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Mutational analysis of the simian virus 40 replicon: Pseudorevertants of mutants with a defective replication origin

(DNA replication/T antigen/regulatory mutants)

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ABSTRACT **The circular genome of simian virus 40 is a model mammalian replicon, containing a unique origin of replication (*ori*) and coding for a protein (SV40 T antigen) known to be involved in initiation of viral DNA replication and to bind *in vitro* to the origin region. Mutations within the *ori* sequence lead to defective viral DNA replication and the formation of small viral plaques after infection of a cell monolayer. Second-site revertants (pseudorevertants) of *ori* mutants were isolated by random local mutagenesis of mutant DNA followed by transfection of cultured cells and the selection of large plaques. In each case, reversion of the plaque phenotype was associated with an increased rate of viral DNA replication. The second-site mutations that suppressed the replication defects were localized by *in vitro* recombination or marker rescue experiments to the gene for T antigen. Their map positions differ from those of previously described T antigen mutants, possibly reflecting a specific *ori*-binding domain of T antigen. From these results we infer that T antigen interacts with the *ori* signal during virus development as it does *in vitro* and that this interaction regulates the rate of viral DNA replication.**

The small circular genome of simian virus 40 (SV40) is a relatively simple mammalian replicon, a DNA molecule that contains a signal for the origin of DNA replication plus gene(s) specifying protein(s) involved in initiating DNA synthesis at the origin (1). SV40 DNA replicates in the nucleus of infected monkey cells, replication commencing at a specific site in the DNA located between the start of early and late genes and proceeding bidirectionally from that site (2, 3). That SV40 DNA codes for a protein involved in the initiation of viral DNA replication was inferred from the observation that tsA mutants of SV40 are defective in initiation of DNA replication at the nonpermissive temperature (4). The mutational alterations in tsA mutants map in the gene for the SV40 T antigen (5). Direct interaction *in vitro* of T antigen with the SV40 replication origin region (6, 7) suggests that specific T antigen binding to the origin signal may be the initial event in SV40 DNA replication.

Recently we have undertaken a mutational analysis of the SV40 replication origin in an attempt to define this regulatory element at the nucleotide sequence level. By means of site-specific local mutagenesis (8), a number of replication-defective mutants have been generated with single base pair substitutions within the DNA segment that contains the replication origin and the *in vitro* T antigen binding site (9). Because these mutations are in a *cis*-acting element that regulates the rate of viral DNA replication, it is possible that this element is the origin signal recognized by the T antigen. If this were the case, a compensating change in the T antigen might suppress the replication defect. To test this possibility we have isolated a series of second-site revertants of defective origin mutants and determined the map positions of the suppressing mutations in the SV40 genome. The compensating mutations map in the gene for T antigen, a result indicating that a direct T antigen–origin interaction plays an important role in the regulation of viral DNA replication.

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MATERIALS AND METHODS

Cell Lines and Viruses. SV40 and its mutants were propagated in monolayer cultures of BSC-40 African green monkey kidney cells (10). The replication origin mutants used are described in ref. 9. Plaque assays were carried out as described (9).

Mutagenesis. Random local mutagenesis of SV40 DNA was carried out by first nicking viral covalently closed, circular, duplex DNA (form I DNA) with pancreatic DNase in the presence of ethidium bromide (11), a reaction that introduces a single nick at random positions in the DNA. The resulting circular duplex DNA with one single-strand break (form II DNA) was treated with *Micrococcus luteus* DNA polymerase I to convert the nick into a small gap and then with bisulfite under conditions estimated to deaminate 30% of cytosine residues in the single-strand gap (8). The gapped, mutagenized DNA was used directly for transfection of BSC-40 cell monolayers (12).

