstable at the *neo* locus. LMtk$^-$ clones containing origin deletion mutants were more stable but were not as stable as the X1 clones containing the same plasmid DNA. We conclude that the SV40 origin of replication and early control region are sufficient viral components for the genomic instability at sites of SV40 integration and that SV40 T Ag is not required.

Simian virus 40 (SV40) DNA integrated into the host genome of nonpermissive cells shows ongoing genomic instability characterized by both duplications and deletions of SV40 and host flanking DNA (4, 24, 27, 42, 43, 57, 64). In the overreplication model, based on the onion skin model (6, 58) and frequently invoked to explain the instability, SV40 large T antigen (T Ag) would bind to the SV40 core origin and initiate unscheduled DNA replication, leading to overreplication of the origin and flanking sequences. The rearrangements would then be the result of recombination between the amplified DNA strands. Alternatively, the viral DNA may be excised from the chromosome, replicate in the form of an extrachromosomal element, and reintegrate into the host DNA at the same or a new chromosomal site (11, 15, 52). However, SV40 extrachromosomal elements have not been isolated from most nonpermissive cell lines containing integrated SV40 DNA (3, 4, 27, 57).

In the cell line X1, isolated as a rearranged subclone of the BALB/3T3 cell line SVT2, the SV40 DNA is highly unstable, with new rearrangements detected in almost every subclone (24). The rearrangements are observed more frequently in the DNA sequences closest to the SV40 origin of replication (2, 3). The enhanced instability of X1 cells, as compared with that of SVT2 and other cell lines with integrated viral DNA, provides an opportunity to readily examine the genomic instability.

In this study we have examined the dependence of the genomic instability of SV40 DNA in X1 cells and another nonpermissive cell line on the SV40 origin of replication and SV40 T Ag. We first determined the stability of the SV40 wild-type origin of replication and auxiliary sequences from a reporter plasmid at several sites of integration in X1 cells and compared this with the stability of a reporter plasmid with a mutated SV40 origin region. This was accomplished by transforming X1 cells with pSV2neo, which has the wild-type SV40 origin of replication, or with pSV2oriΔ plasmids, which have small deletions in the SV40 core origin, and monitoring for restriction fragment length polymorphisms by using Southern blots. Second, we tested the role of T Ag in inducing rearrangements by analyzing pSV2neo in LMtk$^-$ cells, which do not express T Ag. Our results indicate that the presence of the SV40 core origin of DNA replication along with the flanking auxiliary regions and early promoter is sufficient to induce genomic instability.

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MATERIALS AND METHODS

Plasmids used in cloning studies. The plasmid pLTRneo (Fig. 1A) contains the long terminal repeats (LTRs) of the avian sarcoma virus, the Tn*5* *neo* gene, the herpes simplex virus thymidine kinase polyadenylation sequences, and pBR322 vector sequences. pLTRneo was constructed from pRMH140 (29) by deleting the SV40 DNA originally found 3′ to the herpes simplex virus thymidine kinase polyadenylation sequences.

pSV2neo (63) was obtained from the American Type Culture Collection (ATCC 37149). As illustrated in Fig. 1B and C, pSV2neo contains the 342-bp *Pvu*II-to-*Hin*dIII SV40 DNA region consisting of the origin of DNA replication, early and late promoters, and enhancer sequences (SV40 nucleotides 1 to 270 and 5171 to 5243) and a second SV40 fragment with the early polyadenylation signal and splice sites. Both 5′ and 3′ untranslated Tn*5* *neo* sequences, which are not found in pLTRneo, are present in pSV2neo, accounting for the difference in size of the *neo* region found in the two plasmids.

The replication activity of the SV40 origin in pSV2neo was inhibited by making small deletions in T Ag binding domain II,

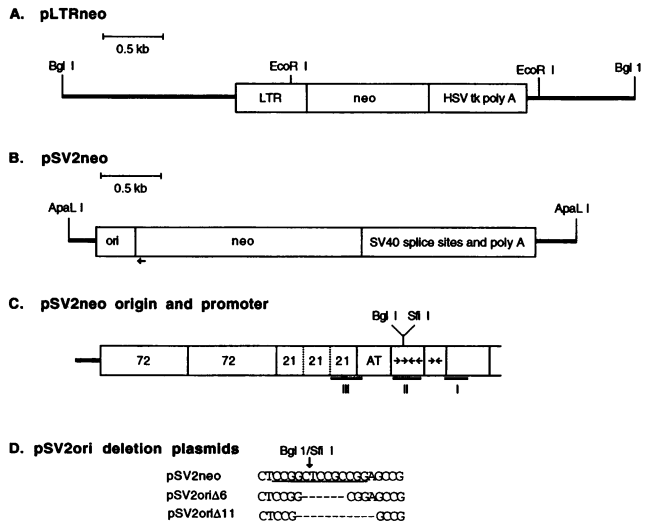

FIG. 1. Plasmid maps. (A) The 4.7-kb pLTRneo plasmid linearized at the unique *Bgl*I site. The Rous sarcoma virus LTRs, Tn*5* *neo* gene, and herpes simplex virus thymidine kinase (HSV *tk*) polyadenylation signal are indicated. The pBR322 vector sequences are illustrated by the heavy lines. (B) The 3.97-kb *Apa*LI fragment of pSV2neo, illustrating the SV40 control region (ori), the Tn*5* *neo* gene, the SV40 early splice sites and polyadenylation region, and pBR322 vector sequences. The location of the primer used in sequencing the pSV2oriΔ plasmids is marked by the arrow under the *neo* gene. The pBR322 vector sequences are illustrated by the heavy lines. (C) Enlarged view of the SV40 control region. The 72-bp enhancers, the 21-bp repeats, and T Ag binding sites III and I constitute the SV40 early promoter region. The SV40 core origin consists of the AT-rich region, T Ag binding site II (box with the *Bgl*I site and *Sfi*I sites), and the early palindrome (imperfect inverted repeat) region. T Ag binding sites are underlined and labeled I, II, and III. (D) The DNA sequence of the *Sfi*I-*Bgl*I site in the SV40 origin of replication in pSV2neo. The *Sfi*I recognition site ($CCGGN_5CCGG$) is underlined, and the *Sfi*I-*Bgl*I cleavage site marked. The nucleotides deleted in pSV2oriΔ6 and pSV2oriΔ11 are indicated.

