JOURNAL OF VIROLOGY, Mar. 1988, p. 954–961
0022-538X/88/030954-08$02.00/0

Vol. 62, No. 3

Mechanisms of Synthesis of Virion Proteins from the Functionally Bigenic Late mRNAs of Simian Virus 40

SYLVIA A. SEDMAN AND JANET E. MERTZ*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received 28 July 1987/Accepted 15 November 1987

The late 19S RNAs of simian virus 40 (SV40) are functionally polycistronic, i.e., all encode both VP2 and VP3. The VP3-coding sequences are situated in the same reading frame as the VP2-coding sequences, within the carboxy-terminal two-thirds of the VP2-coding sequences. To test whether VP3 is produced by proteolytic processing of VP2, we introduced a variety of deletion and insertion mutations within the amino-terminal end of the VP2-coding sequences. Genetic and biochemical analysis of the proteins synthesized in cells transfected with these mutants indicated that VP2 and VP3 were synthesized independently of each other. A leaky scanning model for the synthesis of VP3 was tested by the insertion of a strong initiation signal (CCAACATGG) upstream of the VP3-coding sequences. When the signal was placed in the same reading frame as VP3, synthesis of VP3 was reduced by a factor of 10 to 20, whereas synthesis of the expected VP3-related fusion protein occurred at a rate similar to that observed for VP3 in cells transfected with wild-type SV40 DNA. Insertion of this strong initiation signal at the same site, but in a different reading frame, resulted in the synthesis of VP3 at one-third of the wild-type rate. Mutation of the VP2 initiator AUG resulted in a small but reproducible (1.6-fold) increase in VP3 accumulation. From these experiments we conclude that (i) VP3 is synthesized predominantly by independent initiation of translation via a leaky scanning mechanism, rather than by proteolytic processing of VP2 or direct internal initiation of translation; (ii) a strong initiation signal 5′ of the VP3-coding sequences can significantly inhibit synthesis of VP3, but does not act as an absolute barrier to scanning ribosomes; (iii) approximately 70% of scanning ribosomes bypass the VP2 initiator AUG, which is present in a weak context (GGUCCAUGG), and initiate at the VP3 initiation signal located downstream; and (iv) reinitiation of translation appears to occur on the SV40 late 19S mRNAs at an efficiency of 25 to 50%.

Most eucaryotic mRNAs studied to date encode a single protein (20). Since mRNAs contain many AUG codons, it is of interest to understand how ribosomes recognize and initiate translation at the appropriate ones. From a survey of known initiation signals, Kozak hypothesized that the utilization of an AUG codon as an initiation signal for translation is determined both by its location within the mRNA molecule and by the sequences surrounding it (16). Her scanning model for the initiation of translation of eucaryotic mRNAs states that 40S ribosomal subunits bind at or near the 5′ end of a mRNA and scan toward the 3′ end until they encounter an AUG codon; if the AUG codon is situated within a favorable sequence context, the 40S subunit will stop scanning, the 60S subunit will attach to it, and protein synthesis will begin. Combining a survey of initiation signals used in cellular and viral mRNAs with studies involving the determination of the relative efficiencies of initiation at AUG codons in which the sequences surrounding them were varied systematically (19), Kozak concluded that the optimal sequence context for initiation of translation is 5′-CCACCAUGG-3′, with the −3 and +4 positions being of primary importance for the efficient recognition of the AUG as an initiation signal. However, if a pyrimidine is present at −3 or +4, ribosomes fail to recognize efficiently the AUG codon and frequently scan past it, initiating instead at AUG codons located downstream (i.e., 5′ distal). During the past few years, numerous examples have been reported in which AUG codons located 3′ to other AUG codons function as initiation codons (20).

The late mRNAs of the small primate DNA tumor virus simian virus 40 (SV40) encode the virion proteins VP1, VP2, and VP3, as well as the leader-encoded protein, LP1 (also called agnoprotein) (Fig. 1). Most of them contain the coding sequences for two to four proteins. Each of the late 19S RNA species is functionally bigenic, making both VP2 and VP3 when translated (11). These two proteins are encoded in the same reading frame, with the VP3 sequences contained within the carboxy-terminal two-thirds of VP2. However, although the initiation signal used for the synthesis of VP2 is situated upstream (i.e., 5′ proximal) of the one used for synthesis of VP3, the rate of synthesis of VP2 is only half that of VP3 (11).

There have been numerous reports of bicistronic mRNAs in the literature (20). Members of our laboratory have been interested in determining the mechanism(s) of synthesis of proteins from these RNAs. The primary aim of the studies described here was to determine the mechanism of VP3 synthesis from the bicistronic 19S mRNAs of SV40. To test whether VP3 is produced by proteolytic processing of VP2, a variety of deletion and insertion mutations were introduced within the amino-terminal end of VP2. Genetic and biochemical analysis of the proteins synthesized in cells transfected with these mutants indicated that VP2 and VP3 were synthesized independently of each other. A leaky scanning model for the synthesis of VP3 was tested by the insertion of a strong initiation signal (CCAACAUGG) upstream of the VP3-coding sequences. When the inserted AUG was in the VP2/VP3 reading frame, VP3 synthesis was reduced 10- to 20-fold, whereas synthesis of the expected fusion protein occurred at a rate comparable to VP3 synthesis in cells transfected with wild-type (WT) SV40 DNA. When the same initiation signal was placed in a different reading frame in which ribosomes initiating translation at the inserted AUG encounter a termination codon 60 bases upstream of the

* Corresponding author.

