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Mutational Analysis of the ICP4 Binding Sites in the 5′ Transcribed Noncoding Domains of the Herpes Simplex Virus 1 U_L49.5 γ_2 Gene

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A previous report (P. Mavromara-Nazos and B. Roizman, Proc. Natl. Acad. Sci. USA 86:4071–4075, 1989) demonstrated that substitution of sequences of the thymidine kinase (*tk*) gene, a β gene, extending from −16 to +51 with sequences extending from −12 to +104 of the γ_2 U_L49.5 gene in viral recombinant R3820 conferred upon the chimeric gene γ_2 attributes in the context of the viral genome in a productive infection. The U_L49.5 gene sequences extending from −179 to +104 contain four DNA binding sites for the major regulatory protein ICP4. Of these sites, two map between nucleotides +20 and +80 within the sequence which confers γ_2 regulation upon the chimeric gene. To determine the role of these ICP4 binding sites in conferring the γ_2 gene attributes, sequences comprising the two ICP4 binding sites were mutagenized and used to reconstruct the R3820 recombinant virus. In addition, a new recombinant virus (R8023) was constructed in which *tk* sequences extending from −240 to +51 were replaced with wild-type or mutated sequences contained between nucleotides −179 to +104 of the U_L49.5 gene. Vero cells infected with the recombinant viruses in the presence or absence of phosphonoacetate, a specific inhibitor of viral DNA synthesis, were then tested for accumulation of *tk* RNA by using an RNase protection assay. The results indicate that in the recombinant R3820, a mutation which destroyed one of the two U_L49.5 ICP4 DNA binding sites significantly reduced the accumulation of *tk* RNA at both early and late times after infection. The effect of this mutation was less pronounced in cells infected with the R8023 virus, whose chimeric *tk* gene contains the two upstream U_L49.5 ICP4 binding sites. None of the mutations affected the sensitivity of the chimeric genes to phosphonoacetate. The mutated site appears to be involved in the accumulation of RNA.

Herpes simplex virus 1 (HSV-1) genes form three major groups designated α, β, and γ, whose expression is coordinately regulated and sequentially ordered in a cascade fashion (26, 27). The α genes are the first to be expressed. Functional α proteins, especially functional products of the α4 gene (encoding the infected cell protein 4 [ICP4]), are required for the expression of β genes, whose products synthesize viral DNA. Lastly, γ genes, whose products are primarily virion structural proteins, form a heterogeneous group whose expression requires functional α genes and is impeded partially (e.g., γ_1 genes) or completely (e.g., γ_2 genes) by inhibitors of viral DNA synthesis (3, 9–13, 16, 20, 23, 26, 27, 31, 34, 37, 48, 50–52, 67, 68). The mechanisms by which both HSV-1 β and γ_2 genes are induced in productively infected cells have been the focus of considerable research effort. A key question is the role of ICP4 in the induction process. This report is based on two series of previous reports from this laboratory.

In attempts to map the *cis*-acting determinants of γ_2 gene regulation, we selected a promoter of a γ_2 gene mapped by Hall et al. (21) in the *Bam*HI D′ fragment of HSV-1 DNA. This model γ_2 gene promoter, designated $\gamma_2$42 or U_L49, was recently shown to regulate a hitherto unsuspected gene designated U_L49.5 (4). In previous studies, it was shown that a chimeric gene consisting of the promoter and 5′ noncoding transcribed domains of the U_L49.5 gene fused to the transcribed noncoding domains of the HSV-1 thymidine kinase (*tk*) gene, a β gene (β-*tk*), was regulated as a γ_2 gene when contained in the environment of the viral genome (recombinant virus R3112) in a productive infection (61). To define more precisely the domain of the U_L49.5 gene which conferred upon the chimeric gene the attributes of γ_2 regulation, several recombinant viruses were constructed in which various 5′ untranscribed and transcribed noncoding domains of the β-*tk* gene were replaced with those of the U_L49.5 gene (43). Expression of the chimeric genes was then analyzed for phenotypic attributes such as (i) sensitivity of expression in cells exposed to sufficient phosphonoacetate (PAA) to block viral DNA synthesis, a property which discriminates γ_2 genes from all other genes, including β genes, (ii) expression early in infection, a property of β genes, and (iii) expression late in infection, a property of γ_2 genes. We reported that the nucleotide sequence extending from −77 to +104 of the γ_2 gene, when replacing the sequence extending from −200 to +51 of the β-*tk* gene, conferred upon the latter all of the tested attributes of γ_2 genes.

A key recombinant virus developed in those studies was designated R3820. In that recombinant, the β-*tk* sequence −16 to +51 was replaced with the γ_2 sequence extending from −12 to +104. The chimeric *tk* gene of R3820 had the attributes of both β and γ_2 genes in that it was partially sensitive to PAA and was expressed both early and late in infection. The extended expression of the *tk* gene was reflected both in higher levels of thymidine kinase activity and in the accumulated levels of β-*tk* mRNA. It is notewor-

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thy that in all of the recombinants tested, the presence of transcribed noncoding domains of the γ_2 gene extending at least from +17 to +104 conferred complete or partial sensitivity to PAA. Conversely, the chimeric gene consisting of β-*tk* sequences downstream of −16 fused in the correct transcriptional orientation to the untranscribed domain of the γ_2 U_L49.5 gene extending from −179 to −12 was at best poorly expressed. Also in these studies, no regulatory role could be ascribed to the β-*tk* gene sequences extending downstream from nucleotide +51. These studies led to the conclusion that the regulatory domains of β genes exemplified by the *tk* gene were upstream of nucleotide +51, while the sequences which conferred γ_2 gene regulation were located downstream of the TATAA box.

The second series of reports dealt with the binding of ICP4 to viral DNA. Previous studies from this laboratory have shown that ICP4 binds to DNA directly and that antibody to ICP4 can bind to ICP4-DNA complexes and retard the migration of the complex in nondenaturing gels (38, 45–47). Of particular interest has been the observation that HSV genes may contain multiple binding sites for ICP4 (6, 14, 29, 35, 45–47, 49, 53, 55, 57, 58–60, 62, 65, 66). In the case of the U_L49.5 gene, these analyses demonstrated at least four binding sites mapping between −170 and −94 (site 1), −70 and −40 (site 2), +20 and +40 (site 3), and +45 and +70 (site 4) relative to the transcription initiation site at +1. At least two of the ICP4 binding sites were in DNA fragments mapping between +21 and +80, i.e., within the region which conferred upon the chimeric gene the attribute of γ_2 regulation. A key question is the contribution of the ICP4 binding sites to the regulatory attributes encoded in this fragment.

