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Repression of the Herpes Simplex Virus 1 α4 Gene by Its Gene Product (ICP4) within the Context of the Viral Genome Is Conditioned by the Distance and Stereoaxial Alignment of the ICP4 DNA Binding Site Relative to the TATA Box

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Infected cell protein no. 4 (ICP4), the major regulatory protein encoded by the α4 gene of herpes simplex virus 1, binds to a site (α4-2) at the transcription initiation site of the α4 gene. An earlier report described the construction of recombinant viruses that contained chimeric genes (α4-*tk*) that consisted of the 5′ untranscribed and transcribed noncoding domains of the α4 gene fused to the coding sequences of the thymidine kinase gene and showed that disruption of the α4-2 binding site by mutagenesis derepressed transcription of this gene (N. Michael and B. Roizman, Proc. Natl. Acad. Sci. USA 90:2286–2290, 1993). This experimental design was used to determine the effect of displacement of the α4-2 binding site on the repression of α4 gene transcription by ICP4. We report the following findings. (i) In the absence of the α4-2 binding site, at 4 h after infection, α4-*tk* RNA levels increased 10-fold relative to the corresponding RNA levels of a gene that contained the α4-2 site at its natural location. Displacement of the α4-2 binding site by approximately one, two, and three turns of the DNA helix, i.e., by 10, 21, and 30 nucleotides downstream of the original site, increased the concentration of α4-*tk* RNA 2.4-, 3.5-, and 5.8-fold, respectively. (ii) Displacement of 16 nucleotides, i.e., approximately 1.5 helical turns, increased the accumulation of α4-*tk* by 5.3-fold, i.e., more than predicted by displacement alone. (iii) At 8 h after infection in the absence of the binding site, the accumulation of α4-*tk* RNA increased 13.6-fold. However, in cells infected with recombinants that carried displaced α4-2 binding sites, RNA accumulation decreased relative to the levels seen at 4 h after infection. The insertion of DNA sequences in order to displace the α4-2 binding site had no effect on accumulation of RNA in the presence of cycloheximide, i.e., in the absence of ICP4, or on maximum accumulation of α4-*tk* RNA in the absence of the α4-2 binding site. We conclude that (i) the inhibition of transcription by ICP4 is affected by both orientation and distance of the binding site from the TATA box, (ii) the model that best fits the available data is that ICP4 bound to DNA at an appropriate distance and orientation interacts with and precludes the transcription complex from transcribing the gene, and (iii) at 8 h after infection, additional factors such as protein binding upstream of the TATA box and/or posttranslational modification of ICP4 strengthen the repressive effect of ICP4 at the α4-2 binding site.

Infected cell protein no. 4 (ICP4) (18; reviewed in reference 39), the product of the α4 gene, is the major regulatory protein encoded by herpes simplex virus 1 (HSV-1). ICP4 affects the expression of virus genes both positively and negatively (35, 39). The mechanism by which ICP4 transactivates the expression of virus genes has been studied in some detail, and the available data suggest that this function of the protein involves interactions with cellular transcriptional factors and possibly alteration in the physical structure of DNA (2, 9, 13, 29, 45). Nothing is known of the mechanism by which the same protein acts as a repressor. This paper is a continuation of studies that have demonstrated that repression of gene expression by ICP4 involves physical interaction between the protein and DNA (26). Relevant to this report are the following points.

(i) The expression of HSV-1 genes is tightly regulated. HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (18, 19). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by a structural protein of the virus designated virion protein no. 16 (1, 5, 6, 31, 33, 44) or α-transinducing factor. The expression of β genes requires functional α gene products, most notably ICP4 (7). γ genes, a heterogeneous group of genes that encode largely structural proteins of the virion, require the onset of virus DNA synthesis for optimal expression (17). ICP4 plays a key role in this process: cells that are infected with viruses that carry temperature-sensitive mutations in the α4 gene and maintained at nonpermissive temperatures express largely α proteins (11, 35). Furthermore, experiments that involved a shift of infected cells from permissive to nonpermissive temperatures confirmed a key role for ICP4 throughout the virus reproductive cycle.