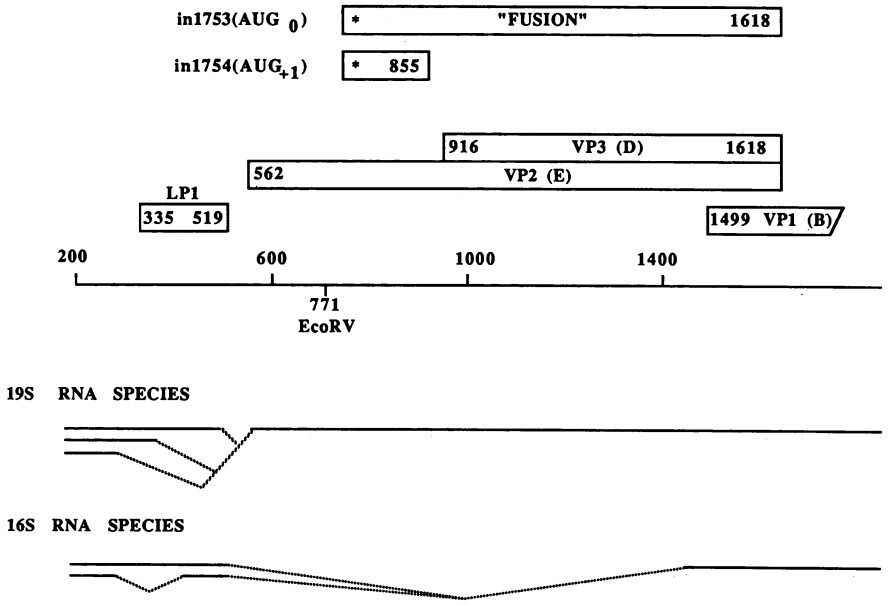


FIG. 1. Map of the late region of SV40. The numbered line represents the late region of the SV40 genome, with the nucleotide residues given in the SV numbering system (33). Below are the structures of the mRNAs made from this region. The 5′ ends of these RNAs are heterogeneous, mapping between nt 28 and nt 450, with the majority mapping to nt 325 (10). ....., Excised intervening sequences. The 3′ ends of these RNAs are identical in sequence and are not shown. The proteins encoded by these mRNAs are indicated by the boxes labeled LP1, VP2, VP3, and VP1, along with the locations of their translation initiation and termination codons. Also shown are the novel polypeptides synthesized in cells transfected with mutants *in*1753(AUG_0) and *in*1754(AUG_{+1}); the precise locations within SV40 and the sequences of the initiation signals for these proteins are presented in Table 1.

VP3-coding sequences, VP3 was synthesized at one-third the wild-type rate. These results indicate that although the initiation signal for synthesis of VP3 is situated approximately 400 bases from the 5′ end of the mRNAs that encode this protein, VP3 is synthesized efficiently by a mechanism in which ribosomes scan from the 5′ end of the mRNA, bypassing the 5′-proximal AUG codons. The removal of the VP2 initiation signal resulted in a 1.6-fold increase in VP3 accumulation, providing further evidence that VP3 is synthesized by a mechanism in which ribosomes scan past 5′-proximal AUG codons. In addition, our finding that reinitiation of translation appeared to occur with 25 to 50% efficiency may explain in part how VP1 is synthesized from the major late 16S mRNA species which encodes LP1 5′ to the coding sequences for VP1. Therefore, by a combination of leaky scanning and apparent reinitiation, SV40 coordinately regulates expression of pairs of genes such that each gene is expressed at the proper molar ratio for virion production.

MATERIALS AND METHODS

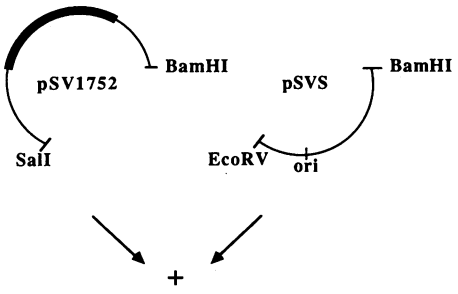
Cell lines and culture conditions. CV-1P cells were used in the complementation experiments. BSC-1 cells were used in transfections performed to analyze viral proteins and mRNAs. Both cell lines, derived from African green monkey kidney cells, were maintained essentially as described by Mertz and Berg (28). Transfections were performed by using a modification (24) of the DEAE-dextran method of McCutchan and Pagano (26). Briefly, viral DNA was excised from the vector sequences and ligated to form monomer circles. The DNA was diluted into Dulbecco modified Eagle medium (DMEM) containing 500 μg of DEAE-dextran (molecular weight 2×10^6) per ml and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [pH 7.1]) and added to BSC-1 cells. After incubation at 37° for 45 min, the cells were washed with DMEM–20 mM HEPES and incubated for 4 h at 37° in DMEM containing 20 mM HEPES, 5% fetal bovine serum, and 100 μM chloroquine. The cells were washed again and incubated at 37° in medium containing 5% fetal bovine serum for the indicated length of time.

Antisera. Polyclonal antisera were raised against gel-purified VP3 in rabbits as described by Kasamatsu and Nehorayan (14). The same method was used to make antisera against VP1.

Synthetic oligonucleotides. To construct the mutants *in*1753(AUG_0) and *in*1754(AUG_{+1}), the oligonucleotides shown in Fig. 2 were synthesized manually by the phosphoramidite method as decribed by Drahos et al. (7).

Construction of mutants. The starting plasmid used in the construction of all mutants described here was pSVS, a plasmid in which the DNA of SV40 WT830 was inserted at the *Eco*RI site of a pBR322-based vector (9). The plasmids corresponding to the deletion mutants *dl*1750 and *dl*1751 were created by sequential treatment of pSVS with *Eco*RV, S1 nuclease, the Klenow fragment of DNA polymerase I, and T4 DNA ligase. Plasmid pSVin1752 was made by the insertion of an 8-base-pair *Sal*I linker at the *Eco*RV site (nucleotide [nt] 771). Mutants pSVin1753(AUG_0) and pSVin1754(AUG_{+1}) were constructed by the three fragment ligations diagrammed in Fig. 2. The subscripts 0 and +1 for mutants 1753 and 1754 indicate that the inserted AUG codon is in the same reading frame used to translate VP2 and VP3 and in the +1 reading frame relative to VP2 and VP3, respectively. The structures of these mutants (Table 1) were confirmed by DNA sequencing (25). In addition, mutants *pm*918 and *dl*1755 were generously supplied to us by L. Barber and P. Good, respectively (see Table 1 for structures).

