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Territorial limits and functional anatomy of the simian virus 40 replication origin

(palindromes and A+T-rich region/21- and 72-base pair repeats/sequential deletions/replication efficiency in COS cells/core and auxiliary regions)

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ABSTRACT **The region at and near the simian virus 40 (SV40) DNA replication origin contains a series of palindromes, a 17-base pair (bp) A+T-rich sequence, three copies of a 21-bp repeat, and two copies of a 72-bp repeat. We have constructed a series of recombinant plasmids containing sequential deletions at the region of SV40 DNA replication origin starting from the end near the repeats. These deletions were introduced by using *in vitro* and *in vivo* techniques. The relative replication efficiency of these recombinant plasmids were directly assayed in COS-1 monkey kidney cells capable of providing the tumor antigen necessary for the replication of these molecules. Recombinants lacking both copies of the 72-bp repeat did not exhibit any reduction in replication efficiency. Recombinants lacking the 21-bp repeats showed decreased replication efficiency; the reduction in replication efficiency was proportional to the number of copies of the 21-bp repeat deleted in these recombinants. A recombinant retaining the palindromes at the region of origin of SV40 DNA replication but lacking the A+T-rich sequence and the repeats failed to replicate. Based on these results, the SV40 DNA replication origin is subdivided into two regions, and their boundaries are defined. One of these two regions is a core region containing the 17-bp, 15-bp, and 27-bp palindromes and, quite likely, the 17-bp A+T-rich sequence which are necessary for replication. The other is an auxiliary region that consists of the 21-bp repeats and has a dose-dependent enhancement effect on replication efficiency.**

The region of simian virus 40 (SV40) DNA located between the start of the early coding region at nucleotide 5091 (map position 0.649) and that of the late genes at nucleotide 191 (map position 0.712) is not known to code for any viral protein. [The SV40 nucleotide numbering system used in this report is that of Reddy *et al.* (1) modified by Piatek *et al.* (2).] This region includes the viral replication origin, sequences corresponding to the 5′ ends of the early and late viral mRNAs, putative transcription initiation signals, and possibly signals controlling the balance between early and late transcription and other yet unknown viral control elements. The nucleotide sequence of this region, determined originally by Subramanian *et al.* (3, 4), exhibits some unusual features. Notable among these, in going from nucleotide 5110 to 168, are a 17-base pair (bp) true palindrome; a 15-bp palindrome; a 27-bp palindrome possessing a perfect 2-fold rotational symmetry; a 17-bp A+T-rich sequence; three copies of a 21-bp repeat, two of which are arranged in tandem; and two copies of a 72-bp repeat arranged in tandem. Assignment of these sequences to the appropriate viral control elements would help to understand the way in which the virus controls its functions *in vivo* and has been the subject of intensive investigation in a number of laboratories.

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The only virus-coded protein identified so far that has regulatory functions is one of the early proteins, the large tumor (T) antigen (referred to here as simply T antigen). T antigen is required for the initiation of each round of viral DNA replication, autoregulates its own synthesis, and may be required for the expression of the late viral genes (reviewed in ref. 5). Mutants defective in the T-antigen coding region replicate their DNA when provided with the early functions by a helper virus. Three T-antigen binding sites have been identified in the region of SV40 DNA between nucleotides 5110 and 22 (6, 7). The direct interaction between T antigen and SV40 DNA in this region is required for viral DNA replication (8).

Studies of viral mutants with either deletions (9, 10) or base-change mutations (8) within the 27-bp perfect palindrome have shown that this sequence forms an integral part of the replication origin. To the left of the 27-bp perfect palindrome, towards the early region of SV40, are located a 15-bp palindrome and a 17-bp true palindrome. The 17-bp true palindrome, or part of it, forms the left extreme of the replication origin (11, 12). To the right of the 27-bp perfect palindrome, towards the late region of SV40, are located the A+T-rich region, followed by the 21-bp and 72-bp repeats. Despite the isolation of deletion mutants lacking portions of the 21-bp repeats (12–14) or the 72-bp repeats (refs. 15 and 16; unpublished data), it still remains uncertain as to what role these repeated sequences play concerning replication.

