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A Single Regulatory Region Modulates Both *cis* Activation and *trans* Activation of the Herpes Simplex Virus VP5 Promoter in Transient-Expression Assays In Vivo

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A detailed analysis of the expression of the bacterial chloramphenicol acetyltransferase gene controlled by the herpes simplex virus major capsid protein (VP5) promoter showed that this promoter can be functionally separated into an 80-base core region, which has the minimal information required to serve as a pol II promoter but which is not fully activated by viral superinfection or by cotransfections with plasmids bearing functional α (immediate-early) genes, and an approximately 100-base regulatory region upstream of the core, which allowed full induction of VP5 promoter-driven chloramphenicol acetyltransferase activity but which repressed the ability of the VP5 core promoter to be *cis* activated by the simian virus 40 enhancer. This was in distinct contrast to the situation with the alkaline exonuclease promoter (a model early promoter) and defined the regions of this promoter which can be used to study the interaction between viral promoters and putative regulatory proteins induced by viral infection.

The pattern of viral gene expression in cells productively infected with herpes simplex virus (HSV) is well characterized and proceeds in at least three temporal phases of mRNA and protein synthesis (11, 30, 35). Individual promoters direct transcription from adjacent structural genes (55), and recognition and usage of viral promoter sequences are a major determinant of the temporal class and relative abundance of individual transcripts (55; Blair and Wagner, unpublished data). Other mechanisms controlling viral protein synthesis, including regulation of mRNA transport from the nucleus to the cytoplasm, splicing, and posttranscriptional regulation, have been described for herpesviruses but are apparently infrequently used in the expression of the majority of HSV genes during lytic infection in cultured cells (14, 32, 35).

Transcription of the genes for α (immediate-early) polypeptides, a group of five proteins with predominantly regulatory functions (20, 44, 57), is regulated in *cis* by several upstream elements including enhancerlike sequences (6, 36, 37). These genes are also *trans* activated by a 65,000-molecular-weight component of infecting virions (2, 8, 29). The cellular transcription factor, Sp1, binds specifically to G + C-rich boxes in the α ICP4 gene promoter and activates transcription from this promoter in vitro (33). Other cellular *trans*-acting factors presumably contribute to the moderate constitutive activities of plasmid-borne α promoters in uninfected cells.

Genes for the β (early) class of viral polypeptides are transcribed under the control of promoters that are active at low levels in uninfected cells or high levels under selection (38, 47). Those β promoters characterized to date appear to be good examples of consensus RNA pol II promoters (55). β promoters are activated by functional ICP4, the major α regulatory protein (44, 57). Transient-expression assays have also implicated another immediate-early protein, ICP0, in the *trans* activation of several promoters for β gene products (22, 41, 45). In addition to catalytic and stoichiometric functions involved in viral DNA replication, β gene products may have positive and negative regulatory functions (25, 31).

Late gene products have been subdivided into two groups, those mRNA species detectable at low levels before the onset of viral DNA synthesis (βγ or γ_1) and those not detectable prior to DNA synthesis (true γ or γ_2) (50, 55). The promoter for the major capsid protein, VP5, has served as a good model for the βγ class of transcripts, and this protein and the mRNA encoding it become one of the most abundant in the infected cell after the inception of viral DNA synthesis (12, 30). The mechanisms regulating the expression of late gene products are less well characterized than are those for α and β gene products. Genes encoding many late mRNA species do not appear to be efficiently expressed in uninfected cells. A HeLa cell nuclear transcription extract did not initiate transcription from the VP5 promoter under conditions where a model β promoter was active (24), and a plasmid expression marker for a late mRNA (VP5) promoter was not active in uninfected cells (13). However, at least some late genes integrated into the host genome can be expressed at low levels in cell lines under selection for linked phenotypic determinants (47, 50). The level of activation of the promoter for the βγ VP5 mRNA in cell lines is directly proportional to the level of plasmid-borne ICP4 expressed in the particular line (43).

Despite the requirement of DNA replication for full expression, the VP5 mRNA promoter has all the features of a typical eucaryotic transcriptional promoter (Fig. 1A); indeed, work by Costa et al. (13) has shown that the VP5 promoter can be readily *trans* activated in a model expression assay either by HSV superinfection or by the presence of a group of expressed α gene products. There appears to be some mechanism whereby recognition of promoter for the VP5 gene and possibly other late gene promoters is prevented at early times of infection or in uninfected cells.

The present study examines in some detail structural features of the VP5 promoter that cause this promoter to be relatively inactive in uninfected cells and yet available for

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considerable *trans* activation after viral infection. This work confirms and extends the work reported by Costa et al. (13), which showed that a core region of approximately 75 bases of the VP5 promoter is capable of being expressed in uninfected cells but does not respond well to viral superinfection. Analyses were carried out in our study by testing the ability of various fragments of the VP5 gene promoter region to respond to strong *cis*-transcriptional activation by simian virus 40 (SV40) enhancer sequences in transient assays, measuring the accumulation of the bacterial chloramphenicol acetyltransferase (CAT). These data allowed the definition of both a core and a regulatory region of the VP5 promoter; it is this latter region which serves as a negative modulator of the VP5 promoter in uninfected cells.

MATERIALS AND METHODS

Cells and virus. Rabbit skin fibroblasts were maintained at 37°C under 5% carbon dioxide in Eagle minimum essential medium containing 5% cadet calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Plaque-purified HSV type 1 (HSV-1) KOS was used for all experiments described. All procedures have been described previously (13, 52). Cell numbers were determined by counting in a hemacytometer.

