Mutational Analysis of the Herpes Simplex Virus Type 1 Strict Late U_L38 Promoter/Leader Reveals Two Regions Critical in Transcriptional Regulation

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The unusual TATA homology TTTAAA at –31 relative to the transcriptional start site of the herpes simplex virus type 1 (HSV-1) strict late (γ) U_L38 gene defines the 5' extent of this promoter in recombinant virus. We have further analyzed this promoter by generating recombinant viruses containing nested deletions 3' of the transcriptional start site and with recombinant viruses containing specific promoter/leader alterations. A recombinant virus containing the U_L38 promoter/leader from –50 to +9 expressed reporter gene enzyme levels at approximately 10% of those from a recombinant containing the full viral promoter/leader (–50 to +99). The accumulation of reporter gene mRNA in infections with the –50 to +9 recombinant was still regulated with γ kinetics. Further removal of U_L38 leader sequences resulted in a nearly complete loss of expression. Analysis of promoter chimera recombinant viruses has shown that sequences downstream of the TATA box and spanning the transcriptional start site of the U_L38 promoter are functionally distinct from those of either the β U_L37 gene or the $\beta\gamma$ VP16 (U_L48) gene; thus, we conclude that sequences from –31 to +9 of the U_L38 gene constitute a core γ promoter. Further deletional and substitutional analyses have also demonstrated the presence of a 14-bp element (the downstream activation sequence) located between +20 to +33 in the nontranslated leader region which is required for full levels of transcription.

Herpes simplex virus type 1 (HSV-1) encodes more than 70 distinct appreciably sized translational open reading frames (ORFs) (27). Proteins encoded by these and smaller ORFs are expressed by as many as 100 discrete viral transcripts (42–45). Individual transcripts are typically expressed under the control of their own promoters. Productive infection is characterized by a temporally regulated cascade in which four distinct kinetic classes of transcripts are expressed in a sequential manner by the host-encoded RNA polymerase II (42). This transcription program is complex and has served as a paradigm for other developmental cascades.

The virus encodes five immediate-early (α) proteins which orchestrate the ensuing transcriptional cascade (35, 43). Under conditions of lytic infection, the α proteins are the only class of HSV proteins which can be expressed in the absence of de novo protein synthesis. Two of these proteins, α 4 and α 27 (U_L54), have been shown to be essential for replication in cell culture and are regulators of transcriptional processes. A third, α 0, facilitates low-multiplicity replication and, hence, plaquing efficiency. A fourth, α 22, is important in determining viral host range.

The early (β) proteins are primarily involved in viral DNA replication. Among these are the nine proteins comprising essential virus-encoded DNA replication machinery (28, 30, 46) and a number of enzymes important in the optimal utilization of deoxyribonucleoside pools. The latter include thymidine kinase (TK; U_L23), the large and small subunits of ribonucleotide reductase (U_L39 and U_L40), and dUTPase (U_L50).

The onset of viral DNA replication triggers a change in the transcriptional program; at this time, transcription of β genes

declines while high-level expression of late gene products begins (42, 45). A majority of these late genes code for viral structural proteins. Late genes can be subdivided into two classes based on their requirement for DNA replication for efficient expression. In contrast to the leaky-late ($\beta\gamma$) genes, which are transcribed at significant levels prior to DNA replication, the transcription of strict late (γ) genes is not readily detected prior to this event.

Researchers have mutagenized promoters representing the different kinetic classes to define viral sequences which act in *cis* to regulate the HSV transcriptional program. Such studies have shown that HSV promoters have elements typical of cellular RNA polymerase II promoters. Most HSV promoters have been shown to have a recognizable TATA homology at approximately –25 to –30 relative to the cap site which is necessary for expression. Promoters of the α , β , and $\beta\gamma$ classes have demonstrated a requirement for upstream sequence elements for full levels of expression (3, 4, 6, 29, 35). These *cis*-acting sites are typically binding sites for cellular transcription factors such as Sp1 and the CAAT-binding protein.

To date, mutagenesis has clearly identified only one *cis*-acting element defining a specific kinetic class of promoters. This element has the consensus sequence TAATGA RAT and is found upstream of the five immediate-early promoters (2, 5, 11, 34, 47). The element interacts with a complex composed of the cellular transcription factor Oct1, at least one other cellular factor, and the viral structural protein VP16 (U_L48 [Vmw65, α TIF, or VSP]) to increase transcription from the α promoters.

Class-specific *cis*-acting elements for β , $\beta\gamma$, and γ promoters have not been clearly defined; however, those γ promoters studied to date have shown that the TATA homology defines their 5' extent. The lack of upstream *cis*-acting sites as well as the requirement for DNA replication for expres-

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sion distinguishes the γ promoters from promoters of other kinetic classes. Model γ promoters have been engineered to contain functional upstream *cis*-acting elements; these modified γ promoters expressed mRNA with $\beta\gamma$ kinetics, indicating that such upstream elements are sufficient to allow expression prior to DNA replication (16, 23).

Interestingly, however, deletion of Sp1 binding sites and the CAAT box of the β TK (U_L23) promoter decreased the levels of message expressed but did not alter the β kinetics of expression (19). Such a result suggests that the expression of transcripts controlled by $\beta\gamma$ and γ promoters involves either specific TATA box sequences or other sequence elements downstream of the TATA box which enable these promoters to be expressed late in infection.

Two independent reports show that the temporal regulation of mRNA expression does not reside within the TATA elements of HSV promoters. Steffy and Weir have shown that the TATA box of the γ glycoprotein H (gH; U_L22) could be readily replaced by the TATA element of either the α ICP4, β TK, or γ gC promoter in the context of the gH promoter (41). Each of these recombinants expressed the reporter gene with γ kinetics. More recently, Imbalzano and DeLuca reported that replacement of the TK TATA element with that of the γ gC promoter in the context of the β TK promoter did not alter the kinetics of expression of TK RNA from this virus (20).

In addition to the upstream TATA homology, full-level transcription from $\beta\gamma$ and γ promoters requires elements within the 5' nontranslated leader region. Deletion or mutation of sequences downstream of the TATA boxes of $\beta\gamma$ (VP5, VP16, and gB) and γ (gC, gH, U_S11, and γ 42) genes has been shown to adversely affect accumulation of reporter gene RNA or enzyme levels in transient expression assays and in recombinant viruses (3, 14, 17, 23, 32, 37, 40). In contrast, deletion of the entire leader (-12 to +189) of the β TK gene has been shown to have no appreciable effect on accumulation of message from this promoter (15).