The object of our investigation was to determine the boundary of the right extreme of the DNA replication origin, specifically to examine whether the 21- and 72-bp repeats and the A+T-rich sequence form part of the replication origin. We cloned in a plasmid vector segments of the region of origin of SV40 DNA replication containing stepwise deletions of the repeats and the A+T-rich sequence. We directly assayed the ability of these recombinant molecules to replicate in COS monkey kidney cells (17) that produce the SV40 T antigen constitutively. The results of these experiments have enabled us to define the territorial limits of the SV40 replication origin and its functional anatomy.

MATERIALS AND METHODS

Materials. COS-1 cells were kindly provided by Y. Gluzman (Cold Spring Harbor Laboratory); SV40 mutant *cs* 1096 cloned in pBR322, by R. Dixon and D. Nathans (The Johns Hopkins University); SV40 mutants *dl* 1409 and *dl* 1449, by T. Shenk (State University of New York at Stony Brook); and plasmid pMK2004 and T4 DNA ligase, by P. Matsumura (University of Illinois, Chicago).

Abbreviations: SV40, simian virus 40; bp, base pair(s); T antigen, tumor antigen.

Restriction endonucleases were bought from New England BioLabs; *Escherichia coli* DNA polymerase I and calf intestine alkaline phosphatase, Boehringer Mannheim; T4 DNA polymerase and T4 polynucleotide kinase, P-L Biochemicals; *Bam*HI linkers, Collaborative Research (Waltham, MA) and radioactive materials, Amersham.

Preparation of Plasmid Recombinants Containing Stepwise Deletions in the Region of Replication Origin in SV40 DNA. All experiments involving recombinant DNA were conducted under P2-EK1 conditions as described in the National Institutes of Health guidelines.

Three of the recombinants (shown in Fig. 1*A*) were made as follows. SV40 mutant *in*1449 that has a *Bgl* II site instead of the *Hin*dIII site at nucleotide 5091 (ref. 18) was cut with *Bgl* II and *Pvu* II, generating a fragment mapping between nucleotides 5091 and 191. By making use of the complementary nature of the *Bam*HI and *Bgl* II cohesive termini, this SV40 fragment was ligated with the *Bam*HI-cut linear DNA of plasmid pMK2004 (ref. 19). Competent *E. coli* C600 were transformed with the ligated products by known procedures (20). Tetracycline-sensitive colonies were picked and probed by hybridization (21) with nick-translated (22) SV40 DNA. Positive clones were analyzed by restriction mapping (23) and DNA sequence determination (24). Recombinants with suitable SV40 inserts were chosen for the replication assay.

The remaining two recombinants (Fig. 1 *B* and *C*) were made as follows. The *Bst*NI–G′ fragment of *dl* 1409 DNA and a *Hin*dII–*Hin*dIII fragment of *cs* 1096 DNA (both spanning the *Bgl* I site at nucleotide 5161) were isolated, and their single-stranded ends were filled with *E. coli* DNA polymerase I or T4 DNA polymerase (25). *Bam*HI linkers were added to the respective fragment, which was cloned at the *Bam*HI site of pMK2004. The recombinant plasmids were tested to ascertain that the inserted SV40 DNA fragment was recovered in full upon *Bam*HI digestion of the respective plasmid.

Determination of the Efficiency of Replication of the Recombinant Plasmids. COS-1 cells (17) grown to about 40% confluence in 60-mm plastic Petri dishes were transfected with 100 ng each of the form I recombinant plasmid DNAs in the presence of 500 μg of DEAE-dextran per ml (26). After 25 min at room temperature, the cells were fed with medium containing 2% (vol/vol) serum and were incubated at 37°C in 5% CO_2/95% air for 48 hr. The cells were harvested and extracted by the procedure of Hirt (27). The low molecular weight plasmid DNAs in the Hirt supernatants were deproteinized by extractions—two phenol and one chloroform—and were precipitated with ethanol. The DNA preparations were fractionated by 0.8% agarose gel electrophoresis, blotted onto nitrocellulose filters by the procedure of Southern (28), and hybridized with nick-translated pMK2004 DNA as the probe. The filters were washed, dried, and autoradiographed (28). Either SV40 or pMK2004 DNAs (100 ng per dish) was used as the positive or negative control, respectively, in this experiment.

