

Biological Activities of Deletion Mutants of Simian Virus 40¹

WALTER A. SCOTT,² WILLIAM W. BROCKMAN,³ AND DANIEL NATHANS⁴

Department of Microbiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Mutants of Simian virus 40 (SV40) with large deletions in the early or late regions of the genome were tested for biological activity. Deletion mutants lacking portions of both late genes (B/C and D), but with an intact early genomic segment, were able to induce T antigen in infected cells, replicate their DNA in the absence of helper virus, stimulate thymidine incorporation into cellular DNA, and transform mouse and hamster cells. Cells transformed by late deletion mutants were shown to contain the mutant genome by a fusion-complementation rescue procedure. Deletion mutants lacking substantial portions of the early genomic region, including those segments where *tsA* mutants map, lacked all of the above activities. However, both early and late deletion mutants interfered with SV40 DNA replication.

INTRODUCTION

Cloned deletion mutants of Simian virus 40 (SV40) have been isolated from two sources: from virus passaged serially at high multiplicity of infection (Brockman and Nathans, 1974; Mertz *et al.*, 1974), and from enzymatically cleaved SV40 DNA (Lai and Nathans, 1974a; Mertz *et al.*, 1974; Carbon *et al.*, 1975). Some SV40 deletion mutants are viable, whereas others are sufficiently defective to require a helper virus for replication. We have been using cloned defective deletion mutants, whose genomes have been physically mapped, to localize SV40 genes (Lai and Nathans, 1974b; 1976) and to assign the various biological activities of the virus to specific regions of the genome (Brockman and Scott, 1975). In this communication we detail our experiments on the activities of

mutants containing large deletions of either the early or late region of the genome. From the results we conclude that the early region plus immediately contiguous segments of DNA are sufficient for viral DNA replication, stimulation of thymidine incorporation into cellular DNA, T antigen formation, and cell transformation. Moreover, cells transformed by a deletion mutant have been shown to yield the mutant genome after cell fusion and complementation. In contrast to the above activities, interference with SV40 DNA replication by a mutant virus does not appear to be dependent on the function of a specific gene product.

MATERIALS AND METHODS

Virus and cells. Wild-type (*wt*) SV40 was derived from plaque-purified small plaque virus (No. 776) grown in the BSC-1 line of African green monkey cells, as detailed previously (Danna and Nathans, 1971). Evolutionary variants *ev*-1117, -1119, and -1114 are defective viruses cloned from serially passaged virus stocks (Brockman *et al.*, 1975); *dl*-1007 is a cloned, constructed deletion mutant (Lai and Nathans, 1974a). Each mutant was recloned at least once prior to preparation of virus stocks, as detailed earlier (Brockman *et*

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² Present address: Department of Biochemistry, University of Miami, School of Medicine, Miami, Florida 33152.

³ Present address: Department of Microbiology, University of Michigan, School of Medicine, Ann Arbor, Michigan 48104.

⁴ Author to whom reprint requests should be addressed.

al., 1975). Temperature-sensitive mutants of SV40 (*tsA28*, *tsB4*, and *tsC219*) were generously supplied by Peter Tegtmeyer (Tegtmeyer, 1972) or Robert Martin (Chou and Martin, 1974). BSC-40 cells (Brockman and Nathans, 1974) were used for the growth of virus at elevated temperature. BALB/c-3T3 cells were obtained from G. Todaro and were grown in minimal Eagle's medium (MEM) with 20% fetal calf serum. Chinese hamster lung (CHL) cells were secondary cultures obtained from Janice Chou and Robert Martin (Martin and Chou, 1975), and were grown in MEM with 10% fetal calf serum.

Purification of deletion mutants. Stocks of SV40 deletion mutants contain complementing *ts* helper virus. To purify the mutant, clarified cell lysates were layered over a CsCl cushion ($\rho = 1.34$ g/ml), and virus particles were centrifuged into the CsCl layer. This entire layer was then added to fresh CsCl solution (final density, 1.34 g/ml), and after equilibrium centrifugation the light virus band was recovered and recentrifuged to equilibrium twice more to exclude most of the denser helper virus. The purified virus was then dialyzed against phosphate-buffered saline, treated with a trace of chloroform, and diluted into minimal medium with 2% fetal calf serum and stored frozen at -30° .

Titration of deletion mutants. The concentration of infectious mutant virus was assayed by infectious center complementation plaque titration in the presence of an excess of a complementing *ts* mutant (Brockman and Nathans, 1974), as noted in the text. The titer is expressed as complementation plaque-forming units (cPFU).

Mutant DNA was labeled with ^{32}P and then purified by Hirt extraction (Hirt, 1967) and electrophoresis in 1.4% agarose gel slabs, as described elsewhere (Brockman and Nathans, 1974).

SV40 T antigen was detected in cells infected with mutant or *wt* DNA (McCutchan and Pagano, 1968) by the indirect immunofluorescence procedure using anti-T serum from Flow Laboratories and fluorescein-tagged anti-hamster serum from Nutritional Biochemicals Corp. For this

purpose about 25 ng of electrophoretically purified DNA was used to infect approximately 10^4 cells growing on a glass slide and about 1000 cells were scored for nuclear fluorescence.

Replication of mutant DNA. BSC-1 cell monolayers in 6-mm microwells were infected with purified mutant virus over a range of input multiplicities in the presence or absence of *wt* SV40 at a multiplicity of 4 PFU per cell. After 24 and 48 hr, 5 μC of [^{32}P]orthophosphate was added, and at 72 hr, viral DNA was extracted by Hirt's procedure. In every case a constant, known amount of [^3H]SV40 DNA was added with the 0.1 ml of lysing solution to monitor recovery of SV40 DNA. After centrifugation of the lysate, RNase treatment, and phenol extraction, 10 μg of tRNA was added, the DNA was precipitated with ethanol, and the precipitate taken up in a small volume of 15 mM NaCl-1.5 mM Na citrate, pH 7.0. An aliquot of each sample was then subjected to electrophoresis in 1.4% agarose gel slabs to separate mutant from helper virus DNA, and all forms of unit length DNA were pooled and counted as were the forms of deleted DNA present. At the same time the recovery of added [^3H]DNA was assessed, and the [^{32}P]DNA values normalized to the original amount of [^3H]DNA present.

Measurement of virus-stimulated incorporation of thymidine into cellular DNA. BALB/c-3T3 cells were uniformly labeled by allowing them to grow in 10-cm dishes for three or four generations in 1 μCi of [^{14}C]thymidine (50 Ci/mol) and then seeded in 6-mm microwells. One day after the culture had reached confluence, the medium was changed to 0.1 ml of MEM supplemented with 2% fetal calf serum which had been heated at 70° for 30 min. Forty-eight hours after this medium change, the first infection was performed by adding 25 μl of diluted virus stock without removing the medium or disturbing the monolayer. Virus stocks were diluted in MEM to give the appropriate virus multiplicity and a final concentration of unheated serum that was not more than 0.2%. Other cultures were infected in the

same way at later times for other time points in the experiment. For the first time point, DNA was pulse-labeled by removing the medium and by adding 50 μ l of MEM containing 2% heated fetal calf serum and 40 μ Ci/ml of [3 H]thymidine (20 Ci/mmol). After 1 hr the cultures were rinsed with cold Tris-buffered saline and harvested by the Hirt method. Culture lysates were transferred into small test tubes using a Pasteur pipet, and the supernatant from the Hirt fractionation procedure, which contained few counts, was discarded. The precipitate was dissolved in a few drops of 3 N NH_4OH , and transferred to Whatman 3 MM filter disks. The disks were dried and counted. ^3H counts from each well were normalized to a constant amount of [14]DNA to correct for variation in recovery of cell DNA during the lysis and fractionation procedures.

Cell transformation. Subconfluent BALB/c-3T3 cells (1.5×10^5 cells) or CHL cells (1×10^5 cells) growing in 1.5-cm wells at 37° were infected at a series of multiplicities with SV40 or a purified deletion mutant. Twenty-four hours later the cells of each well were trypsinized and transferred to a 10-cm petri dish in MEM with 5% fetal calf serum. Three weeks later (3T3) or 4 weeks later (CHL), piled-up colonies were scored as transformants. Several transformed colonies were isolated and cloned twice by plating a dilute cell suspension and selecting colonies originating from single cells.

