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Expression of the Rous Sarcoma Virus *env* Gene from a Simian Virus 40 Late-Region Replacement Vector: Effects of Upstream Initiation Codons

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Expression of the Rous sarcoma virus envelope gene (*env*) from a simian virus 40 (SV40) late-region replacement vector is dependent on the position of *env* within the SV40 late-region sequences. The difference in expression levels appeared to be due to differences in the efficiency with which the *env*-specific transcripts were translated, because transcription levels from different constructions were similar. Deletion of the nucleotides encoding the agnoprotein initiator codon, located upstream of the *env* sequences in the poorly expressed construct, resulted in high levels of *env* expression. The agnoprotein initiator codon and overlapping open reading frame thus act as strong barriers to further ribosome scanning and prevent initiation at the *env* AUG codon. We conclude that AUG codons present in the late region of SV40 can reduce expression of inserted genes positioned downstream. Nevertheless, intrinsic properties of the gene may determine its ultimate level of expression.

Simian virus 40 (SV40), a small DNA virus that can lytically infect or transform cells, has been extensively used as a vector for the expression of inserted DNA in eucaryotic cells (4, 6, 8, 9). SV40 mRNAs are synthesized in two phases during a lytic infection (1). In the early phase, which lasts until the initiation of viral DNA replication, two mRNAs are observed which encode the small t and large T antigens, respectively; these are gene products that are involved in the replication of viral DNA. In the late phase of replication the late promoter is activated to produce two additional classes of mRNAs: a 16S mRNA which encodes the major structural protein VP1 and the agnoprotein (12) and a 19S mRNA which encodes the VP2 and VP3 structural proteins (13).

The presence of unique restriction endonuclease sites in the late region of SV40 that facilitate the excision of the coding sequences for the viral structural proteins and the high level of late transcripts (about 90% of mRNA synthesis) late in infection make this SV40 late promoter particularly useful for the cloning and expression of foreign genes (5, 7, 23, 24). We have recently succeeded in expressing a molecularly cloned copy of the Rous sarcoma virus (RSV) envelope gene (*env*) from this late promoter (26, 27). The glycoproteins produced in this system are synthesized, processed, and transported normally to yield surface expession of the mature viral glycoproteins gp85 and gp37. It thus provides an excellent system to determine the effects of various in vitro constructed *env* mutations on synthesis, maturation, and transport of the RSV glycoproteins (9a, 26, 27; G. L. Davis and E. Hunter, J. Cell Biol., in press; G. L. Davis, K. S. Shaw, and E. Hunter, submitted for publication). In the course of these studies we found that the level of expression of the *env* gene products was significantly lower than that reported for the influenza virus hemagglutinin (HA) (5) and was absolutely dependent on the position of the *env* gene sequences in the late region of SV40 (26). When the 5′ end of the gene was positioned at the SV40 *Kpn*I site (nucleotide 294; Fig. 1A, SV.KB), we observed good levels of expression, whereas when the 5′ end of the gene was positioned at the *Hpa*II site (nucleotide 346; Fig. 1A, SV.CB), expression was drastically reduced. Because the HA of influenza virus was expressed at high levels from the SV40 late promoter using the latter restriction site (5), we attempted to define the basis for these differences in expression of the RSV *env* gene through the construction of the recombinant *env*-SV40 vectors diagrammed in Fig. 1A. Figure 1A shows a simplified scheme of the *env*-SV40 late region replacement vectors used in this study. The SV.KB construction contains the RSV *env* gene positioned between the *Kpn*I and *Bam*HI sites of the late region of the SV40 genome (Fig. 1A). The SV.CB construction contains the RSV *env* gene positioned between the *Cla*I-modified *Hpa*II site and the *Bam*HI site. Compared with SV.KB, therefore, this construction contained an additional 49 nucleotides from the late region of SV40 and an octameric *Cla*I linker (Fig. 1B). This resulted in the insertion of two additional initiator ATG codons (from the agnoprotein gene and linker) upstream of the *env* coding region (Fig. 1B). The ΔSV.CB construction is identical to that of SV.CB, except that the initiator codon for the SV40 agnoprotein gene was deleted by removal of 4 nucleotides at the *Nco*I site at nucleotide 333 of SV40. The SV40 late-region replacement vector pQD was used for deletion of the agnoprotein initiator codon and expression of the *env* gene constructions (Fig. 1B). SV.HA/ENV corresponds to the gene fusion between the HA leader coding sequence and the RSV glycoprotein (gp85 and gp37) coding sequences, which were cloned at the *Hpa*II site of the SV40 vector (Fig. 1A) in an identical manner to the SV40 HA vector described previously by Gething and Sambrook (5). In the SV.HA/ENV construction, the agnoprotein gene ATG, and the HA gene are in a contiguous open reading frame (see below and Fig. 1B).