In our own laboratory, we have investigated the role of *cis*-acting elements of the U_L38 promoter in transcriptional regulation. The U_L38 transcript is expressed with strict late (γ) kinetics (9) and encodes the VP19C capsid protein (33). We have used both transient expression and recombinant viruses to define the 5' extent of this promoter to -31 at the distal extent of an unusual TATA homology, TTTAAA. Further, we have previously used gel retardation assays to demonstrate that sequences from -14 to +99 of the U_L38 cap/nontranslated leader region form complexes from infected cell nuclear extracts which contain the α 4 protein (9, 13).

In this study, we have analyzed the effect of deletion or alteration of sequences downstream of the TATA box of the γ U_L38 gene. These studies reinforce conclusions drawn from studies of other γ promoters and further dissect the late gene leader effect. We have shown that sequence elements downstream of the TATA box and spanning the transcriptional start site of this γ promoter are functionally different from those of a β or $\beta\gamma$ gene. We have also defined an element from +20 to +33 of the U_L38 leader region which increases transcription rates. These analyses suggest that γ promoters such as that controlling the U_L38 transcript consist of a core late promoter from the TATA box to sequences including the transcript start site linked to a downstream activation sequence (DAS) within the nontranslated leader region.

MATERIALS AND METHODS

Cells and virus. HSV-1 strain 17syn⁺ was the parental strain used to generate the recombinant viruses described in the text. Parental and recombinant virus infection of rabbit skin fibroblasts at a multiplicity of infection of 5 PFU per cell was used to generate the protein extracts assayed for β -galactosidase activity and to generate the RNA used for RNase protection assays. Vero cells were used for plaque assays, and both cell types were used during recombinant virus screening and isolation. The cells 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 μ g of streptomycin per ml.

Enzymes. Restriction enzymes were purchased from Boehringer Mannheim Biochemical Corp., Bethesda Research Laboratories, Inc., or New England Biolabs. T7 RNA polymerase and RNasin used in RNase protection assays were purchased from Promega Biotec. T4 polynucleotide kinase (Bethesda Research Laboratories) was used to 5' end label appropriate DNA markers for RNase protection. *Bal* 31 nuclease (slow form) used in the generation of the leader deletions was purchased from IBI. *Taq* DNA polymerase used for the polymerase chain reaction (PCR) analysis of recombinant viruses was purchased from Perkin Elmer-Cetus. Proteinase K, Klenow enzyme, and T4 DNA polymerase were purchased from Boehringer Mannheim.

Recombinant DNA. pUC and pGEM plasmid derivatives were grown in *Escherichia coli* DH5 α or DH1. Growth and purification of plasmids were done essentially as described previously (1, 10, 26). DNA fragments used in the generation of recombinant plasmids or for use as radiolabeled probes were isolated by digestion with the appropriate restriction enzymes followed by either direct excision from SeaPlaque agarose (FMC) following electrophoresis or by electroelution following electrophoresis. Single-stranded oligonucleotides were synthesized at the University of California, Irvine, and then eluted from acrylamide gels following electrophoresis. Probes were uniformly labeled by random priming and synthesis, using Klenow enzyme with [α -³²P] dCTP (3 Ci/ μ mol; Amersham).

Sequencing of double-stranded plasmid templates was performed by using the Sequenase kit (United States Biochemical Corp.). The U_L38 leader deletion constructs were sequenced within pUC19, using the M13 universal primer. All other constructs were sequenced by using a β -galactosidase primer with the sequence, 5'-GTGTTTCGAGGGGAAA ATAGGTTGCGCGAG-3'.

Generation of leader deletion reporter constructs. A bifunctional reporter construct, pCAL5div Δ 17, which contains the promoter/leader of the strict late (γ) gene U_L38 from HSV-1 strain KOS(M) driving expression of the bacterial β -galactosidase gene has been described previously (9, 10). This vector also contains the viral U_L37 promoter/leader driving expression of chloramphenicol acetyltransferase (CAT). An *Xba*I-*Sma*I fragment containing the U_L38 promoter/leader was cloned into pUC19. This plasmid was linearized with *Sma*I and subjected to digestion with slow-form *Bal* 31. T4 DNA polymerase was then used to blunt DNA ends. *Sma*I linkers were added; plasmids were ligated together and then used to transform *E. coli* DH5 α . Miniprep DNA was prepared from transformants, subjected to digestion with *Xba*I and *Sma*I, and run on mini-acrylamide gels. Clones of interest were sequenced to determine exact deletion endpoints.

Promoter/leader deletion fragments of interest were

cloned into pCAL5div Δ 17B as *Xba*I-*Sma*I cassettes. This derivative of pCAL5div Δ 17 was engineered to allow simple insertion of *Xba*I-*Sma*I cassettes into the bifunctional reporter plasmid. This construct was made by subcloning the *Xba*I-*Hind*III fragment from pCAL5div Δ 17 into a derivative of pUC19 in which the *Sma*I site had been disrupted. This fragment contains the promoter/leader of the β U_L37 gene. Within this HSV sequence is a *Sma*I site which would interfere with cloning of the U_L38 promoter/leader deletion fragments. The resultant plasmid was then cut with the *Sma*I isoschizomer *Xma*I, and the overhangs were filled in with Klenow enzyme. The plasmid ends were then ligated. These manipulations destroyed the *Sma*I site within the U_L37 promoter. This modified *Xba*I-*Hind*III fragment was then used to replace the *Xba*I-*Hind*III fragment of pCAL5div Δ 17 to yield pCAL5div Δ 17B.

Transient expression assays. Cultures of rabbit skin fibroblasts were transfected with 10 μ g of form I plasmid DNA, using CaPO₄ precipitation as previously described (9); 24 h later, cells were superinfected with HSV-1 at a multiplicity of 1 PFU per cell and overlaid with medium containing 50 μ g of thymine-1- β -D-arabinofuranoside (AraT) per ml. At 18 h postinfection (hpi), cell extracts were prepared and assayed for protein concentration and β -galactosidase activity as described elsewhere (9). Extracts used for the CAT assays were prepared by heating the β -galactosidase extracts at 65°C for 20 min and removing the precipitate by centrifugation at 4°C for 5 min. CAT activity was determined by using a phase extraction assay as described elsewhere (24), and specific activity (counts per minute per milligram of protein) was calculated for each extract. These CAT specific activities were used to normalize β -galactosidase values for transfection efficiency.

