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Deletions covering the putative promoter region of early mRNAs of simian virus 40 do not abolish T-antigen expression

(*in vitro* deletions/transformation/eukaryotic promoters/recombinant DNA)

C. BENOIST AND P. CHAMBON†

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Médecine, 67085, Strasbourg, France

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ABSTRACT **A recombinant plasmid was constructed by insertion of the early genes of simian virus 40 (SV40) into pBR322. When it was introduced into eukaryotic cells, the SV40 early genes were expressed. We have made deletion mutants of this plasmid, from which the major cap sites of SV40 early mRNAs have been removed along with some of the sequences upstream. The deleted sequences appear to be dispensable for early gene expression, but this does not necessarily imply that they serve no function in the initiation of transcription on wild-type SV40.**

Promoter sequences for prokaryotic genes have been precisely localized preceding the initiation site of transcription (see ref. 1 for review). By analogy, it has been assumed that promoters for eukaryotic RNA polymerase B, responsible for transcription of mRNAs, are situated in similar positions. An A+T-rich sequence, closely resembling T-A-T-A-A-A-T-A (the "Goldberg–Hogness box"), centered at approximately 27 base pairs (bp) upstream from the site coding for the first nucleotide of the mature mRNA (cap site), has been described in most genes whose sequences have been determined (see refs. 2–5 for further refs.), and we have reported another region of sequence homology located further upstream (6). Whether such sequences play a role in the initiation of transcription remains to be demonstrated. Interestingly, these sequences are absent in some cases—for example, in the late genes of simian virus 40 (SV40) and polyoma (7–9) and in the gene for the adenovirus-2 72,000-dalton DNA binding protein (10).

SV40 is a good model system for analysis of the structure of eukaryotic promoter sites because the entire sequence of its small genome (5243 bp) has been determined (7, 8). This DNA tumor virus consists of two sets of genes, one expressed early, the other at later times during lytic infection of permissive monkey cells. The early gene products (T and t antigens) are responsible for the initiation of viral DNA synthesis in these cells and for transformation of other mammalian cells that do not support lytic infection (for review, see ref. 11). The mRNAs for T and t antigens have been precisely mapped on the early region of the SV40 genome. They share common 5′ and 3′ termini, around 0.66 and 0.18 map units, respectively, but differ in their splicing patterns (12–14). Two major 5′ ends have been described for these mRNAs (14), at positions 5164–5165 and 5169–5171 [the numbering throughout the text corresponds to that proposed by Reddy *et al.* (7), modified to take into account an extra 17 bp recently discovered (15) that had been previously overlooked]. Evidence was also provided suggesting the existence of additional minor 5′ ends (14). The region coding for the 5′ end of early mRNAs also corresponds to the origin of DNA replication (positions 5127–5212) (16, 17).

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An A+T-rich region resembling the Goldberg–Hogness box can be found 30–35 bp upstream from the nucleotides coding for the two major 5′ ends of early mRNAs (see Fig. 2). In order to investigate whether this sequence could be part of a promoter site for SV40 early gene transcription, we have constructed a hybrid plasmid, containing pBR322 and the early SV40 genes. When this plasmid (pSV1) is introduced into eukaryotic cells, the early SV40 genes are expressed. Because such plasmids do not require the SV40 origin of replication for their propagation, this approach allows the study of deletions that extend into this origin. We report the construction and preliminary characterization of deletion mutants of pSV1 from which the putative Goldberg–Hogness box has been removed. This sequence appears to be dispensable for early gene expression.

MATERIALS AND METHODS

Enzyme *Bal*-31 was obtained from Bethesda Research Laboratories (Rockville, MD). Sources of other enzymes were as in ref. 18. Hamster antiserum against SV40 T antigen was a gift of R. Wilsnack (Huntingdon Research Center, Brooklandville, MD). Fluorescein-conjugated anti-hamster globulin was obtained from Miles–Yeda (Rehovot, Israel). SV40 DNA was prepared from CV1 monkey cells, propagated and infected with SV40 strain 776 as described (19). A Fisher rat 3T3 fibroblast cell line (FR3T3, a gift of F. Cuzin) was grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and gentamycin (40 μg/ml). The streptomycin-dependent *Escherichia coli* strain N543 (a gift of C. Weissmann) was grown in L-broth in the presence of 300 mg of streptomycin per liter.

