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Functional Organization of the Simian Virus 40 Origin of DNA Replication

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To define the sequence elements involved in initiation of DNA synthesis at the simian virus 40 origin of replication, we determined the relative replication efficiencies in vitro and in vivo of templates containing a variety of mutations within the origin region. Replication of the mutants in vitro was assayed by the cell-free DNA replication system that we recently described (J. J. Li and T. J. Kelly, Proc. Natl. Acad. Sci. USA 81:6973–6977, 1984; J. J. Li and T. J. Kelly, Mol. Cell. Biol. 5:1238–1246, 1985), and replication in vivo was assayed after transfection of the mutant templates into COS-1 cells. The minimal origin of replication defined by both assays included a 15-base-pair (bp) imperfect inverted repeat, a 27-bp perfect inverted repeat, and a 17-bp A/T-rich region. T-antigen binding site I was not required for DNA replication, but its presence increased replication efficiency severalfold both in vitro and in vivo. Although SP1 binding sites and enhancers had little or no effect on replication in vitro, the presence of either element markedly increased replication in vivo. Thus, the biological role of these elements is not restricted to stimulating transcription but may be more general.

Simian virus 40 (SV40) is a simple model system that has proven useful for studying the mechanisms of DNA replication in animal cells (6, 12, 13). The viral genome is a double-stranded circular DNA molecule containing 5,243 base pairs (20, 52). In infected cells, the SV40 genome is complexed with histones to form a minichromosome with a nucleoprotein structure analogous to that of cellular chromatin (9, 27). Initiation of viral DNA replication takes place within a unique region of the viral minichromosome and requires the participation of a virus-encoded protein, the SV40 T antigen (7, 10, 19, 31, 67). The initiation process results in establishment of two replication forks that move in opposite directions from the origin region (10, 19). DNA synthesis at each fork proceeds by a semidiscontinuous mechanism and appears to be mediated by cell-encoded replication proteins (6, 12, 13).

Efficient initiation of SV40 DNA replication requires the presence of specific nucleotide sequences that collectively define the viral origin of replication. The boundaries of the minimal origin of replication have been explored by analysis of the replication of various SV40 deletion mutants in vivo (3, 15, 16, 21, 35, 38, 47, 66). The data indicate that the minimal origin is located within a 65-base-pair (bp) segment of the viral genome (nucleotides 5208 to 30). This segment is approximately centered on a 27-bp perfect inverted repeat and also includes a 15-bp imperfect inverted repeat and a 17-bp sequence of A/T nucleotides (see Fig. 2). The minimal origin contains a high-affinity recognition site for the SV40 T antigen (site II); (11, 36, 60, 68–71), and there exists strong genetic evidence that binding of T antigen to this site is an obligatory event in the initiation of viral DNA replication (43, 62, 74).

The minimal origin of replication lies within a region of the viral genome that is rich in elements known to regulate transcription. On the early side of the minimal origin is a high-affinity binding site for T antigen (site I), which is involved in the repression of early transcription (16, 30, 53, 55, 56). On the late side is a series of G/C-rich repeats that are recognized by the cellular transcriptional factor SP1 (18, 24). Adjacent to the G/C repeats are two tandem copies of a 72-bp sequence that has been characterized as a transcriptional enhancer element (1, 22, 45). Little quantitative work has been done to assess the possible effects of these transcriptional elements on the efficiency of SV40 DNA replication. However, two recent studies have provided evidence that the presence of the G/C repeats has a stimulatory effect on replication in vivo (3, 21).

We have described a cell-free system that is capable of replicating plasmid DNA templates containing the SV40 origin of DNA replication (40, 41). Replication requires proteins isolated from cells permissive for SV40 DNA replication in vivo (e.g., monkey and human) and, in addition, is completely dependent on the presence of the viral initiation protein, T antigen. DNA synthesis in vitro is initiated within the viral origin and proceeds bidirectionally via intermediates that are identical to in vivo replication intermediates (40, 41, 65). To define the sequence requirements for DNA replication in vitro, we determined the relative replication efficiencies of templates containing a variety of mutations within the origin region. For purposes of comparison, we quantified the relative replication efficiencies of the same templates in vivo. The data indicate that the minimal origin includes T-antigen binding site II, the 15-bp imperfect inverted repeat, and the A/T-rich region, in agreement with previous studies. The data also demonstrate that sequences adjacent to the minimal origin have significant effects on the efficiency of replication. The presence of T-antigen binding site I increased the extent of replication by several fold, both in vitro and in vivo. The presence of either the G/C-rich or 72-bp repeats (SP1 binding sites and enhancers, respectively) had little or no effect on replication in vitro but markedly increased the amount of replication in vivo. Although the mechanism by which these elements stimulate

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TABLE 1. Structure of the pOR series of mutant and wild-type SV40 subclones[a]

Mutant	Reference[b]	Mutation within SV40 insert[c]	SV40 insert nucleotide boundaries[d]		Vector	Cloning sites in vector[e]	
			Early	Late		Early	Late
110	MM	S:5189(T→G) D:5177–5187	5171	135	pKP55	*Hin*dIII	*Sph*I
1083	15	D:5177–5197	5171	133	pKP55	*Hin*dIII	*Sph*I
1097	16	D:5178–5208	5171	133	pKP55	*Hin*dIII	*Sph*I
41	35	R:[5177–5214]→ (50 bp of bacterial DNA)	5215	133	pKP55	*Hin*dIII	*Sph*I
43	35	R:[5177–5217]→ (50 bp of bacterial DNA)	5218	133	pKP55	*Hin*dIII	*Sph*I
1085	15	D:5187–5207	5171	133	pKP55	*Hin*dIII	*Sph*I
1-2	MM	D:5193–5208	5171	133	pKP55	*Hin*dIII	*Sph*I
1-65	MM	D:5187–5188	5171	133	pKP55	*Hin*dIII	*Sph*I
1086	15	S:5197 (C→G) D:5193–5196	5171	133	pKP55	*Hin*dIII	*Sph*I
1088	15	S:5188, 5193, 5196, 5197, 5206, 5208 (C→T)	5171	133	pKP55	*Hin*dIII	*Sph*I
1-2.9	MM	S:5204 (G→A)	5171	133	pKP55	*Hin*dIII	*Sph*I
1-4.13	MM	S:5193, 5194 (C→T)	5171	133	pKP55	*Hin*dIII	*Sph*I
1-4.7	MM	S:5194 (C→T)	5171	133	pKP55	*Hin*dIII	*Sph*I
1174	43	D:5239	5171	133	pKP55	*Hin*dIII	*Sph*I
8-4	26	D:5239–5242	5171	133	pKP55	*Hin*dIII	*Sph*I
6-1	26	D:5237–5242	5171	133	pKP55	*Hin*dIII	*Sph*I
6-17	26	D:5238–3	5171	133	pKP55	*Hin*dIII	*Sph*I
TBS2	MM		5229[f]	34[f]	pKP54	*Bam*HI[f]	*Bam*HI[f]
105	MM	I:5228 ↓ 5229 TCA	5171	133	pKP55	*Hin*dIII	*Sph*I
106	MM	I:5228 ↓ 5229 TCACCGGATCCGG	5171	133	pKP55	*Hin*dIII	*Sph*I
107	MM	R:[5217–5228]→CCGGATCCGG	5171	133	pKP55	*Hin*dIII	*Sph*I
1030	61	S:1 (G→A)	5171	133	pKP55	*Hin*dIII	*Sph*I
1180	MM	S:16 (T→G)	5171	133	pKP55	*Hin*dIII	*Sph*I
1181	MM	S:16 (T→G) I:16 ↓ 17 CCCGGGCTGCAG	5171	133	pKP55	*Hin*dIII	*Sph*I
TBS1	MM		5172[f]	5231[f]	pKP54	*Bam*HI[f]	*Bam*HI[f]
1134	51	D:13–314	5171	338	pKP55	*Hin*dIII	*Nco*I
S321	21		5171	25[f]	pKP54	*Hin*dIII	*Sal*I[f]
S312	21		5171	34[f]	pKP54	*Hin*dIII	*Sal*I[f]
HNO	MM		5171	42	pKP55	*Hin*dIII	*Nco*I
S301	21		5171	47[f]	pKP54	*Hin*dIII	*Sal*I[f]
S288	21		5171	58[f]	pKP54	*Hin*dIII	*Sal*I[f]
S274	21		5171	72[f]	pKP54	*Hin*dIII	*Sal*I[f]
HSO	MM		5171	133	pKP55	*Hin*dIII	*Sph*I
HKO	MM		5171	299	pKP55	*Hin*dIII	*Kpn*I
HHO	MM		5171	1051	pKP55	*Hin*dIII	*Hin*dIII
XS1	21	D:35–52	5171	299	pKP55	*Hin*dIII	*Kpn*I
XS2	21	D:35–94	5171	299	pKP55	*Hin*dIII	*Kpn*I
XS3	21	D:35–108	5171	299	pKP55	*Hin*dIII	*Kpn*I
XS14	21	D:59–108	5171	299	pKP55	*Hin*dIII	*Kpn*I
1091	15	D:33–53 R:[30–32]→GCA	5171	133	pKP55	*Hin*dIII	*Sph*I
1094	15	D:56–67	5171	133	pKP55	*Hin*dIII	*Sph*I
1096	15	S:36, 43 (G→A)	5171	133	pKP55	*Hin*dIII	*Sph*I
400	MM	S:36, 43 (G→A) I:34 ↓ 35 CCGGATCCGGCCGGATCCGG	5171	133	pKP55	*Hin*dIII	*Sph*I
4	MM	S:36, 43 (G→A) I:34 ↓ 35 CCGGATCCGG	5171	133	pKP55	*Hin*dIII	*Sph*I

