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Herpes Simplex Virus Virion Stimulatory Protein mRNA Leader Contains Sequence Elements Which Increase Both Virus-Induced Transcription and mRNA Stability

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To investigate the role of 5′ noncoding leader sequences of herpes simplex virus type 1 (HSV-1) mRNA in infected cells, the promoter for the 65,000-dalton virion stimulatory protein (VSP), a beta-gamma polypeptide, was introduced into plasmids bearing the chloramphenicol acetyltransferase (*cat*) gene together with various lengths of adjacent viral leader sequences. Plasmids containing longer lengths of leader sequence gave rise to significantly higher levels of CAT enzyme in transfected cells superinfected with HSV-1. RNase T_2 protection assays of CAT mRNA showed that transcription was initiated from an authentic viral cap site in all VSP-*CAT* constructs and that CAT mRNA levels corresponded to CAT enzyme levels. Use of *cis*-linked simian virus 40 enhancer sequences demonstrated that the effect was virus specific. Constructs containing 12 and 48 base pairs of the VSP mRNA leader gave HSV infection-induced CAT activities intermediate between those of the leaderless construct and the VSP-(+77)-CAT construct. Actinomycin D chase experiments demonstrated that the longest leader sequences increased hybrid CAT mRNA stability at least twofold in infected cells. Cotransfection experiments with a cosmid bearing four virus-specified transcription factors (ICP4, ICP0, ICP27, and VSP-65K) showed that sequences from −3 to +77, with respect to the viral mRNA cap site, also contained signals responsive to transcriptional activation.

The transcriptional control of various temporal classes of herpes simplex virus type 1 (HSV-1) gene products are reasonably well understood. Sequences within the viral promoters contribute not only to temporal class (6, 24, 29, 32, 54), but also to mRNA abundance as a result of "promoter strength" (Blair and Wagner, unpublished observations). Two immediate-early (alpha) polypeptides, ICP4 and ICP27, have been shown to regulate gene expression by a combination of positive and negative interactions (12, 13, 15, 38, 43, 47, 53). Two other *trans*-acting factors, ICP0, an alpha polypeptide with promiscuous transcription-inducing properties (18, 37, 44), and the virion-associated transcription stimulatory protein (VSP-VP16), a beta-gamma polypeptide that specifically activates alpha promoters (3, 7, 38), may also contribute to the regulation of transcription of other viral genes.

Although posttranscriptional and translational control events may contribute to a general selectivity for HSV protein synthesis, there is much less literature on the subject than there is on transcriptional control (e.g., reference 26). Expression of HSV-1 gD has been reported to be subject to posttranscriptional control (25), but more recent data suggest that any such effects are virus strain or cell specific (I. Smith and R. Sandri-Goldin, submitted for publication).

HSV infection of permissive cells results in cytopathic effects which include shutoff of host cell protein and mRNA synthesis (reviewed in references 19 and 46). Shutoff of host macromolecule synthesis appears to be mediated by viral gene products in two stages, early and delayed, since mutants defective in both functions have been isolated and regions involved in shutoff mapped on the viral genome (20, 21, 36, 45).

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Virion-associated shutoff of host protein synthesis appears to involve destabilization of host mRNA and HSV alpha mRNA (20, 34, 35, 39, 45, 48). Viral delayed shutoff also involves specific destabilization of host mRNA (36). It involves HSV beta and/or gamma gene products and may differentiate between host and viral mRNA (46). Thus, a high relative stability of viral mRNA species in the infected cell may be one mechanism by which viral gene expression is maximized.

In addition to host shutoff, two further points are relevant in considering posttranscriptional factors contributing to the predominance of HSV mRNA in infected cells. First, splicing of HSV RNA is both rare and often inefficient (10, 51, 52; Blair and Wagner, unpublished). This is consistent with virus-induced changes in the structure of infected-cell nuclei precluding normal mRNA processing (40). Second, viral mRNA species have leaders, that is, noncoding 5′ sequences, ranging in length from 90 to 700 nucleotides and averaging 200 nucleotides in length (reviewed in reference 51). If these sequences contribute to the selectivity of posttranscriptional mechanisms, they do not appear to require any simple conserved nucleotide sequence, since computer analyses of 19 HSV-1 mRNA leader sequences reveal no significant conserved or consensus sequences among them (E. Wagner, unpublished observation).

The ability to activate the transcription and expression of marker genes, such as bacterial chloramphenicol acetyltransferase (*cat*), controlled by HSV promoters allowed us to independently examine the role of a number of structural parameters on HSV-mediated gene expression. In the present communication we have assessed some of the relative contributions of transcriptional and posttranscriptional mechanisms. As reported below, we found that leader sequences stabilize viral mRNA in the infected cell; further-

