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Mapping Simian Virus 40 Mutants by Construction of Partial Heterozygotes

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Simian virus 40 temperature-sensitive mutants *ts A28, A30, B1, B11,* and *D101* are associated with the region of the genome defined by the restriction endonuclease fragments *Hin*d-I, H, F, G, and E, respectively.

We have used fragments of simian virus 40 (SV40) DNA produced by restriction endonuclease *Hin*dIII (6 fragments) and *Hin*d (11 fragments) (9) to map SV40 temperature-sensitive (*ts*) mutants. Fragments of wild-type DNA were mixed, one at a time, with linear (*Eco*RI endonuclease digested) *ts* DNA plus *Hin*dIII- or *Hin*d-digested *ts* DNA; the mixture was denatured and reannealed. One of the products of reannealing will carry, annealed to a strand of *ts* DNA, a strand of the fragment of wild-type DNA plus strands of fragments of *ts* DNA, in the form of a multiply nicked circular molecule. Virions can be produced from the denatured and reannealed DNA (7), but only if the site of mutation is within the fragment of wild-type DNA added will a large number of wild-type virions arise. In this way five *ts* mutants have been associated with three *Hin*dIII fragments, and further with five *Hin*d fragments of SV40 DNA. Similar results have been obtained by Lai and Nathans (5; submitted for publication).

SV40 strain VA45-54 and its mutants *ts A28, A30, B1,* and *B11* (12, 13) were obtained from P. Tegtmeyer. The mutant *ts D101* (8) was from J. Robb. Small plaque SV40 (11), a derivative of strain 776, was from H. Smith. Virus was propagated in CV-1 cells and SV40 DNA prepared as described previously (3). DNA was digested with excess restriction endonuclease *Eco*RI (4), *Hin*dIII (9), or *Hin*d (10). *Hin*d enzyme was reconstituted from *Hin*dII and *Hin*dIII (9). For some experiments, *Hin*dIII was further purified by passing 150 μg of enzyme over a column (0.9 by 4.5 cm) of carboxymethyl cellulose. Fragments of wild-type DNA produced by *Hin*dIII were separated by continuous elution electrophoresis on a 1.5% agarose gel (6), and *Hin*d fragments were separated by electrophoresis into 4% polyacrylamide gel slabs (0.4 by 15 by 40 cm long) (2). *Hin*d fragments of ^{32}P-labeled DNA were located on gels by autoradiography, and the DNA was eluted by stirring crushed gel bands with 4 volumes of 0.3 M sodium acetate in 0.001 M EDTA-0.01 M Tris-hydrochloride, pH 7.4 (TE). Supernatants from this step, or fractions from the continuous elution apparatus, were freed of contaminating gel by adsorption to 0.4-ml columns of benzoyl napthoyl DEAE-cellulose, washing with 0.3 M sodium acetate in TE, and eluting with 1.5 M NaCl-15% ethanol in TE. After dialysis and concentration by drying, DNA was precipitated with ethanol and resuspended in TE. For denaturation and reannealing, 70 μl containing 0.09 μg of closed-circular or linear DNA, plus fragmented DNA, was mixed with 10 μl of 2.2 M NaOH, neutralized after 10 min with 20 μl of 2 M KH_2PO_4, and incubated for 45 min at 68 C. Cells were infected with DNA by the method of McCutchan and Pagano (7). Reannealed DNA, after dialysis against 1/10× TE and drying, was resuspended in 0.27 ml Tris-buffered Eagle minimal essential medium (7), diluted with an equal volume of medium plus 1.8 mg of DEAE-dextran (Sigma; mol wt = 2×10^6) per ml, and sterilized with $CHCl_3$. CV-1 cell monolayers were washed once with phosphate-buffered saline and inoculated with 0.2 ml of a DNA-DEAE-dextran mixture. After 20 min at room temperature, cells were washed twice with Hanks balanced salt solution and overlaid with 5 ml of minimal essential medium plus 4% fetal calf serum. (Infection by this procedure and assaying for plaque formation gave 10^6 PFU of form I DNA per μg; *Eco*RI linear DNA was 10% as infectious as form I.) After 5 days at 32 C (one cycle of growth), virions were harvested by twice freezing and thawing, and debris was removed by centrifugation.