Analysis of virion proteins. Cells were harvested 40 to 42 h after transfection as described by Barkan et al. (4). Briefly,



1. TTCCAACATGGG
   AAGGTTGTACCCAGCT ⇒ pSVin1753(AUG_0)

2. TCCAACATGGGG
   AGGTTGTACCCCAGCT ⇒ pSVin1754(AUG_{+1})

FIG. 2. Schematic diagram of the method used for construction of mutants *in*1753(AUG_0) and *in*1754(AUG_{+1}). The *Sal*I-*Bam*HI fragment of pSV1752 and the *Bam*HI-*Eco*RV fragment of pSVS were recombined in three-way ligations with the small synthetic restriction fragments 1 and 2 to generate the plasmids pSVin1753 (AUG_0) and pSVin1754(AUG_{+1}), respectively. Symbols: ▬, sequences from pBR322; ——, sequences from SV40.

cells were scraped off the dishes and pelleted by centrifugation. Cells were lysed in a buffer containing 0.01 M Tris (pH 7.4), 4.5% (wt/vol) NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate (SDS), and 1% aprotinin. When the proteins were to be analyzed by immunoprecipitation, the cells were incubated for 2 h in medium lacking arginine, followed by 1 h in medium containing [^{14}C]arginine (10 μCi/ml, 332 mCi/mmol; New England Nuclear Corp.) immediately before being harvested. The immunoprecipitations were performed as described previously (15). The immunoprecipitated proteins were analyzed by electrophoresis through a 12% polyacrylamide (0.35% bisacrylamide) gel containing SDS (21). Afterwards, the gels were treated with En^{3}Hance (New England Nuclear), dried, and exposed to X-ray film. Viral proteins were also analyzed by immunoblotting with ^{125}-protein A (Amersham Corp.) essentially as described by Baichwal and Sugden (1). For both assays, the relative amounts of the virion proteins were determined by quantitative densitometry.

Analysis of viral late mRNAs. Cytoplasmic RNA was isolated from transfected cells in parallel with proteins. Briefly, cells were washed in cold Tris-buffered saline, scraped off the dishes, and pelleted. The cells were suspended in Tris-buffered saline containing 0.5% Nonidet P-40. After incubation on ice for 5 min, the nuclei were pelleted by centrifugation. The supernatant was incubated for 45 min at 37°C with proteinase K (500 μg/ml), extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1 vol/vol/vol), and precipitated twice with ethanol. Quantitative S1 mapping was performed as described previously (3) by using a DNA probe 5′ end-labeled at SV40 nt 1046 to analyze the structures of the 19S RNAs. Correction for recovery of cytoplasmic RNA was done by concurrent quantitative S1 nuclease mapping of cytoplasmic actin mRNA by using a probe with sequences from nt 64 through nt 245 of the human β-actin cDNA clone pHFβA-1 (31). The plasmid was a gift from L. Kedes.

RESULTS

Independent production of VP2 and VP3. Many animal viruses synthesize two or more proteins from one mRNA by proteolytic processing of a polyprotein. Since VP3 is identical in sequence to the carboxy-terminal two-thirds of VP2, it was theoretically possible that VP3 was produced by proteolytic cleavage of VP2.

To test this possibility, we introduced frameshift mutations at the *Eco*RV site situated near the amino-terminal end of the VP2-coding sequences (Fig. 1). Ribosomes translating the VP2-coding sequences from the late 19S mRNAs of these mutants shift reading frames at the site of the mutation and encounter a termination codon before reaching the sequences encoding VP3. If VP3 were produced by proteolytic cleavage of VP2, the frameshift mutants would be unable to synthesize either VP2 or VP3. On the other hand, if VP3 were synthesized independently of VP2 by initiation of translation at its own initiation signal, a frameshift mutation at the N-terminal end of VP2 would not be expected to affect significantly the synthesis of VP3.

As a first assay, complementation tests were performed between the VP2 frameshift mutants *dl*1750, *dl*1751, and *in*1752 and SV40 mutants with functional defects in each of the virion proteins. The data, presented in Table 2 and summarized in Table 1, show that each of the VP2 frameshift mutants failed to complement a mutant defective in VP2 (i.e., *dl*1755), but did complement a mutant defective in VP3 (i.e., *pm*918). The virion proteins made in cells transfected with each of the VP2 frameshift mutants were determined by immunoblot analysis (data not shown). The results confirmed the genetic analysis: VP3 was synthesized at levels similar to that observed in cells transfected with wild-type DNA, whereas VP2 was not detected. Therefore, VP3 is unlikely to be produced by proteolytic processing of VP2. These results confirm the studies of Cole et al. (6) in which mutants that contained deletions within the amino-terminal

TABLE 1. Summary of structures, genetic properties, and proteins synthesized from each of the mutants used in this study

Strain	Nucleotides deleted (inserted)	Genotype	Proteins synthesized	Source or reference
SVS (WT830)		WT	VP1, VP2, VP3	9
*ts*B201	nt 2003 G → A[a]	B$^-$	VP1, VP2, VP3	5
*dl*1755	562–563	E$^-$	VP1, VP3	P. Good
*pm*918	nt 918 G → T	D$^-$	VP1, VP2	L. Barber
*dl*1750	759–778	E$^-$	VP1, VP3	This report
*dl*1751	759–787	E$^-$	VP1, VP3	This report
*in*1752	770(GGTCGACC)771	E$^-$	VP1, VP3	This report
*in*1753(AUG_0)	770(TCCAACATGGGGTCGACC)771	D$^-$, E$^-$	VP1, VP2[b], fusion, VP3[c]	This report
*in*1754(AUG_{+1})	770(TTCCAACATGGGTCGACC)771	E$^-$	VP1, VP2[b], VP3	This report

[a] M. Bina, personal communication.
[b] VP2 is synthesized, but is nonfunctional.
[c] VP3 is synthesized, but at levels too low to complement a VP3$^-$ mutant.

TABLE 2. Time of appearance of plaques after cotransfection[a]

Mutant	Time of appearance (days) with following helper:		
	*ts*B201 (VP1$^-$)	*dl*E1755 (VP2$^-$)	*pm*D918 (VP3$^-$)
*ts*B201	–[b]	8	8
*dl*1755	8	–	9
*pm*918	8	9	–
*dl*1750	8	–	9
*dl*1751	8	–	9
*in*1752	8	–	9
*in*1753(AUG$_0$)	8	–	–
*in*1754(AUG$_{+1}$)	8	–	13

[a] Complementation tests were performed as described previously (27, 28) by cotransfection of monolayers of CV-1P cells with 2 ng of test mutant DNA and 2 to 4 ng of helper DNA followed by incubation at 41°C when *ts*B201 was used and 37°C when it was not used. The numbers in the table indicate the day after cotransfection when plaques were first visible. All monolayers on which plaques appeared contained similar numbers of plaques by 17 days after transfection.

[b] –, Plaques had not yet appeared when the experiment was terminated at 17 days after transfection.

end of VP2 (as determined by altered mobilities of a restriction fragment in a polyacrylamide gel) were able to synthesize VP3. Additional evidence indicating that VP3 is not synthesized by proteolytic processing of VP2 is presented below and in the Discussion.

