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Simian Virus 40 Mutants Carrying Extensive Deletions in the 72-Base-Pair Repeat Region

DIRK GHEYSEN, ANDRE VAN DE VOORDE, ROLAND CONTRERAS, JOSE VANDERHEYDEN, FRED DUERINCK, AND WALTER FIERS*

Laboratory of Molecular Biology, State University of Ghent, B-9000 Ghent, Belgium

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Simian virus 40 mutants were constructed with deletions at the late side of the origin of DNA replication by partial *Bal* 31 digestion at the *Sph*I site or at the *Pvu*II site. Some of these mutants lost virtually all of both 72-base-pair repeat segments ("enhancer" sequences) and exhibited a decrease in viability from 20- to 300-fold; one particular mutant, *dl*1852, even showed a reduction of almost 10^4-fold. The very poorly growing deletion mutants were unstable and gave rise to DNA rearrangements upon further growth. An essential region for viability, at least in the absence of a 72-base-pair repeat, was revealed at the distal side of the 72-base-pair elements (L250 through L272). The effect of the deletions on T-antigen expression was measured, and the decreased viability of the mutants correlated with the impairment of T-antigen expression in all cases. The study of these mutants also revealed that the 72-base-pair repeats are not required for late transcription.

An essential step in our understanding of gene expression is to unravel the nucleotide signals involved in the initiation of transcription. For this purpose, the small DNA tumor viruses such as simian virus 40 (SV40) and polyoma virus have frequently been studied as model systems (51). Functional tests by means of deletion mutants (10, 21, 33) and point mutants (54) in vitro have shown that the TATA box that precedes most, but not all, eucaryotic genes transcribed by polymerase II (3, 29) plays a decisive role in the selection, ~31 nucleotides downstream on the DNA, of the predominant initiation site (3, 20, 21, 33). Functional tests of deletion mutants in vivo have shown that additional distant upstream sequences dramatically modulate or enhance gene expression (4, 11). SV40 early gene expression is not dependent on the presence of the TATA box in vivo (4, 16, 17, 18), but the latter is apparently involved in precise positioning of the 5′ termini of early mRNA (18, 33). Upstream enhancer sequences essential for the expression of the early genes of both SV40 and polyoma virus have been identified (4, 22, 52). The enhancing activity of the SV40 72-base-pair (bp) repeats has been exploited in the construction of high-level expression vectors for mammalian cells (1). The region involved in SV40 and polyoma virus late transcription does not contain TATA box sequences, but these viruses do, nevertheless, have short regions of homology with classical polymerase II promoters (9). The 5′ termini of the late transcripts are heterogeneous and spread out over a distance of more than 300 nucleotides (9, 19, 25, 39). The 5′ termini are capped, and the cap structures correspond to genuine initiation points of transcription (9). One method for the delineation and elucidation of the nature and function of transcriptional regulatory signals is to isolate and characterize viral mutants with altered or deleted signals. Previous studies have indicated that the removal of precisely one 72-bp repeat does not affect the viability of the viral genome (4, 53), but when the deletion is extended into the second repeat, the genome becomes nonviable (22). Moreau et al. (37) reached similar conclusions regarding early transcription by using plasmid constructions containing parts of SV40. Several viable deletion mutants in the late region of SV40 between the origin of replication (*Bgl*I site) and the initiation site of VP2 and VP3 have been reported (2, 3, 43, 44, 46), but only a few viable mutants have been described that lack sequences in the tandem 72-bp repeat region (4, 22, 31). In the present paper we report the construction of a series of mutants with deletions in the region between the *Bgl*I and the *Hpa*II sites of SV40. These were isolated to analyze signals involved in late gene expression and to explore the role of sequences in this region, including the 72-bp repeat, in viability.

MATERIALS AND METHODS

Cell line and viruses. The AP8 cell line of African green monkey kidney cells was used in all experiments

unless otherwise indicated. The cells were grown in Dulbecco modified Eagle minimal essential medium supplemented with 10% newborn calf serum (GIBCO Laboratories), and streptomycin and penicillin were added to give final concentrations of 100 μg/ml and 100 U/ml, respectively. Plaque assays were performed as described previously (36).

Enzymes. Restriction endonucleases *Pvu*II, *Hin*dIII, *Ava*II, *Bgl*I, *Bam*HI, *Hpa*II, *Xba*I, *Msp*I, *Taq*I, and *Eco*RI were purchased from New England Biolabs, and *Hpa*I and *Eco*RI were obtained from Boehringer Mannheim Corp. *Bal* 31 nuclease was from the Bethesda Research Laboratories or made in this laboratory. S1 nuclease was acquired from Miles Laboratories, Inc., and from Sigma Chemical Co., and T4 polynucleotide kinase came from P-L Biochemicals, New England Biolabs, and Boehringer Mannheim. *Exo*VII was made in this laboratory, and *Bam*HI, *Xba*I, and *Sal*I linkers were obtained from Collaborative Research, Inc.

Recombinant plasmids. (i) Construction and sequence of the p894 *Sph*I-*Bal* 31 series. SV40 mutant *dl*894 (and wild-type) viral DNA was linearized with *Eco*RI and inserted into the plasmid vector pBGF1 (3,356 bp) at the unique *Eco*RI site of the chloramphenicol resistance gene; the plasmid pBGF1 was derived from pBR325 by partial *Hae*II deletion of 2,639 bp (positions 5,704 to 2,348) of pBR325 (40) (Gheysen, unpublished data). The resulting recombinant p894 (5 μg) was opened at the unique *Sph*I site and treated with *Bal* 31 nuclease (0.5 U) for either 5, 10, or 20 min at 30°C in a buffer containing 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$, 0.6 M NaCl, 0.05% sodium dodecyl sulfate, and 20 mM Tris (pH 7.9). The three digests were pooled, phenol was added, and uneven ends were repaired with DNA polymerase large fragment (Boehringer Mannheim) in the presence of all four dinucleotide triphosphates. A 20-fold molar excess of *Xba*I linkers was then blunt end ligated to the DNA at 4 to 7°C overnight. After treatment with *Xba*I endonuclease, the DNA was recircularized by ligation at a DNA concentration of 2 μg/ml and cloned in *Escherichia coli* HB101. Sixteen carbenicillin-resistant colonies were picked up and grown in 20 ml of LB broth, and plasmid DNA was isolated by a modification of the Birmboim and Doly method (7). The extent of the deletions was approximately determined by restriction analysis with *Hin*dIII plus one of the following enzymes: *Xba*I, *Bgl*I, or *Eco*RI. Plasmids of interest were grown on a large scale by amplification with chloramphenicol. Plasmid DNA was prepared by lysis with lysozyme and nonionic detergent followed by CsCl-ethidium bromide gradient centrifugation. The deletions in molecules which proved of most interest were sequenced (34) by using a 5′ label at the *Pvu*II site (position L272; numbering as in reference 53) of SV40 DNA or at the *Msp*I site (L348).