DNA transfections and CAT assays. Approximately 6×10^4 cells were seeded into 60-mm tissue culture dishes and were grown for 12 to 16 h to ca. 80% confluence. At 4 h before transfection, culture medium was replaced with Eagle minimum essential medium containing 10% calf serum. Cesium chloride-purified form I plasmid DNA (10 μg) was transfected onto each culture monolayer by calcium phosphate coprecipitation (54). At 6 h posttransfection, cells were treated with 15% glycerol in saline, washed with saline containing 3 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Sigma Chemical Co.], and incubated for a further 24 h. Cells were then superinfected with 0.5 to 1 PFU of HSV-1 per cell and incubated for a further 18 h. Cell extracts were prepared at this time for CAT assays (26) by using 4×10^5 cell equivalents and 0.2 μCi of [^{14}C]chloramphenicol (New England Nuclear Corp.) per reaction. Chloramphenicol was separated from its acetylated forms by ascending thin-layer chromatography with Baker silica gel plates. Acetylated and unacetylated species of chloramphenicol were localized by autoradiography. The percent conversion of chloramphenicol to its acetylated derivatives was measured by determination of radioactivity in individual species as described previously (13).

DNA was extracted from transfected cells, and the amount of CAT sequences in the cells was measured by slot blot hybridization with an M13mp19 *Bam*HI-to-*Hin*dIII probe containing the relevant portion of the bacterial CAT gene. Within a single transfection experiment, the variation of DNA incorporated by individual monolayer cultures never varied by more than 5%. For all experiments reported here, approximately 30 to 50 ng of DNA was taken up by each monolayer, representing transfection efficiencies of 0.3 to 0.5% (data not shown). The same hybridization probe was used to quantify CAT mRNA levels. RNA was extracted from individual transfected cultures; DNA was removed by digestion with RNase-free DNase and 1 U of RNasin (Promega) per μl. In dot blots, RNA levels qualitatively corresponded to the levels of CAT enzyme extracted from individual cell monolayers, in that high levels of enzyme resulted in stronger RNA dot hybridization (data not shown). This is consistent with the development of the CAT assay as a marker of transcriptional activity (26).

Recombinant plasmids. The construction of the parental plasmid pSVOd-CAT and the CAT expression plasmids containing 168, 125, and 75 base pairs of the VP5 mRNA promoter (Fig. 1A) and 240 base pairs of the alkaline exonuclease (AE) mRNA promoter has been described (13; Fig. 1B). An *Xho*I linker (CCTCGAGG) was introduced into the AE promoter at a *Nar*I site 10 base pairs upstream of the CAAT box at nucleotide 125 of the HSV-1 AE gene sequence (21) to facilitate the constructions described below (see Fig. 4 and 5). This insertion did not affect the *cis* or *trans* response of the AE promoter in transient assays. A second parental plasmid, pSVOd-CAT dEnhancer, containing the SV40 enhancer sequences taken from a clone of the SV40 *Hin*dIII C fragment (plasmid pES131; 49), was prepared as follows. The *Nco*I site at SV40 nucleotide 37 was converted to a *Sal*I site by repair with *Escherichia coli* DNA polymerase large fragment and ligation of a *Sal*I linker oligonucleotide, and the *Pvu*II site at SV40 nucleotide 270 was converted to an *Xba*I site by linker addition (this strategy recreated both the *Nco*I site and the 21-base-pair repeat region). This 240-base-pair *Sal*I-to-*Xba*I fragment was inserted into pSVOd-CAT (*Xba*I), a derivative of pSVOd-CAT in which the unique *Ava*I site was converted to an *Xba*I site, to give pSVOd-CAT dEnhancer. *Hin*dIII-to-*Sal*I fragments containing the various lengths of the VP5 promoter and the AE promoter were then introducted into pSVOd-CAT dEnhancer to give a second series of CAT expression plasmids (Fig. 1B). The 360-base-pair SV40 *Hin*dIII-to-*Xba*I SV40 fragment was cloned into pSVOd-CAT (*Xba*I), giving SV40E-CAT, a plasmid used as a standard measure of transfection efficiencies for individual experiments. The structures of all plasmids described were confirmed by chain termination dideoxy nucleotide sequencing of double-stranded alkali-denatured plasmids (10) by using the primers GATAGTCATGCCCCGCGC for the *Sal*I site of pBR322 and CCATTTTAGCTTCCTTAGC for the 5' region of the CAT gene.

Deletion of bases in the VP5–168 CAT plasmid was performed by sequential digestion of plasmid DNA linearized at the *Hin*dIII site by exonuclease III and nuclease S1 according to published protocols (28). *Xho*I linkers were ligated at the deletion endpoints, and bacteria were transformed with reaction products of the required deletion lengths. Plasmid clones from bacterial minicultures with *Xho*I-to-*Sal*I fragments of desired length were sequenced by using the pBR322 *Sal*I site primer, and a deletion (VP5[−50/−168]−pSVOd) was used in the experiments.

Plasmids containing the HSV ICP4 gene (pSG48 K/B) and ICP0 gene (pRS-1) were provided by R. Sandri-Goldin. Plasmid pSG28 (8.8 kilobases) contains the complete ICP4 gene, its promoter, and its upstream regulatory region in pUC18. Plasmid pRS-1 contains the complete ICP0 gene, promoter, and regulatory elements in a 4.8-kilobase fragment in pUC18.

