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Genetics

Viable deletion mutants in the simian virus 40 early region

(*Taq* I site/T antigens/transformation)

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ABSTRACT **For the purpose of isolating *hr-t*-like mutants of simian virus 40, we have constructed variants that have lost the unique site for the restriction enzyme *Taq* I at 0.565. Five mutants have been isolated and characterized by restriction enzyme analysis. All of them produce a normal size T antigen. Four produce a t antigen reduced in size as well as in amount; the fifth one does not seem to make any t antigen at all. The ability of these mutants to transform mouse cells *in vitro*, as tested by anchorage dependence, is clearly altered; however, the defect is only partial. In the same test, the mutants can complement a *tsA* mutant for transformation and therefore define a second complementation group in the simian virus 40 early region.**

The two papovaviruses, simian virus 40 (SV40) and polyoma, are alike in most aspects of their molecular biology. They share equivalent genetic complementation groups except for the *hr-t*, which has been described only in polyoma. *hr-t* mutants were isolated by using polyoma-transformed mouse cells as a permissive host and a normal 3T3 cell as a nonpermissive host (1). They have lost the ability to transform cells *in vitro* (1, 2) and to induce tumors in animals (3). These mutants belong to a single complementation group (2); they map in the 5′ part of the early region (4) and define a second early gene clearly distinct from the A gene (5, 6). A similar host-range selection has not been possible as yet for SV40 essentially because no 3T3-like monkey cell is available. However, Shenk *et al.* (7) have described a series of viable SV40 mutants that map in the 0.54/0.59 region topologically homologous to the locus of the polyoma *hr-t* mutations. These mutants do not show any host range and are fully competent for transformation, as tested by the ability to induce cell growth in a medium containing low amounts of serum (7). In the search for an *hr-t*-like SV40, we have selected a series of SV40 variants that have lost the unique site for the restriction enzyme *Taq* I at 0.565 and that therefore should be genetically close to Shenk's mutants. We report here their initial characterization.

MATERIALS AND METHODS

Cells and Viruses. The monkey CV-1 cell line used to propagate SV40 was grown in minimal essential medium supplemented with 0.3% tryptose phosphate (MCV-1 medium) and 10% calf serum (Sorga). Transformation assays were performed on mouse NIH 3T3 cells. These cells were grown in Dulbecco's modified medium (Gibco H21) supplemented with 10% calf serum. The SV40 wild type was the large-plaque strain provided by H. G. Suarez (8).

Viral DNA Preparation. SV40 DNA was extracted from CV-1 cells infected at low multiplicity [0.001 plaque-forming unit (PFU) per cell]. When at least 75% of the cells showed a cytopathic effect, the cells were lysed according to Hirt (9) and SV40 form I DNA was purified from the high salt supernatant by phenol extraction and two steps of isopycnic centrifugation in CsCl/ethidium bromide gradients.

Enzyme Degradation and Gel Electrophoresis. Restriction enzymes *Hin*dIII, *Hin*fI, *Alu* I, and *Hae* III were purchased from New England Biolabs (Beverly, MA, USA). Digestions were performed at 37° for 1 hr in 50 mM NaCl/6 mM Tris·HCl, pH 7.6/6 mM $MgCl_2$/6 mM 2-mercaptoethanol. The DNA digests were run on 4% acrylamide gels made in 40 mM Tris·HCl, pH 7.8/20 mM sodium acetate/2 mM EDTA. The DNA was stained by ethidium bromide and visualized under short-wave UV light. Bacteriophage λ 5′-exonuclease was prepared as described (10).

Taq I was prepared in this laboratory according to protocol B described by Sharp *et al.* (11) for purification of *Hpa* II. The enzyme was used at 68° for 1 hr in 50 mM NaCl/6 mM Tris·HCl, pH 7.4/6 mM $MgCl_2$/6 mM 2-mercaptoethanol. The *Taq* I linear SV40 molecules were purified by electrophoresis on a 3% acrylamide/0.5% agarose composite gel. The DNA was eluted from this gel by electrophoresis.