Subsequent studies based on footprinting of ICP4 to DNA fragments (46a) narrowed the positions of the binding sites. In the studies described in this report, we mutagenized binding sites 3 and 4, and fragments containing these mutations were used to construct two series of viruses (Fig. 1). The first series consisted of derivatives of R3820 described above. In the second virus series, the β-*tk* sequence extending from −200 to +51 was replaced with the γ_2 U_L49.5 sequence extending from −179 to +104. Thus, whereas the R8023 virus contains all α4 binding sites mapped within the domain of the U_L49.5 gene, the R3820 virus contains only two such sites. We report that specific mutations in one ICP4 binding site in R3820 had a drastic effect on expression of the chimeric gene. This was not the case for the R8023 virus, probably because the additional U_L49.5 gene sequences compensated for the destroyed binding site.

MATERIALS AND METHODS

Cells and viruses. The parent and mutant viruses were grown, and their titers were determined, in Vero cells. Rabbit skin cells, originally obtained from J. McLaren, were used for cotransfection with viral DNAs. The selection for thymidine kinase (tk^+) recombinants was done in the human 143 thymidine kinase-minus ($143TK^-$) cells overlaid with hypoxanthine-aminopterine-thymidine medium. Nuclear extracts were made from HeLa cells maintained in Dulbecco's modified Eagle's medium in 5% (vol/vol) fetal calf serum. Strain HSV-1(F)Δ305 with a deletion in the *tk* gene was derived as described by Post et al. (56) from HSV-1(F), the prototype HSV-1 wild-type strain used in this laboratory. The construction of recombinant R3820 was described previously (43). Recombinant R3112 contains the U_L49.5 sequence extending from −822 to +104 inserted into the *Bgl*II site of the *tk* gene as previously described (61).

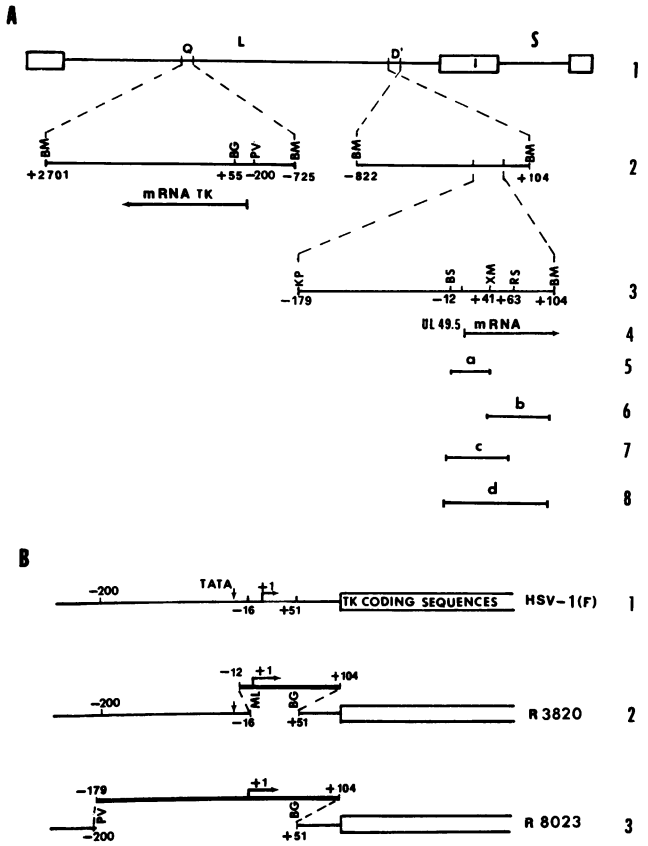

FIG. 1. Sequence arrangements of HSV-1(F) and of recombinant viruses used in these studies. (A) Line 1, sequence arrangement of the HSV-1 genome. The rectangles represent the reiterated sequences flanking the long (L) and short (S) components. Line 2, sequence arrangements of the natural β-*tk* gene and of the U_L49.5 gene. Line 3, expansion of the region showing the U_L49.5 5′ untranscribed and transcribed noncoding domains. Line 4, transcription initiation and direction of transcription of the U_L49.5 open reading frame. Lines 5 through 8, positions of DNA fragments a (*Bss*HII-*Xma*III), b (*Xma*III-*Bam*HI), c (*Bss*HII-*Rsa*I), and d (*Bss*HII-*Bam*HI) used as probes for binding of ICP4. (B) Line 1, sequence arrangement of the *tk* gene in HSV-1(F); lines 2 and 3, structure of the chimeric U_L49.5-*tk* promoter inserted in recombinant viruses R3820 and R8023, respectively. Numbers above the heavy lines refer to nucleotide positions relative to the transcription initiation at +1 of U_L49.5; numbers below the thin lines refer to nucleotide positions of the *tk* gene relative to the transcription initiation site at +1. The dashed lines show the site of insertion of the U_L49.5 sequences in the domain of the *tk* gene. The TATAA box (indicated by letters TATA) is represented by a vertical arrow in the schematic diagram of the *tk* genes of the recombinant viruses. Restriction sites: BM, *Bam*HI; BG, *Bgl*II; PV, *Pvu*II; KP, *Kpn*I; BS, *Bss*HII; XM, *Xma*III; RS, *Rsa*I; ML, *Mlu*I.

Plasmid constructions. The *Eco*RI-*Bam*HI HSV-1(F) DNA fragment from pRB3628 containing the 283-bp (−179 to +104) *Kpn*I-*Bam*HI DNA fragment of the U_L49.5 gene was cloned into M13mp19 to yield pRB3874. pRB3874 was mutagenized as described by Spector et al. (63) with the aid of the Muta-Gene in vitro mutagenesis kit (Bio-Rad, Richmond, Calif.) and 35-mer oligonucleotides synthesized on an Applied Biosystems model 308B DNA synthesizer. All plasmid constructs were verified by sequencing, using Sequenase (United States Biochemical, Cleveland, Ohio).

pRB3823 carries the HSV-1(F) *Bam*HI Q fragment cloned in pUC19 from which the *Pvu*II-*Bgl*II sequence had been replaced with a polylinker. In this plasmid, the *tk* gene sequence extending from nucleotides −200 to +51 relative to the transcription initiation site at nucleotide +1 had been deleted.

The wild-type and mutated *Eco*RI-*Bam*HI sequences from the U_L49.5 gene were cloned into the *Xba*I site of pRB3823 such that the U_L49.5 sequences were inserted in the correct transcriptional orientation relative to the coding sequences of the *tk* gene. The plasmids made with the fragments indicated above were designated pRB8023 (wild-type parent), pRB8018 (mutation A), pRB8019 (mutation B), pRB8020 (mutation C), and pRB8021 (mutation D). Plasmids pRB3820 (wild-type parent [43]), pRB8024 (mutation A), pRB8025 (mutation B), pRB8026 (mutation C), and pRB8027 (mutation D) were made by replacing a portion of the 5′ untranscribed and transcribed noncoding domains of the *tk* gene (*Mlu*I-*Bgl*II fragment of pRB3172) with the parental or mutated *Bss*HII-*Bam*HI (−12 to +104) sequence from the U_L49.5 gene.