using the S1 nuclease deletion method of Gluzman et al. (22). Deletions in the origin were generated first by linearizing pSV2neo with *Sfi*I, which was followed by S1 nuclease attack at the *Sfi*I-*Bgl*I site (Fig. 1C). After S1 nuclease digestion and religation to give circular molecules, plasmids with deletions in the origin region (pSV2oriΔ) were identified by screening for the loss of the *Bgl*I site in the SV40 origin. Twenty-two plasmids screened by *Bgl*I restriction mapping retained the *Bgl*I cleavage site in the pBR322 vector but not in the SV40 origin. The pSV2oriΔ candidates were tested for SV40 origin replication activity in Cos1 cells, as described below. Two replication-defective plasmids, pSV2oriΔ6 and pSV2oriΔ11, were sequenced across the origin with Sequenase 2.1 (United States Biochemical, Cleveland, Ohio) by using the modified protocol of Schuurman and Keulen (62). The oligonucleotide primer used for sequencing hybridized within the *neo* gene and is represented by the arrow in Fig. 1B. A 6-bp deletion spanning the *Sfi*I site was found in pSV2oriΔ6 (Fig. 1D), while pSV2oriΔ11 had an 11-bp deletion. In addition, a second deletion of approximately 250 bp is present in the 5′ untranslated region of *neo* in pSV2oriΔ6.

The *lacZ* expression vector pM4-lacZ, used to optimize cell electroporation conditions, was kindly supplied by Chuxia Deng and Mario Capecchi (Department of Human Genetics, University of Utah, Salt Lake City).

FIG. 2. Map of integrated X1 SV40 DNA. The integrated SV40 DNA in X1 cells is indicated by the bars, and the flanking mouse DNA is indicated by the line. The DNA regions corresponding to the complete coding regions for large and small T Ag are indicated by the hatched bars under the restriction map. ori marks the site of the SV40 origin of replication, enhancers, and early promoter. The *Hin*dIII (H) and *Bgl*I (b) sites present in the integrated SV40 DNA and flanking mouse sequences are marked (24).

Plasmid DNA was prepared by alkaline lysis and purified by polyethylene glycol precipitation (59). The electroporation conditions used to transform *Escherichia coli* MC1061 F′ with supercoiled plasmids, using the Bio-Rad Gene Pulser Apparatus, were 2.45 kV, 400 Ω, and 25 μF. Competent MC1061 F′ cells were prepared by the method of Dower et al. (18).

Cell growth conditions and clone selection. X1 cells, illustrated in Fig. 2, contain one integrated copy of SV40 consisting of the SV40 early coding region, early promoter, enhancers, core origin, and a small duplication, which contains a partial, scrambled section of the early control region and T Ag exon 1 (3, 24). X1 cells were cultured as previously described (24). The geneticin (G-418; GIBCO) concentration used to select X1 clones containing integrated copies of the reporter plasmids was determined by assaying X1 cells for G-418 sensitivity. No spontaneous neomycin-resistant X1 cells were observed in 2×10^7 cells at 400 μg of G-418 per ml.

The clones X1 pSV2neo20 (neo20) and X1 pSV2neo17 (neo17) were selected with 600 μg of G-418 per ml in monolayer culture. The clone X1 pLTRneoA26 (LTRA26) and the remaining X1 clones were selected with 400 μg of G-418 per ml in 0.35% agarose. Subclones of neo20, neo17, and LTRA26 were grown in 0.35% agarose either with or without G-418, as indicated. Individual subclones were picked from the agarose and seeded in monolayer cultures with or without 300 μg of G-418 per ml. All other X1 clones were subcloned as monolayer colonies in the absence of G-418. The protocol for isolating individual clones and subclones is outlined in Fig. 3.

The transformed mouse fibroblast cell line LMtk$^-$ (34) was cultured at 37°C in Dulbecco's modified Eagle minimal essential medium supplemented with 1 mM sodium pyruvate and 5% calf serum (DV-5). The G-418 toxicity curve for LMtk$^-$ cells was determined, and clones containing integrated plasmids were selected in 400 μg of G-418 per ml. LMtk$^-$ clones were grown as monolayers in the presence of G-418. LMtk$^-$ subclones were isolated as monolayer colonies in the absence of selection.

Cos1 cells (21) were grown at 37°C in Dulbecco's modified Eagle minimal essential medium supplemented with 1 mM sodium pyruvate and 10% fetal calf serum (DV-10).

Eukaryotic-cell electroporation. X1 cells were transformed with linearized plasmids by using the Bio-Rad Gene Pulser Apparatus. The initial electroporation conditions for X1 cells were selected by determining the 50% survival rate of the cells, following electroporation either in phosphate-buffered sucrose (PB sucrose) (272 mM sucrose, 7 mM $NaPO_4$ [pH 7.4], 1 mM $MgCl_2$) or in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). X1 cells were trypsinized,

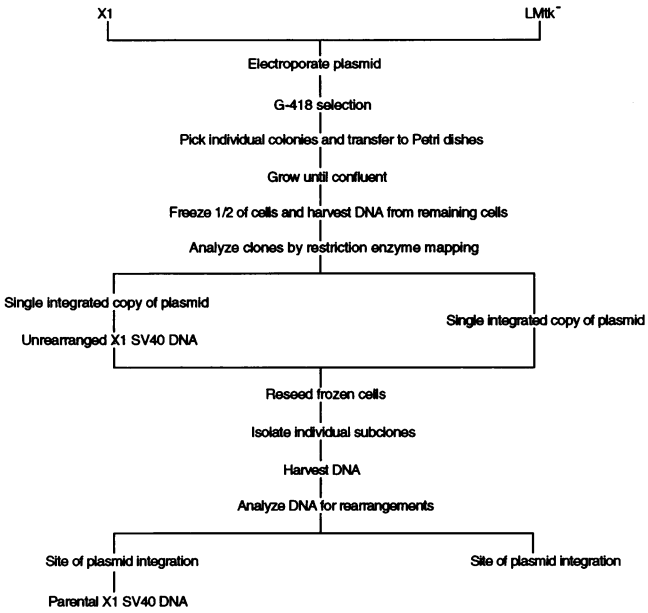

FIG. 3. Cloning and subcloning protocol.

washed in either PB sucrose or PBS, and resuspended in the appropriate buffer to 1.25×10^7 cells per ml. Aliquots of cells (0.8 ml) were transferred to 0.4-cm Gene Pulser cuvettes (Bio-Rad), and 50-V electroporation increments were applied to sequential cuvettes. The cells were incubated on ice for 10 min after electroporation and plated as monolayers at 37°C for 24 to 48 h prior to determination of the percent cell survival. The electroporation conditions for cells resuspended in PBS were optimized in a transient expression assay using the *lacZ* expression vector pM4-lacZ and screening for blue colonies (60).