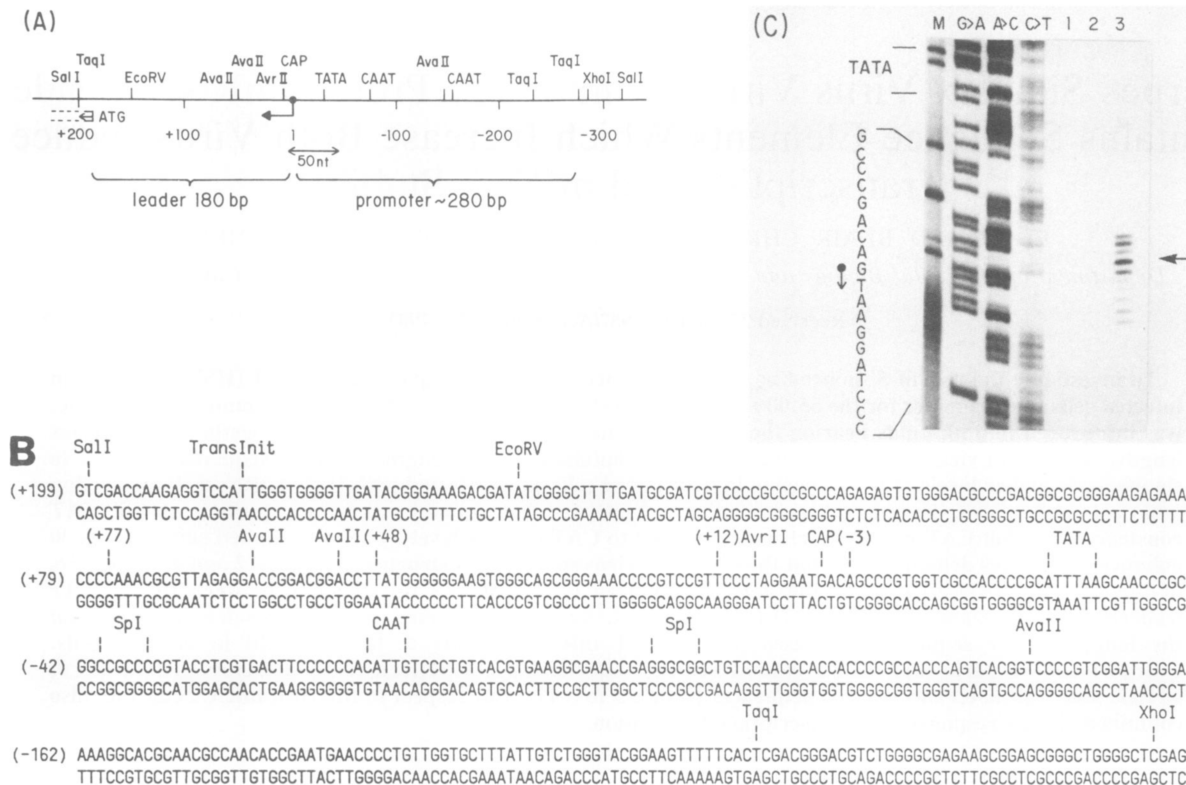

FIG. 1. (A) Relevant features within the VSP gene promoter and mRNA leader region. Restriction sites used in cloning and plasmid analysis are shown. The translation initiation site (CATG,), mRNA cap site (CAP,), TATA box homology, and CAAT box homology are also shown, and numbering of 5′ upstream (negative integers) and 3′ downstream sequences (positive integers) is relative to the mRNA cap site (+1). nt, Nucleotides. (B) Annotated sequence of the VSP mRNA promoter-leader region for HSV-1 (KOS) (K. G. Draper and E. K. Wagner, unpublished). Endpoints of plasmid deletions are shown (+77, +48, +12, and −3). The translation initiation, mRNA cap site, TATA, and CAAT box homologies and transcription factor Spl binding-site homologies are also marked. (C) Fine mapping of VSP mRNA 5′ end with probe end labeled at the *Ava*II site at +52. Lane M is pBR322 end-labeled *Hae*III fragments; lanes G>A, A>C, and C>T are chemical degradation reactions (4) performed on the probe DNA strand. Track 1 contains reaction products of probe DNA without S1 digestion, track 2 is probe DNA hybridized with 20 μg of tRNA followed by S1 digestion, and track 3 is probe DNA hybridized with 8 μg of HSV-1 mRNA followed by S1 digestion. The major protected species is indicated by the arrow, and the left-hand side shows TATA homology and sequence reading around the cap site, with the direction of transcription indicated.

more, there are elements in the leader that are responsive to HSV-induced transcriptional activation.

MATERIALS AND METHODS

Cells, virus, DNA transfections, and CAT assays. All methods have been described in detail (5, 16). Standard cultures of rabbit skin cells (10^6 cells) were transfected with 8 μg of purified plasmid DNA and superinfected or mock infected in the presence of thymidine arabinoside (araT). Transfection efficiency was monitored by Southern blot analysis of DNA from transfected cells with an M13 *cat* probe (5). CAT activity was expressed as the percentage of input chloramphenicol acetylated (to all forms) by an extract from 4×10^5 cells. Consistency between samples was confirmed by doing protein assays on a sample (10^5 cell equivalents) of each extract. The protein assays were done with a commercial protein assay (Bio-Rad Laboratories) with bovine serum albumin as the standard.

Drugs and inhibitors. Actinomycin D was used at a final concentration of 10 μg/ml. AraT and cycloheximide were used at a final concentration of 50 μg/ml.

DNA sequence data. The HSV-1 65,000-dalton (Da) virion stimulatory protein (VSP) gene was chosen for these studies because of the convenient restriction sites it contained, as well as the high activity of its promoter in *trans*-activation assays. The sequence relevant to constructions of various plasmids is shown in Fig. 1B. Sequences for the KOS strain were based on the original data of K. G. Draper and E. K. Wagner (unpublished) and confirmed by comparison of the sequence from the 17syn+ strain (11). Sequences of constructs were confirmed after cloning into *cat* plasmids by chain termination sequencing of double-stranded plasmids with primers specific for the 5′ terminus of *cat* or for the *Sal*I site of pBR322, as described previously (5). Chemical degradation sequencing of 5′-end-labeled DNA was performed by the method of Bencini et al. (4).

Recombinant DNA and plasmids. Salient features of the VSP promoter leader region are summarized in Fig. 1A. Deletions of the VSP leader sequence of lengths described in Results were generated from the *Eco*RV site of *Sal*I fragment X′ of HSV-1 (KOS) DNA (0.681 to 0.689 map units) by sequential digestion with exonuclease III and nuclease S1 as described (23), followed by the addition of *Hin*dIII linkers. This *Eco*RV site was determined to be 153 bases 3′ of the mRNA capsite located by S1 nuclease analysis (Fig. 1C). Various lengths of leader used were +77, +48, +12, and −3 bases from the cap site. The *Eco*RV site at +153 and the *Sal*I