Construction of the U_L49.5-*tk* mutant viruses. Plasmids containing the chimeric U_L49.5-*tk* genes were cotransfected with intact HSV-1(F)Δ305 DNA into rabbit skin cells, and TK^+ progeny were selected on 143TK^- cells as described previously (40, 61). The recombinant viruses were plaque purified four times and checked for purity by hybridization of the electrophoretically separated fragments of the viral DNA digested with *Bam*HI or *Eco*RI after Southern transfer. The *Bam*HI Q′ fragment was used as a probe to differentiate the parental HSV-1(F)Δ305 *tk* sequences from the chimeric U_L49.5-*tk* sequences. The junction sequences involved in the recombination, and the mutated sequences introduced in the recombinant viruses, were sequenced with the appropriate primers after amplification by the polymerase chain reaction as described previously (30).

Preparation of nuclear extracts. Confluent monolayers of HeLa cells were grown in 850-cm^2 roller bottles, mock infected or infected with 5 PFU of HSV-1(F) per cell, and harvested 14 h postinfection. Nuclear fractions were prepared as described previously (15). Protein concentrations of the nuclear extracts ranged from 20 to 30 mg/ml, as determined by the Bio-Rad protein assay.

DNA band shift assay. The DNA probes consisted of the following U_L49.5 DNA fragments: *Bss*HII-*Xma*III (−12 to +41), which contains ICP4 binding site 3 with either the wild-type sequence or mutated sequence A or B; the *Xma*III-*Bam*HI fragment (+41 to +104), which contains ICP4 binding site 4 with either the wild-type sequence or mutated sequence C or D; the *Bss*HII-*Rsa*I fragment (−12 to +63), in which *Rsa*I digestion destroyed ICP4 binding site 4, leaving intact binding site 3 for use with mutated sequence C; and the *Bss*HII-*Bam*HI fragment (−12 to +104), which contains ICP4 binding sites 3 and 4 with mutated sequences A, B, and D. These fragments were purified from polyacrylamide gels, dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and 5′ end labeled with [γ-^{32}P]ATP (>7,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and T4 polynucleotide kinase (United States Biochemical) to an average activity of 50,000 cpm/ng of fragment DNA. The standard competitor DNA used in these assays was synthetic poly(dI-dC)-poly(dI-dC) (Pharmacia P-L Biochemicals, Piscataway, N.J.). DNA-protein binding assays and electrophoresis in nondenaturing gels were done as described previously (38). Anti-ICP4 monoclonal antibody H640, a gift from Lenore Pereira, was described elsewhere (1).

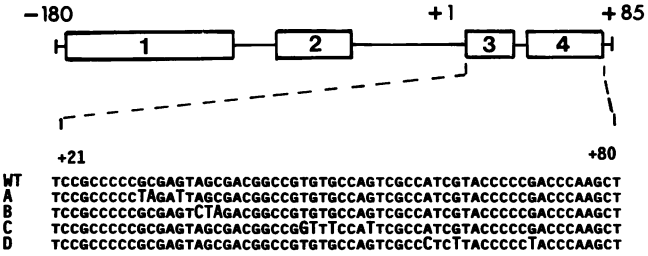

FIG. 2. The 265-bp (−180 to +85) portion of the U_L49.5 untranscribed and transcribed noncoding domains. Rectangles 1 through 4 represent the minimal domains of the ICP4 protein binding. Below are the sequences of the region subjected to mutagenesis. Mutations A and B are located in domain 3, mutation C is located between domains 3 and 4, and mutation D is located in domain 4. The replaced nucleotides are identified by uppercase letters. WT, parental virus defined as wild type in these studies.

Thymidine kinase assay. 143TK^- cells were infected with 5 PFU per cell and incubated in the presence (300 μg/ml) or absence of PAA (a gift from Abbott Laboratories). The cells were harvested at 7, 10, 13, and 16 h postinfection, and the cytoplasmic fractions were assayed for thymidine kinase activity as previously described (39, 56).

RNase protection assay. Plasmid pRB4086 (63) contains the HSV-1(F) *tk* gene sequence *Mlu*I-*Nru*I (+126 to +400) in pGEM-3Z. The 350-bp RNA probe generated for assaying *tk* transcripts was made by cleavage of the vector sequences in pRB4086 with *Eco*RI and transcription with SP6 polymerase (Promega, Madison, Wis.); it protected 274 bp of the *tk* mRNA. The RNA probe for assaying α-*trans* inducing factor (αTIF) transcripts was constructed as follows. Plasmid pRB4392 was made by cloning the 267-bp *Apa*I-*Pvu*I fragment from pRB3623 (44) into the *Sma*I site of pGEM-3Z. Cleavage of pRB4392 with *Eag*I and transcription with T7 polymerase (Promega) generated a 171-bp RNA probe which protected 148 bp of αTIF mRNA. To prepare the test RNAs, replicate 150-cm^2 Vero cell cultures were exposed to 5 PFU of recombinant viruses per cell for 1 h and then overlaid with fresh medium in the presence or absence of PAA. Cells were harvested at 6 and 12 h postinfection, and the cytoplasmic RNA was prepared as previously described (61). RNase protection assays and electrophoretic separation were done as previously described (63).

Radioactivity in protected bands for the αTIF and U_L49.5-*tk* mRNAs from the parental and the recombinant viruses was measured on a model 603 Betagen Betascope blot analyzer as previously described (63).

RESULTS

Generation of mutants and analyses of the mutated fragments for the capacity to form demonstrable complexes with ICP4. A previous report (46) identified at least four ICP4 binding sites detected by gel retardation assays in the promoter-regulatory domain of the U_L49.5 gene (Fig. 1). Of these, two sites were 5′ of the transcription initiation site and two were located in the 5′ transcribed noncoding domain of the gene (46). To determine the role of the binding sites located in the 5′ transcribed noncoding domain of the U_L49.5 gene, four sites of transversion mutations were generated (Fig. 2). The wild-type and mutated fragments were se-

