Molecular cloning and restriction mapping of a Simian virus 40 deletion mutant derived from Simian transformants expressing a non-karyophilic T antigen

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A replication-defective Simian virus 40 genome, with a deletion of about 120 nucleotides in the region encoding the N-terminal fourth of the large T antigen, has been isolated from the DNA of Simian cells transformed by SV40. Both the original transformants, and the murine transformants obtained by transfection with this cloned mutant DNA, produced a large T antigen displaying in immunofluorescence an exclusively cytoplasmic localization. The protein apparent molecular mass (83 kDa) was about 6% smaller than that of normal karyophilic large T. Restriction analysis showed that the deletion eliminated two close *Hin*fI sites, at nucleotides 4459 and 4376 (map unit 0.50).

SV40 large T antigen *Karyophilic protein* *Oncogenic transformation* *Molecular cloning* *Restriction mapping*

1. INTRODUCTION

The large T antigen of SV40 is a multifunctional protein with an apparent molecular mass 88 kDa, encoded by the viral gene A, and predominantly localized in the nuclei of both productively infected and transformed cells (review [1]). We report here the isolation from Simian transformants of a SV40 genome carrying a deletion of about 120 nucleotides at 0.50 map units (i.e., in the N-terminal fourth of the large T). The product of the mutated gene is an 83-kDa large T that has lost its characteristic karyophily, as shown by cytoplasmic localization in immunofluorescence. This large T cannot induce viral DNA synthesis, but has retained the competence to transform cultured cells.

2. MATERIALS AND METHODS

Sources and cultures of CV1 cells, cs3 cells, wt SV40, SV40 tsA239, as well as plaque assays, cell fusion, and T antigen immunofluorescence were as in [2,3]. COS7 cells were kindly provided by D. Smith, NIH-3T3 cells by C. Basilico and SV40 tsBC11 by P. Tegtmeyer. Transfections were carried out by the calcium method [4] with 1 μg of either wt SV40 DNA (linearized with *Bam*HI) or mutant SV40 DNA (excised from recombinant DNA with *Bam*HI) and referred to as 4.85 kb DNA) and 15 μg of cell DNA carrier, in 6-cm dishes containing 6×10^5 cells.

The procedures used for molecular cloning were essentially those described for the establishment of genomic libraries in [5]. λ-Charon 30 arms were isolated, after *Bam*HI digestion of the annealed DNA, by centrifugation in 10–40% sucrose gradients; traces of the 7.4 kb stuffer fragment present in this type of preparation appeared to help the formation of viable recombinants even with relatively small *Bam*HI fragments of foreign DNA. For subcloning in pBR322, the 4.85 kb DNA of a recombinant phage DNA was purified by agarose gel electrophoresis, then ligated into the (phosphatase-treated) *Bam*HI site of the plasmid

Abbreviations: bp, base pair; kb, kilobase; wt, wild-type; SVn, nucleotide number in the SV system [1]

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DNA and used to transform *E. coli* HB101.

T antigen immunoprecipitation was carried out with hamster anti-T serum (Meloy, Springfield, VA) as in [6]; electrophoresis was in 12% polyacrylamide gels as in [7].

3. RESULTS

A line of semipermissive mutants of Simian CV1 cells, cs3 [2,3], was transformed with SV40 tsBC11, a late mutant defective for the coat assembly, but normal for the early functions [8]. Twelve small colonies growing in soft agar were propagated in 2% serum, recloned, then analyzed for the presence of SV40 integrated genomes by Southern blot-hybridization. All but one of these clones exhibited the integration into cellular DNA of either partial of interrupted viral genomes; the remaining clone, called CTS, exhibited a pattern