Rescue of virus from transformed BALB/c-3T3 cells by fusion-complementation. This procedure is based on the complementation-plaquing procedure used for cloning deletion mutants (Brockman and Nathans, 1974). A defective genome in transformed cells is induced by cell fusion with permissive cells and complemented by a *tsA* helper virus, thus leading to the interdependent growth of the defective genome and the helper. In practice, cultures of transformed 3T3 cells at 80–90% confluence were trypsinized and 10^4 cells were seeded in 1 ml of MEM (containing 10% fetal calf serum) in 1.5-cm culture wells. After these cells had attached (6 hr, 37°), 3×10^5 BSC-40 cells were added to each

well. After an additional 12 hr at 37°, the cultures were infected at a multiplicity of 4 with a *tsA* mutant for a period of 2 hr, the medium was removed, and the monolayer was subjected to fusion with uv-inactivated Sendai virus (Microbiological Associates) as described by Davidson (1967). Briefly, the monolayer was washed twice with cold MEM without serum and then exposed to uv-inactivated Sendai virus (200 hemagglutinating units in 0.1 ml of MEM without serum) and incubated on ice for 20–25 min. At the end of the incubation, the Sendai virus was removed and the monolayer again was rinsed twice with cold MEM without serum. MEM (0.2 ml), which had been preequilibrated at 37°, was added and the culture was further incubated at 37° for 30 min, following which 1 ml of MEM with 3% fetal calf serum was added and the incubation continued at 37° for 12–18 hr. At this time the culture was trypsinized, transferred to 6-cm petri dishes, assayed for infective centers at 40°, and the plaques then were surveyed for the presence of deletion mutants by the electrophoretic assay described earlier (Brockman and Nathans, 1974).

Endo R analysis of DNA. The electrophoretically fractionated DNA species prepared by infecting cells with virus from the complementation plaques described above were recovered from agarose gels, dialyzed extensively against 1.5 mM NaCl in 0.15 M Na citrate (pH 7), concentrated 10-fold by evaporation, and subjected to digestion with endonuclease R·*Hind* (Danna *et al.*, 1973). *Hind* fragments were separated by electrophoresis in 4% polyacrylamide gel slabs and fragments visualized by autoradiography.

Virus induction from transformed CHL cells by mitomycin was based on the procedure of Rothschild and Black (1970). Transformed CHL cells were seeded in 1.5-cm wells (3×10^4 cells per culture) and allowed to attach at 37°. Mitomycin-C (Sigma) was dissolved in MEM and added in a darkened room to a final concentration of 2 μ g/ml. The cultures were wrapped in aluminum foil and incubated at 37° for 12 hr. Then the medium was removed and the cultures were rinsed once

with MEM containing 2% fetal calf serum and infected at a multiplicity of 5 with temperature-sensitive helper virus *tsA28* or *tsB4*, or mock-infected. At the end of 2 hr, medium was added and the cultures were incubated for 2 days at 37°, at which time 10^5 permissive BSC-40 cells which had been infected with the same helper virus were added to each culture. When the permissive cells had attached, the cultures were shifted to 40° and incubated for 16 days. After freezing and thawing, the lysates were surveyed for the presence of deletion mutants as described above for virus rescued from transformed 3T3 cells. In one set of experiments defective virus was rescued from transformed CHL cells by the fusion-complementation procedure described above.

RESULTS

Structure of mutant genomes. The genomic structures of mutants used in the studies to be reported are shown in Fig. 1 in relation to the SV40 map (Brockman *et al.*, 1975; Lai and Nathans, 1974a). In sum, *ev-1114* and *dl-1007* have deletions spanning the two known late genes of SV40 (B/C and D), *ev-1114* retaining only a small segment of late DNA (about 450 nucleotide pairs) adjacent to each end of the early region of the genome. Both of these mutants have the entire early region (A gene) intact. The two other mutants (*ev-1117* and *ev-1119*) have deletions which eliminate about 70 and 60% of the early region, respectively, and have the entire late region uninterrupted. All three evolutionary variants, which were derived from serially passaged virus stocks, have duplications of DNA including the origin of DNA replication. In all experiments to be described below, doubly cloned mutant virions were first separated from *ts* helper virus by CsCl centrifugation or purified mutant DNA was prepared by agarose gel electrophoresis, as described in Methods.

Titer of purified mutant virions. To determine the number of biologically active deletion mutant particles in purified virus preparations and the amount of residual *ts* helper virus present, the number of plaque-forming units was determined at

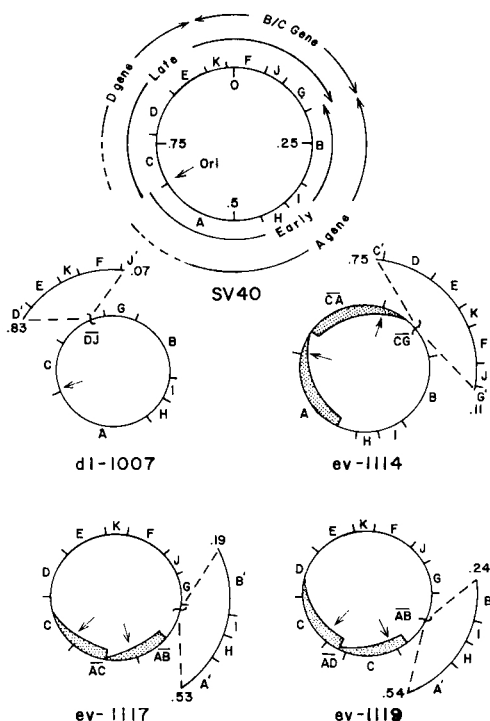


FIG. 1. *Hind*III/III maps of mutant genomes in relation to the map of *wt* SV40 DNA (from Brockman *et al.*, 1975; Lai and Nathans, 1975, 1976). Duplicated regions are indicated by the stippled areas, and deleted regions by the wedge-shaped extension from the circles. In the *wt* map are shown location of templates for early and late mRNA's (Khoury *et al.*, 1975), the three known genes of SV40 (Ozer and Tegmeyer, 1971; Chou and Martin, 1974; Lai and Nathans, 1975), and the origin of DNA replication (→).

40° (nonpermissive for *ts* mutants) by infectious center assay, in the presence of excess *tsA* or *tsB* helper, as described in Methods. The titers, expressed as complementation plaque-forming units or cPFU, are given in Table 1. (To determine the efficiency of complementation, titers of *ts* mutants measured by direct plaque assay at 32° were compared with result of complementation plaque assay; cPFU at 40° were about 30–70% of PFU at 32°.) As seen in Table 1, residual contamination by *ts* helper virus varied from about 2% (*ev-1119*, preparation 1) to about 0.2% (*dl-1007*).

Formation of T antigen in infected cells. Mutant-infected cells were tested for the

presence of T antigen, as described in Methods. To minimize the effect of contaminating helper virus, mutant DNA purified by agarose gel electrophoresis was used. The cells were fixed at 28 hr postinfection and stained for T antigen by the indirect immunofluorescence procedure. DNA from two late deletion mutants, *ev-1114* and *dl-1007*, induced T antigen production in 1.2 and 4.4% of cells scored, respectively, compared to 1.7% for cells infected by *wt* SV40 DNA. DNA from the two early mutants did not induce detectable antigens (<0.1% of cells scored). These

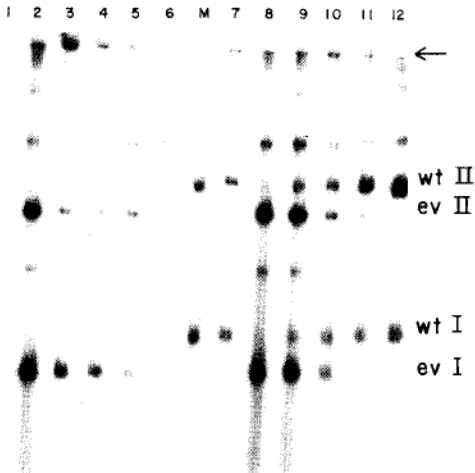
results are consistent with prior conclusions that the genetic determinant of T antigen is in the early region of the genome (Lewis *et al.*, (1974).

