Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 943–947, February 1981
Biochemistry

Simian virus 40 tandem repeated sequences as an element of the early promoter

(deletion mutants/chromatin/RNA initiation/transcriptional regulation)

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Communicated by Hilary Koprowski, November 17, 1980

ABSTRACT **On the late side of the simian virus 40 (SV40) DNA replication origin are several sets of tandem repeated sequences, the largest of which is 72 base pairs long. The role of these sequences was examined through construction of deletion mutants of SV40. A mutant from which one of the 72-base-pair repeated units was removed is viable upon transfection of monkey kidney cells with viral DNA. Extension of this deletion into the second repeated unit, however, leads to nonviability, as recognized by the absence of early transcription and of tumor antigen production. These observations indicate that the 72-base-pair repeated sequences form an essential element in the early viral transcriptional promoter and explain the inability of such a deleted genome to complement an early temperature-sensitive mutant of SV40, tsA, as well as the failure to replicate its DNA. In a parallel experiment it was found that the extended deletion mutant was also unable to complement a late temperature-sensitive mutant of SV40, tsB. This suggests that the extended mutant is also defective in DNA replication or late transcription (or both).**

Elucidation of the nucleotide signals that serve as regulatory elements for the fundamental biochemical processes such as transcription and DNA replication is crucial to an understanding of gene regulation. In this regard, small DNA viruses such as simian virus 40 (SV40) have been valuable model systems. Determination of the complete nucleotide sequence, analyses of the 5′ ends of mRNA molecules, and an extensive examination of the viral genetics have provided a detailed understanding of the organization of the SV40 genome (see ref. 1 for review). The early and late gene coding sequences are situated in opposite halves of the viral genome, transcribed in opposite directions on the antiparallel DNA strands. Located between these sets of gene functions is the origin for viral DNA replication, as well as the 5′ ends of the viral RNAs and the putative control signals (promoters) for their expression. Between the DNA replication origin and the 5′ ends of most late viral mRNAs is a stretch of approximately 200 nucleotides coding for no known viral function and containing several sets of tandem repeated sequences (2, 3), the largest of which is 72 base pairs (bp) (see Fig. 2). The proximity of these repeated sequences to both the replication origin and the 5′ ends of the late mRNAs suggested a potential regulatory function. In an attempt to identify a role for these repeated sequences, we have created two deletion mutants that remove one or more sets of the tandem repeated sequences. Removal of one 72-bp repeating unit is consistent with viability of the viral genome; extension of this deletion, however, leads to a nonviable mutant that can complement neither early nor late temperature-sensitive SV40 mutants.

MATERIALS AND METHODS

Virus Strains and Cell Culture. Small-plaque SV40, strain 776, was used for mutant construction. Helper viruses tsA28 and tsB4 are derived from strain VA-4554. The host cell lines were secondary African green monkey kidney (AGMK) cells.

Preparation of SV40 DNA Fragments and Cytoplasmic RNA. Purified SV40 virion DNA was cleaved with restriction enzymes (as detailed in figure legends) and separated in either 1.4% (wt/vol) agarose gels or 4% (wt/vol) polyacrylamide gels (4). ^{32}P-Labeled SV40 DNA (specific activity 2×10^6 cpm/μg) was obtained as described (5). Restriction enzyme fragments were separated for nuclease S1 analysis in 1.4% alkaline agarose gels (4). Cytoplasmic poly(A)-containing RNA was prepared as detailed (5). Nuclease S1-alkaline agarose gel electrophoresis was carried out by a modification of the technique of Berk and Sharp (6).

Analysis of Viral Proteins. Secondary AGMK cells in 150-cm^2 prescription flasks were incubated for 3 hr with 3 ml of minimal essential medium containing 20 μg of SV40 DNA fragments plus DEAE-dextran (2.5 mg/ml). After incubation, the cells were washed once with medium and further incubated at 37°C for 90 hr. Subsequently, the cultures were stored for 1 hr in methionine-free medium and labeled with 35 μCi of [^{35}S]methionine per ml in methionine-free medium (1 Ci = 3.7×10^{10} becquerels). Viral proteins were extracted and immunoprecipitated as described (7) with an antiserum recognizing both tumor (T) antigen-specific and protein VP-1-specific immunodeterminants. The $NaDodSO_4$/polyacrylamide gel system used was that of Laemmli (8). Radiolabeled protein bands on the gel were detected by fluorography (9).

