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Transcriptional Activation of the Herpes Simplex Virus Type 1 U_L38 Promoter Conferred by the *cis*-Acting Downstream Activation Sequence Is Mediated by a Cellular Transcription Factor

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The herpes simplex virus (HSV) type 1 strict late (γ) U_L38 promoter contains three *cis*-acting transcriptional elements: a TATA box, a specific initiator element, and the downstream activation sequence (DAS). DAS is located between positions +20 and +33 within the 5′ untranslated leader region and strongly influences transcript levels during productive infection. In this communication, we further characterize DAS and investigate its mechanism of action. DAS function has a strict spacing requirement, and DAS contains an essential 6-bp core element. A similarly positioned element from the γ gC gene (U_L44) has partial DAS function within the U_L38 promoter context, and the promoter controlling expression of the γ U_S11 transcript contains an identically located element with functional and sequence similarity to U_L38 DAS. These data suggest that downstream elements are a common feature of many HSV γ promoters. Results with recombinant viruses containing modifications of the TATA box or initiator element of the U_L38 promoter suggest that DAS functions to increase transcription initiation and not the efficiency of transcription elongation. In vitro transcription assays using uninfected HeLa nuclear extracts show that, as in productive infection with recombinant viruses, the deletion of DAS from the U_L38 promoter dramatically decreases RNA expression. Finally, electrophoretic mobility shift assays and UV cross-linking experiments show that DAS DNA forms a specific, stable complex with a cellular protein (the DAS-binding factor) of approximately 35 kDa. These data strongly suggest that the interaction of cellular DAS-binding factor with DAS is required for efficient expression of U_L38 and other HSV late genes.

The coordinated expression of the approximately 80 to 100 transcripts of herpes simplex virus type 1 (HSV-1) is a complex process mediated by the independent function of numerous promoters (reviewed in references 42 and 43). As such, it provides an excellent system for studying the process of regulated transcription by RNA polymerase II in eukaryotic cells. Previous mutational analysis of the strict late (γ) U_L38 promoter revealed the presence of a novel *cis*-acting element, termed downstream activation sequence (DAS), within the 5′ untranslated leader region (14). Here we describe the further characterization of DAS and its effect on promoter activity by use of molecular genetic and biochemical approaches.

One area of research within our laboratory has been to elucidate mechanisms employed by HSV-1 to express its genes in a temporally regulated fashion during productive infection. Specifically, we are interested in the transcriptional "switch" observed during infection, demarcating early and late phases of viral gene expression. The onset of viral DNA replication is coincident with the cessation of transcription of early (β) genes and maximal expression of late genes (16, 35, 43). The late genes have been further subdivided into two classes on the basis of the stringency in their requirement for DNA replication for expression; leaky-late (βγ) transcripts appear at readily detectable levels prior to DNA replication, while strict late (γ) transcripts are poorly expressed prior to this event.

The basis of the nearly mutually exclusive patterns of expression of β and γ genes during infection is not well understood. While it is clear that β and γ genes require the HSV immediate-early (α) proteins for measurable expression, class-specific early or late promoter elements have not been clearly defined (reviewed in references 35, 42, and 43). Despite this, the results of promoter mutagenesis studies and the construction of chimeric promoters demonstrate that there are significant differences between early and late promoters—for example, binding sites for cellular transcription factors such as Sp1 well upstream of the TATA box are important for efficient early expression, while elements near and downstream of the TATA element play a critical role in late expression (14, 17, 20, 38).

In previous communications, we have defined three regions within the γ U_L38 promoter which are critical for regulated transcription. Deletion and mutation analysis showed that the TATA box homology of TTTAAA at position −31 relative to the transcript start site defines the 5′ extent of this promoter and that specific sequences spanning the transcript start site to +9 are required for basal γ gene expression (12, 14). This led us to conclude that sequences from −31 to +9 of the U_L38 gene constitute a core γ promoter. DAS is the third critical *cis*-acting element and is positioned between +20 and +33 within the 5′ untranslated leader region. DAS increases expression from the core promoter approximately 10-fold.

Data presented in this communication indicate that DAS interacts with a cellular transcription factor of approximately 35 kDa to augment transcription initiation at the U_L38 promoter. Since other γ promoters share the general structure of the U_L38 promoter, we suggest that the basis of regulated γ

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transcript expression proposed for U_L38 is common to other strict late promoters.

MATERIALS AND METHODS

Cells and recombinant virus. HSV-1 (17*syn*$^+$) was the parental strain used to generate the recombinant viruses described below. Recombinant viruses were generated and analyzed as described previously (12, 14). Parental and recombinant virus infection of rabbit skin cells 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 or primer extension assays. Vero cells were used for plaque assays, and both cell types were used during recombinant virus screening and isolation. HeLa S3 and mouse neuroblastoma 2A cells were also used. Cells were maintained at 37°C under 5% carbon dioxide in Eagle minimum essential medium containing 5% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Generation of recombinant viruses. A number of sequence modifications of the U_L38 promoter were made and used to generate recombinant viruses for the determination of critical sequences involved in promoter function. Additionally, U_S11 constructs were generated and recombined into the viral genome to determine if the HSV-1 U_S11 promoter contains a functional DAS element. Specific constructs were cloned into the *Xba*I-*Asp*718 "cassette" described for recombination into the gC region of the HSV-1 genome. Details of construction are outlined below; the integrity of all modifications was confirmed by PCR analysis and direct nucleotide sequencing as described previously (14).

For the purpose of consistency in naming recombinant viruses containing U_L38 promoter constructs bearing mutations within DAS, we have changed the name of recombinant $U_L38\Delta+9$/VP5 (14) to $U_L38\Delta+9$/das1. In this communication the lowercase designation das indicates specific alterations within the DAS element. The specific promoter in this recombinant virus replaces U_L38 DAS with a nonhomologous region of the leader from the βγ VP5 gene. As described previously, this modification changes 9 of 14 bases throughout DAS (see Fig. 2A).

The promoter region of recombinant virus $U_L38\Delta+9$/DAS(+42/+55) was constructed as follows. The pGem3 vector containing the *Xba*I-*Asp*718 fragment used to generate recombinant $U_L38\Delta+9$/das1 was linearized with *Ava*I. The double-stranded DAS oligonucleotide (14) was cloned into this site. The net effect of these manipulations is the generation of a U_L38 promoter construct that contains DAS in the correct orientation moved 22 bases downstream of its normal position.

The promoter regions of four recombinant viruses containing specific mutations within DAS (see Fig. 2) were generated as follows. A pGem3 vector containing the *Xba*I-*Asp*718 fragment of the $U_L38\Delta+9$ recombinant was linearized with *Ava*I. Double-stranded oligonucleotides containing mutations within DAS were cloned into the *Ava*I site; aside from specific base changes within DAS (see Fig. 2), these oligonucleotides are essentially identical to the DAS oligonucleotide described previously (14).

The promoter region of $U_L38\Delta+9$/gC was generated as follows. A pGem3 vector containing the *Xba*I-*Asp*718 fragment of the $U_L38\Delta+9$ recombinant was linearized with *Ava*I. A double-stranded oligonucleotide containing sequence from position +20 to +38 of gC (U_L44) and appropriate overhangs was cloned into the *Ava*I site. The sequences of the single-stranded oligonucleotides are as follows: upper strand, 5′-CCGGCGGGAGGCCGCATCGAACGCTCCC-3′; lower strand, 5′-CCGGGGGAGCGTTCGATGCGGCCTCCCG-3′.

U_L38 promoter constructs containing modifications of the wild-type (wt) TATA element were generated as follows. The *Xba*I-*Asp*718 fragments of $U_L38\Delta+9$/DAS and $U_L38\Delta+9$/das1 were cloned into pUC19 in which the vector *Acc*I site had been destroyed. The resultant plasmids were then digested with *Acc*I and *Xba*I. These manipulations released U_L38 promoter sequences from position −50 to −15. A double-stranded oligonucleotide that alters the U_L38 TATA element was cloned into the two vectors. The sequences of the single-stranded oligonucleotides are as follows: upper strand, 5′-CTAGAGGCCCTA TAAAAGTGTGT-3′; lower strand, 5′-ATACACACTTTTAT AGGGCCT-3′. These manipulations result in the generation of U_L38 promoters that are different from the parental promoters in two ways: (i) U_L38 sequences from −50 to −37 are removed, and (ii) the U_L38 TATA boxes are changed from TTTAAAC to TATAAAA. Previous studies have demonstrated that deletion of bases −50 to −32 of the U_L38 promoter does not affect levels of RNA expression (12). The promoter in recombinant U_L38-TATA was generated by releasing the *Acc*I-*Sma*I fragment from $U_L38M\Delta+9$/DAS, blunting the *Acc*I overhang, and ligating the ends. This recombinant does not contain any U_L38 sequences downstream of position −15.

The promoter region of U_L38/U_L37/DAS was generated as follows. The *Xba*I-*Asp*718 fragment of $U_L38\Delta+9$/DAS was cloned into pUC19 in which the vector *Acc*I site had been destroyed. This vector was then digested with *Acc*I and *Bgl*II and purified. A double-stranded oligonucleotide with the following sequences was then cloned into this vector: upper strand, 5′-ATACCGAAGACGCGGGGTACTCGCAACA-3′; lower strand, 5′-GATCTGTTGCGAGTACCCCGCGTCT TCGGT-3′. These manipulations result in the replacement of U_L38 sequences from position −14 to +9 with those of the β U_L37 promoter.

The promoter regions of U_S11 recombinant viruses were generated as follows. Single-stranded oligonucleotides with the following sequences were annealed: upper strand, 5′-CTAGA TCAATAAAAGGGGGCGTGAGGACCGGGAGGCGGC CAGAACCGCCG-3; lower strand, 5′-TGCACGGCGGTTC TGGCCGCCTCCCGGTCCTCACGCCCCCTTTTATTGAT-3′. This resulted in the generation of a double-stranded oligonucleotide that contained U_S11 sequences from position −34 to +15. This double-stranded oligonucleotide contained an *Xba*I overhang upstream of the U_S11 TATA box for cloning purposes and contained an *Apa*LI overhang at the other terminus. The sequences of the *Apa*LI site are contained within the wt U_S11 sequence and were utilized to ligate this double-stranded oligonucleotide to one of two other double-stranded oligonucleotides. One of the downstream double-stranded oligonucleotides contained wt U_S11 sequence from +16 to +36 and an *Ava*I overhang for cloning purposes. The sequences of the single-stranded oligonucleotides are as follows: upper strand, 5′-TGCACGACCCGGAGCGTCCCCT GC-3′; lower strand, 5′-CCGGGCAGGGGACGCTCCGGG TCG-3′. The second downstream double-stranded oligonucleotide was similar to the first except that the 8 bp of the U_S11 sequence that is homologous to DAS was replaced with a *Hin*dIII site. The exact sequences of these single-stranded oligonucleotides are as follows: upper strand, 5′-TGCACGA CAAAGCTTTTCCCCTGC-3′; lower strand, 5′-CCGGGCA GGGGAAAAGCTTTGTCG-3′. These downstream double-stranded oligonucleotides were ligated to the upstream double-stranded oligonucleotide. After ligation, reaction mixtures were phenol-chloroform extracted and ethanol precipitated.