Generation of additional promoter/leader reporter constructs. In addition to the U_L38 leader deletion constructs, five other promoter/leader constructs were generated and recombined into the viral genome. A full-length promoter/leader fragment of U_L38 from pCAL5div Δ 17 was cloned into a modified pUC19 vector (the vector *Acc*I site had been destroyed) as an *Xba*I-*Asp*718 fragment. This plasmid was then cut with *Acc*I and *Ava*I to remove a portion of the U_L38 promoter and the entire leader (-14 to +99). This sequence was replaced with double-stranded oligonucleotides which contained the appropriate overhangs and sequence from -14 to +18 of either the β U_L37 gene or the β VP16 (U_L48) gene. This scheme yielded two promoter/leader chimeric constructs designated U_L38/U_L37 and U_L38/VP16, respectively.

To further analyze the effect of sequence elements within the U_L38 nontranslated leader region, three additional constructs were generated. The *Xba*I-*Asp*718 fragment from the Δ +9 pCAL5div Δ 17 construct was cloned into pGEM3. This vector was then linearized with *Ava*I. Double-stranded oligonucleotides were cloned into this site. The oligonucleotides were designed to regenerate the *Ava*I (*Sma*I) site at only one location to aid in screening of transformants. These oligonucleotides contained requisite overhangs, linker sequences, and additional sequence corresponding to +20 to +33 of the U_L38 leader or +19 to +31 of the VP5 (U_L19) leader. The exact sequences of the double-stranded oligonucleotides and the constructs generated by using this cloning scheme are shown in Fig. 7. All overhangs were equivalent, and therefore constructs which contained inserts in both orientations were generated. The DASR construct contains the wild-type (*wt*) U_L38 oligonucleotide in the inverted

orientation. All constructs mentioned above were sequenced to confirm their integrity.

Generation and analysis of recombinant viruses. All sequence numbers are based on the complete sequence of the 17syn⁺ strain of HSV-1 (27). The construction of a plasmid which allows recombination into the gC open reading frame has been previously described and was used to generate a recombinant virus [U_L38(+99)] which contained the γ U_L38 promoter and nontranslated leader region (-50 to +99 relative to the transcriptional start site) controlling expression of the β -galactosidase reporter gene. Promoter/leader constructs used in this report were cloned into the gC recombination vector as *Xba*I-*Asp*718 cassettes. Resultant plasmids were digested with *Sal*I to release the fragment containing the reporter gene construct and the flanking gC sequences. This plasmid DNA was cotransfected along with infectious 17syn⁺ DNA into 60-mm-diameter plates of rabbit skin fibroblasts, and then recombinant viruses were screened and purified as described elsewhere (13). To ensure that measured promoter activity was not influenced by second-site mutations, independent isolates of recombinant viruses were generated from separate transfections and shown to exhibit equivalent levels of β -galactosidase expression.

DNA from purified recombinant viruses U_L38(Δ -1), U_L38(Δ +9), and U_L38(Δ +33) was analyzed by Southern blotting with a gC probe and a β -galactosidase probe. These recombinants and the others described in this report were also analyzed by PCR under the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 45 cycles, followed by a final extension at 72°C for 10 min. The DNA of recombinant viruses was analyzed by using two primer sets. A recombinant-specific primer set contains one primer from within the gC (U_L44) sequence which is 90 bp upstream of the cloning *Xba*I site (primer 1 in Fig. 2A; 5'-AACCCCGAT TCCAATCCCCACC-3', bases 97744 to 97765 of the HSV sequence), and the other primer (primer 2 in Fig. 2A; 5'-TGTTTCTGGTCTTCACCCACCG-3') lies within the β -galactosidase coding sequence and is 208 nucleotides downstream of the *Sma*I site which defines the boundary of β -galactosidase and U_L38 sequences. The second primer set is used to analyze the purity of recombinant viruses. These primers (primer 1 described above and primer 3 in Fig. 2A [5'-ACCTTCACCTGCCAGATGACG-3', bases 97340 to 97360 of the HSV sequence]) span the region into which the promoter/leader- β -galactosidase constructs are inserted. Under the reaction conditions described above, the *wt* primer set produces a 428-bp gC fragment only in the presence of DNA from *wt* 17syn⁺ or a nonhomologous recombinant. Following PCR, 15% of the products were electrophoresed on 2% agarose gels and visualized after staining with ethidium bromide.

PCR products generated by asymmetric amplification (8) of DNA from all recombinant viruses were directly sequenced by using the Sequenase kit (U.S. Biochemical). Primers 1 and 2 were used to generate the PCR products, and the β -galactosidase primer was used as the sequencing primer.

β -Galactosidase assays of infected cell extracts. Single confluent wells of rabbit skin cells in six-well plates were infected with either 17syn⁺ or the various recombinant viruses at a multiplicity of infection of 5. Cell extracts were prepared at 16 hpi and assayed for protein concentration and β -galactosidase activity.

RNA isolation. Total infected cell RNA was isolated by the guanidium isothiocyanate-caesium chloride method essen-

tially as described previously (12). In some experiments, phosphonoacetic acid (PAA) was used to inhibit viral DNA replication. Cells were incubated with PAA (300 μ g/ml) for 30 min before infection; the drug was present at the same concentration during viral adsorption and infection. Actinomycin D (10 μ g/ml) was used to study the stability of RNAs of interest by its addition 6 hpi. The infection was allowed to proceed for another 2 or 4 h before RNA isolation.

RNAse protection assays. RNAse protection assays were performed as described previously (13). Templates derived from pGEM3 (Promega) were used with T7 RNA polymerase to generate the riboprobes. The amounts of RNA and radiolabeled probe used in each assay are noted in the figure legends. In all cases, control assays (not shown) were performed to ensure that the linearity of the assay was preserved. Total RNA was hybridized with appropriate probes at 55°C overnight in 50 μ l of buffer containing 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, and 1 mM EDTA. The mixture was then digested by addition of 400 μ l of RNase T₁ (5 μ g/ml; Sigma) in 10 mM Tris (pH 7.5)–300 mM NaCl–5 mM EDTA for 1 h at 30°C. The digestion mix was next treated with 60 μ g of proteinase K at 37°C for 15 min, then phenol-chloroform extracted, and finally ethanol precipitated. RNase-resistant material was fractionated on 8 M urea–8% acrylamide gels along with end-labeled DNA markers. It should be noted that use of DNA markers does not allow exact determination of the lengths of RNase-resistant fragments, as RNA can migrate 5 to 10% more slowly than DNA in acrylamide gels (7).