(ii) Construction and sequence of p1836 *Kpn*I-*Xba*I-*Bal* 31 series. The SV40 mutant *in*1836 was created starting from a plasmid pSMboC, which carried the SV40 *Mbo*II fragment C (E236 to L475) cloned by means of *Bam*HI linkers into the *Bam*HI site of pBR322. The plasmid pSMboC was partially cleaved with 2 U of *Alu*I enzyme per μg of DNA for 3 min at 37°C in the presence of ethidium bromide (33 μg/ml). After isolation of linear DNA from a low-melting-point agarose, a 20-fold molar excess of *Xba*I linkers was added; after ligation and *Xba*I digestion, the DNA was recircularized at a low concentration and cloned in *E. coli* HB101. A recombinant plasmid pSMboC9 that lacked the *Pvu*II site of SV40 at 0.72 map units (L272), but that could be cleaved by *Xba*I restriction enzyme, was characterized. The SV40 *Bgl*I-*Kpn*I (0.67 to 0.715 map units) fragment from pMboC9 was ligated into a *Bgl*I-*Kpn*I 5,045-bp agarose-purified SV40 DNA fragment, and this mixture was transfected into AP8 monkey cells (14, 35).

Twelve plaques were chosen for further analysis by preparing ^{32}P-labeled viral DNA from a 20-mm well of a Costar plate. The location of the insertion (*Xba*I linker) was determined by restriction with *Pvu*II plus each of the following enzymes: *Xba*I, *Hin*dII, *Bgl*I, and *Eco*RI. A single plaque isolate, *in*1836, was selected and further characterized by sequencing (34). It appeared that two tandem *Xba*I linkers (16 bp) were inserted at the *Pvu*II site (L272), creating a unique *Sac*I site in this SV40 mutant *in*1836. This was confirmed by restriction analysis. The *in*1836 mutant was subcloned via the unique *Eco*RI site into pBGF1 as described above. The recombinant p1836 was digested either with *Kpn*I or with *Kpn*I plus *Xba*I and treated with *Bal* 31 nuclease as described above. The deletions in isolates that proved of interest were characterized by sequencing (34).

(iii) Construction of the *Xba*I exchange mutants. The mutants p1847 and pS1848, both carrying an *Xba*I linker at the junction of the deletion, were used to create the mutants pS1853 and p1854 by exchanging the appropriate *Xba*I-*Bam*HI fragments (see Fig. 1). This was accomplished by ligation of the agarose-purified *Xba*I-*Bam*HI 2,827-bp SV40 fragment of p1847 into the acceptor *Xba*I-*Bam*HI fragment (5,573 bp) of pS1848, resulting in the new mutant pS1853, and vice versa for p1854. The mutant p1852 was created by exchanging the *Xba*I-*Bam*HI 2,827-bp SV40 fragment of p1847 with the *Bam*HI-*Xba*I 2986-bp fragment of p1836.

Transfection of monkey cells with cloned viral DNA and indirect immunofluorescence assay of SV40 T-antigen. Subconfluent cultures of AP8 cells grown on cover slips (1.3-cm diameter) in 20-mm wells were transfected with a recombinant DNA plasmid by the DEAE-dextran method (35) as previously described (14). The medium was removed by aspiration 60 h posttransfection, and the cells were processed for large T-antigen immunofluorescence. The wells were rinsed once with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-F11 medium, and a solution containing 95% methanol–5% HEPES-buffered saline was added. This methanol solution was replaced by 0.5 ml of fresh cold 100% methanol, and the wells were fixed for 10 min. The methanol was removed; after drying, the microwells were either stored at −70°C or immediately processed for large T-antigen immunofluorescence.

The microwells were rinsed twice with HEPES-buffered saline, 40 μl of pretreated (see below) hamster SV40 T-antigen antiserum (Flow Laboratories, Inc.) was added, and the wells were kept for 2 to 4 h at 37°C with occasional tilting. Then the cover slips were washed three times with HEPES-buffered saline, and 40 μl of fluorescein isothiocyanate-conjugated goat anti-hamster globulin (Progressive Laboratories) diluted (1:10) in HEPES-buffered saline was added for 2 h