[a] The majority of the subclones were created by cleavage at restriction sites in SV40, isolation of the fragment containing the origin of replication, and ligation between the sites flanking the fragment and the identical sites in the vector. Only in two cases (mutant 41 and 43 early sites) was a blunt-end ligation required to fuse vector to insert (see Materials and Methods).

[b] Reference describing construction of the original SV40 mutant for each subclone. MM, Described in Materials and Methods.

[c] D, deletion; S, base substitution; R, replacement; I, insertion. Numbers and letters refer to nucleotide position (4) in the early strand of the SV40 genome.

[d] SV40 nucleotide position (4) that forms the boundary of the insert to the early or late side of the SV40 origin.

[e] Restriction sites in the vector polylinker used to fuse the early- or late-side terminus of the fragment containing the SV40 origin to the vector.

[f] The junction between SV40 and vector sequences was constructed via a restriction site linker (*Bam*HI or *Sal*I) fused to one or both ends of the insert fragment. This linker was subsequently ligated to the corresponding site in pKP54.

replication in vivo is not yet clear, one possibility is that they facilitate the access of initiation factors to the replication origins of SV40 minichromosomes.

MATERIALS AND METHODS

Mutants. Mutants with lesions in the SV40 origin region were obtained from a variety of sources, and a number were constructed de novo. References to original papers describing the construction of mutants used in this study are given in Table 1.

A new series of deletions in site I (e.g., pOR1-2 and pOR1-65) was constructed by the method of DiMaio and Nathans (15) with minor modifications. The starting plasmid for these constructions was pOR1, which contains the fragment of SV40 DNA from the *Hin*dIII site at nucleotide 5171 to the *Sph*I site at nucleotide 128 inserted between the corresponding sites in pBR322. pOR1 DNA was incubated with *Stu*I in the presence of ethidium bromide to introduce single-strand breaks at the *Stu*I site (nucleotide 5190). The resulting nicks were translated in the 5′-to-3′ direction with *Micrococcus luteus* DNA polymerase in the presence of the

four deoxynucleoside triphosphates and di-dTTP. The average distance of nick translation was controlled by varying the ratio of di-dTTP to dTTP. On continued incubation, the nicks were extended into short single-stranded gaps by the 5'-to-3' exonuclease activity of *M. luteus* polymerase. The resulting gapped molecules were digested with S1 nuclease, ligated into circles, and introduced into *Escherichia coli*. Colonies were screened by standard methods for the presence of plasmids with deletion mutations. The precise locations of the deletions were determined by DNA sequence analysis.

pOR110 was constructed by cleavage with *Stu*I (nucleotide 5190) and addition of *Xho*I linkers, followed by digestion with *Hin*dIII and *Xho*I to remove the fragment containing nucleotides 5176 to 5192. After the single-stranded tails of the *Hin*dIII and *Xho*I termini were repaired with the Klenow fragment of DNA polymerase I, recircularization of the plasmid recreated an intact *Hin*dIII site.

Several mutants with base substitutions in T-antigen binding site I (pOR1-4.7, pOR1-4.13, and pOR1-2.9) were generated from pOR1-2 by heteroduplex deletion loop mutagenesis by the method of Peden and Nathans (49). A number of mutants with oligonucleotide insertions in site II were constructed from pOR1 and the analogous plasmid, pOR3, derived from cs1096 (15). Insertion mutants 4 and 400 were generated from pOR3 by insertion of one or two *Bam*HI linkers, respectively, at the *Hin*cII site (nucleotide 32). Mutant 105 was prepared by cleavage with *Dde*I (nucleotide 5228) followed by repair of the single-stranded termini and recircularization. Mutant 106 was made in a similar fashion except that a *Bam*HI linker was added prior to circularization. Mutant 107 was constructed by replacing the *Hin*dIII-*Bam*HI fragment of mutant 106 with an *Hin*dIII-*Mnl*I fragment (nucleotides 5171 to 5216) that had a *Bam*HI linker ligated to the *Mnl*I end. Mutants 1180 and 1181 were constructed by H. Tseng. The former construct, generated by the heteroduplex oligonucleotide mutagenesis method of Morinaga et al. (46), contains a single base substitution (T to G) at nucleotide 16, which creates a new *Pst*I site. Mutant 1181 was then prepared by insertion of a synthetic oligonucleotide of 12 bp at this *Pst*I site.

The plasmids pTBS1 and pTBS2, which contain T-antigen binding sites I and II, respectively, were derived from cs1096 (15). The *Hin*dIII to *Dde*I fragment (nucleotides 5171 to 5228) and the *Dde*I to *Hin*cII fragment (nucleotides 5228 to 32) were isolated, and the single-stranded termini were repaired with the Klenow fragment of DNA polymerase I. After addition of *Bam*HI linkers, each fragment was inserted into pBR322 at the *Bam*HI site.

DNA templates. The DNA templates used in the in vitro and in vivo replication assays were subclones of the SV40 mutants described above. Fragments spanning the origin of replication were inserted into one of two nearly identical vectors, pKP54 and pKP55 (Peden, unpublished data). Both vectors were derived from pBR322 by replacement of nucleotides 35 to 2456 with a multiple restriction site polylinker. Reading from the *Hin*dIII site at nucleotide 30 toward nucleotide 2457, pKP54 has a 27-bp polylinker, *Sal*I-*Bam*HI-*Sma*I-*Kpn*I-*Xba*I, and pKP55 has a 26-bp polylinker, *Nco*I-*Sph*I-*Pvu*II-*Kpn*I-*Xba*I. The latter vector was designed to accept restriction fragments from the SV40 origin region; it contains several restriction sites from this region in their normal order of appearance.

Table 1 lists the nucleotide endpoints for each origin fragment that was subcloned, the vector used for subcloning, and the restriction sites employed for fragment insertion. pIN41 and pIN43 (35) were subcloned with additional bacterial sequences flanking the early side. pIN41 and pIN43 both contain a 66-bp insertion of bacterial DNA between T-antigen binding sites I and II. Digestion of either plasmid with *Dpn*I and *Sph*I released a fragment analogous to the wild-type *Hin*dIII to *Sph*I fragment (nucleotides 5171 to 128) but with site I and parts of site II replaced by approximately 50 bp of the bacterial insert. This fragment was cloned into pKP55 at the *Hin*dIII and *Sph*I sites. For these clones, the *Hin*dIII site on pKP55 was cleaved and repaired with Klenow fragment of DNA polymerase I. Ligation of the resulting blunt end of the vector to the *Dpn*I end of the origin fragment regenerated an intact *Hin*dIII site.

