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Virus-Induced Modification of the Host Cell Is Required for Expression of the Bacterial Chloramphenicol Acetyltransferase Gene Controlled by a Late Herpes Simplex Virus Promoter (VP5)

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The requirements for expression of genes under the control of early (alkaline exonuclease) and late (VP5) herpes simplex virus type 1 (HSV-1) gene promoters were examined in a transient expression assay, using the bacterial chloramphenicol acetyltransferase gene as an expression marker. Both promoters were induced, resulting in the production of high levels of the enzyme upon low-multiplicity infection by HSV-1. S1 nuclease analysis of hybrids between RNA isolated from infected cells containing HSV-1 promoter constructs and marker gene DNA demonstrated normal transcriptional initiation of the marker gene directed by the viral promoters. Viral DNA sequences no more than 125 bases 5′ of the putative transcriptional cap site were sufficient for maximum activity of the late promoter. In contrast to expression controlled by the early gene, the late promoter was not active at a measurable level in uninfected cells until DNA sequences between 75 and 125 bases 5′ of the transcriptional cap site were deleted. Cotransfection of cells with the expression marker controlled by HSV promoters and a cosmid containing HSV α (immediate-early) genes indicated that full expression of both early and late promoters requires the same virus-induced host cell modifications. Inhibition of viral DNA synthesis results in an increased rate of transient expression of marker genes under control of either early or late promoters in contrast to the situation in normal virus infection. These data provide evidence that the normal course of expression of late HSV genes involves negative modulation of potentially active promoters in the infected cell.

Herpes simplex virus type 1 (HSV-1) transcription is temporally regulated in a cascade fashion with controls at both the transcriptional (for review, see reference 60) and the translational levels (36). The first group of transcripts expressed following infection are the immediate-early or α genes (1, 8, 9, 37, 63). Since these transcripts are abundantly expressed in the absence of viral protein synthesis, it is clear that their transcription requires no viral gene expression. Proteins encoded by these genes appear to be regulatory in function (32, 35). One α gene product (ICP4) is absolutely required throughout infection to allow further expression of genes from the viral genome (39, 51, 62). Normal levels of α gene transcription are controlled in part by the presence of regulatory elements in or upstream of the actual promoters. The interaction between components of these regulatory elements and a virion-associated protein(s) results in marked stimulation of transcriptional activity in several marker systems (11, 40, 41, 43, 50, 51).

The early or β genes are expressed after induction of immediate-early proteins in the infected cell. Early transcripts are present at relatively high levels in the absence of viral DNA replication (9, 31). When these promoters are removed from the environment of the viral genome, they can be expressed in an uninfected cell in vitro transcription extract (23, 54). The DNA sequences necessary for expression of thymidine kinase (TK), an early gene, have been studied as a model eucaryotic promoter in uninfected cells (46, 47). The TK promoter and most other early HSV-1 promoters appear to be similar to other eucaryotic RNA polymerase II promoters (14, 19, 60, 61); A. Elkareh, A. J. M. Murphy, T. Fichter, A. Efstratiadis, and S. Silverstein, Proc. Natl. Acad. Sci. USA, in press, and no enhancer or other regulatory elements required for their function during infection have been identified (58; Elkareh et al., in press). However, β promoters can be stimulated by exogenous enhancers. For example, when simian virus 40 enhancer is placed upstream from an early promoter, higher transcriptional activity results (20).

Several research groups have studied the requirements for stimulating expression of early viral genes by HSV superinfection in cell lines containing early genes (55, 58). The DNA sequences required for this stimulation were no greater than 200 bases upstream from the mRNA cap site (58).

Everett (19) has performed several elegant studies with the early gD promoter in a transient transcription assay system and determined that only 83 base pairs 5′ to the transcription cap site are required for induction of expression after viral superinfection. In a similar transient assay system, the TK early promoter was shown to require 109 bases upstream from the cap site for HSV-1-induced transcription (Elkareh et al., in press). The transcriptional stimulation induced by HSV infection is not specific to HSV promoter sequences. It has been demonstrated that herpes virus immediate-early products can induce or increase expression from a number of heterologous eucaryotic promoters. These include the adenovirus E2 promoter (34), the β-globin and simian virus 40 early promoter lacking enhancer sequences (21, 28), and the Moloney murine leukemia virus long terminal repeat promoter lacking its upstream enhancer (R. H. Costa, E. K. Wagner, G. Feuer, and H. Fan, unpublished data).

Late HSV-1 genes can be subdivided into two classes. The βγ or leaky late gene transcripts are detectable prior to viral

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DNA replication. However, they do not reach a normal level of expression until the initiation of viral DNA replication. Qualitatively, the differentiation between a βγ and a β gene is based on the ease of detection of the transcript prior to or in the absence of viral DNA replication (60, 61).

The γ or full late genes require viral DNA replication to observe detectable levels of transcripts and obtain normal expression from their promoters (10, 30, 38). Normal expression of late genes clearly requires a suitable viral transcription template. Alterations in viral nucleoprotein complexes can affect normal transcriptional regulation, which is most clearly seen in studies of temperature-sensitive (*ts*) mutants of the major HSV-1 DNA-binding protein ICP8 (26). Here, at the nonpermissive temperature, late (γ) gene transcription is detectable in the absence of any viral DNA replication.

The DNA sequences and viral factors required for late gene expression have not been extensively investigated. Many late HSV promoters have the form of a nominal eucaryotic promoter (61). In spite of this, at least some late promoters appear to be inactive in uninfected cells (16, 57). Further, the VP5 promoter is inactive in an uninfected in vitro transcription system (23). However, activity is observed when assayed in an in vitro transcription extract isolated from HSV-1-infected HeLa cells (K. G. Draper and E. K. Wagner, unpublished data). Cell lines containing the VP5 promoter linked to the TK gene require HSV-1 superinfection for TK expression (16). This suggests a requirement for HSV-specific host cell modifications to induce transcriptional activity from these promoters.

It is evident from the preceding discussion of factors involved in the differential expression of HSV-1 genes that both transcriptional modification of the host cell and template-controlled modulation of the HSV-1 promoters are important in the temporal control of HSV-1 transcription. A major question concerns the absolute requirements for expression of late viral genes. A transient expression system with a marker gene controlled by model early (alkaline exonuclease) and late (VP5) HSV-1 promoters was used here to examine this problem. It was found that full expression of a gene controlled by a model late promoter requires only the modifications induced by the viral immediate-early genes. As is the case with early promoters, it was found that the VP5 promoter only required sequences from the cap site to between 75 and 125 bases upstream for full stimulation by HSV-1 superinfection. Thus, the late viral genes appear functionally equivalent to early genes in transient assay systems. These data require that models designed to differentiate expression of early and late HSV genes must involve modulatory effects of the viral template as a major factor.

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MATERIALS AND METHODS

Cells and virus. Plaque-purified isolates of the KOS strain of HSV-1 were used. Monolayer cultures of HeLa cells were grown as described previously (1–3, 12–14, 17, 18, 22, 24, 29–31). These cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Enzymes. All restriction enzymes and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories. Bacteriophage T4 polynucleotide kinase (Bethesda Research Laboratories) was used for 5′ end labeling as described by Maxam and Gilbert and previously (12, 14, 17, 18, 22, 24, 29, 45). *Escherichia coli* DNA polymerase I (Klenow fragment) was from Boehringer Mannheim Biochemicals and linkers were from Collaborative Research, Inc. The chosen restriction endonuclease linker was ligated onto blunt DNA ends as described by Maniatis et al. (44). Exonuclease III and S1 nuclease (Boehringer Mannheim) were used to construct one of the HSV-1 promoter deletions following the methods described by J. Nelson of the Scripps Clinic and Research Foundation (personal communication).