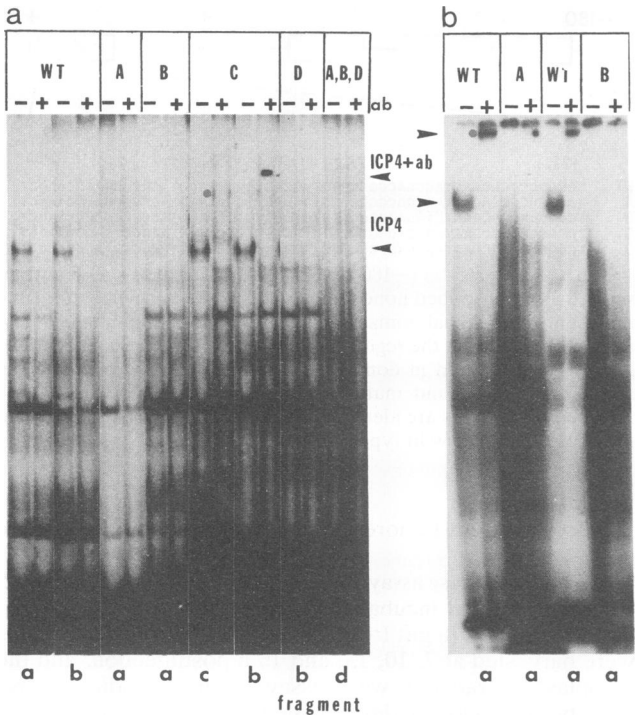

FIG. 3. Autoradiogram of labeled DNA-infected cell protein complexes electrophoretically separated in a nondenaturing gel. DNA fragments a, b, c, and d (Fig. 1A, lanes 5 through 8) derived from wild-type (lanes WT [wild type], A, B, and C) and mutagenized (remaining lanes) fragments and tested for binding are indicated at the bottom. The mutated sites (A through D) for which the DNA fragments were tested are indicated at the top. ab, antibody H640 used to recognize ICP4 in the DNA-protein complexes. Pluses and minuses above the lanes indicate whether antibody to H640 was present or absent during the reaction of the infected cell extract with the DNA fragment. The specificity of the reactivity of this monoclonal antibody with ICP4 was extensively characterized by Kristie and Roizman (38) and by Hubenthal-Voss et al. (28). The position of the ICP4-DNA complex is indicated with an arrowhead; a dot marks the position of the antibody-ICP4-DNA complex.

quenced after mutagenesis in M13 and after cloning prior to recombination into the genomes. In addition, the wild-type and mutated fragments (Fig. 1, lines 5 to 8) were tested for the ability to bind ICP4 as described in Materials and Methods. The sequences selected for mutagenesis were as follows.

(i) Mutation sites A and B (Fig. 2) were selected on the basis of the observation that they spanned the position of an ICP4 binding site (44a). The results of DNA gel shift analyses (Fig. 3) indicate that DNA fragments containing each of the mutagenized sites failed to form demonstrable complexes with ICP4. Mutation sites A and B were of particular importance since they affected the same ICP4 binding site. We should note that verification of the ICP4-DNA complexes is based on the use of a monoclonal antibody which either retards the migration of the complex in the nondenaturing gel or, as occasionally happens, blocks the complex from entering the gel.

(ii) Mutation site C was selected to determine whether mutagenesis of the sequences lying between the known ICP4 binding sites might affect binding of ICP4 or expression of the chimeric gene. Site C is not an ICP4 binding site inasmuch as extensive studies failed to demonstrate binding of ICP4 by DNA fragments containing site C. As expected, mutagenesis of site C did not affect the binding of ICP4 to adjacent sites (Fig. 3a).

(iii) Mutation site D was selected on the basis of the observation that it was a site of binding of ICP4 and because it was the only site homologous to the ICP4 DNA binding consensus sequence derived by Faber and Wilcox (18). As shown in Fig. 3a, the mutagenized fragment failed to form demonstrable complexes with ICP4.

(iv) The three mutation sites A, B, and D account for all of the binding sites within the U_L49.5 fragment replacing the authentic β-*tk* sequences in R3820 and for all of the binding sites in the 5′ transcribed noncoding domain of the U_L49.5 gene inserted into the R8023 recombinant virus.

Construction of recombinant viruses. In our earlier study (43), it was shown that the minimal U_L49.5 sequence tested which imparted upon the chimeric *tk* gene the attributes of a γ_2 gene consisted of the sequence −12 to +104 (recombinant R3820). However, because the ICP4 binding sites of U_L49.5 extended from −179 to +85, it seemed desirable to test the role of the binding sites within the leader sequence also in the context of a chimeric gene in which all four identified ICP4 binding sites were present. Consequently, two series of viruses were constructed. In the first series, the wild-type virus was recombinant R3820, in which the β-*tk* sequence −16 to +51 was replaced with the U_L49.5 sequence −12 to +104. Virus mutants R3820-A through R3820-D were constructed by recombination as previously reported for the R3820 recombinant (43), but using the mutated rather than the wild-type fragments. In the second series, the wild-type virus was recombinant R8023, in which the β-*tk* sequence −200 to +51 was replaced with the U_L49.5 sequence −179 to +104. The parental and mutant viruses, R8023 and R8023-A through R8023-D, respectively, were derived by recombination between HSV-1(F)Δ305 and the fragment containing the chimeric genes as described in Materials and Methods. After purification of the viral progeny carrying the mutations, both the sites of insertion of the U_L49.5 sequence and the mutagenized sites were sequenced to verify that the sequences contained in the viral genome correspond to those shown diagrammatically in Fig. 1 and 2.

Figure 4 shows the accumulation of thymidine kinase activity in 143TK$^-$ cells infected with HSV-1(F), carrying the natural β-*tk*, R3112, described previously (61) as the first U_L49.5-*tk* chimeric gene expressing the attributes of a γ_2 gene, R3820 and R8023. *tk* gene expression in both R3820 and R8023 is grossly inhibited by concentrations of PAA capable of inhibiting viral DNA synthesis.

Accumulation of *tk* RNA in Vero cells infected with recombinant viruses carrying wild-type and mutagenized chimeric *tk* genes. Replicate cultures of Vero cells were infected with 5 PFU of recombinant virus per cell in the presence or absence of PAA. The infected cells were harvested 6 and 12 h postinfection, and *tk* mRNA accumulation was determined by RNase protection assays. To control for variability in extraction and purification of RNA, we simultaneously measured the amount of mRNA of the αTIF gene (5, 8, 22, 54) in each RNase protection assay. For these studies, the virus stocks used to infect cells were carefully controlled to ensure that they were prepared in a uniform fashion and were of identical or similar titers. At least three sets of RNAs were prepared independently for each set of recombinant viruses. The RNase protection assays were done as described in Materials and Methods. Gels containing the electrophoretically separated, protected RNAs were visualized by autora-

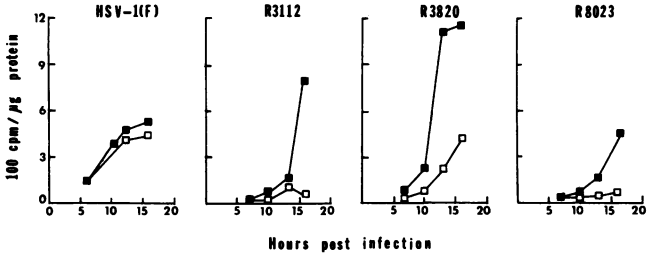

FIG. 4. Specific activities of the thymidine kinase made in cells infected with parental and recombinant viruses in the presence or absence of PAA. Replicate cultures of 143TK⁻ cells were infected with 5 PFU of the indicated wild-type or recombinant virus per cell and incubated in the presence (□) or absence (■) of PAA. The cells were harvested at 7, 10, 13, and 16 h postinfection. The specific activity of thymidine kinase is expressed as counts per minute per microgram of protein. Structures of the chimeric *tk* gene in recombinants R3820 and R8023 are shown in Fig. 1. As previously reported, the chimeric promoter fused to the *tk* gene in R3820 contains elements of both β and γ promoters and is expressed both early and late in infection (43).