Differential expression of *env* from the SV.KB and SV.CB constructions in CV-I cells. When the expression of the RSV *env* gene from the SV.KB and SV.CB constructions was compared in pulse-chase experiments, significant differences could be observed in the amounts of the *env* gene product

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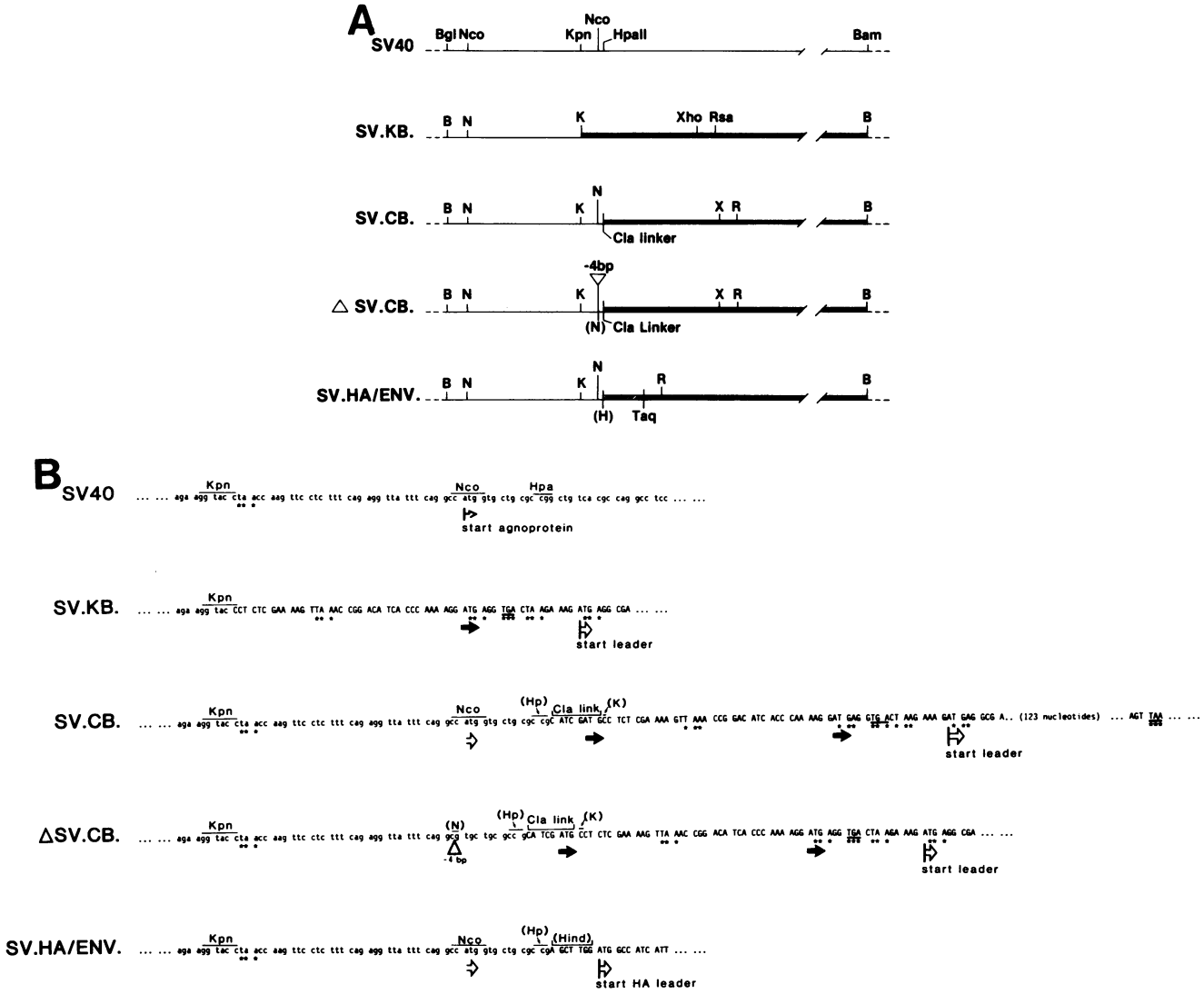

