The Herpes Simplex Virus Type 1 Major Capsid Protein (VP5-U_L19) Promoter Contains Two *cis*-Acting Elements Influencing Late Expression

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The herpes simplex virus type 1 (HSV-1) major capsid protein VP5 gene (U_L19) is expressed with $\beta\gamma$ (γ_1 [leaky late]) kinetics. We have previously described the construction of recombinant HSV-1 in which the VP5 promoter was engineered to control the expression of the bacterial β -galactosidase gene as a reporter (C.-J. Huang, S. A. Goodart, M. K. Rice, J. F. Guzowski, and E. K. Wagner, *J. Virol.* 67:5109–5116, 1993). Here we describe further mutational analysis in recombinant viruses. We have precisely defined the boundaries of the VP5 promoter and identified two regions important for both the level and the kinetics of expression. The 5' boundary was located at –48 relative to the initiation site of transcription by analyzing a series of nested deletions in the upstream sequence, and although a number of *cis*-acting sites influencing transient expression have been identified upstream of this point, these sites have no role in promoter activity during productive infection. Deletion of an Sp1-binding site located between –48 and the TATA box at –30 greatly reduced VP5 promoter activity late but not early after infection. A *cis*-acting element whose sequence resembles the human immunodeficiency virus type 1 initiator was located between –2 and +10 in the VP5 sequence by characterizing a series of deletions and site-directed block mutations downstream the TATA box. This element defines the 3' limit of the VP5 promoter, and like the upstream element, disruption of this element also inhibited promoter activity late in the productive cycle.

The herpes simplex virus type 1 (HSV-1) genome contains more than 80 separate transcription units, each controlled by an individual promoter. Despite this panoply of promoters, HSV transcripts and the proteins that they encode are expressed in two general kinetic classes, early and late, during the productive cycle of infection. The synthesis of early transcripts peaks prior to or concomitant with the onset of viral DNA replication and declines thereafter, while maximal rates of synthesis of late transcripts occur following this “watershed” (reviewed in references 53 and 54). As noted below, both the early and late kinetic classes of transcripts can be further divided into subclasses on the basis of details of individual patterns of expression, and, as with other nuclear replicating DNA viruses, all transcripts are expressed via cellular transcriptional enzymes and factors. It is, thus, of great interest to know how the promoters for the different kinetic classes of transcripts interact with cellular transcriptional machinery and how these interactions change as the infection progresses.

We and many other investigators have been involved in the detailed experimental investigation of such questions for the last decade and a half. It has been suggested that the transcription of early transcripts is mediated by promoters which serve as models for cellular polymerase II promoters in general (49). As exemplified by the, now classic, studies of McKnight and coworkers on the promoter controlling expression of the early (β) transcript encoding the enzyme thymidine kinase (U_L23) (9, 36–38), such promoters contain a canonical TATA homology 25 to 30 bases upstream of the transcript start site which is important in the assembly of the preinitiation complex mediated by TFIID and ancillary factor binding sites, including Sp1 and CAAT binding factor sites located between the TATA box

and a site approximately 100 bases upstream of the transcription start site. If these factor binding sites are not strictly necessary for minimal promoter function, they are clearly important for full promoter activity (22, 25, 57). Additional experimental evidence that promoters controlling early HSV transcripts are canonical polymerase II promoters comes from the observation that a cellular promoter recombined into the viral genome is expressed with normal early (β) kinetics (49, 50).

The expression of a major subclass of early HSV genes, the α (immediate-early) genes encoding important regulatory proteins, is also mediated by canonical polymerase II promoters; these five transcripts are distinguished by their immediate kinetics of expression and expression in the absence of *de novo* viral protein synthesis following infection (reviewed in references 42 and 54). One or more enhancer elements upstream of the core promoter which interact with a complex containing two cellular transcription factors, Oct1 and CCF, and the virus-coded virion-associated α -*trans*-inducing factor (U_L48; α -TIF or VSP) are critical in mediating immediate-early transcription (reviewed in reference 41). These modular enhancer elements in α promoters are functional parallels to those found influencing expression of (immediate) early regulatory genes expressed by smaller DNA viruses.

Strict late (γ) transcripts are either absent or present at very low levels prior to viral DNA replication and generally encode viral structural and assembly proteins (54–56). Structural analysis of several γ promoters, especially that controlling expression of gC (U_L44), has led to the model that γ promoters consist of a TATA box and critical *cis*-acting elements at or near the transcript cap site and extend no more than 30 bases or so (16–18, 24). However, our own detailed investigation of the promoter controlling expression of the γ transcript encoding the U_L38 (VP19C) capsid protein suggests a more complex

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scenario for at least some strict late promoters (10, 15). The U_L38 promoter has a critical length of 65 bases in which two elements, a 40-base core extending from the TATA homology at -31 to sequences spanning the cap site to +9 and the downstream activation sequence extending from +20 to +33, are both required for full activity; some other strict late promoters also exhibit this structure (14). Despite such differences in detail, the structure of γ promoters clearly exhibit significant differences from those of early promoters; these include the absence of *cis*-acting regulatory elements upstream of the TATA box and the presence of critical sequences spanning the transcription start site.

The other major class of late transcripts, the "leaky-late," or $\beta\gamma$, transcripts is readily detectable in the absence of viral DNA replication but is maximally expressed only afterwards (54, 56). Analysis of recombinant viruses containing engineered chimeric model promoters with both β and γ promoter elements has led to the argument that $\beta\gamma$ promoters are natural and functional chimeras of early and strict late promoters in which *cis*-acting elements upstream of the TATA box of the former act to turn on the promoter prior to viral genome replication while elements of late promoters function to obviate shutoff and allow maximal expression (33, 46). But, a complete functional analysis of elements of a wild-type $\beta\gamma$ promoter functioning in the viral genome has been lacking.