site at +199 were also converted to *Hin*dIII sites by *Hin*dIII linker addition. At the latter site, *Hin*dIII linkers of 8 and 12 base pairs (bp) were used to generate in-phase and out-of-phase fusions of the VSP-65K reading frame and *cat* gene reading frame, based on the VSP translation initiation site described by Dalrymple et al. (11). All the VSP-65K promoter-leader constructs were introduced as *Xho*I (−272 bp relative to the cap site)-*Hin*dIII fragments into the *Sal*I and *Hin*dIII sites of two *cat* gene constructs, the pSVOd-CAT (9) or pSVOd-CAT-ENH, a derivative of pSVOd-CAT containing the simian virus 40 (SV40) enhancer sequences (5). An *Avr*II site at +7 was identified by sequencing of the +77 constructs (see Results); it was converted to an *Xho*I site to generate an insertion-spacer mutation of 10 bp. This construct was termed VSP-(+77X)-CAT. This *Avr*II site was also converted to a *Hin*dIII site by using the 8-bp linker, and the 70-bp fragment containing the VSP leader from +7 to +77 was introduced into the *Hin*dIII site of VSP-(−3)-CAT. The orientation of the insertion was determined by sequencing alkali-denatured minipreparations of plasmid DNA. These constructs were termed VSP-(−3/+77)+ve)-CAT (normal orientation) and VSP-(−3/+77−ve)-CAT (reversed orientation). The 8 bp of the *Hin*dIII linker replaced the wild-type sequences from −2 to +6, inclusive, but retained the identical spacing between the upstream and downstream sequences (GGGCTGTCATTCCTAGGG versus GGGCTCAAGCTTGTAGGG) in VSP-(−3/+77+ve)-CAT. These constructs thus introduce seven clustered point mutations around the viral cap site (see Results).

HSV plasmids containing alpha genes. The cosmid clone of HSV-1 17 syn+ *Hin*dIII fragment C (0.647 to 0.876 map units) has been described previously (9, 50). It contains the genes for ICP4, ICP0, ICP27, VSP-65K, dUTPase, and the N terminus of ICP22. Plasmids pSG28(K/B) and pRS-1 carry the individual genes for ICP4 and ICP0, respectively, and have been described elsewhere (5).

SP6 transcription templates. The *Nco*I site at −276 in the VSP promoter region in VSP-(−3)-CAT, VSP-(+77)-CAT, and VSP-(+153)-CAT-ENH constructs was converted to a *Sal*I site. Then the various-length fragments between the *Eco*RI site 250 bp into the *cat* gene and this converted *Sal*I site were cloned into the *Eco*RI and *Sal*I sites of the SP6 transcription template pSP65. The pSP65-VSP-65K-(−3)-, -(+77)-, and -(+153)-CAT recombinants were linearized by digestion at this unique *Sal*I site prior to use in in vitro transcription reactions.

CAT RNA preparation, SP6 probe synthesis, and RNase T_2 protection assays. RNA was prepared from transfected cells by extraction with guanidinium isothiocyanate and phenol as described (5) or by extraction with 0.1% Nonidet P-40 followed by proteinase K digestion and phenol extraction (30). Contaminating DNA in RNA preparations was removed by digestion with 4 U of RQ1 DNase (Promega Biotec) per 60-mm dish equivalents of RNA (less than 5 μg of DNA, less than 100 μg of RNA) in the presence of 80 U of RNasin (Promega Biotec). The SP6 RNA probe was prepared from 1 μg of *Sal*I-linearized plasmid template as recommended by the supplier (Promega Biotec). Template was removed by digestion with 1 U of RQ1 DNase. Incorporation was in the range of 6×10^6 to 13×10^6 cpm/μg of template. Approximately 5×10^5 cpm of probe was hybridized with 50 μg of total RNA for 16 h at 55°C after denaturation at 80°C in 30 μl of 80% Formamide–40 mM PIPES [piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)]–400 mM NaCl– 1 mM EDTA, pH 6.4. Hybrids were diluted with 300 μl of 50 mM sodium acetate–100 mM NaCl–2 mM EDTA, pH 5.0, and digested with 18 U of RNase T_2 (Bethesda Research Laboratories) for 2 h at 30°C. RNase-resistant material was denatured and separated on 8 M urea–5% acrylamide gels. Gels were fixed in 10% methanol–5% glacial acetic acid, dried, and exposed to X-ray film at −70°C with intensifying screens for 2 days.

S1 nuclease analyses and Southern blotting. These methods have been described previously (16, 50). The probes used for S1 analyses were 5'-end labeled at the *Sal*I site (+199) and *Ava*II site (+52) (Fig. 1). The probe used for Southern blot analyses was generated from single-stranded M13mp8 phage containing the *Hin*dIII-*Bam*HI fragment of the *cat* gene (see Fig. 2A).

RESULTS

Definition of the promoter and leader sequences for the VSP gene. Published reports have localized the cap site of the VSP transcript to the region indicated in Fig. 1A (11, 41). The precise location for the cap site of the transcript for the KOS strain of HSV-1 was determined by S1 nuclease analysis of hybrids between poly(A)-containing RNA isolated at 6 h after infection of rabbit skin cells with HSV-1 (KOS) and 5'-end-labeled DNA. The DNA was end labeled at the *Ava*II site at +52 (Fig. 1B) and strand separated. The S1-resistant DNA was fractionated against a sequence from the same *Ava*II site as a size standard, as shown in Fig. 1C. Several minor species, three intermediate species, and one major protected species were observed. The size of the most abundant nuclease-resistant fragment placed the major cap site of VSP mRNA at the G residue labeled +1 in Fig. 1B. This is 1 base 3' of the cap site identified by Dalrymple et al. (11) and is 3' of the group of four residues identified by Pellet et al. (41) as VSP mRNA cap sites. This G residue is located 24 bp downstream of a TATA box and 73 bp downstream of a CAAT box homology (Fig. 1B).

Our previous studies with other promoters and the results of the experiments of others indicate that the majority of HSV promoters are within 200 bases of the mRNA cap (reviewed in reference 51). Therefore, we operationally defined the promoter for the VSP gene to lie within the 280 bases 5' of the cap site defined by the *Xho*I site at 0.688 map units.