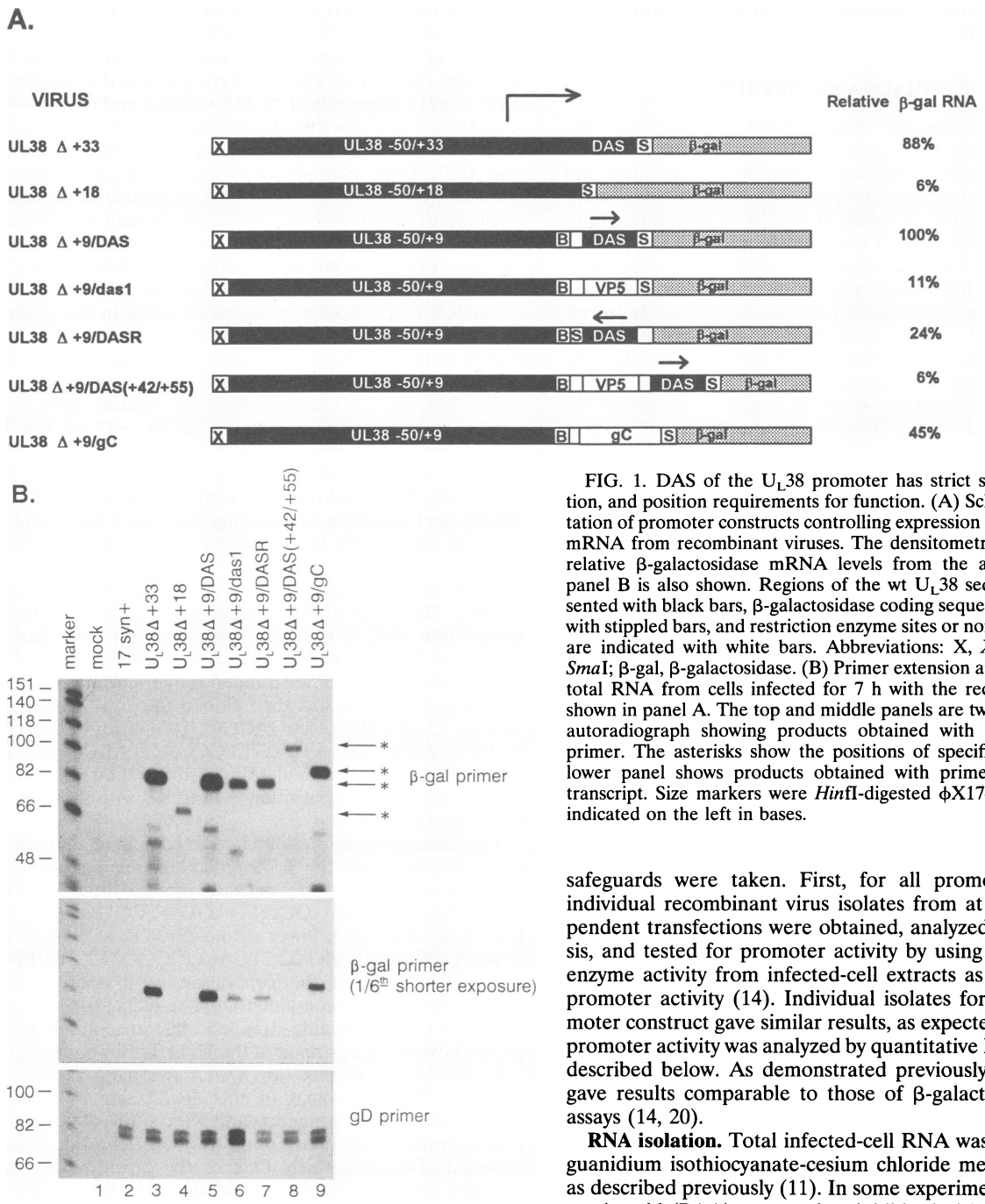


FIG. 1. DAS of the U_L38 promoter has strict sequence, orientation, and position requirements for function. (A) Schematic representation of promoter constructs controlling expression of β-galactosidase mRNA from recombinant viruses. The densitometric quantitation of relative β-galactosidase mRNA levels from the autoradiograph of panel B is also shown. Regions of the wt U_L38 sequence are represented with black bars, β-galactosidase coding sequences are indicated with stippled bars, and restriction enzyme sites or non-U_L38 sequences are indicated with white bars. Abbreviations: X, *Xba*I; B, *Bgl*II; S, *Sma*I; β-gal, β-galactosidase. (B) Primer extension analysis of 10 μg of total RNA from cells infected for 7 h with the recombinant viruses shown in panel A. The top and middle panels are two exposures of an autoradiograph showing products obtained with a β-galactosidase primer. The asterisks show the positions of specific start sites. The lower panel shows products obtained with primer for the wt gD transcript. Size markers were *Hin*fI-digested ϕX174 DNA; sizes are indicated on the left in bases.

Resuspended ligated oligonucleotides were then digested with *Xba*I and *Ava*I. Restriction enzyme digests were separated on an 8% nondenaturing polyacrylamide gel electrophoresis (PAGE) gel to isolate the 74-bp fragments indicative of the correct heterodimeric oligonucleotide ligation product. These fragments were eluted and then cloned into an *Xba*I-*Ava*I-cut and purified pGem3 vector that contained the *Xba*I-*Asp*718 fragment of the $U_L38Δ+9$ recombinant. These manipulations resulted in the placement of the two U_S11 promoter-leader constructs upstream of β-galactosidase sequences. (See Fig. 3 for the exact sequences of the promoter constructs.)

Analysis of promoter activity from recombinant viruses. To ensure careful analysis of promoter activity, the following safeguards were taken. First, for all promoter constructs, individual recombinant virus isolates from at least two independent transfections were obtained, analyzed by PCR analysis, and tested for promoter activity by using β-galactosidase enzyme activity from infected-cell extracts as an indicator of promoter activity (14). Individual isolates for any given promoter construct gave similar results, as expected. Additionally, promoter activity was analyzed by quantitative RNA analysis as described below. As demonstrated previously, RNA analysis gave results comparable to those of β-galactosidase enzyme assays (14, 20).

RNA isolation. Total infected-cell RNA was isolated by the guanidium isothiocyanate-cesium chloride method essentially as described previously (11). In some experiments phosphonoacetic acid (PAA) was used to inhibit viral DNA replication. Cells were incubated with PAA (400 μg/ml) for 30 min before infection; the drug was present at the same concentration during viral adsorption and infection. Actinomycin D (10 μg/ml) was used to study the stabilities of RNAs of interest by its addition at 6 h postinfection (hpi). The infection was allowed to proceed for an additional 2 or 4 h before RNA isolation.

RNA analysis. In most experiments, RNA was analyzed with a commercial primer extension kit (Promega) according to the manufacturer's instructions. Reactions were performed with equivalent aliquots of RNA with either a β-galactosidase primer (5′-GTGTTCGAGGGGAAAATAGGTTGCGCGAG-3′) or a primer for the wt gD transcript as described previously

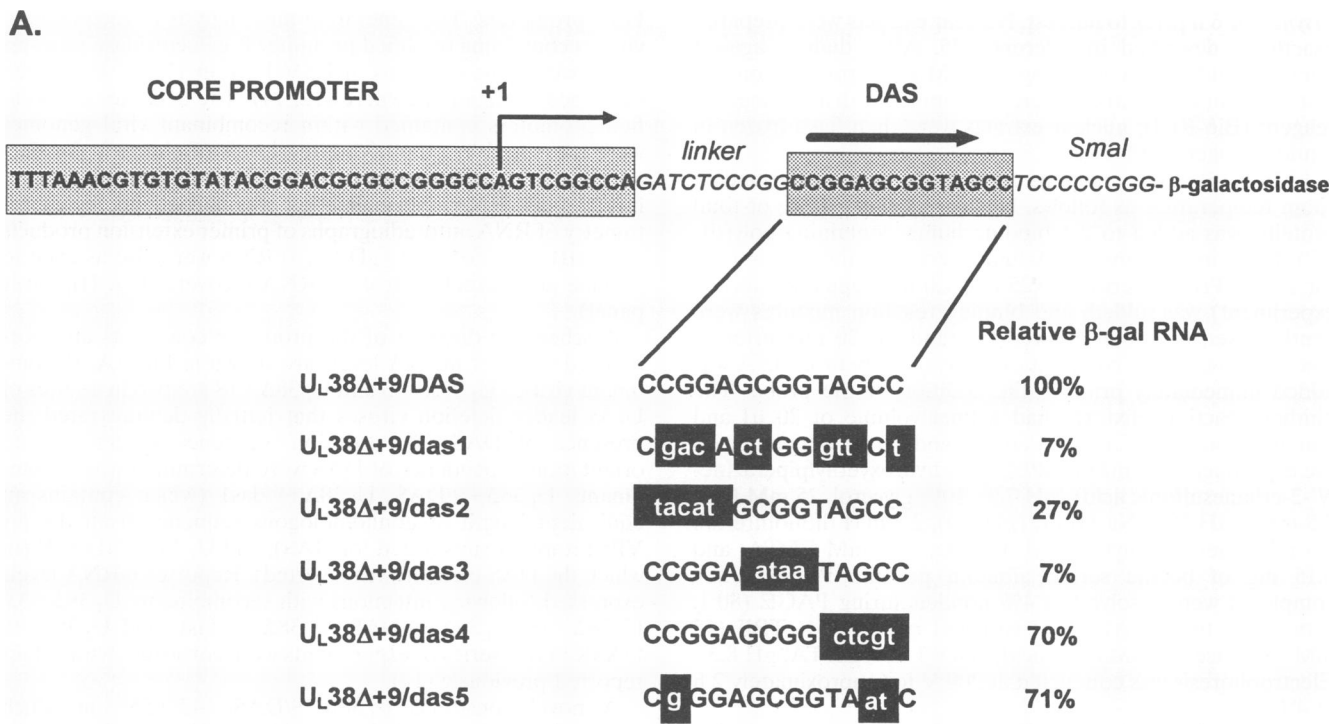


FIG. 2. Mutational analysis of U_L38 DAS. (A) Schematic representation of promoter constructs controlling expression of β-galactosidase (β-gal) mRNA from recombinant viruses. The general structure of the U_L38 promoter is outlined at top. Specific alterations of the U_L38 DAS sequence are blocked in black below. Shown to the right is the densitometric quantitation of relative β-galactosidase mRNA levels from the autoradiograph in panel B. (B) Primer extension analysis of 10 μg of total RNA from cells infected for 7 h with the recombinant viruses shown in panel A. The top panel shows products obtained with a β-galactosidase primer. The lower panel shows products obtained with primer for the wt gD transcript. Size markers are as in Fig. 1B.

(24). In all primer extension assays, 50 fmol of primer was used with 10 μg of RNA. Control experiments (not shown) were performed to ensure that the relationship between added RNA and signal strength was linear in the assay. Primer extension products were analyzed on 8 M urea–8% acrylamide gels. In some experiments, RNA was analyzed by RNase protection assays as described previously (14).