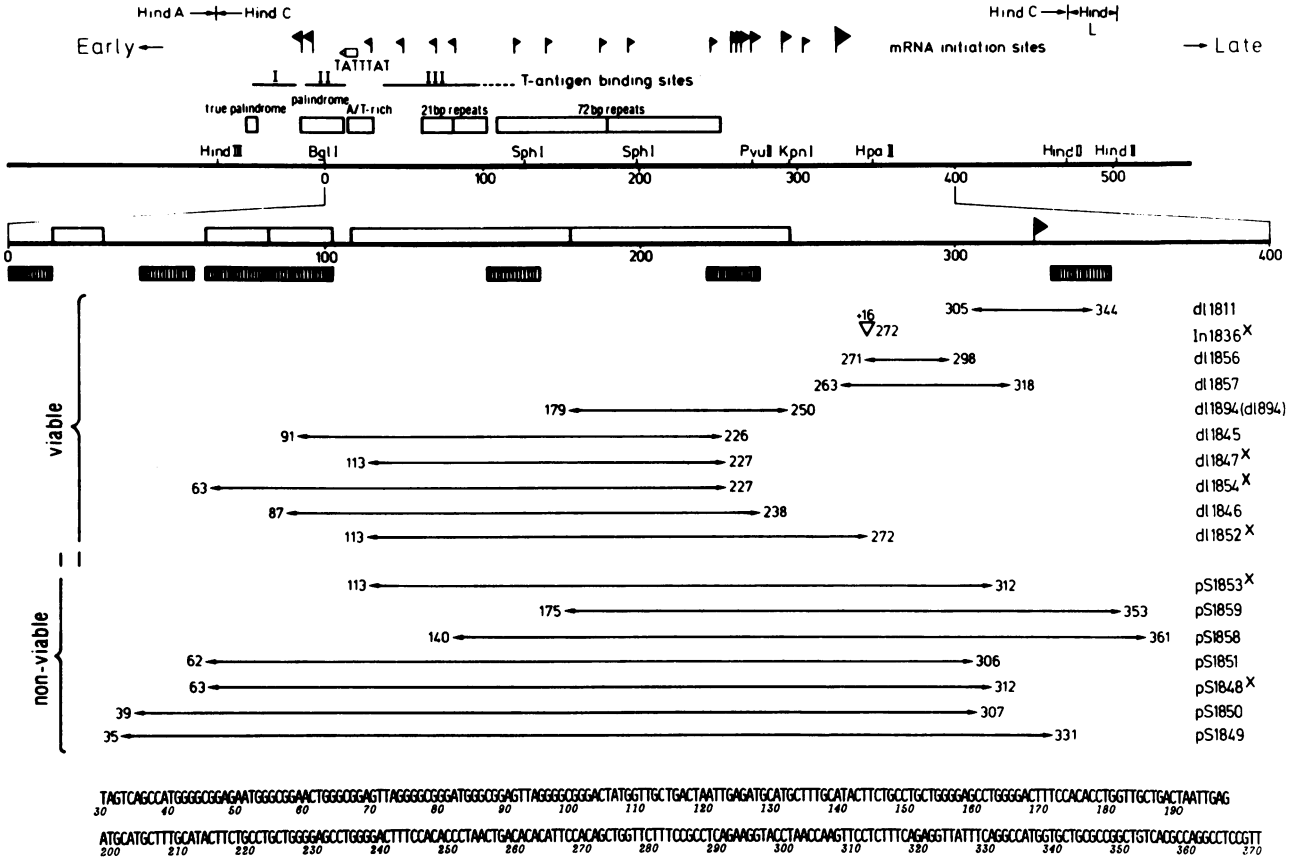

FIG. 1. SV40 promoter control region showing the different constructed 72-bp repeat region deletion mutants. The heavy line represents the relevant segments of the SV40 genome; it is identified by restriction sites above and nucleotide numbering below (13, 53). The nucleotide sequence of this region is shown at the bottom (13, 53). The origin of DNA replication (5, 27, 32, 44, 48) is believed to be coincident with the *BglI* site (nucleotide 0). Early transcription is toward the left (9a, 17, 23), and late transcription is toward the right (9, 19, 25, 39); early and late transcription initiation sites are indicated by leftward- and rightward-pointing flags, respectively, with size of the flag proportional to message abundance. Also represented are the T-antigen-binding sites around the region of replication (26, 50). Palindromes, the AT-rich sequence (17 A/T), the 21-bp repeats (L62 to L82 and L83 to L103), and the two 72-bp repeat sequences (L107 to L178 and L179 to L250) are shown as open boxes. The late region between nucleotides 0 and 400 is shown enlarged with the major transcriptional initiation point (L325) identified by a flag. The hatched areas indicate GC-rich regions (>80% GC over >15 nucleotides). The construction of the different deletion mutants is described in the text. The mutants are ordered according to their decreasing capacity for growth on monkey cells. The endpoints of the different deletion mutations are indicated by the nucleotide sequence number (first and last deleted nucleotide). Mutants which produced no plaques on AP8 monkey cells are denoted by their chimeric plasmid SV40 representation pS followed by the construction number. Mutants which carry an *XbaI* linker at the junction of the deletion are denoted by a superscript X (see the text).

at 37°C with tilting every 15 min. The cover slips were rinsed three times with HEPES-buffered saline before inspection under a Leitz-Dialux incident-light fluorescence microscope. Pretreatment of the SV40 T-antiserum was necessary to avoid artifacts because rounded-off mitotic cells and cells that died before fixation could also bind antibody. This pretreatment was as follows. About 2×10^7 AP8 cells were trypsinized and suspended in 50 ml of medium and then incubated at 21°C overnight with gentle shaking. The cells were collected and washed three times with 50 ml of HEPES-buffered saline. SV40 T-antiserum (0.5 ml) was added to the cell pellet, which was suspended and incubated for 1 h at 37°C and then incubated at 4°C overnight with gentle rotation. The cells were collected by centrifugation, and the supernatant was passed through a 0.22-μm membrane filter (Millipore Corp.).

Southern blotting. The restricted DNA prepared from the individual mutants was analyzed on horizontal 1.4% agarose slab gels (20 by 20 by 0.4 cm), which were run for 15 h at 24 mA in 50 mM Tris-acetate–20 mM sodium acetate–2 mM EDTA (pH 7.8). The DNA was denatured and transferred to nitrocellulose filter paper (Schleicher & Schuell Co.; BA85401180) essentially as described by Southern (46). The ^{32}P-labeled SV40 probe was obtained by nick translation (41). Hybridizations were performed as previously described (49).

S1 nuclease and *Exo*VII nuclease mapping. Late SV40 mRNAs were analyzed by the nuclease S1 or *Exo*VII mapping technique (6, 12) as modified by Favaloro et al. (12) for single-stranded probes. One microgram of polyadenylated RNA isolated from SV40-infected CV1 cells 48 h after infection was hybridized to a single-stranded, 5'-end-labeled *Hpa*II-*Bgl*I wild-type DNA probe derived from pSCL04 as described by Contreras et al. (9a). The SV40 recombinant plasmid contains the *Hin*dII-*Hin*dIII fragments C and L (E67 to L501) encompassing the SV40 origin of replication and promoter control region. This fragment, after addition of a *Bam*HI linker onto the *Hpa*I site (L501), was cloned between the *Hin*dIII and *Bam*HI sites of pBR322.

RESULTS

Construction and characterization of deletion and insertion mutants by using cloned viral SV40 DNA. Full-length, wild-type 776 (*wt*776) or *dl*894 DNA was cloned into the *Eco*RI site of the chloramphenicol resistance gene of pBGF1, a derivative of pBR325 (see above). This enabled us to propagate the SV40 mutant DNA in bacteria and simplified the procedure for the isolation of "pure" mutant clones, particularly those potentially defective in viral DNA replication.

Diagrammatic representations of the mutants used in this study are given in Fig. 1. Most deletions were constructed within the region containing the 72-bp repeats by treatment with *Bal* 31 nuclease at the *Sph*I site of the cloned *wt*776 or the natural mutant *dl*894 (see above).

Plaque-forming capacity of the different deletion mutants in the 72-bp segment region. The relative ability of the SV40 mutants to form plaques on AP8 monkey cells was assessed after excision from their vector with *Eco*RI and recircularization (Table 1). Removal of exactly one 72-bp repeat by the *Sph*I deletion (p1894) did not cause loss of viability, in agreement with previous results with the virus *dl*894 (22). This mutant *dl*894 had approximately the same plaque size as the wild-type virus and caused only a slight reduction (10 to 20%) of virus titers on AP8 cells in a time kinetic experiment. However, *dl*894 produced at least twice as much viral DNA as did *wt*776 when assayed over 92 h (data not shown). The introduction of deletions into the single remaining 72-bp segment of p894 (*dl*1845, *dl*1846, and *dl*1847) caused a drastic reduction in plaque size (up to 3 to 10 times less) when compared on day 19 of plaque assay (Table 1). Further bidirectional extension of these deletions, resulting in the complete removal of the 72-bp segments, the 21-bp repeats, and the sequences lying between the second repeat and extending up to the *Kpn*I site (pS1848 to pS1851 series), eliminated viability (Table 1 and Fig. 1).

It is remarkable that the mutant *dl*1852, which retains only five nucleotides at the *ori*-proximal end of the 72-bp segment, still produced minute plaques at 25 days postinfection, and several more infected foci could be detected microscopically on the basis of the SV40-specific cytopathogenic effect. Mutant pS1853, with a deletion extending further into the late region (L312), produced similar cytopathogenic loci, but here we were never able to detect plaques, even after prolonged incubation for more than 25 to 27 days.

The DNA infectivity was measured as the plaque-forming capacity per microgram of cloned (and excised) viral DNA on AP8 monkey cells. *wt*p776 and *wt*776 viral DNA, purified by CsCl-ethidium bromide centrifugation, displayed nearly the same infectivity (Fig. 2). This means that neither the amplification step in bacteria (*E. coli* HB101) nor the excision of the SV40 genome part from the plasmid followed by dilute ligation (2 μg of DNA per ml) resulted in important loss of viability. The reduction in infectivity varied from two- to threefold for *dl*894 and *dl*1856 to more than 300 times for *dl*1846 (Fig. 2). Mutant *dl*1846 carries only the *ori*-distal 12 nucleotides of the single 72-bp segment. Apparently *dl*1852, although it carries no 72-bp repeats except for the first five nucleotides, is still a viable mutant virus. Its plaque-forming capacity is reduced by a factor of about 10^4 as compared with *wt*776 DNA. This places *dl*1852 in the "twilight" of viability. Viability was completely lost, however, when additional sequences between the *Pvu*II and the *Kpn*I sites were removed (pS1853). These findings are in agreement with the results of experiments on the

TABLE 1. Plaque-forming capacity of deletion mutants[a]