RESULTS

Responses of HSV VP5 and AE promoters to *cis* activation by the SV40 enhancer. Rabbit skin cells were transfected in parallel with plasmids containing various lengths of the VP5 promoter region or the AE promoter region with or without linked SV40 enhancer sequences. Construction of these plasmids is described above (Fig. 1B). The CAT enzyme activity induced by the promoters on these plasmids was

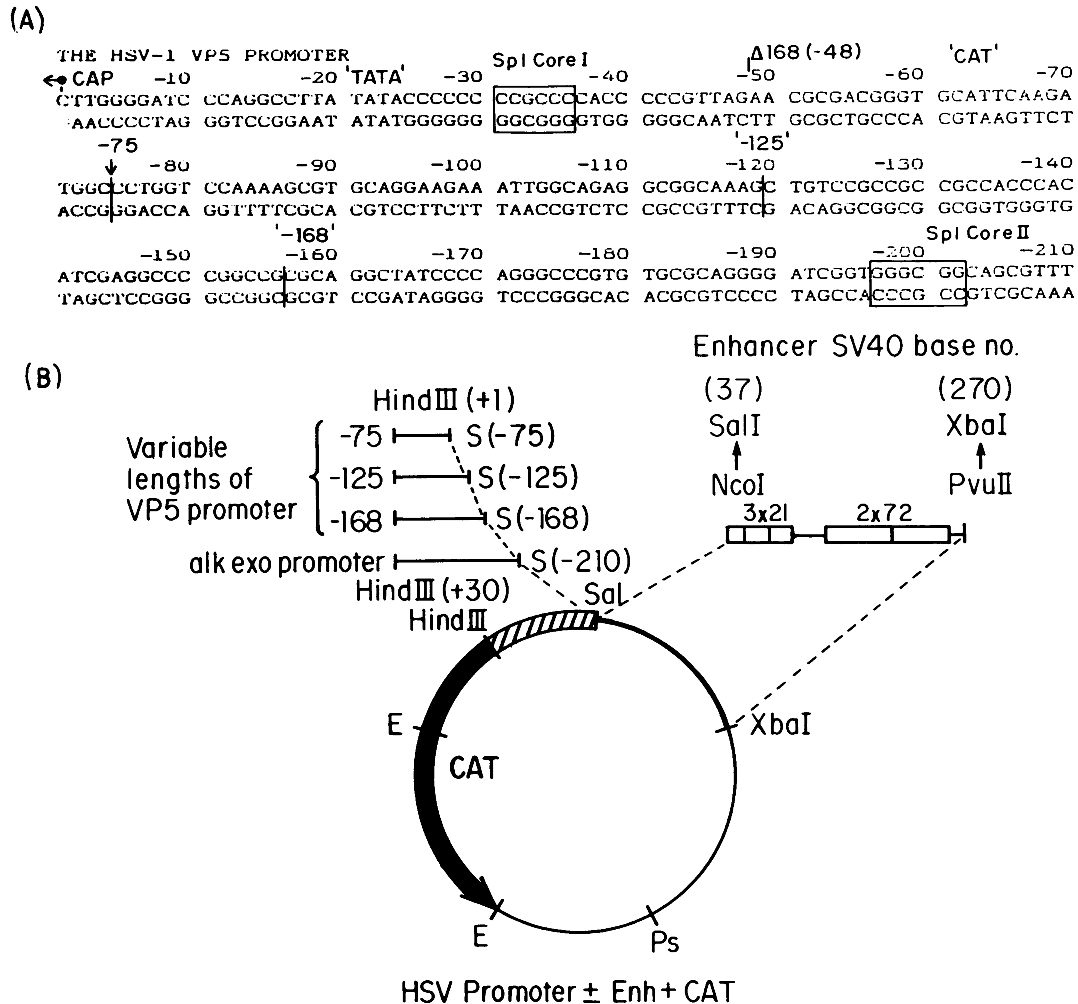

FIG. 1. (A) Relevant features of the VP5 promoter. These include the locations in the sequence where *Sal*I linker oligonucleotides were introduced to generate the –168, –125, and –75 series of promoter deletions and the nucleotide at which the *Xho*I linker was introduced to generate a segment of the VP5 promoter containing predominantly regulatory sequences. Other notable sequence homologies (i.e., the TATA box, CAAT box, and transcription factor Spl binding sites) are shown. Numbering is from the mRNA cap site at –1. (B) Structure of plasmids used to analyze *cis* and *trans* response of the various VP5 promoter segments. Preparation of the parental plasmid pSVOd-CAT has been described previously (13). Modification of this plasmid involved replacement of the pBR322 sequences 650 to 1424 by the SV40 sequences from 37 (*Nco*I) to 270 (*Pvu*II) by conversion to *Sal*I and *Xba*I sites, respectively, as shown. The SV40 sequences include the two 72-base-pair repeats and the three G + C-rich 21-base-pair repeats. The AE promoter segment cloned into the plasmids extends from +30 to −210, and the various lengths of the VP5 promoter were cloned from the *Hin*dIII site at +1 to the appropriate point upstream. E, *Eco*RI; Ps, *Pst*I; S, *Sal*I.

measured in extracts prepared from uninfected cells (i.e., constitutive levels) and from cells infected at low multiplicity with HSV-1. A typical result is illustrated in Fig. 2.

Qualitatively, it was apparent that much greater levels of CAT enzyme were synthesized in uninfected cells transfected with the VP5–75 CAT plasmid containing the SV40 enhancer sequences than in cells transfected with plasmids containing either the VP5−125 or the VP5–168 promoter. Also, the VP5–125 construct induced higher CAT levels than did the VP5–168 plasmid in uninfected cells (compare Fig. 2, tracks ix, xiii, and xvii). We also note that the unenhanced constitutive activity of the VP5–75 promoter in rabbit skin fibroblasts is less than was previously described for HeLa cells (13); such differences have recently been described as a funciton of the cell lines used for transfection (21).

All the enhancer-containing plasmids responded to HSV superinfection with an increased induction of CAT activity; however, the response differed for the different lengths of VP5 promoter. Both the VP5–75 and the VP5–125 plasmids gave higher CAT levels upon superinfection than did their enhancerless counterparts, whereas the VP5−168 plasmid containing the enhancer sequences induced less enzyme activity when superinfected compared with the enhancerless control (compare Fig. 2, tracks viii and xi, xii to xv, and xvi to xix).

In the same series of experiments, it can be seen that the SV40 enhancer increased to extremely high levels the constitutive level of CAT enzyme activity expressed from the AE-promoter-containing CAT vectors. In this case, as with the shorter VP5-promoter-containing constructs, the AE promoter plasmid containing the SV40 enhancer responded to superinfection with HSV-1 to produce somewhat higher levels of enzyme than did the enhancerless constructs.