Recombinant DNA. Our use of map units to locate specific DNA fragments on the HSV genome has been described in numerous recent publications and has been reviewed (60, 61). *Hin*dIII fragment C (0.647 to 0.876 map units [m.u.]) was obtained from a cosmid library constructed with *Hin*dIII-digested DNA from the 17 *syn*$^+$ strain of HSV-1 (59). The chloramphenicol acetyltransferase (CAT) gene (56) was obtained from the pUCCATpA plasmid (a gift from H. Fan, University of California, Irvine) and its construction is described by Linney and Donerly (42). The pSVOD plasmid used in construction of the CAT vector for this study was a gift from Y. Gluzman at the Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. (48). A *Hin*dIII digestion and a partial *Eco*RI digestion were carried out to isolate the intact CAT gene and the polyadenylation site from the pUCCATpA vector. This was ligated into the pSVOD plasmid (48) which was digested with these enzymes to remove the simian virus 40 origin of replication. This marker plasmid contains a convenient *Hin*dIII site at the 5′ end of the CAT gene and a *Sal*I site 631 bases 5′ of the *Hin*dIII site.

The expression marker vector containing the alkaline exonuclease promoter (AE-CAT) was constructed as follows. A *Sal*I linker was inserted at the *Pvu*II restriction site located 200 bases upstream of the mRNA cap site, and a *Hin*dIII linker was inserted at the *Alu*I restriction site located 30 bases 3′ of the start site of the transcript (see Fig. 2A). This promoter was then ligated into the same sites of the CAT vector.

The expression marker containing the VP5 promoter sequences extended up to 650 bases 5′ of the transcript cap site [VP(650)-CAT] was constructed by generating a flush end at the *Bam*HI site 10 bases 5′ of the mRNA cap site (see Fig. 2C) and introducing a 12-base *Hin*dIII linker into this site. The DNA fragment from this site to the *Sal*I site at 0.272 m.u. was then ligated into the appropriate sites of the CAT marker plasmid.

A number of derivatives were made from the VP5(650)-CAT expression marker. The *Sal*I site was converted to a *Hin*dIII site by using linkers. The complete 650-base fragment was then excised and reintroduced in the reverse orientation into the *Hin*dIII site of the CAT plasmid. This construct is termed VP5(650R)-CAT. *Sal*I linkers were introduced into the *Hpa*I site 350 bases 5′ of the mRNA cap site, into the *Bgl*I site 168 bases 5′ of the cap, and into the *Alu*I site 125 bases 5′ of the cap (see Fig. 2C). Thus, the contructions yielded CAT genes containing variable amounts of the VP5 promoter DNA upstream of the normal cap site of the VP5 mRNA. In each case, the sequences upstream of the site of introduction of the *Sal*I linker were discarded. These expression markers were designated VP5(350)-CAT, VP5(168)-CAT, and VP5(125)-CAT. Finally, an expression marker termed VP5(75)-CAT was constructed by exonuclease III-S1 nuclease excision from the *Sal*I site of the VP(350)-CAT construct followed by introduction of the *Sal*I linker at the deleted end. The extent of HSV DNA in the construction was confirmed by nucleotide sequencing.

In several experiments, a second model early HSV-1 promoter was used. This was the promoter for 5.2-kilobase

(kb) early transcript mapping between 0.563 and 0.596 m.u., which has been described previously (2, 18). This transcript is an extremely abundant one and encodes a protein (ICP6) important in the viral ribonucleotide reductase complex (33). A *Hin*dIII linker was introduced into the *Kpn*I site at 0.563 m.u., which is at the cap site of the transcript (23). The *Pvu*II site (0.554 m.u.) 1,300 bases 5′ of the cap site was similarly converted into a *Sal*I site and this fragment was introduced into the CAT plasmid. This construct is called RRductase-CAT.

Transfection and CAT assay. Prior to all transient expression assays, the cells were inspected for their growth properties to ensure that the difference observed in the CAT assays among different HSV-1 expression marker constructions was not due to the cells' viability. HeLa cells (2×10^6 cells) were plated out 24 h prior to DNA transfection into 75-cm^2 tissue culture flasks (Corning Glass Works) containing Eagle minimal essential medium supplemented with 10% calf serum. The cells were plated so that they would reach 50 to 70% confluency before DNA transfection.

The procedure for the calcium phosphate coprecipitation of DNA onto cells was essentially as described by Busslinger et al. (6). At 4 h before addition of the calcium phosphate precipitate to the cells, the medium was removed and replaced with Dulbecco modified Eagle medium containing 10% fetal calf serum. The HSV-1 promoter CAT expression marker plasmid (25 μg) was ethanol precipitated and suspended in 450 μl of 2 mM Tris (pH 7.6)–0.2 mM EDTA, followed by the addition of 50 μl of 2.5 M $CaCl_2$. Then, 500 μl of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpeperazine-*N*-2:ethanesulfonic acid], 1.5 mM Na_2HPO_4, pH 7.15) was added and vortexed vigorously to ensure that a very fine precipitate formed. After 1 h, the precipitate was added to the cells and allowed to incubate for 18 h. The medium containing the precipitate was removed, and the cells were rinsed with 1× Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.6) and then overlaid with Dulbecco modified Eagle medium containing 10% fetal calf serum.

Cells that had been transfected with HSV-1 promoter CAT plasmids were either infected with HSV-1 at a multiplicity of 2 PFU per cell or mock infected 12 to 24 h after removal of the medium containing the DNA precipitant. In the experiment in which HSV-1 promoter CAT vectors were cotransfected with the *Hin*dIII fragment C cosmid, the CAT activity was determined 48 h after the DNA was introduced into the HeLa cells.

The efficiency of transfection with investigated by determining the amount of HSV-1 expression marker DNA present in nuclei of cells that were transfected with expression marker plasmids. Nuclei were isolated by Dounce homogenization in Nonidet-P-40 containing reticulocyte standard buffer, and total cellular DNA was isolated by phenol extraction. Plasmid DNA was quantitated by dot blotting, using a nick-translated CAT construct as a probe. There was little variability in the DNA-transfecting efficiency if the plasmid DNA used for each transfection experiment was always isolated in an identical manner (data not shown). The copy number of the expression marker plasmids were also determined from nuclear DNA of transfected cells in the presence or absence of HSV-1 superinfection. In neither case was a difference found in the amount of CAT expression marker plasmid, thus excluding the possibility that viral superinfection influenced the copy number of inducing plasmid replication (data not shown).

Protein extracts from transfected cells were made and assayed for CAT activity essentially as described by Gorman et al. (27). The standard CAT assay contained 50 μl of supernatant (equivalent to 2×10^6 cells), 40 μl of 0.25 M Tris (pH 7.6), 20 μl of 4 mM acetyl coenzyme A (P-L Biochemicals, Inc.), and 0.25 μCi of [^{14}C]chloramphenicol (58 mCi/mmol; New England Nuclear Corp.). The reaction products were suspended in 20 μl of ethyl acetate and spotted on silica gel thin-layer plates (J. T. Baker Chemical Co.). The products were separated by ascending chromatography, using a 95% chloroform–5% methanol solvent mixture. After autoradiography, spots of the different forms of chloramphenicol were localized, cut out, and counted in scintillation fluid. Relative CAT activity was expressed as a percentage of the amount of acetylated chloramphenicol formed in a standard assay, using 50 μl of cell extract. If the relative CAT activity was >70% acetylation, the extract was diluted and reassayed to obtain a more reliable number. The percent acetylation was then multiplied by the dilution factor to obtain the final standard relative CAT activity. For example, if 5 μl gave 30% acetylation, this was expressed as a relative CAT activity of 300.