Chimeric SV40 plasmid	Nucleotides deleted	Mean plaque diameter (mm) at different times postinfection				
		Expt 1 (day 19)	Expt 2		Expt 3	
			Day 15	Day 19	Day 15	Day 25–27
Viral 776		–	1.90 ± 0.29	X	3.40 ± 0.80	5.40 ± 1.2
p776		5.64 ± 0.80	1.80 ± 0.27	X	–	–
p894(p1894)	72	4.70	1.47 ± 0.30	X	–	–
Viral *in*1836[b]	+16	–	1.27 ± 0.35	1.90 ± 0.36	–	–
p1836[b]	+16	–	1.37 ± 0.37	X	–	–
p1856	28	–	1.25 ± 0.35	2.25 ± 0.22	–	–
p1857	56	–	1.50 ± 0.47	3.75 ± 0.36	–	–
p1845	136	1.80 ± 0.40	NV	0.85 ± 0.33	–	–
p1847[b]	115	1.20 ± 0.50	NV	0.81 ± 0.33	–	–
p1846	152	0.80 ± 0.25	NV	0.58 ± 0.30	0.60 ± 0.30	0.90 ± 0.32
p1854[b]	165	–	NV	X	0.60 ± 0.26	0.90 ± 0.32
p1852[b]	160	–	–	–	–	0.50 ± 0.20
pS1853[b]	200	–	None	None	CPE	CPE
pS1848[b]	250	None	–	–	–	–
pS1849	297	None	–	–	–	–
pS1850	269	None	–	–	–	–
pS1851	245	None	–	–	–	–
pMK16/8-4	4	None	None	None	None	None

[a] The SV40 mutants (see the text) were excised from their plasmid vectors by *Eco*RI digestion and recircularized at higher dilution (2 μg/ml) followed by DEAE-dextran-mediated DNA transfection as described elsewhere (12). The results of several plaque assay (36) experiments are shown. Plaques were scored after the indicated time interval. The replication-negative mutant pMK16/8-4 was obtained from Y. Gluzman (15) and was used as a control. The diameters of the plaques were calculated by counting at least 10 plaques from each DNA dilution on each of four separate plates, except for mutant p1852, for which a maximum of only 20 plaques could be scored. Abbreviations: –, not done; X, cell monolayers destroyed; NV, plaques not visible macroscopically; none, neither plaques nor cytopathic effect visible; CPE, cytopathic effect.

[b] Plasmid mutants carrying an *Xba*I linker at the junction of the deletion (see the text).

nonviable mutant *dl*2356 (*Sph*I-*Kpn*I deletion) reported by Gruss et al. (22).

SV40 late deletion mutations affecting early gene expression. It is possible that mutants lacking part or most of the 72-bp segment had reduced viability because they were impaired in early gene expression. Therefore, we have analyzed these mutants for their ability to express SV40 large T-antigen 60 h after DEAE-dextran-mediated transfection by the T-antigen immunofluorescence assay (36). The quantitative relationship between expression level and the percentage of SV40 T-antigen positive cells is unknown, but is unlikely to be strictly linear (see below). We therefore applied three different concentrations of mutant DNA in the range of 0.3 to 6 ng, 6 to 180 ng, and 2 μg per cell lawn on a cover slip (1.3-cm diameter). As both the total number of transfected cells and the number of T-antigen-positive cells varied greatly between two experiments, we scored T-antigen-positive cells on a scale of +++, ++, +, or − (covering a range of at least 2 orders of magnitude). This procedure was preferred to counting the actual percentage since the statistical error on the latter would be unacceptably high. The results summarized in Table 2 show that for transfections with plasmid DNA of mutants *dl*1852, *dl*1854, pS1858, and pS1859 the number of positive cells increased at higher DNA concentrations, whereas for *wt*p776 or mutants only slightly hampered in the 72-bp segment no effect of DNA concentration was detected (i.e., saturation to maximum number of T-antigen-positive cells was already reached at the lower DNA concentration used).

To demonstrate that the above results could not simply be explained by an impairment in DNA replication rather than a deficient "early" transcription, we included the SV40 plasmid pMK16/8-4 (kindly provided by Y. Gluzman) as a control. This plasmid carries a 4-bp deletion at the *Bgl*I site of SV40, resulting in an SV40 replication-negative phenotype (15). In our assay conditions this mutant gave roughly the same level of large T-antigen-positive cells as did p776 (Table 2) (16).

Mutants *dl*1845 and *dl*1847, which carry extensive deletions in the last remaining 72-bp segment but which retain an intact GC-rich block around the *Eco*RII site and the very *ori*-distal end of the 72-bp segment, display almost the same percentage of large T-antigen-positive cells as does wild-type viral DNA (Table 2). This demonstrates that the *ori*-distal end of the 72-bp repeat is important for enhancer activity (37).