For analysis of reporter gene transcription by the recombinant viruses generated in this study, a β -galactosidase *in vitro* transcription vector which contains the *Asp718-SmaI* β -galactosidase fragment of pCAL5div Δ 17 in pGEM3 was made. Riboprobes made from this vector contain the β -galactosidase antisense sequences and 49 bases of the pGEM polylinker; RNAs generated during infection with the recombinant viruses protect 215 bases of this 264-base probe in RNAse protection assays. The 5' ends of the β -galactosidase transcripts expressed by the U_L38(Δ +9) and U_L38(Δ +33) recombinants were determined by using antisense riboprobes generated from pGEM3 transcription vectors (shown in Fig. 3) containing the *XbaI-Asp718* fragments of the respective constructs. The construction of a transcription vector for the detection of the dUTPase (U_L50) transcript has been described previously (13); this probe protects 95 bases of the 17syn⁺ wt dUTPase (U_L50) mRNA. A pGEM3 vector was also designed for detection of the wt VP16 (U_L48) transcript. This fragment contains 95 bases of the VP16 leader and 278 bases of upstream sequence and was cloned into the vector as an *XbaI-SmaI* fragment. Originally an *XhoI-SalI* fragment (bases 105062 to 105574 of the HSV sequence) was cloned into pUC20. The upstream *XhoI* site was converted to an *XbaI* site with a linker. This plasmid was then subjected to *Bal* 31 digestion and *SmaI* linker ligation as described elsewhere in the text. The *XbaI-SmaI* fragment from the clone containing 95 bases of the VP16 leader was selected for use as the VP16 riboprobe.

RESULTS

Sequence elements 3' of the transcriptional start site of the strict late U_L38 promoter are important for full reporter gene expression in transient expression assays. To examine the role of sequence elements downstream of the TATA box in regulation of the strict late (γ) U_L38 gene, a set of plasmids

FIG. 1. Effects of U_L38 promoter/leader deletions on reporter gene activity in transient expression assays. Aliquots (10 μ g) of the various U_L38 leader deletion reporter plasmids were transfected into rabbit skin cells; at 24 h posttransfection, cells were superinfected with 1 PFU of HSV-1 per cell. Cells were harvested 18 h following superinfection, and β -galactosidase (β -gal) activity was measured and normalized for transfection efficiency as described in Materials and Methods. The values given for the deletion constructs reflect the average of *n* experiments and are given as percentages of the normalized β -galactosidase value for the full-length construct (+99). The value for the Δ +9 construct is taken from a previous publication (9).

containing the β -galactosidase reporter gene controlled by nested deletions of the U_L38 promoter/leader region was generated for transient expression assays. All constructs contained the U_L38 promoter with a 5' end at -50 (base 84344 of the published sequence for strain 17syn⁺) and amounts of the nontranslated leader region ranging from -1 to +99 bases relative to the transcriptional start site. It should be noted that these reporter constructs contain 85 bp of bacterial β -galactosidase leader sequence; this nonspecific leader functions to allow efficient translation of even "leaderless" transcripts. These constructs are shown schematically in Fig. 1.

As mentioned in Materials and Methods and in earlier publications (9, 10), these reporter constructs also contain the unmodified promoter/leader region of the early (β) U_L37 gene driving expression of CAT to serve as an internal standard for normalization of transfection efficiency between experiments. Aliquots of 10 μ g of each reporter construct were transfected into rabbit skin cells, and at 24 h after transfection, plates were infected with HSV-1 strain 17syn⁺. Reporter gene activities of the deletion constructs from superinfected cells is shown in Fig. 1 as percentages of the value obtained with the plasmid containing the longest leader sequence (+99 bases). Plasmids with deletions of up to 66 bases of leader (to +33 bases) evidenced equivalent or slightly increased levels of reporter gene activity; however, removal of an additional 15 bases resulted in an approximate 20% decrease in expression relative to the full-length construct. Removal of 10 additional bases between +18 and +8 had no significant effect, while removal of the next 8 bases covering the entire viral leader and nominal cap site to -1 resulted in a significant further decrease in β -galactosidase activity to a level of approximately 20% of the value of the +99 construct (div Δ 17B). Also shown in Fig. 1 is the Δ +9 construct, which has been previously described (9). Such results show that sequence elements both near the U_L38 transcriptional start site and within the region from +18 to +33 are important for full reporter gene expression of this γ promoter in transient expression assays.

FIG. 2. Analysis of recombinant viruses. (A) Schematic representation of the recombination site in the gC gene of HSV-1. Relevant restriction enzyme sites are indicated, and the locations of the PCR primers used in the analysis of recombinants are shown in the boxes. SV40, simian virus 40; β-gal, β-galactosidase. (B) Southern blot analysis of UL38 leader deletion viruses. Southern blots of *SalI* digests of viral DNA were probed with either a gC probe (corresponding to bases 97477 through 98427 of the HSV sequence) or a β-galactosidase (β-gal)-specific probe. All recombinant viruses show hybridization to both probes by a fragment of ca. 7,300 bp, as expected. The parental 17syn⁺ DNA shows hybridization to a 3,600-bp fragment when probed with the gC probe and fails to hybridize to the β-galactosidase probe, also as expected. Sizes are indicated in kilobases. (C) PCR analysis of DNA from recombinant viruses carried out as described in Materials and Methods with either a primer set specific for the recombinants (R; primers 1 and 2) or a primer set specific for wt 17syn⁺ or nonhomologous recombinants (wt; primers 1 and 3). The panel on the left shows that in all cases, the recombinant genomes produced an expected recombinant PCR product and failed to produce the wt fragment. The panel on the right shows appropriate controls for the recombinant PCR reactions (Δ+8 plasmid) and for the wt PCR reactions (17syn⁺). Sizes are indicated in bases. (D) PCR products from panel C, tracks i, iii, and v, were digested with *SmaI* and then run on a 7% acrylamide gel to gain a higher-resolution analysis of the recombinants. Two fragments are produced: a β-galactosidase (β-gal)-specific fragment of 234 bp and a fragment of variable length [153, 164, and 186 bp for UL38(Δ-1), UL38(Δ+9), and UL38(Δ+33), respectively] corresponding to the UL38 promoter/leader. Sizes are indicated in bases.

Analyses of recombinant viruses confirm the presence of two regions within the UL38 leader important in controlling reporter gene mRNA and enzyme levels. To examine the activity of the UL38 leader deletion constructs from within an authentic genomic context, recombinant viruses were generated and their integrity was analyzed as described in Materials and Methods. We have previously described a recombination scheme which allows us to assay promoter activity within the nonessential gC ORF (13). This locus was chosen because it is devoid of endogenous promoter activity which could interfere with interpretation of results; it also allows us to mutate promoter/leader sequences of essential viral genes such as UL38 without affecting the viability of recombinant viruses.