Primer extension products generated with the β-galactosidase primer and infected-cell RNAs were as follows: (i) for recombinants $U_L38Δ+33$, $U_L38Δ+9$/DAS, $U_L38Δ+9$/das1, $U_L38Δ+9$/das2, $U_L38Δ+9$/das3, $U_L38Δ+9$/das4, $U_L38Δ+9$/das5, U_L38/U_L37/DAS, and $U_L38Δ+9$/DASR, products of 90 nucleotides were expected; (ii) for recombinant $U_L38Δ+9$/gC, a product of 96 nucleotides was expected; (iii) for recombinant $U_L38Δ+9$/DAS(+42/+55), a product of 112 nucleotides was expected; (iv) for recombinants $U_L38Δ+18$ and U_L38/U_L37, products of 75 nucleotides were predicted; and (v) for recombinants U_S11/DAS and U_S11/*Hin*dIII, products of between 86 to 93 nucleotides were predicted on the basis of published U_S11 start sites (23). For in vitro transcription with plasmids $U_L38Δ+18$/pCal5divΔ17, $U_L38Δ+33$/pCal5divΔ17, and VP16 (+6)/pCal5divΔ17, primer extension products of 75, 90, and 63 nucleotides, respectively, were expected with the β-galactosidase primer.

RNA levels were quantitated by densitometry of appropriate exposed autoradiographs with a Bio-Rad model 620 densitometer. Mock-infected RNA lanes were used to establish the background level. When reported as relative β-galactosidase mRNA level, the β-galactosidase signal was normalized to the gD signal for that sample.

Preparation of DNA probes. Double-stranded oligonucleotides used to generate recombinant viruses $U_L38Δ+9$/das1, $U_L38Δ+9$/das3, and $U_L38Δ+9$/DAS (as described elsewhere in this paper and in reference 14) were used as electrophoretic mobility shift assay (EMSA) probes. Single-stranded oligonucleotides were annealed and then filled in by standard procedures with Klenow enzyme. Klenow fill-in reactions were performed with unlabeled deoxynucleoside triphosphates for the generation of competitor oligonucleotides or with unlabeled dATP, dGTP, and dTTP and [α-^{32}P]dCTP for the generation of labeled probes. Klenow reaction mixtures were extracted once with 1 volume of phenol-chloroform (1:1); unincorporated labeled dCTP was removed by using a Sephadex G-25 spin column.

Preparation of nuclear extracts. Rabbit skin cell and HeLa S3 monolayer cultures were grown to approximately 80% confluence. Cells were either mock infected or infected at a multiplicity of infection of 5 PFU per cell with HSV-1 strain

17*syn*$^+$ for 6 h prior to harvest. Nuclear extracts were prepared exactly as described in reference 25. After dialysis against Dignam's buffer D (containing 100 mM KCl), protein concentrations in nuclear extracts were determined with a commercial reagent (Bio-Rad); nuclear extracts were then flash frozen in liquid nitrogen until use.

EMSAs. Binding reactions for EMSA were performed at room temperature as follows. Nuclear extract (10 μg of total protein) was added to 2× binding buffer containing poly(dI-dC) (Boehringer Mannheim); tubes were then allowed to stand for 2 min. Probe (between 0.25 and 1.0 ng, depending on the experiment) was added, and binding reaction mixtures were gently mixed and then allowed to stand for 20 min prior to electrophoresis. For competition assays, competitor DNA was added immediately prior to the addition of the probe. The binding reaction mixtures had a final volume of 20 μl and contained poly(dI-dC) (between 0.25 and 2.0 μg, depending on the experiment), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 10% glycerol, 25 mM KCl, 2.5 mM NaH_2PO_4-Na_2HPO_4 (pH 7.9), 2.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mM EDTA, and 0.15 mg of bovine serum albumin per ml. Protein-DNA complexes were resolved in 4% nondenaturing PAGE (80:1, acrylamide to bisacrylamide) cast and run in 0.5× TBE (45 mM Tris base, 45 mM boric acid, and 0.1 mM EDTA, pH 8.3). Electrophoresis was conducted at 200 V for approximately 2 h at 4°C.

UV cross-linking. The EMSA reactions described above were scaled up twofold. In some experiments, the final KCl concentration was 75 mM. After 20 min of binding, reaction mixtures were subject to UV irradiation (254 nm with a Stratagene Stratalinker 2400) for periods of 1.5 to 4.5 min, which was followed by addition of 2× sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. Cross-linked products were resolved on SDS–10 or 12% polyacrylamide gels and visualized by autoradiography.

In vitro transcription. In vitro transcription was performed by using a commercial HeLa in vitro transcription kit (Promega) according to the instructions of the manufacturer, except that 20 U of RNasin (Promega) was added to each reaction mixture. The final $MgCl_2$ concentration for reactions was 5 mM. $CsCl_2$-purified supercoiled plasmids were used as transcription templates. The construction of plasmids $U_L38\Delta+33$/pCal5divΔ17 and $U_L38\Delta+18$/pCal5divΔ17 has been previously described (9, 14). These plasmids contain the specified U_L38 promoter driving expression of β-galactosidase. As an internal control, a plasmid, VP16(+6)/pCal5divΔ17, which contains sequences from position −278 to +6 of the VP16 promoter driving expression of β-galactosidase, was constructed; this VP16 promoter-leader construct was one of a series of 3′ deletions generated as described previously (14).

In vitro transcription reactions were terminated with the addition of 50 fmol of ^{32}P-end-labeled β-galactosidase primer; this was followed by phenol-chloroform (1:1) extraction and ethanol precipitation. Resuspended material was then subjected to primer extension analysis as described above, except that actinomycin D at a final concentration of 50 μg/ml was added to extension reaction mixtures.

RESULTS

DAS of the U_L38 promoter has strict sequence, orientation, and position requirements for function. We have extended our original observations concerning DAS by generating additional recombinant viruses in which the DAS element was modified, moved, or substituted for similarly located sequences of other HSV promoters. The generation and analysis of recombinant viruses containing modified promoter/β-galactosidase reporter genes within the gC locus of HSV-1 strain 17*syn*$^+$ have been described in detail elsewhere (12, 14). The activities of modified promoters contained within recombinant viral genomes were measured by quantifying the levels of chimeric β-galactosidase transcripts detected by primer extension analysis of infected-cell RNA. Normalization was carried out by densitometry of RNA autoradiographs of primer extension products (Fig. 1B); levels of wt βγ gD (U_S5) RNA were also assayed to provide an internal control for RNA recovery (Fig. 1B, lower panel).

A schematic diagram of the promoter constructs and normalized reporter mRNA levels are shown in Fig. 1A. Recombinant viruses $U_L38\Delta+33$ and $U_L38\Delta+18$ are two in a series of U_L38 leader deletion viruses that initially demonstrated the presence of DAS. The location, sequence specificity, and orientation dependence of DAS were determined with recombinants $U_L38\Delta+9$/DAS, $U_L38\Delta+9$/das1 (which contains an equivalent length of nonhomologous sequence from the βγ VP5 promoter substituted for DAS), and $U_L38\Delta+9$/DASR (in which the DAS element was inverted). Reporter mRNA levels expressed following infections with recombinants $U_L38\Delta+33$, $U_L38\Delta+18$, $U_L38\Delta+9$/DAS, $U_L38\Delta+9$/das1, and $U_L38\Delta+9$/DASR in this series of experiments were consistent with values reported previously (14).

A novel construct, $U_L38\Delta+9$/DAS(+42/+55), in which DAS was moved 22 bp downstream of its native promoter context, expressed levels of chimeric mRNA equivalent to those for infections with mutant $U_L38\Delta+18$, which lacks DAS entirely. This result demonstrates that the spacing between DAS and other elements of the U_L38 promoter is critical for DAS function; moreover, the presence of DAS sequence within the RNA is not sufficient to increase RNA levels with respect to those of DAS null mutants, thus suggesting that DAS has a specific role in transcription.

Promoter activity from another recombinant, $U_L38\Delta+9$/gC, was also analyzed in this series of experiments. In place of DAS, this U_L38 promoter construct contains sequence from position +20 to +38 of the γ gC gene of HSV-1. Mutational analysis by others has led to the suggestion that sequence elements within this region are required for full gC promoter activity (41, 44). The sequence of this region within the gC gene is CgGGAGgccgcatCGaacg, where the bases which match those of U_L38 in the same position are shown in uppercase letters. Analysis of reporter mRNA expression by $U_L38\Delta+9$/gC demonstrates that the gC element can partially substitute for U_L38 DAS in this promoter context, since the amount of RNA expressed was significantly greater than the basal levels expressed by recombinants $U_L38\Delta+18$, $U_L38\Delta+9$/das1, $U_L38\Delta+9$/DAS(+42/+55), and $U_L38\Delta+9$/DASR (Fig. 1B).

Mutational analysis of U_L38 DAS. We further defined the bases critical for DAS function by generating a series of recombinant viruses ($U_L38\Delta+9$/das2, $U_L38\Delta+9$/das3, $U_L38\Delta+9$/das4, and $U_L38\Delta+9$/das5) containing specific mutations within DAS. Infections were performed with these recombinants, the complete DAS mutation recombinant ($U_L38\Delta+9$/das1), and the parental recombinant ($U_L38\Delta+9$/DAS). A diagram of these constructs is shown in Fig. 2A, as are the normalized mRNA levels from the primer extension analysis shown in Fig. 2B.

U_L38 DAS is essentially a direct repeat of G(G/T)AGC, and both copies are required for full activity, as determined from the following observations. Recombinants $U_L38\Delta+9$/das2, $U_L38\Delta+9$/das4, and $U_L38\Delta+9$/das5, which contain mutations