To map mutations into *Hin*dIII fragments, each separated fragment of wild-type DNA was mixed with *Hin*dIII-digested plus *Eco*RI-digested (linear) *ts* DNA, with both total *Hin-*

dIII digest and separated fragment in fivefold molar excess over linear DNA. Each experiment included control reactions using wild-type closed-circular (form I) DNA, *ts* DNA only, the six separated fragments alone, and six separated fragments plus linear *ts* DNA with or without HindIII digest. The presence of the *ts* digest, or remixed separated fragments in controls, avoids the need for extremely pure fragments: there is uniform "enhancement" of the activity of each fragment by the others (cf. [5]). After denaturation and reannealing, the DNA was used to infect CV-1 cells at 32 C. Virions, which presumably arose by the action of cellular

TABLE 1. *Recovery of wild-type SV40 from ts DNA and separated Hind III fragments of wild-type SV40 DNA*[a]

DNA mixtures			PFU/ml at 40 C				
ts DNA		Wild-type DNA					
RI linear	*Hind* III		A28[b]	A30	B1	B11	D101
+	+		≤20;[c] ≤20	220;[d] 20[e]	≤20;[d] ≤20[e]	≤20; ≤20[f]	≤20; ≤20[f]
+	−	A to F[g]	11,000; 6,000	1,100; 1,500	2,150; 2,550	13,500; 750	550; 80
+	+	A to F	6,500; 3,350	1,750; 800	550; 2,300	2,850; 275	1,750; 225
+	+	A	≤20; 10	20; 50	900; 2,550	3,050; 1,250	≤20; ≤20
+	+	B + C	25; 205	ND; ≤20	≤20; 25	≤20; 10	≤20; ND
+	+	D	9,000; 13,500	2,450; 3,150	ND; ≤20	≤20; ≤20	150; 10
+	+	E	10; ≤20	ND; ≤20	≤20; ≤20	≤20; ≤20	1,800; 110
+	+	F	≤20; 5	ND; ≤20	60; ≤20	≤20; ≤20	≤20; 20
−	−	A to F	(≤20)	(145; ≤20)			
−	−	Form 1	(6.5 × 10[6])	(2.0 × 10[6])	(2.0 × 10[5])	(5.0 × 10[5])	

[a] Pairs of numbers give results of two experiments. ND, Not done.
[b] *ts* mutants.
[c] Zero plaques per 0.2 ml is ≤20/ml with 95% confidence.
[d] Wild-type DNA was strain 776.
[e] Adsorption to and elution from benzoyl napthoyl DEAE-cellulose was omitted.
[f] Fragments of wild-type DNA had been stored 4 months at 4 C.
[g] *Hind* III fragments A through F.

TABLE 2. *Recovery of wild-type SV40 from ts DNA and separated Hind fragments of strain VA45-54 wild-type DNA*[a]

DNA mixtures			PFU/ml at 40 C				
ts DNA		Wild-type DNA					
RI linear	*Hind*		A28[b]	A30	B1	B11	D101
+	+		≤20;[c] ≤20	45;[c] ≤20	5;[c] ≤20	20;[c] 20	≤20;[c] ≤20
+	−	A to K[d]	12,000; 3,100	700; 17,000	9,500; 8,500	15,500; 2,100	600; 1,150
+	+	A to K	8,500; 5,600	3,000; 8,500	8,000; 8,000	8,500; 1,300	700; 650
+	+	A	450; 750	≤20; 45	55; ≤20	85; 20	250; 105
+	+	B	5; ≤20	85; 20	5; ≤20	10; 20	250; ≤20
+	+	C	≤20; ≤20	5; 25	≤20; ≤20	5; 20	≤20; ≤20
+	+	D	≤20; ≤20	300; 85	≤20; ≤20	≤20; 20	50; ≤20
+	+	E	≤20; ≤20	70; 10	45; ≤20	100; 20	1,000; 1,600
+	+	F'[e]	≤20	55	1,850	205	450
+	+	F	10; ≤20	10; 650	750; 9,500	195; 20	10; ≤20
+	+	G	≤20; ≤20	≤20; 15	175; ≤20	11,000; 2,150	5; ≤20
+	+	H	500; 30	600; 5,000	≤20; ≤20	≤20; ≤20	≤20; ≤20
+	+	I	10,000; 12,000	55; 15	≤20; ≤20	≤20; ≤20	≤20; ≤20
+	+	J	40; ≤20	≤20; 215	≤20; ≤20	≤20; ≤20	≤20; ≤20
+	+	K	30; 260	≤20; 20	≤20; ≤20	≤20; ≤20	≤20; ≤20
−	−	A to K	(≤20; ≤20)				
−	−	Form 1	(2 × 10[6]; 2.3 × 10[6])		(1.4 × 10[6]; 6.5 × 10[5])		