Viral DNA synthesis. Next we tested the ability of variant genomes to replicate in the absence of helper virus. Since each variant virus preparation contained contaminating helper virus (Table 1), we used a range of variant multiplicities and compared results with and without coinfecting SV40 in order to determine whether variant DNA replication was independent of helper virus. For this purpose two sets of monolayers of BSC-1 cells in 6-mm microwells were infected in parallel with purified deletion mutant virions at different multiplicities of infection, and one set of infected wells was coinfecting with *wt* SV40 at a multiplicity of 4 PFU/cell. Viral DNA was labeled with ^{32}P , and variant and SV40 DNA were isolated from the Hirt supernatant by electrophoresis in agarose, as detailed in Methods and illustrated in Fig. 2. The yield of each [^{32}P]DNA species was then determined by counting dis-

TABLE 1
TITERS OF SV40 MUTANTS

Mutant	Infectious centers (cPFU/ml)		Mutant
	+ <i>tsA28</i>	+ <i>tsB4</i>	
<i>ev-1114</i>	7×10^7	5×10^5	140
<i>ev-1117</i>	2×10^5	4×10^7	200
<i>ev-1119 #1</i>	9×10^5	5×10^7	55
<i>ev-1119 #2</i>	1×10^6	1×10^8	100
<i>dl-1007</i>	3×10^7	6×10^4	500

ev-1114



ev-1117

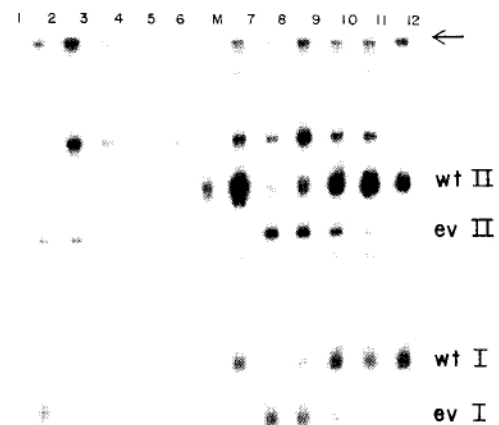


FIG. 2. Replication of viral DNA in mutant-infected cells: autoradiograms of [^{32}P]DNA extracted from infected cells followed by electrophoresis in agarose. The origin is at the top (arrow). On the left are the results with *ev-1114*; on the right are the results with *ev-1117*. Slot 1, no virus; slots 2-6, decreasing multiplicity of mutant; slot 7, *wt* SV40 only; slots 8-12, as in 2-6 plus *wt* SV40. M, reference SV40 DNA. For quantitation, the amount of ^{32}P in short genomes (*ev* I and II) and in *wt* length genomes (*wt* I and II) was measured for each sample and normalized to the recovered [^3H]DNA, as detailed in Methods. Results are plotted in Fig. 3.

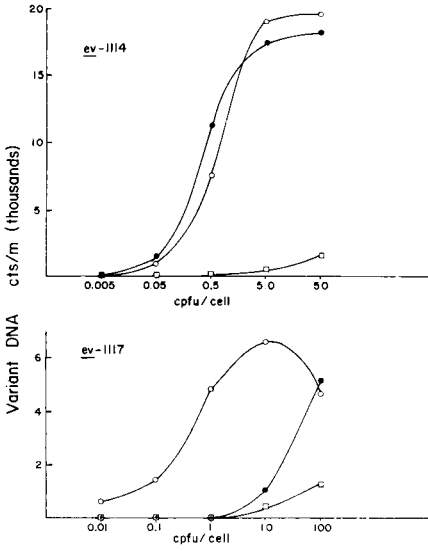


FIG. 3. Replication of mutant DNA in the presence (○) or absence (●) of *wt* SV40. On the ordinate is plotted normalized ^{32}P radioactivity in short genomes and on the abscissa is input multiplicity of deletion mutant virus (cPFU/cell). When added, *wt* SV40 was used at a multiplicity of 4. □, Unit length DNA in absence of added *wt* SV40.

solved gel segments after autoradiographic localization of DNA. As indicated in Methods, all values were normalized to recovered [^3H]SV40 DNA added to infected cells with the lysing solution.

Quantitative results for this series of experiments with *ev-1114* and *ev-1117* are shown in Fig. 3. As seen in this figure, *ev-1114* DNA replication was detectable at a multiplicity of 0.05 cPFU/cell (about 0.0004 cPFU/cell of contaminating *tsA28* helper virus) and was not stimulated by coinfection with SV40 over the entire range of variant multiplicities used. In contrast, *ev-1117* DNA replication was first measurable at a multiplicity of 10 cPFU/cell, under which conditions the cells were coinfecting with about 0.05 cPFU/cell of contaminating *tsB4* helper virus. Moreover, except at the highest multiplicity of variant used, coinfection with SV40 resulted in stimulation of *ev-1117* DNA replication. We conclude that *ev-1114* DNA can replicate independently of helper virus, whereas *ev-1117* DNA replication is dependent on helper. Less detailed experi-

ments with *ev-1119* and *dl-1007* gave results similar to those shown for *ev-1117* and *ev-1114*, respectively.

In this experiment we also scored *cytopathic effect* 72 hr postinfection at the various multiplicities of infection. Cells infected with *ev-1117* showed no visible cytopathic effect at input multiplicities of 10 cPFU/cell, whereas a cell layer infected with *ev-1114* at 0.5 cPFU/cell showed obvious cytopathic effect, similar to that of cells infected with *wt* virus.

Interference. In the course of the above studies of mutant DNA replication in the presence and absence of *wt* SV40, it became apparent that the mutants had inhibited the replication of *wt* virus DNA (Fig. 2). To quantitate this inhibition we determined the yield of *wt* virus and *wt* virus DNA after coinfection of cells with a given mutant and *wt* SV40, as detailed in Methods. To avoid competitive absorption, mutant virus was added 1 hr after *wt* SV40. The results for *ev-1114* and *ev-1117* are presented in Table 2; *ev-1119* gave essentially the same results as *ev-1117*. As seen in Table 2, both *ev-1114* and *ev-1117* inhibited the production of infectious SV40 and also inhibited the formation of SV40 DNA. Since *ev-1114* lacks the major portion of the late region of the SV40 genome while *ev-1117* is missing most of the early region, this interference phenomenon does not appear to be dependent on a specific gene product. One difference in the effect of the two variants is that in *ev-1114*-infected cells, total viral DNA synthesis is undiminished or somewhat greater than in cells infected with SV40, whereas in *ev-1117* cells, total viral DNA synthesis is below that seen in cells infected with SV40 alone. A second difference is seen in the ratios of variant to *wt* SV40 DNA (Table 2). These differences probably reflect the fact that replication of the *ev-1114* genome does not depend on coinfecting *wt* SV40, whereas replication of the *ev-1117* genome does (see Fig. 3).

Stimulation of cellular DNA synthesis. One of the unusual activities of SV40 possibly related to its oncogenic activity is the stimulation of cellular DNA synthesis in both permissive and nonpermissive cells

TABLE 2
YIELD OF SV40 AND OF VIRAL DNA IN CELLS
COINFECTED WITH MUTANT AND WT VIRUS

Mutant	Multi- plicity (cPFU/ cell)	Yield as % of control ^a		Total viral DNA ^b (% of control)	Variant DNA/wt DNA
		wt vi- rus	wt DNA		
<i>ev-1114</i>	5	20	9.7	131	13
	50	2	3.6	127	34
<i>ev-1117</i>	10	25	15	42	1.8
	100	14	4.9	24	3.9

^a Each monolayer, consisting of 5×10^4 BSC-1 cells, was infected with 5 PFU of *wt* SV40 per cell, followed 1 hr later by the indicated mutant at the multiplicity shown. In the absence of mutant (control), the yield of *wt* virus was 4.8×10^7 PFU at 96 hr postinfection and the radioactivity of unit length [³²P]DNA isolated from cell lysate 72 hr postinfection was 2×10^4 cpm.

