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Biochemical Procedure for Production of Small Deletions in Simian Virus 40 DNA

(endonuclease *Eco*RI and *Hpa*II sites)

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ABSTRACT **A simple biochemical procedure for producing small deletions (15 to 50 base pairs) at virtually any location in simian virus 40 DNA has been developed. The steps involved are: cleavage of the closed-circular DNA to produce a linear structure followed by 5′-exonuclease digestion to expose a short single-stranded segment at each 3′ end of the molecule. Mutants containing deletions at the site of the cleavage are obtained by infecting permissive monkey kidney cells with the exonuclease-treated DNA in the presence or absence of a helper DNA (depending upon whether or not the site of cleavage and therefore the deletion occurred in a gene required for vegetative multiplication). In this paper viable mutants with deletions at the *Hpa*II endonuclease cleavage site (0.735 map position) and defective *trans*-complementable mutants with deletions at the *Eco*RI endonuclease cleavage site (0/1.0 map position) were isolated.**

Simian virus 40 (SV40) mutants having substantial alterations in their DNA can be useful for analyzing the structure, expression, and regulation of the viral genome. Naturally arising mutants having specific deletions, duplications, and substitutions in their DNAs have already been cloned and their physical and phenotypic characteristics described (1, 2). Deletion mutants of SV40 have also been constructed *in vitro* by resection of segments bounded by restriction endonuclease sites (3, 4). Here we report a simple biochemical procedure for producing small deletions (15 to 50 base pairs) at virtually any location in SV40 DNA; in this instance deletions have been generated at the *Eco*RI- and *Hpa*II endonuclease cleavage sites [0/1.0 (9, 14) and 0.735 map position (10), respectively] though other restriction and even nonspecific endonuclease cleavage sites provide many potential locations for generating such deletions.

MATERIALS AND METHODS

DNA and Enzymes. SV40 DNA [strain SVS(5)] was extracted by the procedure of Hirt (6) from CV-1 cells [a continuous cell line derived from African green monkey kidney cells (7)] infected at a multiplicity of 0.01 PFU per cell. After deproteinization, the DNA was centrifuged to equilibrium in CsCl (1.56 g/cm^3) containing ethidium bromide (350 μg/ml). The band of covalently-closed, circular [SV40(I)] DNA was collected, and the ethidium bromide was removed by passing the DNA through Dowex 50 (8).

*Eco*RI endonuclease, *Hpa*II endonuclease, *Hin*dII+III endonucleases, and bacteriophage λ 5′-exonuclease were used according to published protocols [*Eco*RI (9); *Hpa*II (10); *Hin*dII+III (11); λ exonuclease (12, 13)].

Preparation of Linear SV40 DNAs [SV40(L)] with Short Lengths Removed from the 5′-Terminal Regions. SV40(I) DNA was cleaved to unit length, linear molecules with either *Hpa*II or *Eco*RI restriction endonuclease, which cleave SV40(I) DNA once at 0.735 (10) or 0/1.0 (9, 14) map units, respectively. The digestions produced >95% SV40(L_{HpaII}) or 99% SV40(L_{EcoRI}) DNA with the remainder being nicked-circular [SV40(II)] DNA.

The 5′-termini from either the SV40(L_{HpaII}) or SV40-(L_{EcoRI}) DNA (50 μg/ml) were digested with the λ 5′-exonuclease (10 μg/ml) at 0° for 30 min, and then the DNA was deproteinized by phenol extraction. Under these conditions about 25 to 30 nucleotides per strand were made acid-soluble, thereby exposing single-stranded 3′-ends of approximately that length [SV40(L_{HpaII}exo) and SV40(L_{EcoRI}exo)DNAs].

The modified DNA was electrophoresed through a 4% agarose plug (1 cm long, 6 mm diameter, 4 mA per gel, 16 hr). Since linear DNA, but not SV40(I) and (II) DNAs, migrates through 4% agarose (see ref. 15), most of the trace of SV40(I) and (II) DNA remaining after incubation with the restriction endonuclease was removed.

Plaque Assays. Plaque assays were performed on monolayers of CV-1P cells with either SV40 virus or DNA plus DEAE-dextran (2).