Procedures for construction of recombinant DNA plasmids, transfection of bacteria, and preparation and sequence determination of plasmid DNA were as described (18, 20). DNA fragments were recovered from agarose gels by electroelution onto DEAE-paper (M. Bellard, unpublished data). For *Bal*-31 digestion, restricted DNA (2 μg) was extracted with phenol/chloroform and precipitated with ethanol. After solubilization in 30 μl of 12.5 mM $CaCl_2$/12.5 mM $MgCl_2$/0.6 M NaCl/20 mM Tris·HCl, pH 7.9, 0.5 unit of *Bal*-31 was added and the sample was incubated at 30°C. Aliquots were removed at 0.5, 1.0, 1.5, and 2 min, and the reaction was stopped by addition of EDTA to 15 mM and $NaDodSO_4$ to 0.2%. After phenol/chloroform extraction, the DNA was precipitated twice with ethanol. Prior to further restriction and ligation, the treated extremities were made perfectly blunt by repair with *E. coli* DNA polymerase I.

Recombinant plasmid DNAs were transfected into CV1 cells by the DEAE-dextran procedure (21). FR3T3 cells were transformed by the calcium phosphate coprecipitation tech-

Abbreviations: bp, base pairs; SV40, simian virus 40.

† To whom reprint requests should be addressed.

nique (22), and transformants were isolated as dense foci growing over the cell monolayer. SV40 T antigen was detected by indirect immunofluorescence as in ref. 23 either after transfection into CV1 cells or in transformed FR3T3 lines.

Biohazards associated with the experiments described in this publication were examined beforehand by the French National Committee; the experiments were carried out under L3-B1* conditions.

RESULTS

Construction of pSV1. The recombinant plasmid, pSV1, containing the SV40 early gene region was constructed by inserting the large *Hpa* II/*Bam*HI fragment from SV40 (0.73–0.15 map unit) into the *Eco*RI site of pBR322 by blunt-end ligation after repair of the extremities with DNA polymerase I (Fig. 1A). When pSV1, which was cloned into *E. coli* N543, is introduced into permissive CV1 monkey cells, the early genes are expressed; i.e., T antigen can be detected by indirect immunofluorescence (see Fig. 4*b*). Moreover, rat fibroblasts can be morphologically transformed by pSV1 by the calcium phosphate coprecipitation technique. This transformation is dependent upon the synthesis of early SV40 proteins (24) and, indeed, T antigen is detected by immunofluorescence in such clones (not shown). Despite the fact that the SV40 origin of replication is present in pSV1, the plasmid replicates poorly in monkey cells, replication apparently being hampered by the presence of pBR322 sequences (unpublished observations).

Construction of Deletion Mutants in the Early Promoter Region of pSV1. We first constructed a plasmid containing the entire SV40 early gene coding region but lacking a functional promoter. pSV1 was digested with *Bam*HI and its extremities were repaired with DNA polymerase I. It was then partially digested with *Hin*dIII, and the second largest fragment, b (arrowed line), was purified by agarose gel electrophoresis (Fig.