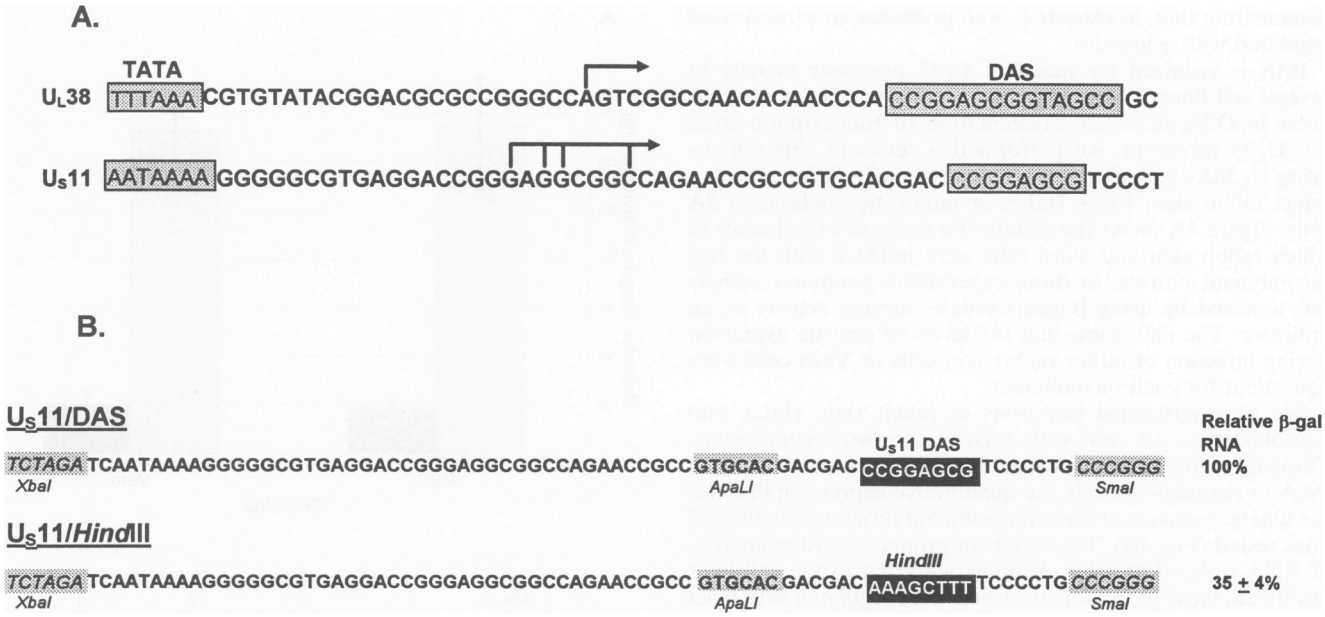


FIG. 3. The U_S11 gene of HSV-1 contains a functional DAS element. (A) Comparison of U_L38 and U_S11 promoters. TATA and DAS elements are indicated. Mapped transcriptional start sites are also indicated (9, 23). (B) Structures of U_S11 promoter constructs in recombinant viruses. Promoter constructs are described in detail in Materials and Methods. Relevant restriction enzyme sites are shown. Densitometric analysis of chimeric β-galactosidase (β-gal) RNA levels expressed by U_S11 recombinant viruses at 6 and 7 hpi is shown on the right; the reported value is the average of two independent experiments. (C) Primer extension analysis of 10 μg of total RNA from cells infected for 3 or 7 h or for 7 h in the presence of PAA with the U_S11 recombinant viruses shown in panel A. The top panel shows products obtained with a β-galactosidase primer. The lower panel shows products obtained with primer for the wt gD transcript. Size markers are as in Fig. 1B.

in only one of the two repeats, express significantly more β-galactosidase mRNA than either $U_L38\Delta+9$/das1 or $U_L38\Delta+9$/das3, which contain mutations within both repeats. Despite this, however, results with $U_L38\Delta+9$/das2 and $U_L38\Delta+9$/das4 indicate that the repeats are not functionally equivalent and that significant activity can be generated with the first copy alone. The structure of the gC DAS-like element, which only partially substitutes for U_L38 DAS, is consistent with the sequence element GGAGCG being the critical functional core; so, too, are the results of analysis of a DAS-like element in the U_S11 promoter (see below).

The U_S11 gene of HSV-1 contains a functional DAS element. Mutational analysis of other late HSV-1 genes has also shown that *cis*-acting sequences near the transcriptional start site and within the 5′ untranslated leader region are required for maximal levels of reporter gene or mRNA expression; a notable example is the U_S11 promoter (14, 20, 24, 33, 41). These leader elements, however, have not been defined to the extent of U_L38 DAS. The leader of the γ U_S11 gene of HSV-1 contains an 8-bp perfect match of U_L38 DAS in the same relative position downstream of the U_S11 TATA box. Figure 3A shows a comparison of the U_L38 and U_S11 promoter-leaders starting at their respective TATA boxes. To determine if the 8-bp element within the U_S11 leader is a functional homolog of U_L38 DAS, two recombinant viruses, U_S11/DAS and U_S11/*Hin*dIII, were constructed. U_S11/DAS contains the U_S11 promoter from position −34 to +37 driving expression of β-galactosidase, while the promoter driving expression of β-galactosidase in U_S11/*Hin*dIII is identical except for replacement of the 8-bp DAS homology with a *Hin*dIII restriction site. The exact sequences of these promoter constructs are shown in Fig. 3B. Promoter activities of U_S11/DAS and U_S11/*Hin*dIII were assessed by measuring levels of chimeric β-galactosidase transcripts from infected-cell RNA (Fig. 3B and C). Mutation of the U_S11 DAS element led to an approximately threefold decrease in U_S11 promoter activity, thus demonstrating that the U_S11 DAS homology is required for maximal expression from the U_S11 promoter.

To further characterize the promoter activity of the U_S11 recombinant viruses, infections were carried out for 3 or 7 h or for 7 h in the continuous presence of the viral DNA replication inhibitor PAA. Total RNA was analyzed by primer extension assays with either a β-galactosidase primer or a primer for the wt gD transcript (Fig. 3C). The kinetics of appearance and the strong sensitivity to PAA of the chimeric β-galactosidase RNA

demonstrate that, as expected, both promoter constructs were regulated with γ kinetics.

DAS is required for maximal U_L38 promoter activity in several cell lines. To examine the role of the cellular environment in DAS-mediated augmentation of transcription from the U_L38 promoter, we performed a series of experiments using $U_L38\Delta+9$/DAS and the DAS mutant $U_L38\Delta+9$/das1 to infect rabbit skin, Vero, HeLa, or mouse neuroblastoma 2A cells. Figure 4A shows the results of a series of experiments in which rabbit skin and Vero cells were infected with the two recombinant viruses. In these experiments promoter activity was assessed by using β-galactosidase enzyme activity as an indicator. The data show that the levels of enzyme expressed during infection of either rabbit skin cells or Vero cells were equivalent for each recombinant.

We also performed infections of rabbit skin, HeLa, and neuroblastoma 2A cells with each of the two recombinants. These infections were performed in the presence or absence of PAA to examine not only the quantitative expression but also the kinetic behavior of these recombinant promoters in the cell lines tested (Fig. 4B). The results of primer extension analysis of RNA isolated at 8 hpi demonstrated that in the cell lines examined, these promoter constructs were regulated with strict late kinetics as indicated by their sensitivity to PAA and that DAS was strongly required for maximal expression.

DAS has a strong effect on promoter activity in in vitro transcription. A number of promoters of cellular origin contain functional *cis*-acting elements sharing a positional similarity to DAS; a partial list of these downstream elements is shown in Table 1, and they are more fully considered in Discussion. To investigate the possibility that DAS interacts with a cellular transcription factor to increase promoter activity, in vitro transcription reactions were performed with uninfected HeLa nuclear extracts and templates containing the U_L38 promoter with or without DAS ($U_L38\Delta+33$ or $U_L38\Delta+18$, respectively). A third template containing the βγ VP16 promoter [VP16(+6)] was included as a control. The products of in vitro transcription were analyzed by primer extension analysis as described in Materials and Methods; results of these experiments are shown in Fig. 5. For comparison, Fig. 5A shows primer extension analysis of steady-state RNA isolated from rabbit skin cells infected for 7 h with either recombinant virus $U_L38\Delta+18$ or $U_L38\Delta+33$. As can be seen in Fig. 5B, in vitro transcription of these promoters demonstrates an effect of DAS equivalent to that seen in vivo (compare lanes 1 and 2 with lanes 4 and 5). In vitro transcription of the strong VP16 promoter serves as an internal control and eliminates the possibility that variations in recovery of product are responsible for the observed effect of DAS on transcriptional activity (Fig. 5C, lanes 6 and 7). These data strongly support the conclusion that DAS augments transcription of the U_L38 promoter by interacting with a cellular transcription factor. Further, these data show that DAS functions at the level of transcription, a result in agreement with our earlier conclusions (see above and reference 14).

U_L38 DAS DNA probe forms a specific, stable complex with a cellular protein. To investigate the specific protein-DNA interactions of DAS, we performed EMSAs. In preliminary experiments (not shown), we found that wt DAS formed a complex that had equivalent mobilities in both uninfected and infected rabbit skin cell nuclear extracts and that this complex did not form with two mutant probes, das1 and das3. The same pattern of complex formation was observed by EMSA with uninfected and infected HeLa nuclear extracts (not shown). The sequences of these altered DAS elements and their effects upon transcription by the U_L38 core promoter are shown in

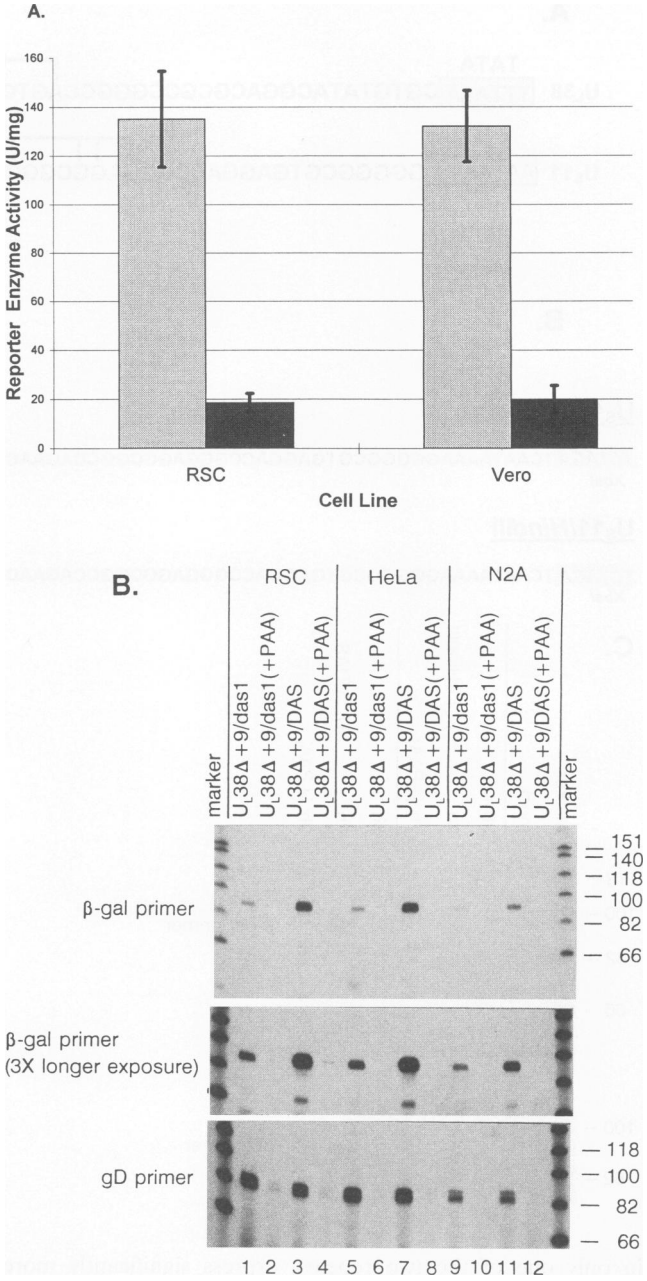


FIG. 4. DAS is required for maximal U_L38 promoter activity in several cell lines. (A) Average reporter enzyme (β-galactosidase) activities obtained from infected-cell extracts harvested at 16 hpi. Values are from three independent experiments. ▨, U_L38+9/DAS; ■, U_L38+9/das1. (B) Primer extension analysis of 10 μg of total RNA from cells infected for 8 h with the indicated recombinant viruses in either the presence or absence of PAA. The top panel shows products obtained with a β-galactosidase primer. The middle panel is the same but exposed three times longer to emphasize weak signals. The lower panel shows products obtained with primer for the wt gD transcript. Abbreviations: RSC, rabbit skin cells; N2A, mouse neuroblastoma 2A cells; β-gal, β-galactosidase. Size markers are as in Fig. 1B.