Inhibition of synthesis of VP3 by insertion of an efficiently utilized translation initiation signal upstream of the VP3-coding sequences. An alternative model for VP3 synthesis is that it occurs via a leaky scanning mechanism in which some of the 40S ribosomal subunits scan past the VP2 initiation signal and instead initiate translation downstream at the VP3 initiator AUG. Consistent with this model is the fact that the AUG codon used for initiation of translation of VP2 is found in a weak context (Table 3).

To test whether VP3 is synthesized by leaky scanning, a pair of mutants, *in*1753(AUG$_0$) and *in*1754(AUG$_{+1}$), were constructed in which the rat preproinsulin translation initiation signal was inserted at the *Eco*RV site within the VP2-coding region, upstream of the VP3-coding sequences (Table 1; Fig. 1). If VP3 were produced by scanning, cells transfected with these mutants would be expected to synthesize VP3 at much lower rates than would cells transfected with WT SV40, because this translation initiation signal conforms closely to the optimal consensus sequence (Table 3) and is utilized very efficiently (17). In both mutants, the sequences from −6 to +5 surrounding this AUG codon are identical to those surrounding it in the rat preproinsulin gene (Fig. 2; Table 1).

In mutant *in*1753(AUG$_0$), this strong initiation signal was placed in the reading frame used in the synthesis of VP2 and VP3. Ribosomes initiating at the inserted AUG codon would be expected to synthesize a 33-kilodalton fusion protein containing VP2 and VP3 sequences (Fig. 1). In addition, since the insertion is 18 bases in length, ribosomes initiating at the VP2 AUG should remain in frame, synthesizing a protein identical to VP2 except for the addition of 6 amino acids due to the insertion. The VP3-coding sequences are not affected by the mutation. Since VP2 and the fusion protein contain amino acid sequences in common with VP3, all should be recognized by antisera raised against VP3.

Complementation tests performed with this mutant indicated that it was unable to complement mutants defective in either VP2 or VP3 (Table 2; summarized in Table 1). To determine the relative rates of synthesis of the proteins translated from the mRNAs made from WT SV40 and mutant *in*1753(AUG$_0$), BSC-1 cells were transfected with DNA of each and pulse-labeled 40 h later with [^{14}C]arginine for 1 h. The VP3-related proteins were purified by immunoprecipitation with antisera raised against VP3 protein and analyzed by electrophoresis through SDS-polyacrylamide gels (Fig. 3A). The mutant- and WT-transfected cells synthesized VP2 at similar rates; however, the rate of synthesis of VP3 was a factor of 10 to 20 lower in the cells transfected with the mutant than in cells transfected with WT DNA (Fig.

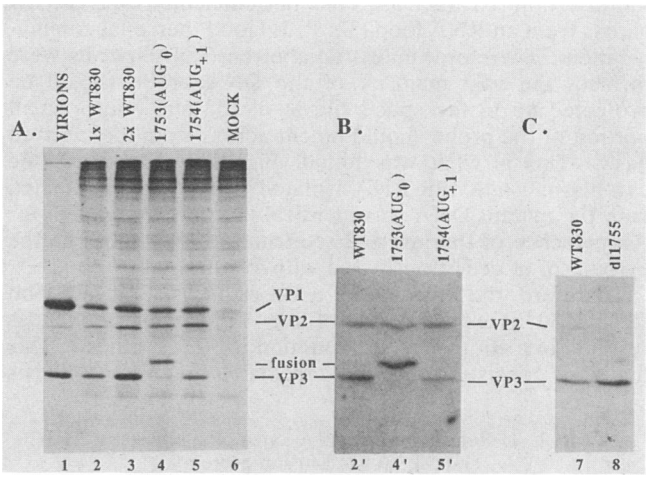


FIG. 3. Autoradiograms of VP3-related proteins synthesized in cells transfected with WT830, the AUG insertion mutants *in*1753(AUG$_0$) and *in*1754(AUG$_{+1}$), and the VP2 AUG$^-$ mutant *dl*1755. (A) Fluorogram of pulse-labeled, immunoprecipitated proteins. At 40 h after transfection with the indicated DNAs, BSC-1 cells were pulse-labeled for 1 h with [^{14}C]arginine and harvested immediately thereafter. The proteins in lysate from 4 × 10^5 cells (lane 3) or 2 × 10^5 cells (lanes 2, 4, 5, and 6) were immunoprecipitated with equal amounts of a polyclonal antiserum raised against VP3 and analyzed by electrophoresis in a 12% polyacrylamide gel containing SDS. Lane 1 was loaded with [^{3}H]leucine-labeled purified SV40 virions for markers. (B) Immunoblot of cells transfected with WT830, *in*1753(AUG$_0$), and *in*1754(AUG$_{+1}$). Proteins from 2 × 10^5 cells were separated by electrophoresis in a 12% polyacrylamide gel containing SDS and transferred to nitrocellulose. The nitrocellulose was blocked by using phosphate-buffered saline containing 1% (wt/vol) powdered milk, incubated with the polyclonal antiserum used in panel A, incubated with ^{125}I-protein A (Amersham), and exposed to X-ray film. (C) Immunoblot of cells transfected in parallel with WT830 and *dl*1755. Immunoblotting was performed as described in panel B. (The faint band which appears between VP2 and VP3 is an irreproducible artifact, not seen in other blots of the same sample or in samples obtained from different transfections with this mutant. It cannot be the result of ribosomes initiating upstream of the VP3 initiation codon, since these 19S mRNAs do not contain any AUG codons in the VP2/VP3 reading frame between the VP2 and VP3 initiation signals.)

TABLE 3. Translation initiation signals of proteins encoded by the late mRNAs of SV40

Protein	Sequence									Reference
			−3			+1			+4	
Consensus	C	C	A	C	C	A	U	G	G	18
VP2	G	G	U	C	C	A	U	G	G	33
VP3	C	A	G	G	A	A	U	G	G	33
LP1	A	G	G	C	C	A	U	G	G	33
VP1	U	G	A	A	G	A	U	G	G	33
Rat preproinsulin	C	C	A	A	C	A	U	G	G	23

3A, lane 4 versus lane 2; Table 4). In place of VP3 was a VP3-related protein (fusion) of the size expected for translation initiating at the inserted AUG codon. Analysis of the relative amounts of these proteins by immunoblotting with VP3 antisera yielded similar results (Fig. 3B), indicating that all of these proteins have similar half-lives. Furthermore, the VP2-to-VP1 ratios were similar for mutant- and WT-transfected cells as well (data not shown). Therefore, the 6-amino-acid insertion in the mutant VP2 proteins does not affect the stability of VP2. These findings, together with the fact that VP2 and VP3 are synthesized from the same mRNAs (11), enabled us to use VP2 as an internal control for calculating the rate of VP3 synthesis in mutant- relative to WT-transfected cells (Table 4).