All of the subclones that were constructed in pKP54 and pKP55 have their origin fragment oriented in the same direction with respect to the vector and almost all are between 2.1 and 2.2 kilobases in length. They were introduced into *E. coli* DH1 (29), a *recA*$^-$ strain, and clones containing exclusively the monomer form of the plasmid were isolated.

pKP45.ori, the reference plasmid used for all in vivo assays, contains the *Hin*dIII to *Sph*I fragment of SV40 inserted into the *Eco*RI site of pKP45 (Peden, unpublished data; 40) with *Eco*RI linkers. It is approximately 2.9 kilobases in length and is easily separated by gel electrophoresis from most of the subclones described above.

DNA was prepared by standard alkali lysis procedures (34) and banded twice by equilibrium centrifugation. All traces of RNA were removed by digestion with RNase A (Calbiochem-Behring) and RNase T_1 (Bethesda Research Laboratories, Inc.). The final samples contained >95% form I monomer DNA.

In vitro replication assays. Extracts were prepared from COS-1 cells (25), and T antigen was immunoaffinity purified by published procedures (17, 40). The protein concentrations of the extracts were approximately 4 mg/ml, and the T-antigen concentration was 1.2 mg/ml as determined by Bio-Rad Laboratories assay (5) with bovine serum albumin and bovine serum gamma globulin, respectively, as standards.

The in vitro replication reactions were performed, and the products were isolated and analyzed as described previously (41) with slight modifications. The standard 50-μl reaction mixture contained 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.2); 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 5 to 10 μCi of [α-^{32}P]dCTP; 25 μM dCTP; 100 μM dATP, dGTP, and dTTP; 50 μM CTP, GTP, and UTP; 4 mM ATP, 40 mM phosphocreatine; 100 μg of creatine phosphokinase (Sigma Chemical Co.; stock solution, 5 mg/ml in H_2O) per ml; 20 mM sodium phosphate (stock solution, 1 M [pH 7.7] at 22°C); 60 ng of form I plasmid DNA; 30 μl (120 μg) of extract; and 2.5 μl (3 μg) of T antigen. All samples were incubated at 37°C for 4 h. After isolation of the products, half of each sample was digested with 2 to 3 U of *Dpn*I and *Eco*RI; the latter enzyme cleaves the products into unit length fragments. Both undigested and digested products were subjected to electrophoresis on a 1.5% agarose gel.

The amount of radioactive label incorporated into full-length linear *Dpn*I-resistant DNA was quantified as previously described (41). All DNA samples were assayed in duplicate, and the average incorporation was determined. This value was expressed as a percentage of the average obtained for pOR.HSO, the subclone containing the *Hin*dIII to *Sph*I fragment (nucleotides 5171 to 128) from wild-type SV40 (strain 776).

Replication of mutant origins in vivo. COS-1 (25) monolayer cultures in 6-cm dishes were cotransfected with 50 ng of mutant DNA and 5 ng of the reference DNA, pKP45.ori, by the DEAE-dextran method (44). At 36 and 48 h after transfection, low-molecular-weight DNA was isolated (32, 51), and replication was assessed by the *Dpn*I assay (50). The DNA was digested with *Dpn*I to cleave unreplicated input molecules to small fragments and with *Sca*I to linearize both the test and reference DNAs. The products were fractionated on a 1.2% agarose gel, transferred to nitrocellulose (63), and hybridized with pKP54 labeled with ^{32}P by nick translation (42, 54). Hybridization and washing of filters has been previously described (48). To quantify the amount of replication, the bands representing *Dpn*I-resistant, full-length, linear test and reference DNAs were cut from the nitrocellulose filter and counted separately in a liquid scintillation spectrometer. The ratio of radioactivity in the mutant DNA band to that in the reference DNA band was calculated. The resulting value was then expressed as a percentage of the corresponding ratio for pOR.HSO to derive the relative in vivo replication efficiency.

RESULTS

The sequence requirements for SV40 DNA replication were defined by measuring the abilities of plasmid templates containing mutant viral origins of replication to support DNA replication in vitro and in vivo. Mutant SV40 DNAs were obtained from a variety of sources (see Materials and Methods), and the origin region of each was subcloned into a common genetic background. The vectors employed for subcloning were pKP54 and pKP55, derivatives of pBR322 in which the DNA segment from nucleotides 35 to 2456 had been replaced by an oligonucleotide polylinker containing multiple restriction enzyme cleavage sites. (The two vectors differed in sequence only within the linker segment.) Plasmid templates constructed in this fashion were assayed for the ability to support DNA replication in vitro under standard reaction conditions (40, 41). Reaction mixtures containing purified SV40 T antigen, extract from uninfected COS-1 cells and [α-^{32}P]dCTP as the radioactive deoxynucleoside triphosphate were incubated for 4 h, and the reaction products were digested wih *Dpn*I and *Eco*RI. Digestion with *Dpn*I eliminates any background due to repair synthesis, and digestion with *Eco*RI cleaves the products of replication to unit-length linear DNA molecules (40, 41, 50). The extent of nucleotide incorporation into full-length, *Dpn*I-resistant template molecules was quantified after agarose gel electrophoresis of the cleaved products. The relative in vitro replication efficiency of a given mutant was defined as the amount of nucleotide incorporation into the mutant template expressed as a percentage of the amount of nucleotide incorporation into the wild-type template, pOR.HSO, under the same conditions.

The same templates were assayed for replication activity in vivo after introduction into COS-1 cells by the DEAE-dextran method (44). At 48 h after transfection, low-molecular-weight DNA was extracted from the cells and digested with *Dpn*I to eliminate background due to unreplicated input DNA (50). After cleavage to unit-length linear DNA molecules with *Sca*I, the products of replication were fractionated by agarose gel electrophoresis, transferred to a nitrocellulose membrane (63), and quantified by hybridization to radioactive vector sequences. To improve the reproducibility of the in vivo data, a small amount of a reference plasmid (pKP45.ori) was mixed with each mutant prior to transfection. In these assays, the mutant and reference DNAs competed for the cellular replication apparatus. To calculate the relative in vivo replication efficiency of the mutant, the ratio of the amount of replication of the mutant template to that of the reference template was determined, and this ratio was then expressed as a percentage of the corresponding ratio for the wild-type template, pOR.HSO. In separate experiments the values for the in vivo replication efficiency of a given mutant were within 10% of each other.

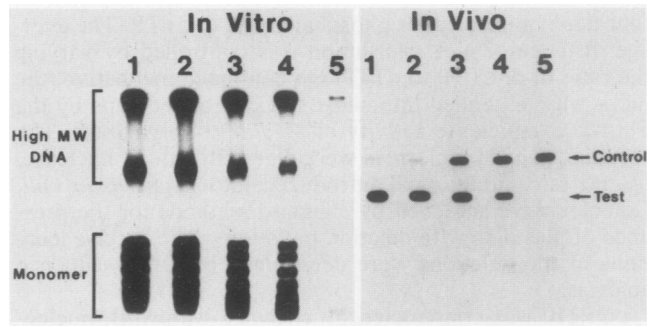

FIG. 1. Replication of site I deletion mutants in vitro and in vivo. Mutants with deletions extending progressively greater distances into SV40 T-antigen binding site I from the early side of the origin region were assayed for replication activity in vitro and in vivo. The mutants, in order of increasing deletion size, are (lanes): 1, pOR.HSO; 2, pOR.110; 3, pOR.1083; 4, pOR.1097; and 5, pOR.43. Left panel, replication in vitro. Reaction mixtures containing extract from uninfected COS-1 cells, T antigen, and the various mutant DNAs were incubated for 4 h at 37°C. The undigested radioactive products were subjected to electrophoresis on a 1.5% agarose gel. The products consisted of a series of topological isomers of mutant DNAs (monomers) and higher-molecular-weight (high MW DNA) tandem arrays of monomers. Synthesis of both products was dependent on the presence of SV40 T antigen (40, 41). Right panel, replication in vivo. The mutant plasmids (test) and an internal reference plasmid (control) were cotransfected into COS-1 cells at a 10:1 weight ratio. After 45 to 48 h, DNA was extracted from the cells, digested with *Dpn*I and *Sca*I, and subjected to electrophoresis on a 1.2% agarose gel. The replication products of the two plasmids migrated as unit length linear DNA molecules (arrows) and were quantified by hybridization to ^{32}P-labeled pKP54 (63). The faint, rapidly migrating bands are fragments resulting from *Dpn*I digestion of residual, unreplicated plasmid DNA.