(ii) ICP4 binds as a dimer (24, 25) with high affinity to the consensus DNA sequence ATCGTCNNNNYCGRC and with lower affinities to a number of other sequences that are not represented by a unique consensus (9, 10, 15, 22, 23, 25, 27). DNA binding sites have been thoroughly mapped in several genes. In α0, a single site that conforms to the consensus is upstream of the cap site (23). In α4, three sites have been mapped. One site (α4-2) whose sequence conforms to the consensus spans the transcription initiation site (15, 21, 22, 25,

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TABLE 1. Description of mutations in the recombinant viruses used in these studies

Virus	ICP4 binding site	Sequence[a]
R4018	α4-2/wt	ctatatgagc ccgaggacgccccgatcgtccacacggag
R4579	α4-2/mut	ctatatgagc ccgaggacgccccgaATTCAcacacggag
R4711	α4-2/+10	ctatatgagc ccgagatctc ccgaggacgccccgatcgtccacacggag
R4712	α4-2/+16	ctatatgagc ccgagatctccgacag ccgaggacgccccgatcgtccacacggag
R4713	α4-2/+21	ctatatgagc ccgagatctccgacagtctgg ccgaggacgccccgatcgtccacacggag
R4833	α4-2/+30	ctatatgagc ccgagatctccgacagtctggtctccgacag ccgaggacgccccgatcgtccacacggag
R4834	α4-2/+10/mut	ctatatgagc ccgagatctc ccgaggacgccccgaATTCAcacacggag

[a] The nucleotide sequence of R4018 is that of the α4 gene, starting with the nucleotide that precedes the TATA box. Capital letters identify nucleotide replacements to destroy the binding of ICP4 to its cognate site. Underlined sequences (beginning at −18) are inserts between the TATA box and the original cap site to displace the ICP4 binding site downsteam from the cap site.

28), whereas two nonconsensus sites, α4-1P (proximal) and α4-1D (distal), are located upstream (approximately −150 and −190) of the cap site (21, 22, 25). In late (γ) genes, binding sites have been found in both 5′ untranscribed and 5′ transcribed noncoding domains (16, 27, 40).

(iii) The rate of synthesis of ICP4 peaks at 2 to 4 h postinfection although the protein accumulates for many hours (18). In cells infected with viruses that carry temperature-sensitive mutations in the α4 gene and maintained at nonpermissive temperatures, ICP4 accumulated at higher levels (11, 35), indicating that ICP4 or some factor that requires the expression of functional ICP4 repressed expression of the α4 gene. Evidence to support the transrepression model whereby ICP4 represses its own transcription and that of several other genes is based on several observations, most notably, the observations that ICP4 binds to the α4-2 site at the transcription initiation site of the α4 gene (25, 28) and that mutations at this site that abolish the binding of ICP4 result in significant increases in the amount of RNA that accumulates during the course of productive infection (26, 38). It has also been reported that ICP4 contains a domain which plays a role in repression of its own synthesis (8, 30, 42, 43).

(iv) Studies of the role of the α4-2 binding site in α4 gene transcription were done by using a chimeric gene that consisted of the short promoter and transcribed noncoding domain of the α4 gene fused to the coding domain of the HSV-1 thymidine kinase gene (*tk* gene) (26; Fig. 1). The *tk* gene served as a reporter gene whose mRNA was quantified in an RNase protection assay. In these experiments, destruction of the α4-2 binding site by nucleotide substitution resulted in a 6-fold increase in the accumulation of chimeric gene mRNA at 4 h postinfection and a 12-fold increase at 8 h postinfection. While the results of these experiments demonstrate a causal role for α4 protein binding to the α4-2 site in autorepression, the mechanisms of this effect remain to be understood. Mutagenesis of the α4-1 sites increased approximately twofold the amounts of mRNA detected at 8 h postinfection but had no appreciable effect at 4 h postinfection.