The structures of such recombinant viruses are shown in Fig. 2A; the UL38 promoter/leader-controlled transcription of the β-galactosidase message is in the opposite direction of gC transcription. In the recombinants, the 3,600-bp *SalI*

fragment is replaced by a ca. 7,300-bp fragment containing the various promoter modifications controlling β-galactosidase and the simian virus 40 polyadenylation signal inserted into the gC sequences. The simian virus 40 bidirectional polyadenylation signal engineered into the recombinants has been shown previously to efficiently terminate both the truncated gC transcript and the chimeric β-galactosidase transcript. Also shown in Fig. 2A are relevant restriction enzyme sites used in cloning and in Southern analysis and the location of primers used in PCR analysis of recombinants.

Figure 2B shows representative Southern blot analyses of *SalI* digests of DNA from the UL38 leader deletion recombinant viruses. Figure 2C shows PCR analysis of the recombinant viruses with use of the primers indicated in Fig. 2A. Pure recombinant viral genomes did not prime synthesis of a 428-bp amplified product when subjected to PCR using the gC wt primer set 1-3 (tracks ii, iv, and vi), while DNA from

FIG. 3. Activities of U_L38 promoter/leader reporter constructs within the viral genome. (A) β -Galactosidase activities of infected cell extracts. U_L38 leader deletion viruses were used to infect rabbit skin cells. At 16 hpi, infected cell extracts were prepared and assayed for β -galactosidase specific activity. The value given for each recombinant is expressed as a percentage of the activity of the full-length (+99) recombinant and represents the average of three to four experiments. (B) Levels of β -galactosidase (β -gal) RNA expressed during infection with U_L38 leader deletion viruses. At 6 hpi, RNA was prepared from infected cells; aliquots (15 μ g) of these RNAs were subjected to RNase protection assays with a β -galactosidase-specific riboprobe (10⁶ cpm of probe per hybridization) as described in Materials and Methods. Sizes are indicated in bases.

the parental 17syn⁺ showed the expected fragment (track x). In contrast, primer set 1-2 yielded appropriate-size fragments with recombinant virus DNA (tracks i, iii, and v) or with a control plasmid (track vii). These PCR products were then cleaved into two unequal pieces with *Sma*I and analyzed on a 7% polyacrylamide gel (Fig. 2D); this procedure provides a high-resolution analysis of the integrity of the inserted promoter sequences, and variations in the length of the leader sequences present are clearly evident.

As a first measure of modified promoter/leader activity, cells were infected with the leader deletion viruses, and at 16 hpi, extracts were prepared and assayed for β -galactosidase activity (Fig. 3A). Similar to the transient expression results, removal of all but 33 bp of viral leader [U_L38(Δ +33)] had no appreciable effect on measured β -galactosidase activity. However, in contrast to the situation with transient expression assays shown in Fig. 1, further deletion of 15 bp resulted in a recombinant virus [U_L38(Δ +18)] exhibiting a 10-fold loss of activity compared with the original recombinant virus, U_L38(+99). Removal of an additional 9 bp to yield the recombinant U_L38(Δ +9) resulted in no further significant loss of β -galactosidase expression. However, and again in marked contrast to the situation observed in transient assays, removal of only one more base yielded recombinant U_L38(Δ +8), which exhibited a further sevenfold loss in activity, while removal of 8 more bases to delete the cap site generated a recombinant virus [U_L38(Δ -1)] expressing essentially no reporter gene activity. While it could be argued that the differences in β -galactosidase levels seen between U_L38(Δ +8) and U_L38(Δ +9) could be due to perturbation of the DNA secondary structure within this region since these two constructs contain different linker sequences just 3' of the U_L38 sequence, the fact that the constructs are equally active in transient expression assays (Fig. 1) militates against this argument.

We confirmed these observations by measuring steady-

FIG. 4. Determination of the 5' ends of chimeric β -galactosidase (β -gal) transcripts expressed from U_L38 leader deletion viruses. RNA isolated 6 hpi with either U_L38(Δ +9) or U_L38(Δ +33) was analyzed by RNase protection assay using one of the two riboprobes shown at the top. Hybridization with the appropriate 5' probe (tracks ii and iv) provides protection to the transcriptional start site, whereas hybridization with the other probe (tracks i and iii) protects only the β -galactosidase sequence present in both transcripts. Sizes are indicated in bases. M1 and M2, molecular size markers.

state levels of chimeric β -galactosidase mRNA accumulating during infection with these leader deletion viruses, using RNase protection assays with a β -galactosidase-specific probe. RNA was harvested from cells at 6 hpi, since at this time late transcripts are readily detected in rabbit skin cells and effects of possible differential transcript stability will be minimized. Figure 3B shows the results of a typical experiment and confirms the strong correlation between β -galactosidase enzyme and message levels.

To ensure that transcripts measured by using the β -galactosidase-specific RNase protection probe utilized the authentic U_L38 transcription start site, an RNase protection assay was performed with probes to detect the 5' ends of the U_L38(Δ +9) and U_L38(Δ +33) transcripts (Fig. 4). Hybridization of the RNA isolated from cells infected with the U_L38(Δ +9) virus with the probe specific for the U_L38(Δ +9) transcript generated a 230-base RNase-resistant fragment corresponding to full-length hybridization to the authentic start site (track ii). Hybridization of this RNA with a probe specific for the U_L38(Δ +33) transcript generated a truncated 215-base RNase-resistant fragment due to the mismatch just past the β -galactosidase sequence (track i). Conversely, hybridization of RNA from cells infected with U_L38(Δ +33) virus protected 252 bases of the U_L38(Δ +33) probe, which confirmed the use of the authentic start site (track iv), but produced the truncated 215-base product when hybridized with the U_L38(Δ +9) probe (track iii).

Removal of leader elements between +9 and +33 decreases mRNA levels but not sensitivity to template replication for U_L38 promoter-controlled reporter gene expression. To determine whether removal of leader sequences between +9 and +33 changed the strict DNA replication dependence seen in expression of the γ U_L38/ β -galactosidase chimeric transcript, we carried out infections with the recombinant viruses in the presence or absence of the replication inhibitor PAA as described in Materials and Methods. The RNA generated in this experiment was then analyzed by RNase protection assay using the β -galactosidase-specific probe. Levels of the $\beta\gamma$ VP16 (U_L48) and β dUTPase (U_L50)

FIG. 5. Expression of RNA by the U_L38 leader deletion viruses in the presence of PAA. RNase protection assays were performed with RNA from cells infected for 6 h in either the presence or absence of PAA. (A) Ten micrograms of RNA hybridized with 10^6 cpm of the β -galactosidase (β -gal) probe; (B) 10 μ g of RNA hybridized with 10^6 cpm of a probe for the *wt* $\beta\gamma$ VP16 (U_L48) transcript; (C) 20 μ g of RNA hybridized with 10^6 cpm of a probe for the *wt* β dUTPase (U_L50) transcript. Sizes are indicated in bases.

transcripts were also measured as controls by using appropriate probes (Fig. 5). As seen in Fig. 5A, the expression of β -galactosidase RNA following infection with the $U_L38(\Delta+9)$ and $U_L38(\Delta+33)$ recombinant viruses was strongly inhibited in the presence of PAA, confirming unaltered strict late (γ) kinetics. As expected, the levels of the *wt* $\beta\gamma$ VP16 (U_L48) transcript were reduced but still present at readily measurable levels (Fig. 5B), while levels of the β dUTPase (U_L50) transcript were essentially unaffected by the presence of PAA (Fig. 5C).

