JOURNAL OF VIROLOGY, Feb. 1991, p. 769–786
0022-538X/91/020769-18$02.00/0

Vol. 65, No. 2

Analysis of the Herpes Simplex Virus Type 1 Promoter Controlling the Expression of U_L38, a True Late Gene Involved in Capsid Assembly†

W. M. FLANAGAN,[1]‡ A. G. PAPAVASSILIOU,[2] M. RICE,[1] L. B. HECHT,[1] S. SILVERSTEIN,[2] AND E. K. WAGNER[1]*

Department of Molecular Biology and Biochemistry, University of California-Irvine, Irvine, California 92717,[1] and Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032[2]

Received 13 July 1990/Accepted 15 October 1990

The cistrons encoding the herpes simplex virus type 1 (HSV-1) U_L37 and U_L38 genes are adjacent to one another but are transcribed from opposite strands of the viral DNA. The U_L37 gene encodes a 1,123-amino-acid protein of unknown function, while the 465-amino-acid U_L38 protein is involved in capsid assembly. Previous work from our laboratory indicated that the transcripts encoding these proteins are expressed with significantly different kinetics in productive infection. In the present communication we confirm the kinetic classes and precisely map the cap sites of the U_L37 and U_L38 mRNAs. A bifunctional reporter gene vector was used to demonstrate that divergent promoters control the expression of these reporter genes in *trans*-activation assays. The U_L38 promoter is functionally separable from that controlling U_L37 in a recombinant virus. We used deletion analysis to demonstrate that as few as 29 bases 5′ of the mRNA cap site are adequate for full activity of the U_L38 promoter in *trans*-activation assays. Finally, we analyzed the protein-binding properties of the U_L38 promoter; several sites that form complexes containing ICP4, with clear homology to those identified in the HSV-1 γ42 promoter, are present. Thus, in general, the properties of this promoter are quite similar to those of other γ promoters.

Herpes simplex virus type 1 (HSV-1) encodes a large number of polypeptides whose expression is coordinately regulated in a sequential cascade (25). Although sequence data suggest 70 to 80 clear open reading frames, differential mRNA processing and partially overlapping transcripts may increase this number to as many as 100. Detailed analysis of the kinetics of expression of mRNA species from each of the major temporal classes indicates that gene regulation is primarily at the level of transcription (48, 57, 59, 62). Immediate-early (α) transcripts are expressed first, and the proteins encoded by these mRNA species are required for the subsequent expression of all other kinetic classes of viral genes expressed during the lytic phase of infection.

Delayed-early (β) genes, many of which encode proteins involved in replication of the viral genome, are maximally expressed prior to or at the peak of DNA replication and are then shut off (62). After expression of the early genes, two subclasses of late genes, βγ (γ1) and γ (γ2), reach maximal levels of transcription. These late genes are distinguished from each other by their requirement for DNA replication. βγ gene expression is moderately reduced, whereas γ gene expression is barely detected in the absence of DNA replication (21).

This complex mechanism of temporal HSV-1 gene expression raises two important questions: (i) what is the nature of the *cis*-acting DNA sequences that are responsible for cellular RNA polymerase II differentiating between different classes of viral promoters, and (ii) what *trans*-acting factors are involved in this differential expression? This problem has been investigated with HSV-1 promoters that control different kinetic classes of transcripts as experimental models. Although details differ, it is clear that transcription from β genes requires a "nominal" polymerase II promoter sequence with a TATA box homology and approximately 100 bp of DNA sequences 5′ of the mRNA cap. These DNA sequences contain binding sites for several cellular transcription factors (38, 48, 55, 57). No consensus virus sequence homologies have been reported in the upstream regions of β genes.

Transcription of the late genes also requires functional α gene products. Studies using temperature-sensitive mutants have shown that ICP4 and ICP27 are essential for full expression of the late promoters (8–10, 42). At least some βγ genes appear to be controlled by promoters that combine the basic properties of RNA polymerase II promoters, similar to early genes, with sequence elements that may bind specific viral factors and that map near the mRNA cap site and into regions of DNA encoding the nontranslated leader sequences (5, 6). In contrast, γ genes require very little sequence information upstream of the cap site to be efficiently expressed. Studies of the γ promoters for U_S11 and U_L44 (glycoprotein C) indicate that they require upstream sequences of only 31 and 34 bp, respectively, for regulated expression (22, 24, 26, 27, 52). A TATA box homology is the only consensus sequence element required 5′ of the cap site. However, as with βγ mRNA promoters, sequences 3′ of the cap site have been implicated as control elements (23, 34, 42).

To further understand the requirements for β and γ

* Corresponding author.

† This paper is dedicated to the memory of Robert W. Honess, whose interest in and unabashed enthusiasm for science made him a friend and colleague to all he met.

‡ Present address: Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305.

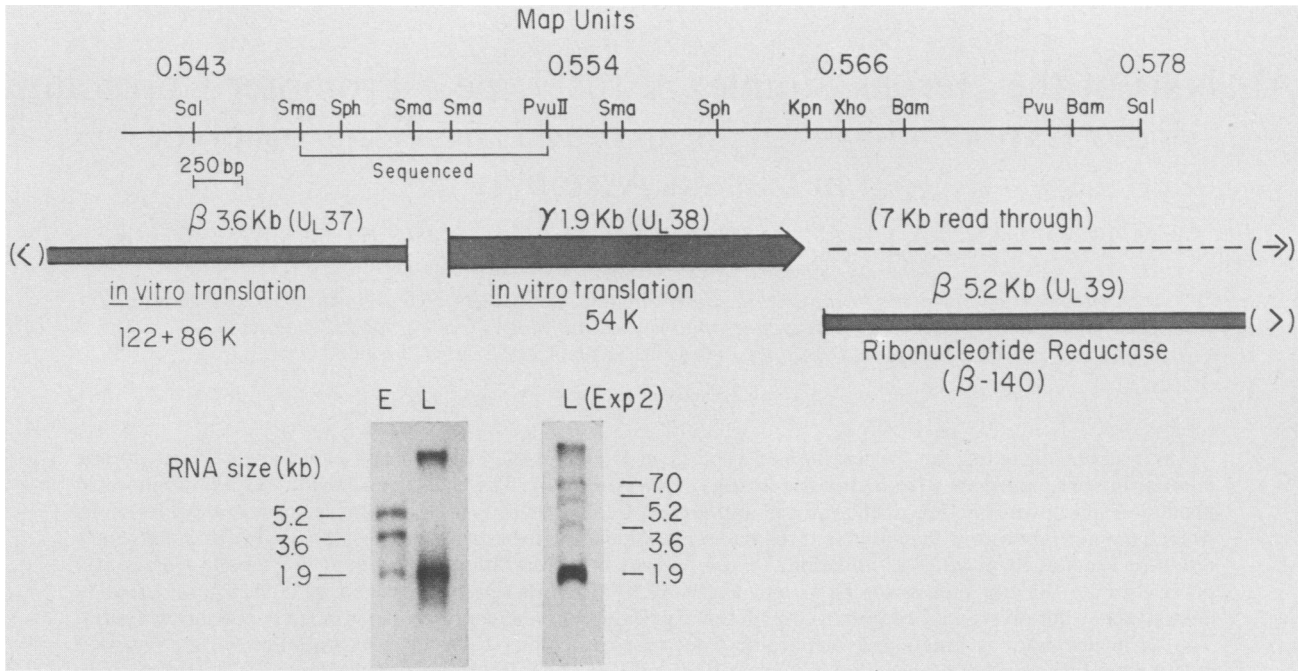

FIG. 1. Northern blot analysis of U_L37 and U_L38 transcripts. Relevant restriction sites and the locations of these transcripts in relation to that encoding U_L39 (ribonucleotide reductase, ICP6) are shown. Northern blot analysis was carried out as described in the text. Lanes E, poly(A)-containing RNA isolated from cells infected in the presence of a DNA synthesis inhibitor; lanes L, RNA isolated 12 to 14 h after infection without any inhibitor. The kinetic classes and sizes of mRNAs in kilobases are shown above their locations, while the sizes of their in vitro translation products are indicated below in kilobases.

promoter expression, we have studied a divergent promoter pair encoding mRNAs of different kinetic classes located near 0.55 map units (m.u.) (2, 62). The head-to-head orientation of these promoters suggested that they would be useful for analyzing the biologic effects of defined modifications to their DNA sequences. Moreover, their close proximity allowed us to define reasonable limits for the region of study. The transcripts expressed from the promoters in question are 3.6 (early) and 1.9 (late) kb, respectively. The former encodes the U_L37 gene product, which is of unknown function, and the latter encodes the U_L38 gene product, a 54-kDa protein required for capsid assembly (46).

In the current study we have further demonstrated the kinetic classes to which these genes belong and precisely mapped the cap sites of the U_L37 and U_L38 mRNAs; used a bifunctional reporter gene vector (pCAL [15]) to show that each promoter controls the expression of reporter genes in *trans*-activation studies; demonstrated that the U_L38 promoter can function independently of the U_L37 promoter in a recombinant virus; and used deletion analysis to show that only 29 bases 5′ of the mRNA cap site are required for full activity of this promoter in *trans*-activation assays. Finally, we have examined the protein-binding properties of the U_L38 promoter-leader; several sites that nucleate DNA-protein complex formation are present, and they have strong homology to those identified for another γ HSV-1 promoter (42). These data identify functional domains of the U_L38 promoter and demonstrate the similarity of this promoter to other γ promoters.

(A portion of this work was submitted by W.M.F. in partial fulfillment of Ph.D. requirements at The University of California-Irvine.)

MATERIALS AND METHODS

Cells and viruses. For Northern (RNA) blot and transactivation analysis, the KOS(M) strain of HSV-1 was used to infect rabbit skin fibroblasts maintained at 37°C under 5% carbon dioxide in Eagle minimum essential medium containing 5% cadet calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (4, 12, 15, 61). HeLa cells infected with the F strain of HSV-1 were used for preparation of nuclear extracts. HeLa cells were propagated as previously described (17, 44).

Enzymes. Restriction enzymes were purchased from Boehringer Mannheim Biochemical Corp., New England BioLabs, or Bethesda Research Laboratories, Inc. All digestions were carried out in buffers recommended by the supplier. S1 nuclease was obtained from Boehringer Mannheim, and bacterial alkaline phosphatase and T4 polynucleotide kinase (Bethesda Research) were used for 5′ end labeling as described by Maxam and Gilbert (35).

Recombinant DNA. Derivatives of the pBR322 and pUC plasmids were grown in *Escherichia coli* JM 101. Details concerning the growth and purification of the plasmids are essentially as previously described (2, 15, 33). All recombinant DNA clones used in experiments described here were derived from *Sal*I fragment L (0.543 to 0.578 m.u.). Linkers were purchased from Collaborative Research, Inc. For synthesis of ^{32}P-labeled DNA probes, the HSV-specific DNA fragment was isolated by digestion of the total plasmid with appropriate restriction enzymes and then subjected to electrophoresis and electroelution. Synthesis of uniformly labeled probes was accomplished by nick translation with [α-^{32}P]CTP (3 Ci/μmol; Amersham).

RNA isolation and fractionation. RNA was isolated from HSV-1 KOS(M)-infected rabbit skin fibroblasts by using the guanidinium-isothiocyanate hot-phenol method (62). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Collaborative Biochemicals) chromatography as described elsewhere (12, 58). RNA was size fractionated by electrophoresis through 1.4% agarose gels containing 10 mM methyl mercury hydroxide (58) and transferred by electrophoresis onto nylon membranes (GeneScreen; New England Nuclear) (Fig. 1). Early RNA was obtained by incubating cells in the presence of 50 μg of thymidine 1-β-D-arabinose furanoside (ara-T; Sigma Bio-Chemical Co., St. Louis, Mo.) per ml for 8 h following infection. Late RNA was isolated at 12 to 14 h postinfection.

Hybridization conditions. RNA transfer blots were hybridized in a volume of 5 ml containing 4×10^7 cpm (Cerenkov) of radiolabeled nick-translated DNA in the presence of 50% formamide, 0.4 M Na^+, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), 0.005 M EDTA, and Denhardt's solution containing 100 μg of denatured calf thymus DNA per ml at 49°C for 40 h. Details and procedures for rinsing were previously described (62).

Nuclease mapping of HSV-1 mRNA. S1 nuclease analysis of RNA was carried out essentially as described previously (3, 7, 13). Briefly, cloned HSV-1 DNA was cleaved at the desired site with the appropriate enzyme and then 5′ end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; ICN Biochemicals) and T4 polynucleotide kinase to a specific activity of 100,000 cpm of DNA per μg (ca. 10,000 cpm/pmol). Labeled DNA was then denatured and strand separated on 5% acrylamide gels as described by Maxam and Gilbert (35). The strand-separated DNA was hybridized with 5 μg of HSV poly(A)$^+$ RNA in 0.1 M Na^+–0.1 M HEPES (pH 8.0)–0.01 M EDTA at 65°C for 16 h in a 30-μl volume, and the hybrids were subjected to S1 nuclease digestion (1,000 U per sample) for 60 min at 37°C. The S1-resistant material was fractionated on 6% acrylamide–8 M urea sequencing gels.