Higher levels of CAT expressed from plasmids carrying longer lengths of VSP mRNA leader sequences. The generation of recombinant *cat* plasmids designed to carry various lengths of viral leader sequences was described in Materials and Methods. We analyzed five of these constructs for the ability to express CAT activity following HSV-1 superinfection (Fig. 2A), including a minimal leader construct, VSP-(−3)-CAT, two with increasing lengths of VSP mRNA leader, VSP-(+77)-CAT and VSP-(+153)-CAT, and two which incorporate the VSP initiation codon at +182 and the first 18 bases of the VSP open reading frame incorporated into the open reading frame of the CAT protein either in phase [VSP-(+199i/p)-CAT] or out of phase [VSP-(199o/p-CAT]. Each of these plasmids generated high levels of CAT activity in transfected cell extracts only after superinfection with HSV-1 (Fig. 2B). The minimal leader construct, VSP-(−3)-CAT, mediated the synthesis of almost twice the level of CAT enzyme seen with the well-characterized promoter for alkaline exonuclease included as a control (Fig. 2B, tracks i and iii) (5, 9, 16). In addition, plasmids with longer leader sequences expressed considerably higher levels of CAT activity than the leaderless construct. Thus, VSP-(+77)-CAT induced four times and VSP-(+153)-CAT ex-

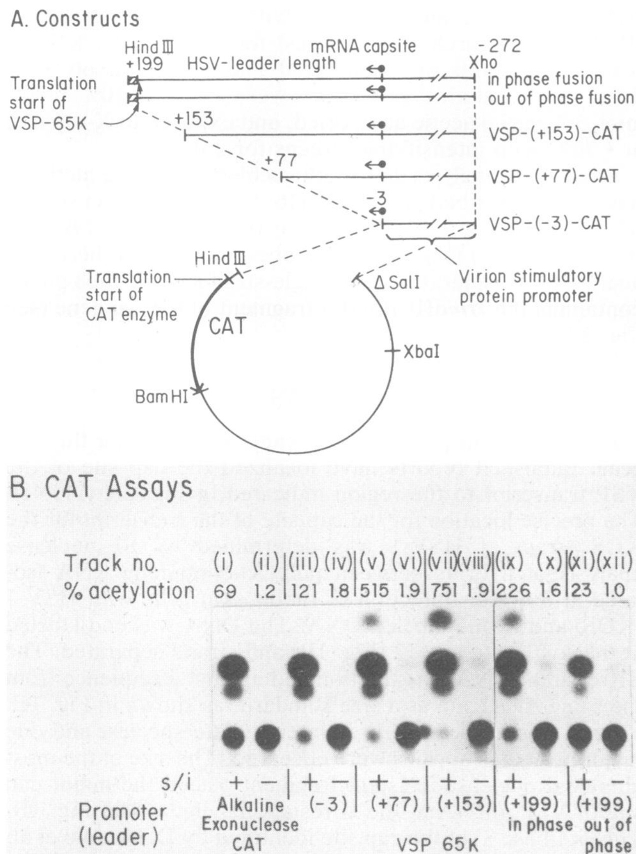

FIG. 2. (A) Schematic summary of *cat* plasmids carrying various leader lengths, including in-phase and out-of-phase CAT-VSP protein fusions. (B) CAT enzyme assays performed on 20 μg of protein extract (3×10^5 cell equivalents) from cells transfected with alkaline exonuclease-CAT (9, 16) (tracks i and ii), VSP-(−3)-CAT (tracks iii and iv), VSP-(+77)-CAT (tracks v and vi), VSP-(+153)-CAT (tracks vii and viii), VSP-(+199i/p)-CAT (tracks ix and x), and VSP-(+199o/p)-CAT (tracks xi and xii). For assays in which conversion of chloramphenicol to its monoacetylated forms exceeded 60%, appropriate dilutions of extract (1:10 to 1:50) were used to obtain an accurate measure of CAT enzyme activity during the 90-min assay period. s/i, Superinfection.

pressed six times the level of enzyme expressed from VSP-(−3)-CAT (Fig. 2B, tracks iii, v, and vii). Less CAT activity was induced with VSP-(+199i/p)-CAT (track ix); this may be due to some translational effects due to initiation of protein synthesis at the VSP initiation codon or it may be due to N-terminal amino acids from the VSP affecting CAT activity or stability. These possibilities have not been addressed in this study. However, VSP-(+199i/p)-CAT induced 10-fold more CAT activity than did VSP-(+199o/p)-CAT (Fig. 2B, tracks ix and xi).

VSP mRNA leader is incorporated into hybrid *cat* transcripts. The structure of the CAT mRNA transcribed from VSP-(−3)-CAT, VSP-(+77)-CAT, and VSP-(+153)-CAT plasmids was analyzed by in vitro-generated SP6 antisense RNA probes (Fig. 3A). We isolated total RNA from cells transfected with VSP-(−3)-CAT and superinfected with HSV-1 or left uninfected. In two separate experiments, a 250-base fragment of the VSP-(−3)-CAT-SP6 probe was protected from RNase T_2 digestion by RNA extracted from infected cells, but not by RNA from uninfected cells (Fig. 3B, tracks iii, iv, v, and vi). The length of the protected fragment, 250 bases, indicated that transcription of the CAT mRNA initiated at or very near the authentic viral cap site. Similarly, RNA from experiments with VSP-(+77)-CAT, the VSP-(+77)-CAT-SP6 probe, yielded a fragment ca. 330 bases in size that was protected from RNase T_2 digestion only by RNA from infected cells (Fig. 3B, tracks ix, x, xi, and xii). Thus, the VSP-(+77)-CAT mRNA also initiated at the authentic cap site, and the 77 bases of viral leader were incorporated into the CAT transcript. In experiments not shown, we found that superinfection of cells transfected with VSP-(+153)-CAT yielded RNA which protected a ca. 400-base-long fragment when hybridized with the VSP-(+153)-CAT-SP6 probe and digested with RNase T_2.