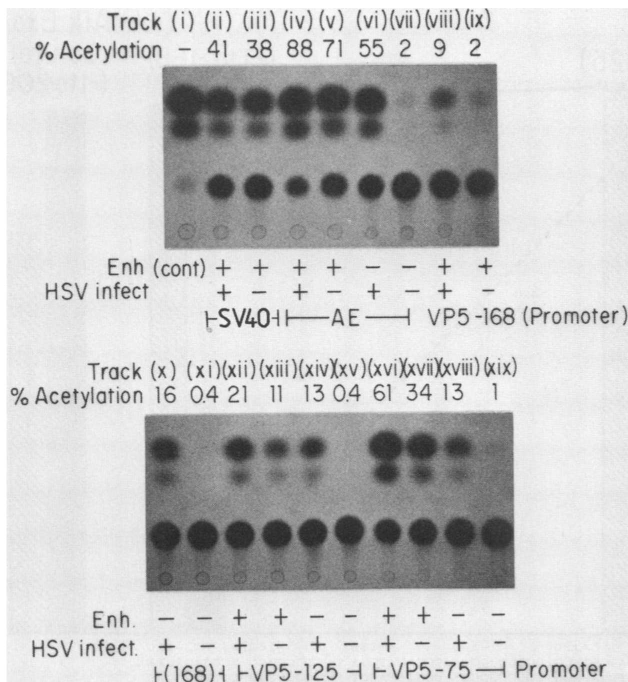

FIG. 2. Differing *cis* and *trans* response of the various lengths of the VP5 promoter. Tracks: i, 0.5 U of CAT enzyme (P-L Biochemicals, Inc.); ii and iii, assays with extracts of cells transfected with plasmids containing the SV40 early promoter (SV40E-CAT); iv to vii, plasmids with the AE promoter; viii to xi, plasmids with +1 to −168 VP5 promoter; xii to xv, plasmids with the +1 to −125 VP5 promoter; xvi to xix, plasmids with the +1 to −75 VP5 promoter. Enh., Plasmids containing (+) or lacking (−) the SV40 enhancers. HSV infect., Extracts prepared from transfected cells after low-multiplicity infection (+; 2 PFU per cell) or mock infection with culture medium alone (−). % Acetylation, Percent conversion of [^{14}C]chloramphenicol to its acetylated forms.

However, CAT enzyme activity was higher in both infected and uninfected cells transfected with this plasmid than in superinfected cells transfected with the enhancerless promoter.

A plasmid containing the full SV40 T antigen (early) promoter sequences, including enhancers, did not respond to HSV-1 superinfection (Fig. 2, tracks ii and iii). This suggested that *trans* activation was specific for HSV promoters in this system.

To ensure that the close juxtaposition of the SV40 enhancer element to the VP5 promoter constructs did not have some nonspecific effect on *cis* activation, a series of experiments were carried out in parallel to those described in the legend to Fig. 2 with constructs in which the SV40 enhancer was introduced into a modified *Ava* site 775 base pairs upstream of the VP5–168 and VP5–75 promoters. Again, the VP5–168 promoter was not activated by the SV40 enhancer sequences, whereas the corresponding VP5–75 construct was *cis* activated. The *trans* responses of these promoters were not affected by the SV40 sequences (data not shown).

The CAT activity expressed as percent conversion of chloramphenicol to its acetylated forms (Fig. 2) was used to calculate the ratio of *cis* or *trans* activation as a function of VP5 promoter length. The effect of VP5 promoter length on the two mechanisms of activation was inversely related. An example of this relationship, representative of four independent experiments with three different isolates of each plasmid, is illustrated in Fig. 3. Thus, on average, the VP5–168-promoter-containing constructs were induced 35-fold in *trans* but only 5-fold in *cis*, whereas the VP–75-promoter-containing constructs were induced 13-fold in *trans* and 34-fold in *cis*.

This inverse relationship of the two activation mechanisms suggested that the differing response of the VP5 promoter containing different amounts of upstream sequences was not determined by the enhancer sequence itself but was related to the actual functional properties of the viral promoter. This differential effect was not seen with the AE promoter (a β promoter); *cis* and *trans* induction of the early AE promoter was 35- and 28-fold, respectively. Such results may indicate differences in the mechanisms responsible for the activation of different kinetic classes of viral promoters.

Regulatory element (–50 to –168) of the VP5 promoter is not readily movable. The failure of the VP5–168 promoter to respond to adjacent SV40 enhancers (Fig. 2) suggested that sequences in the VP5 promoter, from approximately –70 to –170 bases 5′ of the transcription cap site, block activity of the late viral promoter in uninfected cells. These properties resembled those of the silencer element found in the *Saccharomyces cerevisiae* HMR mating type locus (4), which may act as a target sequence for a repressor-type polypeptide. To assess this possibility, we used the strategy of Brent and Ptashne (5), who found that introduction of a repressor-binding site prevented the *cis* activation of a downstream yeast promoter by an upstream yeast enhancer element. In the following experiments, a specific fragment of the VP5 promoter was introduced into the enhancer-containing AE promoter CAT construct (Fig. 4A). The VP5 regulatory fragment, prepared by S1-exonuclease III processive digestion from the *Hin*dIII site of VP5–178 CAT and extending from –50 to –168 of the VP5 promoter (Fig. 1A), was selected for two reasons. First, the terminus of the deletion at –48 eliminated the TATA box and transcription factor SP1 binding-site sequence homologies (Fig. 1A). This prevented the initiation of transcription within the VP5 promoter. Second, because we chose a deletion 20 base pairs away from the approximate endpoint of the regulatory element at –75 bases 5′ of the VP5 cap site, protein recognition sequences within the regulatory element would not be interrupted.