^b Total viral DNA equals unit length plus short genomes, i.e., *wt* + mutant, expressed as % of viral DNA from cells infected with *wt* SV40 alone.

(Hatanaka and Dulbecco, 1966; Gershon *et al.*, 1966). Since cells infected with *tsA* (early) mutants of SV40 show much greater temperature sensitivity of viral DNA replication than of stimulation of cell DNA synthesis (Tegtmeyer, 1972; Chou and Martin, 1975b), it was of interest to determine whether variants containing deletions in the early or late region of the SV40 genome could stimulate cell DNA synthesis. To test for this function, BALB/c-3T3 cells were chosen because of the low background of DNA synthesis in contact-inhibited cells. Cell monolayers prelabeled with [¹⁴C]thymidine were infected with SV40 or with deletion mutant virions as detailed in Methods, and the incorporation of [³H]thymidine into high molecular weight DNA was determined at various times after infection. The results are presented in Fig. 4, each value plotted having been normalized to a constant amount of cellular DNA by means of the ¹⁴C counts in the same sample. As seen in the figure, *ev-1119* (and *ev-1117* in a separate experiment), which is missing much of the early genome region, failed to stimulate thymidine incorporation into cellular DNA. In contrast, *ev-1114* (and *dl-1007* in a separate experiment), which has a deletion of most

of the late region, stimulates thymidine incorporation at least as well as SV40. We conclude that part or all of the early region is clearly necessary for this viral function. On the assumption that the late genome segment present in *ev-1114* DNA does not code for some unknown functional protein, we can also conclude that late gene products are not required. Whether *ev-1114* is actually more active than *wt* SV40 in stimulating thymidine incorporation into cellular DNA, as suggested by the results shown in Fig. 4, is not clear, since in other experiments with different mutant preparations, *ev-1114*- and *dl-1007*-stimulated incorporation was somewhat less than that due to *wt* SV40.

Cell transformation. The ability of mutants to transform cells in culture was tested with nonpermissive BALB/c-3T3 cells and with semipermissive CHL. For this purpose subconfluent cells growing in multi-well dishes were infected at various multiplicities with wild-type or deletion mutant virions, and the number of morphologically transformed colonies was scored following transfer of the cells, as detailed in Methods. Only dense, multi-layered colonies were scored as transformed. The results of representative experiments are presented in Table 3. As

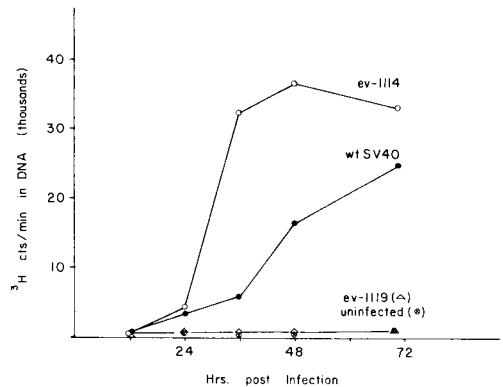


FIG. 4. Incorporation of [³H]thymidine into DNA of 3T3 cells after infection by mutant virus. On the ordinate is plotted the normalized incorporation into high molecular weight DNA vs time after infection. Twenty cPFU or PFU/cell were used in each case. See Methods for experimental details. The results for *dl-1007* and *ev-1117* were similar to those for *ev-1114* and *ev-1119*, respectively.

TABLE 3
 TRANSFORMATION BY MUTANTS

Virus (% helper virus contamination) ^a	PFU or cPFU/ cell	Transformed colonies per 10 ⁵ cells ^b	
		BALB 3T3	CHL
SV40	30 PFU	30	—
	6	15	9
	3	8	9
	1.2	5	—
	0.6	—	2
	0.06	0	0
<i>dl</i> -1007 (0.2%)	5 cPFU	12	—
	1	1	5
	0.1	0	2
<i>ev</i> -1114 (1.0%)	15 cPFU	12	—
	3	1	7
	0.3	0	2
<i>ev</i> -1117 (0.5%)	30 cPFU	0	—
	6	0	0
	3	—	0
<i>ev</i> -1119 (1.0%)	30 cPFU	0	0
	15	0	0

^a The mutant viruses were assayed about 1 month prior to use.

^b Average of two original microwell monolayers.

seen in the table, *ev*-1114 and *dl*-1007 produced morphologically transformed colonies from both 3T3 and from CHL cells, whereas *ev*-1117 and *ev*-1119 had no transforming activity in either cell type at comparable multiplicities of infection. Since similar amounts of contaminating *ts* helper virus were present in the serially diluted variants, it is unlikely that transformation by *ev*-1114 and *dl*-1007 was due to contaminating *tsA* mutant still present in the preparation. Moreover, SV40 at multiplicities similar to that of contaminating *ts* helper virus present in the active virus stocks failed to transform. When transformed colonies were selected from SV40-, *ev*-1114-, or *dl*-1007-infected 3T3 or CHL dishes and recloned twice, the resulting clones all contained nuclear SV40 T antigen detectable by fluorescent antibody staining. We conclude that *ev*-1114 and *dl*-1007, but not *ev*-1117 nor *ev*-1119, can transform mouse and hamster cells, and therefore it is likely that the early region of the SV40 genome is not only necessary but also sufficient for transformation.

Virus production by transformed cells.

Since deletion mutant preparations used to transform CHL and 3T3 cells contained contaminating helper virus (*tsA28*), experiments were carried out to determine whether any of the cloned transformed cell lines contained rescuable virus. In the case of CHL cells, which are semipermissive for SV40 (Martin and Chou, 1975), attempts were made to induce virus production with mitomycin-C at 32°, as described in Methods. In the case of transformed 3T3 clones, virus rescue at 32° by fusion with BSC-1 cells was attempted. For each cell type, two independent clones of *wt* SV40-transformed cells from the experiment shown in Table 3 were used as controls.

As shown in Table 4, both *wt* SV40-transformed CHL cell lines (SV-CHL-1 and 2) yielded small amounts of virus in the absence of mitomycin-C. After drug treatment the yield increased more than 100-fold. However, none of the mutant-transformed CHL cells yielded detectable infectious virus at 32° with or without mitomycin-C treatment. In the case of 3T3 cells both *wt* SV40-transformed cell lines (SV-3T3-11 and 12) and one of the four *ev*-1114-3T3 lines yielded virus after fusion with BSC-1 cells and prolonged incubation, as judged either by cytopathic effect of the cell lysate on a fresh monolayer of BSC-1 cells (Table 4) or by plaque assay (results now shown), whereas the other mutant-transformed lines produced no detectable virus. Presumably, *ev*-1114-3T3-2 had been transformed by the *tsA* helper virus or a derivative thereof. However, the other mutant-transformed CHL or 3T3 cells showed no evidence of an intact SV40 genome by this test.

Rescue of deletion mutants from 3T3-transformed cells by complementation. The rescue and induction experiments just described did not establish that mutant-transformed cells contain the genome of the SV40 mutants used to transform them. In an attempt to determine whether mutant genomes were actually present we applied the complementation plaquing procedure described by Brockman and Nathans (1974) to the rescue of mutant genomes, as detailed in Methods and diagrammed in Fig. 5. In this procedure, cells infected

TABLE 4
 VIRUS RESCUE FROM TRANSFORMED CLONES

Cells	CHL ^a		3T3 ^b	
	Virus production (PFU)		Cells	Virus production (cpe)
	Not induced	Mitomycin induced		
CHL	0	0	3T3	-
<i>ev</i> -1114-CHL-1	0	0	<i>ev</i> -1114-3T3-2	+
<i>ev</i> -1114-CHL-2	0	0	<i>ev</i> -1114-3T3-3	-
<i>dl</i> -1007-CHL-1	0	0	<i>ev</i> -1114-3T3-4	-
<i>dl</i> -1007-CHL-2	0	0	<i>ev</i> -1114-3T3-5	-
SV-CHL-1	100	1.3 × 10 ⁴	<i>dl</i> -1007-3T3-2	-
SV-CHL-2	370	1.0 × 10 ⁵	SV-3T3-11	+
			SV-3T3-12	+

^a A 1.5-cm monolayer containing about 10⁵ cells was treated with mitomycin-C (where indicated), incubated for 6 days at 32°, frozen and thawed, and PFU of lysate was measured on BSC-1 cells at 32°.