X1 clones with integrated plasmid DNA were obtained following the electroporation of 5 to 10 μg of linearized plasmid DNA dissolved in PB sucrose into 10^7 cells. The electroporation conditions for cells suspended in PB sucrose were 420 V and 25 μF with a pulse decay range of 12.8 to 13.3 ms. When the cells were suspended in PBS, the Gene Pulser settings were 500 V and 25 μF with a pulse decay range of 0.4 to 0.6 ms. Forty-eight hours after electroporation, the surviving cells were cloned either in agarose or as monolayer cultures in DV-5 containing 400 μg of G-418 per ml, unless indicated otherwise.

The electroporation conditions for LMtk$^-$ cells were determined as described above for X1 cells. G-418-resistant LMtk$^-$ clones were selected following the electroporation of 5 μg of linearized plasmid into 10^7 cells suspended in 0.8 ml of PBS. The Gene Pulser settings were 950 V and 25 μF with a pulse decay time of 0.4 ms. Forty-eight hours after electroporation, the cells were cloned as monolayer cultures in DV-5 containing 400 μg of G-418 per ml.

Electroporations were conducted with linearized plasmid DNA to increase the efficiency of transformation (19) and to control the site of integration within the plasmid molecule (19, 25, 48). pSV2neo and pSV2oriΔ plasmids were linearized with *Apa*LI, which cleaves within the pBR322 vector sequences on both sides of the SV40 sequences (Fig. 1B), prior to electroporation. Originally, pLTRneo was cleaved with *Eco*RI, which cleaves once within the pBR322 vector sequences and once within the LTR (Fig. 1A). Although G-418-resistant clones such as X1 LTRneoA26 (LTRA26) were obtained with *Eco*RI-cleaved pLTRneo, cutting within the LTR may decrease *neo* expression. Subsequent electroporations were conducted with *Bgl*I-cleaved pLTRneo (*Bgl*I cleaves once within the pBR322 vector sequences [Fig. 1A]), and the clones X1 LTRneoD6 (LTRD6) and X1 LTRneoD8 (LTRD8) were isolated.

The electroporation conditions used to transfect Cos1 cells at 4°C in 0.2-cm Bio-Rad cuvettes were 450 V, 400 Ω, and 3 μF with a pulse decay time of 0.8 ms. The plasmids were resuspended in PB sucrose, and 2 μg of supercoiled DNA was introduced by electroporation into 1.5×10^6 Cos1 cells in 0.1 ml of PB sucrose. The cells were incubated at room temperature for 10 min prior to being reseeded in DV-10.

Cos1 replication assay. The pSV2oriΔ candidates were tested for SV40 origin replication activity in Cos1 cells, which constitutively express SV40 T Ag. The plasmids to be assayed were electroporated into Cos1 cells, and 72 h later cellular DNA was extracted (see below). The DNA was digested with *Eco*RI and *Dpn*I, Southern blotted, and probed with pBR322 to identify replication products (53).

Harvesting of genomic DNA. Chromosomal DNA was harvested from monolayer cell cultures as described previously (24). DNA concentrations were determined by measuring the optical density of the sample at 260 nm or by fluorometric assays (23).

Southern blots. Southern blot analysis was performed as previously described (24) with minor modifications. Briefly, DNA was digested with restriction enzymes at a concentration of 5 U/μg of DNA for 3 to 18 h at the appropriate temperature. The DNA separated in agarose gels was blotted onto nylon (HyBond; Amersham) or nitrocellulose (Schleicher & Schuell) membranes prior to hybridization in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate (pH 6.7)–5 mM sodium pyrophosphate–0.1% sodium dodecyl sulfate–200 μg of the synthetic heparin substitute polyanetholesulfonic acid (Calbiochem) per ml. Nick-translated SV40 DNA, plasmid DNA, or PCR fragments were used as probes, as indicated.

Statistical analysis. To test for significant differences in rearrangement frequencies with each set of clones, contingency table analysis (SAS JMP, 1989; SAS Institute Inc., Cary, N.C.) was applied. Only the clones which were subcloned as monolayer cultures were used in the statistical analysis. The three clones which were subcloned in agar (neo20, neo17, and LTRA26) were not included in the statistical analysis because of the selective pressure on the T Ag coding region.

RESULTS

Genomic rearrangements occur at the site of integration of the reporter plasmid pSV2neo. X1 cells have a high frequency of rearrangement at the SV40 integration site; therefore, we were interested in determining whether a reporter plasmid integrated at a second site in X1 cells would also exhibit a high rearrangement frequency. The *Apa*LI fragment of pSV2neo was introduced into X1 cells by electroporation, as outlined in Fig. 3 and described in Materials and Methods. Clones were selected with G-418 and analyzed for single-copy pSV2neo insertion and unrearranged parental X1 SV40 DNA by restriction enzyme mapping. Clones that met these criteria were subcloned, and the individual subclones were analyzed for restriction fragment length polymorphisms, indicative of chromosomal rearrangements. Restriction enzyme mapping indicated that pSV2neo was integrated at a different chromosomal location in each clone chosen for further analysis (results not shown).