***In Vitro* Recombinants.** *Bcl I*/*Taq* I fragments of viral DNA were separated by electrophoresis in 1.4% agarose gels (10). In the case of *cs* 1031 and its revertants, there is a second *Taq* I site at the origin of DNA replication (9), necessitating the use of partial *Taq* I digestion to prepare the large *Bcl* I/*Taq* I fragment. The appropriate recovered fragments were joined by incubating, for 18 hr at 4°C or at 15°C, approximately 50 ng of each fragment in a 10-μl reaction mixture containing 0.2 unit of T4 DNA ligase (13). After dilution with 15 mM NaCl/1.5 mM Na citrate, the DNA was used to transfect BSC-40 cells by the DEAE-dextran method (12). Each 6-cm dish received from 1 to 5 ng of DNA, which yielded approximately 10 plaques per ng. All enzymes were from commercial sources.

Mapping and Replication Measurement. Mapping by marker rescue was carried out essentially as described (5). Measurement of viral DNA replication was as described in ref. 9.

RESULTS

SV40 *ori* Mutants. In a previous communication (9) we described the construction, by local bisulfite mutagenesis, of SV40 mutants with single base pair substitutions at the origin of viral DNA replication. The base pair substitutions for six

Abbreviations: SV40, simian virus 40; wt, wild type; sr, second-site revertant; form I DNA, covalently closed, circular, duplex DNA; form II DNA, circular duplex DNA with one single-strand break.

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Table 1. *ori* mutants of SV40

Mutant	Susceptibility to *Bgl* I*	Base pair change	Plaque morphology	DNA replication
ar 1026	r	5161 G·C → A·T	wt	wt
shp 1027	r	5155 C·G → A·T	Small, sharp	>wt†
sp 1030	r	5162 G·C → A·T	Small	<wt†
cs 1031	r	5154 C·G → T·A	Small at 32°C wt at 32°C & 40°C	<wt at 32°C† wt at 40°C
cs 1033	s	5158 G·C → A·T	Small at 32°C wt at 37°C & 40°C	<wt at 32°C‡ wt at 40°C
cs 1034	s	5159 G·C → T·A	Small at 32°C wt at 37°C & 40°C	<wt at 32°C‡ wt at 40°C

* r, Resistant; s, sensitive.
† Not corrected by coinfection with wild-type (wt) SV40.
‡ Not tested for correction by wt SV40.

different mutants are shown in Fig. 1, and their properties are summarized in Table 1. In sum, the mutations are in a *cis*-acting element that controls the rate of viral DNA replication—i.e., they are within the operationally defined *ori* signal. It should be noted that four of the mutant DNAs have lost the single *Bgl* I restriction site and, except for the *ar* mutant, each gives rise to plaques distinguishable from those of wild type (wt) SV40. In the experiments to be described below we have concentrated on the two *Bgl* I-resistant mutants with partially or conditionally defective *ori* signals—namely, *sp* 1030 and *cs* 1031.

Isolation of Second-Site Revertants of *ori* Mutants. As a first step in the isolation of second-site revertants (pseudorevertants) of defective *ori* mutants, the DNAs of *sp* 1030 and of *cs* 1031 were randomly mutagenized and used to transfect monolayers of monkey cells. Plaques resembling those of wt virus were then selected at 32°C and tested for the persistence of *Bgl* I-resistant DNA—i.e., for retention of the original *ori* mutation. In order to avoid back mutation at the *ori* site and to minimize multiple mutations at different sites in the DNA, random local mutagenesis with bisulfite was carried out as diagrammed in Fig. 2. For this purpose, mutant form I DNA was randomly nicked once per molecule with pancreatic DNase in the presence of ethidium bromide, the nick was extended into a short gap with *M. luteus* DNA polymerase, and the gapped DNA was treated with sodium bisulfite. The mutagenized DNA was then used to transfect monolayers of BSC-40 monkey cells. Each mutant DNA so treated gave rise to plaques that were larger than those of the original *ori* mutant (Fig. 3). In every case DNA prepared from such cloned virus was still resistant to *Bgl* I; therefore, we infer that each of these large plaque isolates was a second-site revertant of the original *ori* mutant.

Pseudorevertant DNA Replication. Because the small plaque morphology of *sp* 1030 and *cs* 1031 is directly correlated with the lower rates of replication of their respective DNAs in infected monkey cells (9), we assumed that second-site mutations that revert plaque morphology would suppress the replication defect. To test this supposition, rates of replication of second-site revertant DNAs were compared to those of *ori* mutants from which they were derived and also to that of wt SV40 DNA. The rates of replication of sr DNAs exceeded those of their parental *ori* mutants and were approximately equal to or somewhat greater than the rate of wt DNA (Table 2).