RESULTS

Construction of Plasmids Containing Portions of the Region of Origin of SV40 DNA Replication. The plasmid clones isolated in this study were termed the pSVori recombinants be-

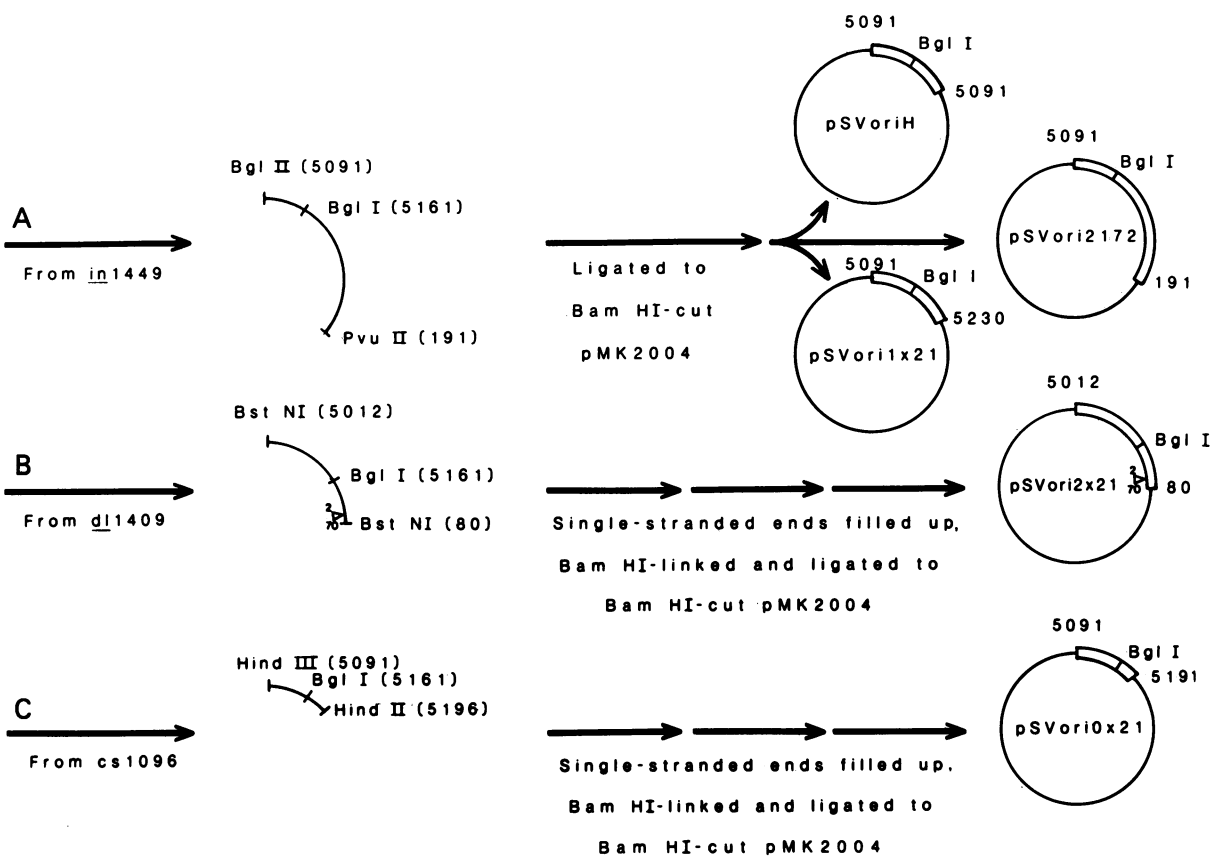

FIG. 1. Strategies used for the generation of stepwise deletions in the region of origin of SV40 DNA replication. The curved lines represent the SV40 DNA fragments spanning the region of replication origin derived from the respective SV40 mutant DNAs (*in* 1449, *dl* 1409, and *cs* 1096) indicated. The ligations to *Bam*HI-cut plasmid pMK2004 DNA were done *in vitro*, and cyclizations took place *in vivo* within *E. coli*. The portions of the region of SV40 DNA replication origin present in the starting fragments and the final cyclized products are indicated by the SV40 nucleotide numbers.

cause the only SV40 sequences carried by them were derived from the region of origin of SV40 DNA replication. All of the recombinants developed for this study are illustrated in Fig. 1. The stepwise nature of the deletions within the region of SV40 DNA replication are pictured in Fig. 2. The extent of SV40 sequences found in each recombinant was determined by restriction mapping (23) and DNA sequence determination (24).