Isolation of total infected cell RNA for in situ RNA blots and S1 nuclease analysis. In this communication, total cellular RNA was isolated at 12 h postinfection (p.i.) by the 4 M guanidinium isothiocynate-hot phenol extraction method, as described by Maniatis et al. (44). Polyadenylic acid-containing mRNA was isolated from total cellular RNA by oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) chromatography. Details of this procedure were presented elsewhere (2, 3, 17, 18, 22, 24, 29).

In situ RNA blots were previously described (14, 17, 24, 29). Hybridization was for 36 h at 50°C in 50% formamide containing 0.4 M Na^+–0.1 M HEPES (pH 8)–0.005 M EDTA–Denhardt solution (15).

Transfected HeLa cells containing HSV-1 promoter CAT plasmids were superinfected with HSV-1 (2 PFU per cell), and total cellular RNA was isolated at 22 h p.i. as described above. Any DNA present in the total cellular RNA preparation was eliminated by pelleting the RNA through a 5.7 M CsCl pad in a Beckman SW50.1 rotor, centrifuged at 35,000 rpm (114,000 × *g*) for 12 h as described in Glisin et al. (25). S1 nuclease analysis was essentially as described by Berk and Sharp (5) and previously (2, 12–14, 17, 18, 22–24, 29). The appropriate HSV-1 promoter CAT expression plasmids were cut at the desired restriction endonuclease site, and the DNA was 5′ end labeled with [γ^{32}P]ATP (3,000 Ci/mmol; ICN Pharmaceuticals, Inc.). Using T4 polynucleotide kinase resulted in a specific activity of 100,000 cpm per μg of DNA. The DNA fragments then were denatured and strand separated on 5% acrylamide gels as described by Maxam and Gilbert (45). The strand-separated DNA (from 10 μg of cloned DNA) was hybridized with 50 to 100 μg of total infected-cell RNA hybrids subjected to S1 nuclease (Boehringer Mannheim) digestion as described before (2, 12–14, 17, 18, 22–24, 29). S1 nuclease material was fractionated on a denaturing 5% acrylamide gel with 5′ end-labeled *Hin*fI- and *Eco*RI-digested pBR322 DNA fragments as size standards.

RESULTS

Expression of a model early (β) and late (βγ) gene during the virus replication cycle. To examine the factors controlling the expression of different temporal classes of HSV transcripts, two model genes were selected. The first was the alkaline exonuclease gene. This is expressed as a normal early gene based on the criterion that it is transcribed at significant levels prior to or in the absence of viral DNA

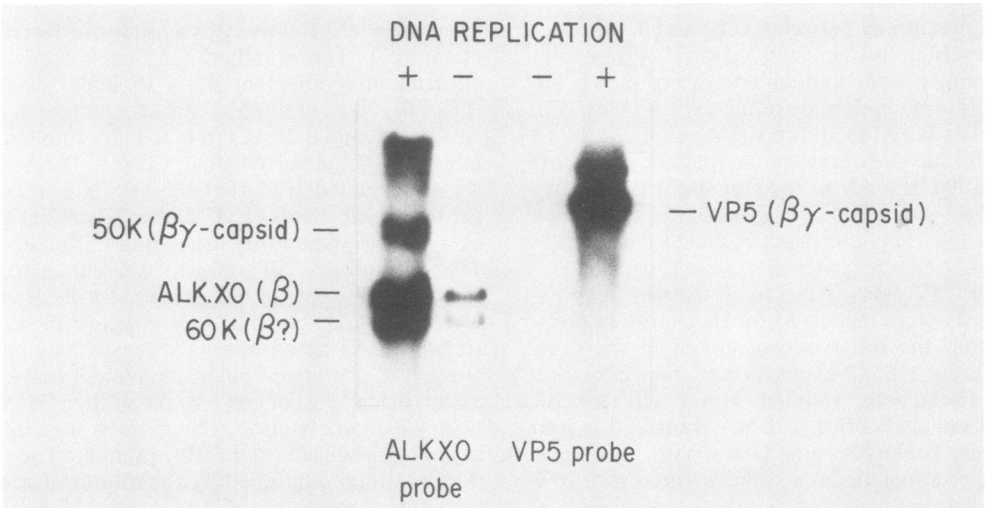

FIG. 1. Effect of HSV DNA replication on expression of model early and late genes following low-multiplicity infection. HeLa cells were infected at a multiplicity of 2 PFU per cell with HSV-1 either in normal Eagle minimal essential medium (DNA replication +) or in Eagle minimal essential medium containing 50 μg of Ara-T per ml as an inhibitor of viral DNA synthesis (DNA replication −). Cells were harvested at 12 h p.i., and total polyadenylic acid-containing RNA was isolated and fractionated on denaturing agarose gels containing 10 mM methylmercury hydroxide. In situ blots of the fractionated RNA were hybridized under our standard conditions with nick-translated ^{32}P-labeled DNA. The probe for hybridization of the alkaline exonuclease transcript was *Bgl*II-*Xho*I fragment O-G (1 kb; 0.164 to 0.171 m.u.). The probe for hybridization to the VP5 transcript was *Hin*dIII-*Bam*HI fragment A-A′ (1 kb; 0.262 to 0.268 m.u.). Specific radioactivity of each probe was 5×10^7 cpm/μg.

replication (14). The second was the late VP5 gene. The mRNA of this βγ gene encodes the major capsid protein and becomes extremely abundant late in infection, although it is readily detected prior to viral DNA replication (12, 13, 30).

The influence of viral DNA replication on HSV gene expression following a low-multiplicity infection (2 PFU per cell) is shown in the experiment of Fig. 1. In this experiment, RNA from cells maintained for 12 h p.i. with 50 μg of thymine arabinoside (Ara-T) per ml was size fractionated in parallel with RNA from cells infected at the same multiplicity of infection but without the drug. The RNA blots were hybridized with nick-translated DNA probes as described in Materials and Methods. The probe for the alkaline exonuclease transcript, as well as for other colinear mRNA, was *Bgl*II-*Xho*I fragment O-G (0.164 to 0.171 m.u.). The probe for the VP5 transcript was *Hin*dIII-*Bam*HI fragment A-A′ (0.262 to 0.268 m.u.). Each probe was approximately 1,000 bases in length and each was labeled to approximately the same specific activity. Therefore, the relative intensity of radioactivity hybridizing to each transcript is a rough measure of the relative steady-state concentration at the time of RNA isolation. The drug Ara-T is comparable to adenosine arabinoside in its efficiency of inhibiting viral DNA replication and does not require the presence of an adenosine deaminase inhibitor for its activity, as does adenosine arabinoside (30; C. Shipman, Jr., R. H. Costa, and E. K. Wagner, unpublished data).