The transcript encoding the major capsid protein (VP5-U_L19) is abundant and expressed with $\beta\gamma$ kinetics, and the sequence of its promoter has features consistent with the chimeric model for $\beta\gamma$ promoters discussed above. There are an excellent TATA box at -30; consensus binding sites for the Sp1 transcription factor at -48, -144, -208, and -268; a site for CAAT-binding protein at -111; and, finally, a YY1 factor binding site at -78. In addition, nominal binding sites for the HSV ICP4 regulatory protein occur at -228 and -353 (1, 20). Results of transient expression assays have suggested that the VP5 promoter can be dissected into three domains: a core promoter domain containing sequences downstream of -83 which is responsible for the basal promoter activity, a second domain between -83 and -125 which negatively regulates basal activity, and a third domain upstream of -125 which allows for expression to be activated by immediate-early (α) gene products (1). Recently, Millette and colleagues have identified the YY1 site as critical for full basal promoter activity (4, 39).

Since the VP5 protein is absolutely required for virus replication, mutational analysis of the VP5 promoter in its normal location in the viral genome is not practical; however, we have recently developed and described a system for analysis of HSV promoters in the context of the viral genome in which the promoter of interest is engineered to control expression of a reporter gene (β -galactosidase) in a standard location in the viral genome (the interior of the U_L44 gene) (13). In a previous communication concerning the VP5 promoter, we described the basic patterns of expression by recombinants in which different extents of a viral leader sequence were included with a large amount of upstream sequence (20). We showed that sequence elements near and/or downstream of the transcription start site are important for the full level mRNA expression with normal $\beta\gamma$ kinetics. Such a result was interpreted as formally consistent with the chimeric model for $\beta\gamma$ promoters in that this promoter contains sequences near the transcript cap site which are analogous to those seen in the γ U_L38 promoter. We also found, however, that substitution of these elements into the β dUTPase (U_L50) promoter did not alter the early kinetics of that promoter. This result would not be predicted by the simplest chimeric model for $\beta\gamma$ promoters.

In the present study, we further define the sequence elements responsible for the wild-type levels and kinetics of expression of a transcript controlled by the VP5 promoter by means of deletional and mutational analysis in the context of the viral genome. Our results demonstrate that this promoter has critical sequence elements which clearly differentiate it from either a nominal early or strict late promoter. This, in turn, suggests that some $\beta\gamma$ promoters are structurally unique, and we conclude that global models of $\beta\gamma$ promoters as simple chimeras of β and γ promoters are invalid.

MATERIALS AND METHODS

Cells and virus. Rabbit skin cells were maintained at 37°C under 5% carbon dioxide in Eagle's minimal essential medium (GIBCO) containing 5% cadet calf serum, 100 U of penicillin, and 100 μ g of streptomycin per ml. Wild-type HSV-1 strain 17syn⁺ and its progeny recombinant viruses were grown in rabbit skin cells, and titers were assayed in Vero cells. These procedures have been described previously (10, 13, 15, 20).

Construction of plasmids. The promoter/leader region of the VP5 gene cloned from the KOS(M) strain of HSV-1 was used for all experiments. The sequence of this region is identical to that of the 17syn⁺ strain except for the number of G residues immediately upstream of the TATA box. Thus, the sequence CG₁₁TATATAA in strain 17syn⁺ is CG₈TATATAA in strain KOS(M) (1, 35).

To make nested deletions in the upstream sequences of the VP5 promoter, the VP5 promoter sequence extending from -364 to +63 in conjunction with part of the β -galactosidase reporter gene was subcloned into pGEM3 (Promega, Madison, Wis.) as an *Xba*I-*Kpn*I fragment from the VP5(Δ +63) plasmid [this plasmid was termed VP5(Δ +73) in the previous paper (20)]. The VP5 upstream sequences were progressively shortened from the *Xba*I site, by using the Bal 31 deletion method (44). The *Xba*I site was reintroduced at deletion endpoints with a linker. Plasmids with *Xba*I-*Kpn*I fragments of desired size were confirmed by dideoxy sequencing with the SP6 primer (Promega). Fragments containing seven representative deletion clones were cloned into the gC (U_L44) recombination vector (13) by replacing the full-length VP5 promoter in VP5(-364/+63) [this recombinant was termed VP5(Δ +72) in the previous paper (20)]; the resulting constructs, VP5(-137/+63), VP5(-104/+63), VP5(-79/+63), VP5(-48/+63), VP5(-42/+63), VP5(-32/+63), and VP5(-25/+63), all contain 63 bases of the VP5 leader sequence and various lengths of the upstream sequence.

Plasmid constructs CH218, CH219, and CH220 were made through oligonucleotide-directed mutagenesis. To this end, the VP5 promoter sequence extending from -364 to +63 was cloned into M13mp18 as an *Xba*I-*Kpn*I cassette with an adjoining β -galactosidase sequence. A single-stranded DNA template was isolated and used for generating mutations in the YY1 site with three mutant oligonucleotides as described previously (19).

Constructs VP5(-364/+4) and VP5(-364/+16) were made by replacing the *Bam*HI-*Xho*I fragment containing VP5 sequence from -13 to +63 in VP5(-364/+63) with appropriate double-stranded oligonucleotides.

Plasmid constructs CH231, CH233, CH235, and CH237 contained block mutations in the region from -15 to +17 relative to the transcript cap site and were made by oligonucleotide-directed mutagenesis as described above for CH218 through CH220.

The UTP-VP5 construct (dUTPase/VP5) was described previously (20). The VP5-UTP construct was generated by

substituting the *XbaI*-*Bam*HI fragment of the VP5 promoter for the *XbaI*-*Sma*I dUTase promoter fragment in a dUTase/ β -galactosidase construct (21).

Generation of recombinant viruses. Plasmids containing modified promoters were cotransfected with infectious HSV DNA into fresh rabbit skin cells, and the recombinant viruses were screened and analyzed as described previously (13, 20). For each pure recombinant virus, the sequence around the insertion site was confirmed by direct PCR sequencing (20). Separate isolates of specific recombinant viruses were generated by separate cotransfections and screenings.