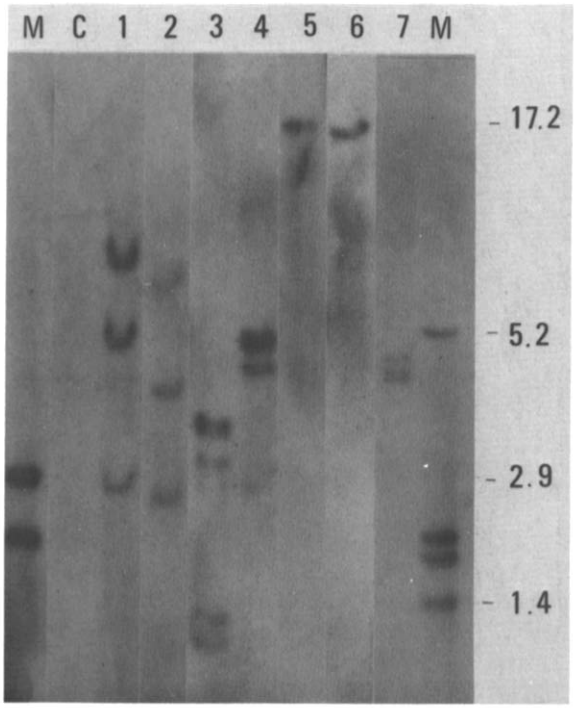

Fig.1. Southern blot hybridization of CTS cell DNA digested with various restriction endonucleases. (1) *Eco*RI; (2) *Eco*RI + *Bam*HI; (3) *Eco*RI + *Bgl*I + *Bgl*II; (4) *Bgl*I + *Bgl*II; (5) *Bgl*II; (6) *Eco*RV; (7) *Hha*I + *Bgl*II; (M) markers; (C) cs3 cell DNA, digested with *Eco*RI. The probe was nick-translated total SV40 DNA. Numbers on the right indicate kilobases.

indicating a tandem head-to-tail insertion of at least 2 copies of nearly the entire SV40 genome (fig.1). The digestion of cellular DNA with endonuclease *Eco*RI, which cuts once into SV40 DNA, originated 3 bands (track 1). The intermediate band, migrating as a 4.85 kb fragment, was found by digestion with a variety of enzymes (only a few of which are shown here) to represent the repeated insert of an SV40 genome lacking about 350 nucleotides in the late region surrounding the *Eco*RV site (0.79 map unit). Digestion with enzymes such as *Bgl*II, that do not cut into SV40 DNA, or *Eco*RV, whose site is deleted, showed that the viral insert was contained into a single band of about 17 kb, and that no free copies of viral DNA were present in cells (tracks 5,6). At this level of resolution no alteration of the early region was clearly detectable, but finer analysis (see below) showed the presence of a smaller, and more important, deletion at about 0.50 map unit.

The analysis of T antigen production in CTS cells by indirect immunofluorescence showed negative nuclei but brightly positive cytoplasms (fig.2A). Under the same conditions, the nuclei of Simian transformants COS7 [9], producing a normal T protein, and of CV1 cells infected with the original transforming virus, were stained as expected (fig.2B,C).

The electropherograms of the T antigen immunoprecipitates from CTS cells (fig.3A) showed a main band comigrating, at 83 kDa, with the second of the 2 or 3 forms of large T commonly detected in lytically infected cells [6]. In view of the deletion in the T antigen gene described below, this result seems likely to reflect the production of a natively smaller protein (83, instead of 88 kDa), rather than the effect of endogenous proteolysis. Two additional data, not illustrated here, deserve to be mentioned: (i) a large T of a size identical to that shown above (83 kDa) was immunoprecipitated from 3T3 cells transformed by the cloned 4.85 kb DNA (see below); (ii) a normal small t antigen band, comigrating at 17 kDa in all cell types, could be observed by slightly overloading the gels, or avoiding a double immunoprecipitation of cell extracts.