It is clear that only the alkaline exonuclease transcript and the partially colinear 1.9-kb mRNA encoding the 60,000-dalton polypeptide are present in readily detectable amounts in the absence of DNA replication (Fig. 1, ALKXO probe, DNA replication −). That the 1.9-kb transcript is expressed at such a high level in the absense of viral DNA replication indicates that this transcript should be classified as an early transcript instead of a late (βγ) one as was suggested earlier (14). Transcripts encoding the VP5 protein as well as the transcripts identified by this laboratory as encoding the 50,000-dalton capsid protein (14) are detectable without DNA replication. However, at this low multiplicity of infection, their steady-state level is only a fraction of that of the alkaline exonuclease transcript. In the presence of DNA replication (Fig. 1, DNA replication +), there is a significant increase in the steady-state concentration of both the early and the late model transcripts. Densitometer tracings indicate that the amounts of alkaline exonuclease mRNA increased by a factor of 6 while the VP5 transcript increased by a factor of 30 after DNA replication. Indeed, after DNA replication the level of the VP5 transcript is greater than that of the alkaline exonuclease mRNA. Such data suggest that the continued synthesis of early transcripts after viral DNA replication involves the same type of templates that are involved in the expression of the late transcripts, yet late transcription is greatly enhanced. This observation is of importance when suggesting models to explain late transcription during HSV replication.

HSV infection induces high levels of viral promoter-controlled CAT activity. To examine the role of promoter specificity in the expression of early and late HSV-1 genes, several expression markers were constructed by using the promoters of the model genes described above. The sequence of the promoter region of the alkaline exonuclease gene was presented previously (14). Convenient restriction endonuclease cleavage sites and characteristic features, including "TATA" and "CAAT" boxes, as well as the early "AC" string, are located schematically in Fig. 2A. The promoter for the VP5 gene has been located, and the sequence of the 100 or so bases immediately 5′ of the transcript cap site has been reported (12). A more extensive sequence of the DNA extending 350 bases 5′ of the cap site is shown in Fig. 2B. A restriction endonuclease cleavage map of the DNA sequences immediately 5′ of the VP5 transcript cap site and essential features of the promoter, including, TATA and CAAT boxes and an AT string, are shown schematically in Fig. 2C. General features of early and late HSV promoters have been reviewed (60, 61).

Promoters were constructed directly 5′ of the bacterial

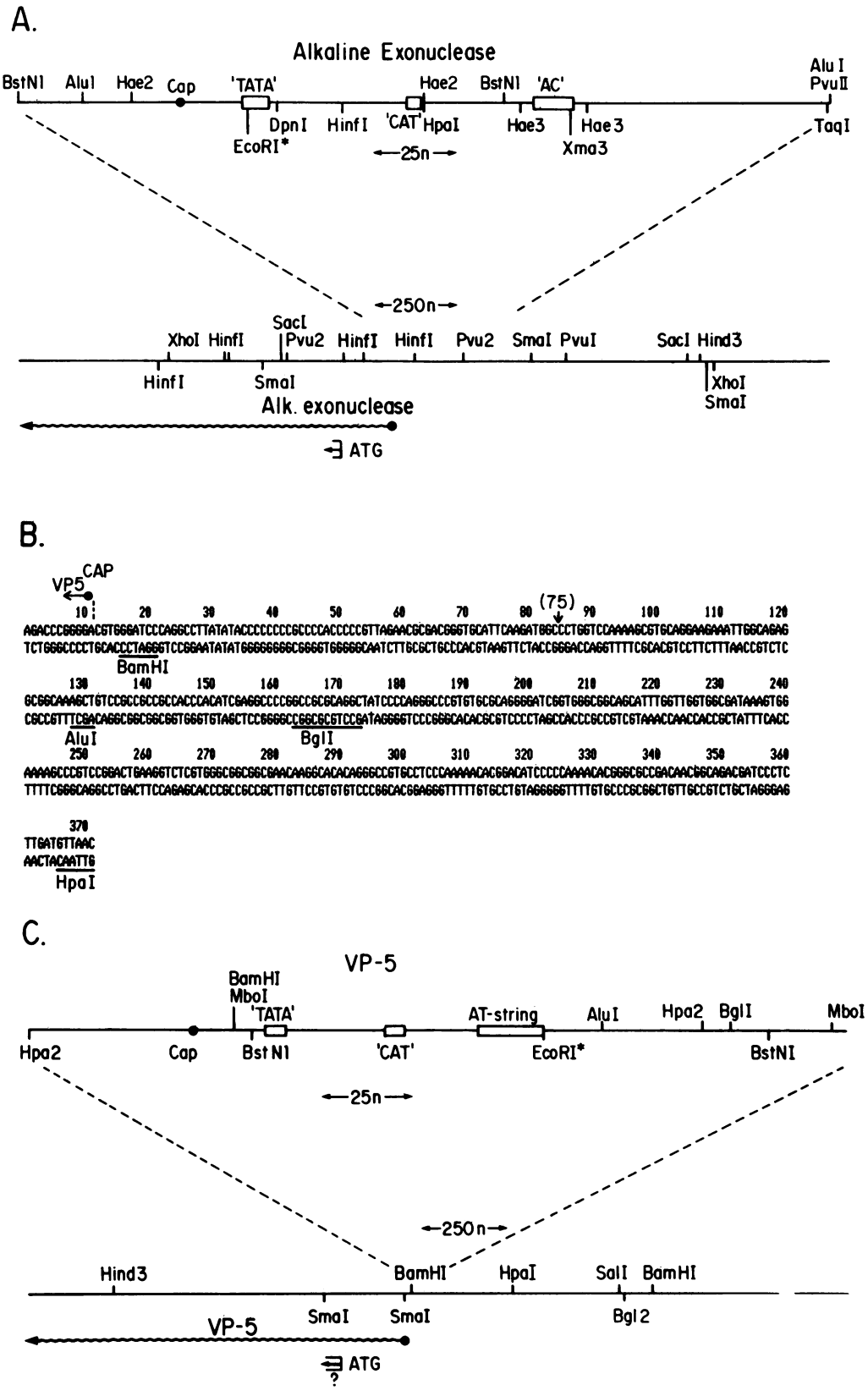

FIG. 2. Summary restriction endonuclease cleavage maps of the promoter regions of the alkaline exonuclease and VP5 transcripts of HSV-1. The schematic data of (A) were based on nucleotide sequence data published previously (14). (B) contains an annotated nucleotide sequence of the 350 bases 5′ of the cap site for the HSV-1 VP5 transcript. This information is schematically summarized in (C).