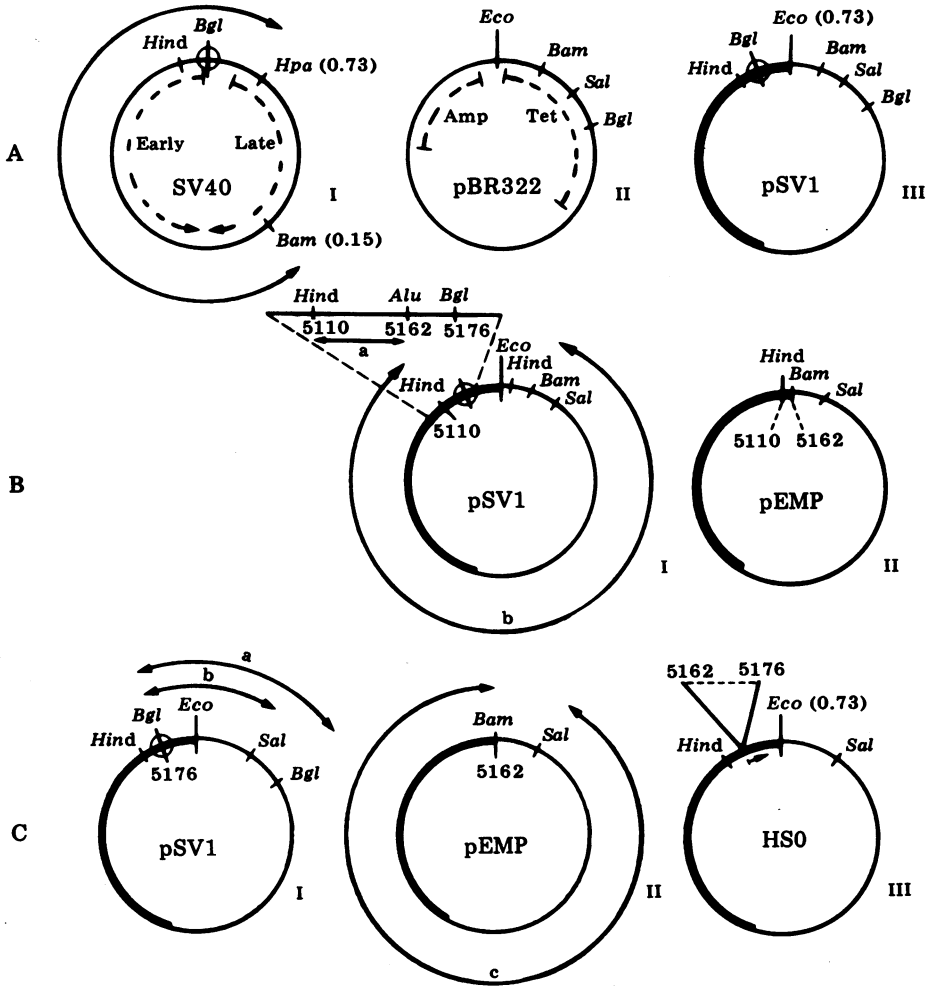

FIG. 1. Diagram of the construction of deletion mutants of recombinant plasmid pSV1. The various steps in the construction of HS mutants are shown. *Eco*, *Hin*d, *Bam*, *Sal*, *Bgl*, and *Hpa* represent cleavage sites for restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, *Sal* I, *Bgl* I, and *Hpa* II, respectively. The various plasmids and the location of their restriction enzyme sites are not drawn to scale. Only the relevant restriction sites are shown. SV40 sequences inserted in the plasmids are shown as thick lines. The symbol ∅ represents the SV40 origin of replication. Arrowed lines around the plasmids indicate the fragments used for each step. (*A*) Construction of pSV1. Insertion of the *Bam*HI/*Hpa* II fragment of SV40 (I) into the *Eco*RI site of pBR322 (II). The direction and extent of early and late genes of SV40 are shown by dashed arrows, as are the genes for ampicillin (Amp) and tetracycline (Tet) resistance of pBR322. (*B*) Construction of plasmid pEMP, which contains the entire early gene amino acid coding region without the putative early promoter region and some amino acid noncoding nucleotides. The SV40 electrophoretically purified *Hin*d/*Alu* fragment a (positions 5110–5162, I) was ligated to the *Hin*dIII/*Bam*HI fragment b derived from pSV1 by *Bam*HI and partial *Hin*dIII digestions (arrowed line, I). Because pEMP does not contain the sequences between the *Eco*RI and *Bam*HI sites of pBR322, bacteria carrying this plasmid are tetracycline sensitive. (*C*) Construction of HS recombinants by ligating the *Bgl* I/*Sal* I fragment b (I), with or without previous treatment of the *Bgl* extremity with *Bal*-31 (see text), with the fragment c (II) derived from pEMP (see text). The resulting deletion mutant HS0 (without *Bal*-31 treatment) is shown in III, where the position of the *Alu* I/*Bgl* I deletion (positions 5162–5176) is indicated by a dotted line. The clockwise arrow indicates the direction of the deletions extending further upstream in the HSn series (see text).