Preparation of Cell Extracts, Immunoprecipitation, and Electrophoresis. CV-1 cells (5×10^6) were plated in 10-cm petri dishes and infected 24 hr later with SV40 at an input multiplicity of 20 PFU per cell. Forty-eight hours after infection the cells were labeled for 3 hr with 50 μCi of [^{35}S]methionine per ml (Amersham, 400–550 Ci/mmol) in methionine-free medium containing 2% dialyzed calf serum, the cells were then scraped, washed twice with ice-cold phosphate-buffered saline, and suspended at a concentration of 10^7 cells per ml in Tris-buffered saline, pH 8 (12), containing 0.5% Nonidet P-40, 10% glycerol, and 2 mM diisopropylfluorophosphate (Serva) and sodium *p*-hydroxymercuribenzoate (Sigma) as protease inhibitors. After 20 min at 4°, nuclei were removed by centrifugation for 10 min at $800 \times g$ and the cytoplasmic extract was clarified by centrifugation (1 hr at $105{,}000 \times g$). The supernatant was brought to 0.5 mg/ml with bovine serum albumin/2 mM methionine and then incubated with 2 μl of hamster anti-T serum or 2 μl of a control hamster serum and 20 μl of settled protein A-Sepharose CL 4B (Pharmacia) as described (13). The anti-T serum used was a pool of sera from Syrian hamsters bearing tumors induced by inoculation of SV40-transformed hamster cells (TSV-5 Cl. 2). Control serum was a pool of sera from 20 normal adult Syrian hamsters. All sera had been exhaustively adsorbed with extracts of spontaneously transformed syrian hamster cells (EHB) and normal calf serum powder. After overnight incubation at 4° with constant agitation, the Sepharose beads were washed three times with 0.2 ml of 0.1 M Tris·HCl, pH 8.8/0.5 M LiCl/1% 2-mercaptoethanol. Immune complexes were then eluted from the Sepharose beads with 50 μl of electrophoresis sample buffer [80 mM Tris·HCl, pH 6.8/2% sodium dodecyl sulfate/5% 2-mercaptoethanol/15% (wt/vol) glycerol/0.001% bromophenol blue], incubated at 100° for 10 min, and analyzed by electrophoresis in polyacrylamide gels as described by Laemmli (14). Running gels were either 6 or 14% in acrylamide. Electrophoresis was carried out at room

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Abbreviations: SV40, simian virus 40; MCV-1 medium, minimal essential medium supplemented with 0.3% tryptose phosphate; PFU, plaque-forming unit.

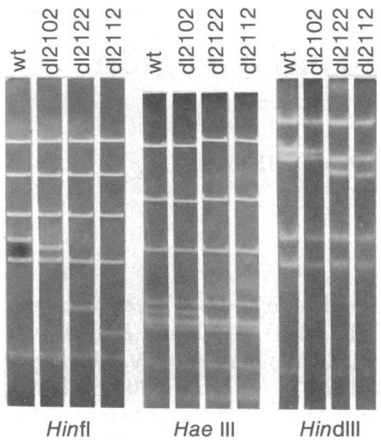

FIG. 1. Four percent polyacrylamide gel electrophoresis of restriction enzyme digests.

temperature at 20 mA for 6–8 hr. The gels were stained with Coomassie brilliant blue, destained, dried, and exposed to Kodirex x-ray films usually for 2–4 days. The following molecular weight standards were used to calibrate the gels: RNA polymerase (β' 165,000; β 155,000; σ 87,000; α 39,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), aldolase (40,000), chymotrypsinogen (25,700), trypsin inhibitor (21,500), and β-lactoglobulin (18,400).

Plaque Assay. DNA infection was by the method of MacCutchan and Pagano (15). Virus stocks were titrated by infecting 2.5×10^5 CV-1 cells in 35-mm dishes with serial dilutions. After 1 hr of adsorption, the plates were overlayed with 3 ml of agar medium (MCV-1, 0.9% agar, 0.5% calf serum, and antibiotics). After 12 days at 37°, 1.5 ml of neutral red agar medium (MCV-1, 0.9% agar, and 0.2% neutral red) was added; plaques were counted the following day.

Transformation Assay. The ability to induce growth of nonpermissive cells in agar was the only property tested. NIH 3T3 cells (5×10^4) infected in suspension for 1 hr at 37° were suspended in soft agar as described (16), with MCV-1 medium containing 5% calf serum in the top and bottom agar layers.