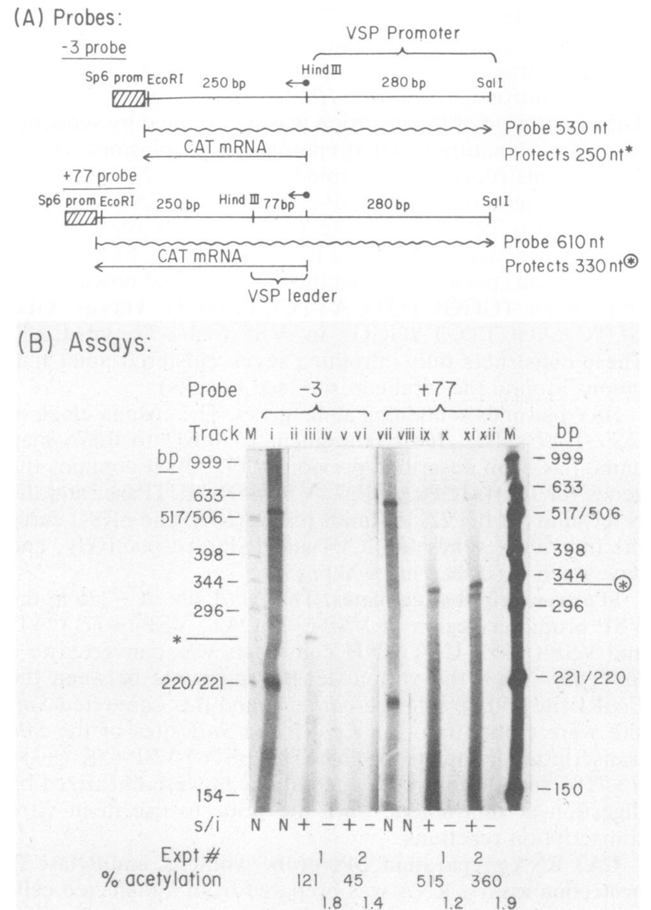

FIG. 3. RNase T_2 analyses of CAT mRNA. (A) Description of SP6 templates and probes for mRNA expressed from VSP-(−3)-CAT and VSP-(+77)-CAT plasmids. The SP6 polymerase-specific promoter (prom) is indicated by the hatched box, and restriction sites for cloning and template linearization are also shown. Predicted sizes of probe RNA and probe protected by CAT mRNA are indicated. nt, Nucleotides. (B) Autoradiogram of polyacrylamide gel separating RNase T_2 reaction products with tracks i to vi for VSP-(−3)-CAT mRNA and viii to xii for VSP-(+77)-CAT mRNA. Tracks M, end-labeled DNA marker fragments from pBR322 digested with *Hin*fI and *Eco*RI with sizes given. Tracks i and vii, SP6 probes prior to RNase T_2 digestion; tracks ii and viii, probes plus tRNA plus RNase T_2; tracks iii to vi and ix to xii, probes plus mRNA from infected (+) and uninfected (−) transfected cells plus RNase T_2. The percentage of chloramphenicol acetylation from the corresponding transfection experiment (1 or 2) is also shown. Track M (left) and tracks i and vii were exposed for 6 h, and the remaining tracks were exposed for 48 h. s/i, Super infected; N, not done; *, proper band sizes.

Judged by the band intensity of probe protected, the amount of infected-cell CAT RNA was greater in the VSP-(+77)-CAT transfection than in that with VSP-(−3)-CAT. This indicated that the levels of CAT activity accurately measured the amount of RNA expressed from these constructs (Fig. 3B, tracks iii and v versus ix and xi). Precise quantitation of VSP-(−3)-, VSP-(+77)-, and VSP-(+153)-CAT mRNA was achieved by using a common SP6 antisense probe and a common M13mp19 *cat* antisense S1 probe. An example of data obtained with the common SP6 probe is summarized in Fig. 4B. All experiments showed that CAT mRNA levels driven by the different VSP-CAT constructs covaried with the CAT activity driven by each construct. Thus, differences in the level of CAT activity induced in infected cells was not due to more efficient translation of CAT mRNA mediated by sequences in the viral leader.

Leader effect specific to infected cells. The basal (uninfected) level of CAT enzyme induced by several different HSV promoters can be increased by the addition of SV40 enhancer sequences to the constructs (5). This allowed us to determine that the leader effect described in Fig. 2 was, in fact, virus specific. The enhancer sequences were first linked adjacent to the VSP −3 promoter, and it was found that this promoter was effectively *cis*-activated by the SV40 sequences. This is in contrast to another beta-gamma promoter, the promoter for the HSV-1 major capsid protein (VP5) gene, which cannot be *cis*-enhanced by the same SV40 sequences without removal of sequences between 75 and 125 bases upstream of that transcript's cap site (5).

The SV40 enhancer was also ligated to the VSP-(+77)-CAT and VSP-(+153)-CAT constructs; all three constructs were tested in parallel with the corresponding enhancerless constructs for the ability to induce CAT enzyme synthesis in infected and uninfected cells. There was little difference in the *cis*-response of each promoter/leader length; i.e., all constructs were enhanced to give essentially equivalent levels of CAT activity in uninfected cells (Fig. 4A). In the same experiment, the corresponding enhancerless constructs responded to superinfection (*trans*-activation) to give levels of CAT activity equivalent to those seen in Fig. 2. However, the *cis*-linked SV40 sequences appeared to affect the *trans*-response of the enhancer-containing VSP-(+77)-CAT and VSP-(+153)-CAT constructs, since the levels of CAT synthesized in cells transfected with these plasmids and then superinfected were considerably lower than in cells transfected with the enhancerless constructs (compare tracks vii and ix with xi and xiii in Fig. 4A). We have not investigated this effect further. The differences in the *cis*- and *trans*-effects of the VSP leader sequences determined with several experiments are summarized in Fig. 4B. The *trans*-response of plasmids with longer leaders was substantially greater than that of the leaderless plasmids, i.e., the VSP-(−3)-CAT construct, whereas all three promoter-leader recombinants responded equivalently to *cis*-activation by the SV40 enhancers. These differences in *trans*-activation, as measured by CAT enzyme levels, paralleled the differences measured by CAT mRNA levels (Fig. 4B). Thus, the leader effects are limited to infected cells and appear to occur at a pretranslational level. From these data we can define an HSV activation index as the ratio of virus-induced CAT activity in infected cells to the basal level in uninfected cells.