Nucleotide sequencing. All recombinant DNA was from the KOS(M) strain of HSV-1; fragments were cloned into M13 vectors as described elsewhere (33, 39). Sequence analysis was carried out using the dideoxy chain termination method of Sanger et al. with single-stranded DNA cloned in M13 (50).

Construction of transient expression vectors. The construction of pCAL4 and pCAL5, which are pBR322-based plasmids containing both β-galactosidase and chloramphenicol acetyltransferase (CAT) as expressible markers, has been described before (15). Note that the only difference between pCAL4 and pCAL5 is the orientation of the polylinker inserted between the two marker genes.

The transient expression vectors pCAL4 Div and pCAL5 Div contain the promoters for the U_L37 and U_L38 promoters (Fig. 2A) and were constructed as follows (Fig. 3A also). The U_L37 promoter was cloned into pCAL4 and pCAL5 as a *Hin*dIII-to-*Sma*I fragment by ligating the DNA between the *Acc*I at bp 49 and the *Sma*I site at bp 219 into pUC9 and then using the *Hin*dIII site from the pUC9 polylinker. The U_L38 promoter contained within the *Sma*I fragment, spanning bases 219 to 421, was then ligated into each pCAL vector containing the U_L37 promoter. The orientation of the U_L38 promoter was determined by using diagnostic restriction enzyme digestions and confirmed by DNA sequencing using an oligonucleotide primer that binds to the CAT gene (5′-CCATTTTAGCTTCCTTAGC-3′) or the β-galactosidase gene (5′-CGCTCATGTGAAGTGTC-3′).

The pCAL4 Div-17 construct was obtained by digesting

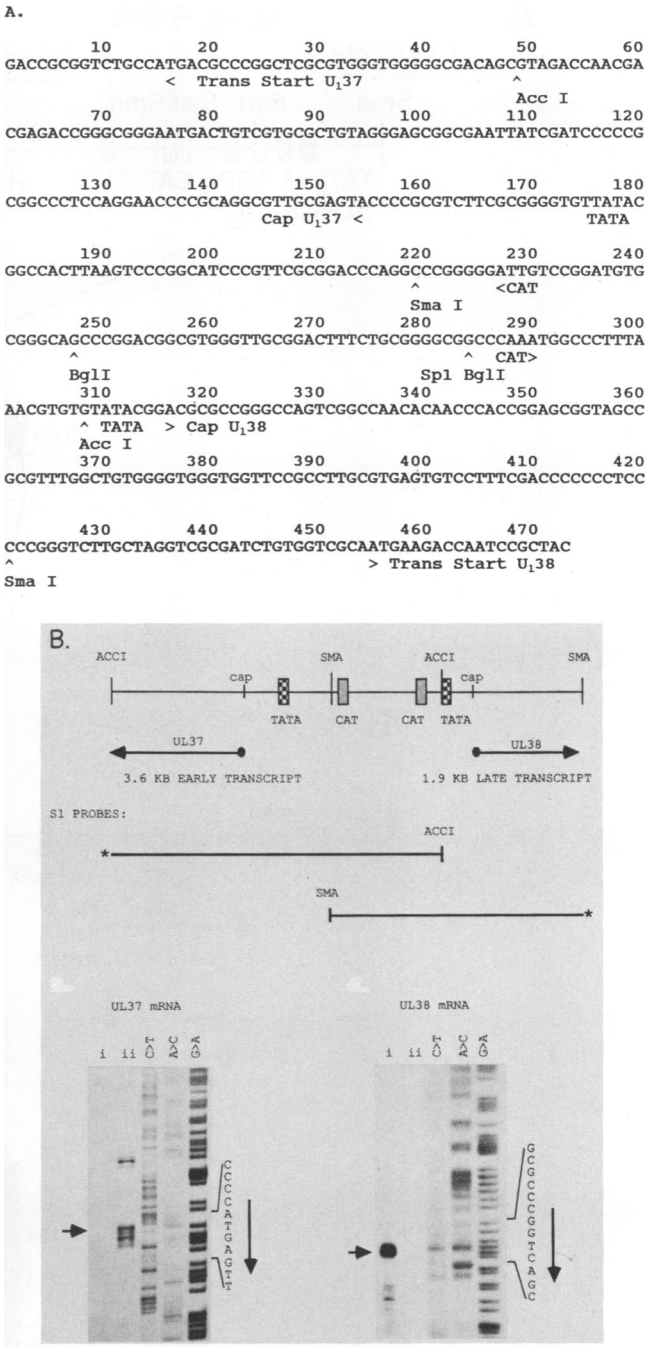

FIG. 2. Location of cap sites of U_L37 and U_L38 mRNAs. (A) Sequence of HSV-1 KOS(M) in the vicinity of the mRNA cap sites. (B) Schematic representation of the sequence data from panel A showing the locations of the caps relative to the S1 nuclease probes. The site of 5′ labeling of each probe is indicated with an asterisk. Also shown are the sizes of S1 nuclease-protected DNA fragments run on a sequencing gel. See the text (especially Materials and Methods) for details.

pCAL4 Div with *Bgl*I, which released a 34-bp fragment. This fragment was then replaced with a synthetic oligonucleotide linker (5′-CTCTAGAGGGGGCGGCCCAAA-3′) that partially restored the deleted sequences and inserted a unique *Xba*I site, resulting in a 17-bp deletion and a 5-bp linker

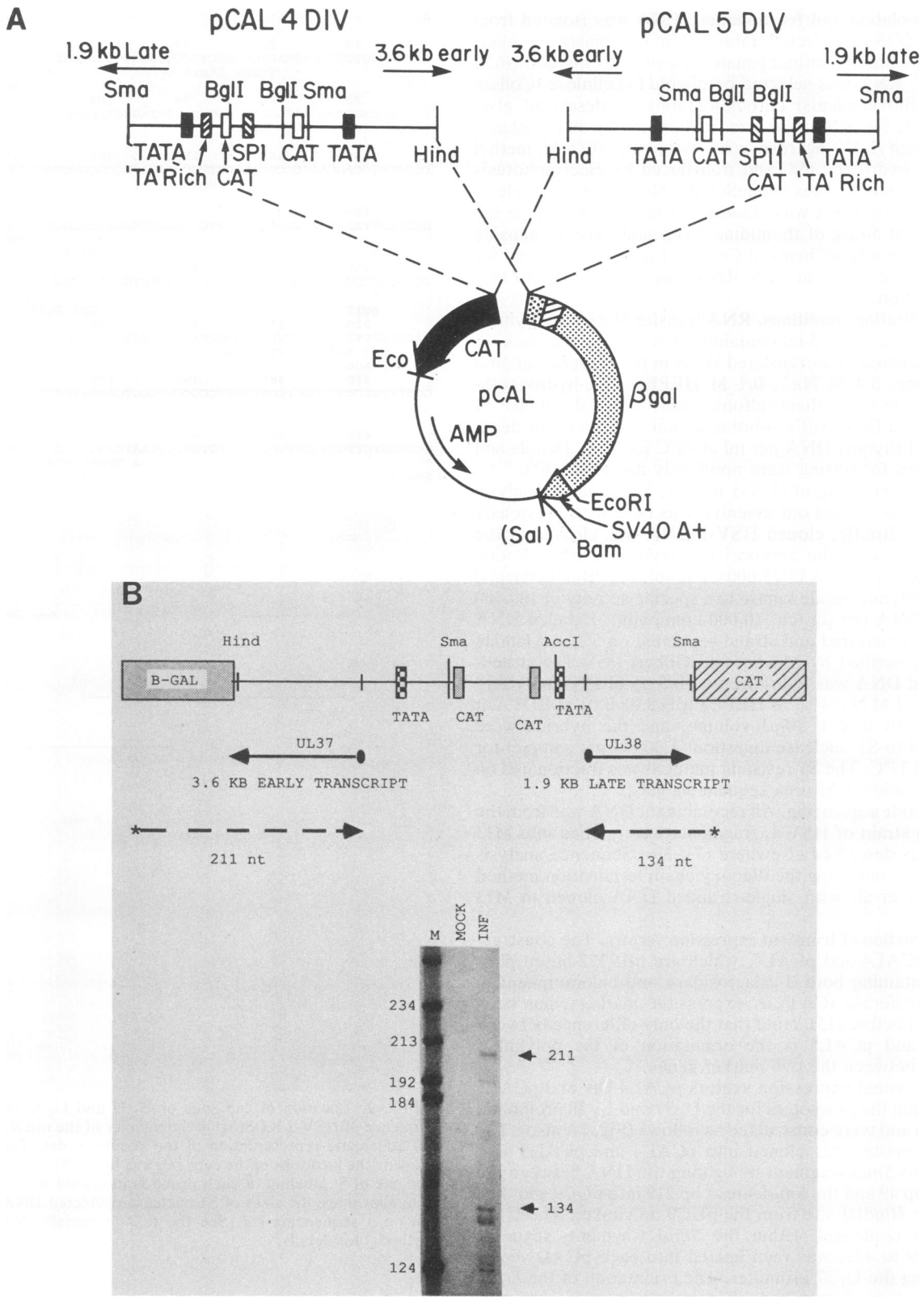

FIG. 3. Transient expression of CAT and β-galactosidase genes in a dual transient expression vector (pCAL). The construction of pCAL has been described previously (15). (A) Schematic description of the construction of pCAL4 Div and pCAL5 Div containing the divergent U_L37 and U_L38 promoters. Details are given in the text. SV40, simian virus 40. (B) Primer extension analysis of β-galactosidase and CAT mRNAs expressed following pCAL4 Div *trans* activation. RNA was isolated from transfected, superinfected cells and subjected to simultaneous primer extension analysis with one primer specific for the CAT gene and one specific for the β-galactosidase gene as described in the text. nt, Nucleotide.

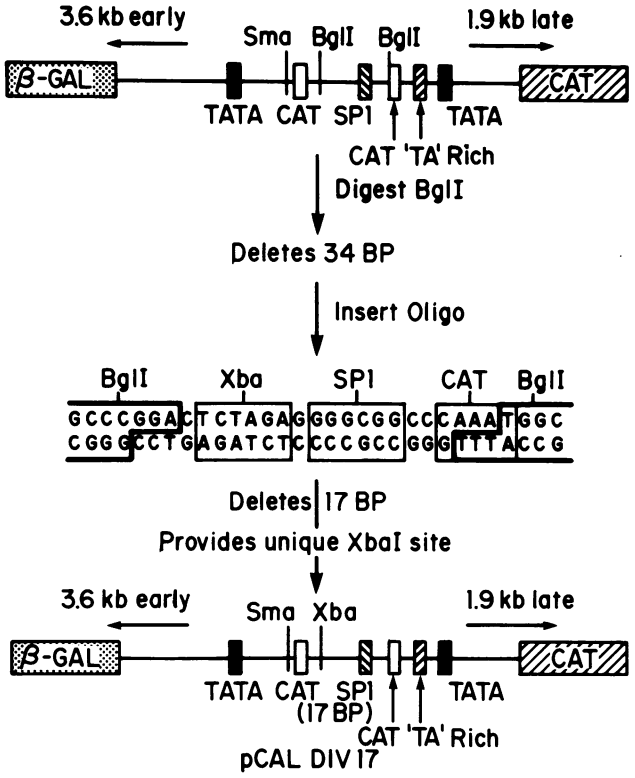

FIG. 4. Construction of pCAL Div-17 containing a 17-bp deletion between the promoters for the U_L37 and U_L38 transcripts. Details are given in the text and especially in Materials and Methods.

"mutation" because of the insertion of a *Xba*I site (Fig. 4A). Deletion mutants of the U_L38 promoter were constructed by sequential digestion of pCAL4 Div-17 linearized at the *Xba*I site by using exonuclease III (Boehringer Mannheim) or Bal 31 nuclease (New England BioLabs) and mung bean nuclease (New England BioLabs) according to published protocols (20) and as described by the suppliers. *Xba*I linkers were ligated to the deletion endpoints and transformed into *E. coli* JM 101. The extent of the deletions was determined by restriction enzyme digestion of plasmid DNA. Mutant clones that contained deletions of the desired length (see Fig. 6 for a list) were then sequenced to determine the precise endpoints as discussed above. The 79-bp U_L38 leader deletion mutant was made by digesting the promoter with *Acc*I and *Xma*I and inserting a synthetic oligonucleotide linker (5'-ATACGGACGCGCCGGGCCAGTCGGCCAGATCTC-3'), which recreates the U_L38 TATA box and cap site plus 10 bp of the untranslated leader sequence and inserts a unique *Bgl*I site next to the *Sma*I site at residue 421.