To rule out the possibility that synthesis of the fusion protein occurred from aberrant mRNAs that lacked the VP2 initiation signal (e.g., RNAs processed via the utilization of a cryptic splice site), the structures of the late mRNAs made from mutant *in*1753(AUG_0) were analyzed by S1 mapping (Fig. 4). Since the DNA probe used in this experiment was isolated from WT DNA (Fig. 4B), hybrids formed between it and viral RNA made in cells transfected with the insertion mutants should contain an 18-base loop of RNA at the site of the insertion. S1 nuclease does not efficiently cleave DNA across from an RNA loop (32; P. J. Good, personal communication). Therefore, unless an aberrant RNA species were formed, the vast majority of the DNA probe should be protected up to the splice site at nt 558 and only a small portion of the probe should be cut across from the inserted bases. This is what was found (Fig. 4A). Therefore, the cytoplasmic late 19S RNA isolated from cells transfected with the mutant DNA was identical in structure, except for the presence of the inserted sequence, to that found in the cytoplasm of cells transfected with WT DNA.

Therefore, the presence of a strong translation initiation signal 150 bases upstream of the VP3-coding sequences resulted in a 10- to 20-fold reduction in VP3 synthesis. This finding is consistent with the conclusion that VP3 synthesis

TABLE 4. Summary of relative rates of synthesis of VP3 and VP3-related fusion protein[a]

DNA	Relative rate of synthesis of:	
	VP3	VP3-related fusion protein
WT830	1[b] (1)	
*in*1753(AUG_0)	0.07 ± 0.01 (ND)[c]	0.46 ± 0.04 (1.2)
*in*1754(AUG_{+1})	0.31 ± 0.04 (0.3)	
*dl*1755	–[d] (1.6 ± 0.1)[e]	

[a] The data were obtained by quantitative densitometry of autoradiograms of gels similar to the ones presented in Fig. 3. VP3 synthesis was determined relative to the amount of VP2-like protein synthesized concurrently in the same cells. All VP3/VP2 ratios were normalized to the VP3/VP2 ratio obtained from cells transfected in parallel with WT830 DNA excised from pSVS. Each number is the mean and standard error of the mean from three independent pulse-labeling experiments performed as described in Materials and Methods. The numbers in parentheses were obtained from two different immunoblotting experiments.

[b] The VP3/VP2 ratio for WT ranged from 2.9 to 3.9 in different experiments.

[c] ND, Not detected.

[d] –, Not done.

[e] Since VP2 is not synthesized by cells transfected with *dl*1755, VP2 could not be used as an internal standard for VP3 synthesis. Instead, the amount of VP3 synthesized in cells transfected with *dl*1755 was determined by normalization to the amounts of cytoplasmic SV40 late 19S RNA present in cells transfected in parallel with *dl*1755 and WT830. Recoveries of cytoplasmic RNA were controlled by normalization to the amounts of actin mRNA present in the same samples (see Materials and Methods for details.) This experiment was repeated three times.

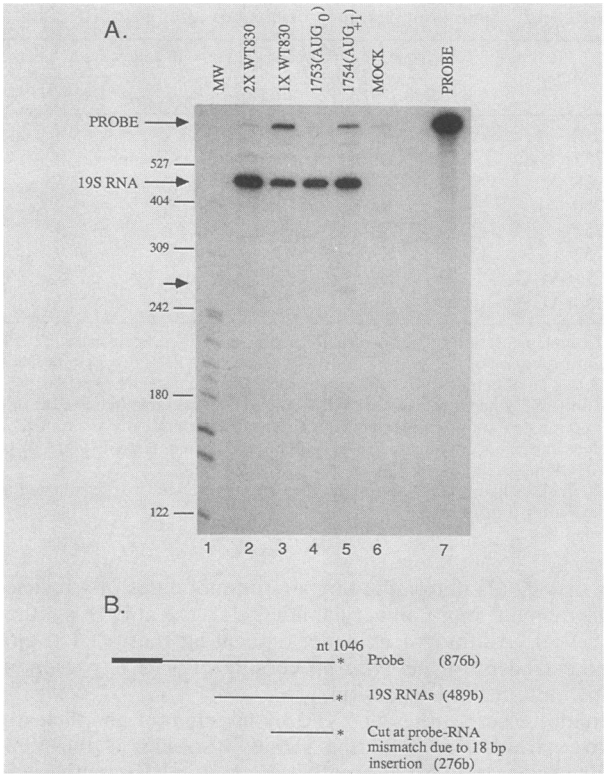


FIG. 4. Analysis of the structure of the cytoplasmic late 19S RNAs. Cytoplasmic RNA was isolated from cells transfected in parallel with those analyzed in Fig. 3. RNA from 2×10^5 cells (lane 2) or 1×10^5 cells (lanes 3 to 6) was incubated at 50°C for 14 h with the probe indicated in panel B and then incubated with S1 nuclease as described by Barkan and Mertz (3). Molecular weight standards are indicated at the left. Lane 7 was loaded with 10% of the amount of probe used in each hybridization reaction. The large arrows on the left indicate the positions of reannealed probe and DNA protected by 19S RNA. The small arrow indicates the size of DNA protected by 19S RNA, but cleaved by S1 nuclease opposite the 18-base insertion present in the RNAs transcribed from the mutants but missing from the probe. (B) Strategy used for S1 nuclease mapping. The probe was derived from a vector that contains WT SV40 sequences from nt 346 to nt 1046. The probe was 5′-end-labeled at nt 1046 (a *Hin*dIII site) and contains 175 nt of vector DNA 3′ to the radiolabeled SV40 5′ end. Abbreviations: b, base; bp, base pair.

occurs predominantly via a leaky scanning mechanism. It also confirms the conclusion that VP3 is not made by proteolytic processing of VP2, since cells transfected with this mutant synthesized a VP2-like protein at a rate comparable to that observed in cells transfected with WT DNA, yet they synthesized VP3 at a greatly reduced rate. It is also noteworthy that the insertion of 6 amino acids into the N-terminal end of VP2 did not reduce the stability of this protein, even though it resulted in the inactivation of its function as determined by complementation analysis (Tables 1 and 2).