The relative in vivo replication efficiencies of the various mutants were independent of several changes in the assay conditions (data not shown). In particular, the values were unchanged when the amount of mutant DNA introduced into the cells was decreased by a factor of 10, so that there was no significant competition between mutant and reference DNAs. Similarly, the relative replication efficiencies measured 36 h after transfection were approximately the same as those measured after 48 h. Finally, most of the mutants were tested in a second monkey cell line (MKSV6; Peden, unpublished data) that expresses SV40 T antigen, and the results obtained were very similar to those obtained with COS-1 cells.

Replication of mutants with lesions in T-antigen binding site I. The highest-affinity binding site for SV40 T antigen (site I) is located between nucleotides 5184 and 5212 on the viral genome as determined by DNase I protection experiments (see Fig. 2) (68–70; D. Rawlins and T. Kelly, unpublished data). The interaction of T antigen with this site has been directly implicated in the repression of SV40 early transcription (16, 30, 53, 55, 56). A number of deletion and substitution mutants affecting site I were assayed for ability to support replication in vitro and in vivo by the methods described above. Except for the indicated lesions in site I,

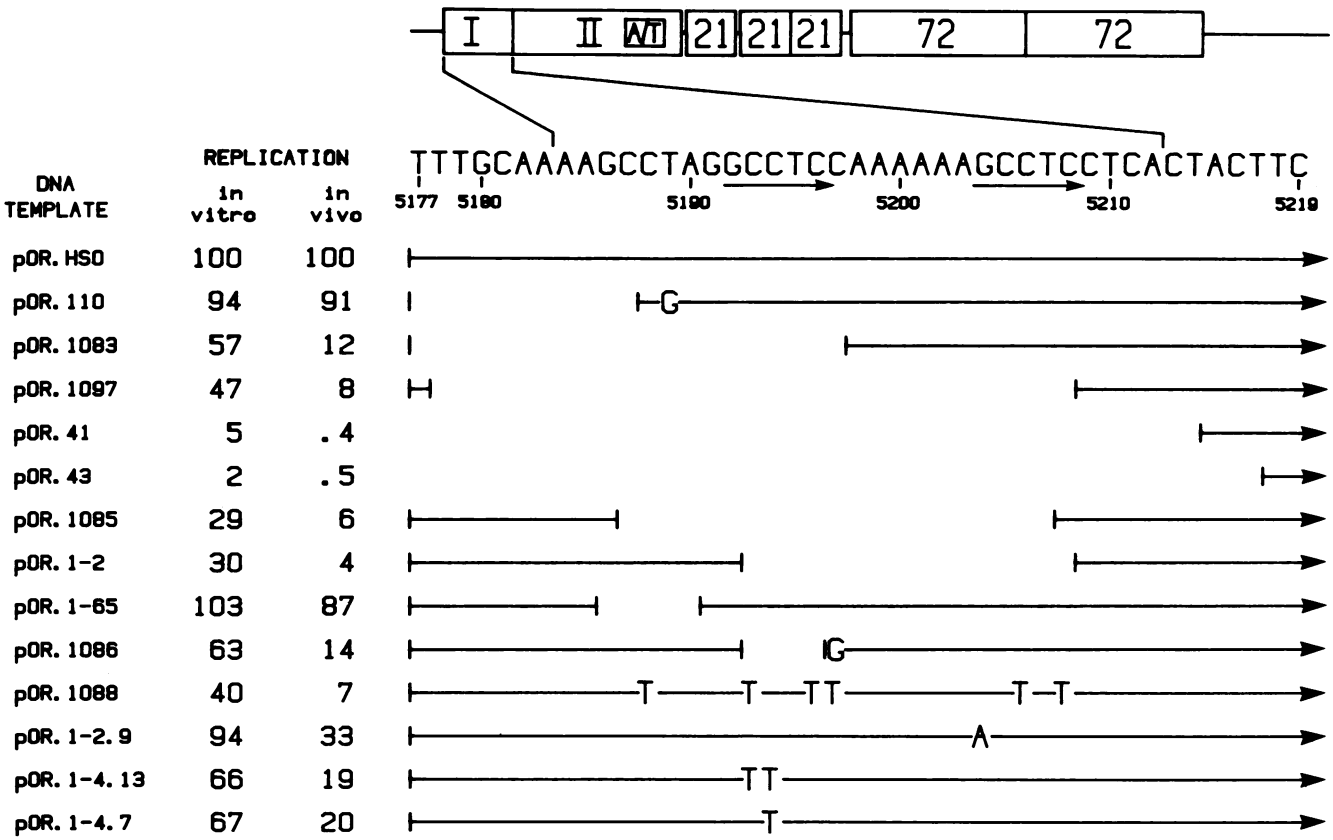

FIG. 2. Summary of the replication activities of site I mutants in vitro and in vivo. Origin-containing plasmids with mutations affecting T-antigen binding site I were assayed for replication activity as described in Materials and Methods. Both the in vitro and in vivo activities are expressed as a percentage of the activity determined for wild-type plasmid pOR.HSO (relative replication efficiency). The relative replication efficiency of the plasmid vector alone was 0.01% in vitro and 0.5% in vivo. The diagram at the top shows the important sequence elements in the SV40 origin region (nucleotides 5171 to 294). The region containing site I has been expanded to show the wild-type nucleotide sequence of the early strand in the 5'-to-3' direction. Arrows indicate pentanucleotide sequences implicated as important components of the recognition site of T antigen. Except for the indicated deletions or substitutions, each mutant plasmid contained the segment of the SV40 genome from the *Hin*dIII site at nucleotide 5171 to the *Sph*I site at nucleotide 128 (see Table 1 for complete structural information).

the mutant templates all contained the segment of the viral genome extending from the *Hin*dIII site at nucleotide 5171 to the *Sph*I site at nucleotide 128. This segment includes the G/C-rich repeats on the late side of the minimal origin but lacks a functional enhancer. (As shown below, deletion of enhancers alone had little or no effect on the efficiency of replication in vitro or in vivo.)

Figure 1 shows the results of replication assays carried out with a series of unidirectional deletions that extend various distances into site I from the vicinity of the *Hin*dIII site at nucleotide 5171. The relative replication efficiencies of these and other site I mutants are summarized in Fig. 2 together with the structures of the mutant templates. A mutant lacking site I sequences to nucleotide 5197 (pOR.110) replicated with approximately the same efficiency as the wild-type template (pOR.HSO). However, templates with deletions extending further into site I (pOR.1083 and pOR.1097) replicated with reduced efficiency, both in vitro and in vivo. The deletion in pOR.1097 (nucleotides 5177 to 5208) removes essentially all of site I, including all known essential T-antigen contacts (11, 36, 57, 71). This mutant replicated 2- to 3-fold less efficiently than the wild-type template (pOR.HSO) in vitro and approximately 10-fold less efficiently in vivo. Large internal deletions in site I (pOR.1085, pOR.1086, and pOR.1-2) or multiple base substitutions (pOR.1088) reduced replication efficiency to a similar extent (Fig. 2). Several mutants with smaller internal deletions (pOR.1-65) or less extensive base substitutions (pOR.1-2.9, pOR.1-4.7, and pOR.1-4.13) had intermediate effects on replication efficiency.