RNase protection and primer extension assays. Total RNA was isolated from HSV-1-infected rabbit skin cells by the guanidium isothiocyanate-cesium chloride method (12). RNA samples were prepared at times (in hours) postinfection (hpi) as indicated in the figures. For the RNase protection assay (20), 10 μ g of total RNA was hybridized with 0.5×10^6 to 1.0×10^6 cpm of probe at 55°C in 50 μ l of buffer containing 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.4), 400 mM NaCl, and 1 mM EDTA. The mixture was digested with 1,000 U of RNase T₁ (Sigma) for 1 h at 30°C, the enzyme was removed by phenol-chloroform extraction, and protected RNA was fractionated on 8 M urea-8% polyacrylamide gels along with appropriate DNA size markers. The chimeric VP5/ β -galactosidase mRNA was measured by using a riboprobe that protects 215 bases of the β -galactosidase gene leader sequence for all recombinant viruses. The VP5-specific probe covering VP5 sequences extending from -75 to +139 protects 139 bases of the wild-type VP5 transcript as well as VP5-specific sequences within this region in chimeric VP5/ β -galactosidase transcripts. All antisense riboprobes used in this study were made from pGEM vectors (Promega). Primer extension was carried out with avian myoblastosis virus reverse transcriptase as described previously (10, 15); recently, we have used a Promega kit, with good effect. Extension was carried out from a primer beginning 57 bases into the β -galactosidase reporter gene (5'-GTGTTTCGAGGGGAAAATAGGTTGCGCGAG-3'). When used, densitometry was performed with a Bio-Rad model 620 video densitometer.

β -Galactosidase assays. Rabbit skin cells were infected with specific recombinant viruses at a multiplicity of infection of 5 PFU per cell. Infected cell extracts were prepared at 18 hpi and assayed for β -galactosidase activity as described previously (10). Enzyme activity was measured in units per milligram of protein in which 1 U cleaves 1 nmol of *o*-nitrophenylgalactopyranoside (ONPG) per min at 37°C. Typically, recombinant virus containing the VP5 promoter extending from -362 to +226 expressed 1,200 U of activity per mg of protein in infected cell extracts isolated at 18 h p.i.

DNase I footprinting. Sp1 was partially purified from HeLa cell nuclear extracts by wheat germ agarose-affinity chromatography as described by Jackson and Tjian (23); it was kindly provided by Timothy Osborne. Probes used for DNase I footprinting assays are described in the legend to Fig. 3; 3'-end-labeled probes were prepared by Klenow reactions with [α -³²P]deoxynucleoside triphosphates. DNA-binding reaction mixtures included 2 to 5 fmol of ³²P-end-labeled DNA fragment in 50 μ l of binding buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6], 2 mM MgCl₂, 1 mM EDTA, 20% glycerol) along with either no extracts or 2 or 10 μ l of Sp1-containing extracts and 1 μ g of poly(dI-dC). Binding was allowed to proceed for 20 min on ice. DNase I (5 to 20 ng/ml, depending on the amount of extract used) was then added with 2.5 mM CaCl₂ and 5 mM MgCl₂. After 1 min of incubation at room temperature, the reaction was stopped by the addition of an equal volume of stop buffer

(1% sodium dodecyl sulfate [SDS], 70 mM EDTA, 200 mM NaCl, 25 μ g of yeast tRNA per ml). The nucleic acids were extracted once with buffered phenol-chloroform and once with chloroform, subjected to ethanol precipitation, and electrophoresed on 6% sequencing gels along with sequencing ladders as size markers.

DNA mobility shift assays. Nuclear extracts were prepared (28) from uninfected rabbit skin cells or from such cells at 7 hpi with HSV-1 (17syn⁺) at a multiplicity of infection of 5 PFU per cell. Electrophoretic mobility shift assays with such extracts and the specific HSV promoter fragment were carried out by standard methods (5). Each assay contained 2 μ g of nuclear extract protein, 0.2 ng of ³²P-labeled DNA fragment, and 1 μ g of poly(dI:dC) in a total volume of 10 μ l. The binding reactions were allowed to proceed for 25 min at room temperature, and the complexes were separated on 4.5% polyacrylamide gels in TBE (40 mM Tris-borate, 1 mM EDTA [pH 8.3]). For competition experiments, the unlabeled DNA fragment or oligonucleotides were added in a 100- or 300-fold molar excess of the radiolabeled DNA probe.

The DNA fragment used for mobility shift assays was a *Hind*III-*Xho*I fragment containing the VP5 promoter sequence spanning -48 to +28 from the cap site and was ³²P labeled with Klenow fragment DNA polymerase and [α -³²P]dCTP. The competing double-stranded oligonucleotides used were 5'-ATTCGATCGGGGCGGGGCGAGC-3' (normal Sp1 site) and 5'-ATTCGATCGGTTTCGGGCGAGC-3' (mutant Sp1 site). They are shown in Fig. 4.

RESULTS

Deletional analysis of promoter elements upstream of the TATA box. Although transient expression assays have suggested the presence of critical VP5 promoter elements upstream of the TATA box, it is well documented that the expression patterns observed for HSV late genes in transient expression systems do not necessarily reflect the patterns observed when those genes are expressed within the viral genome (7, 15, 34, 47, 48). We, therefore, generated recombinant viruses containing serial deletions of portions of the DNA from -364 relative to the transcription start site of the VP5 mRNA (Fig. 1A). Our previous work with this promoter localized the transcript start site by using nucleic acid protection assays of hybrids between viral mRNA and relatively large probes generating protected species on the order of 200 bases in size (20). In order to precisely locate the transcript cap site for the deletional and mutational analysis described here, we carried out primer extension analysis using a primer within the β -galactosidase sequence of the chimeric mRNA expressed. Results shown in Fig. 1B illustrating the extension product of chimeric reporter mRNA expressed by recombinant VP5(-364/+63) comigrating with a dideoxy sequencing ladder generated from the same primer locate the transcript start site 30 bases downstream of the TATA box; this site is 8 bases 3' of our previous estimates.