Recombinant pSVori 2172 is a product of the cloning of *Bgl* II–*Pvu* II fragment of the SV40 mutant *in* 1449 (Fig. 1*A*). The *Bgl* II end (nucleotide 5091) of this fragment is connected to one of the *Bam*HI ends of linear pMK2004. The flush *Pvu* II end (nucleotide number 191) of this fragment was found, by restriction and sequence determination studies, to have fused end-to-end with the other *Bam*HI end of the plasmid *in vivo*, with the result that the *Bam*HI site in this location was regenerated. This recombinant contains all of the sequences at the region of origin of SV40 DNA replication, including all copies of the repeats (Fig. 2).

dl 1409 is a viable SV40 mutant received from Shenk (29) that essentially lacks one 21-bp and one 72-bp repeat (unpublished data). Plasmid pSVori2×21 was produced by cloning the *Bst*NI G′ fragment of *dl* 1409 (Fig. 1*B*). This recombinant contains, in addition to the palindromes and the A+T-rich sequence, two of the three copies of the 21-bp repeat. Both copies of the 72-bp repeat (but for a stretch of 9 nucleotides remaining in one of them) are deleted in this clone (Fig. 2).

Recombinant pSVori1×21, a product of the cloning of the *Bgl* II–*Pvu* II fragment of *in*1449 (Fig. 1*A*) contains the palindromes, the A+T-rich sequence, and only one of the three copies of the 21-bp repeat; the rest of the repeats, including both copies of the 72-bp repeat, are deleted (Fig. 1). This deletion occurred, presumably, during the *E. coli* mediated cyclization of the linear DNA product of the *in vitro* ligation shown in Fig. 1*A*.

SV40 mutant *cs* 1096, isolated by DiMaio and Nathans (12), contains a base-change mutation giving rise to a new *Hin*dII site at nucleotide 5196. Plasmid pSVori0×21, produced by cloning of a *Hin*dII–*Hin*dIII fragment of SV40 mutant *cs* 1096 (Fig. 1*C*), contains the palindromes and 16 out of 17 bp of the A+T-rich sequence but none of the repeats (Fig. 2).

Plasmids pSVoriH (H for hairpin) is a product of the cloning of the *Bgl* II–*Pvu* II fragment used in this study. The SV40 insert in this recombinant is found, by restriction and sequence determination studies, to be an inverted repeat of the sequences from the *Bgl* II site (nucleotide 5091) to the *Bgl* I site (nucleotide 5161) of *in*1449 DNA. This recombinant arose by ligation *in vitro* of one SV40 fragment to each end of the linear plasmid by way of *Bgl* II◄---►*Bam*HI joining, followed by the cyclization of the DNA by recombination at the midpoint of the 27-bp perfect palindrome *in vivo*, resulting in the deletion of the SV40 sequences from this point up to the *Pvu* II end of both copies of the SV40 fragment (unpublished data). pSVoriH contains two copies each of the 17-bp true palindrome and 15-bp palindrome occurring as inverted repeats, with one copy of the 27-bp perfect palindrome located in the middle. It lacks the 17-bp A+T-rich sequence and all copies of the 21- and 72-bp repeats (Fig. 2).

Relative Efficiency of Replication *in Vivo* of the pSVori Recombinants. The replication of the pSVori recombinants was studied using COS-1 cells, a line of monkey kidney cells transformed by origin-defective SV40 DNA (17). Equivalent amounts of the recombinants were used in the transfections.

Myers and Tjian (11) have done a time course of the transfection of COS cells with a form I recombinant plasmid containing the SV40 replication origin. At zero time, forms I (supercoiled), II (relaxed circular), and III (linear) DNAs are all observed. At 12 hr after infection, only forms II and III persist. At 50 hr after transfection, a marked increase in the amount of supercoiled form I DNA is observed relative to form I levels seen at time zero and 12 hr after transfection. Thus, the presence of form I DNA at late times (≈50 hr) after transfection is due to newly synthesized DNA and is a clear indication of the ability of the DNA to replicate.