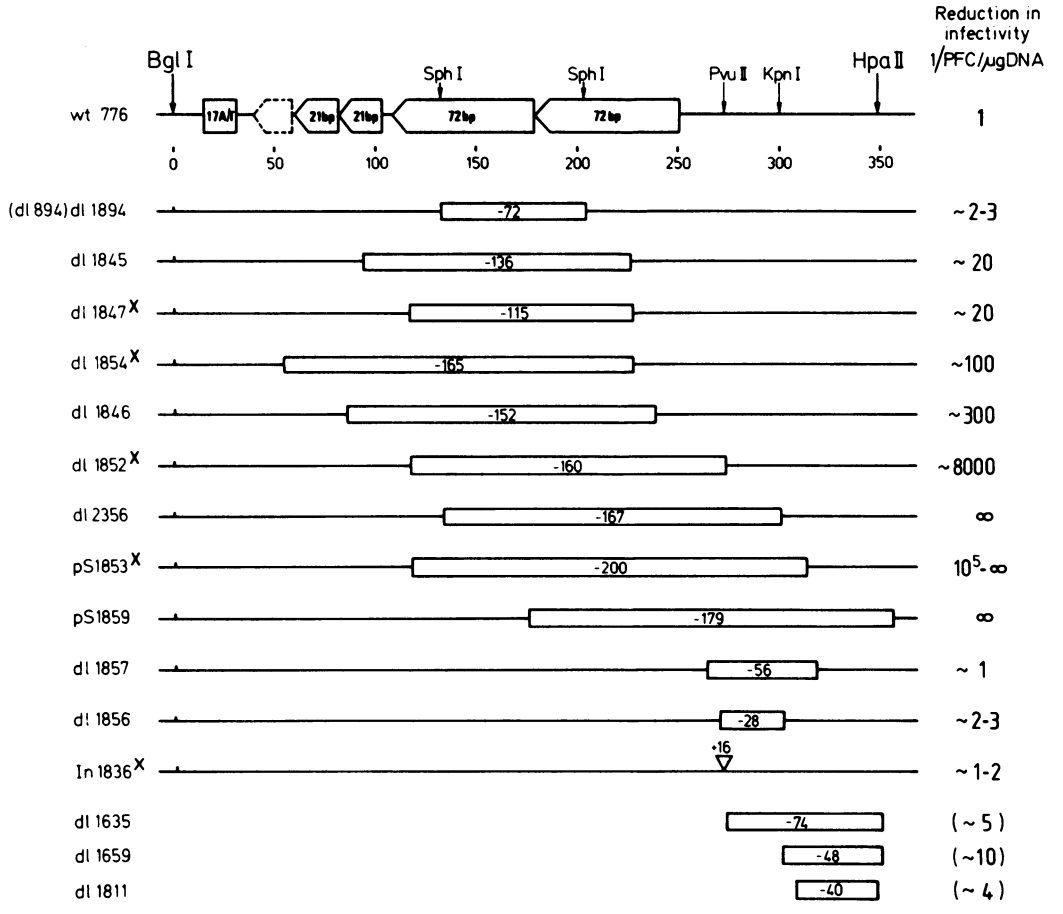

FIG. 2. Correlation between map position of the deletions and the reduction in infectivity. *wt*776 DNA with some characteristic features is shown at the top (nucleotide numbering as described in references 13 and 53). The different deletion mutants were compared with wild-type DNA for their relative ability to form plaques on AP8 monkey cells (experimental details as in footnote *a* of Table 1) as shown on the right. The mutants that carry an *Xba*I linker (see the text) at the junction of their deletion are denoted by a superscript X. The data for *dl*1635, *dl*1659, and *dl*1811 were obtained by assaying the supernatant medium of infected cells for PFU. Other data were from the following sources: *dl*2356, Gruss et al. (22); *dl*1635 and *dl*1659, leader deletion mutants of Subramanian (47); *dl*1811, major initiation deletion mutant of Haegeman et al. (24).

Further removal of sequences involved in the aforementioned GC-rich block, however, as in *dl*1846, caused a drastic decrease in the number of large T-antigen-positive cells of almost 2 orders of magnitude at low DNA concentration (Table 2), although the reduction in T-antigen-positive cells at moderate DNA input was almost 1 order of magnitude. Extension of the deletion to remove this GC-rich block and the very *ori*-distal end of the 72-bp repeat, as well as some additional sequences up to the *Pvu*II or the *Kpn*I site (or both), resulted in mutants that became almost T-antigen negative (*dl*1852) or T-antigen negative (pS1853) at moderate DNA concentrations. Curiously, pS1853 displayed a severely reduced T-antigen immunofluorescence intensity which was at the very limit of detection. The reason that we were able to observe a faint T-antigen immunofluorescence is due to the special pretreatment of the T-antiserum (see above), which resulted in a very low background of (auto)immunofluorescence of the cell monolayer. However, these mutants could still induce T-antigen-positive nuclei to the same extent as *wt*p776 when a large amount of DNA (2 μg per 1.3-cm cover slip was applied in the transfection assay.

None of the non-plaque-forming mutants, pS1851, pS1850, pS1848, and pS1849, produced large T-antigen (considering the detection limit, this means at least 100 to 1,000 times less than p776). Again, if a high input of DNA was used, a

TABLE 2. Ability of deletion mutants to induce T-antigen-positive cells[a]

Chimeric SV40 plasmid	No. of T-antigen-positive cells	Amt of DNA (ng)[b]	No. of T-antigen-positive cells	Amt of DNA (ng)[b]	No. of T-antigen-positive cells	Amt of DNA (ng)[b]
Virus 776	+++		+++		+++	
Viral 776 DNA	+++	9	+++	180	+++	2,000
p776	+++	3	+++	60	+++	2,000
p894	+++	0.9	+++	18	+++	2,000
p1836	+++	0.3	+++	6	+++	2,000
Viral *in*1836	+++	3	+++	60	+++	2,000
p1856	+++	0.6	+++	12	+++	2,000
p1857	+++	0.3	ND[c]		ND	
p1845	+++	0.6	+++	12	+++	2,000
p1847	+++	6	+++	112	+++	2,000
p1846	−	1.8	++	39	+++	2,000
p1854	−	0.9	++	18	+++	2,000
p1852	−	6	+	112	+++	2,000
pS1853	−	0.3	−[d]	6	+++[e]	2,000
pS1859	−	6	+	112	+++	2,000
pS1858	−	6	+	60	ND	
pS1851	−	3	−[d]	39	−[d]	2,000
pS1850	−	1.8	−[d]	39	−[d]	2,000
pS1848	−	1.8	−[d]	39	−[d]	2,000
pS1849	−	1.8	−	39	−[d]	2,000
pMK16/8-4[f]	ND		+++	6	+++	2,000

[a] Subconfluent AP8(CV1) cells growing on 1.3-cm cover slips were transfected by the DEAE-dextran procedure (14, 35) with different amounts of the chimeric mutant DNAs (see the text). The SV40 plasmid mutants were excised from their vectors by *Eco*RI digestion and subsequently recircularized by ligation at higher dilution. At 60 h after transfection, the cells were prepared for T-antigen immunofluorescence (see the text). Three ranges of mutant DNA, 0.3 to 6 ng, 6 to 18 ng, and 2 μg, were applied to AP8 cells on cover slips. The data on T-antigen immunofluorescence of the individual cloned mutant DNAs were obtained from repeated runs of seven separate experiments and are averages taken for two or three cover slips. The columns give the capacity of the mutants to induce T-antigen-positive cells, according to an arbitrary scale for +++ to −, covering a difference of at least 2 orders of magnitude of T-antigen-positive cells (see the text). The amount of *wt*p776 DNA used in the transfection assay represented in the first line (3 ng per cover slip) is saturating. Therefore, the same numbers of T-antigen-positive cells were scored even when larger amounts of DNA were used.

[b] Amount of DNA applied per cover slip (see the text).

[c] ND, Not determined.

[d] A few positive nuclei were scored.

[e] Drastic reduction in the intensity of T-antigen immunofluorescence to the limit of detection.

[f] The origin mutant pMK16/8-4, which is replication negative (15), was included as a reference.

small number (<2 to 10) of large T-antigen-positive cells could be detected with pS1851, pS1850, and pS1848. This was not the case for pS1849, which contained the most extensive deletion (nucleotides L35 to L331; see Fig. 1 and below). At these unusually high DNA input levels, we are possibly observing rare events (integration, rearrangement).

The results with the nonviable mutant pS1859, which has an extensive deletion in the sequences both upstream and downstream from the predominant late 5′ cap site (9, 19, 25), were particularly striking. This mutant (deletion L175 to L353) contained almost an intact 72-bp segment, except for the last four *ori*-distal nucleotides, and did not contain the region beyond the 72-bp segment over and beyond the *Hpa*II site. This displayed a drastic reduction in the number of large T-antigen-positive cells of between 1 and 2 orders of magnitude (Table 2). This severe reduction in T-antigen-positive cells may be attributed either to the *ori*-distal extreme end (four nucleotides) of the single remaining 72-bp segment or to a function of the region beyond the 72-bp enhancer segment which becomes important in the absence of a complete 72-bp segment (or to both). The importance of the *ori*-distal end of the 72-bp repeat was shown already by analysis of the TB101 and TB202 mutants by Moreau et al. (37). It may be noted that there was a discrepancy between the microinjection experiments and the DEAE-dextran transfection assays, as the former experiments did not reveal an important loss of enhancer activity (37). In this respect, our T-antigen immunofluorescence data obtained with high amounts of input DNA

(Table 2) are perhaps more comparable to data from microinjection experiments.

Mutants with deletions in the region beyond the enhancer segment, such as *dl*1856, *dl*1857, and *dl*1811, and other mutants, such as *dl*1635 and *dl*1659 (47), do not (greatly) affect early T-antigen expression, but *dl*1852 (L113 to L272) shows more than a 10-fold reduction in the number of T-antigen-positive cells relative to *dl*1847 (L113 to L227). This points again to the importance (in the absence of a functional 72-bp segment) of the region encompassing the *ori*-distal end of the 72-bp segment or the sequences up to the *Pvu*II site at L272 (or both).