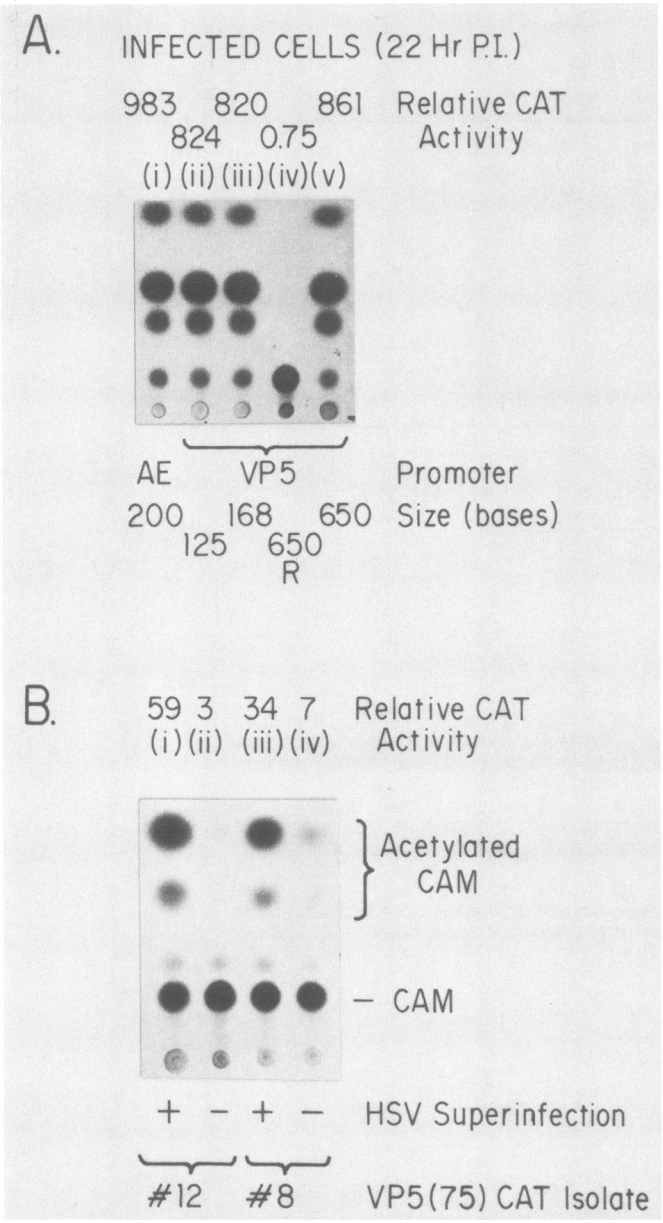

FIG. 3. CAT expression under the control of various HSV promoters with viral superinfection. HSV promoter CAT plasmids were transfected into HeLa cells and superinfected with HSV-1 at a multiplicity of 2 PFU per cell. HeLa cell extracts were then assayed for CAT activity 22 h p.i. as described in Materials and Methods. Relative CAT activity is based on the percent acetylation of chloramphenicol (CAM) as explained in Materials and Methods. (A) shows results with the standard model AE-CAT marker which contains the 200 bases 5′ of the alkaline exonuclease mRNA cap site and results with various amounts of HSV DNA 5′ to the cap site of the VP5 transcript. (B) shows expression of CAT activity under control of the 75 bases 5′ of the VP5 transcript cap site either superinfected (+) or mock infected (−) with HSV-1.

CAT gene (56) as described in Materials and Methods. This marker gene was then used to assay promoter activity either before or after infection with HSV. CAT enzyme activity was expressed as a relative conversion of radiolabeled chloramphenicol into acetylated forms as described in Materials and Methods. When high activities were obtained, dilutions of the standard aliquots from a fixed number of cells were assayed. In such cases, the resulting activity was expressed as the product of the percent acetylation of chloramphenicol and the dilution factor from the standard concentration.

As shown in the next section, depending on the exact promoter used, the CAT expression markers were active at a low or undetectable level in uninfected HeLa cells. When HeLa cells transfected with HSV promoter-controlled CAT expression markers were superinfected with low multiplicities of HSV-1 (2 PFU per cell), high levels of enzymatic activity were induced. An experiment illustrating this is shown in Fig. 3A. Here (tracks i, ii, iii, and v), the AE-CAT and various VP5-CAT expression markers were induced to express very high levels of the enzyme. The multiplicity of infection used in the experiment shown was determined to be optimum. Infections with higher multiplicities resulted in less total CAT activity being induced in the cell population (data not shown). These results are consistent with previous reports that HSV-induced stimulation or marker genes carried in stably transformed cells is multiplicity dependent (16, 55, 58).

The activity of the different VP5-CAT expression markers was tested to estimate the minimum size of a promoter region required for induction of transcription by HSV superinfection. The data of Fig. 3A demonstrated that no more than 125 bases of HSV sequence was sufficient (track ii). In a second experiment (Fig. 3B, tracks i, iii), it was found that the VP5(75)-CAT was sufficient to allow expression with HSV-1 superinfection. It appears that this smallest promoter is somewhat less active, however, since the total amount of CAT activity induced is at least 10-fold less than was seen when the larger DNA fragments were used as promoters.

The VP5(650R)-CAT marker was not induced by HSV superinfection (Fig. 3A, track iv). This result suggests that the induction seen requires an actual viral promoter and is not a nonspecific effect of the construction. S1 nuclease analysis of hybrids between infected-cell RNA from transfected HeLa cells and the CAT marker gene was used to confirm that transcription of the CAT gene is efficiently initiated at the proper location 3′ of the promoter. An example of such an experiment is shown in Fig. 4. Here, strand-separated DNA, 5′ end labeled at the *Eco*RI site, 250 bases 3′ of the *Hin*dIII site of the expression marker extending through the VP5 promoter to the *Sal*I site at 0.272 m.u., was used as a hybridization probe. The location of this site in relation to the TATA box of the VP5 promoter in the VP5(650)-CAT expression marker is shown in Fig. 4A. DNA was hybridized with 100 μg of total cell RNA isolated 22 h after an HSV infection of HeLa cells transfected with VP5(650)-CAT. An S1 nuclease-resistant DNA fragment 250 bases in length is the most abundant hybridization product. (see Fig. 4B, track s). This indicates that the majority of the transcripts induced in the VP5(650)-CAT expression marker were initiated at the proper location 3′ of the VP5 promoter. The other minor DNA fragments are consistent with previous findings from this laboratory of several relatively minor transcription starts 5′ of the cap site of the major VP5 transcript cap site (12, 23). Results consistent with accurate initiation of transcripts from the viral promoter were also found with the AE-CAT expression marker (data not shown).

Total cellular RNA was also isolated 22 h after HSV-1 superinfection from HeLa cells transfected with the CAT plasmid lacking a viral promoter. When this RNA was used for hybridization with the same DNA probe discussed

above, no S1 nuclease-protected fragments were observed (Fig. 4B, track c). This indicates that the 250-base pair fragment seen in the S1 nuclease experiment (Fig. 4B, track s) is due to hybridization to HSV-1 promoter-driven CAT transcripts and not to some nonspecific effect from the viral infection.

Low levels of CAT activity can be induced by HSV-1 promoters in unmodified HeLa cells. Extracts from HeLa cells contain no detectable CAT activity. Less than 0.2% of chloramphenicol was converted to acetylated forms in the standard assay (data not shown). A similar situation was seen if a CAT plasmid containing no promoter region was introduced into HeLa cells by calcium phosphate precipitate-mediated DNA transfection. An example of such data is shown in Fig. 5 (track i). Early HSV promoters induced a low but readily detectable level of CAT activity. Acetylation (ca. 1.5 to 2%) was consistently found in HeLa cells transfected with AE-CAT. An example of this observation is shown in Fig. 5 (track ii). As shown in the next section, this situation appears to be general for the CAT gene controlled by early HSV-1 promoters. Such results are entirely consistent with numerous examples of low levels of transcriptional activity controlled by the early HSV TK promoter (46, 58; ElKareh et al., in press).