Analysis of Complementation and Replicating Viral Molecules. Cloning of mutants and complementation analysis were performed as described (10). Secondary AGMK cells in 6-cm-diameter dishes were incubated for 70 min with SV40 DNA fragments (60 pg/dish) as described for analysis of viral proteins. At 0, 24, 48, 72, and 90 hr after infection, DNA was harvested (11) and an aliquot was electrophoresed in a 1.4% agarose gel. The DNA was transferred to a nitrocellulose filter (Schleicher and Schuell, 0.1-μm) (12), and the blotted DNA was hybridized to a nick-translated SV40 DNA with a specific activity of 2×10^8 cpm/μg. Radiolabeled bands were detected after exposure to x-ray film.

RESULTS

Generation of Deletion Mutants. To determine the function of the late SV40 repeated sequences, deletion mutants were positioned in this segment of the genome. When this study was initiated, *Ava* X (recognition sequence, A-T-G-C-A-T), which cuts SV40 at only three sites, was the preferred restriction enzyme (see Fig. 1). *Sph* I (a kind gift from R. Roberts), which cleaves SV40 DNA at only two sites, once in each repeated sequence, was used in later experiments. SV40 DNA was digested

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Abbreviations: SV40, simian virus 40; bp, base pairs; AGMK cells, African green monkey kidney cells; T antigen, tumor antigen.

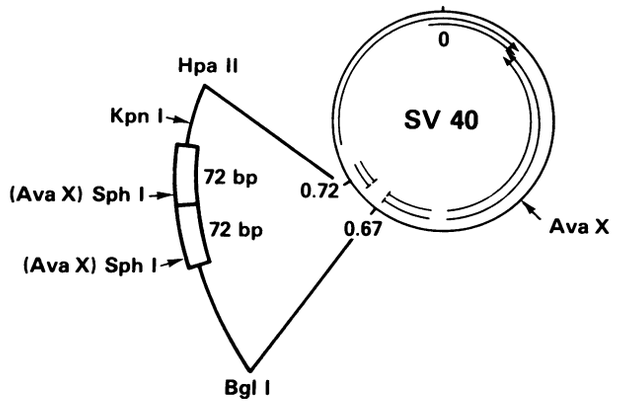

FIG. 1. Schematic representation of the method for construction of SV40 mutants lacking either one repeated sequence, dl-2355 (*Sph* I or *Ava* X cleavage), or both repeated sequences, dl-2356 (*Sph* I plus *Kpn* I cleavage).

to completion with *Ava* X (kindly provided by G. Roizes), and the two largest DNA fragments, purified by agarose gel electrophoresis, were ligated for transfection into secondary AGMK cells. By this experimental approach (Fig. 1), precisely one 72-bp repeated sequence was removed from the SV40 genome and the DNA was prepared for transfection of AGMK cells. Transfection of linear molecules, even those with sticky ends, leads to the production of extended deletion mutants with a frequency of up to 50% in addition to the parental DNA (ref. 13 and unpublished results). These extended deletions most likely result from the action of cellular nucleases on DNA molecules prior to circularization. In anticipation of isolating extended deletions, parallel experiments involved DNA transfection with either tsA28 or tsB4 as potential helper viruses. Nevertheless, the number of plaques observed in complementation studies with the temperature-sensitive (ts) helper viruses were the same as with transfection of the linear *Ava* X DNA alone (data not presented). All of the deletion mutants isolated appeared to retain a single copy of the 72-bp segment and preserved the *Ava* X cleavage site. One of these, referred to as dl-2355, was used for subsequent experiments. DNA sequence analysis (14) of three viable mutants (Fig. 2) indicated that precisely one 72-bp repeated sequence had been removed; these mutants thus resemble viable mutants described by others (17–19). One of the three mutants contained, in addition, a single base-pair transition (G·C to A·T; Fig. 2), the source and significance of which are unclear. The absence of extended deletion mutants in spite of complementation with temperature-sensitive helper viruses suggested a critical role for the one remaining 72-bp DNA segment. In a subsequent experiment we therefore attempted to extend this *Ava* X deletion by biochemical means (see Fig. 1). The recently described restriction enzyme *Sph* I was used with restriction endonuclease *Kpn* I. Cleavage of SV40 DNA with both of these enzymes results in the deletion of approximately 170 nucleotides, including 122 bp of the 144 bp in the two repeated sequences. The additional nucleotides that are removed from the second repeated sequence (*Kpn* I to *Sph* I) are from a dispensable region of the SV40 genome (20) to the late side of the tandem repeated sequences (see Fig. 1). Purified DNA from this double restriction cleavage (to be called mutant dl-2356) was transfected into secondary AGMK cells, which were subsequently analyzed for plaque formation by an infectious center assay. In previous studies, it was shown that linear SV40 DNA molecules with noncohesive ends can transfect cells with approximately 20% the efficiency of linear DNA containing cohesive termini (ref. 21 and unpublished data). With two different concentrations of DNA in transfections, essentially no viable mutants were detected (Table 1). To locate the potential defect in this deletion mutant, we attempted to complement the DNA transfection with early or late SV40 temperature-sensitive mutants. No bona fide plaques were observed even in the presence of coinfecting helper virus. This result was in part anticipated from the absence of extended deletion mutants in earlier transfection experiments with *Ava* X-cleaved SV40 DNA. It suggests that the 72-bp repeated sequence may contain a *cis*-acting element that is required both for early and late SV40 gene expression and, thus, for complementation of early and late SV40 temperature-sensitive mutants.