RESULTS

Deletions at the *Hpa*II endonuclease cleavage site in SV40 DNA

*Hpa*II endonuclease-cleaved SV40 DNA [SV40(L_{HpaII})] can infect monolayers of CV-1P cells and produce plaques at about 30% the efficiency of SV40(I) DNA (1.5×10^6 PFU/μg compared to 4×10^6 PFU/μg). The plaques produced by the cleaved DNA are wild-type in appearance and yield viral DNA which is circular and sensitive to cleavage by *Hpa*II endonuclease. Unexpectedly, SV40(L_{HpaII}) DNA, treated with λ 5′-exonuclease to remove 25 to 30 nucleotides from the 5′ termini [SV40(L_{HpaII}exo) DNA], still yields plaques on CV-1P monolayers (3.1×10^5 PFU/μg). However, these plaques are much smaller and appear later than those produced by SV40(L_{HpaII}) DNA. Several of the slow-growing mutants were plaque-purified three times and DNA preparations were made from each. In each case, the DNA was resistant to cleavage by *Hpa*II endonuclease (Table 1), indicating that the mutant DNA no longer contained an intact *Hpa*II endonuclease cleavage site.

Abbreviation: SV40, simian virus 40.

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TABLE 1. *Resistance of HpaII-site mutants to cleavage by HpaII endonuclease*

SV40 strain	Number of molecules		
	SV40(I)	SV40(II)	SV40(L)
Wild-type (minus enzyme)	92	8	0
Wild-type (plus enzyme)	0	19	81
Mutant *860*	92	5	3
Mutant *dl 861*	96	4	1
Mutant *dl 862*	91	6	3

After treatment with enzyme, DNA was spread for electron microscopy by the aqueous method of Davis *et al.* (22) and examined in a Phillips EM300.

Naturally arising deletion mutants that lack a small DNA sequence in the region of the *Hpa*II endonuclease cleavage site (16), as well as mutants that contain an insert of poly(dA·dT) at this site†, also show this phenotype.

To test whether these mutants contained a deletion at the *Hpa*II restriction site, the size of the fragments produced by treatment of the mutant DNAs with the *Hin*dII+III restriction endonucleases [these enzymes cleave SV40(I) DNA at 11 specific sites (17)] was examined. If the *Hpa*II endonuclease cleavage site of the mutant is deleted, *Hin*dII+III fragment C (0.655 to 0.760 on the SV40 map), which contains the *Hpa*II endonuclease cleavage site, should be smaller than the corresponding wild-type fragment. This proved to be the case for mutants *dl 861* and *dl 862* (Fig. 1). The *Hin*dII+III fragment C generated from the DNA of each of these mutants migrated faster on acrylamide gels than the wild-type fragment, indicating that the mutant fragment was smaller. Since the difference in the relative mobilities of *Hin*dII+III fragment C from mutant and wild-type DNAs reflects the difference in their molecular length, we can estimate the approximate size of the deletions. Mutants *dl 861* and *dl 862* lack 32 and 53 base pairs, respectively.

Mutant 860, however, gave an anomalous result (Fig. 1). The C fragment produced in the *Hin*dII+III digest of this mutant migrated more slowly than the wild-type C fragment, suggesting that the C fragment of the mutant is about 15 base pairs larger than the wild-type fragment. Consistent with this view is the observation that one of the three fragments produced by digestion of the mutant DNA with the *Hpa*I endonuclease [this enzyme cleaves SV40(I) DNA at three specific sites (10)] migrated slightly slower than the corresponding fragment from wild-type DNA. The precise nature of the modification in *mutant 860* is not understood at the present. We cannot rule out the possibility that this mutant is derived from an *Hpa*II endonuclease-resistant SV40(I) DNA present in the original wild-type DNA.

Deletions at the *Eco*RI endonuclease cleavage site in SV40 DNA

When SV40 DNA cleaved at the *Eco*RI restriction site [SV40(L_{EcoRI}) DNA] was digested with λ 5′-exonuclease so as to remove 25 to 30 nucleotides from the 5′-termini, the specific infectivity of the DNA dropped to a very low value (2×10^3 PFU/μg). DNA prepared from infections with the virus from these plaque isolates was as sensitive to *Eco*RI endonuclease cleavage as wild-type DNA; most likely, these plaques were produced from residual uncleaved or undigested SV40 DNA.