diography and analyzed in a model 603 Betagen Betascope blot analyzer. The radioactivity was measured in each protected band and also in the area above and below each band (background) a total of three times. After each measurement, the grids were reset to minimize errors resulting from the placement of coordinates on the actual bands and to obtain background counts. The average counts for each *tk* RNA band were normalized with respect to the value for the corresponding αTIF RNA. Figures 5 and 6 show the autoradiographic images of electrophoretically separated protected RNAs from one of the experiments. The average values obtained for each of the independently derived RNAs for each virus series are shown in Tables 1 and 2.

As would be expected for a γ gene, αTIF RNA accumulated at both 6 and 12 h postinfection in much smaller amounts in cells treated with PAA than in untreated cells. Both chimeric *tk* genes were sensitive to PAA and accumulated in smaller amounts late in infection. At 6 h postinfection, the chimeric *tk* gene in R8023 appeared to be more sensitive to PAA than was the chimeric *tk* gene contained in R3820. This result is not entirely unexpected. As noted previously, the R3820 chimeric gene shares properties of both β and γ genes in that it is expressed both early and late in infection, whereas the R8023 chimeric gene, like that present in R3112 reported earlier (61), resembles a γ gene in its temporal pattern of expression.

Comparison of the expression of the chimeric *tk* gene in R3820 with that of the natural β-*tk*, reported earlier, showed that the chimeric gene was expressed late in infection to a higher level and was more sensitive to PAA than was the native *tk* gene (43). A key question is whether the mutations introduced into the U_L49.5 sequences of the chimeric gene alter its expression relative to that of the nonmutagenized, wild-type parent. The results, summarized in Tables 1 and 2, were as follows.

(i) In cells infected with the R3820 recombinant virus series, at 6 h postinfection and in the absence of PAA, the B mutation reduced the concentration of *tk* RNA by a factor of 6. The reduction in the *tk* RNA observed in cells infected with the B mutant was consistent and of similar magnitude in all experiments. Although the *tk* RNA levels observed in cells infected with the D mutants were the next lowest, the decrease in *tk* RNA levels noted in cells infected with all

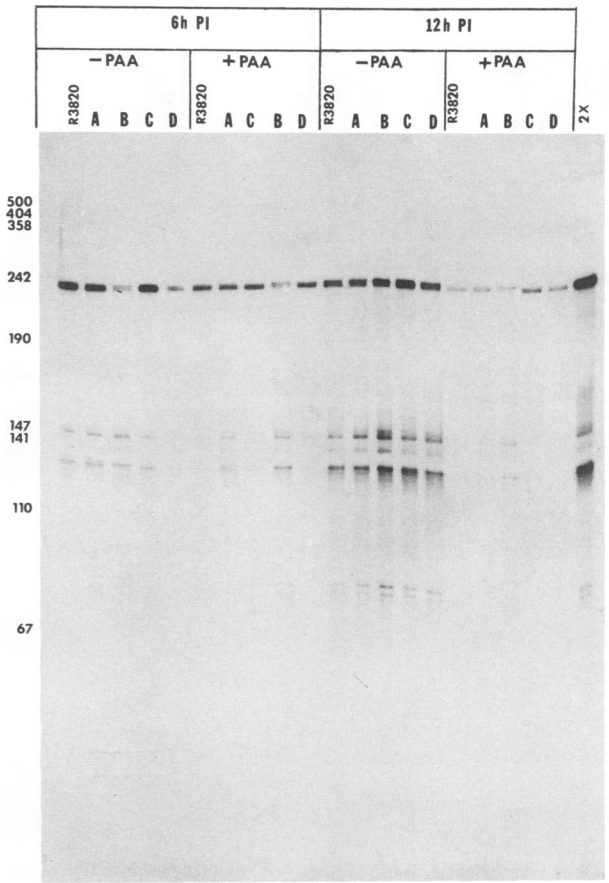

FIG. 5. Autoradiographic image of electrophoretically separated, in vitro-synthesized, labeled probe RNA protected from RNase degradation by U_L49.5-*tk* and αTIF mRNAs. The test RNAs were extracted from Vero cells infected with the R3820 series of recombinant viruses at 6 and 12 h postinfection (PI). The virus used to infect cells is indicated above each lane. R3820 refers to the nonmutagenized parent virus. A, B, C, and D refer to the mutations at sites shown in Fig. 2 and recombined into the viral genome as described in the text. The *tk* probe measured transcription of the chimeric *tk* reporter gene, whereas the αTIF probe measured the accumulation of αTIF mRNA transcripts for normalization of the results. Fragment lengths in nucleotides of *Msp*I-digested, end-labeled plasmid pGEM-3Z are shown at the right as standards. Lane 2× contains twice the amount of RNA added to the other lanes to verify labeled RNA probe excess under the assay conditions.

mutants other than B was much less drastic, more variable from experiment to experiment, and potentially less significant. The effect of the B mutation largely disappeared or was much reduced in cells harvested at 12 h postinfection in the absence of PAA. In this series of experiments, the *tk* RNA levels observed in cells infected with the C mutant were closest to those obtained with the wild-type parent R3820 virus.

(ii) In the presence of PAA, cells infected with the B mutant of the R3820 series again accumulated significantly less *tk* RNA than did cells infected with the wild-type parent virus or other mutant viruses. In this instance, the decrease was nearly identical at both 6 and 12 h postinfection and the reduction in accumulation of *tk* RNA was consistent and reproducible in all experiments. All other mutations resulted