DNA transfections. Rabbit skin cells were seeded at 6×10^5 cells per 60-mm-diameter plate and then grown for 18 h to 80% confluence (1×10^6 to 1.4×10^6 cells). At 4 h prior to transfection, culture medium was replaced with new culture medium. Cultures were then transfected with 5 μg of form I plasmid DNA. Transfection was by $CaPO_4$ precipitation as described elsewhere (56). At 6 h following transfection, the cells were subjected to shock with 15% glycerol and washed with saline and 3 mM EGTA. Fresh medium was then added, and after 24 h cells were infected with HSV-1 at a multiplicity of 1 to 2 PFU per cell and overlaid with medium containing 50 μg of ara-T per ml. At 18 h after infection, cells were harvested and extracts were prepared according to the method of Gorman et al. (19). Protein concentration in the extracts was determined by using a commercial (Bio-Rad) protein assay kit with bovine serum albumin as a standard. CAT activity, measured as percentage of conversion of unacetylated to acetylated substrate per milligram of protein, was determined as described elsewhere (4–6, 19). β-Galactosidase activity was also assayed as described previously (15). Specific activity of β-galactosidase is expressed as units of enzyme activity per milligram of protein, where 1 U of β-galactosidase cleaves 1 nmol of ONPG per min at 30°C and 1 nmol of the product (*o*-nitrophenol) per ml has an A_{414} of 0.0045. CAT activity from modified U_L38 promoters was normalized for transfection efficiency by comparing total β-galactosidase activity expressed from the unmodified U_L37 promoters in each construct; normalization never changed data more than ±20%.

Generation of recombinant virus. A recombination vector extending from the *Hpa*I site just outside the IR_L (base 117,006; 0.770 m.u. [36]) to the *Sal*I site at base 120,902 (0.795 m.u.) and containing a polylinker with *Xba*I and *Bam*HI sites replacing the 1,807 bases between the *Pst*I site at base 118,659 and the *Hpa*I site at base 119,466 was constructed in two steps by using appropriate DNA clones from the $17syn^+$ strain of HSV-1. This construct (5',3'-ΔLAT) eliminates the promoter and 5' regions of the gene encoding the latency-associated transcript, which is nonessential for lytic virus growth (51). The *Xba*I-*Bam*HI fragment of pCAL5 Div-17, containing 45 bases of U_L38 promoter 5' of the cap site and 103 bases within the leader region controlling the β-galactosidase gene (Fig. 3A and 4A), was ligated into this vector to produce the recombinant plasmid shown in Fig. 5A. The plasmid was used to make recombinant viruses by cotransfection with infectious $17syn^+$ DNA, and single virus plaques were screened in 96-well plates by hybridizing aliquots of infected-cell DNA with a ^{32}P-labeled β-galactosidase probe in a dot blot assay. Wells containing β-galactosidase DNA were then plaque purified three more times, and seven positive isolates were analyzed by Southern blot analysis as shown in Fig. 5C. Two of these recombinants have the complete promoter–β-galactosidase construct inserted into both the IR_L and TR_L regions of the HSV-1 genome, and one (FLA5) was used in this report.

Preparation of nuclear extracts for DNA-binding studies. Approximately 5×10^6 actively growing HeLa cells were seeded in 150-mm-diameter culture dishes in 20 ml of Dulbecco's modified Eagle's medium containing 8% calf serum and 2% fetal calf serum (HyClone). The medium was replaced every 24 h, and when the monolayer was confluent, the cells were either mock infected or infected at a multiplicity of infection of 5 PFU per cell. Infections were allowed to proceed for 2, 4.5, or 10 h at 37°C before the cells were harvested for extract preparation. Nuclear extracts were prepared by a modification of the procedure of Dignam et al. [11, 44], frozen as aliquots in liquid nitrogen, and stored at −80°C. The protein concentration varied between 4 and 6 mg/ml and was determined by using a Bio-Rad protein assay kit.

Origin and preparation of probe and competitor DNAs for DNA-binding studies. All probes (Fig. 6B) used in the mobility shift assays were digestion products of the *Xba*I-*Eco*RI restriction fragment obtained from plasmid pUC18 containing bases 276 through 426 of the sequence illustrated in Fig. 2A (−48 to +103 relative to the cap site of the U_L38 gene;

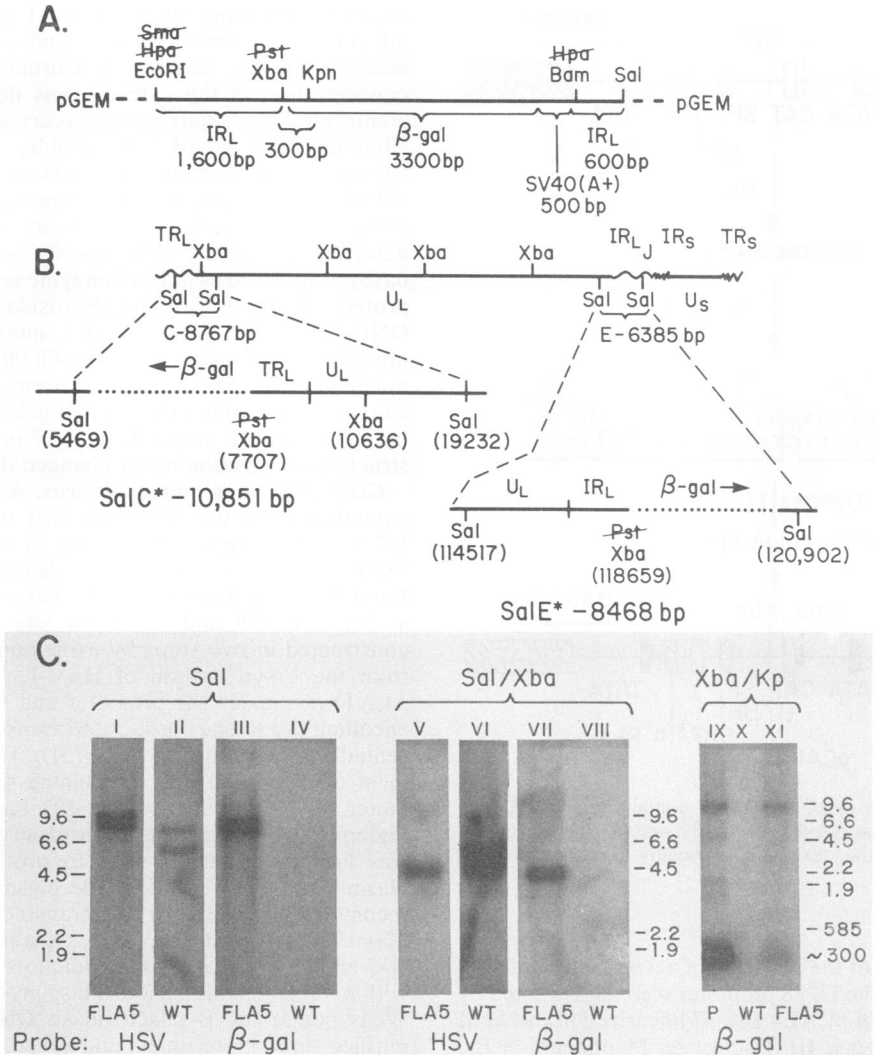

FIG. 5. Construction of the FLA5 recombinant. (A) Schematic representation of the recombination vector derived from pGEM containing the β-galactosidase (β-gal) gene terminated by the simian virus 40 (SV40) poly(A) region (Fig. 3A) controlled by the +45 to −103 promoter region of the U_L38 gene inserted into DNA containing a deletion of the promoter and 1.8 kb of DNA containing the 5′ portion of the latency-associated transcript. (B) Expected structure of the recombinant with the β-galactosidase gene recombined into both the TR_L and IR_L regions of the HSV-1 genome. Insertion of the β-galactosidase gene into both the TR_L and IR_L generates two new *Sal*I fragments containing novel *Xba*I sites. These *Sal*I fragments are each about 2,100 bp larger than the parental ones because of the deletion of 1,800 bp of HSV-1 DNA and the insertion of the 3,900-bp of β-galactosidase–promoter construct. (C) Diagnostic Southern blots demonstrating the insertion of the β-galactosidase fragment into both sites. Digestion of FLA5 DNA with *Sal*I generated fragments migrating at ca. 11 and 8.5 kb that hybridized with either a β-galactosidase-specific or an HSV-1-specific DNA probe (lanes I and III). The latter probe was the 600-bp *Hpa-Sal* fragment from cloned wild-type (WT) viral DNA (A). In contrast, wild-type DNA yielded 8.7- and 6.4-kb DNA fragments detectable only with HSV-1-specific probe (lanes II and IV). Digestion with *Xba*I in addition to *Sal*I generated a 3.9-kb fragment hybridizing to both probes with recombinant virus DNA and 6.3- and 5.2-kb fragments seen only with viral probe with wild-type DNA (lanes V, VI, VII, and VIII). As expected from the structure shown in panel A, digestion of FLA5 DNA with *Kpn*I and *Xba*I generated a 360-bp DNA fragment migrating with the same size as that seen from the recombination plasmid DNA (P) (lanes IX and XI) when hybridized with β-galactosidase-specific probe.

originally −48 to +99, with 4 bp acquired from the polylinker after digestion with *Eco*RI). Each probe was first released from the *Xba*I-*Eco*RI fragment by digestion with the appropriate restriction enzyme(s), labeled by using the Klenow fragment of *E. coli* DNA polymerase I and the appropriate α-^{32}P-labeled deoxynucleoside triphosphates dNTPs (3,000 Ci/mmol; 1 Ci = 37 GBq), and then chased with an excess of all four cold deoxynucleoside triphosphates (to ensure that the reaction generated blunt-ended molecules). Following polyacrylamide gel electrophoresis and a brief exposure to X-ray film, the desired fragment was located by direct comparison to a labeled *Msp*I-cut pBR322 marker ladder and eluted from the gel matrix. Unlabeled competitor DNAs were prepared after digestion of the *Xba*I-*Eco*RI fragment (homologous competitors) or other plasmids (heterologous competitors) with the appropriate restriction enzymes and filling in of their termini by using the Klenow fragment of *E. coli* DNA polymerase I and an excess of all four cold deoxynucleoside triphosphates and isolated after a preparative polyacrylamide gel was stained with ethidium bromide. Recovery and purity of the isolated fragments were assessed by gel electrophoresis in agarose adjacent to known amounts

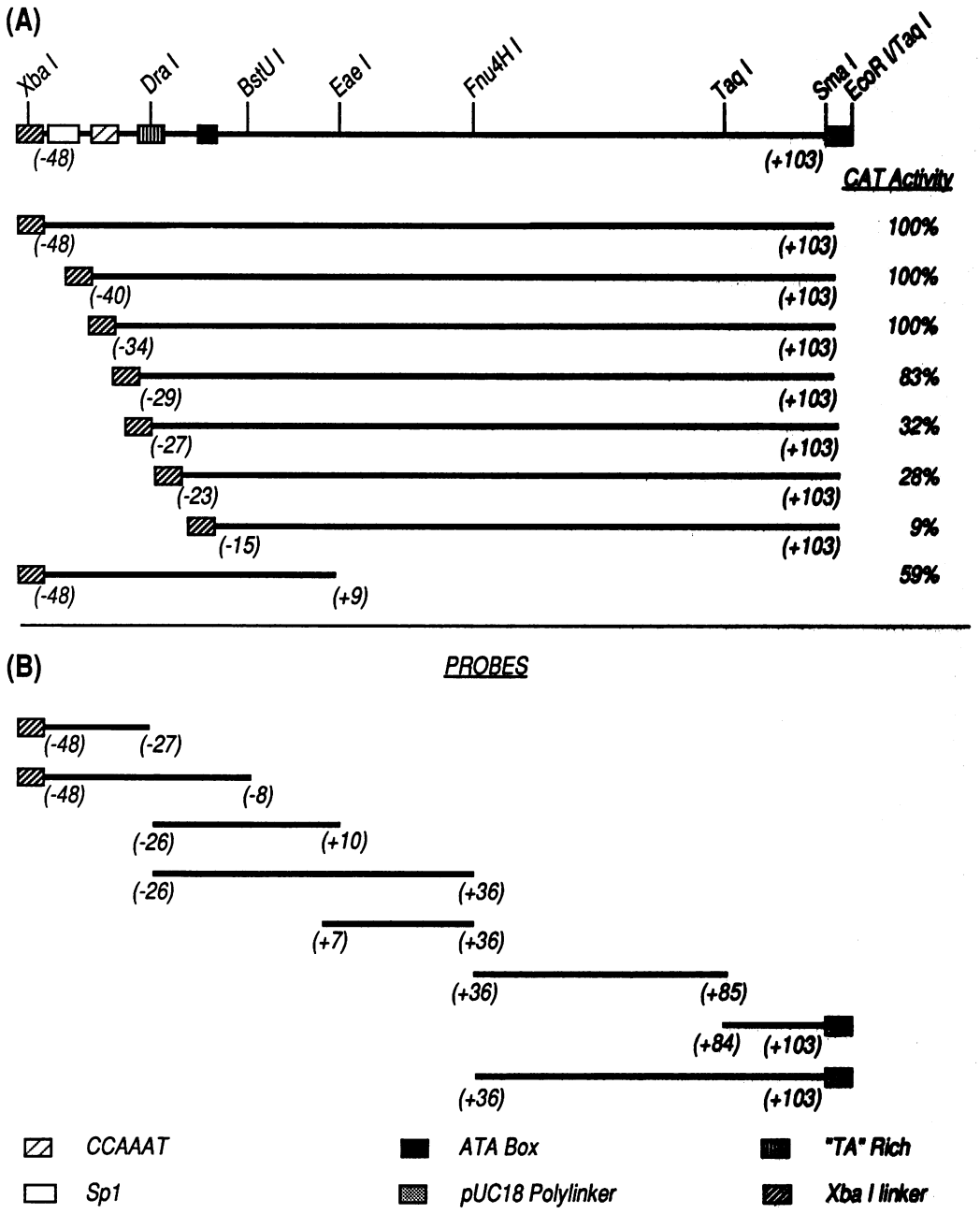

FIG. 6. Deletion analysis of the U_L38 promoter-leader region. (A) Various deletions of the U_L38 promoter were constructed as described in the text and then assayed for the amount of CAT activity expressible via a *trans*-activation assay. CAT activity was normalized to U_L37 promoter-controlled β-galactosidase activity as described in the text. (B) Locations of various portions of the promoter used in protein-binding studies.

of *Msp*I-cut pBR322 DNA. The heterologous competitors used in this study were as follows: (i) β-TK (HSV-1 MP; bases −16 to +56 and +53 to +140 [TK C] [*Mlu*I-*Bgl*II and *Bgl*II-*Mlu*I] relative to the cap site of the thymidine kinase [TK] gene [45]); (ii) α27 promoter-leader (HSV-1 F; bases −132 to +54 [*Rsa*I-*Hin*fI] relative to the cap site of the α27 gene), which contains a homolog of the high-affinity ICP4-binding site (ATCGTC) and forms a complex containing ICP4 (30); (iii) α4 regulatory region (HSV-1 F; bases −196 to −138 [*Hha*I-*Nae*I] relative to the cap site of the α4 gene), which forms a complex that contains ICP4 (30, 41); (iv) α4 promoter-leader (HSV-1 strain F; bases −17 to +32 [*Ava*I-*Bam*HI] relative to the cap site of the α4 gene), which contains the sequence ATCGTC that is present in high-affinity ICP4-binding sites (14, 31, 43, 45).