Effect of the reading frame of the inserted initiation signal on the efficiency of synthesis of VP3. A second mutant, *in*1754(AUG_{+1}), containing an 18-base-pair insertion at the *Eco*RV site, was also constructed. This insertion is identical in sequence to the one present in mutant *in*1753(AUG_0), except that the ATG is displaced by 1 base pair to put it in the +1 reading frame relative to VP2 and VP3. Therefore, ribosomes initiating translation at this inserted initiation

signal encounter a termination codon 60 bases upstream of the VP3 initiator AUG after translating 32 codons (Fig. 1).

Complementation tests indicated that this mutant, unlike *in*1753(AUG_0), can complement mutants defective in VP3 (Table 2). Therefore, cells transfected with *in*1754(AUG_{+1}) must synthesize VP3 in quantities sufficient for virion formation. Analysis of the proteins produced in cells transfected with this mutant confirmed this result (Fig. 3, lanes 5 and 5′; Table 4). Despite the insertion of the same strong translation initiation signal at an almost identical location to that in mutant *in*1753(AUG_0), the rate of synthesis of VP3 in cells transfected with *in*1754(AUG_{+1}) was only threefold lower than the rate of synthesis of VP3 in cells transfected with WT DNA. Therefore, the reading frame of the inserted initiation signal significantly affects the efficiency of synthesis of VP3.

Effect of mutation of the VP2 initiation codon on VP3 accumulation. If VP3 is produced from the late 19S mRNAs by scanning, removal of the initiation signal for VP2 should result in an increase in VP3 synthesis. To test this prediction, we measured VP3 synthesis in cells transfected with DNA from the WT and a mutant, *dl*1755, which contains a deletion removing the first two bases of the VP2 initiation codon. Cells were transfected with the DNAs, and the amount of VP3 accumulated was measured by immunoblot analysis (Fig. 3C). These data show that cells transfected with mutant DNA did not synthesize VP2, as expected, but did accumulate a somewhat larger amount of VP3 than cells transfected with WT DNA. To quantify accurately this change in VP3 accumulation, the amount of VP3 synthesized in cells transfected with *dl*1755 versus WT DNA was determined by densitometric analysis of immunoblots similar to the one shown in Fig. 3C and normalized to the amount of SV40 late 19S RNA found in the cytoplasm of cells transfected in parallel with these DNAs. The results obtained from three separate experiments indicated that cells transfected with *dl*1755 accumulated 1.5-, 1.5-, and 1.8-fold more VP3 than cells transfected with WT DNA (Table 4). Therefore, the removal of the VP2 initiator AUG resulted in a small but reproducible increase in VP3 synthesis.

DISCUSSION

VP3 is synthesized predominantly via a leaky scanning mechanism. The studies presented here were conducted to determine the mechanism of synthesis of VP3, the downstream-encoded protein on a naturally occurring, functionally polycistronic mRNA. To test the hypothesis that VP3 is produced through proteolytic cleavage of VP2, we initially created frameshift mutations in the amino-terminal end of VP2. Cells transfected with these mutants failed to synthesize VP2, but made normal amounts of VP3. Cole et al. (6) and L. Barber (personal communication) have also noted that VP3 can be made in the absence of VP2. These other studies were not definitive, because VP3 may have been synthesized in cells transfected with these mutants by reinitiation of translation or by a simple scanning mechanism when the VP2 initiator AUG is mutated. We also found that cells transfected with mutant *in*1753(AUG_0) made a VP2-like protein at levels comparable to those in cells transfected with WT DNA, yet made greatly reduced quantities of VP3 (Fig. 3; Table 4). In addition, cells transfected with mutant *pm*918, which differs from the WT solely by a point mutation in the VP3 methionine initiation codon, synthesize normal amounts of VP2, but no detectable VP3 (P. J. Good, Ph.D. thesis, University of Wisconsin, Madison, 1987). Therefore, although it is formally possible that the 6-amino-acid insertion present in the VP2-like protein made from mutant *in*1753(AUG_0) or the 1-amino-acid substitution in the VP2-like protein made from mutant *pm*918 might inhibit cleavage of VP2, it is unlikely that both alterations would do so. Taken together with the above data, these results provide strong evidence that VP3 is not synthesized by proteolytic processing of VP2. Also, since inserted sequences located 150 bases upstream of the VP3-coding region had profound effects on VP3 synthesis (Fig. 3; Table 4), it is unlikely that VP3 is synthesized by a mechanism in which ribosomes bind directly to the VP3 initiator codon.

The insertion upstream of VP3 of an efficiently utilized translation initiation codon in the VP2/VP3 reading frame in mutant *in*1753(AUG_0) resulted in a 10- to 20-fold reduction in VP3 synthesis (Fig. 3; Table 4). In its place, a novel protein was synthesized which was of the size expected from translation initiating at the inserted initiation signal. Immunoblot analysis of the viral proteins accumulated in cells transfected with *in*1753(AUG_0) indicated that the fusion protein was present at levels similar to those found for VP3 in cells transfected with WT DNA (Table 4). Quantitation of the pulse-labeled VP3-related proteins made in cells transfected with this mutant indicated that the fusion protein was synthesized at half the rate of VP3 in WT-transfected cells (Table 4). This result was unexpected, since the inserted initiation signal should be, by Kozak's rules (18, 19), stronger than the VP3 initiation signal. One possible explanation for this twofold discrepancy is that the antiserum raised against VP3 may recognize VP3 in an immunoprecipitation reaction more efficiently than it recognizes the fusion protein and therefore quantitatively precipitate the former, but not the latter.

Regardless, our results are consistent with synthesis of VP3 via a leaky scanning mechanism in which approximately 70% of scanning 40S ribosomal subunits bypass the VP2 AUG and instead initiate translation at VP3. Also consistent with a leaky scanning mechanism was our finding that the removal of the VP2 initiation codon resulted in a small but reproducible (1.6-fold) increase in VP3 accumulation (Table 4). Since the VP2 initiation codon is in a weak context, one might have predicted that it would have little effect on VP3 synthesis. The 1.6-fold increase is remarkably consistent with the 0.4:1 ratio of VP2 to VP3 synthesis observed in cells transfected with WT virus, i.e., one in three ribosomes initiates at the VP2 initiator AUG; therefore, its removal should allow 50% more ribosomes to initiate translation at VP3. Others (2, 19) have also shown that the insertion of a weak initiation signal upstream of an open reading frame has little or no effect on translation of the open reading frame.