The results of the Southern blot hybridization experiments on the replication efficiency of the pSVori recombinants used in this study are shown in Figs. 3 and 4. Except for pSVoriH, the other recombinants were found to replicate to various degrees. pSVori2172, which contains all of the pertinent se-

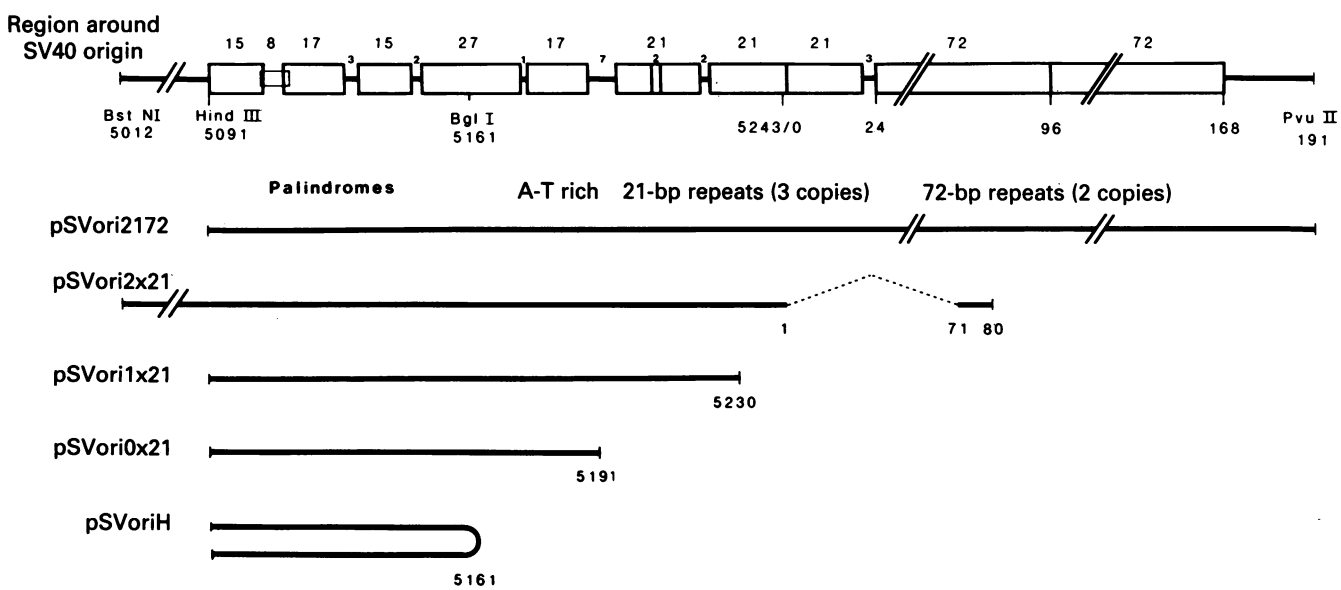

FIG. 2. The structural features of the region of origin of SV40 DNA replication (shown as boxes) and the portions of this region contained in the five pSVori recombinants (shown as bold horizontal lines below the boxes). The sequential nature of the deletions can be seen. The numbers above the boxes show the sizes in nucleotides of the respective sequences. Representative restriction sites and their nucleotide numbers in the SV40 sequence are provided for orientation.

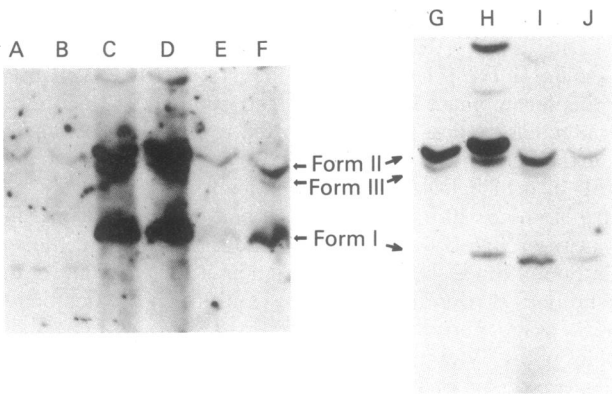

FIG. 3. Autoradiographs of a Southern blot hybridization performed to assay the replication efficiency of the pSVori recombinants in COS-1 cells. Nick-translated parent plasmid pMK2004 DNA was used as the probe. Bands appearing in the form I position indicate newly replicated DNA (11). Lanes: A, pMK2004; B, pSVoriH; C, pSVori2×21; D, pSVori2172; E, pSVori0×21; F, pSVori1×21; G, pSVoriH; H, pSVori2×21; I, pSVori2172; J, pSVori1×21.

quences of the region of SV40 DNA replication origin was found to replicate with ≈1/10th of the efficiency of form I SV40 DNA (data not shown). A similar observation was also made by Myers and Tjian (11). This reduction is due to the inhibition by the plasmid of the SV40 DNA replication origin-dependent replication in monkey cells observed in a number of laboratories (30). However, because of the high sensitivity of the hybridization probe, the efficiency of detection of the replicated DNA is more than adequate.