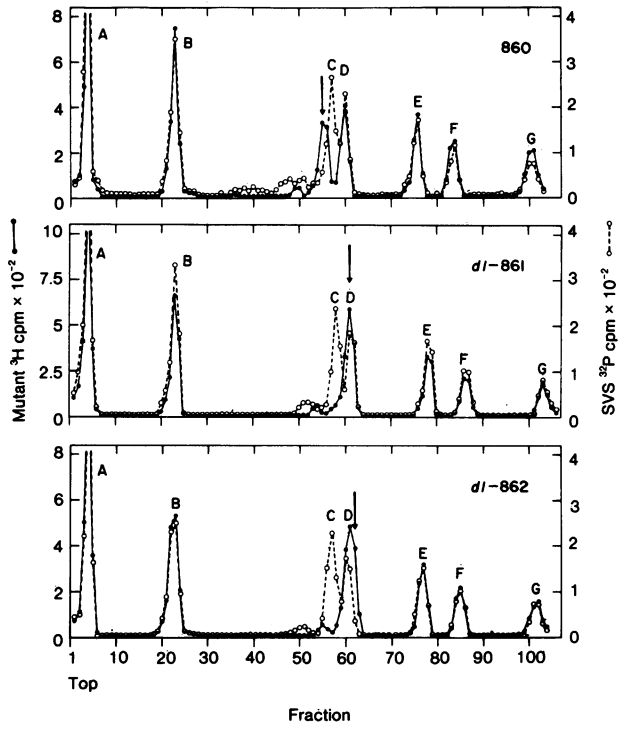

FIG. 1. *Hin*dII+III fragments produced by cleavage of *Hpa*II endonuclease-resistant DNAs. Mixtures of ^{3}H-labeled mutant DNA (●——●) and ^{32}P-labeled wild-type DNA (O- - -O) were digested with the *Hin*dII+III endonucleases and then analyzed on polyacrylamide gels. (Strain SVS is the wild-type parent SV40.) The gels (0.6 cm in diameter; 23 cm long) contained 5% acrylamide and 0.25% *N,N′*-methylene bis-acrylamide in Tris–borate buffer (89 mM Tris-OH, 89 mM boric acid, 2.5 mM EDTA, pH 8.2). Electrophoresis was at 150 V for 18 hr. Slices (1.5 mm) were cut, treated with 0.3 ml of NCS Solubilizer (Amersham/Searle) at room temperature overnight, and then analyzed in a scintillation counter. The arrows indicate the position at which the altered C fragments of the mutants migrate. In this experiment the four smallest *Hin*dII+III fragments had migrated off the end of the gels but in shorter runs the mutant and wild-type fragments H, I, J, and K were found to co-migrate.

SV40 mutants having deletions of the *Eco*RI restriction site can be grown and serially plaque-purified if the cells are co-infected at 41° with an SV40 mutant temperature-sensitive for the expression of an early function [*tsA30* (18)] (2). Accordingly, a mixture of SV40(L_{EcoRI}exo) and *tsA30* DNA was used to infect CV-1P monolayers at 41°. Under these conditions, the specific infectivity of the SV40(L_{EcoRI}exo) DNA increased to 1×10^5 PFU/μg. After three successive plaque purifications (as mixed plaques) viral DNA was prepared from three clones. In each case the DNA was only partially cleaved by *Eco*RI endonuclease (Table 2). This would be expected if the DNA was a mixture of *Eco*RI-sensitive *tsA30* DNA and of DNA that lacked the *Eco*RI endonuclease cleavage site.

The mixture of DNAs was subjected to three successive cycles of cleavage with the *Eco*RI endonuclease. After each

† J. Carbon, T. E. Shenk, and P. Berg, "Construction *in vitro* of mutants of SV40: Insertion of a poly(dA·dT) segment at *Hemophilus parainfluenzae* II restriction endonuclease-cleavage site," manuscript submitted.