Fig. 2. The specificity of the complex observed was confirmed by competition assays with ^{32}P-labeled wt DAS probe and unlabeled competitors (Fig. 6). In the absence of competitor, DAS formed a complex with uninfected and infected nuclear

TABLE 1. Eukaryotic promoters with downstream control elements[a]

Organism	Gene	Location[b]	Functional analog of DAS[c]	Sequence homology to DAS[d]	Reference(s)
HSV	U_L38	+20 to +33		+19-CCGGAGCGGTAGCC-+34	14
	U_S11	+25 to +32	Yes	+19-ACGACCCGGAGCGT-+34	24[e]
	gC (U_L44)	+20 to +38	Yes	+19-CGGGAGGCCGCATC-+34	44[f]
	gB (U_L27)	+21 to +35	Yes	+20-CCGGTGCGGGGAGCT-+36	33
	L/ST	+18 to +33	?	+17-CGGAGCTCCCGGGAGC-+34	3, 45
	$U_L49.5$	+27 to +40	Yes	+26-CCCGCGAGTAGCGA-+41	13
PRV	LAT	+23 to +32	?	+22-CCGGAGCGGTCCAT-+33	19
Human	GFAP	+10 to +40	Yes	Related motif, GGAGA repeats	29
D. melanogaster	hsp70	+18 to +33	Yes	No obvious homology	34
Mouse	c-*mos*	+16 to +27	Yes	No obvious homology	31
Mouse	rpL32[g]	+13 to +45	Yes	No obvious homology	28
D. melanogaster	vermilion[g]	+19 to +36	Yes	Homologous to GFAP	10
Mouse	DHFR[g]	+15 to +61	Yes	No obvious homology	8

[a] Abbreviations: PRV, pseudorabies virus; LAT, latency-associated transcript; DHFR, dihydrofolate reductase.
[b] Relative to transcription initiation (cap) site.
[c] i.e., mutation or deletion of element decreases promoter activity.
[d] For herpesvirus promoters, occurrences of the U_L38 DAS core sequence, G(G/T)AGCG, are indicated by underlining.
[e] Also see Fig. 3.
[f] Also see Fig. 1.
[g] TATA-less promoters.

extracts that had the same mobility (Fig. 6; compare lanes 1, 8, and 15). In the presence of excess unlabeled DAS, formation of this complex was readily blocked (Fig. 6, lanes 2, 3, 4, 9, 10, and 11). As expected, the competition profile was the same for both uninfected and infected nuclear extracts. The specificity of this complex was further demonstrated by the lack of competition observed with the das3 unlabeled competitor (Fig. 6, lanes 5, 6, 7, 12, 13, and 14).

To further characterize the protein-DAS complex observed (Fig. 6), we used EMSA to perform dissociation rate analysis. As seen in Fig. 7, the specific protein-DAS complex was quite stable once it was formed. The lack of competition seen when a 250-fold molar excess of unlabeled DAS competitor was added after complex formation (Fig. 7, lanes 2 through 5) strongly contrasts with the pattern seen when unlabeled DAS competitor was added with the EMSA probe (Fig. 6). The more rapidly migrating nonspecific complex seen in lanes 1 and 6 of Fig. 7 was sometimes observed; it also formed with das1 and das3 mutant probes (not shown) and could be eliminated by performing binding reactions with higher KCl concentrations (not shown). Further, as seen in lanes 2 through 5 of Fig. 7, this nonspecific interaction was not kinetically stable.

UV cross-linking shows that DBF is a cellular protein of approximately 35 kDa. To further characterize the cellular protein interacting with DAS, we performed UV cross-linking experiments. EMSA binding reactions between the DAS probe and either uninfected or infected nuclear extracts were UV cross-linked, and then the cross-linked products were resolved by using SDS-PAGE. With both uninfected and infected nuclear extracts, the dominant cross-linked species migrated with a mobility of approximately 35 kDa (Fig. 8A, lanes 1, 5, 6, and 10). When increasing amounts of unlabeled DAS competitor were added, this major species disappeared, while minor background bands did not (Fig. 8A, lanes 2, 3, 4, 7, 8, and 9). These results show that in both uninfected and infected nuclear extracts, a protein of approximately 35 kDa specifically interacts with DAS. We have termed this protein DAS-binding factor (DBF).

Additional evidence of the specificity of the interaction between DBF and DAS is provided in Fig. 8B. Binding reactions with the DAS probe and infected nuclear extracts were performed with various cross-linking times and KCl concentrations and with both specific and nonspecific competitors. As seen in lanes 2, 3, 4, and 5 of Fig. 8B, increasing the KCl concentration did not affect specific complex formation, but (as noted above) it decreased formation of nonspecific complexes (data not shown). Competition with a 40-fold molar excess of unlabeled DAS (Fig. 8B; compare lanes 6 and 7 with lanes 2 and 3), but not das3 (compare lanes 8 and 9 with lanes 2 and 3), further demonstrates the specificity of the interaction between DBF and DAS.

Genetic evidence that DAS functions at the preinitiation step. In vitro transcription, EMSA, and UV cross-linking results are consistent with DAS-bound DBF augmenting transcription initiation via the formation of the preinitiation complex at the core promoter, or at a point just following this, to increase the efficiency of transcriptional elongation. These two possibilities can be differentiated by assessing the role of DAS in transcription from modified promoters with altered initiation or TATA sequences. As demonstrated below, such analyses suggest a role of DAS in the formation and stabilization of the preinitiation complex.

(i) DAS confers significant promoter activity to an otherwise inactive promoter. Recombinant virus U_L38/U_L37 contains a chimeric promoter consisting of bases −50 to −15 of the γ U_L38 promoter fused to sequences from −14 to +18 of the β U_L37 promoter controlling expression of the β-galactosidase gene; previously, we have demonstrated that this chimeric promoter was inactive in recombinant virus (14). To examine the effect of DAS on transcription from a chimeric U_L38/U_L37 promoter, we engineered recombinant virus U_L38/U_L37/DAS, which is essentially identical to $U_L38\Delta+9$/DAS except for the replacement of U_L38 sequences from −14 to +9 with those of U_L37. To characterize the activity and regulation of the promoter constructs shown in Fig. 9A, infections with the recombinant viruses were carried out for 3 or 7 h or for 7 h in the continuous presence of PAA. These results are shown in Fig. 9C, and the quantitation of RNA levels from the 7-h time point is given in Fig. 9A. Several important conclusions can be made: (i) in the absence of DAS, no specific transcription was detected from recombinant U_L38/U_L37 (Fig. 9C, lanes 2, 3, and 4), while $U_L38\Delta+18$ (lanes 5, 6, and 7) expressed chimeric

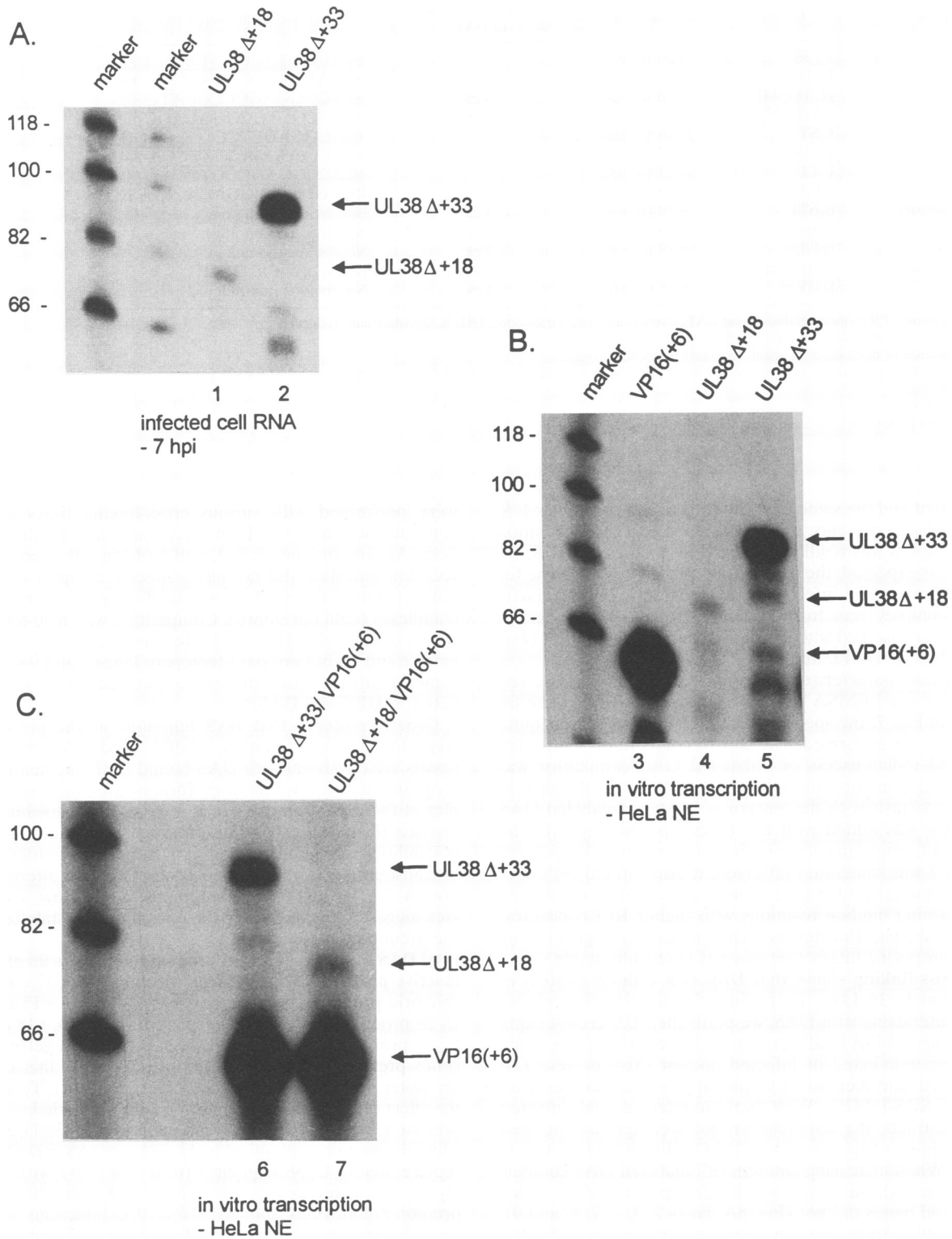


FIG. 5. Effect of DAS on in vitro transcription of the U_L38 promoter. (A) Primer extension analysis (β-galactosidase primer) of 10 μg of total RNA from cells isolated at 7 hpi with the recombinant viruses indicated. (B) Transcription products obtained by incubation of 500 ng of the indicated plasmid templates with uninfected HeLa cell nuclear extract (NE) as described in Materials and Methods. These products were detected by primer extension analysis with the β-galactosidase primer described in Materials and Methods. (C) Same as panel B, except that the VP16 plasmid was included as an internal control with the two test plasmids for transcription. Size markers are as in Fig. 1B.