Our finding that a small insertion containing an AUG codon can be recognized efficiently as a translation initiation signal supports Kozak's definition of a strong initiation signal. However, some VP3 was still synthesized in cells transfected with a mutant containing this signal in frame and upstream of VP3. Therefore, some ribosomes were able to scan past the inserted AUG codon even though it is in an optimal context. This conclusion appears to contradict the results of a study by Kozak in which translation of a synthetic construct containing several copies of the preproinsulin initiation signal arranged in tandem initiated only from the AUG codon located closest to the 5′ end of the mRNA (17). Possible reasons for this apparent discrepancy include (i) sequences outside of the −6-to-+5 region surrounding the AUG codon contribute to the efficiency of utilization of the AUG codon as an initiation signal; (ii) the

secondary structure of the mRNA influences the efficiency of utilization of these initiation signals; (iii) VP3 is also synthesized at a low efficiency by a mechanism in which 40S subunits bind directly to the VP3 initiator AUG; and (iv) initiations downstream of the 5′ proximal AUG were below the limit of detection in the experiments reported by Kozak.

Studying translation of 19S-like mRNAs made from artificial constructs derived from the late region of SV40, Peabody et al. (30) found that ribosomes initiate translation at AUG codons located downstream of the VP3 AUG. Good et al. (11) have also shown that VP1 is synthesized from the SV40 late 19S mRNAs when the number of AUG codons situated upstream of the VP1-coding region is decreased; consistent with a scanning mechanism, the amount of VP1 synthesized is inversely proportional to the number of AUG codons located upstream. We have shown here that 5 to 10% of ribosomes can scan past an AUG codon in a near-optimal context. These findings suggest that ribosomes are able to scan past any AUG codon.

The site of termination of translation or length of an open reading frame affects the efficiency of translation of 5′-distal open reading frames. When cells were transfected with the mutant in which the inserted AUG codon was placed in a translation reading frame different from the one used in synthesis of VP2 and VP3, VP3 was synthesized at a significantly higher rate than was observed in cells transfected with the mutant with the in-frame insertion (Table 4). The mRNAs made by the two mutants are identical in sequence, except for a 1-base shift in the location of the inserted initiation signal (Table 1). No significant differences were seen by S1 mapping analysis in either the quantity or the structures of the late mRNAs made in cells transfected with these two mutants (Fig. 4). However, the mRNAs of these two mutants are translated differently: ribosomes initiating at the inserted AUG codon in the mRNAs synthesized from mutant *in*1754(AUG_{+1}) translate different and fewer codons than do ribosomes initiating at the inserted AUG codon in the mRNAs synthesized from mutant *in*1753(AUG_0), with termination of translation occurring 60 bases upstream of the VP3 initiation codon, rather than at the VP3 termination codon (Fig. 1). The difference in the rates of synthesis of VP3 from these mRNAs can be explained by either of two hypotheses. According to an occluded-scanning hypothesis, 40S ribosomal subunits are unable to scan past translating ribosomes (3; J. E. Mertz, G. Gelembiuk, and J. M. Kane, manuscript in preparation). Therefore, the long open reading frame that follows the inserted AUG codon in mRNAs made from mutant *in*1753(AUG_0) prevents most ribosomes from scanning as far as the VP3 initiation signal, because one or more actively translating ribosomes are present within this region of the mRNA. On the other hand, the short, novel open reading frame present in 19S mRNAs made from mutant *in*1754(AUG_{+1}) enables a significant percentage of ribosomes to scan as far as the VP3 initiation signal because it frequently does not contain actively translating ribosomes owing to its short length. Alternatively, according to a hypothesis that assumes reinitiation, ribosomes reinitiate translation efficiently on the 19S mRNAs made from mutant *in*1754(AUG_{+1}), but not on those made from mutant *in*1753(AUG_0), because ribosomes terminate upstream of the VP3 initiation signal on the former mRNA, but several hundred bases downstream on the latter. Since the sequences surrounding the inserted AUG codons in mutants *in*1753(AUG_0) and *in*1754(AUG_{+1}) are identical from −6 to +5, it is reasonable to assume that ribosomes initiate translation from the 19S mRNAs made from the two mutants at identical frequencies at the inserted AUG codons. Given this assumption, the apparent efficiency of reinitiation at the VP3 initiator AUG after termination of translation from the inserted AUG codon in mutant *in*1754(AUG_{+1}) can be expressed as follows: apparent efficiency of reinitiation = (relative rate of synthesis of $VP3_{1754}$ − relative rate of synthesis of $VP3_{1753}$) ÷ relative rate of synthesis of $fusion_{1753}$. Taking these numbers from Table 4, we calculate an apparent efficiency of reinitiation of 25 to 50%.

Other reports in the literature also describe results consistent with either occluded scanning or reinitiation of translation after termination upstream (8, 12, 13, 22, 29). Most of these reports showed that when an open reading frame precedes the coding region of interest, a termination codon must also precede the 5′-distal open reading frame to translate the latter efficiently. The requirement for a termination codon implies that ribosomes either translate the 5′-distal open reading frame by a reinitiation mechanism or are prevented from scanning as far as the 5′-distal open reading frame by ribosome occlusion when the 5′-proximal open reading frame is long.

The apparent efficiencies of reinitiation range from 0 to 50% or more (13, 29, 34; this study; unpublished data). This variation is probably not solely an artifact of the methods used to determine reinitiation efficiencies. Other factors probably include (i) the specific sequences surrounding the downstream AUG codon; (ii) the distance between the termination and AUG codons; (iii) the length of the 5′-proximal open reading frame (Mertz et al., in preparation); (iv) the cells used in the experiment; and (v) the secondary structures of the mRNAs.