Sequence elements from -14 to +18 of the U_L38 promoter/leader are functionally distinct from those of the β U_L37 or $\beta\gamma$ VP16 (U_L48) promoter/leader. As shown by the comparison of reporter gene activity expressed by the $U_L38(\Delta-1)$, $U_L38(\Delta+8)$, and $U_L38(\Delta+9)$ viruses (Fig. 3), an element spanning the transcriptional start site to +9 is critical for basal expression from the U_L38 promoter. We next examined whether this element has a role in the γ kinetics of accumulation of this transcript. We constructed two recombinant viruses in which the U_L38 sequences from -14 to +18 (HSV bases 84384 to 84417) were replaced with analogous sequences from β and $\beta\gamma$ promoters. Recombinant U_L38/U_L37 contained DNA sequences corresponding to -14 to +18 relative to the cap site of the early (β) U_L37 gene (HSV bases 84233 to 84202), and recombinant $U_L38/VP16$ contained equivalent sequences of the leaky-late ($\beta\gamma$) VP16 (U_L48) promoter/leader region (HSV bases 105240 to 105271); these constructs are shown schematically in Fig. 6A. The DNA sequences of the relevant promoter regions of the $U_L38(\Delta+18)$, $U_L38(\Delta+9)$, $U_L38(\Delta+8)$, $U_L38(\Delta-1)$, U_L38/U_L37 , and $U_L38/VP16$ recombinants are shown in Fig. 6B; it is clear that all recombinants except the $U_L38(\Delta-1)$ construct contain canonical transcription initiator (INR) elements.

The results of β -galactosidase assays performed with cell extracts prepared at 16 hpi with these recombinants are also shown in Fig. 6B; while low but readily detectable levels of β -galactosidase are seen in the $U_L38/VP16$ -infected cell

FIG. 6. Sequences from -14 to +18 of the U_L38 promoter/leader are functionally distinct from analogous sequences of the β U_L37 and the $\beta\gamma$ VP16 (U_L48) promoter/leader. (A) Schematic diagram of recombinant viruses $U_L38(\Delta+18)$, U_L38/U_L37 , and $U_L38/VP16$. Construction of these recombinants is described in Materials and Methods. β gal, β -galactosidase. (B) Sequences of the promoter/chimera and selected U_L38 leader deletion reporter constructs from bases -31 to +33. Shown at the top is the INR consensus described elsewhere (36, 38). The recombinant viruses containing these promoter constructs were used to infect rabbit skin cells. At 16 hpi, cell extracts were prepared and β -galactosidase (β -gal) specific activity was determined. The values shown at the right are given as percentages of the specific activity expressed by the virus bearing the full U_L38 leader [$U_L38(+99)$] and represent the averages of three to four experiments. (C) Expression of chimeric β -galactosidase (β -gal) RNA during infection with $U_L38(\Delta+18)$, U_L38/U_L37 , and $U_L38/VP16$. RNase protection assays were performed with RNA from cells infected for 6 h in either the presence or absence of PAA. The top panel shows the result of an RNase protection assay using 10 μ g of RNA and 10^5 cpm of the β -galactosidase probe. Shown below is an RNase protection assay using 5×10^5 cpm of the *wt* VP16 probe and 2- μ g aliquots of the RNAs specified above. Sizes are indicated in bases.

extracts, appreciable β -galactosidase activity was not seen in the U_L38/U_L37 -infected cell extracts. To examine the DNA replication dependence of the promoter/leader reporter constructs within these recombinant viruses, rabbit skin cells infected in either the presence or absence of PAA were harvested at 6 hpi, and RNA was extracted. This RNA was then analyzed by RNase protection analysis; each

	-30	-20	-10	+1	+10	+20	+30	Relative β-gal activity (% of U _L 38(+99))
U _L 38 (Δ+33)	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAAACACACCCACCGGAGCTAGCCTCCCCCGGGA							108±15
U _L 38 (Δ+18)	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAAACACACCCCTCCCCGGGAGCTTGGGATCTCTAT				BglIII	SmaI		11±4
U _L 38 (Δ+9)	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAGATCTCTCCCGGAGCTTGGGATCTCTATAATCTCG							9±2
	AAATTGCACACATATGCCTGCGCGGCGCGGTCAGCCGGTCTAGAGGGCCCTCGAACCTAGAGATATTAGAGC							
U _L 38 (Δ+9) / DAS	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAGATCTCT						CCGGGA	97±21
	AAATTGCACACATATGCCTGCGCGGCGCGGTCAGCCGGTCTAGAGGGCC						CT	
U _L 38 (Δ+9) / DASR	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAGATCTCT						CCGGGA	8±1
	AAATTGCACACATATGCCTGCGCGGCGCGGTCAGCCGGTCTAGAGGGCC						CT	
U _L 38 (Δ+9) / VP5	TTTAAACCTGTGTATACGGACGCGCGGGCCMGTCGGCCAGATCTCT						CCGGGA	13±2
	AAATTGCACACATATGCCTGCGCGGCGCGGTCAGCCGGTCTAGAGGGCC						CT	

FIG. 7. Presence of a specific 14-bp *cis*-acting element within the U_L38 nontranslated leader region. Recombinant viruses which contained the U_L38 promoter/leader bearing specific leader alterations controlling expression of β-galactosidase (β-gal) were generated; the sequence from -31 to +43 is shown. Bases in italics represent either linker or β-galactosidase (bacterial) sequence. The double-stranded oligonucleotides cloned into the *Ava*I (*Sma*I) site of the Δ+9 construct (see Materials and Methods) are shaded. The 14 bases which constitute DAS are shown in boldface. Recombinants were used to infect rabbit skin cells, and infected cell extracts were prepared at 16 hpi and assayed for β-galactosidase activity. Each value shown at the right is given as a percentage of the specific activity expressed by the virus bearing the full U_L38 leader [U_L38(+99)] and represents the average of three to four experiments.

sample was probed with a β-galactosidase-specific probe and with a *wt* VP16 (U_L48) probe as a control. The results of a typical experiment are shown in Fig. 6C; as expected from the results of the β-galactosidase assays, the U_L38/U_L37 recombinant did not express any chimeric β-galactosidase mRNA under either condition of infection. Also consistent with the measured enzyme levels, chimeric U_L38/VP16 β-galactosidase RNA was expressed at measurable but reduced levels compared with that seen in infections with the U_L38(Δ+18) recombinant. However, and in contrast to the situation during infections with U_L38(Δ+18) (Fig. 6C) and U_L38(Δ+9) (Fig. 5A) recombinants, the U_L38/VP16 recombinant exhibited a relaxed sensitivity to PAA. As treated more fully in Discussion, such a result suggests that specific DNA sequences within the basal U_L38 promoter have a role in the requirement for template replication for γ promoter expression.