FIG. 1. (A) SV40-*env* recombinant constructions. In the SV40 late region, late transcription extends from left to right. In SV.KB, the RSV *env* gene was inserted between the *Kpn*I (nucleotide 294) and *Bam*HI (nucleotide 2533) restriction sites of the SV40 late region. In SV.CB, the RSV *env* gene was inserted between the *Hpa*II site (nucleotide 282) and the *Bam*HI site of the late region. Both the *Hpa*II site of the SV40 DNA and the *Kpn*I site of the *env* gene were modified by the addition of a *Cla*I oligonucleotide linker. For ΔSV.CB, this construction was identical to that of SV.CB described above, but it contained a 4-base-pair deletion that removed the *Nco*I site (nucleotide 333) and the agnoprotein initiator codon in the late region of SV40. For SV.HA/ENV, this construction fused the leader sequence of the HA gene of influenza A virus in frame with coding sequences of RSV *env* gene. The hybrid gene was inserted between the *Hpa*II and *Bam*HI restriction sites with a *Hin*dIII linker. (B) Analysis of the 5′ sequences of the different SV40-*env* recombinants. The nucleotide sequence for each of the constructs described in panel A is shown. Open arrows indicate the ATG initiator codon of the RSV *env* gene and the ATG codon of the SV40 agnoprotein gene. Asterisks indicate termination codons. Black arrows indicate the upstream initiation codons of reading frames that terminate before the *env* ATG (termination codons for these ATGs are underlined). The SV40 late-region replacement vector pQD contains the entire early coding region of SV40 with a deleted late coding region (in which sequences between the *Hpa*II site and the *Bam*HI site have been replaced by a *Cla*I linker) cloned into the *Bam*HI site of pXf3 (kindly provided by M. Gething and J. Sambrook, University of Texas Health Science Center, Dallas, Tex.). This plasmid was used for deletion of the agnoprotein initiator codon and expression of the *env* gene constructions. The pQD plasmid was partially digested with *Nco*I (the recognition site for this enzyme at nucleotide 333 included the agnogene ATG). The linear molecules were gel purified, made blunt-ended with Mung Bean nuclease (19), and then religated. The new late-region replacement vector (pQP) lacking the agnoprotein initiation codon was sequenced by the method of Maxam and Gilbert (20) to confirm the loss of the *Nco*I site and then used to clone the RSV *env* gene sequence. The resulting SV40-*env* vector was designated ΔSV.CB. A late-region replacement SV40 vector containing the influenza A HA gene cloned at the *Hpa*II site (5) was digested with *Kpn*I and *Sal*I to remove the coding region for the HA signal peptide and 52 nucleotides of the SV40 late region. This *Kpn*I-*Sal*I fragment was substituted for the *Kpn*I-*Xho*I fragment (which encodes the *env* signal sequence described by Hunter et al. [10]) of the SV40-*env* late-region replacement vector (SV.KB). In this way, an in-frame gene fusion that encodes the signal peptide of the influenza virus HA and RSV glycoproteins gp85 and gp37 was obtained.