As described in Materials and Methods, we performed Bal 31 exonuclease digestion and then placed the VP5 promoter retaining various amount of upstream sequences in conjunction with 63 bases of leader sequence in front of the bacterial β -galactosidase gene and recombined these constructs into the gC region of the HSV-1 genome as previously described (13, 20). Each recombinant virus harboring deletions ranging from -364 to -25 was used to infect rabbit skin cells, and the expression of β -galactosidase activity was monitored 18 hpi. Figure 1A summarizes the results and shows that deletion of upstream sequences to -79 (removing three Sp1 consensus

FIG. 1. HSV-1 VP5 (U_L19) promoter. (A) Expression of β -galactosidase activity expressed following infection with recombinant viruses carrying deletions in the upstream sequence of the VP5 promoter. Rabbit skin cells were infected at a multiplicity of infection of 5 PFU per cell with each of the recombinant viruses shown on the left. Cell extracts were prepared, and the β -galactosidase activity was measured at 18 hpi as described previously and in Materials and Methods (10). Activity was expressed relative to VP5(-364/+63) virus. Values shown are means and standard deviations of three to five separate experiments, at least one of which was done with a separate independent isolate. (B) Determination of transcription initiation site of HSV-1 VP5 transcripts by primer extension analysis. The oligonucleotide primer complementary to the β -galactosidase sequence starting 57 bases from the VP5/ β -galactosidase junction was used to prime the reverse transcription reaction with 5 μ g of total RNA isolated at 7 hpi from rabbit skin cells infected with VP5(-364/+63) virus. The DNA sequence ladder shown on the left was generated by the dideoxy chain termination method with plasmid VP5(-364/+63) and the same primer used for the primer extension assay.

sites, both ICP4-binding sites, and the CAAT site) did not have any effect on promoter activity assayed in this way. Further deletion to sequence at -48 (removing the YY1 site) resulted in a minimal decrease in reporter enzyme activity, while deletion to -42 (disrupting the remaining Sp1-binding site) reduced the recovered reporter enzyme activity by more than half. A deletion which extended to -32 and removed all specific sequence elements upstream of the TATA box resulted in a drop in the level of reporter gene activity to 10% of the maximal levels. Finally, as expected, removal of the TATA element (deletion down to -25) essentially abolished reporter enzyme activity.

We then measured levels of the chimeric β -galactosidase encoding mRNA isolated 7 hpi from cells infected with recombinant viruses, each carrying a specific deletion in VP5 upstream sequences, by using RNase protection assays. As shown in Fig. 2A and quantified by densitometry, the effect of upstream sequence deletions extending to +48 on steady-state RNA accumulation was similar to that observed by assaying β -galactosidase reporter gene activity. The steady-state RNA levels observed at 7 hpi with viruses VP5(-137/+63), VP5(-104/+63), VP5(-79/+63), and VP5(-48/+63) were 94, 89, 88, and 86%, respectively, of the level observed with VP5(-364/+63). Again, the effect of the removal of sequences to -32 and -25 on the steady-state RNA levels was similar to that expected from β -galactosidase assays; virus VP5(-32/+63) expressed 12% of the RNA seen with VP5(-364/+63), and virus VP5(-25/+63) expressed essentially no reporter mRNA. However, the expression of reporter mRNA during infection with VP5(-42/+63) virus showed a significantly different pattern. This virus expressed approximately 40% of the maximum levels of reporter enzyme activity, and yet the steady-state RNA level observed at 7 hpi was only about 10%

FIG. 2. Expression of mRNA from recombinant viruses carrying deletion in VP5 upstream sequence. (A) RNA was isolated 7 hpi from rabbit skin cells infected with specific recombinant viruses as indicated. Aliquotes (10 μ g) of RNA were analyzed for chimeric β -galactosidase (β -gal) mRNA by using RNase protection assays with a riboprobe specific for β -galactosidase containing mRNA as described in Materials and Methods. MW, molecular weight. (B) Analysis of mRNA kinetics of expression during infection by recombinant viruses carrying deletions in the VP5 upstream sequence. Rabbit skin cells were infected with each of the recombinant viruses indicated, and total RNA was isolated at 3 hpi or 7 hpi and analyzed by RNase protection assays using riboprobes specific for both β -galactosidase (β -gal) containing mRNA and VP5 mRNA.

FIG. 3. DNase I footprinting of the VP5 promoter with transcription factor Sp1. DNase I protection analysis was carried out (see Materials and Methods) with VP5 probes. The corresponding naked probe digestion pattern (lane 1) is shown next to protected patterns seen with 2 (lane 2) or 10 (lane 3) μl of purified HeLa cell Sp1. Regions protected from DNase I digestion by Sp1 are indicated, along with their positions relative to the transcription initiation site. (A) DNase I footprinting patterns observed with wild-type VP5 promoter probes. The coding-strand probe was an *XbaI-KpnI* fragment covering the VP5 sequence extending from -137 to +63. The noncoding-strand probe was a *PstI-XhoI* fragment covering the VP5 sequence from -364 to +63. The probes were end labeled at either the *XbaI* or *XhoI* site with $[\alpha\text{-}^{32}\text{P}]$ deoxynucleoside triphosphates. Areas protected by Sp1 are indicated by solid lines. (B) DNase I protection patterns observed with probes which contain a modified Sp1 site. The coding-strand probe was a *BfrII-KpnI* fragment, and the noncoding strand was a *PstI-XhoI* fragment taken from a pCAL5 vector (10) containing the VP5 sequence extending from -42 to +63 with an *XbaI* linker. Both the *BfrII-KpnI* fragment and the *PstI-XhoI* fragment contain the U_L37 promoter sequence juxtaposed to the VP5 sequence with the *XbaI* linker. The sequence of the disrupted Sp1 site (marked with dashed lines) is TCTAGAGGGCGGGGGGGT versus the sequence shown in the figure. The probes were end labeled at either the *BfrII* or *XhoI* site. (C) Summary of Sp1 binding on VP5 promoter. Nucleotides protected from DNase I digestion by Sp1 on the coding and noncoding strands are indicated by shadowing, along with the position relative to transcription initiation site. Endpoints for upstream sequence deletions at positions -48 and -42 and the position of the TATA box are indicated with arrows.

of the level observed with VP5(-364/+63) virus, which carried the full-length of the upstream sequence.