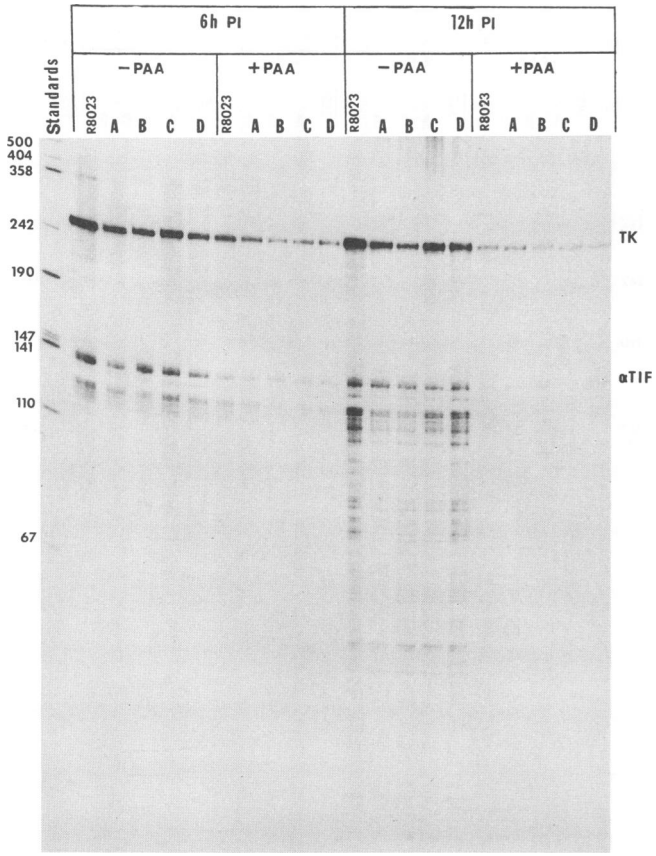

FIG. 6. Autoradiographic image of electrophoretically separated, in vitro-synthesized, labeled probe RNA protected from RNase degradation by U_L49.5-*tk* and αTIF mRNAs. The test RNAs were extracted from Vero cells infected with the R8023 series of recombinant viruses at 6 and 12 h postinfection (PI). The virus used to infect cells is indicated above each lane. R8023 refers to the nonmutagenized parent virus. A, B, C, and D refer to the mutations at sites shown in Fig. 2 and recombined into the viral genome as described in the text. For other details, see the legend to Fig. 5.

in minimal reduction (mutation A) or no significant reduction (mutations C and D) of RNA expression.

(iii) The accumulation of *tk* RNA in cells infected with the R8023 series of viruses and maintained in the absence or presence of PAA was less affected by the mutations than was that of the R3820 series of viruses. While the cells infected with the B mutants accumulated less *tk* RNA than did the cells infected with the parent virus, the difference in this instance was smaller than that seen in the case of the R3820 series of recombinant viruses.

DISCUSSION

For the past several years, a number of laboratories have reported that the *cis*-acting determinants of HSV-1 γ gene expression map at or downstream of the TATAA box (7, 17, 19, 24, 25, 32, 33, 36, 69). The studies described in this report center on the observation that the chimeric *tk* gene encoded by the R3820 recombinant virus was expressed both early and late in infection and accumulated levels of thymidine kinase activity higher than those expressed by the natural β-*tk* gene. Furthermore, the accumulation was more sensitive to PAA than was that observed in cells infected with the wild-type parent.

The objectives of the studies described in this report centered on the role of the ICP4 DNA binding site and were twofold. The first objective was to determine whether the U_L49.5 sequences contained in the chimeric *tk* gene and which bound ICP4 played a role in rendering expression of the chimeric gene sensitive to PAA. The rationale for examining this issue rests on the possibility that the ICP4 binding sites in 5′ transcribed noncoding domains could have both positive and negative functions. Thus, sensitivity to PAA implies that viral DNA synthesis is required for optimal expression of the gene. Previous studies have indicated that viral DNA synthesis exerts its effect in *cis* rather than in *trans* (42). One model described in earlier reports is that a viral protein binding to 5′ transcribed noncoding domains of γ genes blocks transcription until the onset of DNA synthesis dislodges the bound protein (2, 42). ICP4 may have both functions; it may, for example, act as the block in transcrip-

TABLE 1. RNase protection studies on RNA extracted from cells infected with the R3820 series of HSV-1 mutants

Treatment	Mutant	Result in expt no.[a]				
		1	2	3	4	Mean
6 h, no PAA	Wild type	100 ± 4	100 ± 1	100 ± 1	100 ± 1	100
	A	97 ± 8	73 ± 3	45 ± 1	55 ± 2	68 ± 23
	B	27 ± 1	17 ± 1	11 ± 1	13 ± 1	17 ± 7
	C	107 ± 6	57 ± 3	84 ± 1	91 ± 2	85 ± 21
	D		80 ± 4	33 ± 1	54 ± 2	56 ± 19
12 h, no PAA	Wild type	100 ± 8	100 ± 7	100 ± 3	100 ± 3	100
	A	84 ± 7	95 ± 5	27 ± 1	57 ± 2	66 ± 30
	B	62 ± 6	73 ± 5	41 ± 1	48 ± 1	56 ± 14
	C		88 ± 6	72 ± 5	126 ± 5	95 ± 28
	D	80 ± 19	71 ± 6	67 ± 6	68 ± 3	72 ± 6
6 h, plus PAA	Wild type	100 ± 2	100 ± 3	100 ± 2	100 ± 5	100
	A	76 ± 4	71 ± 6	67 ± 3	43 ± 4	64 ± 15
	B	20 ± 2	16 ± 1	15 ± 1	14 ± 1	16 ± 3
	C	72 ± 3	33 ± 4	40 ± 2	80 ± 5	56 ± 23
	D	63 ± 2	82 ± 3	44 ± 1	59 ± 5	62 ± 16
12 h, plus PAA	Wild type	100 ± 6	100 ± 3	100 ± 1	100 ± 1	100
	A	73 ± 1	76 ± 14	60 ± 2	65 ± 4	69 ± 8
	B		18 ± 3	11 ± 1	19 ± 1	16 ± 4
	C	63 ± 1	95 ± 14	84 ± 3	164 ± 11	102 ± 44
	D	52 ± 8	87 ± 3	153 ± 6	97 ± 51	102 ± 15

[a] Values were calculated as follows: (mRNA initiated from mutant promoter/mRNA initiated from wild-type promoter) × 100.

TABLE 2. RNase protection studies on RNA extracted from cells infected with the R8023 series of HSV-1 mutants

Treatment	Mutant	Result in expt no.[a]			
		1	2	3	Mean
6 h, no PAA	Wild type	100 ± 2	100 ± 2	100 ± 4	100
	A	96 ± 8	103 ± 4	153 ± 7	117 ± 31
	B	57 ± 8	45 ± 4	51 ± 1	51 ± 6
	C	91 ± 11	83 ± 7	55 ± 2	76 ± 19
	D	67 ± 2	58 ± 2	63 ± 4	71 ± 11
12 h, no PAA	Wild type	100 ± 1	100 ± 3	100 ± 3	100
	A	55 ± 2	59 ± 3	90 ± 3	68 ± 19
	B	40 ± 1	55 ± 2	45 ± 1	47 ± 8
	C	104 ± 3	96 ± 8	88 ± 4	96 ± 7
	D	52 ± 3	62 ± 2	69 ± 3	61 ± 8
6 h, plus PAA	Wild type	100 ± 1	100 ± 5	100 ± 7	100
	A	103 ± 4	121 ± 9	154 ± 5	126 ± 26
	B	55 ± 1	55 ± 2	51 ± 2	54 ± 2
	C	83 ± 2	90 ± 1	102 ± 4	92 ± 10
	D	82 ± 2	79 ± 2	95 ± 1	85 ± 9
12 h, plus PAA	Wild type	100 ± 1	100 ± 1	100 ± 3	100
	A	123 ± 9	117 ± 16	106 ± 5	115 ± 8
	B	48 ± 3	46 ± 7	52 ± 4	49 ± 3
	C	110 ± 4	89 ± 12	109 ± 6	103 ± 12
	D	51 ± 3	68 ± 9	77 ± 3	65 ± 13

[a] Values were calculated as follows: (mRNA initiated from mutant promoter/mRNA initiated from wild-type promoter) × 100.

tion, and it may transactivate expression of the gene. In this instance, we could expect that mutagenesis of the sequence which renders the gene sensitive to PAA would result in increased accumulation of *tk* RNA in infected cells treated with PAA.