VSP leader increases the stability of CAT mRNA. Two effects, separately or together, had the potential to modulate the increase in the HSV activation index with longer leaders shown in the data of Fig. 4. First, the longer leader lengths

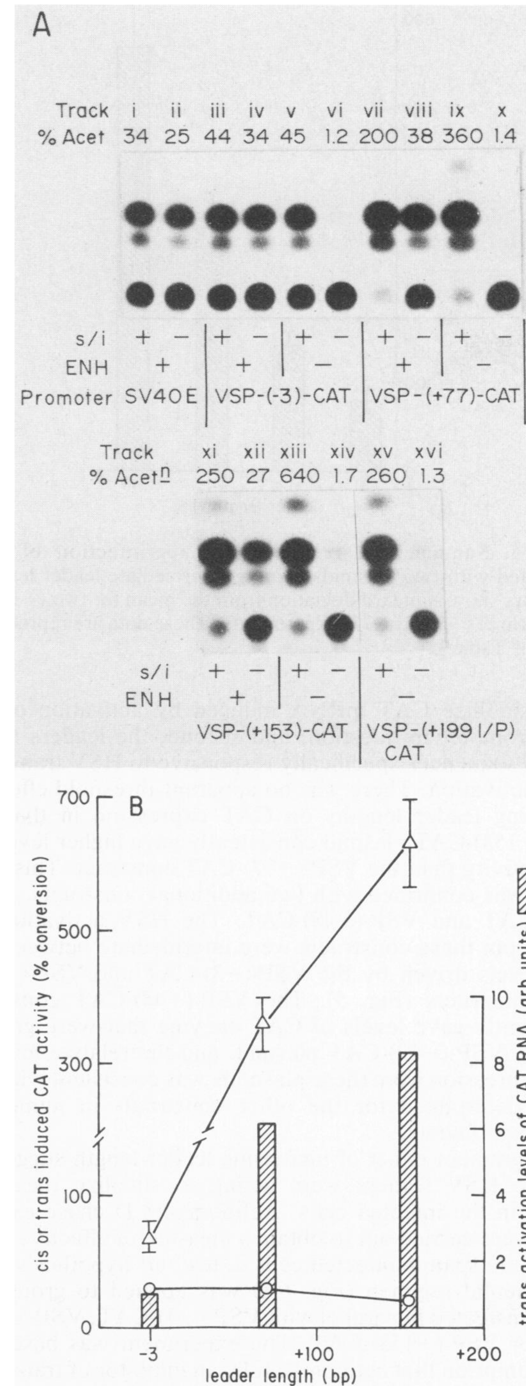

FIG. 4. (A) Level of CAT enzyme driven from various leader constructs in *cis* by SV40 enhancer sequences (as indicated by + or − adjacent to ENH) and/or in *trans* by virus superinfection (s/i) + and − symbols adjacent to s/i). The various CAT constructs are indicated below the enzyme assays; SV40E-CAT was described previously (5). (B) Summary of *cis*- (○) and *trans*-activation (△) data (the latter derived from four independent experiments, with standard deviation from mean value shown by bars). Also shown is CAT mRNA quantitation from densitometer tracings of an autoradiogram of a common SP6 probe RNA protected from RNase T_2 digestion by mRNA from infected cells previously transfected with VSP-(−3)-CAT, VSP-(+77)-CAT, and VSP-(+153)-CAT as indicated along the *x*-axis. Note break in ordinate axis.

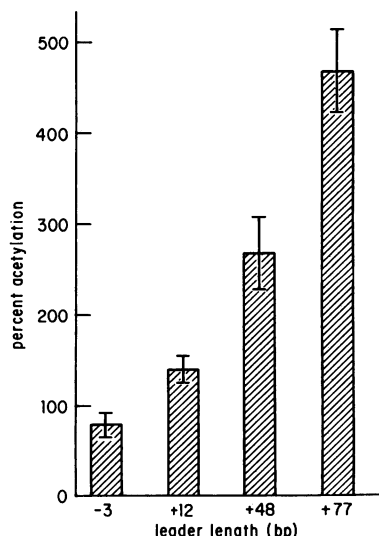

FIG. 5. Summary of response to superinfection of cells transfected with two plasmids bearing intermediate leader lengths. Error bars show standard deviation from the mean for two (+12 and +48) or three (−3 and +77) experiments. These data are reproduced in part of Table 1.

might stabilize CAT mRNA induced by activation of the HSV promoter in question, and second, the leaders might contain sequences specifically responsive to HSV transcriptional activation. There was no apparent threshold effect of increasing leader lengths on CAT expression in that the VSP-(+153)-CAT plasmid consistently gave higher levels of CAT activity than the VSP-(+77)-CAT construct. This continuity was confirmed with two additional constructs, VSP-(+12)-CAT and VSP-(+48)-CAT. The HSV activation indexes from these constructs were intermediate between the CAT levels driven by the VSP-(−3)-CAT and VSP-(+77)-CAT constructs (Fig. 5). The VSP-(+48)-CAT construct consistently gave levels of CAT enzyme that were greater than the VSP-(+12)-CAT plasmid, and the relative level of CAT expression from these plasmids was consistent with the values determined for the other constructs in numerous other experiments.

This gradient effect of increasing leader length suggested that the HSV leaders were acting to stabilize the CAT mRNA in the infected cells. Actinomycin D chase experiments were carried out to obtain a measure of effective CAT mRNA life span in infected cells to test our hypothesis. The experimental regimen (Fig. 6A) was applied to groups of cells transfected in parallel with VSP-(−3)-CAT, VSP-(+77)-CAT, or VSP-(+153)-CAT. The experiment was based on the assumption that actinomycin D, an inhibitor of transcription, added at 10 h postinfection would not affect the translation of preexisting mRNA. However, mRNA species of greater stability would be available for translation for longer periods of time than less stable mRNA, giving rise to higher levels of CAT enzyme at a subsequent time of assay. To determine the level of enzyme present at 10 h postinfection (the time of addition of actinomycin D), cycloheximide, an inhibitor of protein synthesis, was added in parallel to a third group of infected cells. Extracts were prepared from cells transfected with each VSP construct at 20 h postinfection, and the levels of CAT enzyme were assayed (Fig. 6B). In cultures transfected with the VSP-(−3)-CAT, VSP-(+77)-CAT, or VSP-(+153)-CAT constructs, 24, 42, and 38%, respectively, of the maximum CAT activity for each construct was recovered from cells treated with actinomycin D (tracks i and iii, v and vii, and ix and xi). One-third or less of this activity was recovered from the corresponding cycloheximide-treated cultures (7 to 9% of control values, tracks iv, viii, and xii). Thus, CAT activity was increased at least twice as much with the longer leaders as with the leaderless construct after the addition of actinomycin D (33 and 31% of control versus 16% of control). These data suggest that mRNA stabilization does play a role in the increased HSV activation index of the constructs with the longer leaders. We suggest that the twofold value indicated by the actinomycin chase experiment is a minimal one due to interruption of the viral infection cycle by the addition of the drug at 10 h postinfection.