Map Positions of Second-Site Mutations. We expected that, for some pseudorevertants, the second mutations might be lo-

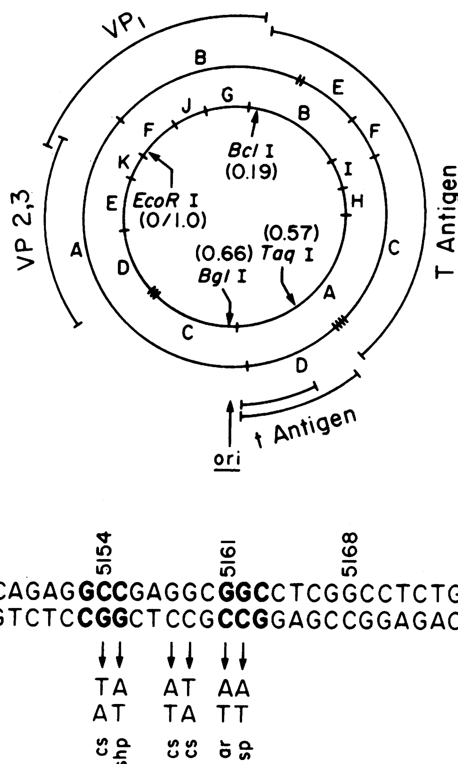

FIG. 1. Map of the SV40 genome and the nucleotide positions of base substitutions of *ori* mutants. The map shows the cleavage sites and map coordinates of restriction endonucleases *Eco*RI, *Bgl*I, *Bcl*I, and *Taq*I; *Hin*dII + III (inner circle) and *Hinf* (outer circle) cleavage sites; origin of DNA replication (*ori*); and the segments that code for T antigens and viral structural proteins VP1, -2, and -3. Shown below is the nucleotide sequence at the origin of replication (14) and base substitutions found in *ori* mutants (9). *cs*, Cold-sensitive mutant; *shp*, sharp plaque mutant; *ar*, altered restriction mutant; *sp*, small plaque mutant. Bold letters represent the *Bgl* I recognition sequence (9).

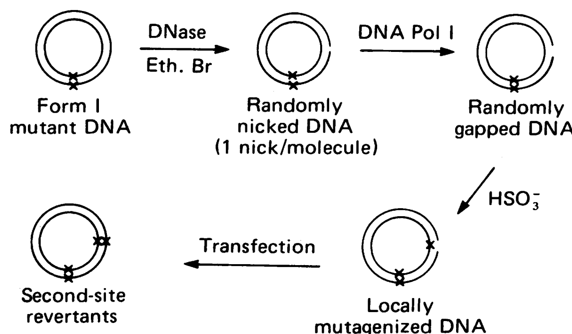

FIG. 2. Scheme for random local mutagenesis, as described in text.

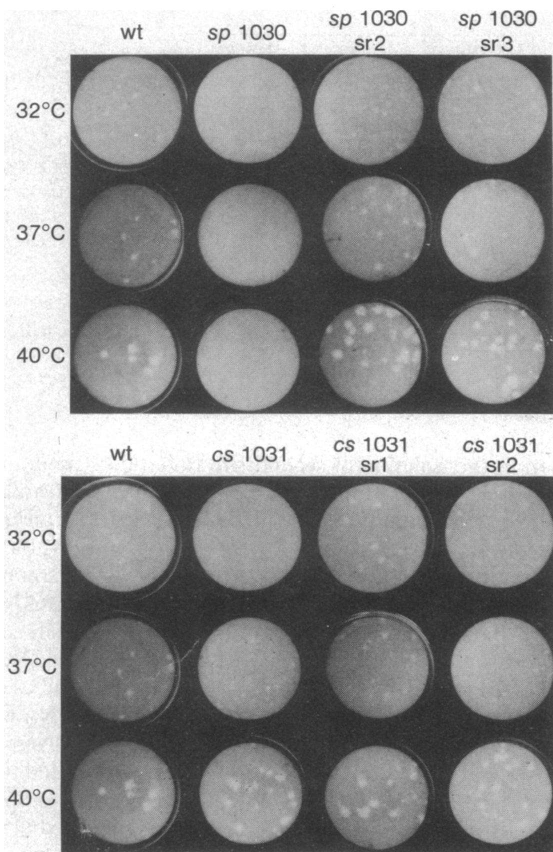

FIG. 3. Representative second-site revertant (sr) plaques of *sp* 1030 and *cs* 1031.