^b Three × 10⁴ BALB 3T3 cells were fused with 3 × 10⁵ BSC-1 cells using uv-inactivated Sendai virus, and the mixture was plated in a 10-cm petri dish. Following incubation at 32° for 22 days, the entire undiluted frozen and thawed cell lysate was then used to infect BSC-1 cells at 32°, and cytopathic effect was scored after 10 days.

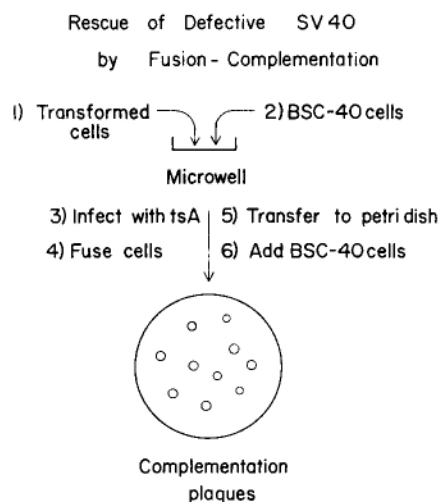


FIG. 5. Diagram of defective virus rescue by fusion-complementation.

with two complementing mutants are plated, and excess indicator cells are then added to allow plaque formation. Plaque formation should be dependent on complementation or recombination between a rescued genome and the added helper *ts* genome in a single heterokaryon; revertants in the *ts* virus stock would also produce plaques. In this series of experiments we concentrated on analyzing clones *ev*-1114-3T3-5 and *dl*-1007-3T3-2.

The results of complementation plaqu-

TABLE 5
VIRUS RESCUE FROM TRANSFORMED CELLS AT 40°^a

Cells	Infectious centers after fusion		
	No helper	+ <i>tsA</i> 28	+ <i>tsC</i> 219
3T3	0, 0	2, 1, 1	0, 0
<i>ev</i> -1114-3T3-5	0, 0	11, 13, 14	0, 0
<i>dl</i> -1007-3T3-2	0, 0	4, 0, 1	0, 0
SV-3T3-11	3, 1	2, 2, 4	1, 0

^a Each value is the yield from 10⁴ transformed or nontransformed 3T3 cells. Multiple values refer to replicate dishes.

ing are shown in Table 5. As controls, untransformed 3T3 and *wt* SV40-3T3-11-transformed clones were included, and plaque formation was also assessed with a late mutant (*tsC*219), instead of *tsA*28, as helper virus. As seen in the table, *tsA*28 with fused 3T3-BSC-40 cells resulted in a small number of plaques. These are probably revertants in the *tsA* stock (see below), since 10⁶ PFU were used in each experiment. In the case of SV-3T3 clones, fusion alone resulted in plaque formation with no apparent increase in plaques due to superinfection by *ts* mutants. In contrast, both *ev*-1114- and *dl*-1007-transformed clones produce plaques only in the presence of *tsA* helper virus; *tsC* could not substitute for *tsA*. These results suggest that complementation and fusion resulted in rescue of

a resident mutant genome. As shown below, this inference was substantiated by analysis of individual plaques.

Analysis of complementation plaques. To determine whether a putative complementation plaque contained the input defective genome in addition to helper virus, plaques were screened by infecting BSC-40 cells grown in 1.5-cm wells with a few drops of plaque suspension at 40°. This temperature favors the replication of genomes containing a normal early region over the *tsA* helper, since the multiplicity of infection is low. ³²P viral DNA was prepared from the infected cells and subjected to electrophoresis in agarose gels to separate short genomes from unit length helper virus DNA as described in Methods. To determine whether short genomes were derived from the mutant used for

transformation or from the *tsA* helper, recovered DNA was analyzed by digestion with endo R·*Hind*II/dIII and electrophoresis of digest products. In some cases plaque suspensions were first used to prepare virus stocks in order to obtain larger quantities of DNA.

Plaques from tsA-infected untransformed 3T3/BSC heterokaryons. Three plaques formed by the *tsA*-infected untransformed 3T3-BSC cells (see Table 5) all yielded full-length SV40 DNA; the *Hind*II/dIII digest of one of these was identical to a digest of *tsA* DNA (Lai and Nathans, 1974b). These plaques therefore appear to be derived from revertants of *tsA*.

Plaques from tsA-infected ev-1114-3T3-5/BSC heterokaryons. Eight plaques formed by *tsA*-infected *ev-1114-3T3-5/BSC* cells were analyzed (Fig. 6 and Table 6).

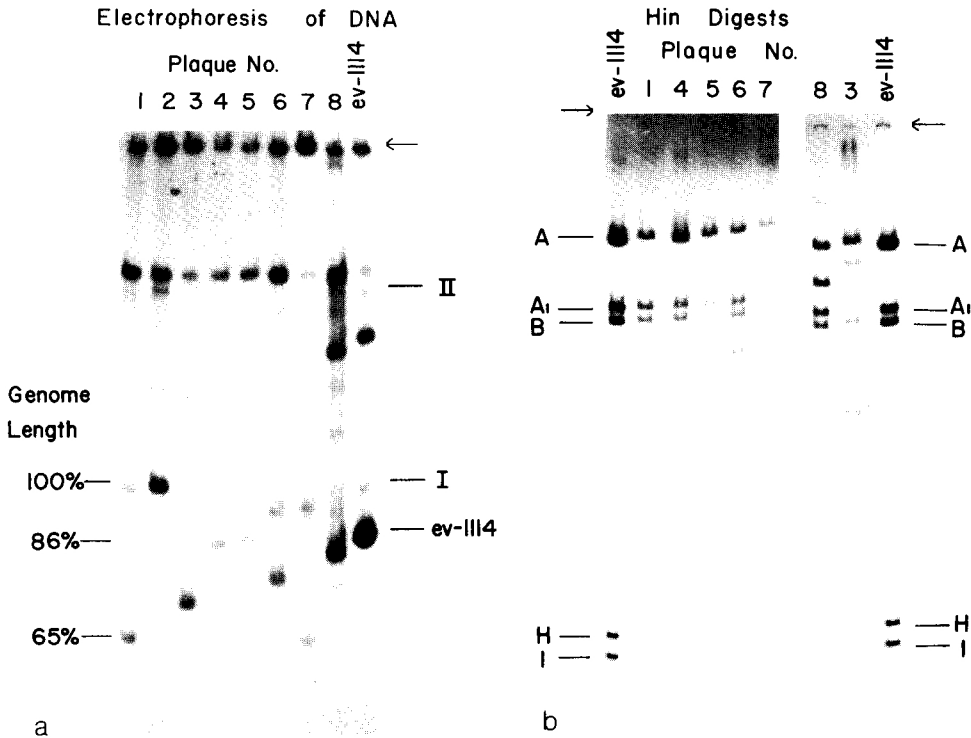


FIG. 6. (a) Agarose gel electrophoresis of viral DNA rescued from *ev-1114-3T3-5*. The numbers at the top refer to individual plaques, as described in the text and Table 6. The genome lengths recorded at the left are based on electrophoretic mobilities (Brockman *et al.*, 1975). I, II refer to the positions of *wt* DNA forms I and II, respectively. (b) Representative *Hin* digest patterns of dominant genomes shown in Fig. 6a: autoradiogram of [³²P]DNA fragments following electrophoresis. Numbers at the top refer to individual plaques noted in Fig. 6a and Table 6. The three lanes on the right are from a different electrophoretic run than those on the left. Fragment A₁ is the CG fusion fragment (Fig. 1).