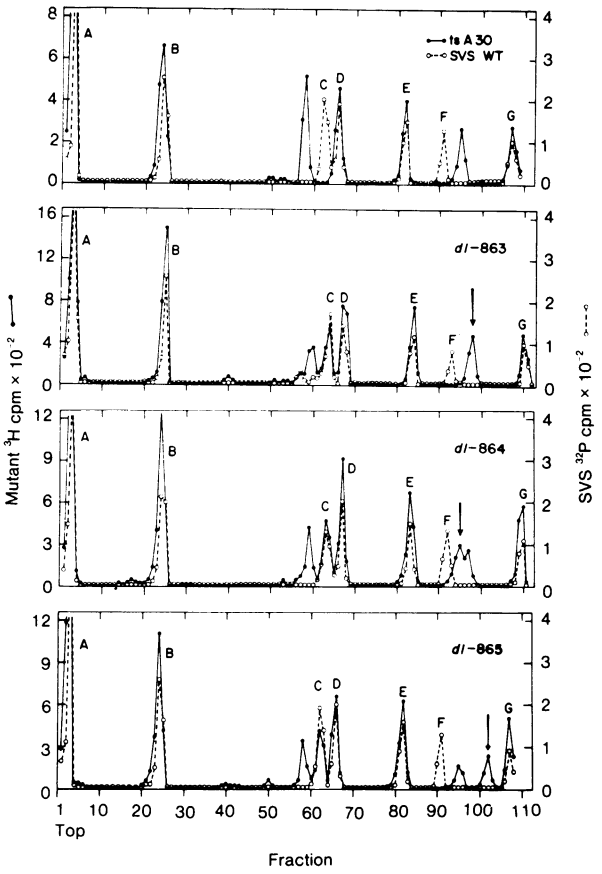

FIG. 2. *Hin*dII+III fragments produced after cleavage of *Eco*RI endonuclease-resistant DNAs. Mixtures of ^{3}H-labeled mutant DNA (●——●) and ^{32}P-labeled wild-type DNA (O- - -O) were digested with the *Hin*dII+III endonucleases and analyzed on 5% polyacrylamide gels. In the top panel, *tsA30* DNA is compared to SVS DNA. These DNAs differ most markedly in fragments C (*tsA30* fragment C migrates slower) and F (*tsA30* fragment F migrates faster). Therefore, in experiments in which mutant DNA has been cleaved with the *Hin*dII+III endonucleases (mutant DNA is a mixture of *Eco*RI endonuclease-resistant DNA and *tsA30* DNA), additional C and F fragments are found due to the presence of *tsA30* DNA. The arrows indicate the position at which the altered *Eco*RI-endonuclease-resistant F fragments migrate. Again, mutant and wild-type fragments H, I, J, and K were found to co-migrate in other experiments.

cycle, mutant DNA, resistant to the endonuclease, was separated from cleaved *tsA30* DNA by equilibrium centrifugation in CsCl/ethidium bromide gradients (see *Materials and Methods*). As expected, infection with *Eco*RI-endonuclease resistant DNAs alone produced no plaques in a DNA plaque assay (Table 3). However, plaques were produced if the monolayer was coinfected with *tsA30* DNA, showing that *tsA30* can complement the defective function in the *Eco*RI-endonuclease-resistant DNA. On the other hand, DNA from a mutant defective in a late function, *tsB4* (18), could not complement the *Eco*RI-resistant DNAs. We conclude that the alteration of the *Eco*RI endonuclease site affects the B cistron function but does not alter expression of the A function.

The modifications at the *Eco*RI endonuclease cleavage site also proved to be deletions as evidenced by examination of *Hin*dII+III digests of these DNAs (Fig. 2). With each of the *Eco*RI-resistant DNAs, the *Hin*dII+III-generated F fragment (0.985 to 0.06 on the SV40 map), which contains the *Eco*RI endonuclease cleavage site, migrated faster than the corresponding F fragment from wild-type DNA. The increase in mobility of the mutant fragment indicates that the sizes of the deletions are approximately 23, 15, and 47 base pairs in mutants *dl 863*, *dl 864*, and *dl 865*, respectively.

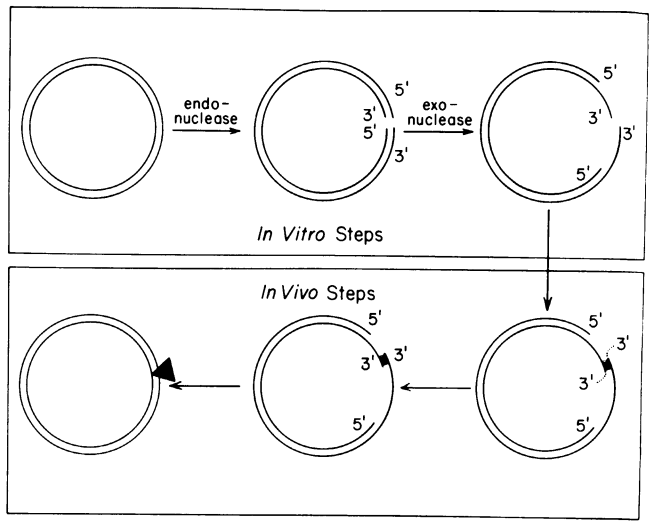

FIG. 3. Biochemical and cell-mediated steps in production of small deletions in SV40 DNA. The cell-mediated pathway is hypothetical.