Since all recombinant viruses described here express the same chimeric mRNA encoding the reporter enzyme, such a result could not be due to some alteration in mRNA stability during infections with the two viruses. This suggested that the deletion between -48 and -42 might disrupt a *cis*-acting element important in maintaining maximal levels of mRNA at late times. We confirmed this hypothesis by analysis of steady-state levels of reporter gene mRNA isolated at 3 and 7 hpi (Fig. 2B). Like the situation with the VP5(-48/+63) recombinant, VP5/ β -galactosidase mRNA levels expressed during infection by the VP5(-42/+63) recombinant at 3 hpi was at least equivalent to the level of wild-type VP5 mRNA. Unlike infections with the VP5(-48/+63) recombinant virus, however, the level of chimeric reporter mRNA expressed by the VP5(-42/+63) recombinant did not show the increase ex-

FIG. 4. DNA mobility shift assays. Nuclear extracts from mock-infected (A) or HSV-1-infected (B) rabbit skin cells were incubated with the ^{32}P -labeled double-stranded VP5 promoter extending from -48 to +28 relative to the transcript start site. Competition was carried out with unlabeled promoter probe, a double-stranded oligonucleotide (oligo) containing the canonical Sp1 site and flanking bases, or a mutant double-stranded oligonucleotide in which the Sp1 site was disrupted. Competitor was present at 100- and 300-fold molar excess. The sequence of the probe and the competing oligonucleotides are shown in panel C. Locations of DNA/protein complexes and free probe are indicated.

pected for a normal $\beta\gamma$ transcript. Indeed, the steady-state levels of RNA seen at the late (7-h) time point were more similar to those seen for a nominal β (early) transcript.

The pattern of reporter gene expression seen during infections with this set of upstream deletion mutants defines the boundary for upstream sequence elements important for both maximal levels of mRNA accumulation and the normal kinetics of expression of the VP5 promoter. It is notable that this minimal promoter is significantly different in extent from that defined by transient expression assays. Further, the effect of deletion of the critical region from -48 to -42 relative to the cap site has an effect opposite to that expected from the chimeric model of a $\beta\gamma$ promoter; i.e., deletion of a putative cellular transcription factor binding site affects mRNA levels late rather than early following infection. To verify that the canonical Sp1-binding site between -48 and the TATA box does, indeed, bind this transcription factor, we carried out DNase I footprinting analysis using authentic Sp1 protein purified from HeLa cell extracts and VP5 promoter fragments containing this Sp1-binding site. We also used a VP5 promoter probe containing a modified Sp1 site in which the *XbaI* linker has been inserted at -42 (Fig. 3C); since it is known that the context of a canonical Sp1-binding site has a significant effect upon total affinity (6), we would expect the linker insertion at -42 to affect binding. The results of DNase I protection analysis shown in Fig. 3A clearly demonstrate that the G/C-rich region upstream of the TATA box can be protected by the Sp1 protein, while the parallel experiment using the probe in which the -48 to -42 region has been changed to a less-canonical Sp1 site resulted in significantly reduced binding, as shown by the increased nuclease sensitivity in the critical region (Fig. 3B).

Having demonstrated that the G/C-rich region upstream of the TATA box can be protected from DNase digestion by Sp1

YY1 Consensus	cGACATtTTGc		Relative Reporter Activity
VP5 YY1 Site	GGCCATCTTGA	VP5(-364/+63)	100
Mutant YY1 Site	GAATTCCTTGA	vCH218	117 ± 6
	GGCCAGATCTGA	vCH219	213 ± 27
	GAATTCACCCGA	vCH220	97 ± 13

FIG. 5. Expression of β -galactosidase activity from recombinant viruses carrying block mutations in the YY1 site. Block mutations in the YY1 site were generated by site-directed mutagenesis as described in Materials and Methods. The altered nucleotides are indicated by underlining. Rabbit skin cells were infected at a multiplicity of infection of 5 PFU per cell with each of the recombinant viruses shown on the left. The β -galactosidase activity was measured at 18 hpi as described in the text and expressed relative to VP5(-364/+63) virus. Values shown are means and standard deviations of three to five separate experiments as described in the legend to Fig. 1A.

protein, we next assayed the binding of this putative sequence element to Sp1 in mobility shift assays. As shown in Fig. 4B, interaction of nuclear extract prepared from cells isolated at 7 hpi with a VP5 promoter probe extending from -48 to +28 generated one complex (C_1) which comigrated with the major complex observed when partially purified Sp1 protein was used in a similar binding reaction. The formation of this DNA/protein complex was abolished by competition with unlabeled probe or double-stranded oligonucleotide containing the Sp1-binding site but was able to form completely when competition was carried out with a double-stranded oligonucleotide containing a mutant Sp1 site. The sequences of the probe and the two competitors are shown in panel C. A similar pattern was seen with nuclear extract from uninfected cells, but two additional mobility shift complexes (C_2 and C_3) were observed (Fig. 4A). These additional complexes were not diminished by competition with either the Sp1 or mutant Sp1 site containing oligonucleotides but were diminished by competition with the full unlabeled probe, suggesting that they represent complexes between uninfected cell nuclear proteins and other regions within the region used as the probe. Finally, we carried out Western immunoblot analysis of nuclear extracts with commercial antibody to Sp1 protein and confirmed the presence of normally migrating Sp1 protein following infection with HSV-1 (data not shown). Such results are fully consistent with our conclusion that Sp1 protein functions in the expression of the VP5 promoter during the course of infection.