cated in the gene for T antigen, giving rise to a mutant T antigen that compensates for the defective *ori* signal. To test this possibility we mapped the reverting mutations in two independently derived pseudorevertants of each of the above-described *Bgl* I-resistant defective *ori* mutants. In an initial series of experiments, *in vitro* recombinant genomes were constructed consisting of a *Bcl*/*Taq* restriction fragment of a given *ori* mutant DNA and a *Bcl*/*Taq* restriction fragment of DNA from one of its pseudorevertants. (See Fig. 1 for the map positions of the two *Bcl*/*Taq* fragments.) Each recombinant was then used to transfect cell monolayers, and the plaques were scored as mutant or sr type. The results are shown in Table 3. The two pseudorevertants of *sp* 1030 have their second-site mutations within the small *Bcl*/*Taq* fragment (between map coordinates 0.19 and 0.57)—i.e., in the gene for T antigens (see Fig. 1). The same was true for the pseudorevertants of *cs* 1031.

Table 2. Pseudorevertant DNA replication

Mutant	DNA replication, % of wt rate
sp 1030	6.9
sp 1030-sr2	160
sp 1030-sr3	210
cs 1031	35
cs 1031-sr1	132
cs 1031-sr2	151

DNA replication was measured in infected BSC-40 cells (9) by determining the amount of [^{3}H]thymidine incorporated into viral DNA between 24 and 48 hr after infection at 37°C (for *sp* series and its controls) or between 48 and 96 hr at 32°C (for *cs* and its controls). Each value is the mean of duplicate determinations.

Table 3. Mapping by *in vitro* recombination

Source of *Bcl*/*Taq* fragments: Large fragment	Source of *Bcl*/*Taq* fragments: Small fragment	Plaque morphology of recombinant
sp 1030	*sp* 1030	*sp* 1030
sp 1030-sr2	*sp* 1030-sr2	*sp* 1030-sr2
sp 1030	*sp* 1030-sr2	*sp* 1030-sr2
sp 1030-sr2	*sp* 1030	*sp* 1030
sp 1030-sr3	*sp* 1030-sr3	*sp* 1030-sr3
sp 1030	*sp* 1030-sr3	*sp* 1030-sr3
sp 1030-sr3	*sp* 1030	*sp* 1030
None	*sp* 1030	No plaques
None	*sp* 1030-sr2	No plaques
None	*sp* 1030-sr3	No plaques
cs 1031	*cs* 1031	*cs* 1031
cs 1031	*cs* 1031-sr1	*cs* 1031-sr1
cs 1031	*cs* 1031-sr2	*cs* 1031-sr2
None	*cs* 1031	No plaques
None	*cs* 1031-sr1	No plaques
None	*cs* 1031-sr2	No plaques

Plating was at 37°C for *sp* 1030 recombinants and at 32°C for *cs* 1031. Reciprocal recombinants of *cs* 1031 and its pseudorevertants were not tested.

To map the second-site mutations more precisely, we utilized the marker rescue procedure in which a restriction fragment of DNA from a pseudorevertant was hybridized to single-strand circles of DNA from the parent *ori* mutant and the resulting partial heteroduplex was used to transfect cell monlayers (5). The appearance of sr type plaques would indicate that the second-site mutation was in the particular fragment whose heteroduplex gave rise to such plaques. Due to the leakiness of *cs* 1031, this procedure could be used only for mapping *sp* 1030 pseudorevertants. The results of these experiments are given in Table 4. In the case of *sp* 1030-sr2 and -sr3, the mutations that "correct" the plaque morphology defect of *sp* 1030 map in fragments *Hin* A and *Hinf* C (see Fig. 1 for the map positions of these fragments). Therefore, each mutation maps in the overlapping viral DNA segment present in these fragments—i.e., between map coordinates 0.43 and 0.50, in the gene for large T antigen.