Mobility shift electrophoresis assays. For the mobility shift electrophoresis assays, 3.5 μg of nuclear extracts was mixed with 1 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia, Inc.) for 2 min prior to the addition of 0.25 ng of each probe. Binding reactions (with 20 μl of reaction mixture) were carried out in

a buffer containing 10 mM Tris hydrochloride (pH 7.5), 2 mM dithiothreitol, 1 mM EDTA, 10% (vol/vol) glycerol, and 45 mM NaCl. After 30 min at room temperature (25°C), the reaction mixtures were assayed for complex formation by electrophoresis through a native, low-ionic-strength 4% polyacrylamide gel (acrylamide/bisacrylamide weight ratio of 29:1) containing 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1 mM sodium EDTA (54). The gel was prerun for 1.5 h at 5 V/cm, and electrophoresis was conducted at the same voltage gradient for 2 to 5 h (depending on the size of the probe) at room temperature with constant buffer recirculation. The gel was then transferred to Whatman 3MM paper, dried, and autoradiographed at −70°C with an intensifying screen for 14 to 16 h. For competition experiments, the conditions were exactly as described above, except that competitor DNAs were added to the reaction mixture (in amounts detailed in the appropriate figure legends) at the same time as the DNA probe.

For the relative dissociation rate analysis, DNA probe-protein complexes formed in extracts from mock-infected and 10.5-h-infected cells were allowed to equilibrate for 30 min, and the off-rate time course was started by adding (with gentle mixing) a 250-fold molar excess of *Xba*I-*Eco*RI unlabeled competitor fragment. Samples were then loaded onto a running polyacrylamide gel at various times after the chase.

Antibodies and supershift assays. Monoclonal antibodies (1 μg/μg of nuclear extract) were added to the preformed complexes, and incubations were continued for an additional 40 min prior to mobility shift analysis (30). H640, a monoclonal antibody specific for ICP4, was the generous gift of L. Pereira, and HC-1, specific for glycoprotein C, was provided by P. G. Spear.

Copper-phenanthroline footprint analysis. Binding reactions with the −48/+103 probe (labeled in either the coding strand at the *Eco*RI site or the noncoding strand at the *Xba*I site; see Fig. 10) were carried out with five times as much material as described above in an 100-μl final volume for 30 min at room temperature (25°C), and mixtures were fractionated by polyacrylamide gel electrophoresis (6 h) as described above. The free and bound (see legend to Fig. 7 for exact descriptions of the various bound forms used) DNAs were then digested in situ by the nuclease activity of the 2:1 1,10-phenanthroline–cuprous complex by using the procedure of Kuwabara and Sigman (32), as detailed previously (44). Chemical cleavage was allowed to proceed for 25 min at room temperature, after which the gel was carefully rinsed with distilled water, and free and bound DNAs were electrophoretically transferred to an NA45 membrane (Schleicher & Schuell, Inc.) for 8 h in TBE buffer (100 mM Tris base, 100 mM boric acid, 2 mM EDTA) at a constant current (0.5 A). DNAs from the free and bound fractions were visualized by autoradiography at 4°C, and radioactive bands were eluted from the corresponding area of the membrane in 450 μl of 20 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA–1 M NaCl at 68°C for 2 h. Glycogen (10 μg per sample; Boehringer Mannheim) was then added, and the DNA was extracted with phenol-chloroform-isoamyl alcohol, precipitated twice with ethanol, washed, dried, and resuspended in 5 μl of denaturing buffer. Samples were then heated at 95°C for 5 min and quickly chilled in ice water, and equal Cerenkov counts per minute of the bound and free fractions recovered from the gel together with the same amount of a G+A Maxam-Gilbert sequencing ladder of the labeled DNA were loaded immediately on a standard 8% polyacrylamide–8.3 M urea sequencing gel in TBE buffer and electrophoresed at constant power (75 to 80 W). After electrophoresis, the gel was fixed in a 10% acetic acid–10% methanol solution for 20 min, transferred to two pieces of Whatman 3MM paper, dried, and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

RESULTS

Identification of promoters controlling expression of transcripts encoding U_L37 and U_L38. Analysis of the HSV-1 DNA sequence reveals the presence of potential TATA box homologies just 5′ of the open reading frames identified as encoding the U_L37 and U_L38 near 0.55 m.u. (36, 37). The 5′ ends of these mRNAs and their kinetics of expression were examined by using Northern blot and S1 nuclease analysis combined with sequence analysis of the KOS(M) strain of HSV-1 used in the present studies. An example of Northern blot analyses of early and late RNAs is shown in Fig. 1, with *Sal*I fragment L used as a probe. Major species detected were the early (β) 5.2-kb ribonucleotide reductase (U_L39 or ICP6) mRNA, the 3.6- and 1.9-kb transcripts encoding U_L37 and U_L38, and the 7-kb transcriptional readthrough product of the 1.9-kb mRNA. The steady-state level of the U_L37 mRNA is equivalent to that of the ribonucleotide reductase (U_L39) transcript in the presence of ara-T, and both are much reduced at late times after infection, while the U_L38 mRNA is present at low levels early and is quite abundant late. These data are consistent with our earlier measurements of mRNA abundance and rates of transcription from these genes (1, 62).

We determined the sequence of HSV-1 KOS(M) DNA encompassing the 5′ ends of the U_L37 and U_L38 mRNAs and compared it to sequences in two previously sequenced strains (17syn$^+$ and A44) to ensure that no major differences occurred (37, 46). The sequence between the *Sma*I site at 0.547 m.u. and the *Pvu*II site at 0.554 m.u. is shown in Fig. 2A; this region roughly corresponds to bases 84069 through 84552 in the sequence of the 17syn$^+$ strain. Although the sequences of the three strains in this region are highly homologous, strains A44 and KOS(M) were more similar to each other than either was to 17syn$^+$. These differences result from the addition or deletion of bases and are summarized in Table 1.

The sequence data were used to select appropriate DNA probes to determine the precise locations of the mRNA start sites. For the early 3.6-kb transcript, a 259-bp *Acc*I fragment spanning the region at 0.55 m.u. was used, while a 201-base *Sma*I fragment served to locate the 5′ end of the 1.9-kb late transcript (Fig. 2B). DNA probes were end labeled and strand separated, and both strands were hybridized to early or late mRNA. The DNA-RNA hybrids were subjected to S1 nuclease digestion, and nuclease-resistant fragments were observed by electrophoresis on denaturing polyacrylamide gels against appropriate size markers and the DNA sequence of the protected strand. For the U_L37 mRNA, the major protected species was 98 bases (UL37, lane ii). This located the RNA cap site 25 bp 3′ of an excellent TATA box homolog (TATAA). Analogous experiments (UL38, lane i) located the 5′ end of the U_L38 mRNA 16 bp 3′ of a possible TATA homology (TATA) and 28 bp 3′ of a more striking homology (TTTAAA). An additional S1-resistant fragment was sometimes observed upstream of the 5′ end of the 3.6-kb transcript; it is not clear whether this protected fragment resulted from occasional incomplete S1 digestion or represents an alternate start site.

Functional identification of promoters controlling expression of U_L37 and U_L38 mRNAs. The data in Fig. 2 place the

TABLE 1. Comparison of the U_L37-U_L38 promoter regions in three strains of HSV-1

Promoter region in HSV:		
17syn+[a]	KOS(M)	A44ts+[b]
84105–84106	36–38	—
TG	TGG	—
84108–84109	41–43	—
GG	GCG	—
84210	134	86
C	A	A
84348–84350	282–284	234–235
CGG	CGG	CG
84417–84419	351–352	302–304
AGG	AG	AGG
84432	365	317
T	T	G
84477–84494	410–418	362–371
$AC_{12}TC_3T$	AC_7T	AC_8T
84523	447	400
G	T	T

[a] Data are from reference 37. Sequence numbers are based on the complete sequence released by McGeoch et al. (37).
[b] Data are from reference 46. —, Sequence not present.

TABLE 2. *trans*-Activation analysis of pCAL4 and pCAL5 containing the U_L37 and U_L38 Div promoters

Construct[a]	Enzyme activity[b]	
	CAT	β-Galactosidase
pCAL4		
Uninfected	3.0	0.3
HSV-1 infected[c]	48.0	109
pCAL5		
Uninfected	1.0	0.7
HSV-1 infected[c]	95.0	56.0

[a] In pCAL4 the U_L38 promoter drives CAT expression and the U_L37 promoter drives β-galactosidase expression. In pCAL5 the orientation is reversed (Fig. 3A).
[b] Transient expression of CAT and β-galactosidase. pCAL4 Div and pCAL5 Div were transfected into ~10^6 rabbit skin cells. Enzyme activity is defined as units per milligram of protein isolated from the infected cell mass. CAT activity was measured as percentage of conversion of unacetylated to acetylated substrate per milligram of protein, and 1 U of β-galactosidase cleaves 1 nmol of σ-nitrophenyl-β-D-galactopyranoside per min at 30°C (see Materials and Methods).
[c] Cells were subsequently infected with 2 PFU of HSV-1 per cell in the presence of 50 μg of ara-T per ml, and relevant enzyme activities were determined 20 h later as described in the text.

5′ ends of the mRNAs encoding U_L37 and U_L38 just 170 bp apart. In addition to TATA box homologies, this region contains other recognizable promoter elements, including potential CAT box homologies and at least one Sp1 binding site (Fig. 2A). These elements are similar to those found in other HSV promoters from various kinetic classes. The ability of this fragment of DNA to serve as a promoter for the divergent mRNAs was confirmed by transient expression assays. As described in Materials and Methods, the *Acc*I site at base 49 was ligated to a polylinker containing a *Hin*dIII site, and the 397-bp DNA fragment between this *Acc*I site and the *Sma*I site (base 423) was ligated into the double transient expression plasmids pCAL4 and pCAL5 (Fig. 3A) (15). This DNA fragment contains the upstream regions of the divergent promoters as well as 126 bp of the U_L37 and 101 bp of the U_L38 untranslated leader sequences.

These divergent promoter expression constructs, pCAL4 Div and pCAL5 Div, were tested for their abilities to direct expression of the two marker genes before and after HSV-1 infection. The results of a typical experiment are shown in Table 2. Both promoters generated low basal levels of enzyme activity. These basal levels of activity were significantly increased following infection with HSV-1, as shown by the 300-fold increase of β-galactosidase expression and the 16-fold increase of CAT activity when pCAL4 Div was used. Results for pCAL5 Div, in which the early promoter U_L37 controlled the CAT gene, are essentially the same as those for pCAL4 Div, in which this promoter controlled the expression of β-galactosidase. The data on induced enzyme levels also reveal that the early promoter is more active than the late promoter controlling the same marker gene.

Experiments described above show that the divergent promoters direct expression of a linked marker gene. To demonstrate that induction of enzyme activity correlated with accurately initiated transcripts from these promoters, the transiently expressed mRNA was analyzed by primer extension. Cells transfected with pCAL4 Div were infected with HSV-1, and total RNA was extracted 18 h later. Two ^{32}P-5′-end-labeled oligonucleotide primers that bind to the CAT gene (5′-CCATTTTAGCTTCCTTAGC-3′) or the β-galactosidase gene (5′-CGCTCATGTGAAGTGTC-3′) were hybridized to total RNA isolated from transfected cells that were either mock infected or infected with HSV-1. The primers were extended by using avian myeloblastosis virus reverse transcriptase, and the products were analyzed by polyacrylamide gel electrophoresis (Fig. 3B). Primer extension of total RNA from transfected cells infected with HSV-1 showed major cDNA products of 211 and 134 bases, which are the sizes expected for the transcription start sites mapped in Fig. 2. No extension products were observed in the mock-infected RNA sample. The shorter fragment of about 124 bases may represent the same truncated product as was seen with U_L38 RNA in the S1 experiment of Fig. 2B, but a larger U_L37 product was not seen.