With respect to this objective, none of the mutations introduced into the U_L49.5 sequence contained in either of the chimeric *tk* genes reduced the effect of PAA on expression of the chimeric genes. These data argue that the two ICP4 binding sites contained within the U_L49.5 sequence contained in the chimeric *tk* gene are not each individually sites of putative repression of transcription that is alleviated by the onset of viral DNA synthesis. Furthermore, the results obtained in this study neither support nor refute the model described above.

The second objective was to determine whether the ICP4 binding sites played a role in expression of the chimeric-*tk* gene and specifically whether the ICP4 binding site was the *cis*-acting site responsible for the increase in the expression of the chimeric *tk* gene contained in the recombinant R3820. If this hypothesis were tenable, mutagenesis of one or more ICP4 binding sites within the domain of the U_L49.5 gene contained in the chimeric gene would be expected to reduce the expression of the chimeric gene.

The experiments described in this report indicate that in the context of the R3820 recombinant virus, at least one of the two ICP4 binding sites is involved in the accumulation of the *tk* RNA in a fashion similar to those of γ genes. Specifically, mutagenization of the B site in the U_L49.5 sequence inserted into the chimeric *tk* gene (Fig. 2) significantly reduced the accumulation of the *tk* RNA in cells infected with the R3820 virus. The findings are particularly significant since the mutation abolished only one of the two known ICP4 binding sites. The effect of mutagenesis of either site A or site D was less pronounced. The significance of the results of mutagenesis of sites A and D is uncertain. The results obtained with the mutated site A are particularly puzzling inasmuch as both sites A and B appear to destroy the ICP4 binding site which these sequences flank. It is conceivable that site A is not completely destroyed or that this site has other functions inapparent in our assays.

The effect of the site B mutation in the context of the R8023 virus was less drastic. One explanation for the observed effect of this mutation in R8023 rests on the observation that the *cis*-acting sites within regulatory domains of HSV genes are highly redundant. This redundancy is particularly noteworthy for the αTIF response elements located upstream of α genes (41) and for the ICP4 binding sites found in several genes. In the case of the αTIF, recent studies have shown that the response elements are additive and complementary (63, 64). Inasmuch as the U_L49.5 sequence contained within the chimeric gene encoded in R8023 contains two additional ICP4 binding sites upstream from the transcription initiation sites, it is conceivable that one or both of those sites act to replace the site destroyed by mutagenization of site B. Parenthetically, the evidence linking ICP4 DNA binding sites with transcriptional activation in the context of genes contained within viral genomes introduced into cells by infection is paradoxically weak. Although the enthusiasm for linking binding sites with transactivation remains unabated, a recent paper reported that the destruction of all apparent binding sites in the promoter-regulatory domains of the glycoprotein D gene failed to affect significantly its expression in productive infection (62).

This report is in effect the first evidence linking the destruction of an ICP4 binding site with down regulation of the accumulation of a transcript. A key issue, which remains unresolved, is the relationship between the destruction of the ICP4 binding site and the decreased accumulation of *tk* RNA. Because the binding site is within the 5′ transcribed noncoding domain, mutagenesis of the ICP4 binding site could affect either transcription or the stability of the mRNA. Technically, the resolution of the two alternatives is particularly vexing since nuclear RNA runoff, which could measure transcription rates, is notoriously unreliable in the case of HSV γ genes (2, 61). Further studies on other γ genes may resolve this problem.

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REFERENCES

1. **Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman.** 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. J. Virol. **53**:108–118.
2. **Arsenakis, M., G. Campadelli-Fiume, and B. Roizman.** 1988. Regulation of glycoprotein D synthesis: does α4, the major regulatory protein of herpes simplex virus 1, regulate late genes both positively and negatively? J. Virol. **62**:148–158.
3. **Arsenakis, M., J. Hubenthal-Voss, G. Campadelli-Fiume, L. Pereira, and B. Roizman.** 1986. Construction and properties of a cell line constitutively expressing the herpes simplex virus glycoprotein B dependent on functional α4 protein synthesis. J. Virol. **60**:674–682.
4. **Barker, D. E., and B. Roizman.** 1992. The unique sequence of the herpes simplex virus 1 L component contains an additional translated open reading frame designated UL49.5. J. Virol. **66**:562–566.
5. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
6. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected-cell polypeptide α4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83**:4016–4020.
7. **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.
8. **Campbell, M. E., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus sequences which encode a transacting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–15.
9. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. **37**:191–206.
10. **DeLuca, N. A., A. M. McCarthy, and P. A. Shaffer.** 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.
11. **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**:1997–2008.
12. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense polypeptides. Nucleic Acids Res. **15**:4491–4511.
13. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62**:732–743.
14. **DiDonato, J. A., J. R. Spitzner, and M. T. Muller.** 1991. A predictive model for DNA recognition by the herpes simplex virus protein ICP4. J. Mol. Biol. **219**:451–470.
15. **Dignam, J. D., R. M. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
16. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36**:189–203.
17. **Everett, R. D.** 1983. DNA sequences required for regulated expression of the HSV-1 glycoprotein D gene lie within 83bp. of the RNA cap sites. Nucleic Acids Res. **11**:6647–6666.
18. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **14**:6067–6083.
19. **Flanafan, W. M., A. G. Papavassiliou, M. Rice, L. B. Hecht, S. Silverstein, and E. K. Wagner.** 1991. Analysis of the herpes simplex virus type 1 promoter controlling the expression of UL38, a true late gene involved in capsid assembly. J. Virol. **65**:769–786.
20. **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.
21. **Hall, L. M., K. G. Draper, R. J. Frink, R. H. Costa, and E. K. Wagner.** 1982. Herpes simplex virus mRNA species mapping in *Eco*RI fragment I. J. Virol. **43**:594–607.
22. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Protein specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
23. **Holland, L. E., K. P. Anderson, C. Shipman, Jr., and E. K. Wagner.** 1980. Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology **101**:10–24.
24. **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.
25. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ2) gene lie within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.
26. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of the three groups of viral proteins. J. Virol. **14**:8–19.
27. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72**:1276–1280.
28. **Hubenthal-Voss, J., R. A. Houghten, L. Pereira, and B. Roizman.** 1988. Mapping of functional and antigenic domains of the α4 protein of herpes simplex virus 1. J. Virol. **62**:454–462.
29. **Imbalzano, A. N., A. A. Sheperd, and N. A. DeLuca.** 1990. Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. J. Virol. **64**:1–14.
30. **Innis, M. A., B. K. Myambo, D. H. Gelfand, and M. A. Brown.** 1988. DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction amplified DNA. Proc. Natl. Acad. Sci. USA **85**:9436–9440.
31. **Johnson, P. A., and R. D. Everett.** 1986. DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucleic Acids Res. **14**:3609–3625.
32. **Johnson, P. A., and R. D. Everett.** 1986. The control of herpes simplex virus type late gene expression: a TATA box cap-site region is sufficient for fully efficient regulated activity. Nucleic Acids Res. **14**:8247–8264.
33. **Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Dalziel, and R. D. Everett.** 1986. The product of gene US11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67**:871–883.
34. **Jones, P. C., and B. Roizman.** 1979. Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. **31**:299–314.
35. **Kattar-Cooley, P., and K. W. Wilcox.** 1989. Characterization of the DNA-binding properties of the herpes simplex virus regulatory protein ICP4. J. Virol. **63**:696–704.
36. **Kibler, P. K., J. Duncan, B. D. Kleith, T. Hupel, and J. R. Smiley.** 1991. Regulation of herpes simplex virus true late gene expression: sequences downstream from the US11 TATA box inhibit expression from an unreplicated template. J. Virol. **65**:6749–6760.