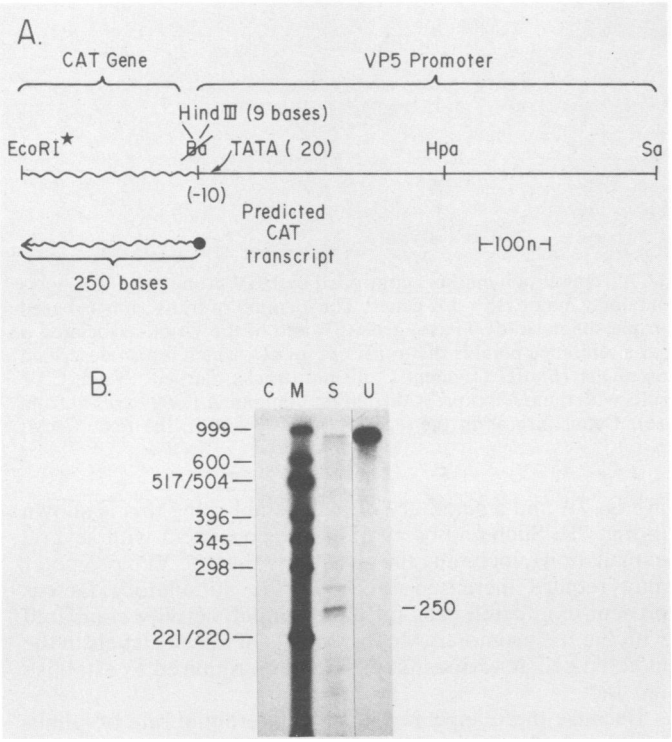

FIG. 4. S1 nuclease analysis of CAT transcripts expressed under the control of the VP5 promoter. (A) indicates the position of the major transcript expressed from the VP5(650)-CAT expression marker following superinfection with HSV-1. The *Eco*RI site used for 5′ end labeling for the single-stranded hybridization probe is shown with the star. (B) shows fractionation of S1 nuclease-resistant DNA isolated by digestion of hybrids between the single-stranded DNA hybridization probe and total cell RNA isolated at 22 h p.i. from cells transfected with VP5(650)-CAT superinfected with HSV-1 (S) as described in Materials and Methods. The track marked (U) contains undigested DNA. Track (M) contains DNA size markers derived by *Hin*fI and *Eco*RI digestion of pBR322 as described previously (12, 14, 18, 24). Track (C) shows the result of control S1 nuclease digestion of a hybridization mixture of CAT probe DNA and total cell RNA isolated 22 h after HSV-1 superinfection of HeLa cells which had been transfected with a CAT gene plasmid lacking an HSV promoter.

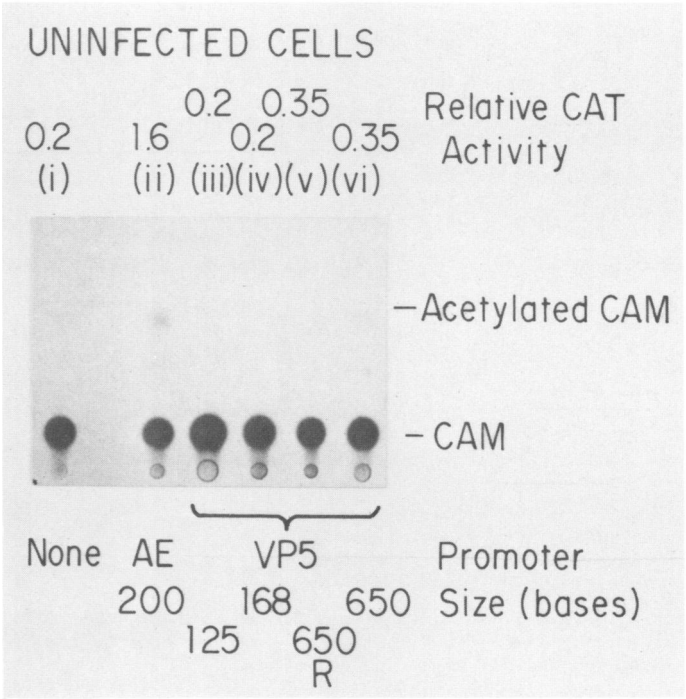

FIG. 5. Expression of CAT activity in uninfected HeLa cells transfected with various CAT expression markers containing early and late model HSV promoters. Details are as described in the legend to Fig. 3 and in the text. CAM, Chloramphenicol.

In contrast to the situation with early HSV-1 promoters, most of the VP5 promoter-controlled CAT expression markers were completely inactive in transfected HeLa cells in the absence of HSV-induced modifications (Fig. 5, tracks iii to vi). The VP5(75)-CAT marker did, however, induce measurable CAT activity in uninfected HeLa cells as is shown in the experiment illustrated in Fig. 3B (tracks ii and iv). This observation was quite reproducible. However, different isolates of the construct appeared to differ somewhat in the total amount of activity induced. This result suggested that some feature of the HSV DNA sequence between bases −75 and −125 of the VP5 promoter blocked the uninfected cell's ability to recognize the VP5 promoter in the CAT expression marker. These data also suggest that the low level of CAT activity induced in cells containing the VP5(75)-CAT marker upon superinfection (Fig. 3B, tracks i and iii) was not due to some nonspecific contaminant in the DNA used since the level of activity in uninfected cells was of the same level or higher than those seen with early HSV promoter-containing CAT expression markers [ca. 3 to 5% acetylation with VP5(75)-CAT versus 1.5 to 2% with AE-CAT]. From this, one can suggest that the region of the VP5 promoter important in blocking activity in uninfected cells may be involved in achieving full activity from this promoter after HSV superinfection.

Gene products encoded by *Hin*dIII fragment C are sufficient to induce the expression of CAT activity controlled by early and late HSV promoters. Three complete HSV-1 immediate-early (α) genes (reviewed in reference 61), as well as the gene for the stimulatory virion-associated protein (7), are found in the 34-kb *Hin*dIII fragment C (0.647 to 0.876 m.u.). This