Establishment of limits of minimal U_L38 promoter. Two separate approaches were used to demonstrate that all the promoter elements necessary for the expression of the U_L38 mRNA were contained within 45 bases of the cap site. First, as described above, a 17-bp deletion was made within the *Bgl*I sites spanning bases 247 to 294 of the divergent promoter region and an *Xba*I site was introduced at base 277 (Fig. 4). This mutated sequence was introduced into pCAL4 (pCAL4 Div-17), and cells were transfected with it or with a plasmid containing the complete promoter (pCAL4 Div) for comparison. We found that deleting 17 bp of the upstream region from the divergent promoters had no effect on promoter activity under these assay conditions (data not shown). In other experiments, in which levels of transfected DNA were not measured, all U_L37 promoter elements upstream of the *Xba*I site were deleted, and U_L38-controlled CAT expression was at least 50% of the level seen in Table 2.

We next assayed this minimal U_L38 promoter in a recombinant virus (FLA5). In order to do this, the Div-17 promoter construct was introduced into pCAL5, and the complete *Xba*I-*Bam*HI fragment containing the β-galactosidase 5′,3′-ΔLAT gene terminating with the simian virus 40 poly(A) signal was excised and ligated into the 5′,3′-ΔLAT recombination vector, as described in Materials and Methods. This construct was used to generate recombinants in which the latency-associated transcript (LAT) gene was replaced in

TABLE 3. Expression of β-galactosidase from a recombinant virus containing the U_L38 promoter[a]

Virus	MOI[b]	Time (h) after infection[c]	β-Galactosidase activity[d]
Mock		0	5
		8	7
17syn+	10	0	7
		4	10
		8	8
FLA5	5	0	8
		2	7
		4	1
		6	25
		8	38
FLA5	10	0	7
		2	6
		4	12
		6	24
		8	32

[a] The structure of the recombinant is shown in Fig. 5A.
[b] MOI, Multiplicity of infection (PFU per cell).
[c] After addition of virus to culture.
[d] Units per milligram from lysate of 2×10^4 infected cells. One unit of β-galactosidase cleaves 1 nmol of o-nitrophenyl-β-D-galactopyranoside per min at 30°C (see Materials and Methods).

both long repeat regions and the genomic structure of the recombinants was analyzed by Southern blot hybridization (Fig. 5). Complete characterization of the kinetics of expression of β-galactosidase mRNA controlled by this and other truncated forms of the U_L38 promoter is currently in progress. However, the enzyme activity expressed by FLA5 shows that the promoter fragment used is functional in the environment of the viral genome and that its expression is generally as expected for a gene controlled by a functional HSV γ promoter; i.e., levels increase significantly with time after infection (Table 3). This result is consistent with those from the transient expression assays.

Deletion analysis of U_L38 promoter. Because both the pCAL4 Div and pCAL4 Div-17 reporter constructs had comparable activity in the HSV-1 *trans*-activation assay, we pursued the analysis of the minimal sequences required for U_L38 mRNA transcription by using the U_L37 promoter-controlled expression of β-galactosidase as an internal control for transfection efficiency. A series of deletions within the U_L38 promoter were obtained after Bal 31 digestion at the *Xba*I site of the Div-17 promoter, and these were religated into the *Xba*I-*Sma*I sites of the pCAL4 expression vector, replacing the parental Div-17 promoter. The ability of the mutant promoters to respond to HSV-1-induced *trans* activation of CAT gene expression was tested and compared with that of either the full Div promoter element or the Div-17 element and normalized to the amount of β-galactosidase expressed after transactivation of the unmodified U_L37 promoter. The data from a number of experiments are summarized in Fig. 6A. The normalized amount of CAT activity from the inducible U_L38 promoter deletion constructs is presented as a percentage of the maximum CAT activity observed. The absolute induced level of β-galactosidase activity was always within 20% of the average values; thus, normalization for transfection efficiency never significantly changed the values.

These data indicate that only 29 bp of DNA 5′ of the U_L38 mRNA cap site is required for nearly full U_L38 promoter *trans* activation after HSV-1 infection. However, a critical element lies in the TA-rich sequence very near this point, because a further deletion of 2 bp resulted in significant loss of promoter activity in this assay. Deletion of the putative TATA box for U_L38 (−15 to +103) decreased promoter activity to that seen in uninfected controls (9% of maximum levels). Even this low level of activity was still significantly above the background level seen with promoterless constructs. Finally, deletion of sequences 3′ of the cap site reduced inducible CAT activity to 59% of unmodified control levels. It is not yet known whether this leader effect results from destabilization of mRNA or loss of important transcription signals or both. However, data presented in the following sections indicate that the leader region forms specific DNA-protein complexes in infected-cell extracts that contain ICP4. Biochemical analyses of temperature-sensitive mutants demonstrated a requirement for functional ICP4 to initiate γ gene expression. We suggest that the protein complexes which form about the leader sequence and contain ICP4 may play a role in transcriptional control of this gene.

Binding properties of DNA fragments encompassing the U_L38 promoter-leader region. In order to correlate sequence requirements for transactivation of the U_L38 promoter with protein-binding sites in the sequences that compose this promoter-leader, we dissected this region (−48 to +103) (Fig. 6A) and examined the abilities of the corresponding fragments to participate in complex formation with nuclear proteins extracted from mock-infected and infected cells. The infected cells were harvested at 2.5, 5, and 10.5 h postinfection, and nuclear extracts were prepared at each time. These times were chosen to correlate with the immediate-early, early, and late phases of virus gene expression. This analysis revealed that when the fragment from −48 to +103 was used as probe, it formed complexes in all four extracts (Fig. 7A). Although the abundance of the novel complexes obtained in 2.5- and 5-h extracts differed significantly, the binding patterns were identical. However, a unique slowly migrating species formed in the 10.5-h extracts (Fig. 7A, C3). Complexes formed in each of the infected-cell extracts contained ICP4, as demonstrated by their changes in mobility after further incubation with H640, a monoclonal antibody specific for ICP4 (Fig. 7A, lanes c, f, and i). Control reactions using a monoclonal antibody specific for glycoprotein C (U_L44, a virus glycoprotein) demonstrated the specificity of this assay (Fig. 7A, lanes d, g, and j).

To localize the sites in this region that were required for the formation of ICP4-containing complexes, we analyzed the complex-forming abilities of each of the probes shown in Fig. 6B. A summary of these analyses is shown in Table 4, and representative autoradiograms of the mobility shift assays using probes that encompass the upstream promoter (−48 to −27), proximal promoter-leader (−26 to +36 and −26 to +10), 5′ leader (+7 to +36), and 3′ leader (+36 to +85) regions are shown in Fig. 7B, C, D, E, and F, respectively. The upstream promoter probe formed complexes with identical electrophoretic mobilities and abundance after incubation in all of the extracts except that prepared at 10.5 h. None of these complexes contained ICP4 (Fig. 7B). A similar analysis performed with the proximal promoter-leader probe revealed that it, too, formed complexes after incubation in all four extracts. The amount of probe present in the novel complexes increased in proportion to the length of time postinfection before the extract was

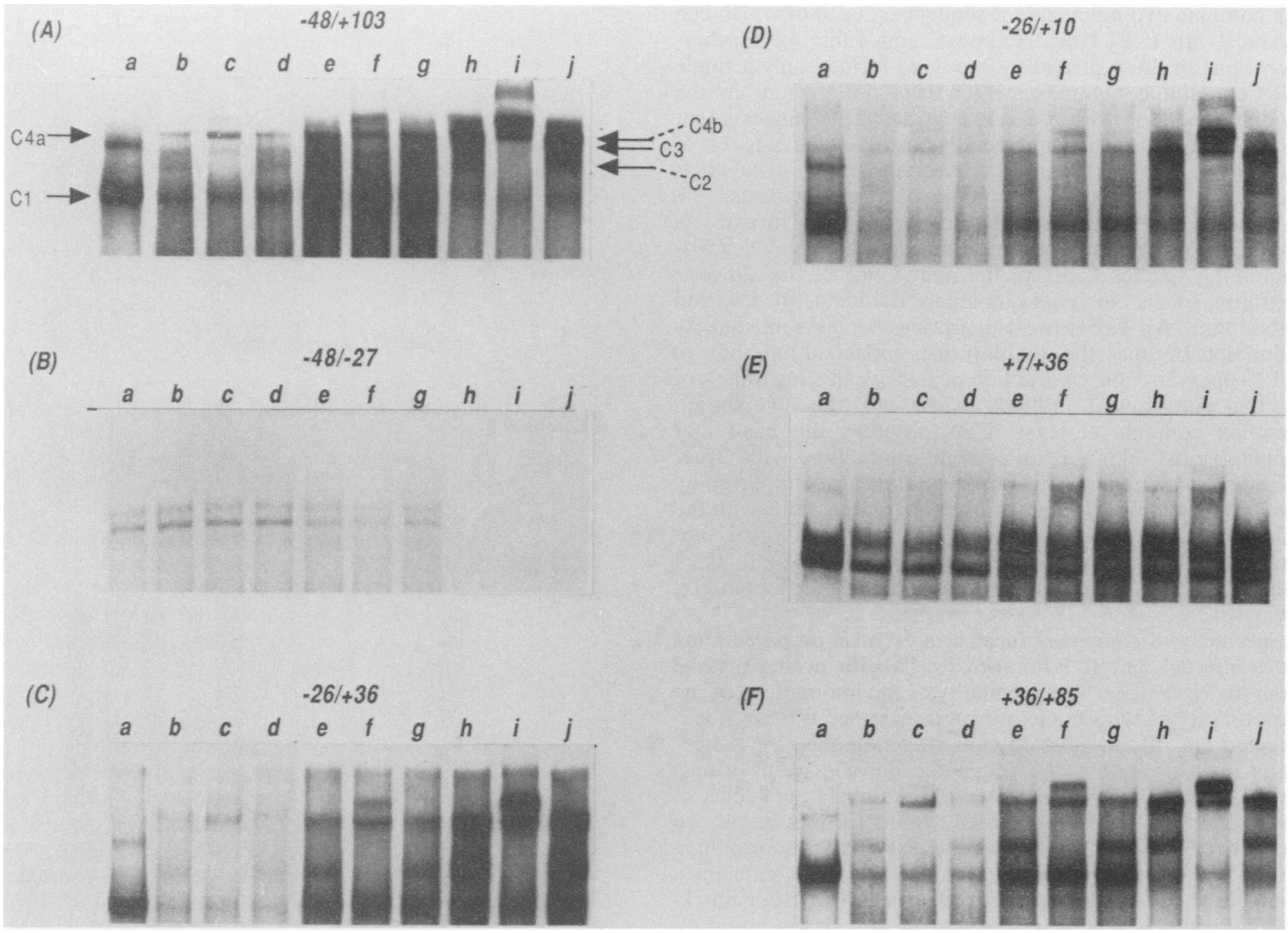

FIG. 7. Identification of ICP4 in complexes formed between DNA sequences encompassing the promoter-leader region of the U_L38 gene and nuclear extracts from HSV-1-infected cells. Binding reactions with the −48 to +103 (A), −48 to −27 (B), −26 to +36 (C), −26 to +10 (D), +7 to +36 (E), and +36 to +85 (F) probes were performed with nuclear extracts prepared from mock-infected cells (lanes a) or from cells infected with HSV-1 for 2.5 h (lanes b, c, and d), 5 h (lanes e, f, and g), or 10.5 h (lanes h, i, and j). Monoclonal antibodies specific for ICP4 (lanes c, f, and i) or glycoprotein C (lanes d, g, and j) were added after incubation of the probes with each extract. The reaction mixtures were loaded onto native, 4% low-ionic-strength polyacrylamide gels, and complex formation was monitored by the mobility shift electrophoresis assay. Only the regions of the gels containing complexes are shown. The arrows in panel A (C1 through C4b) point to the complexes analyzed by the chemical nuclease protection assay (Fig. 5A and B).

prepared. In each instance, ICP4 was present in at least one of the complexes (Fig. 7C and D, lanes c, f, and i). We noted (as with the −48 to +103 probe) the appearance of an additional slowly migrating species in the lanes showing binding reactions performed in the 10.5-h extracts (Fig. 7C and D, lanes h). In this instance, three of the species were shifted to more slowly migrating forms after reaction with the monoclonal antibody specific for ICP4 (Fig. 7C and D, lanes i). Analysis of the 3′ leader probe revealed that it also formed complexes in each extract; these complexes increased in abundance in relation to the time postinfection at which the extract was prepared, and some of the complexes formed in extracts from infected cells contained ICP4 (Fig. 7F). Moreover, as described above, an additional ICP4-containing species appeared in the extracts prepared at late times postinfection (Fig. 7F, lanes h and i).