In this paper, we report on a series of experiments that were designed to test the relative importance of the ICP4 binding site position with respect to the transcription initiation site. These experiments indicate that both absolute distance and relative stereoaxial alignment of the binding site with respect to the TATA box determine the extent of repression. From these studies, we conclude that the model which best fits our data is one whereby repression involves spatially defined interactions between ICP4 and proteins bound at another site.

MATERIALS AND METHODS

Viruses. HSV-1 strain F [HSV-1(F)] is the prototype strain used in this laboratory (12). HSV(F)Δ305 carries a 501-bp deletion in the 5′ transcribed noncoding and coding domains of the *tk* gene and served as the parent of all the other recombinants generated in this study (33). All of the recombinant viruses used in this study (Table 1) were made by cotransfecting plasmid DNAs that carried the appropriate mutations with intact HSV(F)Δ305 DNA into rabbit skin cells (33, 34). tk^+ progeny viruses were selected on 143tk$^-$ cells. The recombinants were plaque purified, and electrophoretically separated restriction enzyme digests of DNAs were analyzed by hybridization with appropriate probes for the correct insertion of mutated sequences and for the absence of parental virus contamination. The sequence arrangement of the HSV genome and the locations of the virus genes relevant to these studies are depicted in Fig. 1A.

Plasmids. Plasmids pRB4018 and pRB4579 have been previously described (26). R4018 is the parental α4-*tk* construct upon which all mutations were made to generate the new constructs used in this study. Briefly, R4018 (α4-2/wt) contains the sequence −332 to +311 of the α4 gene, which encompasses a functional minipromoter of the gene that contains α-transinducing factor binding sites, the transcribed noncoding sequence, and the first 9 nucleotides (nt) of the coding sequence. This sequence was substituted for nucleotides −200 to +117 of the *tk* gene, which correspond to the promoter, the transcribed noncoding sequence, and 9 nt of the latter gene. This fusion was made possible by creation, through mutagenesis, of a *Bgl*II restriction site on the α4 sequence and a *Bam*HI site on the *tk* sequence; mutagenesis resulted in the conversion of codon 4 of *tk* from tyrosine to aspartate. pRB4579 (α4-2/mut) was made by mutagenesis of the α4-2 binding site to replace the sequence TCGTC with ATTCA. Table 1 contains a list of all of the substitutions made in the studies described in this report. Briefly, pRB4711 (α4-2/+10), pRB4712 (α4-2/+16), pRB4713 (α4-2/+21), and pRB4833 (α4-2/+30) were created by inserting oligonucleotides of 10, 16, 21, and 30 bp, respectively, into the *Ava*I site at position −18 in the α4-*tk* parental construct pRB4018. Correct insertions of oligonucleotides were confirmed by DNA sequence analyses (data not shown). pRB4834 (α4-2/+10/mut) corresponds to pRB4711 except that the ICP4 α4-2 binding site was mutagenized to replace the sequence TCGTC with ATTCA as was done in plasmid pRB4579. Recombinant viruses generated from these plasmids are denoted by the letter R and carry the same number (Table 1).

Gel retardation assays. Gel retardation assays were done as previously described (22, 23, 26). Briefly, 1 μg of mock- or HSV-1(F)-infected nuclear extracts was reacted with 2×10^4 cpm of probe DNA in 20 mM Tris (pH 7.6)–50 mM KCl–0.05% Nonidet P-40–5% glycerol–1 mM EDTA–1 mg of bovine serum albumin per ml–10 mM β-mercaptoethanol. Probes were *Eco*RI-*Bam*HI fragments (135 bp) of each plasmid, except for constructs that carried a mutagenized α4-2 site, which contained an additional *Eco*RI site. For these, *Nco*I-*Bam*HI fragments (257 bp) were used. DNA fragments were dephosphorylated by alkaline phosphatase treatment and 5′ end labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. Poly(dI-dC) (27-7875-02; Pharmacia) was added to each reaction mixture in 3-μg amounts to serve as competitor nucleic acid. ICP4-DNA complexes were supershifted as first described by Kristie and Roizman (22) by the addition of monoclonal antibody H943 to reaction mixtures. Complexes were electrophoretically separated on nondenaturing acrylamide gels.