A leader element positioned between +20 to +33 relative to the U_L38 cap site influences levels of mRNA expression. The marked reduced expression of β-galactosidase by the U_L38(Δ+18) virus compared with that of the U_L38(Δ+33) virus suggested that a specific element influencing message levels had been disrupted or completely deleted. Three viruses were generated to test the specificity of this element and to more precisely define its boundaries. These constructs (Fig. 7) were generated by ligating double-stranded oligonucleotides into the *Ava*I (*Sma*I) site of the Δ+9 construct.

All constructs contain the U_L38 promoter from -50 to +9 and a linker region to +19. The Δ+9/DAS construct contains U_L38 *wt* sequence from +20 to +33, while the Δ+9/DASR construct contains this same sequence but in an inverted orientation. The Δ+9/VP5 construct replaces the U_L38 sequence with nonhomologous sequence from the βγ VP5 (U_L19) leader region (+19 to +31 relative to the cap site; bases 40756 to 40744 of the HSV sequence). Viruses containing these modified promoter/leader regions controlling β-galactosidase expression were used to infect cells, and enzyme assays of infected cell extracts were then performed.

The values shown in Fig. 7 are given as percentages of U_L38(+99) specific activity and represent averages of three to four experiments. These results clearly show that a specific element increasing expression from the U_L38 promoter lies between +20 and +33 of the viral leader and that this element exhibits an orientation dependence for its

function. We have termed this element the downstream activation sequence or DAS.

U_L38 DAS has no measurable effect on reporter mRNA stability. Steady-state mRNA levels for a given transcript are a function of the transcriptional activity of the promoter mediating its expression and the mRNA's stability within the cell. Conceivably, DAS could function to increase transcription rates or increase message stability. We investigated any possible differences in the half-lives of the chimeric transcripts from the U_L38(Δ+9)/DAS and U_L38(Δ+9)/DASR viruses, using an actinomycin D chase experiment. Cells were infected with either of the two recombinants for 6 h, at which time cells were harvested or incubated with medium containing the transcriptional inhibitor actinomycin D for an additional 2 or 4 h. RNA isolated from each time point was analyzed by RNase protection assays using either a β-galactosidase or a *wt* VP16 (U_L48) probe.

As seen in Fig. 8, the control VP16 (U_L48) mRNA decayed with a half-life in the range of 2 to 4 h during the actinomycin chase; in striking contrast, β-galactosidase re-

FIG. 8. The orientation of U_L38 DAS has no effect on the stability of chimeric β-galactosidase transcripts. At 6 h after infection with the indicated virus, cells were harvested or treated with medium containing actinomycin D (10 μg/ml) for an additional 2 or 4 h. (A) RNase protection assay performed with 10 μg of RNA from each time point, using 5×10^5 cpm of β-galactosidase (β-gal) probe; (B) RNase protection assay performed with 2-μg aliquots of the same RNAs, using 5×10^5 cpm of *wt* VP16 (U_L48) probe. Sizes are indicated in bases.

porter transcripts expressed by either of the recombinants were stable. The decay of the control VP16 (U_L48) mRNA validates the utility of this technique for measuring differential transcript stability within the infected cell. In another experiment (not shown), 30- and 60-min actinomycin D chases were performed on cells infected with $U_L38(\Delta+9)$ and $U_L38(\Delta+33)$ viruses; again, the β -galactosidase transcripts were stable. These results demonstrate that DAS deletion or inversion does not adversely affect transcript stability, and we conclude that DAS increases the transcriptional activity of the U_L38 promoter.

DISCUSSION

In a previous report, we have shown that the unusual TATA homology TTTAAA defines the minimal 5' sequence required for U_L38 promoter activity within the gC locus used in this study (13). In the same study, we demonstrated that the identical U_L38 promoter/leader sequences were sufficient for regulated γ kinetics of expression within the R_L region of the genome. We have also generated recombinant viruses containing promoter/leader constructs of the β class (dUTPase; U_L50) and the $\beta\gamma$ class (VP5; U_L19) controlling expression of β -galactosidase within the same gC locus as reported in the accompanying report (18); these recombinants express chimeric reporter mRNA with kinetics identical to those of the parental promoters. Such results demonstrate that the gC locus used in this study is kinetically neutral for transcription and that the sequence element(s) necessary for regulated γ kinetics of the U_L38 promoter lies within -31 to +99.

The data presented in this report show that the U_L38 regulatory region consists of a core late promoter spanning the transcriptional start site linked to a DAS within the 5' nontranslated leader region. The elimination of basal β -galactosidase expression seen during infection with the $U_L38(\Delta-1)$ virus (Fig. 3) shows that sequences at and/or just 3' of the transcriptional start site are critical for basal expression from this late promoter. The very low level of β -galactosidase expressed from $U_L38(\Delta+8)$ compared with $U_L38(\Delta+9)$ suggests that the 3' limit of the core promoter is at +9. Data in Fig. 5 show that the accumulation of the β -galactosidase RNA during infection with the $U_L38(\Delta+9)$ recombinant is strictly dependent on viral DNA replication; however, substitution of DNA sequences from the $\beta\gamma$ VP16 (U_L48) promoter relaxes this dependence. These results taken together lead us to conclude that sequences from -31 to +9 of the U_L38 promoter are necessary and sufficient for regulated basal γ gene expression.

Data for other HSV γ promoters also indicate that sequence elements downstream of and including the TATA box play a critical role in the regulation of γ genes. Homa and colleagues used promoter/leader deletions to infer that a 15-bp sequence element of the gC (U_L44) promoter containing the gC TATA box was sufficient for regulated γ gene expression within its native genomic context (16). Kibler et al. (23) recombined this same element into the TK (U_L23) locus and showed that it and other minimal TATA elements were sufficient to direct appreciable levels of transcription, but with $\beta\gamma$ kinetics of expression. Inclusion of the strict late (γ) U_S11 promoter/leader sequence from -16 to +40 downstream of the minimal TATA element was required to confer γ kinetics of regulation.