RESULTS

Isolation of *Taq* I-resistant mutants

The method of Carbon *et al.* (17) was followed. Purified *Taq* I-linear SV40 DNA (50 μg/ml) was exposed to λ 5′-exonuclease (20 μg/ml) at 0° for 30 min in 67 mM glycinate buffer, pH 9.4/2.5 mM $MgCl_2$. The modified DNA was then directly used for plaque assay on CV-1 cells. DNA infectivity was 1/10th the original value as a result of the 5′-terminal digestion. The actual reasons for this loss of infectivity are not clear; however, the fact that it is limited suggests that the integrity of the region around the *Taq* I site is not absolutely required for lytic growth on CV-1. A similar observation has been made after the removal of a few unessential nucleotides at the *Hpa* II site (17). By reference to the growth properties of most of the dl 54/59 mutants isolated by Shenk *et al.* (7) and also to the plaque phenotype of most of the polyoma *hr-t* mutants, we selected small-sized plaques and were able to pick 5 clones (out of 12) whose DNA was *Taq* I resistant. In order to get extensively purified DNA from wild-type viruses, the DNA from these four clones was treated by *Taq* I and the resistant fraction was purified on a 1.4% agarose gel. The eluted DNA was used to infect CV-1 cells at high dilution, plaques were picked, and the virus was cloned a third time. DNA was prepared from the five isolates for restriction enzyme analysis.

Restriction enzyme analysis

The DNAs from the five isolates were *Taq* I resistant, as expected. Four enzymes, namely, *Hin*dIII, *Hin*fI, *Alu* I, and *Hae* III, were used for a detailed analysis of the part of the genome carrying the *Taq* I site. Part of this analysis is illustrated in Fig. 1, which shows the *Hin*dIII, *Hin*fI, and *Hae* III restriction patterns of dl 2102, dl 2122, dl 2112, and wild-type DNAs. In all instances the differences observed between the wild-type and the mutants can be explained on the basis of a single deleted segment. The three mutants show *Hin*fI D and *Hin*dIII B fragments shorter than those from the wild type. No difference between dl 2102 and the wild type could be detected on the basis of the *Hae* III restriction pattern because the deletion is small and does not affect the mobility of the large *Hae* III A fragment. On the contrary, the *Hae* III A/E junction at 0.590 has been deleted in dl 2122 and dl 2112, causing the appearance of a fused (A + E) fragment migrating slightly ahead of the original A fragment. *Alu* I (patterns not shown) was used to define the 3′ limit of the deletion. In all the mutants but dl 2112 the *Alu* I B fragment was shorter than in the wild type. *Alu* I B and C fragments were missing in dl 2112 and, instead, a fused (B + C) fragment slightly larger than *Alu* I B appeared. Therefore, the deletion in dl 2112 extends over the *Alu* I B/C junction at 0.545. The two other mutants, dl 2132 and dl 2121, were analyzed in the same way. Taken together, these data give a reasonably accurate localization of the deletions although their exact limits on both sides are still undetermined.

The deletions vary in size from 20 to 310 base pairs (Table 1), as calculated from the electrophoretic mobility of the *Hin*fI fragments (Fig. 2). They can be sorted into two extreme classes: a small-sized deletion class represented by dl 2132 and dl 2102, in which 20–30 base pairs have been deleted; and a large-sized deletion class represented by dl 2121, dl 2122, and dl 2112, in which 250–310 base pairs have been deleted. No variant showing a deletion of an intermediate size was found. A fine map of the region showing the limits of the deletion is presented on Fig. 3.

The DNAs from dl 891 and dl 883, two of the deletion mutants isolated by Shenk *et al.* (7), were analyzed with the same set of restriction enzymes. Shenk *et al.* (7) had mapped dl 891 at 0.590 and estimated the deletion to be 41 base pairs long. In agreement with the mapping, we find a shorter *Hae* III E fragment, but our electrophoretic mobility data suggest a larger alteration (100 base pairs). For dl 883 we also find a larger deletion (90 base pairs instead of 23), which causes the loss of the *Alu* B/C function of 0.545. Therefore, dl 891 and dl 883 overlap the 5′ and 3′ ends of our dl 2112 deletion, respectively.