This regulatory fragment was inserted between the AE promoter and the SV40 enhancer sequences in both orientations to determine whether there was any polarity in the effects of the VP5 sequences on *cis* activation. Enhancerless plasmids were also prepared containing the VP5 element, again in both orientations, and were inserted upstream of the AE promoter to determine effects of the VP5 element on *trans* activation.

Neither orientation of the VP5 element was able to prevent the *cis* activation of the AE promoter by the linked SV40 enhancers (Fig. 4B). Thus, whereas the enhanced AE promoter plasmid expressed sufficient CAT enzyme to give 49% acetylation in uninfected cells (track v), plasmids containing the same promoter but with the VP5 element inserted in the positive or negative relative orientation could induce 69 and 71% acetylation, respectively, as a result of *cis* enhancement (tracks ix and xi). It is unclear whether these differences were significant, but such differences have been observed in two of three independent experiments. Moreover, the levels of CAT enzyme expressed by these plasmids were very much greater than the 4% acetylation induced by the enhancer-containing VP5–168 CAT plasmid in uninfected cells (track xv).

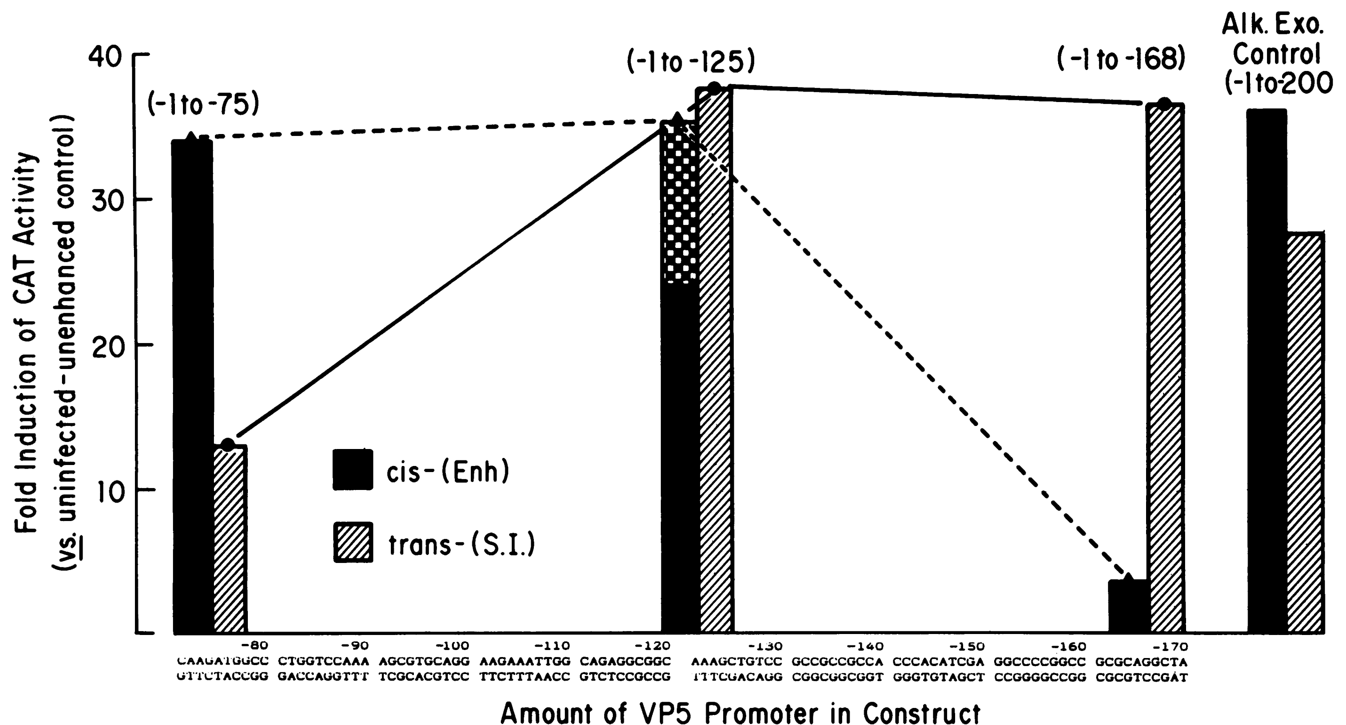

FIG. 3. Schematic summary from four independent experiments illustrating the differences in the *cis* and *trans* responses of the various VP5 promoter sequences. The fold level of activation is the ratio of CAT enzyme activity (as percent conversion of chloramphenicol) induced from the enhancer-containing plasmids in uninfected cells (*cis*) or from enhancerless plasmids in superinfected cells (S.I.; *trans*) to the CAT activity driven by the corresponding unenhanced plasmid in uninfected cells. Data for the VP5−75, −125, and −168 plasmids and AE promoter are shown. Checkered area in the *cis* response of the VP5−125 CAT plasmid indicates scatter of response found only with this particular construct. The VP5 promoter sequences are provided below the graph for reference.

Insertion of the VP5 regulatory element in the tandem (positive) orientation with the AE promoter in the enhancerless plasmid (Fig. 4A) did not affect the *trans* response of the AE-CAT plasmid to superinfection (data not shown). A small but reproducible effect was seen when the VP5 element was introduced in the opposite orientation in the AE promoter. Here, the level of CAT enzyme induced by this plasmid was lower than that seen from the parental AE-CAT plasmid (59 versus 74% acetylation; Fig. 4B, tracks xii and vi). This suggested that the VP5 regulatory sequence interferes to some extent with the activity of the AE promoter.