produced (26) (Fig. 2). CV-1 cells that were infected for 48 h with viral stocks of the two SV40-*env* late-region replacement vectors were pulsed for 15 min with [^{3}H]leucine, and the cells were lysed immediately after the labeling period. Equivalent amounts of cell lysates were immunoprecipitated with a rabbit antibody against both RSV glycoproteins, and the immunoprecipitated proteins were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. The fluorograph of the gel (Fig. 2) shows that labeled Pr95^{env} is considerably less abundant (approximately 10 to 20% of that seen with SV.KB; Fig. 2, lane 1) in the cells infected with the SV.CB construction (Fig. 2, lane 2). This difference in expression did not reflect differences in the titers of the viral stocks because the level of infection for both vectors was comparable, as assessed by immunoprecipitation of the T antigen (data not shown), analysis of Hirt extracts of cells infected in parallel (data not shown), and RNA analyses (see below). In addition, the results obtained from these pulse-chase experiments paralleled those obtained from immunofluorescent antibody staining experiments, in which infected cells were fixed, permeabilized, and stained with rabbit antibody to gp85. Fig. 3 shows the difference in fluorescent staining between the constructions (SV.KB and SV.CB). The following experiments therefore were designed to determine the basis of the defect in SV.CB.

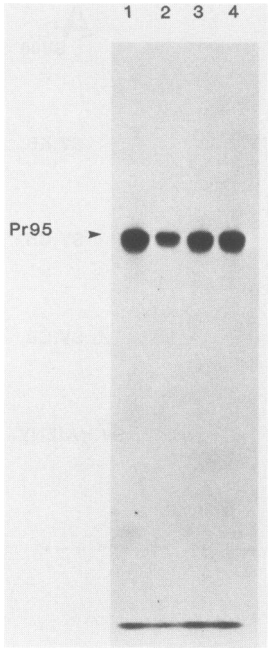

FIG. 2. Rate of synthesis of the RSV *env* gene product from the SV40-*env* constructions. At 48 h postinfection CV-1 cells were pulsed with [^{3}H]leucine for 15 min. The cells were lysed immediately after this period, and the immunoprecipitates obtained from these cell lysates were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel, as described previously (10). Lane 1, SV.KB; lane 2, SV.CB; lane 3, ΔSV.CB; lane 4, SV.HA/ENV. The major band observed in all the lanes corresponds to the primary *env* product Pr95^{env}.

Quantitation of the amount of SV40-*env* late transcripts. To examine if the differential expression of the RSV *env* gene was at the transcriptional level, cytoplasmic RNA was purified from cells infected with virus containing the different SV40 and *env* constructions. After quantitation of the cytoplasmic RNA, dilutions were spotted onto nitrocellulose filters and hybridized to a ^{32}P-labeled *env* gene probe. A comparison of the amount of specific transcripts present in the infected cells 48 h after infection with the two *env* late-region replacement vectors is shown in Fig. 4. It is clear from these results that the level of *env*-specific RNA is similar in both cases, indicating that the differential expression is not at the level of RNA transcription.

Because in other studies we have found that an excess of the helper component (or wild-type recombinant SV40 virus) can reduce the level of expression of *env* seen in these experiments (data not shown), we hybridized the same filter (after we completely removed the *env* probe) to ^{32}P-labeled helper virus (dl1055 [22]) sequences. Similar levels of transcripts were seen, indicating that this was not the cause of reduced expression from the SV.CB construct.

Deletion of the SV40 agnoprotein gene initiator ATG restores high-level expression from the SV.CB construct. An analysis of the SV40 sequences present in SV.CB and absent in SV.KB (Fig. 1B) revealed the presence of an ATG codon, in the correct context, to be a strong initiator codon (15, 16) at the beginning of an open reading frame that overlapped the start of the *env*-coding sequence. To determine whether this (agnoprotein gene) ATG codon, which is upstream from *env*, caused the low expression observed with the SV.CB construction, we deleted this initiator codon from the late-region replacement vector (Fig. 1B). The resulting SV40-*env* vector was designated ΔSV.CB. The level of expression from this modified vector was determined by immunoprecipitation of the *env* product from pulse-labeled cells and by indirect immunofluorescent staining of infected cells. The data presented in Fig. 2 (lane 3) show that the level of synthesis of *env* gene products from the ΔSV.CB construct is equivalent to that of SV.KB. These results were confirmed by using indirect immunofluorescent antibody staining. Cells infected with the construction ΔSV.CB, which carries the deletion of the agnogene initiator codon, show levels of immunofluorescent staining comparable to those of cells infected with the construction SV.KB (Fig. 3). We conclude from the results of these studies that the agnoprotein gene ATG codon constitutes a very strong barrier to most of the ribosomes, which stop scanning the mRNA at this point and are unable to initiate from the internal ATG codon at the start of the open reading frame for the RSV *env* gene.