Mutational analysis of YY1 site. Consensus YY1 sites are

present in several HSV-1 late genes, and (as noted above) the YY1 site located at -78 was found to be required for the full expression from VP5 promoter in cells using a transient expression system (4, 39). To verify the difference between the results of transfection assays and those using recombinant viruses in which the presence of the YY1 site had little, if any, effect, we introduced deleterious mutations into the YY1 site using site-directed mutagenesis and then generated recombinant viruses containing modified VP5 promoters. The results of determinations of reporter β -galactosidase activity expressed following infection are shown in Fig. 5; the data confirm our conclusion based upon deletional analysis that mutations which significantly modify the YY1-binding site did not result in any significant reduction of VP5 promoter activity. Interestingly, the mutations harbored in mutant vCH219 actually resulted in increased β -galactosidase expression following infection. This might be due to the gaining of a positive *cis*-acting sequence element in the promoter, but we have not analyzed this phenomenon further at this time.

Deletional analysis of sequences downstream of the VP5 TATA box important in promoter activity. In previous work, we found that sequences between -13 and +27 contained one or several *cis*-acting elements required for maximal levels of mRNA expression (20). We further defined this region by using recombinant viruses containing two more-extensive leader deletions. A summary of measurements of reporter β -galactosidase activity expressed following infection with a number of recombinant viruses containing leader deletions is shown in Fig. 6A; all recombinants contained sequences up to -364 relative to transcription initiation site. Deletion of leader sequences from +226 to +16 did not result in any major reduction in reporter gene activity, whereas removal of another 12 bases to +4 led to a sixfold decrease. Further deletion to -13 did not have any further effect on levels of reporter enzyme expressed. This indicates that the VP5 promoter sequence between +16 and +4 contains at least part of a *cis*-acting element that is important for full-level expression.

These altered levels of reporter gene expression correlate with altered temporal patterns of mRNA expression. This is demonstrated by the data shown in Fig. 6B in which RNA isolated at both 3 and 7 hpi from cells infected with recombinant viruses VP5(-364/-13), VP5(-364/+4), and VP5(-364/+16) was analyzed. During infection with VP5(-364/+16) virus, expression of β -galactosidase encoding chimeric reporter mRNA retained the typical leaky-late kinetics of expression

FIG. 6. Promoter activity from recombinant viruses carrying deletions in the VP5 promoter/leader region. (A) β -Galactosidase reporter activity. Rabbit skin cells were infected with each of the recombinant viruses shown on the left. Extracts were prepared, and β -galactosidase activity was determined and expressed relative to VP5(-364/+63) virus. Values given are means and standard deviations of three to five individual experiments as described in the legend to Fig. 1. (B) Kinetics of mRNA accumulation during infection by recombinant viruses carrying deletion mutations in the VP5 leader. Total RNA was isolated at 3 and 7 hpi and analyzed by RNase protection assays using riboprobes specific for both β -galactosidase (β -gal) and VP5 mRNA.

observed with wild-type VP5 RNA, whereas the reporter mRNA accumulation pattern during infections by either VP5(−364/−13) or VP5(−364/+4) virus was altered so that RNA no longer accumulated to high levels at late times during the infection. Thus, by using deletion analysis, we identified a *cis*-acting element downstream of the mRNA start site in the VP5 promoter that is important for $\beta\gamma$ kinetics of expression. We confirmed that deletions in the region identified have an effect on the $\beta\gamma$ kinetics of VP5 mRNA expression by measuring the effect of inhibition of viral DNA replication following infections with recombinant viruses VP5(−364/−13), VP5(−364/+4), and VP5(−364/+16) (data not shown). Chimeric reporter RNA expressed at reduced levels by either VP5(−364/+4) and VP5(−364/−13) virus at 7 hpi was not sensitive to inhibition of viral DNA replication; in contrast, β -galactosidase encoding reporter mRNA expressed during infections with VP5(−364/+16) virus was clearly sensitive to inhibition of viral DNA replication.

Site-directed mutagenesis of VP5 leader region. To pinpoint the *cis*-acting element which is critical for mediating normal levels and kinetics of mRNA expression from the VP5 promoter, we introduced four blocks of mutations, each with four to seven mismatches, into the region between −15 and +17 and studied the effect of these block mutations on promoter activity in the context of viral genome, using gC recombinant viruses. Each recombinant virus constructed for this purpose contains VP5 promoter sequences extending from −364 to +63. Reporter activity at 18 hpi relative to that observed with VP5(−364/+66) virus is shown in Fig. 7A. All mutants except vCH235 exhibited nearly wild-type levels of activity; however, mutant virus vCH235 expressed fourfold-less reporter enzyme activity. This result was confirmed by analysis of 7-hpi steady-state RNA levels (Fig. 7B).

To examine the effect of block mutations on kinetics of mRNA accumulation, cells were infected with the appropriate recombinant viruses, and RNA was extracted at 3 and 7 hpi and analyzed by RNase protection analysis. As shown in Fig. 7C, unlike the situation following infection with the mutant virus vCH233, expression of chimeric reporter mRNA in virus vCH235 did not increase at late times after infection compared with wild-type VP5 transcripts. Hence, analogous to the upstream element, a *cis*-acting element in the leader region between −2 and +10 as identified with this set of four block mutations is required for proper $\beta\gamma$ kinetics of VP5 mRNA accumulation.