The results of two types of experiments suggested that the mutant cytoplasmic T antigen was not able to support viral DNA replication. First, the ability was tested of CTS cells to complement

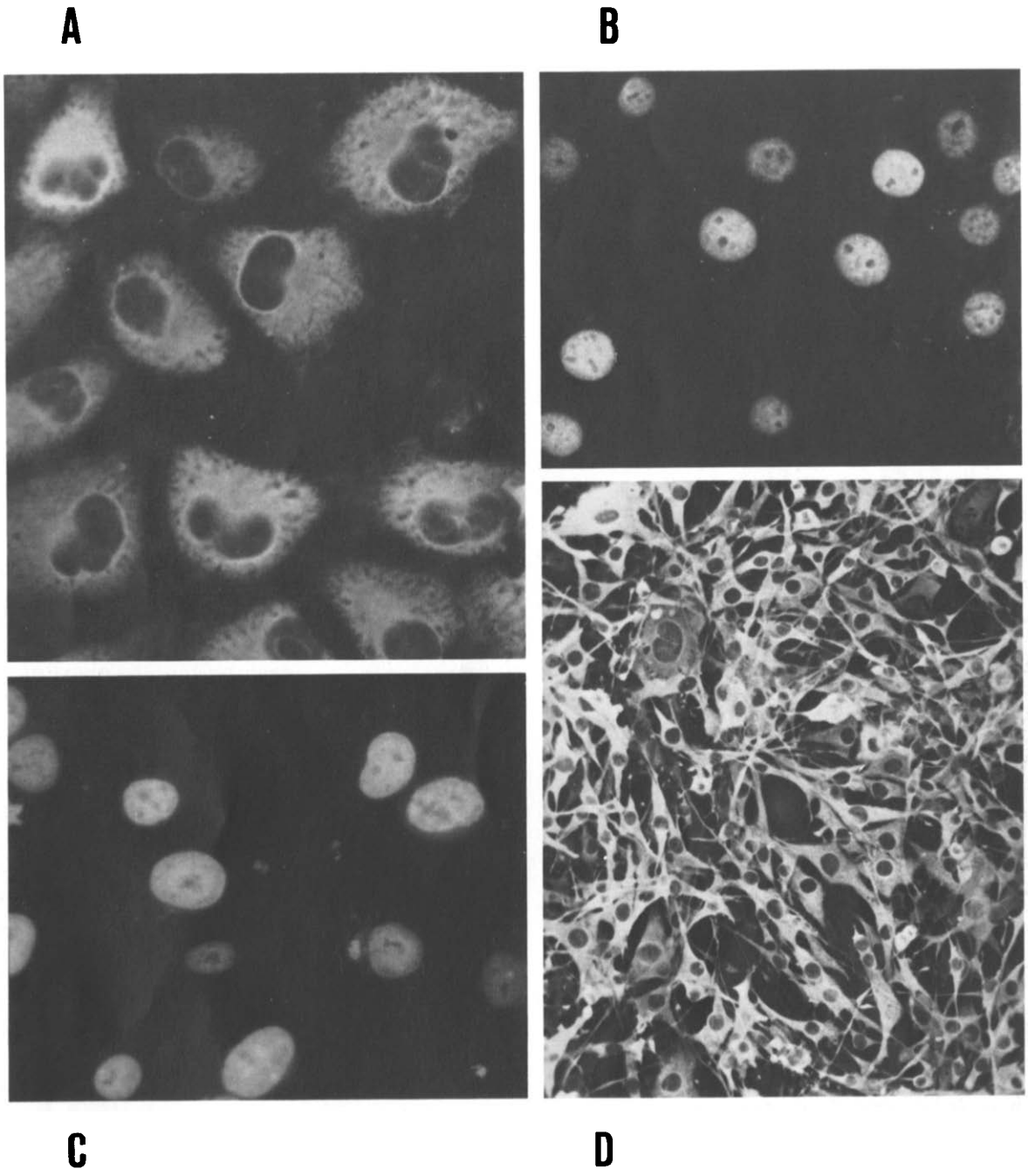

Fig.2. Indirect immunofluorescence assay of SV40 T antigen. (A) CTS cells (400×), (B) COS7 cells (400×), (C) CV1 cells infected with SV40 tsBC11 (400×), (D) CTM cells (0.3% agar-selected colony of NIH3T3 cells transformed by the SV40 deletion mutant DNA. 160×).

the growth at high temperature of an SV40 tsA mutant (tsA239). It was found that while the tsA virus grew in CTS cells at 33°C (albeit very slowly: the input was only 6-fold multiplied 5 days post infection), at 39°C it did not grow at all. In the second kind of experiment, we induced the fusion