[a] Pairs of numbers are results of two separate experiments.
[b] *ts* mutants.
[c] *Hind* digestion of *ts* and wild-type DNA used *Hind* III not passed over carboxymethyl cellulose.
[d] *Hind* fragments A through K.
[e] In one preparation of separated fragments, the radioactivity expected in band *Hind*-F was in two bands between E and G. The upper, minor, band was called F', the lower was called F, and the two were treated as separate fragments.

TABLE 3. *Recovery of wild-type SV40 from ts DNA and separated Hind fragments of strain 776 wild-type DNA*

DNA mixtures			PFU/ml at 40 C				
ts DNA		Wild-type DNA	A28[a]	A30	B1	B11	D101
RI linear	Hind						
+	+		≤20	≤20	≤20	≤20	≤20
+	−	A to K[b]	1,700	1,400	750	3,000	4,200
+	+	A to K	2,300	1,300	1,450	3,200	10,000
+	+	A	165	≤20	≤20	80	35
+	+	B	≤20	≤20	5	≤20	85
+	+	C	90	≤20	≤20	≤20	80
+	+	D	≤20	≤20	≤20	≤20	45
+	+	E	≤20	≤20	15	25	3,600
+	+	F	≤20	≤20	2,100	≤20	450
+	+	G	≤20	≤20	≤20	3,750	≤20
+	+	H	35	3,700	5	≤20	≤20
+	+	I	9,500	≤20	5	≤20	≤20
+	+	J	≤20	≤20	≤20	≤20	≤20
+	+	K	≤20	50	≤20	≤20	≤20
−	−	A to K	(≤20)				
−	−	Form 1	(2.5×10^6)				

[a] *ts* mutants.
[b] *Hind* fragments A through K.

repair enzymes, were harvested after one cycle of growth. Wild-type virions among these were assayed on TC-7 cell monolayers; assay dishes were incubated for 22 h at 32 C and then 10 days at 40 C. The period at 32 C and the one cycle of growth at 32 C were intended to avoid interference by possible *trans*-dominant mutations (work of others [5] in which DNA mixtures were used directly for plaque assay suggests the mutations are in fact not *trans*-dominant). For each mutant, a particular fragment of wild-type DNA gave a high titer of wild-type virus (Table 1). We therefore associate *ts A28* and *A30* with *Hind*III-D, *ts B1* and *B11* with HindIII-A, and *ts D101* with *Hind*III-E.

To refine the mapping, exactly analogous experiments were done with fragments produced by *Hind* endonuclease (Table 2). Again, each mutant can be associated with a particular fragment: *ts A28* with *Hind*-1, *A30* with H, *B1* with F, *B11* with G, and *D101* with E.

The order of *Hind* fragments has been determined for SV40 strain 776 (2). Two methods were used to compare the DNA and restriction endonuclease fragment sequences of strain 776 with those of strain VA45-54. (i) Heteroduplexes formed by DNA of strain VA45-54, strain 776, and by an equimolar mixture of each, were spread and examined by electron microscopy (methods described in [1]). No deletions, additions, or substitutions were detected in one strain relative to the other. (ii) It was possible that fragments were interchanged in gel position by way of additions and deletions too small to be seen by electron microscopy. However, each *ts* mutant mapped in the same fragment of strain 776 DNA (Table 3) as strain VA45-54 (Table 2). We conclude that at least fragments E, F, G, H, and I are in the same order in the two strains. The results presented here thus confirm and are in full agreement with those of Lai and Nathans (5).

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