Linker scanning mutagenesis of the γ promoters for gC (U_L44) and gH (U_L22) has also shown that sequence elements at the transcriptional start site are important for γ

gene expression (40). The only known consensus *cis*-acting element within this region of RNA polymerase II promoters is the INR element as first described by Smale et al. (38) and later given the consensus sequence YΔYTCYY (where A denotes the transcript start site) by Roy et al. (36). Despite this, our examination of the effect of substitution of sequence elements spanning -14 to +18 from β and $\beta\gamma$ promoter/leaders for those of U_L38 (Fig. 6B and C) indicates that the lack of expression of chimeric mRNA by the $U_L38(\Delta-1)$ recombinant cannot be simply due to lack of an INR element since essentially no mRNA is expressed from the U_L38/U_L37 promoter. The relaxed requirement for DNA replication seen with the chimeric $U_L38/VP16$ promoter indicates that one or more other sequence elements within the core U_L38 promoter between -14 and +9 relative to the cap site are required for γ kinetics of mRNA accumulation. These results, along with the data on other γ promoters reviewed above, indicate that such elements are a general feature of γ HSV promoters.

In addition to the elements which constitute the core U_L38 promoter, we have identified a DAS important for full-level expression of reporter gene mRNA. A specific 14-bp element positioned between +20 and +33 of the U_L38 leader increases expression from the core promoter approximately 10-fold (Fig. 3 and 7). The $U_L38(\Delta+9)/DAS$ recombinant contains seven point mutations in the region from +10 to +19 relative to the *wt* U_L38 sequence. The essentially *wt* levels of β -galactosidase expressed during infection with this virus (Fig. 7) strongly suggest that this region acts as a relatively nonspecific spacer between the core promoter and DAS. The results with $U_L38(\Delta+9)/DASR$ show that DAS exhibits orientation dependence for its function. Actinomycin D chase experiments with $U_L38(\Delta+9)$ and $U_L38(\Delta+33)$ (data not shown) and with $U_L38(\Delta+9)/DAS$ and $U_L38(\Delta+9)/DASR$ (Fig. 8) indicate that DAS modification or deletion does not alter the stability of the reporter gene transcript, suggesting that DAS influences the accumulation of message at the transcriptional level.

An additional line of evidence militates against DAS functioning to influence cytosolic stability of chimeric transcripts. In transient expression assays, β -galactosidase levels of the $\Delta+18$ construct are 56% of those for the $\Delta+33$ construct (Fig. 1); however, when these same constructs are recombined into the viral genome, the β -galactosidase results become sharply different: $U_L38(\Delta+18)$ expresses only 8% of the activity shown by $U_L38(\Delta+33)$ (Fig. 3A). If the deletion exerted its effect solely at the level of differential mRNA stability, the effect should be equivalent in the two systems; this is clearly not the case.

Regions of the U_L38 promoter/leader have previously been used as probes in gel retardation assays using mock-infected and infected cell extracts (9, 31). These studies have shown that the U_L38 leader from -14 to +99 forms complexes which contain the $\alpha 4$ protein; this interaction is not direct but requires mediation by cellular factors. It has also been shown that only phosphorylated $\alpha 4$ participates in formation of these complexes. Footprint analysis of the U_L38 promoter from -50 to +99 using infected cell nuclear extracts demonstrated defined protected regions (9). Data demonstrated in the present report show that two of these areas, -17 to +9 within the core promoter and +24 to +39, which includes DAS, contain critical *cis*-acting sequences required for promoter activity in recombinant virus. However, the third area of protection (+57 to +71) seen in the footprints appears to be dispensable for reporter gene ex-

FIG. 9. Regions of the U_L38 promoter/leader important for reporter gene expression largely overlap areas which form complexes with infected cell nuclear extracts. The figure summarizes data shown here and elsewhere (9, 13). Ovals show areas protected during footprint analysis of the U_L38 promoter/leader with use of infected cell nuclear extracts. Shown in the striped box is the core U_L38 promoter. The 5' limits of the promoter are at the TATA homology (TTTAAA) at -31; this element is strictly required for reporter gene expression. The 3' limit of the core promoter is at +9. U_L38 DAS shown in the cross-hatched box is necessary for *wt* reporter gene levels. See text for details.

pression in recombinant virus. The results are summarized in Fig. 9.

DAS could conceivably function to increase transcription from the U_L38 promoter by functioning as a binding site for a protein factor or factors which recruit necessary transcription factors to the promoter to aid in template commitment and/or initiation of transcription. Alternatively, a *trans*-acting protein(s) could interact with DAS at the level of either DNA or RNA to increase the processing of RNA polymerase II elongation complexes through this region. It may be of significance that U_L38 DAS shares 5 bp of perfect homology (GTAGC) with a similarly located region in the leader of the strict late γ 42 (U_L49.5) gene. Further, mutation of this sequence within the γ 42 leader has been recently shown to eliminate α 4 binding to this DNA probe *in vitro* and reduce accumulation of message levels during infection with recombinant viruses (14). The location of DAS within the nontranslated leader region and its orientation dependence are reminiscent of the *cis*-acting Tat response element of human immunodeficiency virus. The interaction of human immunodeficiency virus *trans*-acting protein Tat with the Tat response element is believed to be important in increasing the processing of elongating RNA polymerase II through this region (22); such a role for DAS is plausible.

Although the transient expression assays shown in Fig. 1 suggest the importance of DAS for full expression, clearly this method of analysis is much less sensitive to its action than is analysis from within the viral genome. It is of interest to consider reasons for the differences seen between results of promoter mutagenesis studies using transient expression assays and those using recombinant viruses reported here and elsewhere (21, 39). Knipe et al. have demonstrated the presence of replication compartments within infected cell nuclei which contain the regulatory proteins α 0 and α 4 and probably other proteins or protein assemblies involved in regulated viral transcription (25). Thus, reporter gene constructs resident within the viral genome would be exposed to a different transcriptional environment than would those resident on plasmids introduced via transfection. For U_L38 DAS to be fully functional, it may require interaction with specific complexes within the replication compartments that the plasmid-borne promoter constructs are not exposed to.

Clearly more work must be done to better understand how U_L38 DAS functions to increase message levels from the U_L38 promoter. Although direct and indirect interactions between the α 4 protein and late gene leaders have been documented above, the significance of these interactions is not clear at this time. Elucidation of the interactions between cellular transcription factors and the HSV α proteins is

necessary to gain a clearer understanding of transcriptional regulation during infection by HSV.

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