Table 1. Size of deletions and their consequence on molecular weight of t antigen

	Mutant				
	dl 2132	dl 2102	dl 2121	dl 2122	dl 2112
Location of deletion on physical map	0.565	0.565	0.55–0.60	0.55–0.60	0.54–0.60
Size of deletion (base pairs)	20	30	270	250	310
Expected M_r loss on t	700	1000	9000	8300	10,300
Actual M_r loss on t	500	500	2500	2000	No detectable t

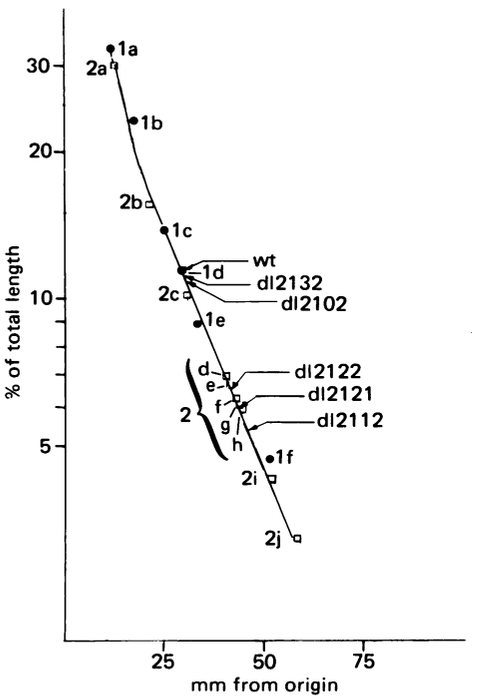

FIG. 2. Semilogarithmic plot of fragment mobility against percent of total genome length. The *Hin*fI (●) and *Hae* III (□) fragments from our wild type (LP) were used as reference. Arrows indicate the positions of the *Hin*fI D fragments from deletion mutants.

Size of early antigens

Sera from animals bearing an SV40-induced tumor immunoprecipitate two major polypeptides from SV40-induced monkey cell as well as from SV40-transformed cell extracts (20–23). The apparent molecular weights of these two polypeptides, as measured by polyacrylamide gel electrophoresis, are 90,000 for T and 17,000–19,000 for t. They share common NH_2-terminal sequences (24). The synthesis of these two polypeptides *in vitro* is specifically directed by early SV40 mRNA, which strongly suggests that they actually represent primary gene(s) products of the early region (20, 22, 23). The existence of deletion mutants in this region provides a good tool for exploring the expression of the early functions. CV-1 cells infected by the wild type or *Taq* I deletion mutant viruses were labeled for 3 hr with [^{35}S]methionine at 48 hr after infection. Cell extracts were prepared under conditions that minimize proteolysis, and immunoprecipitation was performed. Fig. 4 shows the analysis of the immunoprecipitates by sodium dodecyl sulfate/acrylamide gel electrophoresis. On Fig. 4*A*, a 14% acrylamide gel is shown. In two different wild-type-infected cell extracts (wt 776 and our LP wild type), the immune serum specifically precipitates two polypeptides with M_r of 90,000 and 19,000, respectively. These polypeptides are totally absent in mock-infected cell extracts and are not precipitated by preimmune sera (Fig. 4*B*).

The T antigen is precipitated in extracts of cells infected by all the mutants tested, dl 2102, dl 2101 (a subclone isolated from the original dl 2102 plaque), dl 2121, dl 2122, dl 2112, and dl 891. No alteration of M_r of this polypeptide can be detected on this gel. A more accurate test for M_r alteration of large polypeptides was performed by using a 6% acrylamide gel on extracts from cells labeled with ^{32}P (Fig. 4*C*). On this type of gel the apparent M_r of the T antigen was 86,000, as opposed to 90,000 on a 14% gel. However, no difference could be detected between any of the mutants and the wild-type T antigen. In dl 2122 and dl 2121, 250-base-pair deletions would be expected to reduce the size of the T antigen by 9000, a difference which should certainly have been detected on a 6% gel.