Both the 5′ and 3′ leader regions of U_L38 form ICP4-containing complexes. Also, two probes (−26 to +10 and +7 to +36), which overlap by only 3 bp, both form complexes that contain ICP4, suggesting that the region near the mRNA

TABLE 4. Complex-forming ability and reaction with antibody to ICP4 of various regions of the U_L38 promoter-leader

Probe	Mock infection	Reactions at: 2.5 h p.i. Complex	2.5 h p.i. Reaction with +αICP4[a]	5 h p.i. Complex	5 h p.i. Reaction with +αICP4[a]	10.5 h p.i. Complex	10.5 h p.i. Reaction with +αICP4[a]
−48 to +103	+	+	+	+	+	+	+
−48 to −27	+	+	−	+	−	−	−
−48 to −8	+	+	−	+	−	−	−
−26 to +10	+	+	+	+	+	+	+
+7 to +36	+	+	−	+	+	+	+
−26 to +36	+	+	+	+	+	+	+
+36 to +85	+	+	+	+	+	+	+
+84 to +103	−	−	−	−	−	−	−
+36 to 103	+	+	+	+	+	+	+

[a] After complexes were allowed to form, the reaction mixtures were incubated with H640, a monoclonal antibody specific for ICP4, and then electrophoresed through a nondenaturing gel.

cap contains two independent sequences, each of which can interact with ICP4 (Fig. 7D and E and Table 4). Furthermore, one of these probes (+7 to +36) formed only a single ICP4-containing complex, which was not present in the binding reactions with the 2.5-h extract. This suggests that recognition of this sequence occurs only at elevated levels of ICP4. A careful analysis of the autoradiograms reveals that the mobility shift profiles of complexes formed with the −48 to +103, −26 to +36, and +36 to +85 probes in extracts prepared at late times postinfection contain a novel ICP4-containing species and that the abundance of the slowest-migrating species increases (compare the bands for C4a and C4b in Fig. 7A). The slowest-migrating species is most likely a doublet, because the addition of monoclonal antibody to ICP4 supershifts only a fraction of this species regardless of the concentration of antibody. Therefore, the supershifted complex contains at least ICP4, whereas the band that remains most likely results from interaction with other infected-cell proteins. This observation is also supported by the lower yield of this complex in binding reactions with the 2.5- and 5-h extracts and the fact that its mobility is not altered after addition of the monoclonal antibody to ICP4 (Fig. 7C and F, lanes c and f; see also the same effect in Fig. 7A).

Specificity of complexes formed in extracts prepared from mock-infected and 10.5-h-infected cells with probes derived from the U_L38 gene. The specificity of the interactions of the various probes with nuclear proteins extracted from mock-infected and 10.5-h-infected cells was examined by competition analysis with DNA fragments derived from various regions of the HSV genome. We first examined the fidelity of the interactions between the −48 to +103 probes in the two extracts (Fig. 8A). This analysis revealed that the proximal promoter-leader sequence (−26 to +36) was as efficient a competitor for complexes formed in extracts from mock-infected cells as the entire region (Fig. 8A, lanes b and c). However, the same competitor was less efficient at inhibiting complex formation in extracts from infected cells (Fig. 8A, lanes h and i). The 3′ leader sequence (+36 to +85) did not compete as efficiently as the proximal promoter-leader sequence in either extract (Fig. 8A, lanes d and j). Moreover, a heterologous leader sequence (−16 to +56) from the TK gene (a β gene), which forms specific complexes in extracts from mock-infected cells and ICP4-containing complexes in extracts from 5-h-infected cells (44, 45), competed to the same extent in both extracts as the +36 to +85 sequence (Fig. 8A, lanes e and k). In contrast, no effect on complex formation was seen with a DNA fragment containing a region from the β-TK gene (+53 to +140) that was previously shown to be unable to form complexes in either mock-infected or infected-cell extracts (Fig. 8A, lanes f and l) (44).

Because both the proximal promoter-leader and the 3′ leader regions competed (albeit to different extents) in both extracts, we suggest that they utilize common factors for complex formation in each extract. To test this, we examined the ability of each of these sequences to compete with the other and with heterologous fragments from the virus genome to form complexes in extracts from mock-infected and infected cells (Fig. 8B and C). The proximal promoter-leader and 3′ leader regions efficiently competed with one another, although the −26 to +36 element was a more potent competitor than the 3′ region (compare Fig. 8C, lane c, with B, lane d, and Fig. 8C, lane i, with B, lane j). This difference supports the demonstration that the proximal promoter-leader region has multiple binding sites that are recognized

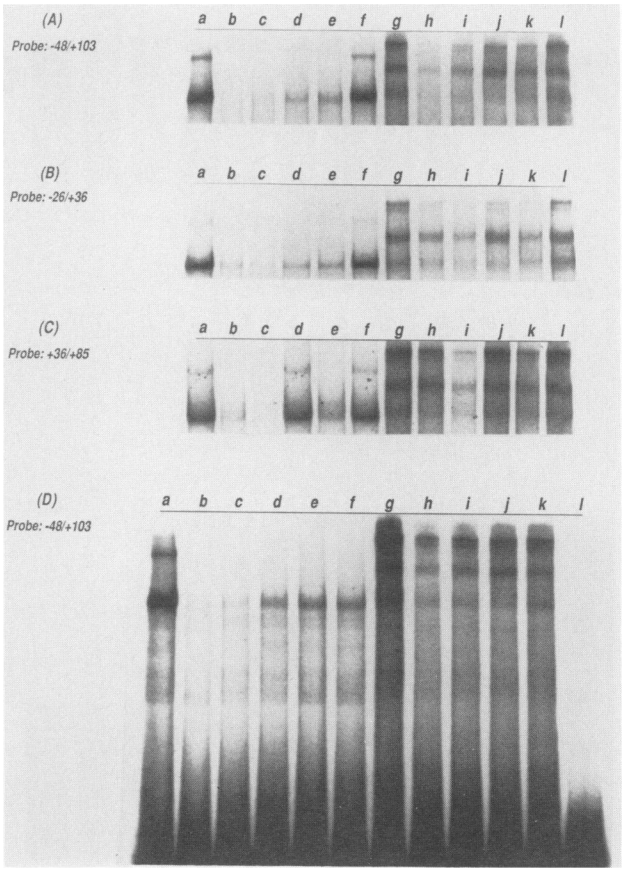

FIG. 8. U_L38 promoter-leader region complexes formed in the presence of homologous or heterologous HSV-1 DNAs. Nuclear extracts prepared from mock-infected (lanes a through f) or 10.5-h-infected (lanes g through l) cells were preincubated in the presence or absence unlabeled competitor DNAs as follows. (A) Lanes a and g, No competitor added; lanes b and h, 5-fold molar excess of self DNA; lanes c and i, 5-fold molar excess of −26 to +36 DNA; lanes d and j, 5-fold molar excess of +36 to +85 DNA; lanes e and k, 5-fold molar excess of β-TK DNA; and lanes f and l, 50-fold molar excess of TK C DNA. (B) Lanes a and g, No competitor added; lanes b and h, 2.5-fold molar excess of self DNA; lanes c and i, 5-fold molar excess of self DNA; lanes d and j, 5-fold molar excess of +36 to +85 DNA; lanes e and k, 5-fold molar excess of β-TK DNA; and lanes f and l, 50-fold molar excess of TK C DNA. (C) Lanes a and g, No competitor added; lanes b and h, 5-fold molar excess of self DNA; lanes c and i, 5-fold molar excess of −26 to +36 DNA; lanes d and j, 5-fold molar excess of β-TK DNA; lanes e and k, 10-fold molar excess of β-TK DNA; and lanes f and l, 50-fold molar excess of TK C DNA. (D) Lanes a and g, No competitor added; lanes b and h, 5-fold molar excess of self DNA; lanes c and i, 5-fold molar excess of β-TK DNA; lanes d and j, 5-fold molar excess of α27 promoter-leader DNA; lanes e and k, 5-fold molar excess of α4 regulatory region DNA; and lanes f and l, 50-fold molar excess of α4 promoter-leader DNA. The indicated probes were then added, and the incubation was continued for 30 min. Binding reactions with the −48 to +103 probe in panel D were performed with twice as much probe DNA as was used in panel A. The reaction mixtures were analyzed for complex formation by the mobility shift electrophoresis assay, and dried gels were exposed to X-ray films. Only the regions of the gels containing complexes are shown.

by nuclear proteins present in both mock-infected and infected-cell extracts. Furthermore, these experiments show that these regions are recognized by common factors that are shared by the β-TK leader region.

Competition for U_L38 promoter-leader binding with defined ICP4-binding sites. The ability of the β-TK leader sequence to compete for complex formation with both the proximal promoter-leader and 3′ leader probes was similar to results from a previous study in which we demonstrated that complex formation with the β-TK leader probe was competed by a fragment of virus DNA that contained a high-affinity ICP4-binding site (44). Therefore, sequences known to form complexes that contain ICP4 were examined for the ability to compete with the U_L38 promoter-leader (−48 to +103) complexes. Competition analyses with nuclear proteins from mock-infected and infected cells revealed that the β-TK leader region (Fig. 8D, lanes c and i), a degenerate homolog of the high-affinity ICP4-binding site found in the promoter for the α27 gene (Fig. 8D, lanes d and j) (18, 30), an upstream region from the α4 gene that was previously shown to nucleate complexes that contain ICP4 (Fig. 8D, lanes e and k) (31, 42), and a high-affinity ICP4-binding site present in the promoter-leader region of the α4 gene (Fig. 8D, lanes f and l) (14, 30, 43, 44) all inhibited complex formation with the probe in both extracts to different extents. However, the α4 promoter-leader DNA, which is bound directly by ICP4 (28, 60), completely abolished complex formation in extracts from infected cells (Fig. 8D, lane l). Thus, sequences that form complexes with ICP4 through cell intermediates and those that are directly bound by the protein effectively compete for complex formation with the U_L38 proximal promoter-leader probe. These competition data further suggest that ICP4 is a component of complexes formed with this promoter-leader probe.

Kinetic stability of γ-leader complexes. To examine the kinetic stability of the complexes formed in extracts from mock-infected and 10.5-h-infected cells with the U_L38 probe (−48 to +103), the complexes were allowed to equilibrate for 30 min (16) and were then chased with a 250-fold molar excess of the unlabeled probe for various periods before being analyzed by the mobility shift electrophoresis assay. The complexes formed in extracts from mock-infected cells began to dissociate as early as 1 min after the chase and were undetectable after 10 min (Fig. 9). Thus, the U_L38 probe does not form stable complexes with nuclear proteins from uninfected cells. In contrast, the complexes formed in nuclear extracts from infected cells were stable for at least 20 min. The same qualitative results were obtained when the challenging sequence was derived from the α-promoter-leader and β-leader regions (data not shown). These results support the conclusion from the competition analyses (Fig. 8D) and demonstrate that U_L38 complexes that contain ICP4 and other infected-cell proteins are stable.

Footprint analysis of gene complexes. To determine what sequences are involved in complex formation, we examined the chemical nuclease cleavage pattern of the U_L38 promoter-leader probe (−48 to +103) labeled in either the noncoding (Fig. 10A) or coding (Fig. 10B) strands in complexes formed in mock-infected and infected cell extracts. Accordingly, complexes were formed in extracts from mock-infected cells and separated on nondenaturing gels. The gels were treated in situ with the chemical nuclease activity of Cu^+–1,10-phenanthroline (32), and DNAs corresponding to free DNA and the most abundant complex (C1 in Fig. 7A) were extracted and electrophoresed through a sequencing gel. The nuclease cleavage patterns of these samples (Fig. 10A and B, lanes b and c) reveal two regions of protection that map from −15 to +11 and from +60 to +74. Control reactions using a rapidly migrating nonspecific species isolated from the same gel demonstrated the specificity of the assay (Fig. 10A and B, lanes d).

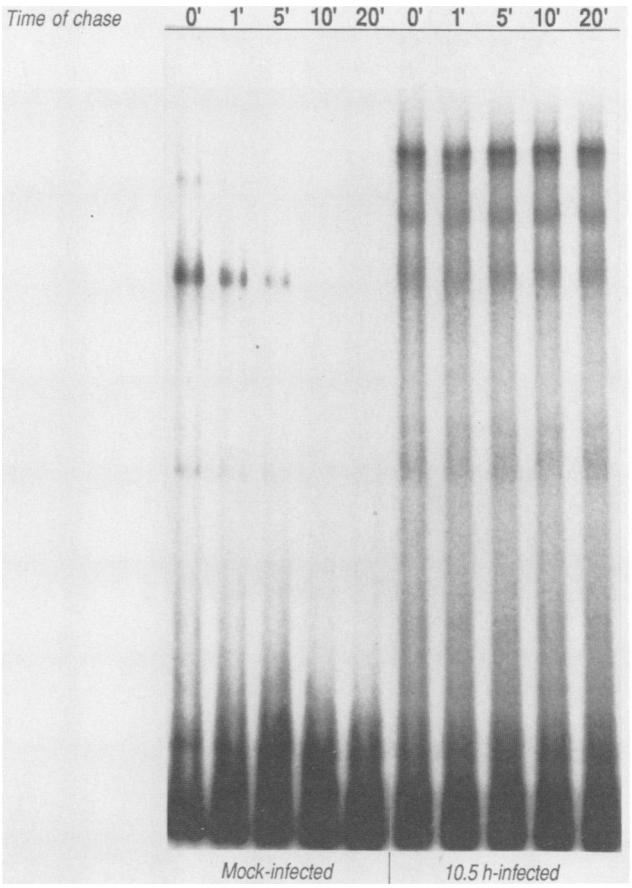

FIG. 9. Relative dissociation kinetics of complexes formed with the U_L38 −48 to +103 probe. DNA-protein complexes were allowed to form under standard reaction conditions (30 min) in extracts prepared from mock-infected or 10.5-h-infected cells with the −48 to +103 DNA probe from the U_L38 gene. At the end of the incubation period, a 250-fold molar excess of unlabeled −48 to +103 DNA was added to each reaction, and samples were withdrawn at 1, 5, 10, or 20 min after the chase and applied directly to a running, 4% low-ionic-strength polyacrylamide gel. The 0′ denotes a control reaction not chased but incubated for a total period of 50 min.