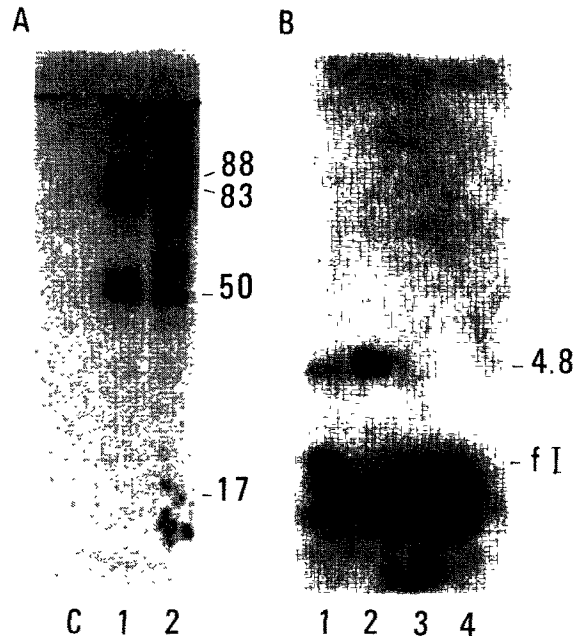

Fig.3. (A) SDS-polyacrylamide gel fluorogram of SV40 T antigen, immunoprecipitated from cells labeled with [^{35}S]methionine (250 μCi/ml) for 3 h. (C) nonimmune serum; (1) CV1 cells infected with wt SV40; (2) CTS cells. Numbers on the right indicate kilodaltons. (B) Southern blot hybridization of Hirt extracts of heterokaryons of CTS cells and either COS7 (1,2) or CV1 (3,4) cells. Each extract (approximately 6×10^5 cells) was electrophoresed half as such (1,3), half after *Eco*RI digestion (2,4), in a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide. The probe was nick-translated total SV40 DNA. On the right, the linear (4.8) and supercoiled (fI) forms of the 4.85 kb DNA are indicated.

with polyethylene glycol of CTS cells to either CV1 cells or COS7 cells. As shown in fig.3B, synthesis of free viral DNA occurred only upon fusion to COS7 cells, but not to CV1 cells. This result indicated that the origin of the viral cytoplasmic-T genome was indeed able to function, if a normal T antigen was made available. In this case the normal T was supplied by the SV40 ori$^-$ genome integrated into COS7 DNA, a genome unable to replicate but encoding a regular T protein [9].

The enrichment in SV40 mutant DNA (4.85 kb DNA), thus obtained in heterokaryons, was exploited for its molecular cloning. The Hirt extract illustrated in fig.3B was treated with endonuclease *Bam*HI, then placed in a ligation reaction with λ-Charon 30 arms and packaged. Upon hybridization with a nick-translated SV40 DNA, positive plaques were detected with a frequency around 10^{-4}. The 4.85 kb DNA derived from the *Bam*HI digest of a recombinant λ DNA was eventually subcloned into the *Bam*HI site of plasmid pBR322.

Transfection of NIH3T3 cells with the cloned mutant DNA efficiently originated stable transformants (CTM cells), with production of foci in monolayers and colonies in semisolid medium. Fig.2D shows the typical cytoplasmic-T pattern exhibited by colonies of these cells selected in soft agar. A detailed description of the transforming properties of this deletion mutant will be reported elsewhere.

The early region of the mutant SV40 genome was then examined for possible DNA alterations. Preliminary digestions, not shown here, with endonuclease *Hin*dIII, directed the attention to the only early fragment that did not comigrate with its wild-type equivalent. This was *Hin*dIII-fragment B, which contains 1169 bp in wt SV40 DNA (see [1] for SV40 general restriction map, and fig.4C for details of this DNA region), but appeared to be reduced by about 10% in the mutant DNA (fig.4A, tracks 1 and 2).