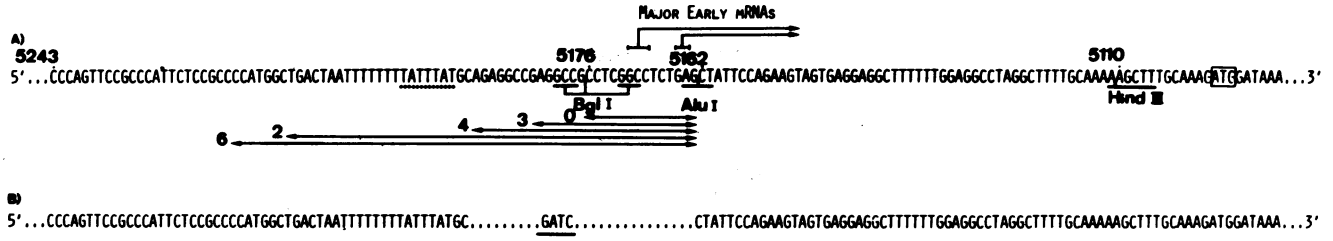

FIG. 2. SV40 DNA sequence around the cap sites of early mRNAs. (*A*) The sequence (from refs. 7 and 8) shows the early noncoding strand of SV40 DNA, positions 5243–5080. Positions follow the numbering system of Reddy *et al.* (7) modified according to ref. 15. The 5′ ends of major early mRNAs are shown by brackets with arrows in the direction of transcription. Restriction enzyme sites are underlined, and the AUG initiation codon is enclosed by a box. Dots underline the putative Goldberg–Hogness box. Double-pointed arrows under the sequence indicate the extent of the deletions in the various mutants of the HS series (0, 3, 4, 2, and 6), as deduced from Maxam and Gilbert sequencing after 5′ end labeling at the *Hin*dIII site (position 5110). (*B*) Sequence of mutant HS4, given as an example. The underlined sequence is the tetranucleotide insertion that originates from the repaired *Bam* site used in construction of the mutants.

1*B*, I). This fragment was ligated to *Hin*dIII/*Alu* I SV40 fragment [positions 5110–5162 (Fig. 1*B*, I)] corresponding to most of the 5′ untranslated region of early mRNAs (see Fig. 2). Transfection of this recombinant into *E. coli* N543 yielded the plasmid pEMP, which contains the early SV40 coding region starting at position 5162 and thus lacking both the putative promoter region and a few nucleotides corresponding to the very 5′ end of major early mRNAs (positions 5164 and 5170, see Introduction and Fig. 2; the AUG codon is at position 5098). As expected, the early functions were not expressed when this plasmid was introduced into monkey or rat cells (not shown). The blunt-end ligation of an *Alu* I extremity onto a repaired *Bam*HI extremity has regenerated a *Bam*HI site at the junction between the pBR322 sequences and the SV40 sequences (position 5162 in Fig. 1*B*, II). This site can now be used to introduce DNA sequences which might be recognized as promoters by eukaryotic RNA polymerases, rendering the early genes functional.

A vector was prepared from pEMP by digestion with *Bam*HI followed by repair with DNA polymerase I and secondary digestion with *Sal* I (arrowed line c in Fig. 1*C*, II). pSV1 was digested with *Bgl* I, and the fragments were treated with the enzyme *Bal*-31, which possesses both exonuclease and single-stranded nuclease activities (25), to remove up to 60 nucleotides from the ends of fragment a (Fig. 1*C*, I, arrowed line a). (The *Bal*-31 treatment was omitted for the construction of recombinant HS0.) The DNA was digested with *Sal* I and then ligated with fragment c to yield recombinants HS, which were transfected into *E. coli* N543. The HS recombinants were selected on agar plates containing tetracycline because only those recombinant plasmids, having incorporated the pSV1 fragment b, can confer tetracycline resistance (see Fig. 1*C*).

The series (HSn) of plasmids thus obtained have the same overall structure as pSV1 (Fig. 1*A*) (except for a 4-bp insertion), but have a deletion of at least 14 bp between the *Alu* I site at position 5162 and the *Bgl* I cutting site at position 5176. This minimal deletion is present in recombinant HS0 (Fig. 1*C*, III), which was constructed from a fragment a (Fig. 1*C*, I) not treated with *Bal*-31. Other HS mutants have larger deletions, also starting at site 5162 but extending further upstream (see Fig. 1*C*, III, and Fig. 2). Screening of these mutants by restriction enzyme analysis revealed deletions ranging from 20 to 65 bp.