Effects of substituting a β promoter cap site and leader into its cognate position in VP5 promoter. We have previously described a recombinant virus containing the β dUTPase (U_L50) promoter and VP5 cap site and leader in its cognate position; this construct expressed chimeric β -galactosidase encoding mRNA with characteristic early kinetics, i.e., relatively abundant expression prior to DNA replication and reduced expression following this (20). Thus, the VP5 leader alone cannot confer $\beta\gamma$ kinetics upon an early promoter. The structure of this recombinant promoter is shown in Fig. 8A. In contrast to this, replacing the authentic VP5 leader sequence with the leader sequence from the dUTPase promoter clearly alters its temporal pattern of accumulation so that it behaves like a β promoter also (Fig. 8B). This result is expected from the fact that the VP5/UTPase promoter lacks the critical element located between −2 and +10 relative to the transcript start site, which is required for proper levels of accumulation of reporter mRNA following DNA replication. However, substitution of the dUTPase leader did not effect a complete shutoff of transcription as observed for expression from dUTPase promoter or UTP-VP5 hybrid promoter. We also used the

FIG. 7. (A) Expression of β -galactosidase activity from recombinant viruses carrying block mutations in the VP5 promoter/leader region. Block mutations in the VP5 promoter region were generated by site-directed mutagenesis, and the altered nucleotides are underlined. Rabbit skin cells were infected with each of the recombinant viruses shown on the left. The β -galactosidase activity was measured at 18 hpi as described in the text and expressed relative to VP5(−364/+63) virus. Values shown are means and standard deviations of two to three separate experiments done with two independent isolates. (B) Expression of mRNA from recombinant viruses carrying block mutations in the VP5 promoter. RNA was isolated 7 hpi from rabbit skin cells infected with VP5(−364/+63), vCH231, vCH233, vCH235, and vCH237 virus. For each specific recombinant virus, a 10- μ g aliquot of RNA sample was analyzed by using RNase protection assays with riboprobes specific for β -galactosidase (β -gal) containing mRNA. MW, molecular weight. (C) Analysis of kinetics of mRNA accumulation during infection by recombinant viruses carrying block mutations in the VP5 promoter. Total RNA was isolated at 3 and 7 hpi and analyzed by RNase protection assays using riboprobes specific for both β -galactosidase containing and VP5 mRNA.

VP5-specific probe spanning −75 to +139 relative to the cap to demonstrate that these similar kinetics of mRNA accumulation are from the different viruses tested. Thus, the VP5/UTPase recombinant virus expresses wild-type VP5 mRNA with authentic $\beta\gamma$ kinetics; however, infections with the UTPase/VP5 recombinant express VP5 containing mRNA both early and late, as expected. Taken together, these data suggest that the leader sequences alone can not confer $\beta\gamma$ kinetics on an early promoter.

DISCUSSION

Investigation of expression of transcripts controlled by chimeric promoters in both transient expression assays and infections with recombinant viruses have been useful in identifying common and specific promoter elements that participate in the regulation of both the levels and kinetics of expression. It has been demonstrated that a TATA element is essential for expressing strict late (γ) HSV-1 genes (13, 16, 17, 24, 51), and TATA elements taken from several HSV-1 promoters can effectively replace one another without modifying temporal gene expression (22, 46, 51, 52). As noted in the Introduction, chimeric promoters containing early promoter sequence elements upstream of the TATA box and late promoter elements downstream of this have been constructed; mRNA under the control of such chimeras demonstrates $\beta\gamma$ kinetics of accumulation (33, 46). Such a result has lead to a reasonable and testable model for the structure of wild-type $\beta\gamma$ promoters in

FIG. 8. Substitution of dUTPase promoter leader sequence and VP16 promoter leader sequence into the VP5 promoter. (A) Diagram of the substitution mutants constructed as described in Materials and Methods. (B) Analysis of kinetics of mRNA accumulation during infection by recombinant viruses carrying chimeric VP5/dUTPase promoters. Total RNA was isolated at 3 and 7 hpi and analyzed by RNase protection assays using riboprobes specific for β -galactosidase (β -gal) containing mRNA or VP5 mRNA. It should be noted that the elevated level of VP5 mRNA observed at 3 hpi with UTP-VP5 virus is expected because of expression of the VP5/ β -galactosidase chimeric mRNA from the UTP-VP5 hybrid promoter.

which modular elements of both β and γ promoters are present (33, 34). Such a structure would predict that deletions and/or mutations of critical early elements of a $\beta\gamma$ promoter would generate a promoter displaying strict γ kinetics.

Detailed functional analysis of the VP5 (U_L19) promoter controlling expression of the major capsid protein indicates that this model does not accurately describe its structure and thus cannot be generalized. Sequences upstream of the TATA element in the $\beta\gamma$ VP5 promoter contain potential binding sites for several well-characterized cellular transcription factors including Sp1, CAAT-binding factor, and YY1; such a situation is reminiscent of the upstream cellular *cis*-acting elements in β promoters. Despite this, we have shown that deletion and/or modification of sequences upstream of -48 removing three Sp1 sites, the CAAT site, two ICP4 sites, and the YY1 site did not have any significant effect on either the levels or the kinetics of expression from the VP5 promoter (Fig. 1, 2, and 5). Therefore, we conclude that the upstream boundary for full and regulated expression from VP5 promoter lies within 48 bases of the transcript cap site.

If the recognizable cellular transcription binding sites in the region upstream of -48 in the VP5 promoter have no role in the levels or kinetics of expression of the transcript controlled, why are they there? Of course, their presence may be due to just a statistical accident, but the occurrence of such sites in analogous locations of other $\beta\gamma$ promoters and in this promoter in closely related herpesviruses argues against this. It is possible that the presence of these upstream sites has a role in promoter function only in certain critical tissues or under certain conditions of viral gene expression, perhaps early during the reactivation process. Such a role might correlate with the observed role of these sites seen in transient expression assays in which the transcription template is clearly different from that present on the viral genome during productive infection. This model leads to the testable proposition that

mutation or deletion of these upstream sites would result in a virus with normal replication properties in cultured cells but with altered pathogenicity or ability to reactivate in one or another animal model.