VSP leader contains sequence elements responsive to transcriptional activation. In infected cells, the VSP-(+77)-CAT construct typically gave levels of CAT enzyme activity four times greater than the VSP-(−3)-CAT plasmid, whereas the stability factor for this longer leader length did not exceed twofold in three different experiments. This result suggested to us that sequences responsive to transcriptional activation might lie within the leader sequences. To test this hypothesis, cells were transfected with either VSP-(−3)-CAT or

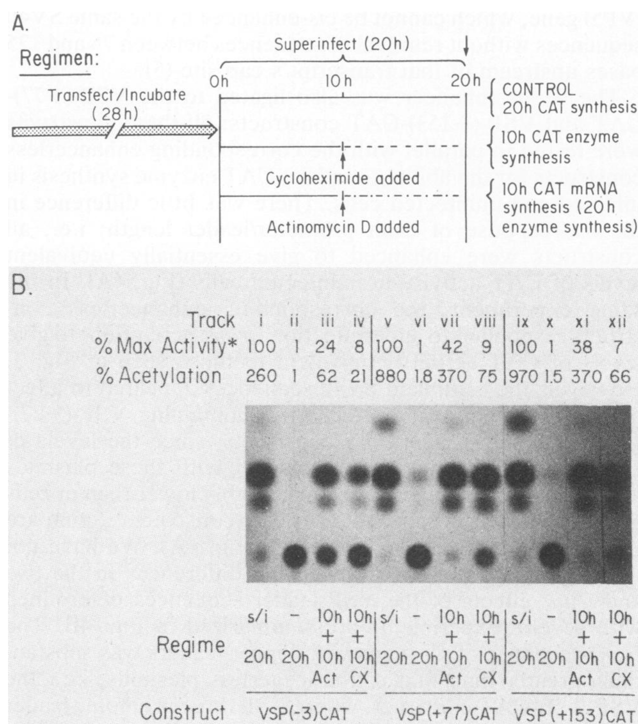

FIG. 6. Analysis of CAT mRNA stability. (A) Experimental regimen showing period of transfection (48 h total), superinfection (20 h), and time of inhibitor addition. (B) CAT enzyme assays performed on samples (20 μg; 3× 10^5 cell equivalents) of protein extracted from cells harvested at 20 h after infection or mock infection and 48 h posttransfection with the CAT constructs indicated. Regimens with actinomycin D (Act) or cycloheximide (CX) or no inhibitor are indicated. CAT enzyme activity as a percentage of chloramphenicol acetylation was accurately measured by dilution of extract (see legend to Fig. 1) and converted to a percentage of the level of acetylation measured at 20 h postinfection with no inhibitor treatment (*). For example, the 24% value in track iii is derived from the value of CAT activity of track iii (62% acetylation) divided by the CAT activity of lane i (260% acetylation). s/i, Superinfection.

TABLE 1. *trans*-Activation response of various VSP leader lengths to HSV-1 transcriptional activation

Construct	Viral leader		CAT activity induced[a] (% acetylation)				
	Length (bp)	Orientation	Expt no.	*Hin*dIII-C[b]		HSV-1[c]	
VSP-(−3)		Normal	1	89	60	70	75
			2	41		65	
			3	49		91	
VSP-(+12)	12	Normal	2	45	53	156	141
			3	61		126	
VSP-(+48)	48	Normal	2	94	92	266	217
			3	90		169	
VSP-(+77)	77	Normal	1	114	92	445	430
			2	68		466	
			3	94		386	
VSP-(−3/77+ve)	77	Normal, modified cap site	1	118	84	480	380
			2	65		280	
			3	71			
VSP-(−3/77−ve)	77	Reversed, modified cap site	2	11	16	70	78
			3	21		85	
VSP-(+77X)	87	Normal, spacing altered	2	45	45	319	313
			3	46		307	

[a] Percent acetylation of chloramphenicol in three experiments; average value given.
[b] Harvested 48 h after cotransfection with 5 μg of HSV-1 *Hin*dIII fragment C (controls received 5 μg of pUC19).
[c] Superinfected; harvested 48 h posttransfection (20 h postinfection with 1 to 2 PFU of HSV-1 per cell).

VSP-(+77)-CAT and plasmids containing either the ICP4 and ICP0 genes singly or together. The results (not shown) clearly indicated that the 77 bases of leader incorporated into the VSP-(+77)-CAT construct were activated to a higher level, as measured by induced CAT activity, than was the leaderless construct.

To carefully quantitate the effect and to ensure that molar equivalent amounts of activating genes were introduced into the cotransfected cells, a cosmid clone of HSV-1 *Hin*dIII fragment C (0.647 to 0.876 map units) was used for further cotransfectional activation studies. As described previously (9, 50), this cosmid carries functional copies of the genes for ICP4, ICP0, and ICP27, three alpha polypeptides with known transcriptional regulatory activity. Levels of CAT activity induced from the marker plasmids by *Hin*dIII-C cotransfection were compared with activity induced by superinfection (Table 1). *Hin*dIII fragment C consistently induced a higher activation index for the VSP-(+77)-CAT construct than for the VSP-(−3)-CAT construct by an average of 1.5-fold. The VSP-(+12)-CAT construct was not responsive to transcriptional activation by the *Hin*dIII fragment C clone. This suggested that the sequences responsive to activation required some element between 11 and 77 bases in the leader.

To ensure that differences in the values of the activation index for the different constructs were not due to changes in upstream sequences arising during cloning of the promoter sequences in VSP-(−3)-CAT, the VSP leader sequence from +7 to +77 was introduced into the VSP-(−3)-CAT construct. This construct, VSP-(−3/+77+ve)-CAT, maintained the spacing of upstream and downstream sequences (see Materials and Methods and Fig. 1B). Constructs were also tested in which (i) the leader sequence was introduced in reverse orientation to the upstream region [VSP-(−3/+77−ve)-CAT] and (ii) the spacing of the upstream sequences (−280 to +7) and the downstream sequences (+7 to +77) was increased by 10 bp by insertion of an *Xho*I linker [VSP-(+77XhoI)-CAT]. The results of these transfection and cotransfection experiments are also summarized in Table 1.