A reinitiation mechanism could be used to regulate coordinately the relative rates of synthesis of two or more proteins encoded within a single mRNA. The major late 16S mRNA species of SV40 contains the coding sequences for two proteins, LP1 and VP1, separated by 48 bases (Fig. 1). The LP1 initiation signal is theoretically strong. Therefore, few ribosomes should scan past it and initiate downstream at the VP1 AUG codon. If this is true, VP1 should be synthesized from the major late 16S mRNA species predominantly by reinitiation of translation. This hypothesis has recently been tested by using SV40 mutants that enable us to determine directly the effect of the LP1 open reading frame on the synthesis of VP1 (unpublished data). Our preliminary results indicate that the majority (approximately 65%) of VP1 synthesized from the major late 16S mRNA species appears to be made by reinitiation, with the remaining 35% being made by leaky scanning. Therefore, reinitiation may be the predominant mechanism of synthesis of some proteins.

ACKNOWLEDGMENTS

We thank Larry Kedes for the human β-actin clone, and Leslie Barber and Peter Good for the generous gifts, prior to publication, of the SV40 mutants pm918 and *dl*1755, respectively. We also thank Gregory Gelembiuk and Robert Wisecup for technical assistance; Peter Good and Marilyn Kozak for discussions; and Jeff Ross, Roland Rueckert, Bill Sugden, Howard Temin, and Michael Young for helpful comments on the manuscript.

This research was supported by Public Health Service grants CA-07175 and CA-22443 from the National Institutes of Health. S.A.S was supported by Public Health Service training grant T32 CA-09135 from the National Institutes of Health.

LITERATURE CITED

1. **Baichwal, V. R., and B. Sugden.** 1987. Posttranslational processing of an Epstein-Barr virus-encoded membrane protein expressed in cells transformed by Epstein-Barr virus. J. Virol.

61:866–875.
2. **Bandyopadhyay, P. K., and H. M. Temin.** 1984. Expression from an internal AUG codon of herpes simplex thymidine kinase gene inserted in a retrovirus vector. Mol. Cell. Biol. **4:** 743–748.
3. **Barkan, A., and J. E. Mertz.** 1984. The number of ribosomes on simian virus 40 late 16S mRNA is determined in part by the nucleotide sequence of its leader. Mol. Cell. Biol. **4:**813–816.
4. **Barkan, A., R. C. Welch, and J. E. Mertz.** 1987. Missense mutations in the VP1 gene of simian virus 40 that compensate for defects caused by deletions in the viral agnogene. J. Virol. **61:**3190–3198.
5. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13:**1101–1109.
6. **Cole, C. N., T. Landers, S. P. Goff, S. Manteuil-Brutlag, and P. Berg.** 1977. Physical and genetic characterization of deletion mutants of simian virus 40 constructed in vitro. J. Virol. **24:**277–294.
7. **Drahos, D., G. R. Galluppi, M. Caruthers, and W. Szybalski.** 1982. Synthesis of the nutL DNA segments and analysis of antitermination and termination functions in coliphage lambda. Gene **18:**343–354.
8. **Dixon, L. K., and T. Hohn.** 1984. Initiation of translation of the cauliflower mosaic virus genome from a polycistronic mRNA: evidence from deletion mutagenesis. EMBO J. **3:**2731–2736.
9. **Fromm, M., and P. Berg.** 1982. Deletion mapping of DNA regions required for SV40 early region promoter function in vivo. J. Mol. Appl. Genet. **1:**457–481.
10. **Ghosh, P. K., V. B. Reddy, J. Swinscoe, P. Lebowitz, and S. M. Weissman.** 1978. Heterogeneity and 5'-terminal structures of the late RNAs of simian virus 40. J. Mol. Biol. **126:**813–846.
11. **Good, P. J., R. C. Welch, A. Barkan, M. B. Somasekhar, and J. E. Mertz.** 1988. Both VP2 and VP3 are synthesized from each of the alternatively spliced late 19S RNA species of simian virus 40. J. Virol. **62:**944–953.
12. **Hughes, K. Mellstrom, S. E. Kosik, F. Tamanoi, and J. Brugge.** 1984. Mutation of a termination codon affects *src* initiation. Mol. Cell. Biol. **4:**1738–1746.
13. **Johansen, H., D. Schumperli, and M. Rosenberg.** 1984. Affecting gene expression by altering the length and sequence of the 5' leader. Proc. Natl. Acad. Sci. USA **81:**7698–7702.
14. **Kasamatsu, H., and A. Nehorayan.** 1979. Intracellular localization of viral polypeptides during simian virus 40 infection. J. Virol. **32:**648–660.
15. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115:**1617–1624.
16. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9:**5233–5252.
17. **Kozak, M.** 1983. Translation of insulin-related polypeptides from messenger RNAs with tandemly reiterated copies of the ribosome binding site. Cell **34:**971–978.
18. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start sites in eukaryotic mRNAs. Nucleic Acids Res. **12:**857–872.
19. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**283–292.
20. **Kozak, M.** 1986. Bifunctional messenger RNAs in eukaryotes. Cell **47:**481–483.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Liu, C-C., C. C. Simonsen, and A. D. Levinson.** 1984. Initiation of translation at internal AUG codons in mammalian cells. Nature (London) **309:**82–85.
23. **Lomedico, P. T., and S. J. McAndrew.** 1982. Eukaryotic ribosomes can recognize preproinsulin initiation codons irrespective of their position relative to the 5' end of mRNA. Nature (London) **299:**221–226.
24. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11:**1295–1308.
25. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
26. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41:**351–357.
27. **Mertz, J. E.** 1984. A detailed genetic analysis of the late complementation groups of simian virus 40. Virology **132:**173–185.
28. **Mertz, J. E., and P. Berg.** 1974. Defective simian virus 40 genomes: isolation and growth of individual clones. Virology **62:** 112–124.
29. **Peabody, D. S., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6:**2695–2703.
30. **Peabody, D. S., S. Subramani, and P. Berg.** 1986. Effect of upstream reading frames on translation efficiency in simian virus 40 recombinants. Mol. Cell. Biol. **6:**2704–2711.
31. **Ponte, P., S. Y. Ng, J. Engel, P. Gunning, and L. Kedes.** 1984. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res. **12:**1687–1696.
32. **Sisodia, S. S., D. W. Cleveland, and B. Sollner-Webb.** 1987. A combination of RNase H and S1 nuclease circumvents an artefact inherent to conventional S1 analysis of RNA splicing. Nucleic Acids Res. **15:**1995–2011.
33. **Tooze, J. (ed.).** 1981. DNA tumor viruses. The molecular biology of tumor viruses, part 2, 2nd ed., rev. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Wong, T. C., and A. Hirano.** 1987. Structure and function of bicistronic RNA encoding the phosphoprotein and matrix protein of measles virus. J. Virol. **61:**584–589.