The replication efficiency of the other plasmids relative to that of pSVori2172 can be seen in Fig. 3. pSVori2×21, which has none of the 72-bp repeats and has two of the three copies of the 21-bp repeat, replicated almost as well as pSVori2172. This result shows that the 72-bp repeats, which are implicated in the promotion of transcription (16, 31), play no role at all in the initiation of DNA replication.

Plasmid pSVori1×21, which contains only one copy of the 21-bp repeat and no 72-bp repeats, replicated about 1/2 as efficiently as did pSVori2×21 and pSVori2172 (Fig. 3). Plasmid

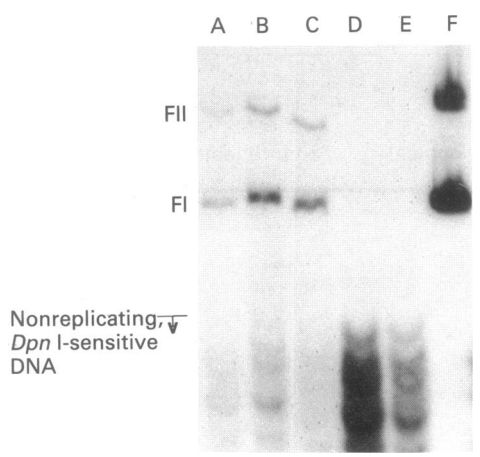

FIG. 4. Autoradiograph of Southern blot hybridization performed to assay the relative replication efficiency of the pSVori recombinants. DNA samples isolated from COS cells 48 hr after transfection were digested with *Dpn* I prior to electrophoretic fractionation and Southern blotting. Bands appearing in the form I and II positions (FI and FII) indicate newly replicated *Dpn* I-resistant DNA. Lanes: A, pSVori0×21; B, pSVori2×21; C, pSVori1×21; D, pSVoriH; E, PMK2004; F, PMK2004 undigested marker.

pSVori0×21, which contains the palindromes and 16 out of 17 bp of the A+T-rich sequence but none of the repeats, registered a further drop in replication efficiency; it replicated about 1/4th to 1/6th as efficiently as did pSVori2172. Plasmid pSVoriH, which contains the palindromes but not the A+T-rich sequence or the repeats, did not replicate at all, fully resembling in its lack of replication the parent plasmid pMK2004 used as a negative control in the experiments. Though the actual ratios varied from one experiment to another, the overall pattern of the relative replication efficiencies described above was observed consistently and reproducibly in five separate experiments with four different sets of DNA preparations.

In the experiment shown in Fig. 4, the Hirt supernatant DNA samples isolated from COS cells after transfection were digested with *Dpn* I before Southern blotting. *Dpn* I cleaves the sequence G-A-T-C only if the A is methylated (32). Because of this property, the input procaryotic-derived DNA, but not the DNA produced by replication within the animal cell, is sensitive to *Dpn* I. pSVoriH and pMK2004 DNA were completely digested by *Dpn* I, showing a lack of replication in COS cells. pSVori2x21, -1x21, and -0x21 gave rise to *Dpn* I-resistant, newly replicated DNA in amounts roughly proportional to the number of copies of the 21-bp repeat that they contained.

DISCUSSION

In SV40 and in other papovaviruses, the control elements for replication and transcription are arranged contiguously and quite likely overlap to some extent. Because T antigen—a product of the early region—is needed for the initiation of DNA replication, it is important to determine whether the deletion of a certain sequence in the replication–origin region affects the DNA replication directly or indirectly through its effect on transcription. The advent of COS cells, which make the viral T antigen constitutively and are capable of supporting the replication of segments of SV40 DNA containing a functional replication origin (17), make this kind of an assay possible. Using this technique, we have directly assayed the effect of stepwise deletions in the SV40 origin region on replication *per se*.

One of the objectives of this study is to define the territorial limits of the SV40 replication origin. Previous efforts in this direction (outlined in the introduction) have been incomplete especially when considered in the light of more recent evidence. Questions remained particularly on the role of the 21- and 72-bp repeats. Our results presented above show categorically that the 72-bp repeat, implicated in the promotion of transcription (16, 31), is not needed for the initiation of DNA replication. This result is consistent with the finding by Gutai and Nathans (33) that a stable evolutionary variant of SV40, *ev* 1104, is capable of replication and does not contain the 72-bp repeats.