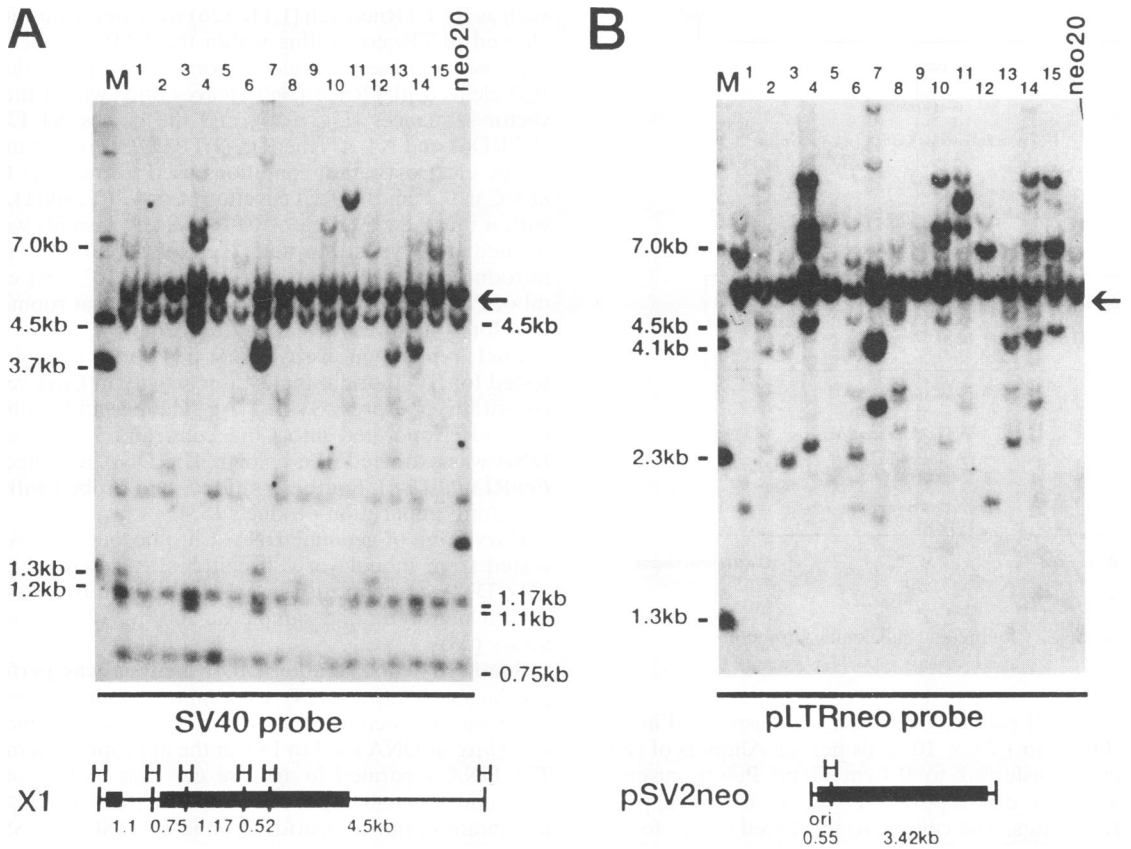

FIG. 4. X1 pSV2neo20 subclone analysis. (A) Southern blot with 2.5 μg of *Hin*dIII-digested genomic DNA per lane isolated from G-418-resistant neo20 (lane marked neo20) and neo20 subclones grown in the presence of G-418 (numbered lanes). The blot was hybridized with an SV40 probe, which hybridizes to parental X1 SV40 DNA and SV40 sequences in pSV2neo. The X1 SV40 *Hin*dIII restriction map is drawn below the autoradiogram. The sizes of the parental X1 SV40 *Hin*dIII bands are listed to the right of the blot. The *Apa*LI pSV2neo fragment in neo20 is indicated by an arrow. The 1.1-kb *Hin*dIII X1 SV40 band is missing in neo20, and novel SV40 bands are present. DNA linear size standards were run in lane M. (B) Southern blot as in panel A but probed with pLTRneo, which hybridizes to *neo* and pBR322 sequences present in the *Apa*LI pSV2neo fragment (Fig. 1A). The genomic instability of the plasmid DNA in this cell line is illustrated by the novel bands present in most of the subclones compared with the single pSV2neo band (marked by arrow) in neo20. H, *Hin*dIII sites.

In the first experiment, X1 pSV2neo clones were selected in monolayer cultures in the presence of 400 μg of G-418 per ml. Two clones, neo20 and neo17, were then subcloned in agarose with and without G-418 selection. Figure 4 shows a Southern blot of genomic DNA isolated from neo20 (lane labeled neo20) and subclones of neo20 (numbered lanes), digested with *Hin*dIII, and probed with SV40 DNA (Fig. 4A) or pLTRneo (Fig. 4B). Bands which hybridize with the SV40 probe but not with the pLTRneo probe represent the X1 SV40 parental DNA. The X1 SV40 1.1-kb *Hin*dIII fragment was missing and additional SV40 restriction fragments were present in neo20, indicating that the X1 SV40 parental DNA had rearranged. Only one integrated copy of pSV2neo was present (arrows in Fig. 4). Both features of neo20 were confirmed with other blots using different restriction enzymes (data not shown).

Restriction fragment length polymorphisms were present in the *neo* gene in most neo20 subclones cultured with G-418 (Table 1; Fig. 4B shows 15 of these subclones), indicating that rearrangements occurred at the site of pSV2neo integration. Fifteen of the neo20 subclones had rearrangements in the parental X1 SV40 DNA (Table 1; Fig. 4A [10 subclones are not shown]). In some of the subclones, rearrangements were observed in the X1 SV40 DNA but not in the pSV2neo DNA

TABLE 1. Summary of rearrangements in X1 clones

X1 clone	Rearrangements[a]			
	No selection		G-418 selection	
	X1 SV40	*neo*	X1 SV40	*neo*
pSV2neo20 (neo20)	20/21[b]	18/21[b]	15/25[b]	20/25[b]
pSV2neo17 (neo17)	4/15[b]	4/15[b]	6/13[b]	8/13[b]
pSV2neoA1 (neoA1)	14/14	2/14		
pSV2neoA5 (neoA5)	11/12	1/12		
pSV2neoB5 (neoB5)	5/10	4/10		
LTRneoA26 (LTRA26)	5/10[b]	0/10[b]	1/9[b]	0/9[b]
LTRneoD6 (LTRD6)	6/9	0/9		
LTRneoD8 (LTRD8)	6/9	0/9		
pSV2oriΔ6B4 (oriΔ6B4)	7/14	0/13		
pSV2oriΔ6C1 (oriΔ6C1)	1/10	0/10		
pSV2oriΔ6C3 (oriΔ6C3)	12/14	0/14		
pSV2oriΔ11 (oriΔ11A1)	3/13	0/13		

[a] Number of subclones with rearrangements/total number of subclones.
[b] Subcloned in agar.

(Fig. 4A, lane 9), while in other subclones, rearrangements were observed at the site of pSV2neo integration but not in the X1 SV40 DNA (not shown).

The stability of the integrated reporter plasmid in the absence of selection was determined by subcloning neo20 without G-418. As summarized in Table 1, most of the neo20 subclones isolated without drug selection had rearrangements at both the pSV2neo and the X1 SV40 integration sites.