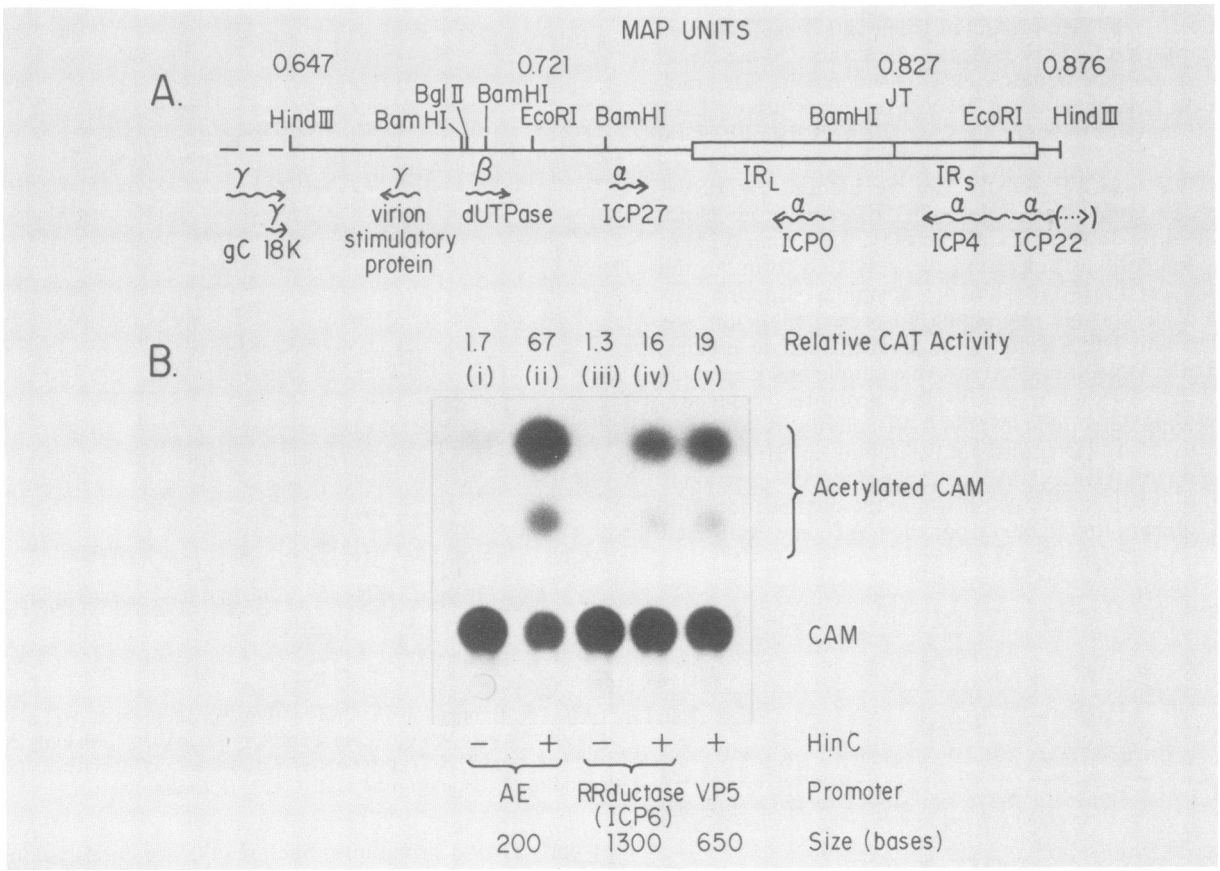

FIG. 6. Effect of cotransfection of α (immediate-early) HSV genes and CAT expression markers controlled by HSV promoters. (A) shows features of the *Hin*dIII fragment C (34 kb; 0.647 to 0.876 m.u.) cosmid-containing major HSV-1 α genes. The location of transcripts is based on data reviewed previously (61). Identification of the deoxypyrimidine triphosphatase (dUTPase) gene (53) and of the virion-associated α gene stimulatory protein (7) are described. Also indicated on the drawing as a reference point is the transcript for gC, which is also described (25). (B) demonstrates the induction of CAT activity upon cotransfection of the *Hin*dIII fragment C cosmid (tracks marked +) and CAT expression markers containing model HSV promoters. Also shown are results with the CAT expression marker containing 1,300 bases 5′ from the ribonucleotide reductase mRNA cap site (23) (marked as RRductase). Details are as in the legend to Fig. 3 and in the text. CAM, Chloramphenicol.

fragment also includes the promoter and 5′ portion of the ICP22 (α) gene. A restriction endonuclease cleavage map of this region of the HSV-1 genome, as well as location of transcripts encoding known biological functions, is shown in Fig. 6A. Cloned *Hin*dIII fragment C was introduced with CAT expression markers containing early and late promoters into HeLa cells by cotransfection. In all cases, the transfection contained equal weights of the two plasmids. The three tested expression markers all induced significant CAT activity (Fig. 6B). The AE-CAT expression marker showed the most activity (track ii). However, its activity in uninfected cells was equivalent to that of RRductase-CAT, another early promoter-driven marker (cf. tracks i and iii). That RRductase-CAT is induced to the same level as VP5(650)-CAT upon cotransfection with *Hin*dIII fragment C (cf. tracks iv and v) suggests that the higher activity of AE-CAT in such experiments is not due to a temporal class-specific differential promoter stimulation.

Inhibition of viral DNA replication appears to increase the rate of CAT expression in superinfected cells. When following the time course of induction of CAT activity after HSV superinfections, it was consistently observed that induction of CAT activity in superinfected cells was more rapid when the AE-CAT marker was used than when the VP5(650)-CAT marker was used. An example of this observation is shown in Fig. 7A and a summary of data establishing this is shown in Fig. 7B. Such an observation was consistent with several explanations, including the possibility that the VP5 promoter may require increased levels of the stimulatory factors present in infected cells for its maximum activity compared with the AE promoter. Measurements of mRNA levels in the infected cell at various times would be required to establish this fact.

Because there appeared to be a differential rate of stimulation of CAT activity depending on whether an early or late viral promoter was used, the effect of inhibition of superinfecting viral DNA replication on the expression of CAT activity controlled by the AE and VP5 promoters was examined. HeLa cells were transfected with AE-CAT or VP5(650)-CAT, and HSV-1 superinfection was carried out in the presence or absence of Ara-T to inhibit viral DNA replication. At 12 h after infection, there was a greater stimulation of CAT activity with both promoters if DNA synthesis was inhibited (Fig. 8, tracks ii and iv). These data reconfirm the conclusion that no virus-induced modifications of the cell requiring viral DNA replication are necessary for transcriptional activity of the model late gene. The decreased stimulation observed from the HSV-1-controlled marker genes when superinfecting viral DNA replication proceeds may be the result of induced transcription activity

from the viral template (cf. Fig. 1). The active transcription of replicated viral templates may compete for some limiting factor required for marker gene expression. This would result in reduced induction of the marker gene in the transient assay.

DISCUSSION

The original description of the CAT expression marker demonstrated that this bacterial gene could be very useful in measuring the transient level of eucaryotic promoter-controlled gene expression (27). The CAT transient expression markers used here were constructed so that very little, if any, of the viral leader sequences 3′ to the promoter being studied were present. This was to eliminate differential effects of viral leader sequences upon translation or stability of the marker gene protein. This lends confidence that measurements of CAT activity in the test cell extracts are a good measure of the steady-state levels of HSV promoter-controlled CAT transcripts. The S1 nuclease analysis of the CAT mRNA expressed in the test cells confirms that the promoter-controlled expression is an accurate model for expression of a viral gene.