Analysis of the late transcription pattern of the different deletion mutants. To evaluate the role of the deleted sequences in late transcription, we performed an S1 and *Exo*VII analysis of mRNA extracted from AP8 monkey cells 48 h after infection with *wt*776, *dl*894, and *in*1836 and with the deletion mutants *dl*1845, *dl*1846, and *dl*1847. Figure 3 shows the banding pattern corresponding to the mRNA start points. The pattern produced by *Exo*VII mapping is similar to that with nuclease S1 but is more discrete.

Although *dl*1845, *dl*1846, and *dl*1847 have extended deletions in the 72-bp repeat sequence (Fig. 1), initiation at any position used in the wild type (including the major initiation site at L325) and not removed by the deletion is apparently still functional (Fig. 3). In the mutant pS1850 most of the sequence upstream of the major initiation site (L325) is deleted up to 18 nucleotides before this site (9a), and yet no interference with transcriptional initiation at this site was observed (data not shown).

*dl*1846 exhibits a new strong band close to the boundary of the junction of the deletion. It could correspond to a new mRNA(s) 5′ end. This band was not found with the analogous (but slightly smaller) deletion mutants *dl*1845 and *dl*1847, which still have most of the GC-rich block present in the 72-bp segment (Fig. 1).

A peculiar feature is the enhancement of some minor wild-type initiation sites found with *dl*894 and *in*1836 (which carries a 16-bp *Xba*I linker insertion at the *Pvu*II site [L272; see above]). Mutant *dl*894, which lacks one 72-bp repeat, shows an enhanced transcription in the region distal to the 72-bp segment. A 16-bp insertion in mutant *in*1836 just distal to the initiation sites around position 264 drastically reduced this transcription, with a concomitant enhancement of the higher bands (L120, L140, and L167) (9a).

Heterogeneity in mutant virus stocks carrying deletions in the 72-bp segment. We have shown that deletions in the remaining 72-bp segment of *dl*894 severely reduced the ability to form plaques on AGMK-AP8 cells (Table 1 and Fig. 2). When randomly chosen plaques picked after DNA-mediated transfection of different mutants were propagated and the resulting viral DNA was subjected to restriction analysis, we observed a marked heterogeneity with plaques derived from *dl*1845, *dl*1846, and *dl*1847. Figure 4 shows the restriction analysis pattern obtained after *Ava*II digestion of the Hirt extracts prepared after a high-multiplicity infection with the first low passage starting from a "unique purified" plaque. These stocks exhibited a reassorted or rearranged pattern mainly characterized by length polymorphism of the *Ava*II fragment encompassing the *Hin*dII-C fragment. This indicates that these low-passage stocks, which are derived from homogeneous bacterial plasmids, must contain duplications or have picked up other DNA sequences during propagation in the monkey cells. Similar heterogeneity was also apparent from Southern blotting experiments (data not shown). Mutant *dl*1846 exhibited the most pronounced DNA heterogeneity and was also the mutant with the most impaired growth capacity when compared with *dl*1845 and *dl*1847 (Table 1 and Fig. 2). None of the seven Hirt supernatants originating from *dl*1846 produced fragments of the length expected from the parent SV40 plasmid in which the deletion was created; after *Ava*II digestion, four of the plaques (Fig. 4a, lanes 2, 3, 4, and 6) even yielded multiple fragments. These were present in submolar quantities, indicating a polymorphism of the *Ava*II-D fragment.

Three of the other plaques had acquired a larger *Ava*II-D fragment of about the same size as that of the wild type. The mutant *dl*1847 displayed an analogous, but less pronounced, heterogeneity. Mutant *dl*1845 exhibited almost no heterogeneity, except in one plaque (Fig. 4b, lane 4), which had acquired an *Ava*II-D fragment with a length slightly longer than that of the wild-type fragment. Control experiments with *dl*894 and *wt*776 revealed that the presence of plasmid sequences in conjunction with the transfection procedure could not account for the observed heterogeneity (data not shown).

Since these results were obtained after a cycle of low-multiplicity and high-multiplicity passages, we have also tried to extract DNA obtained after DEAE-dextran-mediated transfection of excised plasmid DNA followed by direct ^{32}P labeling at the onset of the cytopathic effect. The resulting DNA was purified on low-melting-point agarose, and the form I DNA was subjected to *Bgl*I and *Hpa*II digestions. Under these circumstances we detected a small (5 to 10%) heterogeneity in *dl*1845, *dl*1846, and *dl*1847, whereas no heterogeneity could be found with *wt*p894 or *wt*p776 (data not shown), indicating that heterogeneity is quickly acquired after serial infective cycles.