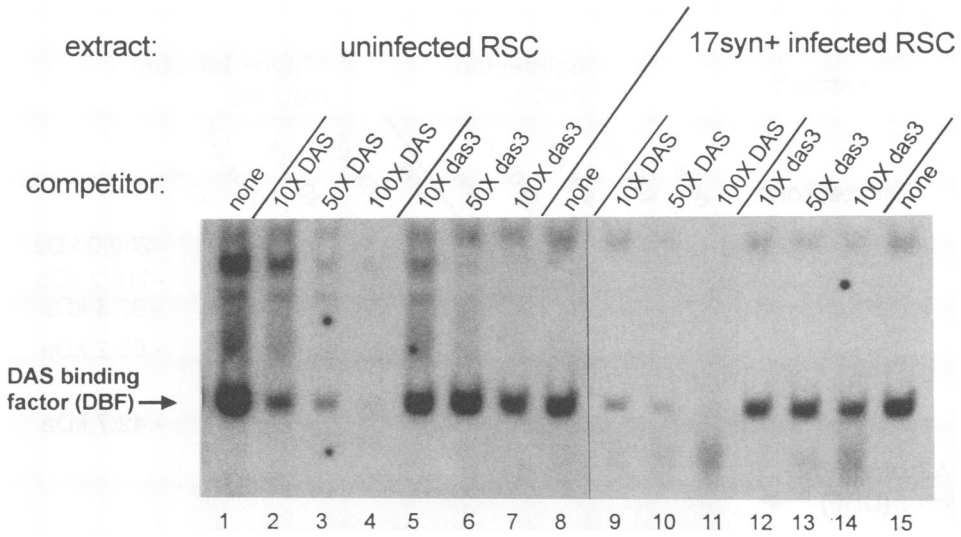


FIG. 6. Competition EMSAs of complexes formed between wt DAS and uninfected or infected rabbit skin cell (RSC) nuclear extracts. Extracts were generated as described in Materials and Methods and were incubated with 1.0 ng of ^{32}P-labeled DAS probe in the presence of 0.25 μg of poly(dI-dC) and the amounts of competing unlabeled probe or mutant probe indicated. Only the region of the gel containing complexes is shown, and the specific complex discussed in the text is indicated with the arrow.

β-galactosidase RNA at 6% of wt levels ($U_L38\Delta+9$/DAS; lanes 11, 12, and 13); (ii) addition of DAS downstream of the minimal promoter containing the U_L37 initiator element (U_L38/U_L37/DAS; Fig. 9C, lanes 8, 9, and 10) increased transcription to readily detectable levels (29% of wt levels); (iii) the kinetic behavior of chimeric β-galactosidase RNA accumulation during infections with $U_L38\Delta+18$, U_L38/U_L37/DAS, and $U_L38\Delta+9$/DAS was essentially identical; and (iv) primer extension products generated from RNA expressed by U_L38/U_L37/DAS migrated with a mobility consistent with utilization of the U_L37 start site in this promoter context. Since the U_L38/U_L37 promoter chimera was essentially inactive, we would not expect to see significant transcript levels expressed by U_L38/U_L37/DAS if DAS functioned following initiation (i.e., by increasing polymerase processivity). The ability of DAS to partially overcome the defect introduced by the U_L37 initiator element replacement is most readily consistent with a model in which DAS and the U_L38 initiator element are involved in the formation of transcription initiation complexes.

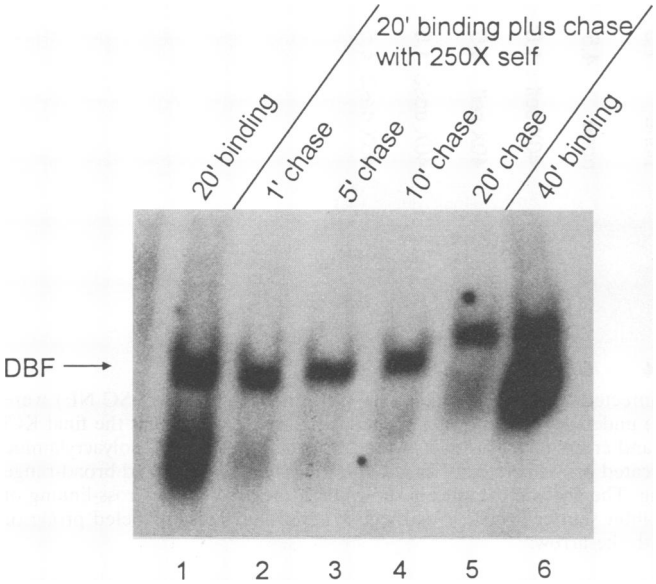


FIG. 7. Dissociation analysis of complexes formed between DAS and infected rabbit skin cell nuclear extracts. Extracts were generated as described in Materials and Methods and were incubated with 1.0 ng of ^{32}P-labeled DAS probe and 0.25 μg of poly(dI-dC) for 20 min prior to addition of a 250-fold molar excess of cold competitor probe for the times indicated. Samples were then loaded directly onto a running low-ionic-strength 4% polyacrylamide gel. Only the region of the gel containing complexes is shown, and the specific complex of interest is indicated with the arrow.

Five of nine bases at the 5′ end of the chimeric β-galactosidase transcript expressed by U_L38/U_L37/DAS were changed relative to those in $U_L38\Delta+9$/DAS (Fig. 9B). To examine whether these alterations affected mRNA stability, we analyzed the kinetic stability of transcripts by using an actinomycin D chase experiment essentially as previously described (14). Densitometric analysis of reporter mRNA demonstrated that in the absence of the transcriptional inhibitor actinomycin D, levels of β-galactosidase mRNA expressed by both recombinants doubled from 6 to 10 hpi, while reporter RNA levels remained constant within this period during the actinomycin D chase (Fig. 9D). These results show that U_L37 sequence elements within the chimeric transcript expressed by U_L38/U_L37/DAS do not alter RNA stability, and they confirm that alterations near the transcription start site of the U_L38 promoter decrease promoter activity.

(ii) DAS influences RNA levels in a TATA box-dependent manner. To examine the relationship, if any, between DAS and the TATA box in regulating transcription from the U_L38 promoter, we designed two recombinant viruses, $U_L38M\Delta+9$/DAS and $U_L38M\Delta+9$/das1 (see Materials and Methods for details of construction). The promoters in these recombinant viruses are essentially identical to those driving β-galactosidase expression in recombinant viruses $U_L38\Delta+9$/DAS and $U_L38\Delta+9$/das1 except that the U_L38 wt TATA box (TTTAAAC) was changed at two positions to change the sequence to the strong

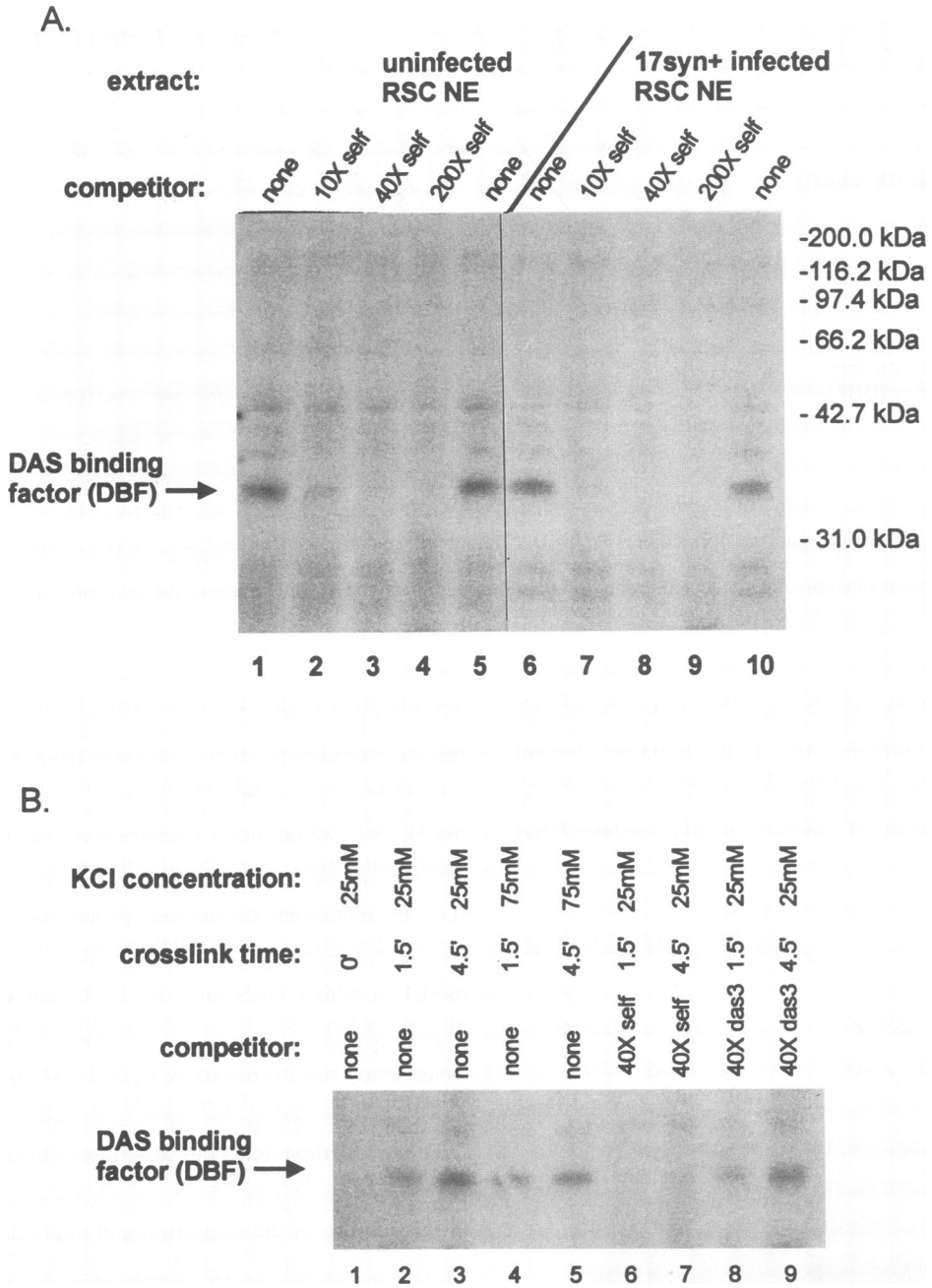
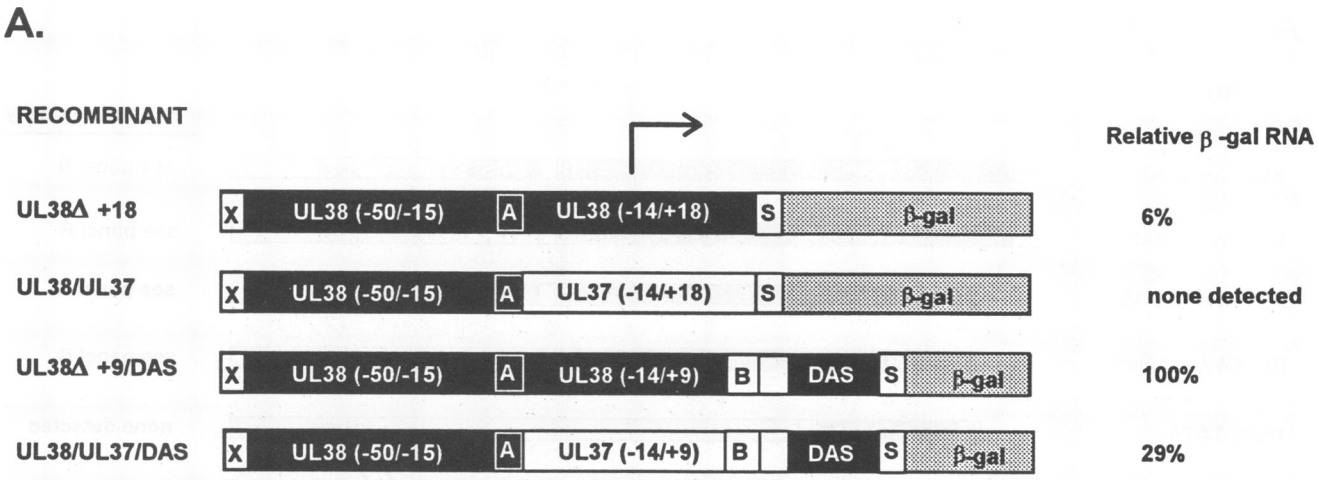