TABLE 6

VIRAL GENOMES RESCUED FROM *ev*-1114-3T3-5 BY FUSION-COMPLEMENTATION

Plaque number	Lengths of genomes ^a (% of SV40 DNA)	Inferred structure of predominant genome
1	<u>65</u> , 78, 86, 90, 100	<i>ev</i> -1114 minus duplication
2	<u>100</u> , 88	Variant of <i>ev</i> -1114 or recombinant
3	<u>69</u> , 90, 100	Variant of <i>ts</i> helper
4	<u>86</u>	<i>ev</i> -1114
5	<u>86</u>	<i>ev</i> -1114
6	<u>78</u> , 65, 86, 92, 100	Variant of <i>ev</i> -1114
7	<u>65</u> , 76, 86, 94	<i>ev</i> -1114 minus duplication
8	<u>82</u> , 65, 76, 92, 100	Variant of <i>ev</i> -1114

^a The predominant genome length is underlined.

Six of these yielded more than one size class of DNA, but each had a predominant species. Only one plaque (plaque no. 2 in Table 6) produced unit length DNA as the major species; *Hin* digestion revealed a complex fragment pattern suggesting derivation from *ev*-1114 DNA. A shorter genome (88% of full length) formed from the same plaque was a variant of *tsA*, as judged by its fragment pattern. Two plaques (Nos. 4 and 5 in Fig. 6a and Table 6) produced predominantly a DNA species of 86% of unit length, each of which gave an *Hin* digest pattern identical to that of *ev*-1114 DNA (Fig. 6b). Two plaques (Nos. 1 and 7) produced predominantly a DNA species of 65% of unit length, each of which gave a *Hin* digest pattern qualitatively identical to the *ev*-1114 DNA pattern, but with only one molar equivalent corresponding to the *Hin*-A fragment (Fig. 6b), indicating loss of the duplication present in *ev*-1114 (see Fig. 1). Of the remaining three plaques analyzed (nos. 3, 6, and 8), one produced a predominant DNA species 69% of unit length which was derived from *tsA* DNA. The other two (nos. 6 and 8) produced predominantly genomes of 78 and 82% of unit length, respectively, each of which showed *Hin* digest patterns resembling the 65% DNA present in plaques 1 and 7, except that each digest had one additional new fragment (Fig. 6b). It is of interest that each of the last two plaques

also produced a smaller amount of a DNA species 65% of full length (Fig. 6a).

From the foregoing results we conclude that clone *ev*-1114-3T3-5 harbors the *ev*-1114 genome, and after fusion-complementation the original mutant genome or a derivative thereof replicates and becomes encapsidated (see Discussion).

Plaques from *tsA*-infected *dl*-1007-3T3-2/BSC heterokaryons. Five plaques formed by *tsA*-infected *dl*-1007-3T3-2/BSC cells were analyzed (Fig. 7 and Table 7). Four plaques (Nos. 2 to 5 in Table 7) yielded DNA which included molecules 76% of SV40 DNA length, i.e., the length of *dl*-1007 DNA (Fig. 7a). However, this DNA species was the major one in only one case (no. 2); *Hin* digestion of the 76% DNA from plaque no. 2 yielded an electrophoretic fragment pattern identical to *dl*-1007 DNA (Fig. 7b). The unit length DNA species appeared to be derived from the *tsA* helper virus, as judged by their length or *Hin* digestion (Fig. 7b). On the basis of the results with rescued plaque no. 2, we conclude that clone *dl*-1007-3T3-2 harbors the *dl*-1007 genome.

Rescue of deletion mutants from transformed CHL cells. Similar analyses of virus rescued from three transformed CHL clones were carried out, in this case in the presence of mitomycin-C and without cloning, as detailed in Methods, or by complementation-fusion as with 3T3 cells. After superinfection with the *tsA*28 mutant (but not with *tsB*4), *dl*-1007 virus was rescued from *dl*-1007-transformed CHL cells (1007-CHL-1 and -2). Identification of the rescued mutant was carried out by electrophoretic isolation of its genome followed by analysis of *Hin* digests, as described above for virus rescued from transformed 3T3 cells. Similarly, from *ev*-1114-transformed CHL cells (*ev*-1114-CHL-2) superinfected with *tsA*28 and fused with BSC-40 cells, mutant genomes 65% of the length of SV40 DNA were detected. This species yielded the *Hin* digest pattern of *ev*-1114 DNA minus its duplicated segment. Thus, mutant-transformed clones of CHL cells as well as mutant-transformed clones of 3T3 cells produced the original transforming virus or a derivative thereof.

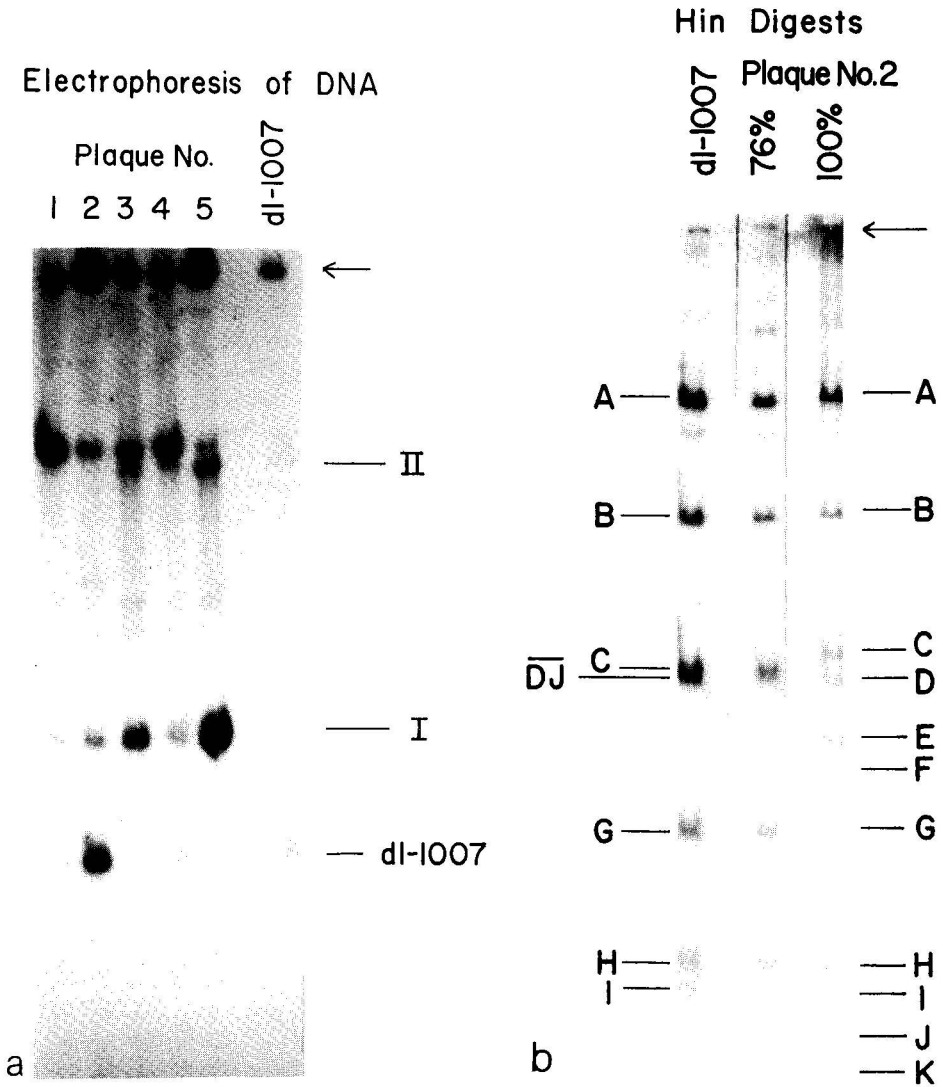


FIG. 7. (a) Agarose gel electrophoresis of viral DNA rescued from *dl-1007-3T3*. Numbers at the top refer to individual plaques, as described in the text and Table 7. I, II refer to the positions of *wt* DNA forms I and II, respectively. (b) *Hin* digest patterns of genomes shown in Fig. 7, plaque no. 2: autoradiogram of [³²P]DNA fragments following electrophoresis. Notations at the top refer to the 76% genome and the unit length genome from plaque no. 2.