Biochemical Analysis of Deletion Mutants. A series of biochemical experiments was performed to locate the defect in the extended deletion mutant dl-2356 more precisely. AGMK cells were inoculated with gel-purified DNA from mutant dl-2356 or, as a control, with DNA from mutant dl-2355, which is missing precisely one 72-bp repeated sequence (Fig. 3). At 48 hr after infection, cells were labeled with [^{35}S]methionine for 2 hr.

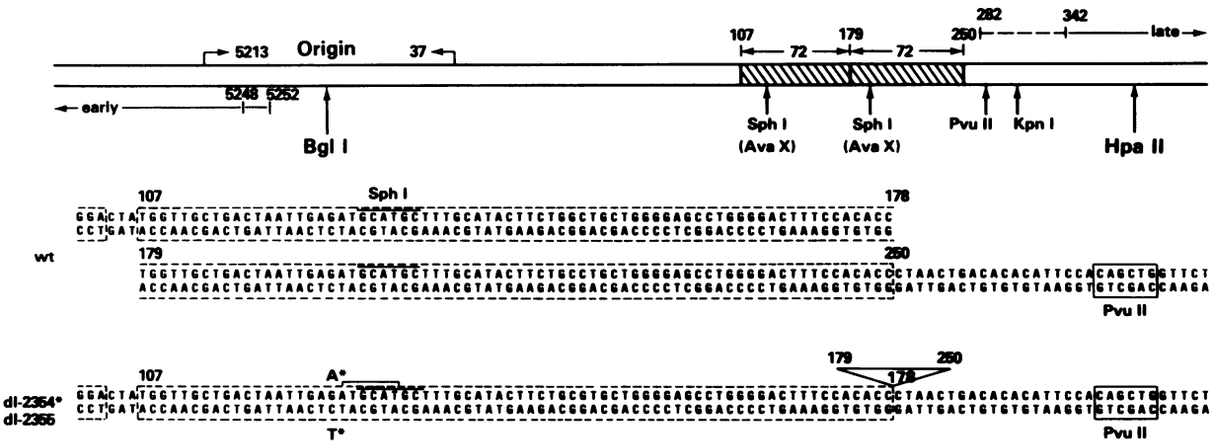

FIG. 2. Nucleotide sequence of deletion mutants lacking one repeated sequence. Sequence of two deletion mutants, dl-2354 and dl-2355, is pictured below the sequence from the same region of the wild-type (wt) viral genome. Both of these mutants are missing nucleotides 179–250, inclusive. In addition, dl-2354 contains a single base change indicated by an asterisk. The diagram above the sequence shows the positions of the replication origin (15), two major early 5′ ends (16), and the late transcriptional units with reference to the region of the repeats. The numbering system is adapted from ref. 1.

Table 1. Complementation analysis between deletion mutants and temperature-sensitive mutants of SV40

Mutant DNA	Mutant		
	tsA28	tsB4	None
Sph I/*Kpn* I deletion			
10 ng/plate	0, 0, 0, 0, 1	1, 2, 0, 0, 2	1, 1, 0, 0
50 ng/plate	1, 0, 0, 0, 0	1, 0, 0, 0, 0	2, 2, 0, 0
pBR-2350 (*Bam*)			
10 ng/plate	28	ND	ND
20 ng/plate	70	ND	ND
SV40 t (*Eco*RI)			
20 ng/plate	ND	ND	85, 92
None	0, 0	1, 0	—