Insertion of the +7 to +77 region increased the transcriptional response of the minimal VSP promotor by the same factor of 1.5 seen in the unmodified VSP-(+77)-CAT construct. This confirmed our conclusion that the +7 to +77 region of the VSP leader appears to carry all the signals required to increase the responsiveness of the VSP −3 promoter. Addition of the +7 to +77 leader sequences in the reversed orientation strongly reduced the level of CAT enzyme expressed after transcriptional activation by cotransfection with *Hin*dIII fragment C. In other experiments (Table 1), it was found that after herpesvirus superinfection, cells carrying this construct were able to produce as much CAT activity as those transfected with the VSP-(−3)-CAT construct. This result suggested that the stabilization of CAT transcripts by the extra leader lengths is not sequence dependent in any simple way.

Finally, spacing of upstream and downstream sequences also appeared to be critical for the transcriptional response since the plasmid VSP-(+77X)-CAT, in which an *Xho*I linker added 10 bases to the leader, was significantly less responsive to cotransfected *Hin*dIII-C than either VSP-(+77)-CAT or VSP-(−3/+77+ve)-CAT (Table 1). Here, too, herpesvirus superinfection gave a significant increase in CAT activity compared with the VSP-(−3)-CAT construct, suggesting that mRNA stabilization effects of the 77 bases of HSV leader were unaltered by the 10-base insertion.

DISCUSSION

This communication describes two effects on viral gene expression that are mediated wholly or in part by sequences contained within the nontranslated 5′ leader of the VSP-65K gene. The stabilization of viral mRNA in the infected cell as measured by the actinomycin D chase experiments (Fig. 6) and the transcriptional activation data (Table 1) suggest that the longer leader lengths used in the VSP promoter-driven *cat* constructs should have threefold greater activity than the leaderless construct utilizing the same promoter. This value is in good agreement with the total herpesvirus activation index of 4 to 6 seen in Fig. 4 and 5.

The first effect of the VSP leader sequences operates to stabilize CAT mRNA in the infected cell. The CAT induction system is ideal for an analysis of this type of leader effect, since even small effects on mRNA stability are

evident against the background instability of the basic CAT transcript (28). The presence of 77 and 153 bases of VSP leader increased the apparent stability of CAT mRNA by a minimum of twofold, specifically in infected cells, since there was no detectable difference in the level of CAT enzyme *cis*-enhanced from each leader construct in uninfected cells.

Transient expression assays have also been used to show that the +62 to +142 region of a human cytomegalovirus (HCMV) beta mRNA leader exerts a *cis*-dominant posttranscriptional effect on β-galactosidase gene expression (22), although the level at which this regulation operated was not determined. In addition, from this report it appears that the HCMV leader regulates the temporal expression of the β-galactosidase enzyme, but we do not know if the same is true for the VSP leader. Leader sequences in the polyomavirus late mRNA leader contribute to transcript stability, and defects in the leader give rise to nonviable viruses (1; G. R. Adami, C. W. Marlor, and G. G. Carmichael, Cold Spring Harbor Symp. Tumor Viruses 1986, p. 197). The adenovirus 72K DNA binding protein apparently affects mRNA stability (2). Also, the leader from the first intron on the murine c-*myc* gene affects mRNA stability in response to certain inducers (17, 42). Therefore, differential stability conferred on viral mRNAs by their leader sequences may be an important determinant of the predominance of virus gene expression and the progression of the productive replication cycle to completion in infected cells.

In this paper we found a second effect of nearly equivalent magnitude at the level of transcription; it was found that sequences between +7 to +77 modulate the response of the VSP gene to HSV alpha gene transcriptional activation. Although this effect was modest (1.5-fold activation over leaderless constructs), it was readily detectable against a background of transcriptional activation mediated by upstream (−3 to −280) promoter sequences. Based on previous detailed analyses of the promoter functions of HSV alpha (6, 29, 54), beta (8, 18, 32), and beta-gamma (9, 14) genes, we would predict that the predominant transcription regulatory elements for the VSP gene lie 5′ of the mRNA cap site. Thus, the leader control element may be only a small component of the whole transcription regulatory complex. The topography of this element vis-a-vis upstream elements clearly is important given the sensitivity of the transcriptional response to spacing and orientation of the leader sequences. The importance of the proper spacing between elements in viral promoters has probably been best characterized in the SV40 system (27, 49). However, Muesing et al. (33) have shown that spacing and orientation of the human immunodeficiency virus (HIV) leader are important for *tat*-III mediated *trans*-activation in terms of mRNA structure and stability rather than transcriptional activation. We found by computer analysis some weak potential stem-loop structures within the first 50 bases of the VSP leader, but poor evidence for inverted or direct repeats and no homology with the HIV leader inverted repeat.

Since the sequences between +11 and +77 in the leader seem to be required for activation to be evidenced, the leader element(s) important in transcriptional activation has downstream limits different from those described by Homa et al. (24) for the HSV gamma gC promoter, which lies within 14 bases of the cap site. The limits of the VSP transcriptional control region appear to be somewhat similar to those mapping between +33 and +190 in the adenovirus late transcription unit tripartite leader (31).

Although McKnight and co-workers have reported that the thymidine kinase (*tk*) promoter sequences required for viral transcriptional activation are within the classic promoter region (i.e., within 200 bases 5′ of the cap site [8, 32]), a linker-scanning mutation affecting sequences from +5 to +15 substantially reduced *tk* mRNA expression from a recombinant virus template (8). Thus, sequences downstream of the cap site may also regulate the expression of TK in response to immediate-early gene products. Transcriptionally responsive leader elements may occur in a number of other HSV genes since we find that similar leader effects operate on the expression of CAT enzyme from the VP5 promoter-leader in response to superinfection (Blair and Wagner, unpublished data). The type of transient expression assays developed for the study of HSV promoters should be useful in assessing the limits of such promoter elements within the viral leader as well as in determining their relative importance in the full and temporally regulated expression of the genes in question. In addition, these assays offer a relatively rapid means of identifying targets in HSV DNA sequences which will be fruitful for modification and reinsertion into the viral genome to assess template effects on observed transcriptional control signals.

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