Rearrangements were also observed at the sites of pSV2neo and X1 SV40 integration in agarose subclones of neo17, as summarized in Table 1. Restriction enzyme mapping of the parental X1 SV40 DNA and pSV2neo in neo17 indicated that the parental X1 SV40 DNA was unrearranged but at least two copies of pSV2neo were integrated in tandem (results not shown). Eight of 13 G-418-selected subclones of neo17 showed rearrangements at the site of pSV2neo integration, while 6 of the 13 subclones had rearrangements at the X1 SV40 site of integration. Rearrangements at the pSV2neo and the X1 SV40 DNA sites were also observed in the absence of drug selection (Table 1). The frequency of rearrangements at the X1 SV40 and the pSV2neo integration sites varied from clone to clone. neo20 had a very high rearrangement rate associated with the single, integrated copy of pSV2neo, while only 4 of 15 agarose subclones of neo17 showed rearrangements at the site of integration with two tandem copies of pSV2neo.

In the second experiment, G-418-resistant X1 pSV2neo clones were selected in agarose rather than in monolayer culture, to minimize the number of clones with rearrangements in the parental X1 SV40 DNA (24). Three X1 pSV2neo clones, neoA1, neoA5, and neoB5, were identified that met the criteria of having one integrated copy of pSV2neo and unchanged X1 SV40 DNA. The clones were subcloned as monolayer cultures in the absence of G-418. Rearrangements were observed at the sites of pSV2neo integration and at the X1 SV40 DNA integration site in subclones of neoA1, neoA5, and neoB5, as summarized in Table 1. The fraction of neoA1, neoA5, and neoB5 subclones with rearrangements at the parental X1 SV40 DNA site was significantly higher ($P < 0.001$) than the fraction with rearrangements at the three independent pSV2neo insertion sites.

A number of conclusions can be drawn from the analysis of integrated pSV2neo in X1 cells. First, rearrangements were observed at the site of integration of a reporter plasmid with the entire SV40 control region, including the SV40 origin of replication, early and late promoter sequences, and enhancers. Second, these rearrangements were independent of selection with G-418. Third, the rearrangements were observed independent of the site of chromosomal integration. Finally, the frequency of rearrangement appeared to vary from clone to clone.

A plasmid that does not have SV40 DNA is stable in X1 cells. To demonstrate that the instability was not a common feature of plasmid integration in X1 cells, we analyzed the stability of a transfected plasmid entirely lacking SV40 sequences. Three X1 clones transfected with pLTRneo, clones LTRA26, LTRD8, and LTRD6, were isolated in agarose cultures and examined for rearrangements at the site of the X1 SV40 DNA and at the pLTRneo integration site. Restriction enzyme mapping indicated that one copy of pLTRneo was integrated at an independent site in each of the three clones and that the parental SV40 DNA was unrearranged (results not shown).

Rearrangements were not observed at the pLTRneo site of integration in nine G-418-resistant LTRA26 subclones isolated in agar, and only one of the nine subclones had a rearrangement at the parental X1 SV40 site (Table 1). Rearrangements were also not observed at the site of pLTRneo integration in 10

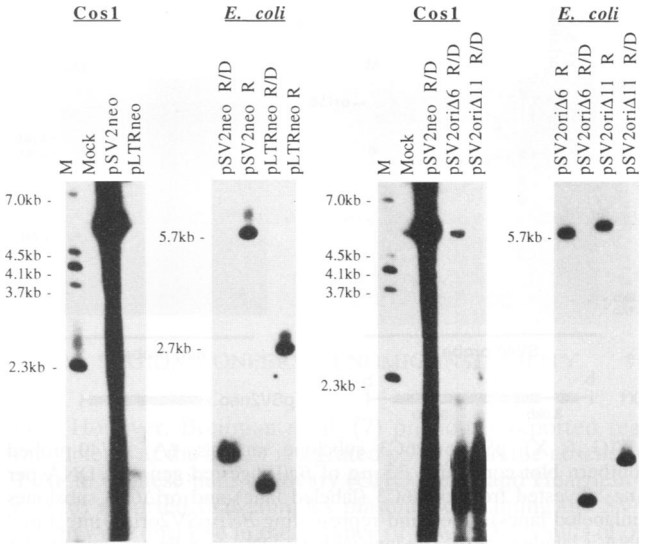

FIG. 5. Cos1 replication assay. Southern blots probed with pBR322 are shown. Lanes in the panels labeled Cos1 contained 4 μg of Cos1 DNA digested with *Eco*RI (R) and *Dpn*I (D). The Cos1 cells had been mock transfected or transfected with pSV2neo, pLTRneo, or the pSV2oriΔ plasmids, as indicated above the lanes. Lanes in the panels labeled *E. coli* contained 200 ng of plasmid DNA isolated from *E. coli* and digested with *Dpn*I (D) and/or *Eco*RI, as indicated.

LTRA26 subclones isolated in the absence of G-418 (Table 1); however, 5 of the 10 subclones had rearrangements at the SV40 integration site.

LTRD6 and LTRD8 subclones were isolated as monolayer cultures in the absence of G-418 selection. Rearrangements were not observed at the pLTRneo integration site in nine subclones of LTRD6 and in nine subclones of LTRD8. Rearrangements were observed, however, at the site of X1 SV40 DNA integration in six of the nine subclones of both LTRD6 and LTRD8, as reported in Table 1. The results of analysis of the pLTRneo clones indicate that integration of plasmid DNA in X1 cells is not sufficient to confer genomic instability at the site of integration.

Plasmids with small deletions in the core origin are stable in X1 cells. To determine the relationship between the rearrangements observed at the site of pSV2neo integration in X1 cells and the presence of the SV40 origin of replication in pSV2neo, we made small deletions in the SV40 replication origin. Two deletion plasmids, pSV2oriΔ6 and pSV2oriΔ11, which have deletions of 6 and 11 bp, respectively, at the *Bgl*I site in T Ag binding site II (Fig. 1C and D), were tested for replication activity in Cos1 cells (Fig. 5). Plasmid DNA isolated from *E. coli* shows the typical *dam* methylation pattern of sensitivity to *Dpn*I digestion (Fig. 5, panels labeled *E. coli*). This pattern would be retained if the plasmid DNA did not replicate in Cos1 cells. However, plasmid DNA replicated in Cos1 cells is insensitive to *Dpn*I digestion. All Cos1 DNA samples were digested with both *Eco*RI and *Dpn*I so that replicated molecules, which are not cut by *Dpn*I, would be linearized. The *Eco*RI plasmid DNA bands are shown in Fig. 5 in the *E. coli* lanes marked R.