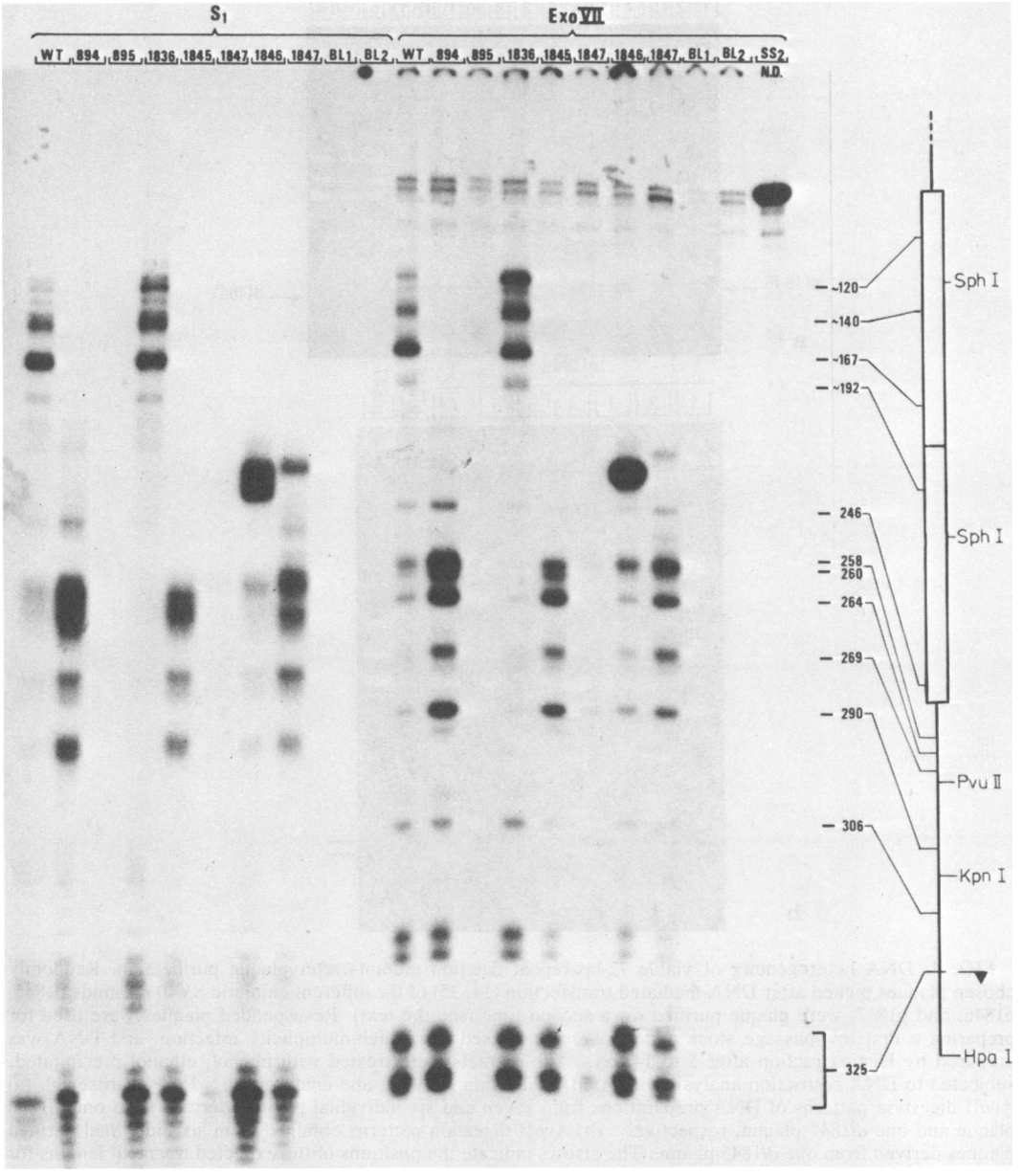

FIG. 3. Mapping of late mRNA start points of *wt*776 and the constructed viable deletion mutants. Mutant virus from the first DEAE-dextran plaque isolates was used for infection of AP8 cells. At 48 h postinfection, cytoplasmic polyadenylated mRNA was prepared. About 1 μg was hybridized against a wild-type single-stranded 5′-^{32}P-labeled *Hpa*II-*Bgl*I (L348) probe (see the text). The RNA-DNA hybrid was digested by S1 or *Exo*VII nuclease as described elsewhere (9a, 12), and the protected DNA segments were analyzed on an 8% polyacrylamide gel. The various mutants used are identified at the top. BL1 indicates the single-stranded probe incubated with S1 or *Exo*VII nuclease without polyadenylated mRNA, BL2 indicates the single-stranded probe incubated with polyadenylated mRNA from uninfected CV_1 cells, and SS_1 indicates the single-stranded probe without digestion. The right side of the figure is a schematic representation of the wild-type initiation points and major restriction enzyme sites. The flag indicates the major late SV40 initiation site at L325.

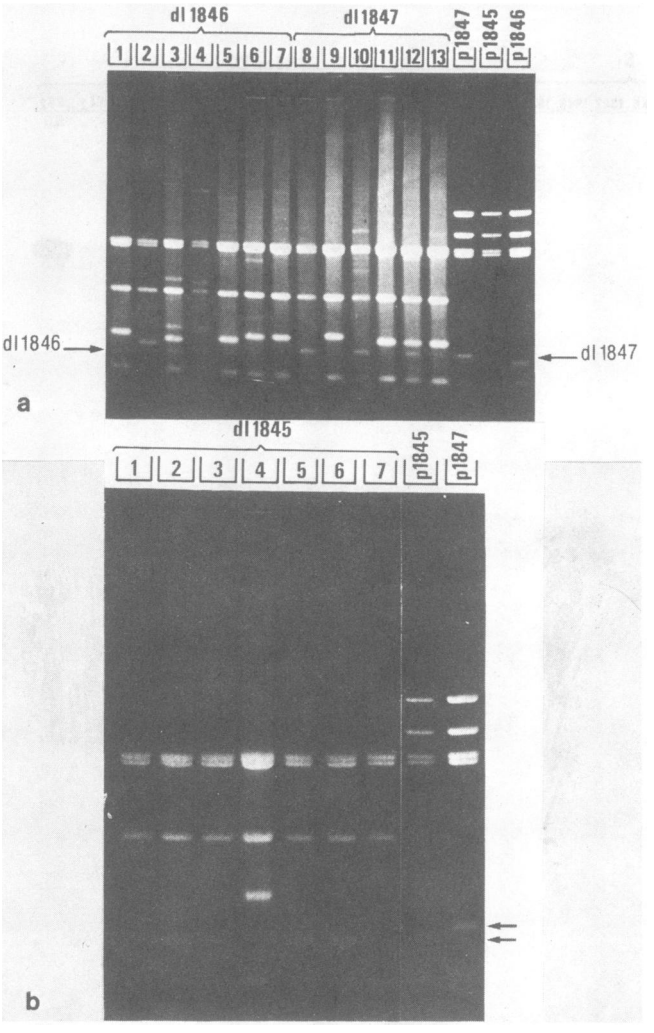

FIG. 4. DNA heterogeneity of viable 72-bp repeat deletion mutants after plaque purification. Randomly chosen plaques picked after DNA-mediated transfection (14, 35) of the different chimeric SV40 plasmids p1845, p1846, and p1847, were plaque purified for a second time (see the text). Resuspended plaques were used for preparing a first low-passage stock. These were then used for a high-multiplicity infection, and DNA was prepared by Hirt extraction after 5 to 14 days. The extracts were treated with phenol, ethanol precipitated, subjected to DNA restriction analysis with *Ava*II restriction enzyme, and analyzed on a 1.4% agarose gel. (a) *Ava*II digestion patterns of DNA preparations from seven and six individual plaques derived from one *dl*1846 plaque and one *dl*1847 plaque, respectively. (b) *Ava*II digestion patterns obtained from six individual purified plaques derived from one *dl*1845 plaque. The arrows indicate the positions of the expected fragment lengths for the individual mutants.

DISCUSSION

Effect of deletions in the 72-bp repeat segment on infectivity and mutant genome stability. The introduction of deletions into the single remaining 72-bp segment of *dl*894 (Fig. 1) has a dramatic effect on the size of the plaques formed by these mutants (Table 1). Their size decreases 3- to 10-fold as compared with *wt*776, and their time of appearance is also delayed, to up to 23 days for *dl*1852 versus 11 to 13 days for *wt*776. This imposes some constraints on the evaluation of viable and nonviable mutants because of the practical difficulties of maintaining healthy monkey cell monolayers covered under agar at 37°C for such long periods.

Concomitant with the observed reduction in plaque size, removal of information from the remaining 72-bp segment also resulted in a 20-fold reduction in DNA infectivity (*dl*1845 and

*dl*1847) (Fig. 2). Comparing *dl*1845 and *dl*1847 with *dl*1854 (Fig. 2), it may be further concluded that removal of sequences in the 21-bp repeat causes an additional fivefold reduction. This effect may well be related to a role of the 21-bp repeats as an amplifier in DNA replication (5) or as a regulatory region for the expression of early T-antigen (26).