DNA Sequence Analysis of HS Mutants. To establish precisely the extent of deletions in the HS mutants, we performed sequencing studies on recombinants HS0, HS2, HS3, HS4, and HS6. All sequences were obtained by the technique of Maxam and Gilbert (26), after 5′-end labeling at the *Hin*dIII site (at position 5110, Fig. 2) and secondary restriction with *Eco*RI. Autoradiograms of the sequencing gels for mutants HS4 and HS6 are shown in Fig. 3. The A+T-rich region extending from position 5193 to position 5209 is conserved in HS4 but absent in HS6. Also visible is the extra sequence 5′-G-A-T-C-3′, present

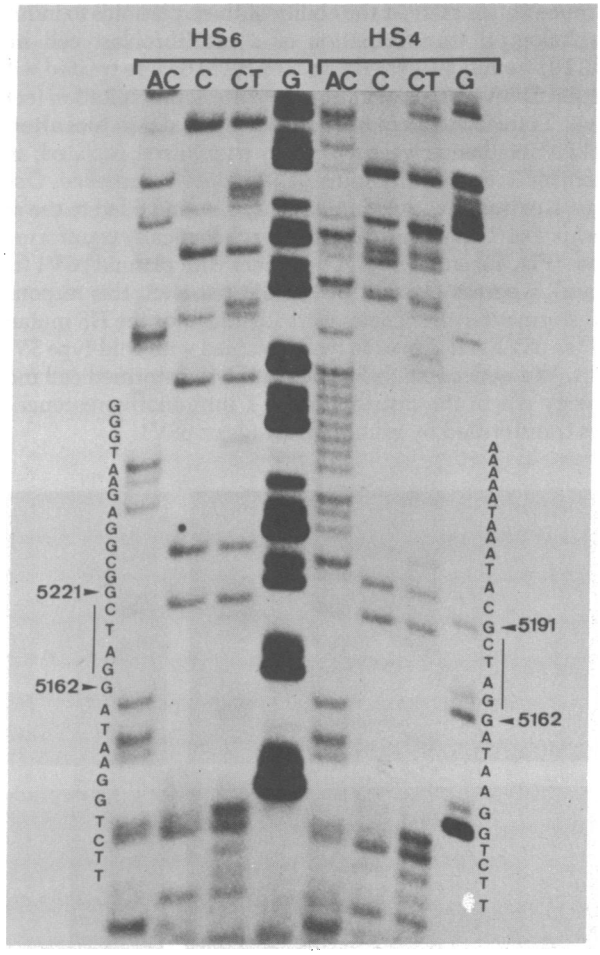

FIG. 3. Autoradiogram of a sequencing gel for mutants HS4 and HS6. Plasmid DNA of mutants HS6 and HS4 was digested with *Hin*dIII, 5′-end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase, and secondarily digested with *Eco*RI. After purification on acrylamide gel, the sequence of the *Hin*dIII/*Eco*RI fragment encompassing the site of the deletion (see Fig. 1*C*, III) was determined by the technique of Maxam and Gilbert (26). Vertical lines indicate the position where the deleted sequences have been replaced by the tetranucleotide G-A-T-C (see text). The sequence obtained is complementary to that shown in Fig. 2.

at the position of the deletion (shown by vertical lines in Fig. 3), which originates from the repaired *Bam*HI site (see above and Fig. 1*C*, II) used for the construction of the HS mutants. The nucleotides coding for the two major 5′ ends of early mRNA have been deleted in all the HS mutants. The extent of the deletions present in the various recombinants, as deduced from the sequencing data, is indicated schematically in Fig. 2.

HS Deletion Mutants Support SV40 Early Gene Expression in Eukaryotic Cells. Purified DNAs from the various HS mutants were transfected into CV1 cells by the DEAE-dextran procedure. Fifty hours after transfection, early gene expression was assayed by indirect immunofluorescence with a hamster antiserum against SV40 T antigen. The results are shown in Fig. 4. Whereas pEMP did not induce the appearance of T-antigen-positive cells, such cells were observed with all the HS recombinants described above (Fig. 4 *c* and *d*). The fluorescence obtained with these deletion mutants was at least as intense as that obtained with the undeleted plasmid pSV1 and could first be detected 15–17 hr after transfection in both cases. No difference in the distribution of the fluorescent staining within the cell nucleus was noted.