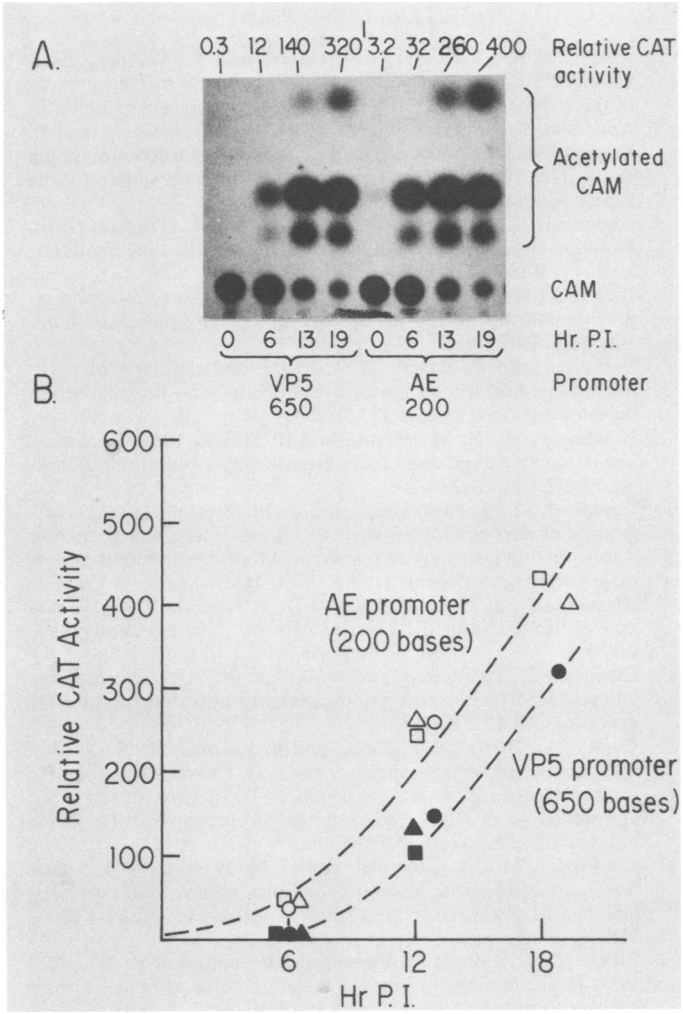

FIG. 7. Differential rate of expression of CAT activity in HSV-1-superinfected cells containing CAT expression markers controlled by early and late model HSV-1 promoters. (A) shows a typical time course experiment indicating the slower expression of CAT activity under control of the VP5 promoter compared with the alkaline exonuclease promoter. (B) summarizes the results of three experiments. Open symbols indicate CAT activity expressed by the AE-CAT marker. Solid symbols indicate CAT expression from the VP5(650)-CAT marker. CAM, Chloramphenicol.

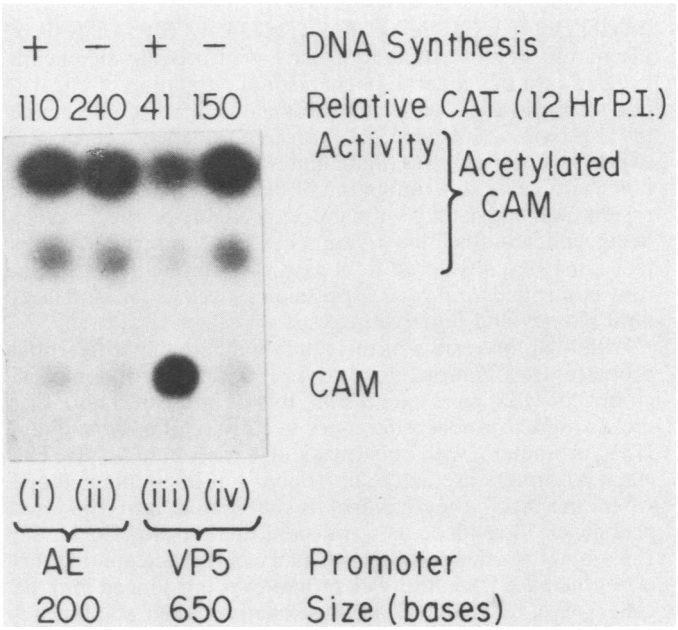

FIG. 8. Effect of inhibiting superinfecting HSV-1 DNA synthesis on CAT activity from model early and late expression markers. Increased expression of CAT activity in the absence of superinfecting viral DNA synthesis (designated with −) for both model promoters is shown. HeLa cells containing the HSV expression markers were superinfected with HSV-1 and maintained in either the presence or the absence of Ara-T, which inhibits viral DNA replication. The cells were then harvested at 12 h p.i. and analyzed for CAT activity as described in Materials and Methods. Note that replication of these expression marker vectors does not occur in either mock-infected or superinfected HeLa cells (data not shown). CAM, Chloramphenicol.

These data demonstrate that, based on transient expression assays, there is no clear difference in activity of an early or late HSV promoter following viral superinfection. Indeed, activity of the two model promoters used in this study are quite similar when viral DNA synthesis is inhibited. Such activity is reminiscent of the activity of such promoters inferred from mRNA levels in infected cells after DNA replication has begun. This result indicates that all modifications to the host cell's transcriptional machinery required for full expression of the VP5 gene is accomplished by immediate-early or early gene products or both. It is also evident that the extent of DNA sequence required in the late promoter for its activity is of the same order as that required for the activity of early HSV genes, between 75 and 125 bases (19, 47; ElKareh et al., in press). Such results support other data suggesting that much of the discrimination between early and late genes seen in infected cells prior to or in the absence of viral DNA replication is due to the environment of the promoter in the viral transcription template, not to inherent differences in the ability of the cell's transcriptional machinery to recognize different temporal classes of promoters (57).

These data also provide further confirmation of the important role of the α proteins in activation of cellular transcrip-

tional activity reported by others (28, 34, 49, 55). Analysis of the nature of this stimulation may well provide significant insights into the general mechanism of activation of eucaryotic transcription. No attempt was made here to fully define the role of a specific HSV protein independent of the expression of other α (immediate-early) genes as well as other viral genes in stimulation of cellular transcription. Our results are consistent with the expression of the α genes being sufficient for full activation of HSV transcription. This fact must be considered in constructing models for temporally controlled viral gene expression as well as for models of viral latency and reactivation.

Although the results of this study indicate that differential promoter recognition alone is not the basis of temporal control of HSV gene expression, it is of interest to note that there are measurable differences in activity of early and late HSV promoters with the transient expression assay. The early promoters are active or "open" in the uninfected cell when the gene they control is integrated into the host genome or introduced as a transient marker (46, 49, 55, 58; ElKareh et al., in press). These data clearly indicate that this is not the case when the VP5 promoter is introduced into the cell. The VP5 promoter is also inactive when stably integrated into the host cell until that cell is infected with HSV (16). As recently reviewed (60), this lack of activity of late HSV promoters is common, but exceptions have been noted. It is suggested here that the general lack of activity of late HSV promoters in uninfected cells is a reflection of aspects of the biology of HSV-1 replication that are, as yet, unknown.

In the case of the VP5 promoter, the blockage of its activity in uninfected cells appears to be due to some feature of the DNA sequences lying between 75 and 125 bases 5′ of the cap site. These sequences may also be important in full activity of the VP5 promoter following virus-induced host modifications, but they do not appear to be absolutely required. Recent preliminary experiments carried out by this laboratory with G. Feuer and H. Fan suggest that the lack of activity of the VP5 promoter in uninfected cells is correlated with the inability of this promoter to respond to enhancer sequences. If these observations are confirmed, this would provide another difference between the VP5 promoter and early HSV promoters since the latter promoters have been shown to respond to upstream enhancer sequences (20).

Our data strongly suggest that in the transient assay system, the VP5 promoter does not require regulatory sequences further upstream than 125 bases for HSV transcriptional activation. Several HSV-1 early promoters have been analyzed and they also do not appear to contain regulatory sequences upstream of the promoter which are required for virus-induced activation (19, 46, 47, 58; ElKareh et al., in press). At the present time, however, it is now clear that this is the complete general case. O'Hare and Hayward recently suggested that the induction of transcription by HSV superinfection, using the strong early HSV-2 promoter for the ribonucleotide reductase gene in a transient assay, requires specific upstream regulatory sequences (49). Their observations could be the result of the strength of the early promoter chosen (20-fold greater than TK in uninfected cells) and be specific to that particular promoter. The system studied may also be important since O'Hare and Hayward observed that the simian virus 40 early promoter was not induced by HSV superinfection and they suggested that HSV-induced transcriptional stimulation is specific to only HSV promoters. This latter result is in contrast to the studies involving several heterologous eucaryotic promoters whose activity was increased by herpesvirus immediate-early products (21, 28, 34; Costa et al., unpublished data).

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