The wild-type-sized t antigen is totally absent from extracts of cells infected by the deletion mutants (Fig. 4*A*). New polypeptides with M_r 18,500 (dl 2102), 17,000 (dl 2122), 16,500 (dl 2121), and 12,500 (dl 891) were clearly observed instead. Their amount relative to the amount of the 90,000 M_r protein is clearly reduced as compared to wild-type-infected cells. We fail to detect any t antigen in dl 2112-infected cells.

The absence or low yield of t antigen might result from a rapid turnover due to the altered structure of the mutant polypeptides. This possibility was tested by analyzing extracts of [^{35}S]methionine-labeled dl 891-, dl 2112-, and LP-infected cells. The results are shown in Fig. 4*C*. A 30-min pulse neither reveals t antigen in dl 2112 nor strikingly improves its labeling in dl 891. We then examined the synthesis of both wild-type and mutant t antigens in mixed infections at equal multiplicity (20 PFU per cell). In this experiment the infected cells were labeled for 3 hr and, therefore, the patterns shown in Fig. 4 *A*

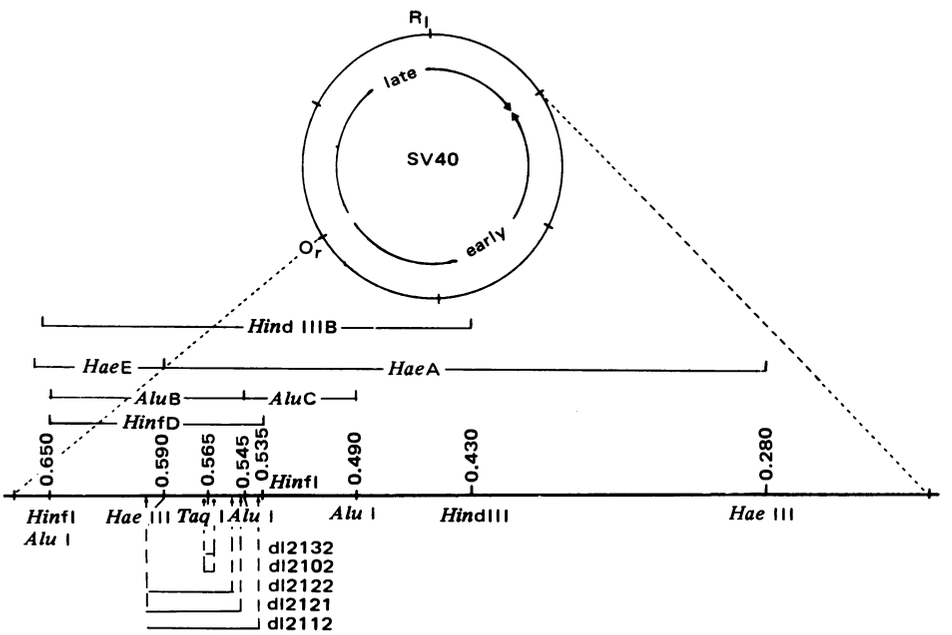

FIG. 3. Physical map of the SV40 early region. The sequences missing in the deletion mutants are indicated.