Secondary AGMK cells (4×10^4) were transfected with DNA fragments of wild-type (wt) SV40 linearized at the *Eco*RI site, dl-2356 (the *Sph* I/*Kpn* I mutant lacking most of both 72-bp repeated sequences), or dl-2350 (the defective late SV40 deletion mutant lacking precisely the intron for 16S mRNA). This mutant is incapable of complementing tsB4 (7). In parallel experiments, transfected cells were superinfected with a helper virus, either tsA28 or tsB4 (1–5 plaque-forming units/cell). Numbers represent plaques counted per plate. The amount of DNA used for transfection is indicated. Duplicate or quadruplicate infections were carried out for each analysis. The occasional plaques arising from cells infected with *Kpn/Sph* SV40 (dl-2356) were shown to result from incomplete cleavage or reversion of a temperature-sensitive mutant. ND, Not determined.

Proteins were extracted and immunoprecipitated with a hamster antiserum that recognizes both T antigen and VP-1 (see *Materials and Methods*). As expected on the basis of its viability, mutant dl-2355 induces the production of both T antigen and VP-1. Neither of these polypeptides could be detected in an extract of cells infected with mutant dl-2356. This analysis confirms the complementation studies and indicates that removal of more than one 72-bp repeated sequence results in the loss of expression of both early and late SV40 gene functions. In a parallel study, AGMK cells similarly infected by both mutants were examined for the production of SV40-specific RNA (Fig. 4). Between 90 and 96 hr after infection, cytoplasmic poly(A)-containing RNA was isolated from AGMK cells and examined by the nuclease S1 technique with SV40-specific probes. Both early and late transcripts were detected in cells infected with

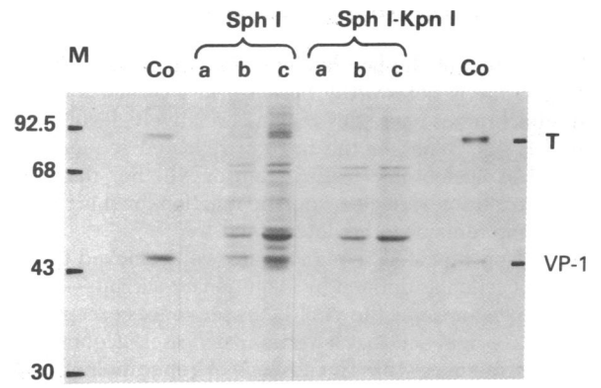

FIG. 3. Analysis of proteins after infection of cells with dl-2355 and dl-2356 DNA. SV40 DNA was cleaved to completion (5-fold excess) with either *Sph* I (dl-2355) or *Sph* I and *Kpn* I (dl-2356). Cells were infected and proteins were extracted. Three different concentrations of proteins were used for immunoprecipitation: lanes a, from 0.25×10^6 cells; lanes b, from 1×10^6 cells; lanes c, from 3×10^6 cells. M is a protein marker (Bethesda Research Laboratories); Co is a control protein extract from wild-type SV40-infected cells. Sizes are given in kilodaltons.

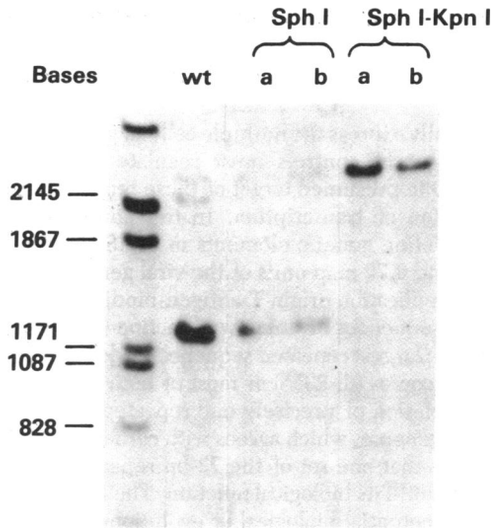

FIG. 4. RNA analysis of cells transfected with dl-2355 or dl-2356 DNA. Secondary AGMK cells (3×10^7) were infected with DNA fragments (cleaved by either *Sph* I or *Sph* I and *Kpn* I). RNA was purified and analyzed by the nuclease S1 technique (6). Poly(A)-containing RNA at two different RNA concentrations (lanes a = 0.5 and lanes b = 0.2 of the total preparation) was analyzed in parallel with *Hpa* II/*Bam*HI SV40 fragment B (representing the late region of the genome) terminally labeled with [γ-^{32}P]ATP (specific activity, 5×10^6 cpm/μg). Early viral RNA was analyzed in a separate experiment (data not presented). wt, Wild type.