Deletions extending beyond site I to nucleotide 5214 (pOR.41) or 5217 (pOR.43) reduced the amount of replication in vitro to a few percent of that of the wild type and virtually abolished replication in vivo (Fig. 1 and 2). Thus, these deletions appear to enter the minimal origin of replication. We conclude that the early boundary of the minimal origin defined by the in vitro replication assay coincides with that defined by the in vivo replication assay and is located between nucleotides 5209 and 5214. These coordinates are in good agreement with those obtained in previous in vivo studies (16, 35, 47).

Replication of mutant templates with lesions mapping to the late side of the minimal origin. Figures 3 and 4 summarize the effects on DNA replication of progressively larger deletions of sequences from the late side of the minimal origin. The mutant templates employed in this study contain segments of the SV40 genome that extend from a common terminus at nucleotide 5171 to the various deletion endpoints (Fig. 4). Mutants with deletion endpoints at nucleotides 1051, 299, and 133 (pOR.HHO, pOR.HKO, and pOR.HSO, respec-

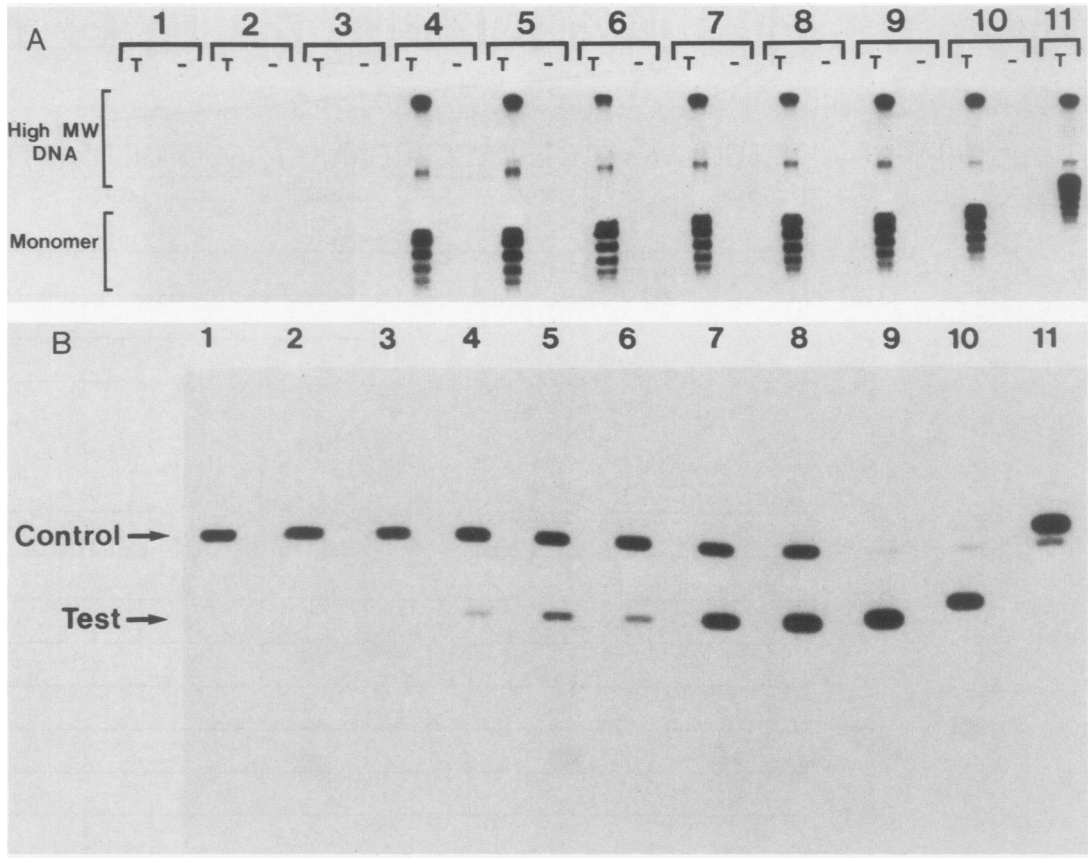

FIG. 3. Replication of mutants with deletions on the late side of the minimal origin. Mutants with deletions extending progressively greater distances into the SV40 origin region from the late side of the genome were assayed for replication activity in vitro and in vivo. The mutants, in order of decreasing deletion size, are (lanes): 1, pOR.TBS1; 2, pOR.1134; 3, pOR.S321; 4, pOR.S312; 5, pOR.HNO; 6, pOR.S301; 7, pOR.S288; 8, pOR.S274; 9, pOR.HSO; 10, pOR.HKO; and 11, pOR.HHO. Panel A, replication in vitro. Reaction mixtures containing mutant DNAs and extract from uninfected COS-1 cells were incubated for 4 h at 37°C in the presence (T) or absence (−) of purified SV40 T antigen (MW, molecular weight). The undigested products of replication were analyzed as described in the legend to Fig. 1. Panel B, replication in vivo. The mutant plasmids (test) and an internal reference plasmid (control) were cotransfected into COS-1 cells at a 10:1 weight ratio and assayed for replication activity as described in the legend to Fig. 1.

tively) replicated efficiently, both in vitro and in vivo. Since pOR.HSO lacks the SV40 enhancer elements, whereas pOR.HHO and pOR.HKO retain them, we conclude that deletion of enhancers alone has little or no effect on the amount of replication under the conditions of these experiments. Templates with deletions that extend into the G/C-rich repeats (pOR.S274, pOR.S288, pOR.S301, pOR.HNO, and pOR.S312) replicated with reduced efficiency in vivo but not in vitro. For example, mutant pOR.S312, which lacks the complete set of G/C repeats, replicated about 30-fold less efficiently than the wild type (pOR.HSO) when introduced into COS-1 cells but was indistinguishable from the wild type in the in vitro system. In addition, mutations that increase the distance between the minimal origin and the G/C-rich repeats (pOR.400 and pOR.4) decreased replication efficiency in vivo but not in vitro. Thus, the G/C repeats are not absolutely required for SV40 DNA replication, but their presence near the minimal origin significantly increases the amount of replication observed in vivo.

Deletions extending past the G/C-rich repeats into the A/T-rich region and beyond resulted in a sharp decline in DNA replication. For example, the replication efficiency of pOR.S321, which lacks six nucleotides of the A/T-rich region, was less than 1% of the wild-type (pOR.HSO) level in vitro and was not significantly above background in vivo. These data place the late boundary of the minimal origin of replication, both in vitro and in vivo, between nucleotides 24 (pOR.S321) and 34 (pOR.S312), which is in good agreement with previous in vivo data (3, 15, 21, 38, 66).

Further characterization of the effects of enhancers and G/C-rich repeats on efficiency of replication. The results described above indicate that optimal replication in vivo can be obtained with a template that contains T-antigen binding site I, the minimal origin, and G/C-rich repeats (e.g., pOR.HSO). Addition of enhancer elements to such a template had little effect on the observed amount of replication. To analyze further the role of sequences on the late side of the minimal origin, we studied several templates that retain both enhancer elements but lack sequences within the G/C-rich repeats (pOR.XS1, pOR.XS2, pOR.XS3, and pOR.XS14) (Fig. 3 to 5). Surprisingly, these templates replicated quite efficiently in vivo. In the extreme case (mutant pOR.XS3), the G/C-rich repeats had been completely deleted, yet the amount of replication remained about one-third of the wild-type level. Comparison of the replication efficiency of pOR.XS3 with that of pOR.S312, which lacks both enhancers and G/C-rich repeats, indicated that the presence of the enhancers stimulated replication about 10-fold (Fig. 3 to 5). We conclude from these data that the efficiency of SV40 DNA replication in vivo was markedly