Although the presence of Sp1-binding sites in the critical region between the TATA box and -48 would be expected from the chimeric model for $\beta\gamma$ promoters being tested, its effect on late, not early, mRNA levels (Fig. 2B) is not. The presence of recognizable transcription factor-binding sites has often been taken as *prima facie* evidence of factor binding, but it is important to demonstrate such binding experimentally; this is especially true in view of the fact that this site is implicated in late not early promoter activity. We have presented two lines of evidence that Sp1 can bind to its recognition site between -48 and the TATA box. (i) DNase I footprint analysis (Fig. 3) demonstrates that this region does, indeed, bind Sp1 protein efficiently *in vitro*. However, disruption of the context of the site by insertion of an *Xba*I linker upstream of the minimal canonical site greatly reduced affinity (Fig. 3B), as expected for an authentic Sp1-binding site (6). (ii) Electrophoretic mobility shift assays indicate that an Sp1-specific complex is formed between the promoter element and nuclear extracts isolated from infected cells (Fig. 4). The molecular mechanism by which Sp1 mediates the regulation of leaky-late kinetics remains to be determined.

The other extreme of the VP5 promoter also contains a critical element important in $\beta\gamma$ kinetics of mRNA accumulation. Our preliminary analysis demonstrated the presence of such a *cis*-acting element (20), and data presented in this communication confirm and extend this conclusion (Fig. 6, 7, and 8). Since all recombinant viruses express nearly identical chimeric mRNAs encoding the reporter gene, it is unlikely that differences in mRNA stability or transport are important in affecting the levels and kinetics of mRNA accumulation observed during infection with VP5(-364/+4) and VP5(-364/+16). The presence of a *cis*-acting element required for wild-type levels and kinetics of expression was confirmed by analysis of site-directed block mutations introduced into the region between -15 and +17 (Fig. 7). Of the four mutant viruses examined, only virus vCH235 which carried mismatch mutations between +3 and +8 exhibited a significant decrease in promoter activity.

Although the critical element upstream of the TATA box has no positional counterpart in γ promoters, both the extent and nature of core elements of the $\beta\gamma$ VP5 promoter more clearly resemble properties of γ promoters than those of β ones. The distance between the essential TATA box and *cis*-acting elements downstream of the cap site is ca. 40 bases, the same distance as that between these elements in the γ U_L38 and other promoters (15). More compelling is the fact that the *cis*-acting element encompassing the transcript cap site is clearly reminiscent of initiator elements found in a number of HSV-1 γ promoters, including those controlling mRNAs encoding gC, gH, and gE (46, 51). The sequence around the cap site (GGGTCTGTTGGG) is similar to an equivalently located critical element in the long terminal repeat of human immunodeficiency virus type 1 (TGGTCTCTCTGG) which mediates interaction of the human immunodeficiency virus cap with cellular transcription factors (8, 30, 31, 43). It is important to determine whether this element in the VP5 promoter has analogous activity.

Despite being critical in controlling normal kinetics of mRNA accumulation, the *cis*-acting element near the transcript start site must act in concert with other cognate elements. As shown in Fig. 8, the substitution of the dUTPase gene leader sequences with VP5 leader sequences did not

change the early kinetics of reporter mRNA expression. It should be remembered, however, that replacement of the VP5 cap site and downstream sequences by the analogous sequences of the dUTPase promoter/leader results in a change in the temporal pattern of reporter mRNA accumulation from $\beta\gamma$ to β (20) (Fig. 8); therefore, the sequence elements near the VP5 cap site are necessary but not sufficient for normal $\beta\gamma$ kinetics of mRNA accumulation.

The presence of two elements critical in transcription kinetics at distal ends of the VP5 promoter is consistent with the interesting possibility of cooperation between them. Such a situation would be analogous to late-phase promoter activity during infections with other DNA viruses. For example, synergistic promoter activation has previously been observed under conditions in which no cooperative DNA binding of the cognate activators occurred during adenovirus late gene expression, and it was suggested that the late-phase-specific activation results from the simultaneous action of factors bound at the upstream and downstream elements onto a common component of the transcriptional machinery (29, 40). This suggests that there are some common structural features in the ability of promoters to be active on replicating templates in infections with nuclear replicating DNA viruses.

Despite the high activity of the VP5 promoter late in infection, $\beta\gamma$ mRNA accumulation requires that appreciable transcription occur during the early phase of lytic infection. Superinfection experiments have shown that viral DNA introduced into infected cells in which late-phase transcription was occurring exhibited no late gene expression until undergoing template replication (32); this and other observations have been cited in the suggestion that replication of viral DNA templates results in a *cis*-modification relieving a structural or conformational block to transcription from late promoters on nonreplicating templates (26). While our analysis of the γ U_L38 promoter are consistent with this (15), our ability to generate modified promoters which express normal amounts of mRNA early but significantly reduced amounts late is not. Clearly, the minimal early promoter differs in extent from that required for late function; therefore, transcription complexes forming on $\beta\gamma$ promoters early compared with those forming late in infection must reflect biochemical differences in the assembled transcription complex itself.

Virus-encoded proteins are intimately involved in such biochemical changes in the transcription complex early and late after infection. For example, alteration in the function of the α regulatory protein ICP27 can affect the early to late transcription switch even when viral DNA replication proceeds (reviewed in reference 45); a function or functions mediated by the β DNA-binding protein, ICP8 (U_L29), are also implicated in the switch (11). It is important to emphasize, however, that no matter how profound the biochemical differences in transcription machinery during the two major stages of the productive replication cycle, there must be great similarities as well. For example, the HSV-1 α proteins ICP4 and ICP0 are important in the normal expression of HSV genes at all times after infection (2, 3, 27), and, as shown here for the $\beta\gamma$ promoter controlling VP5 mRNA expression, these and probably other viral regulatory proteins mediate transcription in a spatially compact region near the transcription start site in association with cellular sequence-specific transcription factors and RNA polymerase II transcriptional machinery at all times during infection. Future experiments will be aimed at further characterizing the *cis*-acting elements within the VP5 promoter and proteins interacting with these elements at different stages of infection.

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