These experiments provided good evidence that the VP5 element does not have the properties of a mobile silencer element. However, it was of interest to know whether the VP5 sequences could transfer *cis* and *trans* responses of the VP5 promoter to another HSV core promoter of the same general size as that defined for the VP5 promoter. Accordingly, upstream sequences of the AE promoter (–79 to –210 bases 5′ of the cap site) were replaced with the VP5 regulatory element (–50 to –168 bases 5′ of the VP5 cap site) in plasmids either containing or lacking the SV40 enhancers. The VP5 regulatory element has just a small effect on the *cis* activation of the AE core promoter by the SV40 enhancer (Fig. 5, tracks iv and viii). A more dramatic effect is seen with constructs in which the enhancer element is placed directly 5′ of the AE core promoter. In this case, the interposition of the VP5 regulatory element can suppress the *cis* activation by at least a factor of four (data not shown). In this case too, however, there is a significant level of *cis* activation which is much greater than that seen in the native VP5–168 promoter. Thus, this element, which serves to silence the *cis* response of the VP5 core promoter, does not possess readily movable properties.

VP5 core promoter responds poorly to cooperative *trans* activation by HSV immediate-early polypeptides ICP4 and ICP0. It was previously shown that the VP5 promoter, extending from –1 to –650 bases 5′ of the transcription cap site, could be *trans* activated by cotransfection of a cosmid (HSV-1 *Hin*dIII fragment C, 0.65 to 0.87 map units) encoding α (immediate-early) genes for ICP4, ICP0, ICP27, and part of ICP22, in addition to several other viral genes (13). It was of interest to know whether ICP4 and ICP0, together or alone, could mediate *trans* activation of the VP5 promoter as had been shown for some other HSV promoters (15, 22, 41, 45). The relative response of the VP5 CAT plasmids bearing various lengths of promoter to these immediate-early gene products was also of considerable interest. Therefore, plasmids containing individual α genes were cotransfected in various combinations with two plasmids, VP5–168 CAT and VP5–75 CAT (Fig. 6).

The combination of ICP4 and ICP0 induced a low level of *trans* activation with both VP5 promoters (tracks iii and vii). The VP5–75 promoter appeared significantly less responsive to the combination of α genes than did the VP5–168 promoter as measured by CAT enzyme levels. In both cases, as shown by the ratios of induced activity, the induction of CAT activity was significantly increased over that seen in cells which contained only the VP5 promoter elements (tracks iv and viii). Cotransfection with the ICP4 gene alone provided *trans* activation of a small amount of CAT activity from both VP5 promoters (tracks i and v), but the induction was less than that seen with the combined genes. This result was as observed by O'Hare and Hayward for the HSV