DISCUSSION

How do these deletion mutants arise? The steps we perform are quite simple: cleavage of the closed-circular DNA to produce a linear structure followed by 5′-exonuclease digestion to expose a short single-stranded segment at each end of the molecule (Fig. 3). The more complex operations are performed by the cell. One plausible explanation is that, after infection with these modified DNA molecules, the cells can generate circular structures by forming base pairs between short complementary sequences in the single-stranded 3′-hydroxyl terminal segments. Once formed, covalently closed circular duplex DNA may be regenerated by removal of unpaired DNA and repair of the gaps (Fig. 3). Presumably all genetic information proximal to the 3′-hydroxyl end from the site of base pair information is lost in the repair process. Consequently, a deletion is introduced at the site at which the circular molecule was originally cleaved to form linear DNA. A similar explanation has been invoked to account for deletions that extend beyond the restriction sites when *Hin*dIII endonuclease-cleaved SV40 DNA is resealed following infec-

TABLE 2. *Resistance of mixed clones to cleavage by EcoRI endonuclease*

SV40 strain	Number of molecules		
	SV40(I)	SV40(II)	SV40(L)
Wild-type	0	2	98
dl 863	60	3	37
dl 864	51	16	33
dl 865	51	8	41

DNA was spread for electron microscopy by the aqueous method of Davis *et al.* (22) and examined in a Phillips EM300.

TABLE 3. *Specific infectivity of EcoRI endonuclease-resistant DNAs*

Complementing DNA	Specific infectivity (PFU/μg)		
	dl 863	*dl 864*	*dl 865*
None	$<10^2$	$<10^2$	$<10^2$
tsB4	$<10^2$	$<10^2$	$<10^2$
tsA30	3.8×10^5	2.8×10^5	1.8×10^5

CV-1P cells were infected with a mixture of *Eco*RI endonuclease-resistant DNA and the indicated *ts* mutant DNA (10 ng), and incubated at 41°. Plaques were counted on day 10. Neither *tsB4* nor *tsA30* DNA produced plaques alone.

tion (3). And the same phenomenon may account for the observation by Murray and Murray (19) that deletions are occasionally generated at the sites where bacterial cells rejoin *Eco*RI endonuclease-generated fragments of bacteriophage λ.

Little published information is available concerning how much base homology is required to permit circularization of structures such as SV40(Lexo). With SV40(L_{EcoRI}) and SV40-(L_{HpaII}), cohesive ends of four A·T base pairs (20) and two G·C base pairs (21), respectively, are sufficient to permit rejoining of the ends. But in these instances the rejoining would be stabilized by the reestablishment of the original base stacking interactions. Circularization requiring base pairing of two single strands, as suggested in our proposal (Fig. 3), may require longer regions of homology. The probability of forming three to four contiguous complementary base pairs anywhere between two random polynucleotide segments about 25 to 30 nucleotides long is greater than 0.9, while the probabilities of forming five or six contiguous base pairs are of the order of 0.5 and 0.1, respectively (S. Goff, T. Landers, and P. Berg, unpublished result). Quite possibly the cell can stabilize such weak interactions sufficiently to permit the enzymatic trimming and repair needed to effect ring closure as visualized in our scheme.

Our model is consistent with the finding that linear molecules with single-stranded "tails" of 25 to 30 bases yielded mutants with deletions of approximately 15 to 50 base pairs. More extensive exonuclease treatment, to expose longer single-stranded "tails," could result in larger deletions and increase the probability of circularization. (Our calculations suggest a probability of about 0.9 for a homologous run of 7 bases between two random sequences of 200 bases.)

Particularly noteworthy is the fact that deletions of this sort can most likely be introduced at any point where the SV40 DNA (or possibly any circular infectious DNA) can be cleaved, whether by restriction enzymes or non-specific endonucleases. For example, a viable SV40 mutant with a deletion of about 25 base pairs at map position 0.17 (Shenk, Carbon, and Berg, unpublished observation) has been isolated from DNA that had been cleaved once with DNase I in the presence of Mn^{2+} (presumably at random locations) and treated with λ exonuclease. [The location of a randomly produced deletion can be precisely determined by a biochemical mapping procedure utilizing S1 nuclease (23).] Cell-mediated circularization can also be effected with a shortened SV40 linear DNA produced by successive cleavages with *Hpa*II and *Eco*RI endonucleases and treatment with λ exonuclease; in this instance, the *Hpa*II restriction site at map position 0.735 is joined to the *Eco*RI restriction site at map position 0/1.0, to produce a molecule with a deletion of about 28% in the late region (M. Dieckmann and P. Berg, unpublished observations).

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