As another test for expression of the SV40 early genes in the HS mutants, we assayed the ability of these plasmids to induce morphological transformation of a rat fibroblast cell line (FR3T3). Semiconfluent cultures of FR3T3 were treated with plasmid DNA by the calcium phosphate coprecipitation technique. Transformed colonies appearing as dense foci after 2 weeks of incubation were selectively trypsinized, replated, and tested for T antigen by indirect immunofluorescence. Once again, treatment with HS mutants 2, 3, 4, and 6 led to the appearance of T-antigen-positive, morphologically transformed clones (Fig. 4 *e* and *f*), as did treatment with plasmid pSV1 (not shown), whereas plasmid pEMP did not elicit this response. Transformation efficiencies were identical for the HS mutants and for pSV1 and similar to those obtained with wild-type SV40 DNA. We noticed no difference in the transformed cell morphology nor in the distribution of T immunofluorescence in lines transformed by mutant plasmids or pSV1.

DISCUSSION

In an attempt to delineate those sequences located at the 5′ end of SV40 early genes that might play a role in the initiation of transcription, we have studied the effect on early gene expression of deletions created *in vitro* in this region. We first constructed a recombinant plasmid, pSV1, containing the SV40 early genes inserted into pBR322. Early functions are expressed when this plasmid is introduced into different eukaryotic cells. We then made *in vitro* deletion mutants of pSV1, from which the nucleotides coding for the major 5′ ends of early mRNAs and some of the sequences upstream have been deleted. Early gene expression is not abolished by the deletion of up to 60 bp upstream from the cap site in mutant HS6. On the other hand, the plasmid pEMP, from which all sequences upstream have been removed, does not support T-antigen expression. We conclude that at least part of the promoter sequences for RNA polymerase B are located upstream from the region coding for the 5′ end of mRNA, as in prokaryotes.

In mutants HS2 and HS6, which act as templates for early gene expression, we have removed a putative Goldberg–Hogness box, an A+T-rich region centered about 27 bp from the cap site of many eukaryotic mRNAs and hypothesized to function in the initiation of transcription. Our results seem to be in contradiction to this hypothesis, but we cannot conclude that the Goldberg–Hogness box is unnecessary for promoter function because at least three other mechanisms can explain early gene expression in HS mutants.

(*i*) It is possible that the T-antigen expression observed originates from minor promoters. In fact, Reddy *et al.* (14) have provided evidence suggesting the existence of minor early mRNAs with 5′ ends both upstream and downstream from the two major 5′ ends. These minor species appear to be present both in lytic infection and in SV40-transformed cell lines. It is unlikely that minor promoters located downstream from the major cap sites are involved in HS plasmid early gene expression because they would also be expected to be functional in pEMP, which contains these sequences. It is, on the other hand, quite possible that minor promoters, initiating transcription upstream from the major cap sites, are responsible for transcription of