DISCUSSION

Three genes have been identified and mapped in the SV40 genome (for a recent review, see Kelly and Nathans, 1976): the A gene which codes for a protein (A protein) of about 100,000 daltons that contains determinants for SV40 T antigen (Lewis *et al.*, 1974; Tegtmeyer, 1974; Rundell *et al.*, 1976); the B/C gene which codes for the major virion protein (VP1) (Prives *et al.*,

1974; May *et al.*, 1975; Khoury *et al.*, 1976; Lai and Nathans, 1976); and the D gene which may code for a minor virion protein (Chou and Martin, 1975b). Based on the properties of *tsA* mutants, which map in the early region of the genome (Lai and Nathans, 1975), it has been inferred that the A protein is required for viral DNA replication (Tegtmeyer, 1972), stimulation of cellular DNA synthesis (Chou and Mar-

TABLE 7
VIRAL GENOMES RESCUED FROM *dl*-1007-3T3-2 BY
FUSION-COMPLEMENTATION

Plaque number	Length of genomes ^a (% of SV40 DNA)	Inferred structure of predominant genome
1	<u>100</u>	<i>ts</i> helper
2	<u>76</u> , 100	<i>dl</i> -1007
3	<u>100</u> , 76	<i>ts</i> helper
4	<u>100</u> , 76	<i>ts</i> helper
5	<u>100</u> , 76	<i>ts</i> helper

^a The predominant genome length is underlined.

tin, 1975), and cell transformation (Brugge and Butel, 1975; Martin and Chou, 1975; Osborn and Weber, 1975; Tegtmeyer, 1975; Kimura and Itagaki, 1975). The experiments reported here with mutants containing deletions within the early region confirm these earlier findings with *tsA* mutants. Of particular note is the complete lack of stimulation of cellular DNA synthesis (assessed by thymidine incorporation into high molecular weight DNA) after infection with *ev*-1117 or *ev*-1119, in contrast to the "leakiness" for this property of *tsA* mutants (Tegtmeyer, 1972; Chou and Martin, 1975). As shown in Fig. 1, both early deletion mutants tested are missing extensive segments of the early region of the genome, including that portion where almost all available *tsA* mutants map. Therefore the possibility of assigning separate functions to specific segments of the early region remains open. For example, it is possible that the A protein is a primary gene product that is subsequently cleaved into polypeptides with distinct functions or that the A protein itself has multiple, distinct activities.

In contrast to the early deletion mutants, the two mutants (*dl*-1007 and *ev*-1114) with intact A gene but extensive deletions of the late genomic segment, including deletions of portions of both the B/C and D genes (Fig. 1), can replicate their DNA without helper virus, stimulate thymidine incorporation into high molecular weight DNA of nonpermissive mouse cells, and transform mouse and hamster cells with about the same efficiency as wild-type virus. Furthermore, we have shown by fusion-complementation that the mu-

tant-transformed cells harbor the mutant genome. Since the *ev*-1114 genome retains only a small segment of DNA outside the early genomic region, we conclude that the early region plus immediately contiguous DNA segments are sufficient for all of the above viral functions. Again, these results agree with earlier studies of *tsB/C* and *tsD* mutants (Tegtmeyer and Ozer, 1971; Martin and Chou, 1975), and with the finding that a linear fragment of DNA derived from the genome segment between 0.15 and 0.74 map units (and thus the entire early region) can transform rat cells (Abrahams *et al.*, 1975). Extension of these several studies by use of other deletion mutants or DNA fragments should establish whether any DNA segments outside the early region are needed (e.g., transcription or replication signals), and also what parts of the early region are indispensable. Recent studies by Shenk *et al.* (1976) with viable deletion mutants already indicate that the genomic segment between 0.54 and 0.59 map units is not necessary for cell transformation.

The finding that the early genome segment of SV40 DNA is sufficient for viral DNA replication indicates that this segment of DNA will be useful as a vector for constructing self-replicating plasmids containing inserted DNA segments. If the recombinant is of appropriate size, it can be encapsidated into an SV40 virion in the presence of a helper mutant and cloned as described for constructed deletion mutants (Lai and Nathans, 1974a).

Uchida *et al.* (1966) first noted that defective particles of SV40 interfere with the replication of *wt* virus. The coinfection of cells with *wt* virus (under conditions which minimize competitive absorption) and either *ev*-1114, *ev*-1117, or *ev*-1119, resulted in inhibition of *wt* DNA replication as well as inhibition of virus production. Since both the early and late deletion mutants produce this inhibition, interference apparently does not depend on the presence of a specific gene product. On the other hand, since all three mutants contain a duplication of the replication initiation site, interference may be related to the number of initiation signals present;

i.e., interference may be due to competition for a limiting initiation protein which interacts at this site. According to this notion, molecules with multiple initiation sites should initiate replication more frequently than molecules with a single site. Consistent with this expectation is the observation that in cells coinfecting at about equal multiplicities with *wt* SV40 and *ev*-1114 (which is not dependent on helper virus for DNA replication), variant DNA accumulates (Table 2). Presumably this is the basis for the evolution of these variants (Brockman *et al.*, 1973). It will be of interest to determine in a more systematic way both rates of replication and interfering activity of SV40 variants as a function of the number of initiation sites per molecule (Lee *et al.*, 1975).

The observation that a single clone of 3T3 cells transformed by *ev*-1114, an evolutionary variant with a tandem duplication of DNA, yielded a variety of genomes related to *ev*-1114 after fusion-complementation is of interest. Among these genomes were molecules lacking the tandem duplication and others with what appear to be new duplications of DNA. In all of the latter instances, molecules the size of those lacking a duplicated segment were also present in the same virus plaque. One possible explanation for these observations is that excision of integrated *ev*-1114 DNA can occur in two ways (see Fig. 8): (1) by reversal of the integration step, i.e., recombination at the site of integration, giving rise to the original variant genome; or (2) by recombination within the duplicated segment, giving rise to a genome without the original duplication, as in the case of tandemly duplicated SV40 DNA in certain adeno-SV40 hybrid viruses (Kelly *et al.*, 1974). In the second mechanism, genomes 65% of the length of SV40 DNA are generated; since this length is probably below the level needed for efficient encapsidation, secondary duplications may be selected during subsequent rounds of virus replication. In fact, further propagation of the plaque suspension containing virus with predominant genomes 65% of unit length leads to the appearance of longer viral DNA molecules and disappearance of the 65% genome. On the other hand, rou-

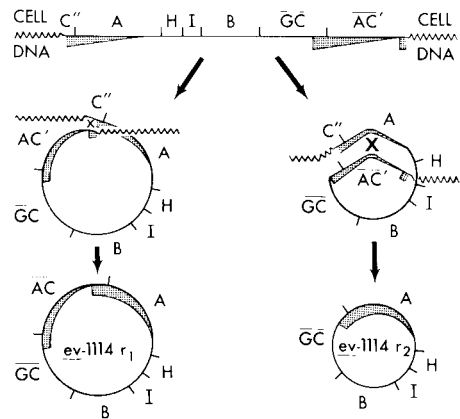


FIG. 8. Two possible modes of excision of integrated *ev*-1114 DNA. See the text for discussion of the model. Duplicated regions are indicated by the stippled segments.

tine propagation of *ev*-1114 in BSC-1 cells does not generate the 65% variant, suggesting that this genome was not derived in the fusion-complementation experiments from free *ev*-1114 DNA. The inferred mechanism of excision of integrated viral genomes containing duplications deserves more detailed and quantitative study.