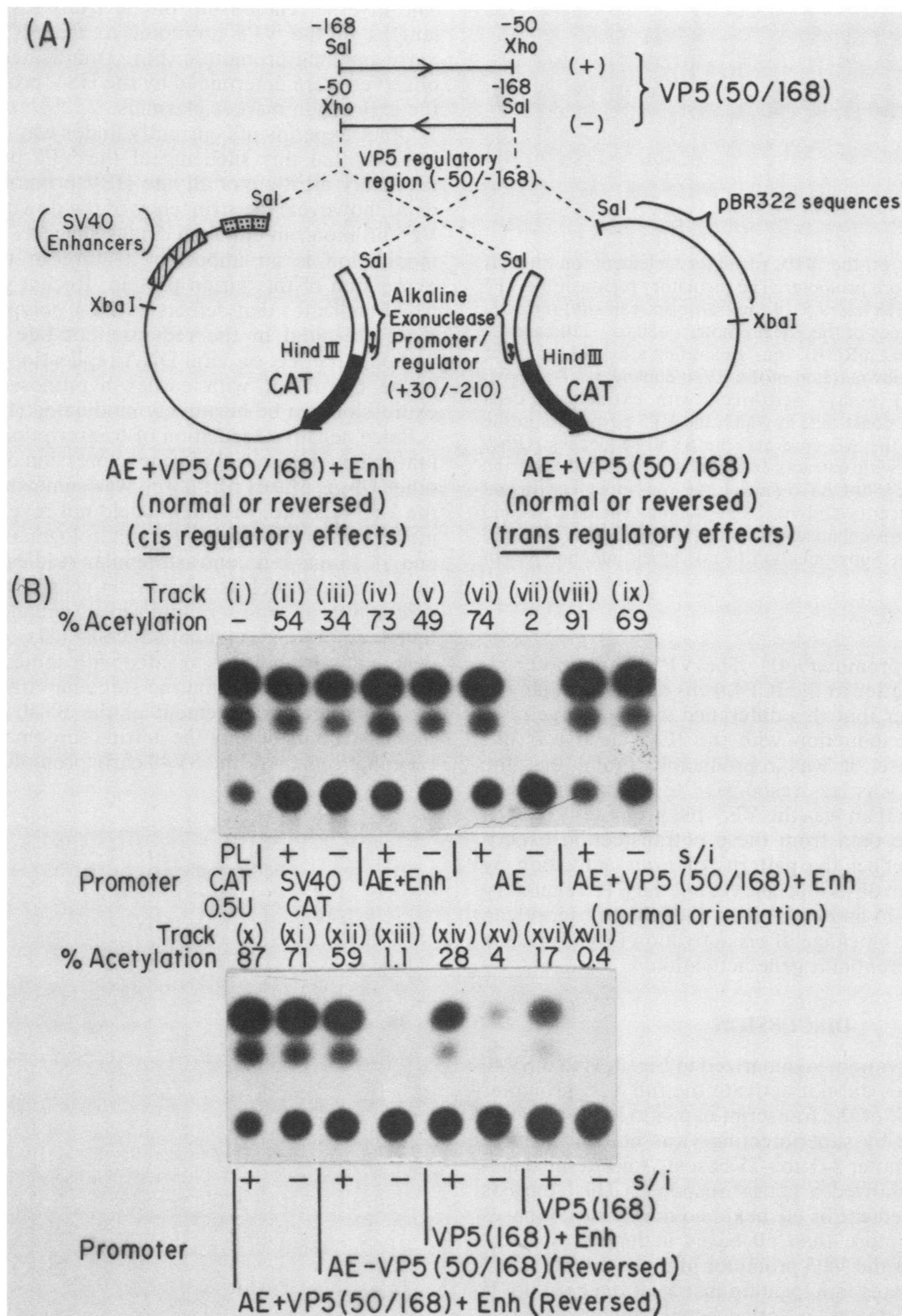

FIG. 4. Effects of the VP5 regulatory region on the *cis* and *trans* response of the complete AE promoter. (A) Illustration of the plasmids used. The VP5 regulatory element (−50 to −168 bases 5′ of the transcript cap site) flanked by *Xho*I and *Sal*I restriction sites was inserted in both orientations (+ and −) immediately upstream of the AE promoter (+30 to −210) and downstream of either the SV40 enhancers containing the three 21-base-pair repeats (▩) and two 72-base-pair repeats (▨) or the *Sal*I site of pSVOd containing only pBR322 sequences. (B) CAT assays. Tracks: i, 0.5 U of CAT enzyme; ii and iii, extracts from cells transfected with SV40-CAT; iv and v, AE promoter plus upstream SV40 sequences; vi and vii, AE promoter with no *cis*-activating enhancers; viii and ix, VP5 regulatory sequences (−50 to −168) inserted in the same orientation as flanking AE promoter and SV40 enhancers; x and xi, VP5 element inserted in reverse orientation to flanking AE and SV40 sequences; xii and xiii, VP5 element inserted in reverse orientation upstream of AE promoter in enhancerless pSVOd-CAT; xiv and xv, VP5–168 promoter adjacent to SV40 enhancers (Fig. 1B); xvi and xvii, VP5−168 promoter with no enhancers. Superinfection (s/i; +) and mock infection (−) of transfected cells are indicated.

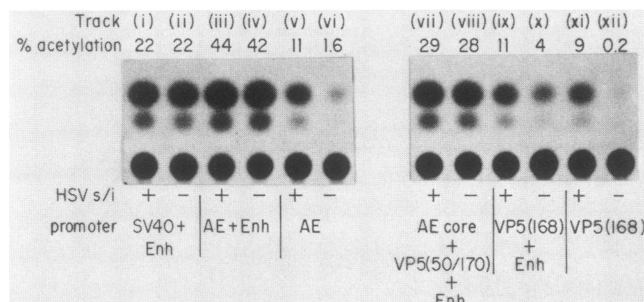

FIG. 5. Influence of the VP5 regulatory element on the *cis* response of the AE core promoter. The regulatory region of the VP5 promoter (−50 to −168 bases 5′ of the transcript cap site) replaced the upstream sequences of the AE promoter (−80 to −210 bases 5′ of the cap site for that mRNA), thus generating a hybrid promoter placed immediately downstream of the SV40 enhancers. Tracks vii and viii show CAT assays performed with extracts of cells transfected with this construct, in which the VP5 element is in the same orientation as the AE core and the SV40 sequences. Other tracks show assays with extracts from cells transfected with the following plasmids: SV40-CAT (i and ii), AE plus enhancer (iii and iv), AE minus enhancer (v and vi), VP5−168 plus enhancer (ix and x), and VP5−168 minus enhancer plasmids (xi and xii) (as illustrated in Fig. 1B). HSV s/i, Superinfection (+) or mock infection (−) of transfected cells.

thymidine kinase promoter (41). The VP5–75 promoter always responded better to the ICP4 *trans* activation than did the larger promoter, but this difference was not large. The situation with the induction with the ICP0 gene was different. In this case, it was reproducibly found that the VP5–75 promoter was less responsive to the action of the ICP0 gene product than was the VP5–168 promoter (tracks ii and vi). Thus, the data from these cotransfection experiments generally reflect the patterns of *trans* activation by virus superinfection described above, but the VP5 regulatory element between –75 and –168 bases 5′ of the transcript cap site defined by the blockage of *cis* activation (Fig. 3) is also detectable by differential α gene activation.

DISCUSSION

The major observation, summarized in Fig. 3, is that SV40 enhancer sequences did not activate the full VP5 promoter (–1 to –168 bases 5′ of the transcript cap site) in the absence of factors induced by superinfecting virus but did activate the VP5 core promoter (–1 to –75 bases). An intermediate-length construct showed a partial response. This suggests that a sequence element or elements no more than 90 bases and probably no more than 50 bases in length mediates down-regulation of the VP5 promoter in the uninfected cell. This element operates in a location just 5′ of the core pol II promoter itself. A strong *cis*-acting signal which is effective in activating at least one model β (early) HSV promoter could not overcome this negative modulation.

The SV40 enhancer was used to increase the low level of endogenous transcriptional activity from the HSV promoters tested. Although there are previous reports of enhancers that were incompatible with heterologous promoters, the determinant of this effect has been the tissue or positional specificity of the enhancer sequences (1, 9, 19). The SV40 enhancer was chosen because of its broad specificity, to eliminate any systemic effects that might complicate the interpretation of the data. This generic enhancer has been shown to be active with many promoters, including several of herpesvirus origin, and in many cell lines (40, 48, 53, 58). The inverse relationship of the *trans* response of various lengths of the VP5 promoter to the *cis* response of the corresponding promoters (Fig. 3) indicated that the effects observed were determined by the HSV promoter segment in the expression marker plasmids.

Other experiments currently under way in our laboratory indicate that this silencing of the VP5 promoter is not a necessary attribute of all late HSV promoters (unpublished data); however, the stringency of the down-regulation of the VP5 promoter in uninfected cells suggests that this negative modulation is an important feature in the regulation of expression of this virion protein. Recent genetic evidence, which indicates that defects in the α polypeptides ICP4 and ICP27 resulted in the reduction of late gene expression without an effect on viral DNA replication (16, 46), appears to be consistent with earlier hypotheses that late gene expression can be negatively modulated (13, 17).