37. **Knipe, D. M., W. T. Ruyechan, B. Roizman, and I. W. Halliburton.** 1978. Molecular genetics of herpes simplex virus: demonstration of regions of obligatory and nonobligatory identity within diploid regions of the genome by sequence replacement and insertion. Proc. Natl. Acad. Sci. USA **75**:3896–3900.
38. **Kristie, T. M., and B. Roizman.** 1986. α4, the major regulatory protein of herpes simplex 1 is stably and specifically associated with the promoter-regulatory domains of α genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83**:4700–4704.
39. **Leiden, J. M., R. Buttyan, and P. G. Spear.** 1976. Herpes simplex virus gene expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. **20**:413–424.
40. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. **58**:583–591.
41. **Mackem, S., and B. Roizman.** 1982. Differentiation between α promoter and regulator region of herpes simplex virus 1: the functional domains and sequence of a movable α regulator. Proc. Natl. Acad. Sci. USA **79**:4917–4921.
42. **Mavromara-Nazos, P., and B. Roizman.** 1987. Activation of herpes simplex virus 1 γ_2 genes by viral DNA replication. Virology **161**:593–598.
43. **Mavromara-Nazos, P., and B. Roizman.** 1989. Delineation of regulatory domains of early (β) and late (γ_2) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes. Proc. Natl. Acad. Sci. USA **86**:4071–4075.
44. **McKnight, J. L. C., P. E. Pellett, F. J. Jenkins, and B. Roizman.** 1987. Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate α-*trans*-inducing factor-dependent activation of α genes. J. Virol. **61**:992–1001.
44a. **Michael, N.** Unpublished data.
45. **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.
46. **Michael, N., and B. Roizman.** 1990. Determination of the number of protein monomers binding to DNA with Fab fragments of monoclonal antibodies to the protein. Methods Mol. Cell. Biol. **1**:203–211.
46a. **Michael, N., and B. Roizman.** Unpublished data.
47. **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.
48. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
49. **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.
50. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
51. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
52. **O'Hare, P., and G. S. Hayward.** 1987. Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded *trans*-acting factors. J. Virol. **61**:190–199.
53. **Paterson, T., and R. D. Everett.** 1988. The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Res. **16**:11005–11025.
54. **Pellet, P. E., J. L. McKnight, F. J. Jenkins, and B. Roizman.** 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing α genes. Proc. Natl. Acad. Sci. USA **82**:5870–5874.
55. **Pizer, L. I., R. D. Everett, D. G. Tedder, M. Elliot, and B. Litman.** 1991. Nucleotides within both proximal and distal parts of the consensus sequence are important for specific DNA recognition by the herpes simplex regulatory protein ICP4. Nucleic Acids Res. **19**:477–483.
56. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24**:555–565.
57. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62**:4307–4320.
58. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex type 1 late (γ_2) glycoprotein C gene: sequences between base pairs −34 to +29 control transient expression and responsiveness to transactivation by the products of immediate early α4 and α0 genes. Nucleic Acids Res. **15**:3097–3111.
59. **Shepard, A. A., and N. A. DeLuca.** 1991. A second-site revertant of a defective herpes simplex virus ICP4 protein with restored regulatory activities and impaired DNA-binding properties. J. Virol. **65**:787–795.
60. **Shepard, A. A., A. N. Imbalzano, and N. A. DeLuca.** 1989. Separation of primary structural components conferring autoregulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **63**:3714–3728.
61. **Silver, S., and B. Roizman.** 1985. γ_2-thymidine kinase chimeras are identically transcribed but regulated as γ_2 genes in herpes simplex virus genomes and as β genes in cell genomes. Mol. Cell. Biol. **5**:518–528.
62. **Smiley, J. R., D. C. Johnson, L. I. Pizer, and R. D. Everett.** 1992. The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein D (gD) promoter are not essential for efficient gD transcription during virus infection. J. Virol. **66**:623–631.
63. **Spector, D., F. Purves, and B. Roizman.** 1990. Mutational analysis of the promoter region of the α27 gene of herpes simplex virus 1 within the context of the viral genome. Proc. Natl. Acad. Sci. USA **87**:5268–5272.
64. **Spector, D., F. Purves, and B. Roizman.** 1992. Role of α-*trans* inducing factor (VP16) in the induction of α genes within the context of viral genomes. J. Virol. **65**:3504–3513.
65. **Tedder, D. G., R. D. Everett, K. W. Wilcox, P. Beard, and L. I. Pizer.** 1989. ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. J. Virol. **63**:2510–2520.
66. **Tedder, D. G., and L. I. Pizer.** 1988. Role for DNA-protein interaction in activation of the herpes simplex virus glycoprotein D gene. J. Virol. **62**:4661–4672.
67. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature (London) **285**:329–330.
68. **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.
69. **Weir, J. P., and P. R. Narayanan.** 1990. Expression of the herpes simplex virus type 1 glycoprotein C gene requires sequences in the 5′ noncoding region of the gene. J. Virol. **64**:445–449.