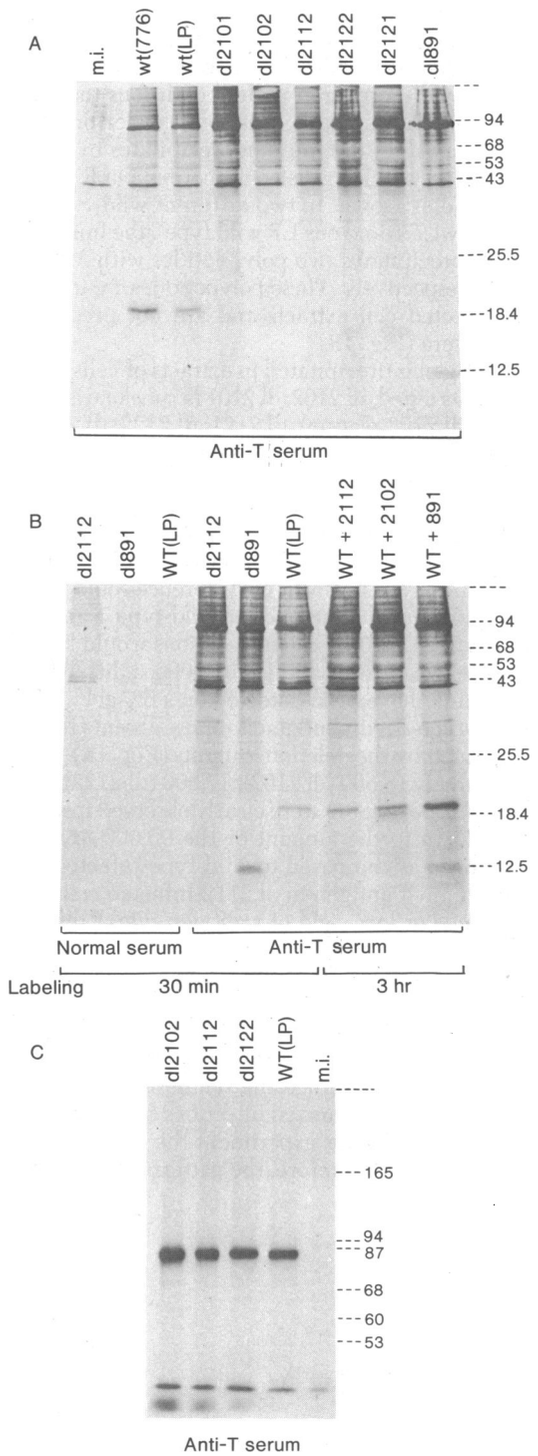

FIG. 4. Polyacrylamide gel electrophoresis analysis of the polypeptides precipitated by a serum from SV40-induced tumor-bearing hamster. Numbers on the right side of the gels indicate the position of M_r markers ($\times 10^{-3}$). (*A*) 14% gel. Infected cells were labeled for 3 hr. m.i., mock infected. (*B*) 14% gel. Labeling, immunoprecipitation, and mixed infections were performed as indicated. (*C*) 6% acrylamide gel. Infected cells were labeled 48 hr after infection for 3 hr with ^{32}P (100 μCi/ml) in phosphate-free minimal essential medium.

and *C* are comparable. The respective yields of wild-type and mutant t antigens are not changed in mixed as compared to single infections. This suggests that no negative interference is exerted by the mutant over the wild type, at least at the level of t antigen production, and that the presence of wild-type t antigen does not stimulate the appearance of t in dl 2112 or

Table 2. Transformation by deletion mutants and complementation for transformation between deletion mutants and tsA-58

Virus	m.o.i.*	Transformation efficiency†
(*A*) Transformation by deletion mutants		
Wild type	20	120
Wild type	2	250
dl 2102	100	1
dl 2112	400	0.1
dl 2122	200	2
(*B*) Complementation for transformation		
Wild type	10	100
dl 2102	10	<2
dl 2122	10	<2
tsA-58	10	<2
dl 2102 + tsA-58	10 + 10	10
dl 2122 + tsA-58	10 + 10	8
dl 2102 + dl 2122	10 + 10	<1

No macroscopic colonies could be detected when 5×10^4 mock-infected cells were plated in agar.
* Multiplicity of infection.
† Transformation units/PFU $\times 10^{-6}$.

increase its yield in dl 2102- or dl 891-infected cells.

The primary conclusion from these data is that the deletions affect a region that is part of the t antigen, but not of the T antigen, coding sequences.

Transforming ability of mutants

The ability of SV40-transformed cells to grow in agar appears to be the transformation character that correlates the best with the ultimate biological oncogenic property, namely, the ability to induce tumors in animals (25). *hr-t* mutants of polyoma are strictly defective for the induction of growth in agar in various cell systems (1, 2). We have tested our dl mutants for this property by using NIH 3T3 cells. The results (Table 2*A*) show that these mutants have at least 1/100 the transforming ability of their wild-type parent. For dl 2112, which carries the most extensive deletion, the ratio goes to 1/1000. The defect is not absolute but was strong enough to be used to test for complementation with an *A*-group mutant. The experiment was done on NIH 3T3 at 39° with dl 2102, dl 2122, and tsA-58 as a representative of the *A* group, at multiplicities of 10 PFU per cell for each virus. While no clone appeared in any of the single infections, complementation was observed between tsA-58 and either dl 2102 or dl 2122 (Table 2*B*), providing evidence for a second complementation group in the early region of SV40.