Four of the infected-cell-specific complexes were analyzed. Three were derived from binding reactions with the 10.5-h extract (C2, C3, and C4b, Fig. 7A), and one was from the 2.5-h extract (C4a, Fig. 7A). DNA isolated from each of the treated complexes revealed the same protected regions as those found in DNA extracted from the complex formed with nuclear proteins from mock-infected cells (Fig. 10A and B, lanes f, h, j, and l). However, the nuclease protection patterns of the C4b complex (Fig. 7A) formed in extracts prepared at 10.5 h postinfection contained, in addition to the previous sequences, a novel protected region from +26 to +42 (Fig. 10A, lane l). Thus, this novel footprint, found only in the slowest-migrating ICP4-containing complex (C4b) that formed in the 10.5-h extracts, occurs only when the concentration of ICP4 is very high (Fig. 7A, lanes b, e, and h). In

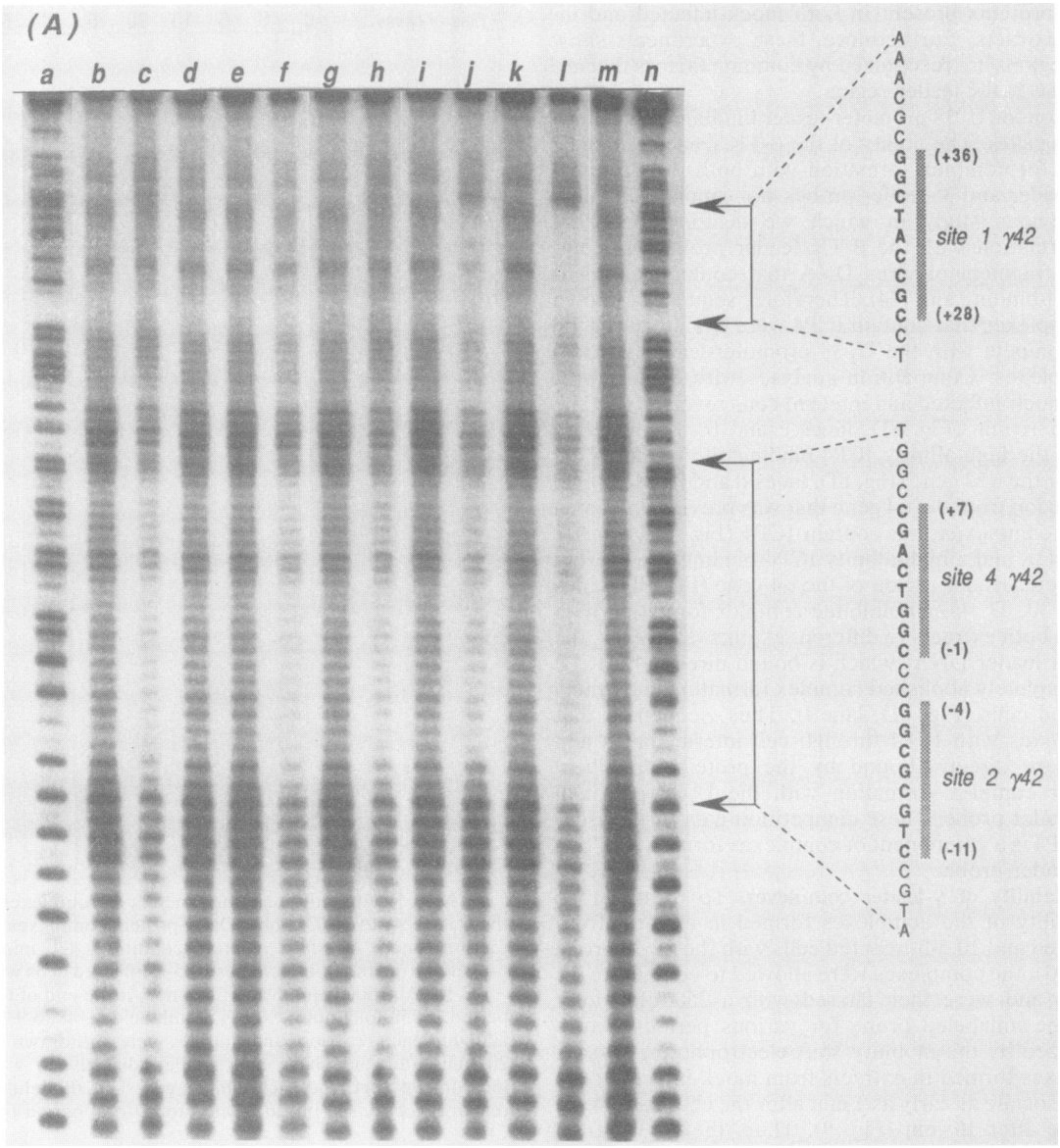

FIG. 10. Chemical nuclease cleavage pattern of complexes formed with the U_L38 −48 to +103 probe. DNA-protein complexes were formed with the −48 to +103 probe labeled at either the noncoding strand (*Xba*I site) (A) or the coding strand (*Eco*RI site) (B) and nuclear extracts prepared from mock-infected, 2.5-h-, or 10.5-h-infected cells in standard binding reactions that were scaled up fivefold. Reaction products were then fractionated by a preparative mobility shift electrophoresis assay. Immediately after electrophoresis, the gel was subjected to chemical nuclease treatment as described in Materials and Methods, and free and bound DNAs were then transferred to an NA45 membrane, eluted, denatured, and displayed on standard 8% polyacrylamide sequencing gels. Lanes: a and n, Maxam-Gilbert G+A sequencing reactions of the corresponding probe DNAs; b, cleaved DNAs isolated from bands corresponding to free DNAs from reactions containing extracts from mock-infected cells; c, cleaved DNAs isolated from the abundant complex (C1; Fig. 2A) formed in reactions containing extracts from mock-infected cells; e, g, i, and k, cleaved DNAs isolated from bands corresponding to free DNAs from reactions containing extracts from 10.5-h-infected (lanes e, g, and k) or 2.5 h-infected (lane i) cells; f, h, j, and l, cleaved DNAs isolated from complexes C2, C3, C4a, and C4b, respectively (Fig. 2A), formed in reactions containing extracts from 10.5-h-infected (C2, C3, and C4b) or 2.5-h-infected (C4a) cells; d and m, cleavage pattern of DNAs isolated from fast-migrating, nonspecific complexes formed in extracts from mock-infected and infected cells, respectively (Fig. 3D). Arrows connected by a line in each panel demarcate the protected regions. Also shown are the sequences of the protected regions; nucleotides in boldface type and the adjacent solid bars indicate homologs or perfect matches with sequences found in the promoter-leader region of the γ42 gene.

addition, the presence of the novel footprint only in C4b and not in C4a, its mobility homolog, demonstrates the existence of two comigrating species in this complex, as the supershift analyses suggested (Fig. 7). Comparative computer-aided densitometric analysis of the common protected regions from the C1 complex formed in mock-infected extracts versus the C4b complex formed in 10.5-h-infected-cell extracts (Fig. 10A, lanes c and l) revealed that 25% more DNA by mass was protected in the C4b complex. These data support the relative dissociation rate analyses, which sug-

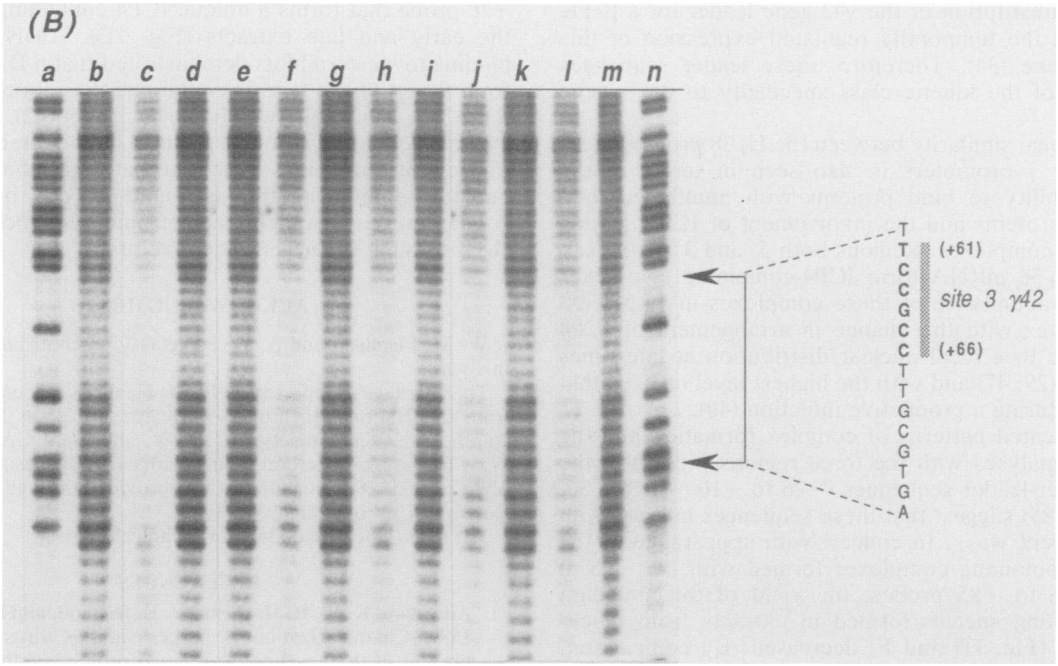

FIG. 10—*Continued.*

gested that the presence of at least ICP4 resulted in stabilization of the γ gene complex that forms in extracts from mock-infected cells.

DISCUSSION

The data presented here indicate that the adjacent and divergent promoters for the U_L37 and U_L38 genes are functionally separable. Thus, only 45 bases of DNA sequence 5′ of the U_L38 mRNA cap is required for reporter gene expression in recombinant virus (Table 3). Indeed, transient expression assays of defined deletions indicate that only 30 or so bases of 5′ sequence are necessary for promoter activity (Fig. 6A).

Transient expression assays do not always accurately reflect the kinetics of expression of individual promoters seen in lytic infection (53). Still, with HSV, analyses of modified α and β promoters in either transient expression assays or in recombinant viruses have given similar results in terms of the sequence requirements for functional expression from these promoters. Moreover, the critical regions of the U_L38 promoter that we have identified are reminiscent of those described for other γ promoters, especially with regard to the extent of upstream sequences and the requirement for sequences 3′ of the cap site for full activity (22, 26, 34).

The physiologic significance of the multiple DNA-protein interactions which we describe is not clear at this time. However, there is a strong correlation between the ability of this leader sequence to form complexes with proteins in infected-cell extracts and its requirement for full transcriptional activation (Fig. 6A and 7). Deletion analysis of the U_L38 promoter reveals that only 29 bp 5′ of the mRNA cap site are required for high-level expression of reporter genes linked to this promoter. Removal of the leader from the U_L38 reporter chimeras results in a significant decrease in expression (Fig. 6A). Previous analyses of γ-gene promoter-leader sequences revealed that the leader sequences are important for the stable accumulation of γ-gene products (4,

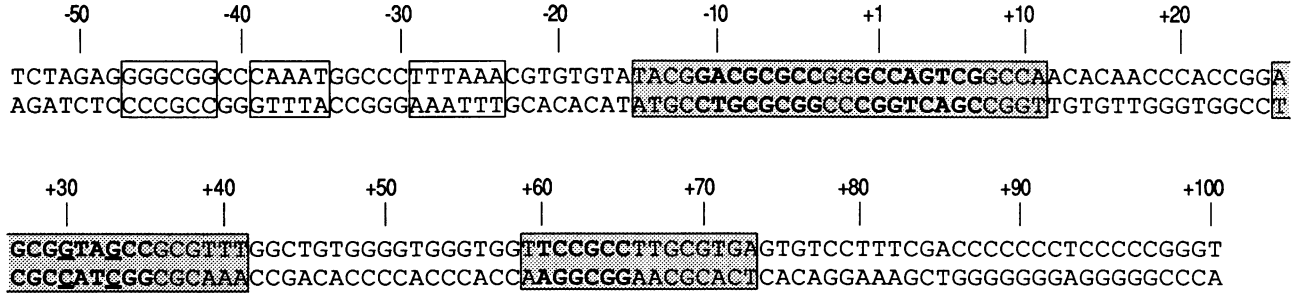

FIG. 11. Summary of footprint data. The regions of the U_L38 promoter-leader protected from chemical nuclease cleavage after complex formation in extracts containing nuclear proteins from 10.5-h-infected cells are shown as stippled boxes. Nucleotides in boldface type are homologous to sequences in the γ42 gene that form complexes containing ICP4 or that bind in Southwestern assays (41, 42); underlined bases denote mismatches. The Sp1, CAT, and TATA homologies present in the promoter are boxed.