The wt *Hin*dIII-fragment B is further split by endonuclease *Hin*fI into the following sequential fragments: 543 bp-fragment D, 25 bp-fragment I, 109 bp-fragment G, 83 bp-fragment H, and a 374 bp-fragment that represents the 5′ half of *Hin*fI-fragment C. As illustrated in fig.4A (tracks 5,6), such a digestion of the mutant DNA showed the presence of a normal 543 bp-fragment D (not unexpectedly, since CTS and CTM cells can synthesize a normal small t), and of a single fragment of about 445 bp substituting for the 109, 83 and 374 bp-fragments. This finding indicated that two *Hin*fI sites, those delimiting fragment H, were missing, along with approximately 120 nucleotides. A deletion thus appeared to have eliminated the entire H fragment with surrounding nucleotides, and fused the rest of fragment G to fragment C. This fusion, in digestions of the mutant DNA with *Hin*fI not preceded by *Hin*dIII, originated an 840 bp-fragment (fig.4, track 4), representing the region between SVn 4568 and 3610 of wt DNA; this fragment was eluted from a gel and further digested with *Alu*I. Fig.4B, track 2, shows that this digestion generated 4 bands migrating, in increas-

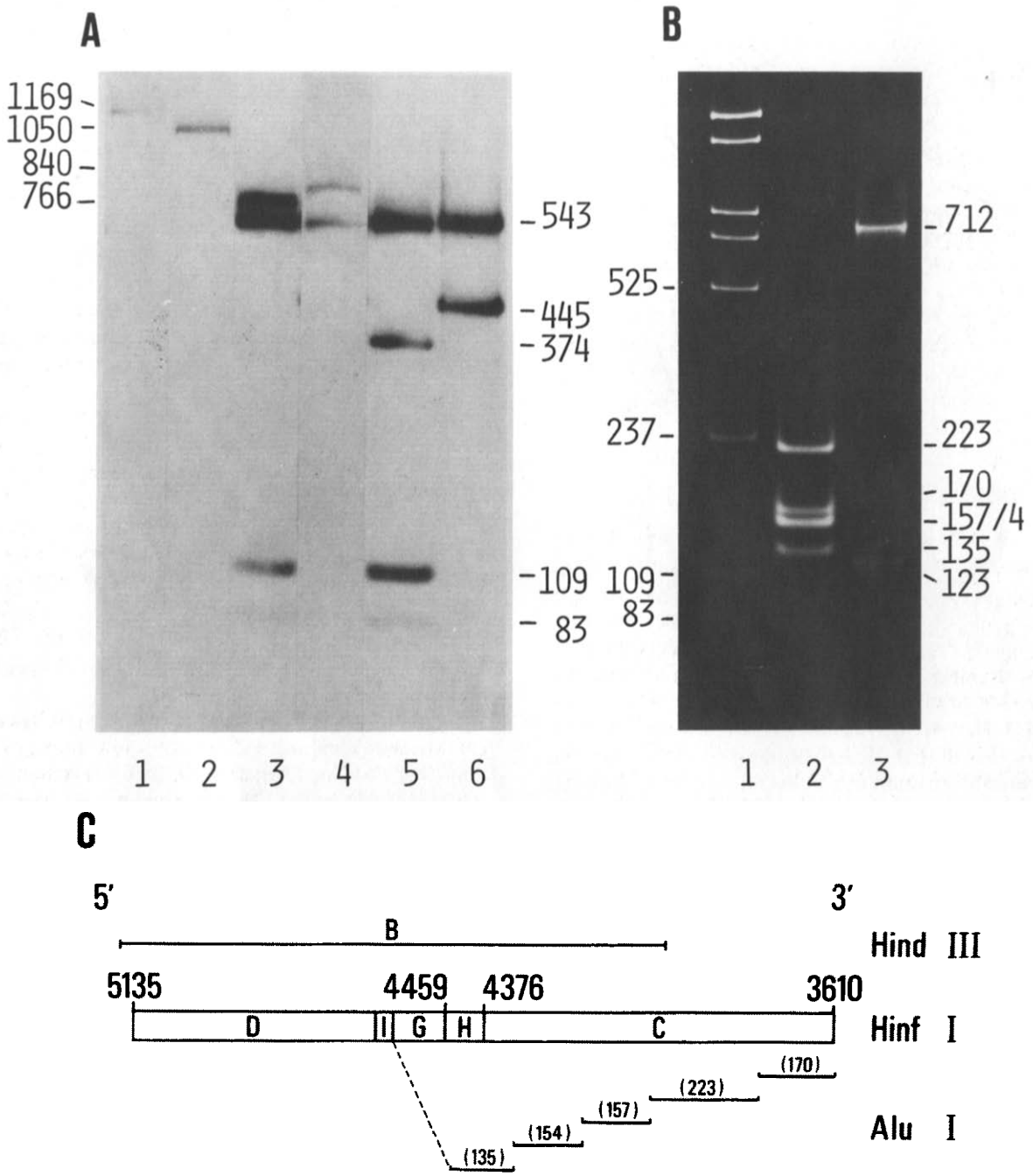

Fig.4. Restriction analysis of the mutant SV40 DNA. (A) After endonuclease digestion, DNAs were electrophoresed in 8% polyacrylamide gels, electroblotted and hybridized with nick-translated SV40 *Hin*dIII fragment B probe. (1,3,5) wt SV40 DNA digested with *Hin*dIII, *Hin*fI, and *Hin*dIII + *Hin*fI, respectively. (2,4,6) 4.85 kb DNA digested with the same enzymes, in the same order. The fragment sizes are indicated in bp. (B) The 840 bp-fragment obtained from *Hin*fI digestion of the 4.85 kb DNA was eluted from a gel and further digested with AluI (lane 2), or *Hpa*I (lane 3). Total wt SV40 DNA *Hin*fI-digest is shown in lane 1. DNA was electrophoresed in 8% polyacrylamide gel and stained with ethidium bromide. (C) Diagram illustrating the SV40 DNA region in which the deletion has occurred. Nucleotide numbers are shown above the *Hin*fI restriction map. Numbers in parentheses indicate fragment sizes in bp (see text).