A strong replication signal was observed with pSV2neo in Cos1 cells (Fig. 5). No replication was detected with pLTRneo, which does not contain the SV40 origin of replication. A faint band corresponding to the pSV2oriΔ6 *Eco*RI band predicted for the replicated plasmid DNA was detected in Cos1 cells

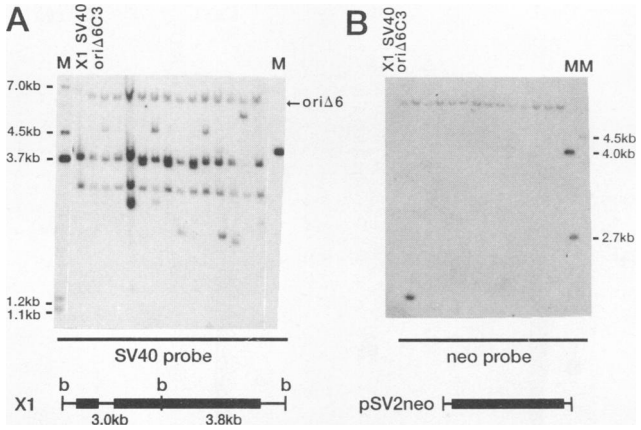

FIG. 6. X1 pSV2oriΔ6C3 subclone analysis. (A) SV40-probed Southern blot containing 2.5 μg of *Bgl*I-digested genomic DNA per lane harvested from oriΔ6C3 (labeled lane) and oriΔ6C3 subclones (unlabeled lanes). The band representing *Bgl*I pSV2oriΔ6 integrated DNA is labeled oriΔ6. The lane with X1 DNA is indicated, and the X1 SV40 *Bgl*I restriction map is drawn below. DNA linear size standards were run in the lanes labeled M. b, *Bgl*I sites. (B) Blot as in panel A but probed with a *neo*-specific PCR fragment that hybridized only to pSV2oriΔ6 bands. A single pSV2oriΔ6 band is observed in the oriΔ6C3 lane and in all the subclone lanes. The band observed at the bottom of the first subclone lane is an artifact not observed in other blots.

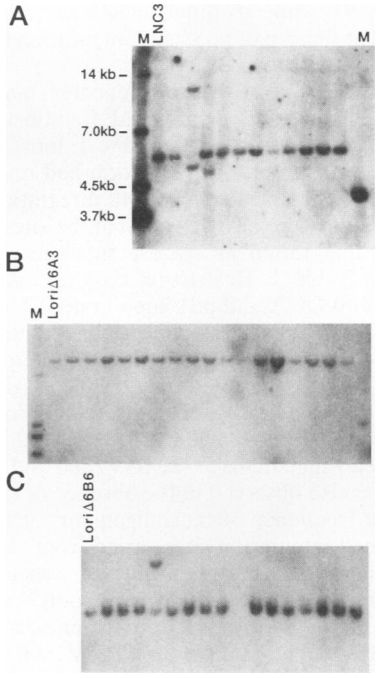

FIG. 7. LMtk⁻ pSV2neo and LMtk⁻ pSV2oriΔ6 subclone analysis. (A) Southern blot containing 2.5 μg of *Eco*RI-digested genomic DNA per lane, harvested from LNC3 and LNC3 subclones (unlabeled lanes). The blot was hybridized with nick-translated SV40, which hybridizes to the SV40 DNA flanking both sides of the Tn*5 neo* gene. The lane labeled M contained linear size standards. (B) Southern blot containing 2.5 μg of *Hin*dIII-*Bgl*I-digested genomic DNA per lane, harvested from LoriΔ6A3 and LoriΔ6A3 subclones. The blot was hybridized with nick-translated pSV2neo. (C) Southern blot containing 2.5 μg of *Eco*RI-digested genomic DNA per lane, harvested from LoriΔ6B6 and LoriΔ6B6 subclones. The blot was hybridized with nick-translated pSV2neo. The subclone DNA in one lane was lost, but another blot demonstrated that the plasmid DNA in this subclone had not rearranged.

(Fig. 5). This replicated band, however, was barely detectable compared with pSV2neo replication in Cos1 cells. Replication products were not detected in genomic DNA isolated from Cos1 cells transfected with pSV2oriΔ11 (Fig. 5), indicating that the deletion disrupted the replication function of the origin.

The *Apa*LI fragment of pSV2oriΔ6 or pSV2oriΔ11 was introduced into X1 cells by electroporation, and G-418-resistant clones were isolated in agarose. Four clones meeting the criteria of parental X1 SV40 DNA and one integrated copy of the reporter plasmid were subcloned in monolayer cultures in the absence of selection. Restriction enzyme mapping during the analysis for single-copy integration of pSV2oriΔ indicated that the plasmid DNA was integrated at a different chromosomal location in each clone.

Analysis of three pSV2oriΔ6 clones and one pSVoriΔ11 clone, oriΔ11A1, revealed that integrated plasmid DNA with small deletions in the SV40 core origin remained stable in X1 cells, whereas rearrangements still occurred at the site of the parental X1 SV40 DNA (Table 1). An example of restriction analysis of 14 subclones of oriΔ6C3 is shown in Fig. 6. Genomic DNA digested with *Bgl*I, which does not cleave within the pSV2oriΔ6 *Apa*LI fragment, gave an identical band for oriΔ6C3 and all of its subclones when hybridized with a *neo*-specific probe (Fig. 6B). The same invariant band was observed with the SV40 probe (Fig. 6A), although 12 of the subclones showed a new pattern of SV40 bands representing rearrangements that occurred at the X1 SV40 DNA site (Fig. 6A and Table 1). Similar results were obtained with oriΔ6B4 and oriΔ6C1 and with oriΔ11A1 (Table 1). These results suggest that specific sequences present within the SV40 origin of replication affect genomic stability.

T Ag is not required for induction of rearrangements at the site of pSV2neo integration in LMtk⁻ cells. It has been difficult to determine the role of T Ag in generating rearrangements in integrated SV40 DNA because the phenomenon was found in SV40-transformed rodent cells that continuously express T Ag (4, 24, 27, 28, 42, 43, 57, 64). Investigation of the genomic instability of pSV2neo integrated into a cell line lacking the T Ag gene presented an opportunity to directly test the role of T Ag.