DISCUSSION

We have isolated a series of viable deletion mutants of the early region of SV40 by selecting variants that have lost the unique *Taq* I site. The high freqency of appearance of such mutants strongly suggests that the region carrying the *Taq* I site is dispensable for lytic growth on CV-1 cells. A concordant observation had been made previously by Shenk *et al.* (7). We show that as many as 300 base pairs can be removed in this region without altering the viability of the resulting virus. Unlike the polyoma *hr-t* mutants, these SV40 deletion mutants have not yet exhibited any host-range effect. By analogy with polyoma (2, 26), this could imply that all the monkey cells tested can complement these mutants by permanently providing a set of functions required for lytic growth.

Biochemical evidence has been recently provided that establishes the existence of post-transcriptional processing of SV40 mRNAs. It has been shown that the original early transcript

gives rise to two prominent species of mRNA having two different spliced sequences. One of these mRNAs lacks the sequence between 0.54 and 0.59; the other lacks the sequence between 0.54 and 0.545 (27). The existence of two polypeptides coded for by the early region of SV40 is largely documented (20–23). Our data support the concept that T antigen is coded by the mRNA lacking the 0.54/0.59 transcript. Since deletions in this region do not affect the size of T polypeptide, it seems reasonable to conclude that the 0.54/0.59 region is not represented in T mRNA. Crawford *et al.* (19) have reached similar conclusions and Schaffhausen *et al.* (18) have recently shown analogous results with respect to normal T antigen coded by deleted polyoma *hr-t* mutants.

The size of the dl 2112 deletion that totally removes this region is an indication of the minimal size of the sequence absent in T mRNA. With the exception of dl 2112, which fails to produce t, the deletions in the 0.54/0.59 region do cause measurable modifications of the M_r of t antigen. This suggests that this polypeptide is actually coded by the other mRNA that only lacks the 0.54/0.545 region. A serious discrepancy exists, however, between the observed and the predicted values of the M_r of the t synthesized by the various mutants (Table 1). Actually a 25- or a 275-base-pair deletion has almost the same consequence. The most likely explanation of this paradox is that the large deletions cause a frameshift. In the new reading frame, beyond the end of the deletion the nonsense codon that terminates t antigen translation is no longer recognized as a stop. The translation can proceed until it finds a nonsense codon further downstream, probably around map position 0.52. A testable consequence of such a model is that the COOH-terminal part of mutant t antigens should be abnormal. The same kind of explanation holds for dl 891: in this case a nonsense codon is read just beyond the 3'-end of the deletion, causing the release of a 12,500 M_r polypeptide. This M_r fits with the value predicted from the physical localization of the dl 891 deletions.

The existence of such viable frameshift mutants is consistent with the notion that the COOH-terminal part of t antigen is dispensable for lytic growth. Another possibility is that this polypeptide is not required at all in the lytic cycle in cultured cells. On the contrary, it is almost certainly required in some aspects of transformation, since its alteration has a drastic effect on the ability of the virus to induce cell growth in agar. However, since transformation is not totally abolished, it could be argued that the retention of a partial biological activity in our deletion mutants can be attributed to the synthesis of a polypeptide that retains the NH_2 terminus of t.

Besides the absence of host-range restriction, this leakiness for transformation is a major difference between the SV40 0.54/0.59 deletion mutants and the polyoma *hr-t* mutants, which are strictly defective for transformation (1, 2). This observation is rather puzzling since in both types of mutants the genetic alterations affect the same region. These phenotypic differences might result from more profound genetic differences reflecting the selection procedures used. The polyoma *hr-t* mutants have been isolated in a biological way, namely, on the basis of a host-range selection. However, the SV40 0.54/0.59 deletion mutants have been artificially constructed and selected for their viability. Alternatively, the rodent cells used in all the transformation experiments might respond differently to a rodent virus (polyoma) or a primate virus (SV40).

As shown, the *Taq* I deletion mutants can complement tsA-58 for transformation, as measured by the ability to stimulate cell growth in agar. This clearly suggests the existence of a second gene in the early region of SV40.

Note Added in Proof. Sleigh *et al.* [(1978) *Cell* **14**, 79–88] have independently isolated a series of dl 54/59 mutants that seem to be phenotypically much like ours.

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