ing order, as: the 223 bp-band expected between *Alu*I sites at SVn 4003 and 3780 of wt DNA (see fig.4C for alignment); the 170 bp-band expected between SVn 3780 and 3610; a thicker band, containing the unresolved 154- and 157 bp-fragments expected between SVn 4314, 4160 and 4003, respectively; and a remaining band of about 135 bp. This fragment replaces the residual length of 254 bp between the *Alu*I site at SVn 4314 and the *Hin*fI site at SVn 4568, in agreement with the above indications that a deletion of 120 bp encompassing the two *Hin*fI sites at SVn 4459 and 4376 had occurred. A single deletion of this size, considering the limits posed by the missing restriction sites, cannot extend beyond SVn 4496 upstream and SVn 4339 downstream.

4. DISCUSSION

The cytoplasmic localization of the large T antigen is a rare SV40 mutation. Only another cytoplasmic-T mutant, derived from an SV40-Adenovirus 7 hybrid virus, has been described and biochemically well characterized [10]. Very little is known about the determinant(s) responsible for the karyophily of normal large T (see [11] for a recent review on karyophilic proteins). Recent results have shown that the hybrid-derived mutant carries a base substitution at SVn 4434, a point within our deletion (R. Lanford and J. Butel, personal communication). The origin-specific DNA-binding domain of SV40 large T extends from the deleted region described here towards the N-terminal end of the protein [12]; this is consistent with the inability of the mutant T protein to support viral DNA replication.

A major point of interest for this deletion mutant is the finding that the SV40 large T antigen can suffer the loss of about 40 amino acids, in a region relatively close to the N-terminal end, without significantly impairing the ability to transform established cell lines such as NIH3T3. We observed, however, that in primary cultures of rat embryo fibroblasts this mutant induced less dense and greatly delayed foci, compared to those induced by wt SV40 DNA (in preparation). It is possible that the competence to transform established cell lines (*ras*-like function), and to immortalize primary cultures (*myc*-like function), which in Polyoma virus appear to be separated between the middle T and the large T [13,14] may reside in different domains of the SV40 large T protein.

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