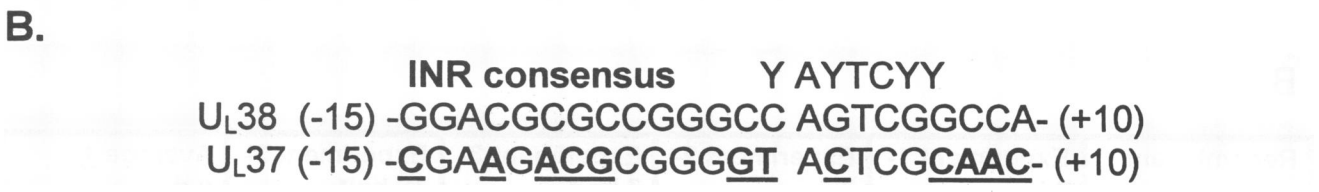
FIG. 8. UV cross-linking analysis of DAS-associated protein. (A) Uninfected or infected rabbit skin cell nuclear extracts (RSC NE) were incubated with 0.5 ng of ^{32}P-labeled DAS probe and 1.0 μg of poly(dI-dC) under the conditions described for EMSA, except that the final KCl concentration was 75 mM. Unlabeled competitor was added as indicated, and cross-linked products were fractionated on a 12% polyacrylamide gel containing 0.1% SDS as described in Materials and Methods. The indicated protein sizes are based upon migration of Bio-Rad broad-range size standards (catalog no. 161-0217) visualized by Coomassie blue staining. The specific complex is shown with the arrow. (B) Cross-linking of products of EMSA binding reactions between DAS probe and infected rabbit skin cell nuclear extracts. Where indicated, unlabeled probe or mutant (das3) competitor was added. The specific complex is indicated with the arrow.

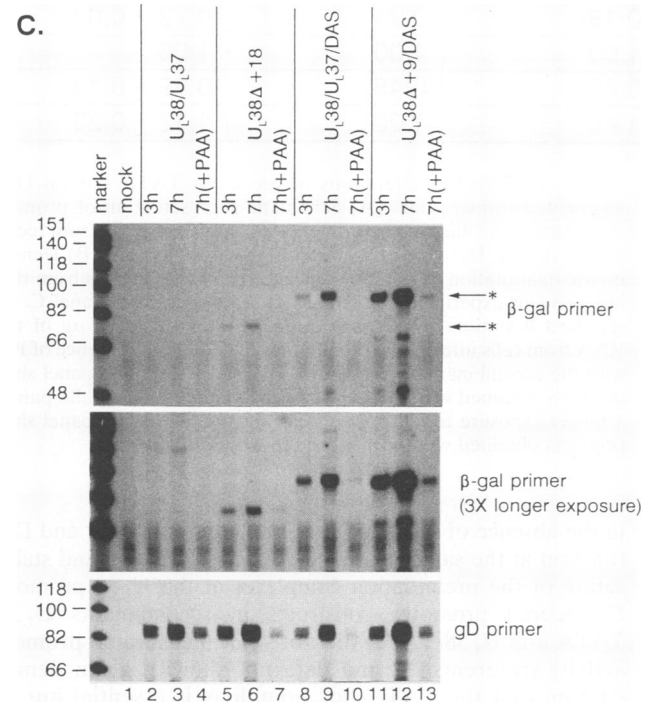
consensus TATAAAA. We reasoned that this change would increase transcription only if the formation of preinitiation complexes was limiting.

U_L38Δ+9/DAS, U_L38Δ+9/das1, U_L38MΔ+9/DAS, U_L38MΔ +9/das1, and two additional control promoter constructs are shown schematically in Fig. 10A. The construction of recombinant virus U_L38ΔTATA, which had the TATA box deleted and contains U_L38 sequences from position −25 to +99 fused to β-galactosidase coding sequences, has been described previously (12). An additional recombinant, U_L38-TATA, was generated to analyze the contribution of the modified TATA box alone to transcription (see Materials and Methods for details of construction).

Reporter mRNA expressed during infections with the







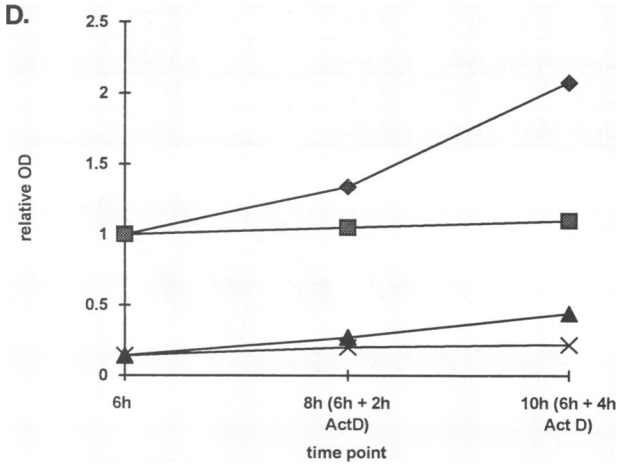
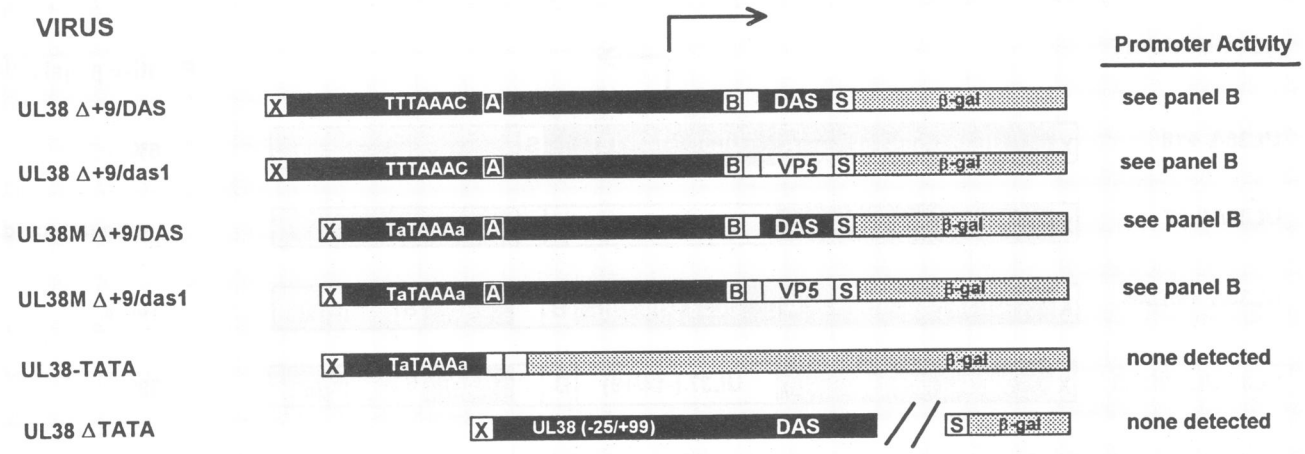


FIG. 9. Effect of DAS on activity of a chimeric U_L38 promoter containing the U_L37 transcription start site. (A) Schematic representation of promoter constructs controlling expression of β-galactosidase mRNA from recombinant viruses. Densitometric quantitation of relative β-galactosidase RNA levels for the autoradiograph shown in panel C is also indicated. Bars and abbreviations are as in Fig. 1A. (B) Comparison of U_L38 and U_L37 transcription start site sequences. Bases of the U_L37 sequence which do not match that of U_L38 are underlined and in boldface. The consensus mammalian initiator sequence (INR) is also shown (22, 37). (C) Primer extension analysis of 10 μg of total RNA from cells infected for 3 or 7 h or for 7 h in the presence of PAA with the recombinant viruses shown in panel A. The top panel shows products obtained with a β-galactosidase primer. Asterisks indicate specific transcript start sites. The middle panel is a longer exposure to emphasize weak signals. The lower panel shows products obtained with primer for the wt gD transcript. Size markers are as in Fig. 1B. (D) Densitometric analysis of the relative stability of β-galactosidase-encoding mRNAs expressed by recombinant viruses during actinomycin D (ActD) chase as described in the text. Symbols: ◆, U_L38+9/DAS; ■, U_L38+9/DAS plus actinomycin D; ▲, U_L38/U_L37/DAS; ×, U_L38/U_L37/DAS plus actinomycin D. OD, optical density.

above-described recombinant viruses was measured by using RNase protection or primer extension assays of total infected-cell RNA at 6, 7, or 16 hpi (Fig. 10B and C). When the U_L38 TATA box was mutated to TATAAAA in the absence of functional DAS ($U_L38MΔ+9$/das1), an approximately twofold increase in chimeric RNA expression compared with that in

A.



B.