The *Apa*LI fragment of pSV2neo was introduced by electroporation into mouse LMtk⁻ cells, a cell line completely lacking SV40 DNA. G-418-selected clones were analyzed for single-copy integration of pSV2neo by restriction mapping. Five LMtk⁻ pSV2neo clones with one integrated copy of pSV2neo were chosen for further analysis. Restriction enzyme mapping indicated that pSV2neo was integrated at a different site in each of the five clones (results not shown). The LMtk⁻ pSV2neo clones were subcloned in monolayer cultures in the absence of G-418. DNA harvested from the subclones was digested with restriction enzymes, Southern blotted, and hybridized with SV40- or *neo*-specific probes.

Rearrangements were observed at the pSV2neo sites in all five LMtk⁻ clones despite the absence of T Ag. A Southern blot of *Eco*RI-cleaved genomic DNA from LNC3 and from 11 subclones of LNC3 hybridized with SV40 DNA is shown in Fig. 7A. Two of the 11 subclones have new bands. The results were identical when the blot was hybridized with a *neo*-specific probe instead of SV40 (data not shown), confirming that the bands were derived from the *Apa*LI pSV2neo fragment. Bands were not observed with genomic DNA from LMtk⁻ cells hybridized with either probe (data not shown). Rearrange-

TABLE 2. Summary of rearrangements in LMtk$^-$ clones

LMtk$^-$ clone	*neo* rearrangements[a] (no selection)
pSV2neoA3 (LNA3)	3/11
pSV2neoA6 (LNA6)	6/17
pSV2neoB6 (LNB6)	6/17
pSV2neoC1 (LNC1)	4/13
pSV2neoC3 (LNC3)	2/11
pSV2oriΔ6A3 (LoriΔ6A3)	0/16
pSV2oriΔ6B6 (LoriΔ6B6)	1/16
pSV2oriΔ6C5 (LoriΔ6C5)	3/15
pSV2oriΔ11A4 (LoriΔ11A4)	0/13

[a] Number of subclones with plasmid rearrangements/total number of subclones.

ments were observed at the sites of pSV2neo integration in LNA3, LNA6, LNB6, and LNC1 (Table 2). Each set of subclones was analyzed by using at least two restriction enzymes to verify that new bands were due to rearrangement and not to incomplete restriction enzyme digestion (data not shown).

The genomic instability observed at the site of the integration of a reporter plasmid containing the SV40 origin of replication did not require T Ag in LMtk$^-$ cells. These results indicate that the rearrangements must be induced by the DNA alone or by some factor present in LMtk$^-$ cells.

Deletions in the SV40 core origin increase the genomic stability of integrated plasmid DNA in LMtk$^-$ cells. To determine whether the rearrangements were dependent on a functional origin of replication, pSV2oriΔ6 and pSV2oriΔ11 were introduced into LMtk$^-$ cells by electroporation, and G-418-resistant colonies were selected and analyzed for single-site insertions. Four clones, LoriΔ6A3, LoriΔ6B6, LoriΔ6C5, and LoriΔ11A4, with a single pSV2oriΔ plasmid integrated at a different site were chosen for subcloning.

Rearrangements were not observed in any of the 16 subclones of LoriΔ6A3 (Fig. 7B) or in any of the 13 subclones of LoriΔ11A4 (Table 2). Rearrangements were observed, however, in 3 of the 15 subclones of LoriΔ6C5 (Table 2) and in 1 of the 16 subclones of LoriΔ6B6 (Fig. 7C). Each set of subclones was analyzed by using at least two restriction enzymes to verify that the new bands were due to rearrangement and not to incomplete restriction enzyme digestion (data not shown). A contingency table analysis indicated that the frequency of pSV2oriΔ subclones with rearrangements in LMtk$^-$ cells was significantly lower than that of pSV2neo subclones with rearrangements ($P < 0.01$). Small deletions within the core origin of replication that diminished replication activity increased the genomic stability of the integrated plasmid DNA; however, the deletions did not totally inhibit rearrangements. These results, combined with the results presented above for the X1 cells, indicate that the wild-type SV40 origin region induced genomic instability at the site of chromosomal integration. Small deletions in the core origin disrupted this function.

DISCUSSION

The instability of integrated SV40 DNA in nonpermissive cells has been observed in many different cell lines (4, 24, 27, 28, 42, 43, 57, 64). In each of these studies, SV40 T Ag was expressed in the cells and the predominant models invoked the replication functions of T Ag and the SV40 origin of replication. However, Breitman et al. (7) previously reported rearrangements at the site of integrated pSV2gpt in the absence of T Ag in Chinese hamster ovary (CHO) cells, and Heartlein et al. (26) reported that complex plasmids containing the SV40 region were unstable in the absence of T Ag. The results of our study prove that the SV40 control region is required for the observed genomic instability but that T Ag is not required.

Genomic instability is correlated with the presence of the SV40 origin of replication in X1 cells. To analyze the role of the SV40 origin of replication in the genomic instability of integrated SV40 DNA in nonpermissive cell lines, pSV2neo was integrated into the SV40-transformed cell line X1. In the five independent pSV2neo clones analyzed, rearrangements were observed at each site of plasmid integration. Rearrangements were not observed at three independent pLTRneo integration sites in X1 cells, indicating that integration of foreign DNA into the cellular genome is not sufficient for generating rearrangements. Small deletions within the SV40 core origin in pSV2neo decreased the rearrangement frequency, indicating that sequences present in the *Pvu*II-to-*Hin*dIII SV40 origin fragment of pSV2neo are the maximum sequences required to confer genomic instability to the integrated DNA.

The instability of the X1 SV40 DNA was maintained in the X1 pSV2neo clones, X1 pSV2oriΔ clones, and X1 pLTRneo clones, indicating that independent events were occurring at the two separate integration sites. The chromosomal site of the X1 SV40 DNA, in the absence of integrated viral DNA, is stable (2); therefore, the SV40 DNA is not integrated at a preexisting chromosomal recombination hot spot. In addition, seven cellular genes were stable in X1 cells, indicating that the instability associated with the site of SV40 DNA integration is not due to a high rearrangement rate throughout the genome (24).

T Ag is not essential for the genomic instability of integrated SV40 DNA. The presence of the SV40 control region, including the origin, enhancer, and promoter sequences, was sufficient to cause genomic instability at the site of integration of pSV2neo in five independent LMtk$^-$ clones in the absence of T Ag. There is no statistical difference ($P > 0.05$) between the fraction of LMtk$^-$ pSV2neo subclones with rearrangements at the *neo* locus in the absence of T Ag and the total fraction of X1 pSV2neo subclones with rearrangements at the *neo* locus in the presence of T Ag, indicating that the presence of T Ag does not increase the genomic instability of pSV2neo.