Factors other than the initiation codon and upstream sequences determine the ultimate level of *env* gene product expression. Because the influenza HA gene is expressed at very high levels from the late region of SV40 (5), we used a hybrid HA-*env* gene to determine whether the 5′ region of the HA gene could confer a higher translation efficiency on the RSV *env* gene. This construct retains the HA initiation codon in the same context as that described by Gething and Sambrook (5) and fuses the signal peptide and 16 amino acids of HA1 to Pr95^{env} that lacks five amino-terminal amino acids (Fig. 1B) (J. W. Wills, M. J. Gething and E. Hunter, manuscript in preparation). Surprisingly, the level of expression of the hybrid polypeptide in each of the assays (Fig. 2, lane 4; Fig. 3, SV.HA/ENV) was only equivalent to but no better than that observed with SV.KB, suggesting that the translational initiation signals alone do not necessarily confer high levels of expression on a gene.

The high-level expression of foreign genes from mammalian expression vectors requires efficient utilization of transcriptional and translational signals within the vector and the gene itself. The experiments described here demonstrate that even when good levels of RNA transcription are achieved, upstream sequences derived from the vector can

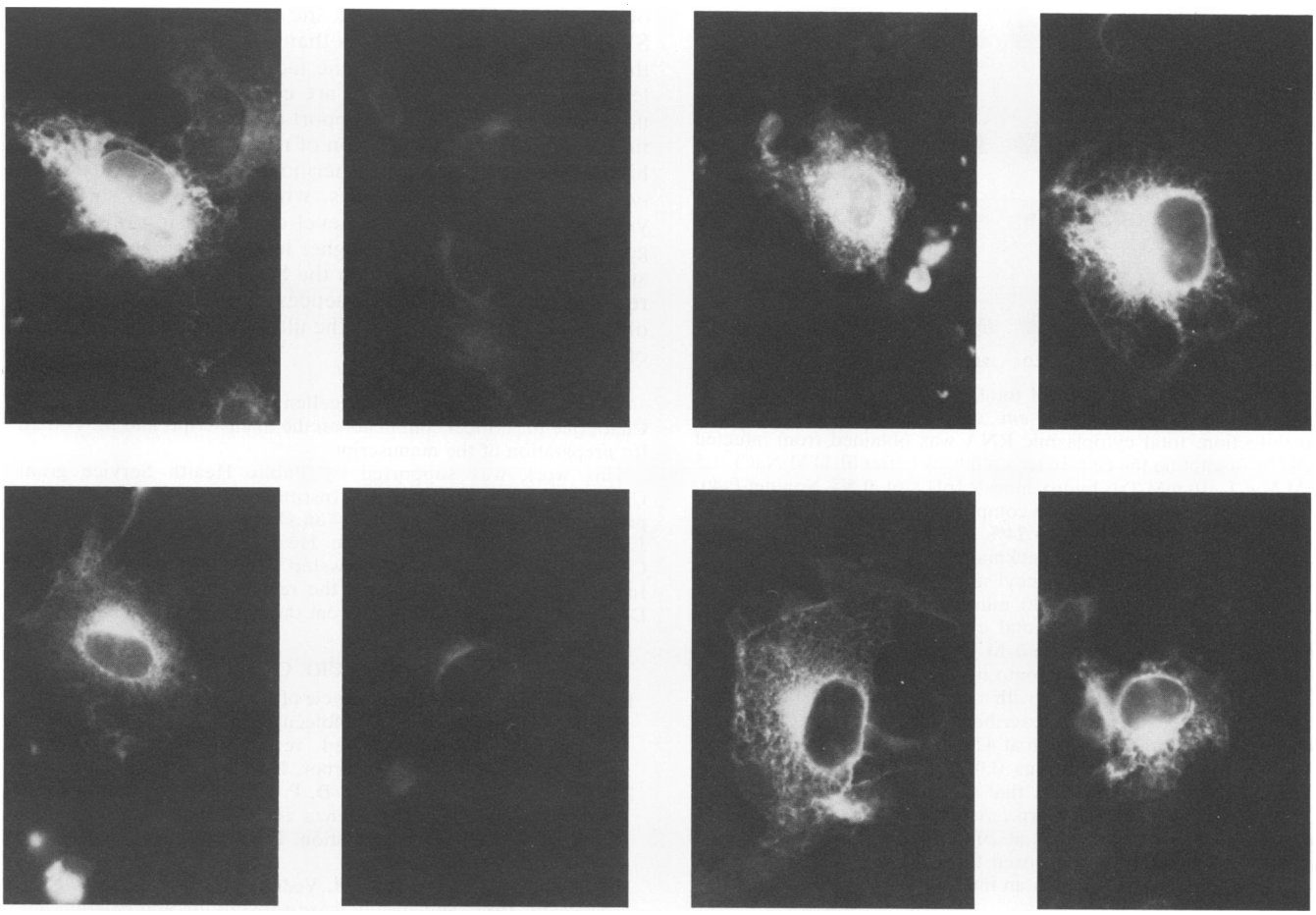

FIG. 3. Indirect immunofluorescent staining of infected cells with the SV40-*env* constructions. CV-1 cells, which were infected for 48 h, were fixed, permeabilized, and stained by the indirect immunofluorescent antibody technique with a rabbit anti-gp85 antibody and fluorescein-conjugated goat anti-rabbit immunoglobulin G as described previously (10). The panels show fluorescent antibody staining of CV-1 cells infected with SV.KB, SV.CB, ΔSV.CB, and SV.HA/ENV, from left to right, respectively.

significantly interfere with the efficient translation of the gene product.