22, 23, 34). Substitution of the γ42 gene leader for a β-TK leader altered the temporally regulated expression of this hybrid TK gene (34). Therefore, these leader sequences impart some of the kinetic class specificity to the γ-gene family.

This functional similarity between the U_L38 promoter and that of other γ promoters is also seen in terms of the promoter's ability to bind proteins with uninfected- and infected-cell proteins and the involvement of ICP4 in such DNA-protein complexes. Regions both 5′ and 3′ of the cap site of the U_L38 mRNA form ICP4-containing complexes (Fig. 7). The abundance of these complexes in 10.5-h extracts correlates with the change in arrangement of ICP4 from a diffuse to a focal nuclear distribution at late times postinfection (29, 47) and with the highest level of ICP4 that accumulates during a productive infection (40).

The complicated patterns of complex formation and the competition analyses with the three regions present in the U_L38 promoter-leader sequences (−26 to +10, +7 to +36, and +36 to +85) suggest that these sequences interact with ICP4 in different ways. In concert with appearance of the novel ICP4-containing complexes formed with the −26 to +10 and +36 to +85 probes, the yield of the abundant rapidly migrating species formed in extracts from mock-infected cells (Fig. 7D and F) decreased. By comparison, only a single ICP4-containing complex formed with the +7 to +36 probe. This occurred without any change in abundance of the complexes with electrophoretic mobilities identical to those seen in extracts from mock-infected cells (Fig. 7E). Therefore, it seems likely that ICP4 interacts directly with the +7 to +36 sequence, whereas its participation in complex formation with the −26 to +10 and +36 to +85 regions appears to be mediated through other proteins.

In previous work using competition experiments with extracts from mock-infected cells, we showed that regions that interact with ICP4 share cell factors (44). We have extensively analyzed two of the competing sequences used in these experiments and shown that the TK β-leader probe forms extremely labile complexes in infected-cell extracts, whereas complexes formed with the ICP4 α-promoter-leader probe are extremely stable (44). These analyses, along with the results shown in Fig. 8, suggest that the U_L38 gene probe contains low-affinity sites that are involved in complex formation with ICP4 and that the complexes formed are relatively stable because only the high-affinity binding site is a strong competitor.

Finally, DNAse footprinting analysis (Fig. 10) also clearly shows the similarities between functional elements in at least some HSV-1 γ promoters. As summarized in Fig. 11, examination of the three protected regions revealed that each contains a homolog or perfect match with the HSV γ42 (U_L49) promoter sequences previously shown to form ICP4-containing complexes in extracts from infected cells (41, 42). Interestingly, this promoter, too, is close to a β promoter—that encoding dUTPase (U_L50 [37]). The +60 to +74 region contains the sequence 5′-TCCGCC-3′, which is present at site 3 (+21 to +26) of the γ42 gene. The sequence 5′-GCTACCGC-3′, present in the +26 to +42 region, is found in the opposite orientation at site 1 (−133 to −125) of the γ42 gene and matches at 7 of 9 positions. The furthest upstream footprint, −15 to +11, contains two sequences, 5′-CGAC TGGC-3′ and 5′-GGCGCGTC-3′, that match sequences present at sites 4 (+50 to +57) and 2 (−67 to −60), respectively, of the γ42 gene. The footprint of the site 1 homolog was detected only in complexes formed in late extracts. Moreover, this sequence is present in the +7 to +36 probe that forms a unique ICP4-containing complex in the early and late extracts (Fig. 7E). Analysis of DNA binding to western blots demonstrated that a DNA fragment from the γ42 gene (site 1) containing the sequence found in the +7 to +36 probe was bound by ICP4 (42). By analogy, we believe that this sequence is also bound directly by ICP4. These footprint analyses suggest that U_L38 contains at least one low-affinity site that is bound directly by ICP4 in a concentration-dependent fashion and sequences that bind ICP4 through the aegis of cell factors.

ACKNOWLEDGMENTS

W. M. Flanagan and A. G. Papavassiliou contributed equally to this work.

This work was supported by Public Health Service grant CA11861 from the National Institutes of Health and grant MV372 of the American Cancer Society to E.K.W. and grant CA17477 to S.S. W.M.F. was a predoctoral trainee with carcinogenesis training grant T32-Ca-09054. Further support was provided through the UC Irvine Research Unit in Animal Virology.

We thank Sheryl Goodart for technical help.

REFERENCES

1. **Anderson, K. P., R. H. Costa, L. E. Holland, and E. K. Wagner.** 1980. Characterization of herpes simplex virus type 1 RNA present in the absence of *de novo* protein synthesis. J. Virol. **34**:9–27.
2. **Anderson, K. P., R. J. Frink, G. Devi Rao, B. Gaylord, R. Costa, and E. K. Wagner.** 1981. Detailed characterization of the mRNA mapping in the *Hin*dIII fragment K region of the HSV-1 genome. J. Virol. **37**:1011–1027.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S_1 endonuclease-digested hybrids. Cell **12**:721–732.
4. **Blair, E. D., C. C. Blair, and E. K. Wagner.** 1987. Herpes simplex virus virion stimulatory protein mRNA leader contains sequence elements which increase both virus-induced transcription and mRNA stability. J. Virol. **61**:2499–2508.
5. **Blair, E. D., and E. K. Wagner.** 1986. A single regulatory region modulates both *cis* activation and *trans* activation of the herpes simplex virus VP5 promoter in transient-expression assays in vivo. J. Virol. **60**:460–469.
6. **Costa, R. H., K. G. Draper, G. Devi Rao, R. L. Thompson, and E. K. Wagner.** 1985. Virus-induced modification of the host cell is required for expression of the bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus promoter (VP5). J. Virol. **56**:19–30.
7. **Costa, R. H., K. G. Draper, T. J. Kelly, and E. K. Wagner.** 1985. An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus DNA. J. Virol. **54**:317–328.
8. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J. Virol. **52**:767–776.
9. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56**:558–570.
10. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997–2208.
11. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
12. **Draper, K. G., G. Devi Rao, R. H. Costa, E. D. Blair, R. L. Thompson, and E. K. Wagner.** 1986. Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. **57**:1023–1036.
13. **Draper, K. G., R. J. Frink, and E. K. Wagner.** 1982. Detailed

characterization of an apparently unspliced beta herpes simplex virus type 1 gene mapping in the interior of another. J. Virol. **43**:1123–1128.
14. **Faber, S. W., and K. W. Wilcox.** 1988. Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Res. **16**:555–570.
15. **Flanagan, W. M., and E. K. Wagner.** 1987. A bi-functional reporter plasmid for the simultaneous transient expression assay of two herpes simplex virus promoters. Virus Genes **1**:61–71.
16. **Fried, M. G., and D. M. Crothers.** 1984. Kinetics and mechanism in the reaction of gene regulatory proteins with DNA. J. Mol. Biol. **172**:263–282.
17. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
18. **Gelman, I. H., and S. Silverstein.** 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. J. Virol. **61**: 3167–3172.
19. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
20. **Guo, L. H., and R. Wu.** 1983. Exonuclease III: use for sequence analysis and in specific deletions of nucleotides. Methods Enzymol. **100**:60–96.
21. **Holland, L. E., K. P. Anderson, C. Shipman, Jr., and E. K. Wagner.** 1980. Viral synthesis is required for the efficient expression of specific herpes simplex type 1 mRNA species. Virology **102**:10–24.
22. **Homa, F. L., J. C. Glorioso, and M. Levine.** 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev. **2**:40–53.
23. **Homa, F. L., A. Krikos, J. C. Glorioso, and M. Levine.** 1990. Functional analysis of regulatory regions controlling strict late HSV gene expression. *In* E. Wagner (ed.), Herpesvirus transcription and its regulation, in press. CRC Press, Boca Raton, Fla.
24. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.
25. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
26. **Johnson, P. A., and R. D. Everett.** 1986. The control of herpes simplex virus type-1 late gene transcription: a 'TATA-box'/cap site region is sufficient for fully efficient regulated activity. Nucleic Acids Res. **14**:8247–8264.
27. **Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Dalziel, and R. D. Everett.** 1986. The product of gene U_S11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67**:871–883.
28. **Kattar-Cooley, P., and K. W. Wilcox.** 1989. Characterization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. J. Virol. **63**:696–704.
29. **Knipe, D. M., D. Senecheck, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J. Virol. **61**:276–284.
30. **Kristie, T. M., and B. Roizman.** 1986. α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of alpha genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83**:3218–3222.
31. **Kristie, T. M., and B. Roizman.** 1986. DNA-binding site of major regulatory protein α4 specifically associated with promoter-regulatory domains of α genes of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:4700–4704.
32. **Kuwabara, M. D., and D. S. Sigman.** 1987. Footprinting DNA-protein complexes *in situ* following gel retardation assays using 1,10-phenanthroline-copper ion: Escherichia coli RNA polymerase-lac promoter complexes. Biochemistry **26**:7234–7238.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 109–121. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Mavromara-Nazos, P., and B. Roizman.** 1989. Delineation of regulatory domains of early (β) and late (γ2) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes. Proc. Natl. Acad. Sci. USA **86**:4071–4075.
35. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
36. **McGeoch, D. J.** 1989. The genomes of the human herpesviruses: contents, relationships and evolution. Annu. Rev. Microbiol. **43**:235–265.
37. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
38. **McKnight, S. L.** 1983. Constitutive transcriptional control signals of the herpes simplex virus tk gene. Cold Spring Harbor Symp. Quant. Biol. **47**:945–958.
39. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19**:269–276.
40. **Metzler, D. W., and K. W. Wilcox.** 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329–337.
41. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex virus major regulatory protein to viral DNA. Proc. Natl. Acad. Sci. USA **86**:9808–9812.
42. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein α4 of herpes simplex viruses. Science **239**: 1531–1534.
43. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. **61**:858–865.
44. **Papavassiliou, A. G., and S. J. Silverstein.** 1990. Characterization of DNA-protein complex formation in nuclear extracts with a sequence from the herpes simplex virus thymidine kinase gene. J. Biol. Chem. **265**:1648–1657.
45. **Papavassiliou, A. G., and S. J. Silverstein.** 1990. Interaction of cell and virus proteins with DNA sequences encompassing the promoter/regulatory and leader regions of the herpes simplex virus thymidine kinase gene. J. Biol. Chem. **265**:9402–9412.
46. **Peruiset, B., M. Boccara, J. Cebrian, N. Berthelot, S. Chousterman, F. Puvion-Dutilleul, J. Sisman, and P. Sheldrick.** 1989. Physical mapping and nucleotide sequence of a herpes simplex type 1 gene required for capsid assembly. J. Virol. **63**:2169–2179.
47. **Randall, R. E., and N. Dinwoodie.** 1986. Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP4 is associated with progeny virus DNA. J. Gen. Virol. **67**:2163–2177.
48. **Roizman, B., and A. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. Fields, D. Knipe, et al. (ed.), Virology, 2nd ed. Raven Press, New York.
49. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55**:796–805.
50. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
51. **Sedarati, F., K. M. Izumi, E. K. Wagner, and J. G. Stevens.** 1989. Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. J. Virol. **63**:4455–4458.
52. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex virus type 1 late (gamma 2) glycoprotein C gene: sequences between base pairs −34 to +29 control transient expression and responsiveness to transactivation by the products of the immediate early alpha 4 and 0 genes.

Nucleic Acids Res. **15**:3097–3111.
53. **Snowden, B. W., E. D. Blair, and E. K. Wagner.** 1988. Transcriptional activation with concurrent or non-concurrent template replication has differential effects on transient expression from herpes simplex virus promoters. Virus Genes **2**:129–145.
54. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **377**:889–901.
55. **Su, L., and D. M. Knipe.** 1987. Mapping of the transcriptional initiation site of the herpes simplex virus type 1 ICP8 gene in infected and transfected cells. J. Virol. **61**:615–620.
56. **Van der Eb, A. J., and F. L. Graham.** 1980. Assay of transforming activity of tumour virus DNA. Methods Enzymol. **65**:826–839.
57. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), Herpesviruses. Plenum Publishing Corp., New York.
58. **Wagner, E. K., G. Devi Rao, L. T. Feldman, A. T. Dobson, Y. F. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62**:1194–1202.
59. **Weinheimer, S. P., and S. L. McKnight.** 1987. Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195**:819–833.
60. **Wu, C.-L., and K. W. Wilcox.** 1990. Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA-binding protein. Nucleic Acids Res. **18**:531–538.
61. **Zhang, Y. F., G. B. Devi Rao, M. Rice, R. M. Sandri Goldin, and E. K. Wagner.** 1987. The effect of elevated levels of herpes simplex virus alpha-gene products on the expression of model early and late genes in vivo. Virology **157**:99–106.
62. **Zhang, Y. F., and E. K. Wagner.** 1987. The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes **1**:49–60.