Another possible regulatory sequence is the 21-bp repeat that constitutes the third T-antigen binding site (6, 7). The three copies of this repeat contain six copies of the G-C rich octanucleotide G-G-G-C-G-G-R-R. This octanucleotide occurs at or near the DNA replication origins of polyoma virus, BK virus, and a few adenoviruses (reviewed in ref. 34). SV40 evolutionary variant *ev* 1104 contains two SV40 replication origins within its monomer unit. One of these contains two and the other five copies of the G-G-G-C-G-G-R-R sequence (33). It is not known whether the origin, having five copies of this sequence, is more efficient than the one having two copies.

Our results shown in Fig. 3 and 4 indicate an interesting proportionality between the number of copies of the 21-bp repeat present in the DNA and its replication efficiency. The recombinant having two copies of the 21-bp repeat, replicates almost as well as the one having all three copies of the 21-bp

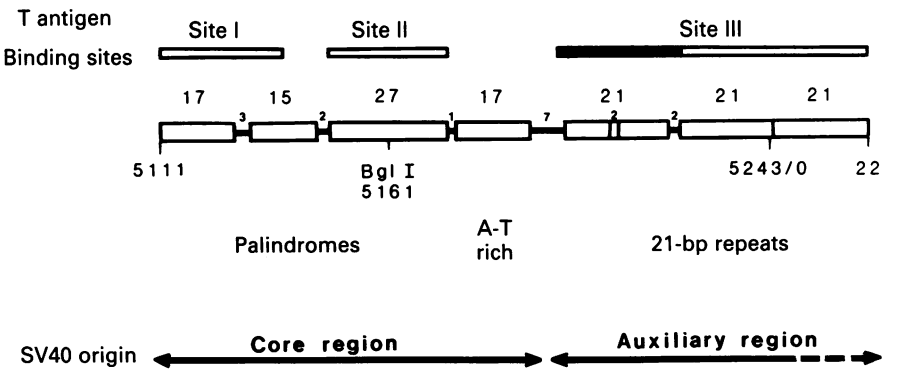

FIG. 5. Definition of the boundaries of the SV40 replication region. Boxes show the features of the sequences contained in the respective subdivision. The numbers below the boxes are SV40 nucleotide numbers. Numbers above the boxes denote sizes in nucleotides. T antigen is the SV40 large T antigen. T-antigen binding sites I and II are positioned as described by Tjian (6) and Shalloway *et al.* (7). In the case of T-antigen binding site III, Shalloway *et al.* reported that T antigen binds essentially to all three of the 21-bp repeats; however, Tjian reported that the T antigen binds to only a portion of those repeats, as denoted by the darkened area.

repeat. The recombinant having one copy of the 21-bp repeat shows a reduction in replication efficiency by a factor of 2. The recombinant devoid of the 21-bp repeat exhibits a further reduction in replication efficiency (by a factor of 4–6) compared to the one having all copies of this sequence. These results show that the 21-bp repeat constituting the third T-antigen binding site, though not an absolute requirement for replication, has an enhancement effect on the replication efficiency. This enhancement appears to be dose-dependent because DNA molecules having two or more copies of this repeat replicate better than those having one, which in turn replicate better than those having none. Preliminary studies on the time course of replication of these recombinant plasmids in COS cells (unpublished data) show that this enhancement is from an increasing rate of replication.

The SV40 insert in plasmid pSVoriH is an inverted repeat of the early side of the replication origin located between the *Bgl* II site and the midpoint of the 27-nucleotide palindrome. Plasmid pSVoriH lacks the 17-bp A+T-rich region totally. Assuming that the increased length of the palindromic sequence in this recombinant does not hinder replication, the inability of this plasmid to replicate shows that the A+T-rich sequence, which also contains the "T-A-T-A" box component of the early promoter (10, 31), may be required for replication. As proposed a few years ago (3), this A+T-rich sequence might help the initiation of replication by contributing to the unwinding of the DNA at this location.

On the basis of our results, we propose that the SV40 replication origin consists of (*a*) a core region containing the 17-, 15-, and 27-bp palindromes and quite likely the 17-bp A+T-rich sequence and (*b*) an auxiliary or enhancement region consisting of two or more copies of the 21-bp repeat (Fig. 5).

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