mutant dl-2355; no stable early or late SV40-specific RNA was found in cells infected with the extended deletion mutant dl-2356. To confirm the analysis of viral RNA and proteins induced after DNA transfection of mutants of dl-2355 and dl-2356, we assayed parallel sets of transfected AGMK cells (22) for viral DNA replication (Fig. 5). As anticipated, the dl-2355 DNA was actively replicated whereas dl-2356 DNA was not. The absence of both early and late transcripts, as well as the inability of dl-2356 to complement tsA and tsB mutants or to replicate its DNA, suggest that at least one repeat unit is required for early

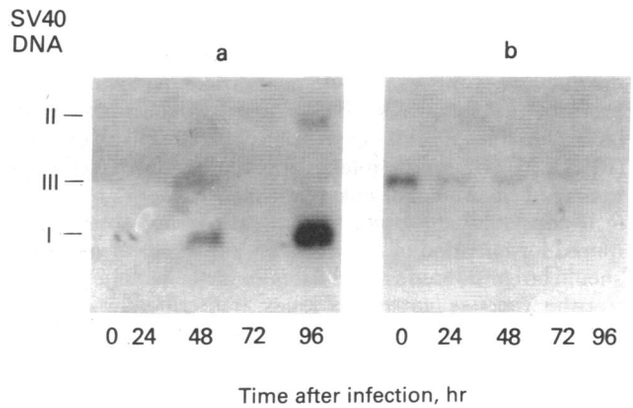

FIG. 5. Replication of viral DNA molecules. Secondary AGMK cells in 6-cm-diameter dishes were infected with 60 pg of either (*a*) dl-2355 (lacking one 72-bp repeated sequence) or (*b*) dl-2356 (the *Sph* I/*Kpn* I fragment lacking most of or both 72-bp repeated sequences). At the indicated times after infection, DNA of one dish each was harvested (11). Half of each preparation was subjected to electrophoresis in a 1.4% agarose gel, and the DNA was transferred to a nitrocellulose filter and hybridized with a nick-translated DNA probe (12, 22). The positions of superhelical (I), relaxed circular (II), and linear (III) SV40 DNA are indicated. (Note that the sample at 72 hr in *a* was lost.)

transcription and for DNA replication or for late transcription of the SV40 genome.

DISCUSSION

To differentially express the multiple cellular genetic programs, an intricate set of controls must regulate eukaryotic gene expression. One presumed target of these regulatory functions is the initiation of transcription. In this study, we have attempted to define genetic elements in the SV40 genome between 0.67 and 0.72 map units of the viral genome; this region harbors the replication origin T-antigen-binding sites and a set of repeated sequences of unknown function (1). We examined the role of the largest repeated sequence by deleting either one of the 72-bp copies (dl-2355) or most of both 72-bp sequences (dl-2356). Deletion of precisely one repeated unit results in a viable SV40 genome, which agrees with earlier studies (17–19). This suggests that one set of the 72-bp repeated sequences is sufficient to fulfill its biological function. The role of the second copy and its potential biological or evolutionary advantage are unclear.

Extension of the single repeated sequence (dl-2355) deletion into the second repeated sequence resulted in a complete loss of early and late gene activities, as seen by the failure to complement early (tsA28) or late (tsB4) temperature-sensitive mutants. Furthermore, direct biochemical analysis confirmed the biological assay: neither early nor late cytoplasmic mRNA was detected after inoculation of dl-2356 DNA into AGMK cells. This is also reflected in the absence of early and late SV40 polypeptides (T antigen and VP-1), as analyzed by immunoprecipitation. Although we cannot rigorously exclude the possibility that SV40 RNA is synthesized and rapidly degraded, a logical interpretation of these data is that the 72-bp repeated sequence is crucial to the initiation of early gene transcription. A similar conclusion comes from recent data of Benoist and Chambon (personal communication and ref. 23). By analogy to prokaryotes (24), one would expect a certain amount of structural similarity among eukaryotic promoters required for the basic operation of the RNA polymerase II. Indeed, regions resembling genetic elements preceding prokaryotic 5′ ends of mRNAs have been described. There is a T-A-T-A-A sequence (Goldberg–Hogness box) preceding the cap site by approximately 25 nucleotides which closely resembles the sequence 10 nucleotides upstream from the prokaryotic mRNA start point (24). Although the structural similarity is evident, preliminary data suggest that the function of this genetic element differs. As opposed to the prokaryotic Pribnow box, the Goldberg–Hogness box seems to be dispensable for *in vivo* expression of certain eukaryotic genes (16, 23, 25). One of its roles is apparently related to the positioning of the 5′ end of eukaryotic mRNA.