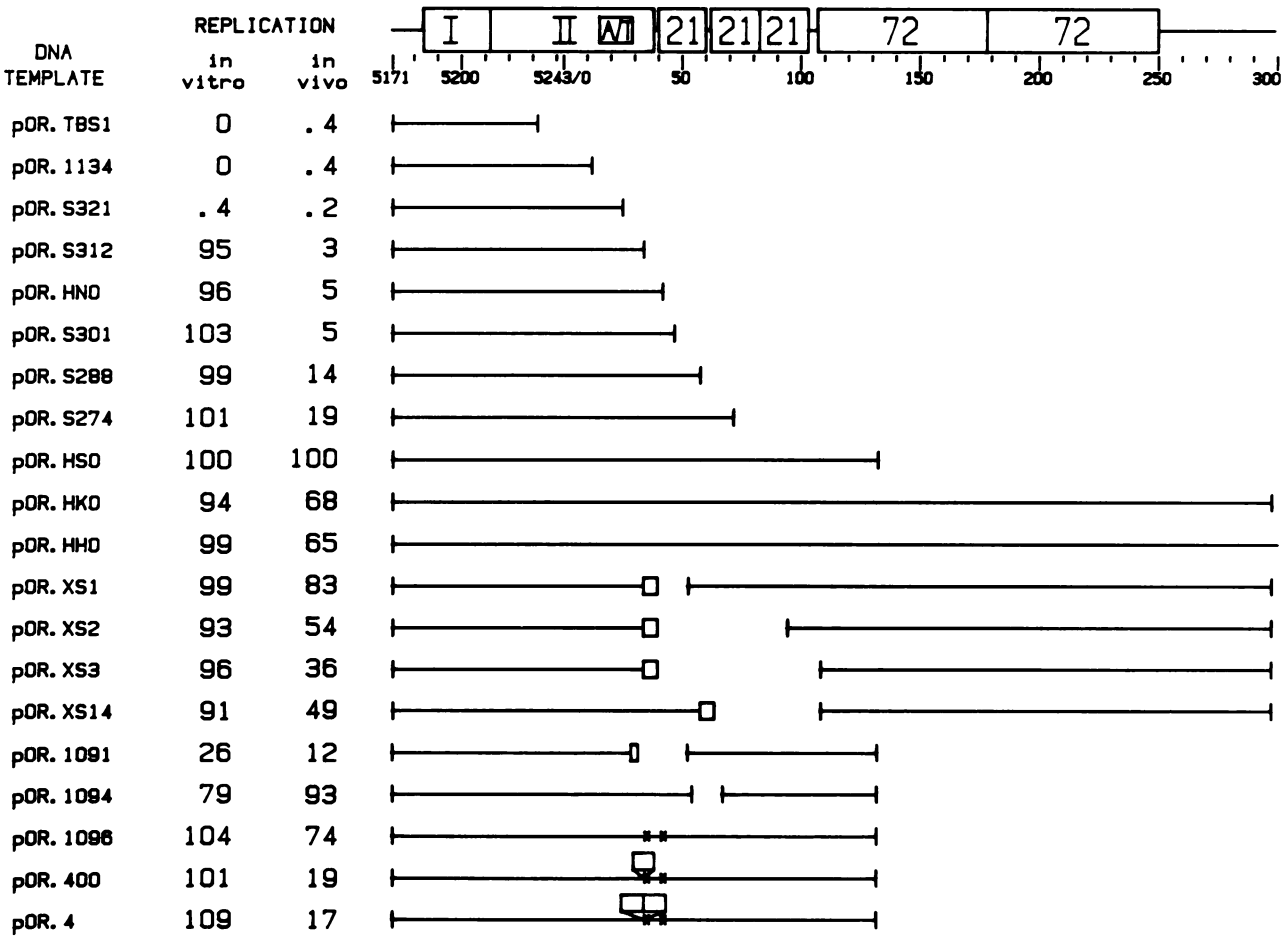

FIG. 4. Summary of the replication activities of mutants with lesions affecting the late side of the origin region. Origin-containing plasmids with mutations in the late side of the origin region were assayed for replication activity as described in Materials and Methods and the legend to Fig. 2. The segments of SV40 DNA in all of the mutant plasmids shared a common endpoint on the early side of the origin region (the *Hin*dIII site at nucleotide 5171). Boxes denote either replacement (pOR.XS1, pOR.XS2, pOR.XS3, pOR.XS14, and pOR.1091) or insertion (pOR.400 and pOR.4) of sequences. Both in vitro and in vivo activities are expressed as percentages of the activity determined for wild-type plasmid pOR.HSO (relative replication efficiency). The relative replication efficiency of the plasmid vector alone was 0.01% in vitro and 0.5% in vivo. The diagram at the top shows the important sequence elements in the origin region: I, T-antigen binding site I; II, T-antigen binding site II; A/T, 17-bp A/T-rich region; 21, 21-bp repeats (also referred to as G/C-rich repeats or SP1 binding sites); and 72, 72-bp repeats (or enhancers).

increased by the presence of either enhancers or G/C-rich repeats, but when one element was present the effect of the other was less apparent. Neither element had any significant effect on replication in the in vitro system.

Replication of mutants with lesions in the minimal origin of replication. Within the boundaries of the minimal origin of replication there are several discrete sequence elements of interest (20, 52). Proceeding toward the late region, these include a 15-bp imperfect inverted repeat, a 27-bp perfect inverted repeat, and a 17-bp A/T-rich region. All three elements appear to contain sequences that are required for SV40 DNA replication, both in vitro and in vivo. As described above, deletions that enter the 15-bp inverted repeat or the A/T-rich region from outside the minimal origin greatly reduce the ability of the template to support replication. In addition, mutant pOR.TBS2, which contains only the 27-bp inverted repeat and the A/T-rich region, failed to replicate to any significant extent (Fig. 6). Finally, mutations within the 27-bp inverted repeat produced severe replication defects (Fig. 6) (26). For example, introduction of a single-base-pair deletion (pOR.1174) or substitution (pOR.1030) in this element reduced the efficiency of replication in vitro and in vivo more than 100-fold relative to that of the wild-type template (pOR.HSO). The spatial relationship of the various sequence elements within the minimal origin of replication may also be crucial for its function, as suggested by the effects of spacer mutations on replication efficiency (Fig. 6). Insertion of a 13-bp oligonucleotide between the 15-bp inverted repeat and the 27-bp inverted repeat (pOR.106) reduced the amount of replication to less than 1% of the wild-type level. Insertion of a 3-bp oligonucleotide at the same site (pOR.105) also reduced replication efficiency, but to a lesser extent. Similarly, the introduction of a 12-bp spacer between the 27-bp inverted repeat and the A/T-rich region virtually abolished replication in vitro and in vivo.

Effect of T-antigen concentration on replication of mutant templates in vitro. As described above, the presence of T-antigen binding site I increased the efficiency of replication from the SV40 origin in vitro and in vivo. One possible explanation for this phenomenon is that binding of T antigen to site I increases the affinity of T antigen for the minimal origin (site II) either by altering the conformation of the

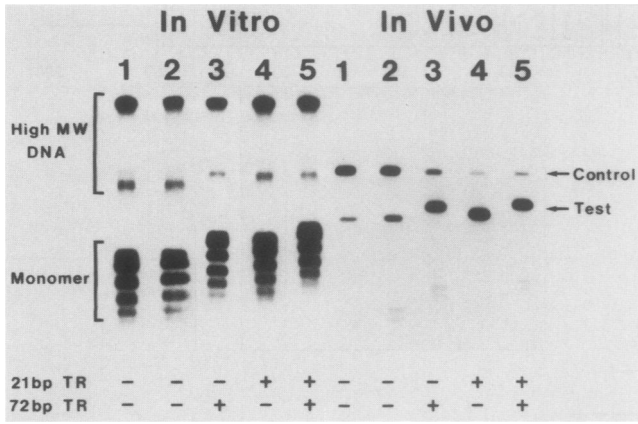

FIG. 5. Effect of enhancers and G/C-rich repeats on SV40 DNA replication in vitro and in vivo. Origin-containing plasmids with deletions affecting the transcriptional elements on the late side of the origin region were assayed for replication activity in vitro (left) and in vivo (right) as described in Materials and Methods. All of the mutants contained T-antigen binding site I and the minimal origin of replication but differed according to the presence (+) or absence (−) of enhancers (72 bp) and G/C-rich repeats (21 bp). Lanes: 1, pOR.312; 2, pOR.HNO; 3, pOR.XS3; 4, pOR.HSO; and 5, pOR.HKO. MW, molecular weight; TR, tandem repeats.