It should be noted that by deleting sequences from the 72-bp enhancer segment, new sequence elements are juxtaposed at the endpoints of the deletions. This may be relevant to the observation that mutant *dl*1845, with a somewhat larger deletion than *dl*1847 and lacking part of a 21-bp repeat, nevertheless grows slightly better than *dl*1847 (Table 1). Perhaps the difference of one nucleotide into the *ori*-distal end of the 72-bp repeat is also important. It may be noted that *dl*1845 restores (to some extent) the GC-rich block at the *ori*-distal end of the 72-bp repeat (Fig. 1). Removal of this GC-rich block resulted in a 10-fold decrease in infectivity (compare *dl*1846 with *dl*1845; Fig. 2). It may be noted that mutants *dl*1857 and *dl*1856, with deletions around the *Pvu*II (L272) and *Kpn*I (L299) sites but with intact 72-bp segments (Fig. 2), showed no significant decrease in plaque-forming capacity. However, extension of the deletion as present in *dl*1847 to that present in *dl*1852 leads to a drastic reduction in viability (which becomes 0.01% of the wild-type viability), and further extension of the deletion, as present in pS1853, leads to total loss of viability (Fig. 1 and 2). These results suggest that sequence information distal to the end of the 72-bp segment (L250) and extending to beyond the *Pvu*II site (L272) plays an important role in viability when the 72-bp segments have been deleted. It may be noted that this segment contains the sequence TAACTGAC$(N)_{2\text{-}3}$ACATT (L252 to L266), which has been conserved in polyoma virus DNA (45). But perhaps also significant is the strong homology (88%) of a 17-bp sequence, 5′ $ACT_{A}TTCCACAC_{G}CTGGTT$ 3′, appearing at position L167 to L183 in the 72-bp repeat segments, which is repeated in the region around the *Pvu*II site at position L262 to L278. The first 12 bp of this homology sequence correspond to the important *ori*-distal end of the 72-bp repeat.

As shown above, deletion mutants *dl*1845, *dl*1846, and *dl*1847 exhibit a marked degree of heterogeneity after passage of plaque-purified mutant virus. The *Ava*II restriction analysis revealed a reassortment or rearrangement resulting in an increased restriction length polymorphism of the *Ava*II-D fragment, encompassing the initiation of transcription region (Fig. 4). In several of the plaques isolated from *dl*1846 and *dl*1847, we found multiple new *Ava*II-D fragments. These newly generated fragments were larger than the deleted fragment carried by the parental mutant and in some cases were even slightly larger than the corresponding *wt*776 *Ava*II fragment. Undoubtedly, upon infection there is a strong selection in favor of better-growing variants. The rearrangements or reassortments might therefore restore a reduced function perhaps specified by a sequence-length relationship.

Reduction in viability and nonviability correlated to the amount of T-antigen expression. Deletion mutants *dl*1845, *dl*1847, and *dl*1854 have virtually the same *ori*-distal deletion endpoint (leaving the GC-rich block near the end of the 72-bp repeat intact; Fig. 1), but differ in the position of their *ori*-proximal endpoint. Mutants *dl*1845 and *dl*1847, which retain at least one 21-bp repeat unit, have approximately the same numbers of T-antigen-positive cells as does wild-type DNA. Mutant *dl*1854, lacking both 21-bp repeat sequences, shows a decrease of more than 1 order of magnitude in T-antigen-positive cells.

Deletion mutants *dl*1845 and *dl*1846 have almost the same *ori*-proximal end (4-bp difference), but *dl*1846 lacks distally a further 12 bp, thus eliminating the GC-rich sequence near the end of the 72-bp repeat (Fig. 1). Despite this apparently small diffference, *dl*1846 displays a reduction of more than 1 order of magnitude in number of T-antigen-positive cells. These results indicate that, at least in the absence of a 21-bp repeat, loss of the GC-rich block near the end of the single remaining 72-bp segment leads to a severe reduction in the number of T-antigen-positive cells.

Compared with *dl*1847 (with a normal number of T-antigen-positive cells), mutant *dl*1852 has the same *ori*-proximal deletion endpoint but has an extension of the deletion at the *ori*-distal end, removing the terminal 22 nucleotides of the 72-bp repeat and the sequences up to the *Pvu*II site at L272. This additional deletion causes a reduction of almost 2 orders of magnitude, and increasing the deletion to include the *Kpn*I site (*dl*1853) results in virtual loss of T-antigen-positive cells (100 times fewer T-antigen-positive cells than *wt*p776). These results indicate that, in addition to the necessity of a 72-bp segment for T-antigen expression, an extension of this region up to the *Kpn*I site also has an important role (at least in the absence of a functional 72-bp segment). It is remarkable that when the region around the *Pvu*II site and the *Kpn*I site is removed while leaving the 72-bp repeat structure intact, no effect on the number of T-antigen-positive cells is observed (cf. *dl*1856, *dl*1857, *in*1836, *dl*1811, *dl*1635, and *dl*1659) (2, 24, 47). A further illustration of the importance of sequences upstream of the 72-bp repeat for T-

antigen expression comes from results of experiments with the nonviable pS1859 mutant, which has an almost complete 72-bp repeat except for the four terminal *ori*-distal nucleotides. The *ori*-distal end of the deletion maps at L353, i.e., beyond the *Hpa*II site. Although pS1859 lacks only the *ori*-distal end (4 bp) of the single remaining 72-bp segment, this mutant results in a reduction of the number of T-antigen-positive cells by approximately 2 orders of magnitude (Table 2). Hence, concomitant removal of only 4 bp at the *ori*-distal end of the single remaining 72-bp segment, together with upstream sequences in the region of the *Pvu*II-*Kpn*I sites, has severe effects on T-antigen expression.

These results provide evidence for another, remote control sequence situated at a distance of more than 250 bp from the early transcriptional initiation sites (9a, 17, 20, 23). This remote control region, which is important for early gene expression (as measured by T-antigen immunofluorescence in permissive cells), is located between the second 72-bp segment until position L353. We therefore propose that this region resembles a remote control sequence with (partial) properties of an auxiliary enhancer only and solely observable in the absence of a complete 72-bp segment (removal of the first 72-bp segment as well as the *ori*-distal 4 bp of the single remaining 72-bp segment). We pointed out above the important role of at least part of this control segment (region L250 to L312) for viability (cf. *dl*1852 and pS1853); we conclude here that this role involves (at least) T-antigen expression. The reduced viability of the mutants reported in the present study can thus be correlated to their reduced capacity to induce T-antigen-positive cells such that they become almost replication negative. The nonviability of mutants pS1848, pS1849, pS1850, and pS1851 can likewise be explained by the fact that they induce hardly any T-antigen (at least 2 to 3 orders of magnitude less than the wild type). This does not exclude the possibility that, in addition, other essential functions are also controlled by these deleted regions.

The detailed role of the 72-bp repeat region in T-antigen expression is at present unknown. It could be a bidirectional entry site for *Pol*II (37), or the SV40 72-bp repeats and neighboring sequences could have pleiotropic functions such as reorganizing the chromatin structure or directing the DNA template to nuclear matrix sites where viral DNA and transcriptional complexes (8, 30, 42) are apparently located.

Effects of deletions in the 72-bp repeat segment on late transcription. The main conclusion to be drawn from the S1 or *Exo*VII mapping experiments of SV40 late polyadenylated mRNA reported in this paper is that the initiation patterns downstream from the deleted sequences were qualitatively almost unchanged in comparison with the wild-type 5′ termini (Fig. 4) (for a detailed discussion, see also 9a). Taken together, our results and those of Contreras et al. (9a) allow us to conclude that the deletions in the upstream region of the principal late SV40 initiation site L325, including the 72-bp repeat segment, do not interfere appreciably with the synthesis and exact positioning of the remaining RNA species, although with some mutants (*dl*894, *in*1836) particular minor wild-type initiation sites are enhanced. The reason for this peculiar feature is not obvious. As discussed elsewhere (9a), we have no evidence that promoter elements essential for late transcription are located in the region covered by the series of deletions used in this study. Rather, late transcriptional expression is correlated with activation of the DNA replication origin, which functions as a new type of polymerase II promoter.

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