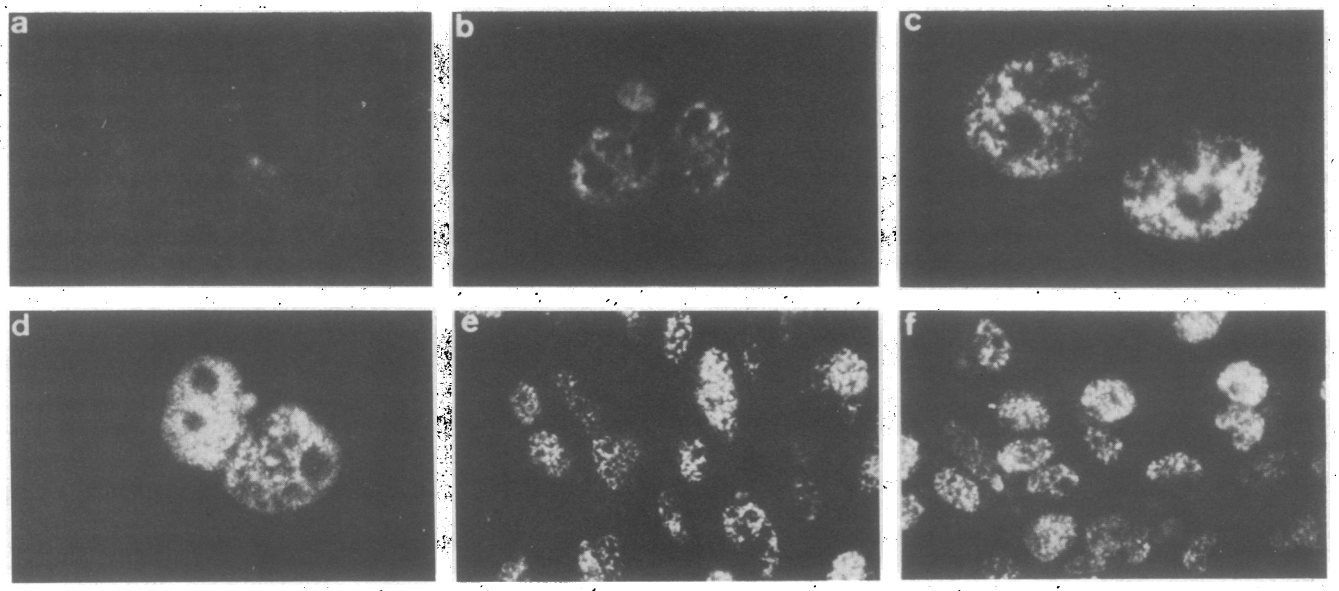

FIG. 4. Detection of SV40 T antigen in cells transfected with recombinant plasmids. T antigen was detected by indirect immunofluorescence by using hamster antiserum against SV40 T antigen followed by fluorescein-labeled antiserum against hamster globulin. (*a–d*) CV1 monkey cells stained after mock transfection (*a*) or transfection with plasmids pSV1 (*b*), HS2 (*c*), and HS4 (*d*). (*e* and *f*) Clones of FR3T3 rat cells transformed by plasmids HS0 (*e*) and HS3 (*f*). Clones transformed by the other HS mutants had the same appearance after immunofluorescent staining.

early genes of HS mutants. Such a situation has been described for SV40 late genes, where removal of sequences coding for the 5′ end of the major 16S mRNA lead to enhanced expression of minor species of late mRNA (27, 28).

We can essentially rule out that pBR322 sequences located upstream from the early gene are fortuitously recognized as promoters by eukaryotic RNA polymerases; when HS mutant DNA from which the pBR322 sequences have been excised by digestion with *Hha* I (there is an *Hha* I site at position 0.729, see Fig. 1) is transfected into monkey CV1 cells, T-antigen-positive cells still appear.

(*ii*) One can also hypothesize that in SV40 or pSV1 initiation of early transcription occurs not at the positions coding for the capped nucleotides of early mRNAs as we have assumed up to now, but further upstream. Subsequent cleavage would then create the major 5′ ends normally found. The deletions in HS plasmids would then remove only the processing site but not the actual promoter sequences. In adenovirus 2 major late genes, Ziff and Evans (4) have shown that such a mechanism does not occur; i.e., that the capped nucleotide in mature mRNA corresponds to the initiation site. However, such evidence is lacking elsewhere, particularly for SV40 early genes. It is quite conceivable that SV40 represents a special case; for example, the initiation of transcription could occur differently on linear and circular genomes.

(*iii*) It is conceivable that the Goldberg–Hogness box is only part of a larger promoter sequence and that its removal drastically affects, but does not completely eliminate, promoter function. A remaining low level of transcription could possibly account for the transformation of rat cells or the positive immunofluorescence pattern in monkey cells. However, preliminary blotting hybridization analysis of the RNA synthesized in rat cell lines transformed by HS mutants indicates that messengers for both T and t antigens are present in amounts at least as high as those found in a line transformed by pSV1. Deletions in HS mutants remove portions of the sequences at which binding of T antigen has been hypothesized to repress early mRNA synthesis (29, 30). Thus, such deletions could have the two opposing effects of diminishing promoter efficiency and eliminating T-antigen repression, which would lead to normal or even increased rates of early mRNA synthesis.

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