template or by providing stabilizing protein-protein contacts. If this explanation were correct, it might be expected that the effects of site I mutations could be overcome by increasing the T-antigen concentration. Therefore, we measured the relative replication efficiencies of wild-type and mutant templates in vitro as a function of T-antigen concentration (Fig. 7). For all of the templates tested, DNA synthesis reached a maximal level when the reaction mixture contained 4 μg of T antigen, which corresponds to a monomer concentration of approximately 8×10^{-7} M. The replication activities of site I mutants (e.g., pOR.1097 and pOR.1085) remained one-third to one-half that of the wild-type template, even at elevated T-antigen concentrations. Thus, the effect of site I mutations was independent of T-antigen concentration, suggesting that the increased replication efficiency of templates containing site I is not simply due to effects on the affinity of T antigen for the minimal origin. We carried out similar studies on several templates with lesions mapping in the minimal origin and to the late side of the minimal origin (data not shown). As expected, the effects of these mutations on relative replication efficiency were also found to be independent of T-antigen concentration. In particular, mutant pOR.S312, which lacks G/C-rich repeats but retains the entire minimal origin, replicated as efficiently at all T-antigen concentrations as pOR.HSO, which also contains the G/C-rich repeats. Mutant pOR.S321, which lacks 6 bp of the A/T-region, failed to replicate at any T-antigen concentration.

DISCUSSION

Whereas the detailed mechanism of initiation of SV40 DNA replication is not understood, it is clear that initiation occurs within a specific region of the viral genome and requires the presence of specific nucleotide sequences. The recent development of a cell-free system that is capable of initiating DNA replication on plasmid DNA templates containing the SV40 origin of replication (40, 41) provides a means to analyze the biochemistry of the initiation reaction. As the first step toward this goal, we studied the sequence requirements for SV40 DNA replication both in vivo and in vitro. For this purpose, we measured the effects on replication efficiency of a variety of mutations within and adjacent to the viral origin region. The same cell line (COS-1) was used for analysis of replication in vivo and as the source of extract for in vitro replication assays. To ensure a constant genetic context for this analysis, DNA fragments containing the mutant origin regions were subcloned into identical or closely related plasmid vectors prior to analysis. The subclones were constructed so that they lacked functional T-antigen genes to avoid possible indirect effects on replication in vivo by mutations that affect T-antigen expression. In addition, the inherent variability of in vivo replication assays was overcome by measuring the replication efficiencies of the mutant templates relative to that of a wild-type template introduced into the same cells. It should be noted that, because the reaction conditions in the cell-free system may differ significantly from the conditions present in intact cells, it is probably not meaningful to compare directly the values for relative replication efficiency measured in vitro with those measured in vivo. Nevertheless, with a few interesting exceptions discussed below, the results obtained with the two assays are qualitatively quite similar. Our data clearly indicate that there are a number of sequence elements in the origin region that affect the overall efficiency of DNA replication. It has been shown previously that sequences within a 65-bp region centered on the 27-bp inverted repeat sequence at nucleotides 5230 to 13 are absolutely required for origin function in vivo (3, 15, 16, 21, 35, 38, 47, 66). We demonstrated in the present study that the same sequences are required for T-antigen-dependent replication in the cell-free system. In addition, we found that replication efficiency was markedly increased, particularly in vivo, by the presence of sequence elements that map outside of this minimal origin.

The boundaries of the minimal origin of SV40 DNA replication that we derived by analysis of replication in the cell-free system coincide with those derived from this and previous analyses of replication in vivo (3, 15, 16, 21, 35, 38, 47, 64, 66) as well as with a recent in vitro study (64). The minimal origin contains several potentially important sequence elements. The 27-bp inverted repeat contains nucleotides essential for binding of T antigen as determined by genetic and biochemical studies (26, 35, 36, 39, 61, 69). Although there is good evidence that binding of T antigen to this element is required for initiation of DNA replication (43, 62, 74), the precise biochemical function of T antigen in the initiation process is not known. Sequences immediately adjacent to the 27-bp inverted repeat (the 15-bp imperfect inverted repeat and the 17-bp A/T-rich region) do not appear to be required for high-affinity binding of T antigen to the origin (35, 36) but are essential for replication both in vitro and in vivo. These elements may be binding sites for other proteins involved in initiation of DNA synthesis.

The presence of T-antigen binding site I, which maps to the early side of the minimal origin, increases the efficiency of DNA replication in vitro and in vivo above that observed with the minimal origin alone. Since binding of T antigen to site II is required for SV40 DNA replication, one possible explanation for this result is that binding of T antigen to the higher-affinity site I facilitates subsequent binding to site II. Our data strongly suggest that this simple explanation cannot account for the effect of site I on replication efficiency. This conclusion is supported by previous studies in which attempts to demonstrate cooperative interactions between T-antigen molecules bound at sites I and II were unsuccess-

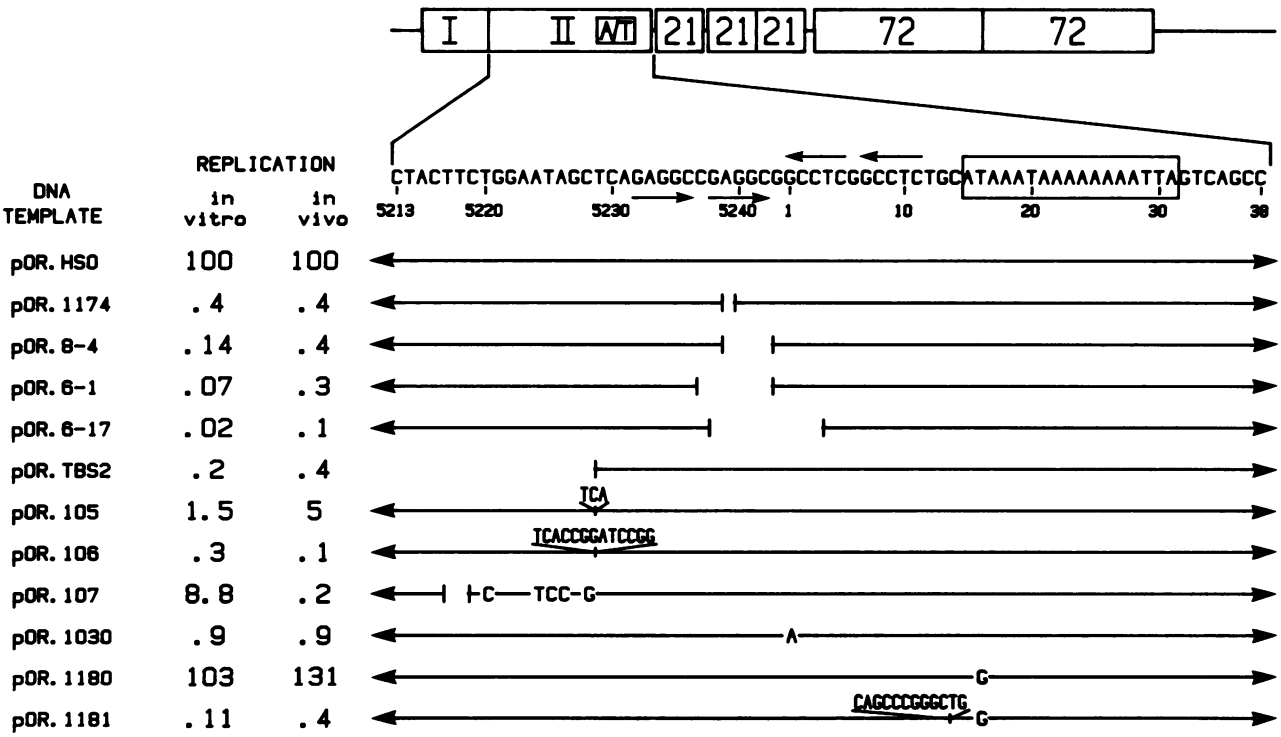

FIG. 6. Summary of the replication activities of mutants with lesions affecting the minimal origin. Origin-containing plasmids with mutations in the neighborhood of T-antigen binding site II were assayed for replication activity as described in Materials and Methods and the legend to Fig. 2. Except for the indicated mutations, each plasmid contained the segment of the SV40 genome from the *Hin*dIII site at nucleotide 5171 to the *Sph*I site at nucleotide 128 (see Table 1 for complete structural information). Both in vitro and in vivo activities are expressed as percentages of the activity determined for wild-type plasmid pOR.HSO (relative replication efficiency). The relative replication efficiency of the plasmid vector alone was 0.01% in vitro and 0.5% in vivo. The region containing site II has been expanded to show the wild-type nucleotide sequence of the early strand in the 5'-to-3' direction. The A/T-rich region is enclosed within a box. The positions and orientations of the pentanucleotide sequences implicated as important components of the recognition site of T antigen are indicated by arrows. These pentamers also form the core of the 27-bp perfect inverted repeat (nucleotides 5230 to 13). To the left (nucleotides 5213 to 5227) is the 15-bp imperfect inverted repeat.