The initiation of protein synthesis in eucaryotes involves the localization of a capped 5′ end of an mRNA by the 40S ribosomal subunit. The 40S subunit, together with a charged $tRNA^{Met}$ and initiation factors, is postulated to scan the mRNA until it encounters an AUG codon, where it can form an initiation complex (14). The 60S ribosomal subunit then binds to this initiation complex and translation begins. When the ribosome finds a termination codon, the two ribosomal subunits dissociate and the protein synthesis is completed. The original scanning hypothesis was consistent with the observation that only a single protein is translated from most eucaryotic mRNAs (11), but a number of exceptions to this rule have required that the original hypothesis be modified (15, 18). Thus, the efficiency with which translation is initiated can be shown to depend to a significant extent on the nucleotides flanking the AUG codon (18a, 21).

The SV40 16S late mRNA, a polycistronic mRNA, is one of the exceptions to the general translational features that are present in more than 90% of the eucaryotic mRNAs (12). This late mRNA is translated into both the agnoprotein, a basic DNA-binding protein of 7.9 kilodaltons, and VP1, the major structural protein of SV40. Both proteins are expressed in large amounts late in infection, indicating that the 16S mRNA is structurally and functionally polycistronic (12). The agnoprotein gene ATG codon is in a nearly optimal context (GCCATGG; Fig. 1B), and therefore, translation of VP1 from the same mRNA has been postulated to involve both occasional skipping of this initiator (leaky scanning) and reinitiation at the VP1 ATG codon after termination of the agnoprotein (18).

The defect in expression from the SV.CB construction is at the level of translation. The fact that comparable *env* mRNA levels were found in CV-1 cells infected with either the SV40 vector, carrying the RSV *env* gene positioned between the *Kpn*I and *Bam*HI sites, or the RSV *env* gene positioned 50 nucleotides downstream, between the *Hpa*II and the *Bam*HI sites, argues against a transcriptional defect as the basis for the low levels of *env* gene product. Interestingly, it also suggests that genes inserted at the *Kpn*I site can be efficiently transcribed, despite the fact that deletions at this site within the SV40 genome down-regulate SV40 late transcription (3).