Small deletions in the SV40 origin of replication decreased the rearrangement frequency in LMtk$^-$ cells. Rearrangements were observed with pSV2oriΔ plasmids at a significantly lower frequency ($P < 0.01$) than observed with pSV2neo. Rearrangements at the site of integration of foreign DNA containing mutations in the SV40 core origin that disrupt the viral replication activity have been previously reported (13, 45–47). The integration of foreign DNA into LMtk$^-$ cells, however, is not sufficient to induce rearrangements (38, 69).

The increased fraction of LMtk$^-$ pSV2oriΔ subclones with rearrangements at the *neo* locus compared with the X1 pSV2oriΔ subclones may be a reflection of a more permissive state for recombination in LMtk$^-$ cells. Gene amplification rates vary among different cells lines (54), and a similar variation in permissiveness for DNA rearrangements may exist. Although the possibility that integration occurred in recombination hot spots cannot be excluded for any of the reporter plasmids that exhibited rearrangements in this study, it is unlikely that pSV2neo integrates into hot spots more frequently than either pSV2oriΔ or pLTRneo.

Flanking DNA may influence the frequency of rearrange-

ments. The fraction of subclones with rearrangements at the site of the parental X1 SV40 DNA varied between clones from 100% of the subclones with rearrangements (neoA1) to only 10% of the subclones with rearrangements (oriΔ6C1), although the number of subclones examined (14 and 10, respectively, in the examples cited) was too small to establish the statistical significance of the variation. Three factors might affect the observed frequency of rearrangement. First, the number of subclones with rearrangements was determined conservatively because faint minor bands were excluded. Second, duplications in the mouse DNA and part of the SV40 early region, mouse d(TG) repeats that are potential Z-DNA tracts, and other repetitive mouse DNA sequences flank the SV40 integration site in X1 cells (2, 24) and may contribute to the different recombination rates. Small changes in these regions might not have been detected by the restriction enzyme analysis used in this study. Third, the cellular environments of the clones may not be identical. Gross chromosomal aberrations, including chromatid breaks, dicentric chromosomes, and translocations, which are frequently observed in transformed cell lines (34, 54, 56, 65), may exist and might affect the mechanisms involved in generating the rearrangements.

The fraction of X1 pSV2neo subclones with rearrangements at the X1 SV40 site was statistically higher ($P < 0.001$) then the fraction of subclones with rearrangements at the site of pSV2neo integration. The SV40 origin of replication and auxiliary sequences are present in both the parental X1 SV40 DNA and pSV2neo, and we assume that both origins are exposed to the same T Ag population in X1 cells. However, the different DNA sequences flanking the integration sites may influence the frequency of rearrangements (9, 10, 35, 39, 40, 47, 49, 52, 55, 72, 74, 76, 77, 78) observed at the X1 SV40 site and the pSV2neo sites. The pSV2neo integration sites have not been characterized.

Models to explain the observed instability associated with the SV40 origin. Many of the models proposed to explain rearrangements associated with integrated SV40 DNA have been based on the viral DNA replication functions of T Ag and the SV40 origin of replication (15, 24, 28, 33). The permissiveness of cells to support viral DNA replication is associated with the ability of the polymerase α/primase to interact directly with T Ag (16, 17, 44). In cells that are nonpermissive for viral replication, T Ag-based replication models require, at some level, that this inhibition of viral replication be circumvented. Previous reports (7, 26–28, 66) and our results reported here indicate that a T Ag-based replication model is not required to explain the genomic instability of integrated SV40 DNA in cell lines nonpermissive for viral replication. However, integration of the SV40 origin of replication into chromatin may create or unmask a cryptic function of the SV40 origin, independent of T Ag, that could activate recombination or repair enzymes, resulting in the observed rearrangements. The activation of a cryptic eukaryotic replication origin by a chromosomal rearrangement has been reported (37).

Different models based on DNA structure rather than origin activity may also explain the instability associated with the SV40 origin region. Secondary structures can arrest DNA replication, leading to genomic instability (1, 14; reviewed in reference 61). The rearrangements, therefore, may be induced by DNA secondary structures present in the SV40 core origin and auxiliary regions, including nuclease-hypersensitive sites present in the 21-bp repeats, the 72-bp enhancers (20, 31, 32), and the major late promoter (81, 82). Furthermore, the SV40 DNA control region is rich in palindromic sequences with the potential of forming cruciforms (5, 51, 67), which have been implicated as recombination hot spots (30, 36, 75, 79, 80). Cruciform structures in SV40 DNA have not been detected in vivo (80); however, integration of the SV40 control region into chromatin may induce or allow cruciform formation. Sequences with the potential of forming Z-DNA, which are also implicated in recombination (8, 70, 71, 73, 74), are present in the SV40 early promoter (50).

Maintaining the palindrome at T Ag binding site II may be important for permitting DNA rearrangements. Rearrangements were observed at the site of integration of an origin-defective plasmid with a deletion that disrupts the perfect palindrome in T Ag binding site II (45) and at the site of integration of a replication-defective construct with a 4-bp insertion (and a 1-bp transition) in T Ag binding site II that only partially disrupted the palindrome (12, 13). We also observed rearrangements at the site of integration of pSV2oriΔ6 and pSV2oriΔ11 in LMtk$^-$ cells. Our results, however, indicate that the presence of small deletions in T Ag binding site II in pSV2oriΔ6 and pSV2oriΔ11 decreased the frequency of rearrangement. The permissiveness of the cell line for DNA rearrangements, in conjunction with residual secondary structure, may explain the rearrangements observed at the pSV2oriΔ6 sites in LMtk$^-$ cells. Alternatively, the expression of T Ag in X1 cells may interfere with recombination in the X1 pSV2oriΔ clones by blocking hot spots for recombination (73). The roles of the early palindrome and other secondary structures associated with the SV40 control region were not investigated in this study.

Transcription has been shown to increase the accessibility of DNA to recombination machinery or nuclease attack (41, 48, 68). In pSV2neo, pSV2oriΔ6, and pSV2oriΔ11, *neo* expression is under the control of the SV40 early promoter. It is not known what effect deletions in the core origin have on the secondary chromatin structure of pSV2oriΔ6 and pSV2oriΔ11 during transcription. At this time, we cannot distinguish between possible contributions of the transcription components (SV40 early promoter and enhancers) and the replication components (SV40 core origin and auxiliary regions) in inducing the rearrangements.

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