Finally, we point out that the procedure used to rescue defective SV40 genomes from transformed cells is similar in principle to that described by Yoshiike *et al.* (1974), which was based on triple fusion of two transformed cells with a permissive cell. In the procedure described in the present communication, complementing functions are supplied by a superinfecting *tsA* mutant rather than a *wt* SV40 viral genome present in transformed cells. Therefore virus growth at elevated temperature is dependent on rescue of the endogenous genome, and infectious centers can be plated directly to isolate clones of rescued virus. This rescue procedure may have other applications, e.g., rescue of viruses from cells suspected of harboring specific defective genomes (e.g., from tumors or certain degenerative lesions), or rescue of misexcised viral DNA from transformed cells.

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REFERENCES

- ABRAHAMS, P. J., MULDER, C., VAN DE VOORDE, A., WARNAAR, S. O., and VAN DER EB, A. J. (1975). Transformation of primary rat kidney cells by fragments of simian virus 40 DNA. *J. Virol.* **16**, 818-823.
- BROCKMAN, W. W., and NATHANS, D. (1974). The isolation of simian virus 40 variants with specifically altered genomes. *Proc. Nat. Acad. Sci. USA* **71**, 942-946.
- BROCKMAN, W. W., and SCOTT, W. A. (1975). Biological activities of cloned evolutionary variants of simian virus 40. *Fed. Proc.* **34**, 526 (Abstract).
- BROCKMAN, W. W., GUTAI, M. W., and NATHANS, D. (1975). Evolutionary variants of simian virus 40: Characterization of cloned complementing variants. *Virology* **66**, 36-52.
- BRUGGE, J. S., and BUTEL, J. S. (1975). Role of simian virus 40 gene A function in maintenance of transformation. *J. Virol.* **15**, 619-635.
- CARBON, J., SHENK, T. E., and BERG, P. (1975). Biochemical procedure for production of small deletions in simian virus 40 DNA. *Proc. Nat. Acad. Sci. USA* **72**, 1392-1396.
- CHOU, J. Y., and MARTIN, R. G. (1974). Complementation analysis of simian virus 40 mutants. *J. Virol.* **13**, 1101-1109.
- CHOU, J. Y., and MARTIN, R. G. (1975a). Products of complementation between temperature sensitive mutants of simian virus 40. *J. Virol.* **15**, 127-136.
- CHOU, J. Y., and MARTIN, R. G. (1975b). DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. *J. Virol.* **15**, 145-150.
- DANNA, K. J., and NATHANS, D. (1971). Specific cleavage of simian virus 40 DNA by restriction endonuclease of *Hemophilus influenzae*. *Proc. Nat. Acad. Sci. USA* **68**, 2913-2917.
- DANNA, K. J., SACK, G. H., JR., and NATHANS, D. (1973). Studies of SV40 DNA. VII. A cleavage map of the SV40 genome. *J. Mol. Biol.* **78**, 363-376.
- GERSHON, D., SACHS, L., and WINOCOUR, E. (1966). The induction of cellular DNA synthesis by simian virus 40 in contact inhibited and X-irradiated cells. *Proc. Nat. Acad. Sci. USA* **56**, 918-925.
- HATANAKA, M., and DULBECCO, R. (1966). Induction of DNA synthesis by SV40. *Proc. Nat. Acad. Sci. USA* **56**, 736-740.
- HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **36**, 365-369.
- KELLY, T. J., JR., and NATHANS, D. (1976). The genome of simian virus 40. *Advances in Virus Research*, Vol. 21. Academic Press, New York, in press.
- KELLY, T. J., JR., LEWIS, A. M., JR., LEVINE, A. S., and SIEGEL, S. (1974). Structure of two Ad2-SV40 hybrids which contain the entire SV40 genome. *J. Mol. Biol.* **89**, 113-126.
- KHOURY, G., CARTER, B. J., GERDINAND, F.-J., HOWLEY, P. M., BROWN, M., and MARTIN, M. A. (1976). Genome localization of simian virus 40 RNA species. *J. Virol.* **17**, 832-840.
- KIMURA, G., and ITAGAKI, A. (1975). Initiation and maintenance of cell transformation by simian virus 40: A viral genetic property. *Proc. Nat. Acad. Sci. USA* **72**, 673-677.
- LAI, C.-J., and NATHANS, D. (1974a). Deletion mutants of simian virus 40 generated by enzymatic excision of DNA segments from the viral genome. *J. Mol. Biol.* **89**, 179-193.
- LAI, C.-J., and NATHANS, D. (1974b). Mapping the genes of simian virus 40. *Cold Spring Harbor Symp. Quant. Biol.* **39**, 53-60.
- LAI, C.-J., and NATHANS, D. (1975). A map of temperature-sensitive mutants of simian virus 40. *Virology* **66**, 70-81.
- LAI, C.-J., and NATHANS, D. (1976). B/C gene of simian virus 40. *Virology*, in press.
- LEE, T. N. H., BROCKMAN, W. W., and NATHANS, D. (1975). Evolutionary variants of simian virus 40: Cloned substituted variants containing multiple initiation sites for DNA replication. *Virology* **66**, 53-69.
- LEWIS, A. M., JR., BREEDEN, J. H., WEWERKA, Y. L., SCHNIPPER, L. E., and LEVINE, A. S. (1974). Studies of hamster cells transformed by adenovirus 2 and the nondefective Ad2-SV40 hybrids. *Cold Spring Harbor Symp. Quant. Biol.* **39**, 651-656.
- MARTIN, R. G., and CHOU, J. Y. (1975). Simian virus 40 functions required for the establishment and maintenance of malignant transformation. *J. Virol.* **15**, 599-612.
- MAY, E., KOPECKA, H., and MAY, P. (1975). Mapping the transcription site of the SV40-specific late 16S mRNA. *Nucleic Acids Res.* **2**, 1995-2005.
- MCCUTCHAN, J. H., and PAGANO, J. S. (1968). Enhancement of the infectivity of SV40 DNA with DEAE-dextran. *J. Nat. Cancer Inst.* **4**, 351-357.
- MERTZ, J. E., CARBON, J., HERZBERG, M., DAVIS, R. W., and BERG, P. (1974). Isolation and characterization of individual clones of simian virus 40 mutants containing deletions, duplications and insertions in their DNA. *Cold Spring Harbor Symp. Quant. Biol.* **39**, 69-84.
- OSBORN, M., and WEBER, K. (1975). Simian virus 40 gene A function and maintenance of transformation. *J. Virol.* **15**, 636-644.
- PRIVES, C. L., AVIV, H., GILBOA, E., REVEL, M., and WINOCOUR, E. (1974). The cell-free translation of SV40 messenger RNA. *Cold Spring Harbor Symp.*

- Quant. Biol.* **39**, 309-316.
- ROTHSCHILD, H., and BLACK, P. (1970). Analysis of SV40-induced transformation of hamster kidney tissue *in vitro*. *Virology* **42**, 251-256.
- RUNDELL, K., COLLINS, J. K., TEGTMEYER, P., LAI, C.-J., and NATHANS, D. (1976). Identification of simian virus 40 protein A. *J. Virol.*, in press.
- SHENK, T. E., CARBON, J., and BERG, P. (1976). Construction and analysis of viable deletion mutants of simian virus 40. *J. Virol.* **18**, 664-671.
- TEGTMEYER, P. (1972). SV40 DNA synthesis. The viral replicon. *J. Virol.* **10**, 591-598.
- TEGTMEYER, P. (1974). Altered patterns of protein synthesis in infection by SV40 mutants. *Cold Spring Harbor Symp. Quant. Biol.* **39**, 9-15.
- TEGTMEYER, P. (1975). Function of simian virus 40 gene A in transforming infection. *J. Virol.* **15**, 613-618.
- TEGTMEYER, P., and OZER, H. J. (1971). Temperature sensitive mutants of simian virus 40: Infection of permissive cells. *J. Virol.* **8**, 516-524.
- UCHIDA, S., WATANABE, S., and KATO, M. (1966). Incomplete growth of simian virus 40 in African green monkey kidney cultures induced by serial undiluted passages. *Virology* **27**, 135-141.
- YOSHIKKE, K., FURUNO, A., and UCHIDA, S. (1974). Rescue of defective SV40 from a transformed mouse 3T3 cell line. *Virology* **60**, 342-352.