An analysis of those sequences that are present in the SV.CB construction and absent in SV.KB revealed that the agnoprotein gene ATG codon present in SV.CB could initiate translation of a peptide which has coding sequences that overlap the *env* gene by 135 nucleotides (Fig. 1B). Because the agnoprotein gene ATG codon is in a nearly

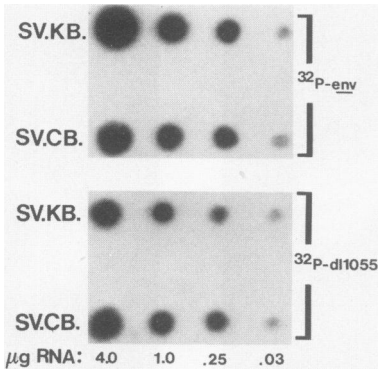

FIG. 4. Dot blot analysis of total cytoplasmic RNA from CV-1 cells infected with the SV40-*env* constructions. At 48 to 60 h postinfection, total cytoplasmic RNA was obtained from infected cells by disrupting the cells in ice-cold lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 8.6], 0.5% Nonidet P-40, 10 mM vanadyl-ribonucleoside complex). After cell lysis the nuclei were pelleted at 4°C through a 24% sucrose cushion in lysis buffer for 20 min at 7,500 rpm in a Beckman JA20 rotor. The supernatant was treated with a sodium dodecyl sulfate (0.5%)-proteinase K (200 μg/ml) mixture at 37°C for 30 min. After two extractions with phenol-chloroform (1:1), the total cytoplasmic RNA was precipitated and then denatured with 1 M glyoxal (Fisher Scientific Co., Fair Lawn, N.J.) and spotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) with a minifold apparatus (Schleicher & Schuell) by the procedure described by Thomas (25). The blotted RNAs were hybridized for 16 h at 42°C in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to *env* gene-containing plasmid DNA that was labeled with [α-^{32}P]dATP (7,000 Ci/mM; Amersham Corp., Arlington Heights, Ill.) by nick translation. After four washes at 20°C, the filters were washed at 42°C for 10 min and then exposed for 4 h to x-ray film (Eastman Kodak, Rochester, N.Y.) with an intensifying screen.

optimal nucleotide context (see above), it would be predicted to cause most ribosomes to stop scanning the SV40 late mRNA at this position and to initiate translation. Because the ribosomes do not terminate translation until 135 nucleotides after the *env* initiation codon, they would not be able to reinitiate translation at that AUG codon. The low level of expression from SV.CB (approximately 10 to 20% that of SV.KB) presumably reflects the leakiness of ribosome scanning at the agnoprotein gene ATG codon and is consistent with the expression predicted by Kozak (18). The increase in *env* expression that accompanies deletion of this initiation codon supports this interpretation of the data. Thus, the results presented here demonstrate that the agnoprotein gene initiator codon can act as a very strong barrier to ribosomes scanning the late RNA transcripts of SV40 and, thereby, can reduce the levels of expression of foreign genes inserted downstream in the SV40 late-region replacement vector. It seems likely that the increased expression of the influenza virus neuraminidase gene product, which was observed after fusion of the agnoprotein gene and neuraminidase gene coding regions (2), resulted from an abolition of this agnoprotein gene ribosome barrier.

It should be noted that just upstream of the *env* initiation codon in the SV.KB construction is an initiation codon followed by a termination codon (Fig. 1B). Thus, ribosomes stopping at the first AUG codon would have to reinitiate to translate the *env* gene product. In contrast, in the HA/ENV construction both the agnoprotein gene ATG codon and the HA initiator are in frame with the *env* gene coding sequences, and no abortive ATG codon precedes them. Thus, one might have expected that the level of expression from SV.KB would have been less than that from SV.HA/ENV; this is not the case, despite the fact that steady-state RNA levels for the two constructs are essentially the same (data not shown). These results support the concept that termination and immediate reinitiation of ribosomes can occur with high efficiency (16–18). Furthermore, despite the fusion to *env* of HA gene 5′ sequences, which have been shown to yield an exceptionally high level of expression of the HA gene in this system (5), no higher levels of *env* gene expression were observed than with the SV.KB construction; this result suggests that other sequences, presumably in the body of the *env* gene, may control the ultimate level of expression of that gene.

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