Since negative regulation of transcription plays an important role in the control of gene expression of herpesvirus and other DNA viruses (23, 42), it was somewhat surprising that the VP5 regulatory element could not reverse the enhancer-mediated *cis* activation of a heterologous promoter (Fig. 4 and 5). In marked contrast, similar studies with other negative elements, such as silencers in yeasts (4, 5) or the murine sarcoma virus long terminal repeat enhancer in undifferentiated embryonal carcinoma cells (27), have revealed a degree of portability associated with some *cis* effectors. The experiments with the murine sarcoma virus enhancer, however, involved replacement of the SV40 enhancers rather than an examination of the murine sarcoma virus silencer on *cis* enhancement of the SV40 early promoter (27). In another

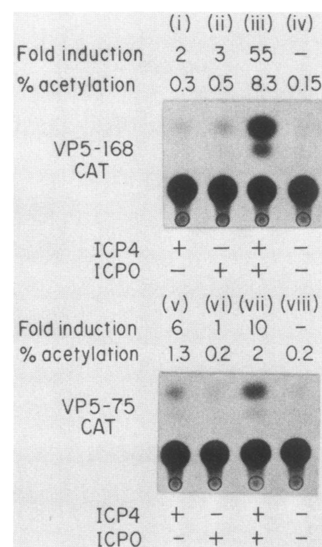

FIG. 6. Comparative response of various VP5 promoter lengths to cotransfected plasmid-borne copies of HSV-1 immediate early genes for ICP4 and ICP0. Cells were transfected with 10 μg of the VP5−168 CAT (tracks i to iv) or VP5−75 CAT (tracks v to viii) plasmids together with either 2 μg of a plasmid carrying the ICP4 gene, 2 μg of the ICP0 gene, or 2 μg of each plasmid-borne immediate-early gene. The total mass content of DNA in each transfection cocktail was adjusted to 14 μg with pBR322 plasmid DNA, and the various regimens of cotransfection for each VP5 CAT plasmid are indicated by + and − symbols. All assays were performed 48 h posttransfection.

instance, transcription and replication of recombinant polyoma genomes in different cell lines were regulated not only by the presence or absence of *cis*-acting substitute positive and negative enhancer elements but also by the relative position of the endogenous polyoma A enhancer and the heterologous enhancers (7). Thus, the interactive arrangement—and therefore the context—of negative and positive transcriptional control elements may have more than a passive influence on the apparent portability of individual elements.

We discuss below several possible reasons why the VP5 regulatory region did not reverse *cis* activation out of context in the complete (-1 to -210) or core (-1 to -79) AE promoter, although still immediately adjacent to the SV40 enhancers (compare Fig. 1B and 4A). It is important to note, however, that the lack of portability of the negative modulation does demonstrate that the silencing effect is not some artifact of the construction of the expression markers such as the incorporation of a cryptic promoter which is dominant over the VP5 promoter upon upstream *cis* activation.

One reason for the lack of mobility of the silencer effect of the VP5 promoter may be that full activity of the VP5 promoter element in silencing *cis*-activated or basal levels of transcription in uninfected cells requires sequences from the region between -1 and -75 bases 5′ of the transcription cap site in addition to the upstream sequences between -75 to -168 bases 5′ of this point. Recently, the *cis* alignment of two distinct elements from the regulatory region of the SV40 genome was shown to be necessary for full SV40 late promoter activity (3), and the same may be true for the constitutive and regulatory elements of the VP5 promoter. Because the VP5 core promoter responds well to *cis* activation, it is clear that the sequences from -1 to -75 are unable to prevent enhancer activity in the absence of the region of the promoter from -75 to -168.

Since the hybrid AE-VP5 promoter responded to *cis* activation but the wild-type VP5 promoter did not, the nucleotide sequences of the AE and VP5 core promoter elements were compared to determine whether some obvious required sequence element was altered in the construct. Given the diversity of HSV promoter sequences (55), it is hardly surprising that there are some significant differences in these elements. For example, the AE promoter CAT box homology at -72 reads CAAT (20), but VP5 CAT homology at -70 reads GAAT (Fig. 1). Interestingly, in the enhancer-containing VP5-75 construct, the relative organization of the Spl binding site and CAT box from the VP5 promoter and the Spl sites from the SV40 sequences is similar to the Spl-CAAT box-Spl arrangement in the HSV thymidine kinase promoter (34, 39) and may fortuitously recreate an early-type promoter responsive to *cis* activation.

Another possible reason for the lack of mobility of the VP5 regulatory region *cis*-silencing effect is that the complete VP5 promoter may simply be a very poor *pol* II promoter such that even the juxtapositioning of the SV40 enhancer sequences with attendant transcription factors does not provide the full complement of transcription factors necessary for activation of the VP5 promoter. This would imply that the VP5 regulatory element behaves as a neutral segment of DNA in uninfected cells and, as such, would not affect SV40 enhancer activation of downstream promoters (18, 56). This scenario is somewhat difficult to accept per se since activity of the core VP5 promoter is equivalent to the activity of the AE and other β (early) promoters in uninfected cells whether the promoters are enhanced, as in this work, or not (13). Finally, our data suggest that the involvement of stereospecific alignment of enhancer and promoter sequences (51) is unlikely.

Although the VP5 promoter regulatory region is not a silencer per se, it is quite clear that virus-induced transcription factors interacting with it are necessary for full activation of the VP5 promoter. These factors may include the α gene products. The comparative response of the VP5-168 and VP5-75 CAT plasmids to cotransfected α genes parallels the response of the two plasmid-borne promoters to virus superinfection (Fig. 6 versus Fig. 2), i.e., the VP5-168 promoter responds significantly better than does the VP5-75 promoter to superinfection and to α gene products (ICP4 and ICP0). However, other factors expressed as early gene products may well be required for a quantitative response equivalent to superinfection. Such a hierarchy of sequentially expressed transcription factors provides a plausible and testable molecular model for the temporal regulation of viral gene expression as an important factor in the biology of the herpesviruses. Such models have, of course, been discussed over the last decade or more (14, 30, 35, 55).

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