DISCUSSION

In this communication we describe the isolation and preliminary characterization of second-site revertants of SV40 mutants with defective replication origins as a way of exploring the interaction of SV40 gene products with the *ori* signal. The *ori* mutants were constructed by site-specific local bisulfite mutagenesis of the genomic segment known to contain the origin of replication and the *in vitro* T antigen binding site. To generate second-site mutations, the DNA of each *ori* mutant was subjected to random local bisulfite mutagenesis. The purpose of this novel procedure was 2-fold: first, to avoid true reversion of the *ori* mutations, all of which are G·C-to-A·T transitions or G·C-to-T·A transversions (see Fig. 1) and therefore not targets

Table 4. Mapping *sp* 1030 pseudorevertants by marker rescue

Source of DNA fragments	Form II DNA, ng	sr plaques/dish, With *Hind* fragments: A	H	I	B	With *Hinf* fragments: C	D+E
sp 1030-sr2	15	12	1	2	0	11	0
	5	2	0	0	0	5	0
sp 1030-sr3	5	7	0	0	0	10	1
	5	5	0	0	0	4	0

for bisulfite mutagenesis; and second, to minimize multisite mutations in a given molecule. The mutants analyzed had second-site mutations in the gene for T antigen, leading to suppression of the replication defect. We interpret this to mean that the mutant T antigens interact with their respective mutant *ori* signals more effectively than does wt T antigen. More generally, the fact that changes in T antigen can suppress the effect of an *ori* mutation strengthens the hypothesis that, within infected cells, T antigen binds specifically at the *ori* site and that this interaction is involved in viral DNA replication. Measurement of binding of mutant T antigens to various *ori* segments *in vitro* would test the hypothesis further.

The precise changes in T antigen associated with suppression of a given *ori* defect are relevant to the recognition of the *ori* sequence by T antigen. Our present data are limited. In the case of *sp* 1030, suppressing mutations mapped between coordinates 0.43 and 0.50, corresponding to amino acid residues 147 and 272 in the 708-amino acid-long large T antigen (14–16). (The suppressing mutations of *cs* 1031 have not yet been mapped as precisely but were nonetheless assignable to a DNA segment coding mainly for large T antigen.) Nucleotide sequence analysis of these and other pseudorevertants should allow us to determine if alteration of specific amino acid residues in T antigen is associated with its ability to recognize modified *ori* sequences. In this regard, we note that the sites of T antigen mutations in *sp* 1030-sr2 and -sr3 differ from those of tsA mutants, which map between coordinates 0.32 and 0.43 (in 12 independent mutants) or between coordinates 0.20 and 0.27 (1 mutant) (ref. 5; unpublished results). Whereas tsA mutations may lead to T antigen inactivation by temperature-dependent changes in overall structure of the protein, the mutations of *sp* 1030 pseudorevertants may affect a domain of T antigen specifically involved in *ori* recognition.

It should be noted that the pseudorevertants described in this report can be viewed as new viral replicons—i.e., DNA molecules with both an altered *ori* signal and an altered gene coding for a protein that interacts with the new signal. Serial mutagenesis or construction of *in vitro* recombinants could result in even more drastically modified replicons. Such new replicons may be useful in understanding the multiple functions of T antigen and SV40 regulatory elements—e.g., their role in cell transformation, integration and excision of viral DNA, and regulation of gene expression. We also point out the generality of the mutational approach to exploring the specific interaction of viral and perhaps cellular proteins with regulatory elements in viral DNA or RNA.

Although we have concentrated in this report on the analysis of pseudorevertants with suppressing mutations in the gene for T antigen, one would expect that additional changes in the nucleotide sequence of the T antigen binding site, or changes in other genetic elements whose products are involved in initiation of DNA replication, might suppress an initial *ori* mutation. Analysis of a larger number of pseudorevertants may thus reveal other interactions at the replication origin.

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