If the Goldberg–Hogness sequence is not absolutely required for initiation of transcription, other sets of nucleotides should be considered as candidates for the eukaryotic promoter. For the *Xenopus borealis* 5S genes, transcribed by RNA polymerase III, a region essential for transcriptional initiation has been found in the middle of the gene (26, 27). In addition to this "promoter," sequences at the 5′ end of the gene seem to be important for modulating the efficiency of transcription (28). Similar results have been obtained for another eukaryotic gene under the control of polymerase III, the adenovirus *Val* gene (29; R. Guilfoyle and R. Weinmann, personal communication).

Although we know less about the genes transcribed by polymerase II, a deletion that maps >100 nucleotides proximal to the 5′ end of the histone H2a initiation site appears to abolish transcriptional activity (25). Thus, although the examples are limited, one might suggest that a putative polymerase II "promoter" element can map more than 100 nucleotides to the 5′ side of the cap site whereas some the elements of the polymerase III promoters are located within their genes. Also, by analogy, the Goldberg–Hogness box, like sequences to the 5′ side of the polymerase III genes, may play a role in modulating the efficiency of transcription, perhaps through positioning the 5′ ends of mRNAs.

One difficulty in searching for analogous sequences in other genes transcribed by polymerase II is our limited understanding of what elements constitute a eukaryotic promoter. Nevertheless, a number of similar repetitive sequences have been found at the 5′ ends of certain genes. Not surprisingly, an analogous 68-bp tandem repeated sequence is found to the late side of the BK virus replication origin (30), but this sequence has no obvious sequence homology to the SV40 repeated sequences. A variant strain of BK virus called MM shares 54 nucleotides of its late tandem repeated sequence with the prototype BK virus strain (31); this may define the minimal number of nucleotides essential for the biological activity of the sequence. It is curious that a related papovavirus, polyomavirus, has no large tandem repeated sequence to the late side of its replication origin. Nevertheless, recent experiments by C. Tyndall and R. Kamen (personal communication) indicate that deletions in this region can prevent the expression of the early polyomavirus RNA and proteins. This suggests that an analogous regulatory element is present in a single copy. Further support for this concept is based on an analysis of polyomavirus mutants that grow in undifferentiated teratocarcinoma cells (32–34). These mutants all appear to contain alterations just to the late side of the replication origin and, in most cases, the alteration involves the duplication of a set of nucleotides that form a tandem repeated sequence analogous to that of SV40.

A recent nucleotide sequence analysis of the ends of the cloned Moloney murine sarcoma virus reveals a 72-bp repeated sequence located 179 nucleotides upstream from one of the major 5′ RNA termini (35). Although its function is unknown, the similarity to the SV40 large repeated sequence in both size and position is striking.

McCutcheon and Singer (36) have isolated clones of monkey cell sequences that share homology with the SV40 replication origin. These clones contain segments homologous not only to the replication palindrome but also to the late tandem repeated sequences. Although it may prove difficult to establish a general biological role for tandem repeated sequences, application of recently developed *in vitro* transcriptional systems may be beneficial (37, 38).

The position of the late tandem repeated sequences in the SV40 genome is noteworthy because, in a certain fraction of viral minichromosomes, this region is relatively devoid of histones (39–42). Whether the tandem repeated sequences are involved in nucleosome organization or whether the nucleosome distribution reflects a specific function for this region of the genome remains to be determined.

The 72-bp repeated sequences are well removed from the SV40 replication as defined by evolutionary variants—mutants that have lost most of the viral DNA sequences except for the replication origin and that have, in many cases, acquired monkey cell sequences (15). The absence of replication of dl-2356 in AGMK cells may be explained simply by its failure to induce T antigen. Nevertheless, the failure of dl-2356 to complement mutant tsB could be related either to the absence of late transcription (e.g., repeated sequences may also play a role in late transcription) or to a fundamental deficiency of replication even in the presence of the functional T antigen provided by tsB4. This latter possibility is not without precedent; the transcriptional activation of replication origins has been described for bacteriophage λ and its host *Escherichia coli* (43).

We thank Monika König and Linda Greenbaum for technical assistance and Myles Brown, Barrie Carter, Neil Goldman, and Gilbert Jay for helpful discussions.

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