ful (16, 17, 35, 69). There are a number of other possible mechanisms that could account for the increased replication efficiency observed in the presence of site I. For example, binding of T antigen to site I may facilitate binding of cellular proteins involved in initiation or alter the structure of the template to a conformation more favorable for initiation. In this context it is of interest that site I appears to be a major locus for initiation of nascent SV40 strands that are complementary to the late strand of the viral genome, while the minimal origin of replication contains initiation sites for nascent chains that are complementary to the early strand (31). Thus, it is possible that T antigen bound at site I may increase replication efficiency by promoting initiation events on the late strand.

Previous in vivo studies have demonstrated that viruses with mutations in site I display a cold-sensitive defect in viral multiplication (15, 16). When infection was carried out at 32°C, such mutants displayed reduced plaque size and decreased rates of accumulation of viral DNA and late viral proteins. Because of these observations, we examined the temperature sensitivities of replication of several site I mutants in our in vitro and in vivo assays. Our data indicated that the replication efficiencies of site I mutants relative to the wild type were about fivefold lower at 32 than at 37°C in vivo but were independent of temperature in vitro (data not shown).

The most striking finding of the present study is that both the G/C-rich and 72-bp repeats had significant stimulatory effects on the efficiency of SV40 DNA replication in vivo. These elements have previously been shown to be required for maximal transcription from the SV40 early promoter (2, 21, 28). G/C-rich repeats represent binding sites for SP1, a cellular transcription factor (18, 24). The 72-bp repeats contain enhancer elements that increase transcription by a mechanism that is relatively independent of orientation and distance (1, 22, 45). Some (3, 21), but not all (38), previous studies have suggested that the presence of G/C-rich repeats increases SV40 replication efficiency. The ability of enhancers to stimulate SV40 DNA replication was not detected previously, probably because most of the enhancer deletions that were studied retained the G/C-rich repeats. Our results indicate that the stimulatory effect of enhancers is most apparent with templates that lack G/C-rich repeats. (Similar observations have recently been obtained by A. DeLucia, S. Deb, K. Partin, and P. Tegtmeyer and by G. Lee-Chen and M. Woodworth-Gutai [personal communications]). A stimulatory effect of enhancers on polyomavirus DNA replication has been reported previously (14). The effect was observed with several different enhancers and was independent of their position and orientation.

The G/C-rich repeats and enhancers have no detectable effect on the efficiency of SV40 DNA replication in the cell-free system under standard conditions. It is not likely that inability to detect such an effect is simply due to lack of sensitivity of the in vitro assay. For example, the effect of deletion of site I on in vivo replication efficiency is signifi-

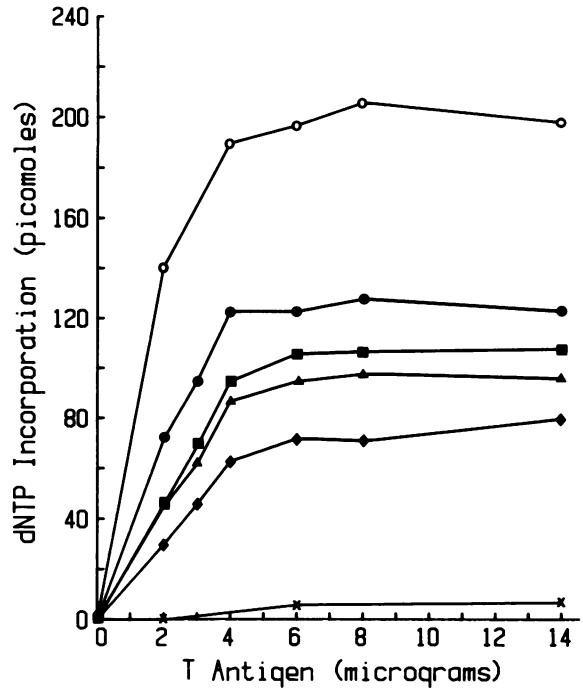

FIG. 7. Replication of site I mutants as a function of T-antigen concentration. Selected plasmids with mutations in T-antigen binding site I were assayed for replication activity in standard reaction mixtures containing extract from uninfected COS-1 cells, 60 ng of plasmid DNA, and various amounts of purified SV40 T antigen. Incorporation of radioactive deoxynucleoside triphosphates (dNTPs) into *Dpn*I-resistant DNA was quantified as described in Materials and Methods. Symbols: ○, pOR.HSO; ●, pOR.1083; ■, pOR.1097; ▲, pOR.1088; ◆,pOR.1085; and ×, pOR.43.

cantly less than the effect of deletion of enhancers and G/C-rich repeats, yet the former effect is readily detectable in vitro. We believe, therefore, that the difference between the in vivo and in vitro data reflects some more fundamental difference in the factors that control replication rates in the two cases.

One possible explanation for the stimulatory effect of enhancers and G/C-rich repeats in vivo is that the presence of these elements in some way facilitates the access of initiation factors to the minimal origin region of SV40 minichromosomes. It seems reasonable to suppose that, in the case of in vivo replication, histones compete with replication factors (and transcription factors) for binding to viral DNA. It follows that some special mechanism may have evolved to minimize such competition. In the case of the in vitro assay, the template is introduced in the form of naked DNA, so it is possible that the need for such a special mechanism may not be as great. The plausibility of this hypothesis is supported by the finding that the origin of replication is preferentially sensitive to endonuclease cleavage in SV40 chromatin isolated from infected cells (8, 59, 72, 73). This nuclease-sensitive region corresponds in position with a nucleosome-free gap that has been observed in SV40 minichromosomes by electron microscopy (58). Several studies suggest that the genetic determinants of the nuclease-sensitive chromatin structure lie within the G/C-rich and 72-bp repeats (22, 23, 33, 37). The observation that either one of these elements is sufficient to induce nuclease sensitivity is of interest in view of our finding that either element was capable of increasing replication efficiency in vivo. Finally, one recent study has shown that insertion mutations that increase the distance between the minimal origin and G/C-rich repeats simultaneously reduce the nuclease sensitivity of the origin and also replication efficiency (33).

Since enhancers and G/C-rich repeats are clearly required for optimal transcription of the SV40 genome, it is possible that the stimulatory effect of these elements on replication may be a result of active transcription through the origin region. Whereas we cannot completely rule out this possibility, it seems unlikely, because deletion of either the G/C-rich or 72-bp repeats had little effect on replication efficiency in vivo when the other element remained intact. On the basis of previously published studies, it would be expected that deletion of either set of repeats would drastically reduce the rate of transcription of the viral genome (2, 21, 28). Thus, it seems more likely that the act of transcription per se is not essential for replication and that binding of specific protein factors to enhancers or G/C-rich repeats increases replication efficiency directly, perhaps by affecting chromatin structure as discussed above. It follows from these considerations that the role of enhancers and G/C-rich repeats in viral multiplication may be rather general. These elements may function to provide access to the origin region for factors involved in a variety of essential processes, including both DNA replication and transcription.

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