Recombinant	Experiment 1-6 hpi	Experiment 2-7hpi	Experiment 3-7 hpi*	Experiment 3-16 hpi*	Average ± s.d.
U_L38Δ+9/das1	0.24	0.23	0.19	0.24	0.22 ± 0.02
U_L38Δ+9/DAS	1.00	1.00	1.00	1.00	1.00
U_L38MΔ+9/das1	0.37	0.21	0.52	0.45	0.39 ± 0.13
U_L38MΔ+9/DAS	0.95	1.07	1.46	1.00	1.12 ± 0.23

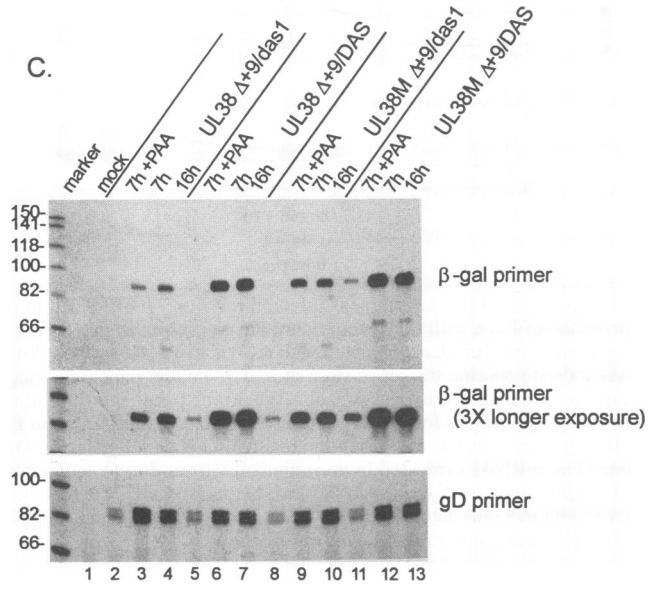


FIG. 10. Effect of alterations of the U_L38 TATA box on DAS-augmented transcription. (A) Schematic representation of promoter constructs controlling expression of β-galactosidase RNA from recombinant viruses. Bars and abbreviations are as in Fig. 1A. (B) Densitometric quantitation of relative β-galactosidase RNA levels from three independent experiments. Asterisks indicate data from panel C. s.d., standard deviation. (C) Primer extension analysis of 10 μg of total RNA from cells infected for 7 or 16 h or for 7 h in the presence of PAA with the recombinant viruses shown in panel A. The top panel shows products obtained with a β-galactosidase primer. The middle panel is a longer exposure to emphasize weak signals. The lower panel shows products obtained with primer for the wt gD transcript.

the parental construct (U_L38Δ+9/das1) containing the wt TATA element was seen; however, the same TATA box alterations had no effect upon reporter mRNA levels when functional DAS was present, as seen following infections with recombinants U_L38Δ+9/DAS and U_L38MΔ+9/DAS. The fact that the TATA box alteration increased promoter activity only in the absence of DAS suggests that the TATA box and DAS function at the same limiting step—the formation and stabilization of the preinitiation complexes at the U_L38 promoter. Engineered promoter constructs in recombinants U_L38Δ TATA and U_L38-TATA did not show measurable promoter activity (reference 12 and data not shown), again demonstrating that the TATA box homology is essential but not sufficient for appreciable expression from the U_L38 promoter.

Additional experiments were performed to examine the kinetics of expression from the promoter constructs mentioned above. The only noticeable alteration of expression observed for the two recombinant viruses containing the strong TATA homology (U_L38MΔ+9/das1 and U_L38MΔ+9/DAS) was a slight but reproducible increase in RNA expression in the presence of the DNA synthesis inhibitor PAA. This is illus-

trated by the experiment shown in Fig. 10C (compare lanes 2 and 8 and lanes 5 and 11).

DISCUSSION

The strict late (γ) U_L38 promoter of HSV-1 is composed of three defined elements: a TATA box, a γ initiator element, and a novel *cis*-acting element, DAS. In the present study, we have identified a cellular transcription factor, DBF, which interacts with DAS to activate transcription. Our search of the available literature does not demonstrate any significant similarity between DAS and well-characterized transcription factor-binding sites; therefore, we suggest that DBF is a novel cellular transcription factor.

Role of DAS in U_L38 promoter activity. U_L38 DAS is a sequence-specific *cis*-acting element with strict orientation and position requirements for activity (14) (Fig. 1 and 2). DAS specifically forms a stable complex with a cellular protein of approximately 35 kDa (DBF) (Fig. 6, 7, and 8). In vitro transcription experiments (Fig. 5) show that unmodified cellular nuclear proteins are competent to initiate transcription from the U_L38 promoter in a DAS-dependent fashion which virtually mimics the pattern observed during productive infection. Further, DAS augments core reporter promoter activity in a number of cultured cell lines, indicating that the protein interacting with it is a common cellular factor. The implications of these results for late gene expression are discussed below.

Several lines of evidence indicate that DAS increases transcription initiation from the U_L38 promoter. Initially, we considered that DAS might function to increase the ability of RNA polymerase to complete nascent RNA molecules in a manner analogous to that of the *trans*-activation response element of human immunodeficiency virus (reviewed in references 5 and 6); however, upon examination of RNAs from $U_L38\Delta+9$/DAS- and $U_L38\Delta+9$/das1-infected cells, we failed to detect short 5′ RNAs which would be indicative of transcriptional pausing or stalling (data not shown). The ability of DAS to confer significant activity on an otherwise inactive promoter (Fig. 9C; compare lanes 3 and 9) also argues against DAS functioning to increase polymerase processivity and is instead consistent with DAS participating in preinitiation complex formation. This conclusion is further supported by experiments presented in Fig. 10; modification of the U_L38 TATA box from TTTAAAC to TATAAAA increases promoter activity twofold in the absence of DAS (Fig. 10B and lanes 3, 4, 9, and 10 of Fig. 10C) but does not significantly alter promoter activity when DAS is present (Fig. 10B and lanes 6, 7, 12, and 13 of Fig. 10C), demonstrating an interdependence between TATA box homology and DAS action.

The U_L38 promoter shares features of other strict late promoters of HSV-1. Mutational analyses of a number of γ promoters has led to a largely consistent model of their architecture: TATA box, γ initiator element, and a DAS-like downstream element. A characteristic which distinguishes the γ promoters from promoters of the α, β, and $\beta\gamma$ classes is the lack of functional upstream *cis*-acting sites, and analyses of the U_L38, U_S11, and gC (U_L44) promoters indicate that a TATA box at approximately position -30 defines their 5′ extents (9, 12, 17, 23). While a TATA box is critical for efficient late gene expression, other *cis*-acting elements must define the differential kinetic behavior of early and late promoters, since specific TATA homologies are interchangeable between promoters of different kinetic classes (21, 40).

Experimental evidence indicates that sequence elements downstream of the TATA boxes of early and late promoters are not functionally equivalent. Studies reported here and elsewhere have shown that perturbation of sequences downstream of the TATA boxes of the U_L38, U_S11, VP5 (U_L19), VP16 (U_L48), gB (U_L27), $U_L49.5$, gH (U_L22), gC, and gE (U_S7) promoters leads to a decrease in RNA or reporter gene levels (1, 2, 13, 14, 18, 20, 33, 36, 41, 44). In contrast, the entire leader and cap site (positions -15 to $+189$) of the β thymidine kinase (U_L23) gene have been deleted without any appreciable effect on RNA levels or kinetics of expression (15). Additionally, chimeric promoter-leader constructs have shown that initiator-leader regions of β genes cannot functionally replace those of either $\beta\gamma$ or γ genes (14, 18, 20, 27).

Specific sequences near the transcriptional start sites of both $\beta\gamma$ and γ transcripts are critical for maximal expression at times after the onset of viral DNA replication; these can be termed late initiator elements (14, 20, 36, 41). Since they have been identified in all late HSV-1 promoters studied to date, it is reasonable to posit that these late initiator elements are common to all late promoters. While the sequences of late initiator elements are clearly important in late promoter function and are related to other reported initiator elements (22, 37), a cursory examination does not show any obvious common consensus sequence which distinguishes them from the generic initiator consensus.

In addition to containing a late initiator element, the strict late U_L38, U_S11, $U_L49.5$, gC, and gH promoters contain additional separate elements between approximately positions $+20$ and $+40$ that are required for maximal levels of expression (13, 14, 41). The general similarities between γ promoters have led us and others to identify the TATA box, the γ initiator element, and a DAS-like downstream element as common elements (41).

DAS-related elements are components of cellular promoters. It is interesting that the basic architecture of HSV γ promoters, so well exemplified by that controlling the U_L38 transcript, is also seen in some cellular promoters. A partial list of herpesvirus and cellular promoters containing downstream *cis*-acting elements positioned in approximately the same location as DAS is shown in Table 1. Several of the promoters listed in Table 1 do not contain TATA boxes, and downstream elements may play a critical role in nucleating or stabilizing preinitiation complexes containing TFIID at such promoters—the fact that DAS has such a marked effect in the context of the "poor" TATA box of the U_L38 promoter is consistent with this possibility. While many of these elements do not have obvious homology with DAS, the positional similarity suggests a common mechanism of action. It is possible that DBF, which interacts specifically with DAS, has a degenerate binding sequence specificity or is a member of a class of transcription factors which interact with downstream elements.

A notable example of the shared architecture between the U_L38 and cellular promoters is the promoter regulating expression of the glial fibrillary acidic protein (GFAP). The GFAP promoter contains the same three *cis*-acting elements, i.e., TATA box, initiator element, and downstream element positioned between $+10$ and $+40$; additionally, the relative contribution of each of these elements to activity of the GFAP promoter is essentially identical to those observed for U_L38 (29). Moreover, the GFAP downstream element interacts with a protein(s) that copurified with TFIID to stabilize TFIID binding and in turn to increase transcription (30). Similarly, sequences from position $+18$ to $+33$ have been implicated in stabilizing TFIID binding to the hsp70 promoter of *Drosophila melanogaster* (34). The interaction of DBF with DAS may serve a similar function in preinitiation complex formation at the U_L38 promoter.

A promoter-biased model for expression of differential levels of γ transcripts before and after HSV DNA replication. All data are consistent with a working model for γ promoters in which the TATA box, γ initiator element, and DAS interact with cellular proteins in a cooperative fashion to form a stable preinitiation complex and potentiate promoter activity. Removal or alteration of any of these three *cis*-acting elements has a dramatic effect on complex formation and hence promoter activity. The TATA box is presumed to interact with the TATA-binding protein (TBP) of the multisubunit general transcription factor TFIID (reviewed in reference 4), and DAS interacts with cellular DBF. In addition, we postulate that the γ initiator elements interact with an as yet uncharacterized cellular protein. This protein could be a TBP-associated factor, in the terminology of Dynlacht et al. (7), and thus a component of a late gene-specific TFIID complex, or it could function independently of TFIID.

Although active in uninfected-cell transcription extracts, the amount of product generated with the U_L38 promoter as a template is significantly less than that seen with an equivalent amount of VP16 template (Fig. 5) or with a model early promoter template (unpublished data). The difference in levels of product generated with the two templates is generally equivalent to the relative activity observed for strict late promoters compared with β and βγ promoters in transient expression assays when reporter gene levels are normalized to the amount of template transfected into cells (39). From this, it is clear that γ promoters are weak compared with those of other kinetic classes.

During viral infection, two events must occur for readily detectable expression from the γ promoters to be seen. The first event is general virus-induced transactivation of the transcription reaction by the interaction of HSV α proteins with cellular transcription factors, and not the promoter directly, to augment transcription. In agreement with this proposal, it has been demonstrated that phosphorylated ICP4 indirectly interacts with the U_L38 promoter-leader via interactions with promoter-bound cellular proteins (32). The second event required for efficient expression is DNA replication, which serves to amplify the number of templates available to be transcribed. The multiplicative effect of these two events leads to the large increase in transcription seen at late promoters after DNA replication.

Although we do not exclude the possibility of active virus-mediated repression of late promoters prior to template replication as has been suggested by others (26), our data are experimentally consistent with the mechanistically simpler model described above. The ability to insert reporter promoters representing β, βγ, and γ classes into the same genomic locus and to express reporter RNA with kinetics identical to those of transcripts driven by the native promoter demonstrates that global template effects do not determine kinetics of expression (12, 18). Mutational analysis of U_L38 and other γ promoters has failed to identify *cis*-acting elements which specifically repress expression prior to template replication; if such elements exist, they must map exactly to areas required for activation at late times. Also, the general equivalence of γ promoter activities in uninfected-cell transcription reactions and in transient expression assays in virus-infected cells argues against cellular or viral proteins acting in *trans* to repress promoter activity.

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