RNase protection assay. Probes uniformly labelled with [α-^{32}P]CTP were generated from plasmids pRB4582 and pRB4583 that had been linearized with *Eco*RI and transcribed with SP6 RNA polymerase. Transcription of pRB4582 generated a 397-nt transcript that represented 347 nt (+51 to +398) of the *tk* gene. This probe hybridizes to a 281-nt sequence of α4-*tk* mRNA. The α22 probe generated by transcription from pRB4583 is 457-nt long and overlaps 225 nt (+1117 to +1342) of the α22 gene. The RNase protection assay was done as described by Sambrook et al. (41). The hybridization step was done with 5×10^5 cpm of each probe and 0.5 μg of total RNA in a 30-μl solution that contained 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 1 mM EDTA (pH 8.0), and 0.4 M NaCl at 55°C overnight. Digestion by RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) was done after the addition to hybridization reactions of 300 μl of a mixture that contained 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 5 mM EDTA (pH 7.5). Digestion products were electrophoretically separated on a 7.5 M urea–4.5% acrylamide gel. The amounts

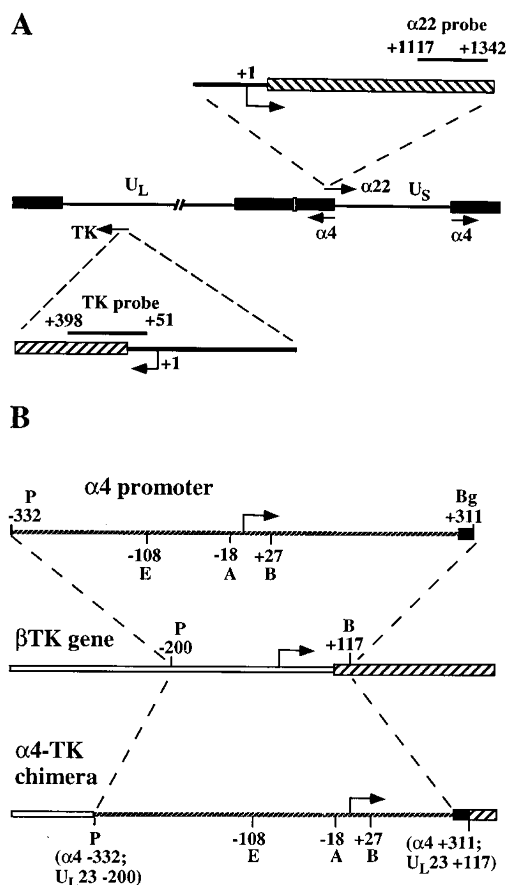

FIG. 1. Diagram of the HSV-1 genome and structure of the α4-*tk* chimeric gene. (A) Organization of the genome and positions of the α4, α22, and natural *tk* genes. The reiterated sequences (filled rectangles) that flank the unique short (U_S) and unique long (U_L) components (lines) and the relative locations and directions of transcription (arrows) are shown. The sequence arrangements of the α22 and *tk* genes relevant to this study are shown above and below the genome, respectively. The initiation of transcription is marked by a bent arrow. The probes used in RNase protection assays are indicated. (B) Sequence arrangement of the α4-*tk* chimera. The α4 minimal promoter is represented by the upper, hatched bar, and the filled rectangle represents the α4 coding domain (three codons) in the chimera. In the middle, the organization of the natural *tk* gene is shown; the open bar represents the untranscribed and transcribed noncoding domains, and the hatched rectangle represents the transcribed coding domain. The bottom line details the organization of the chimeric gene. Bent arrows represent transcription initiation sites. P, *Pvu*II; E, *Eco*RI; A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II. Note that the *Bam*HI site at +117 on *tk* and the *Bgl*II site on α4 were created by mutagenesis and are not present in natural sequences.

of probe RNA protected by α4-*tk* and α22 cytoplasmic RNAs were measured in a betascope (Betagene) as previously described (26).

RESULTS

Experimental design. The objective of these studies was to determine the effect of ICP4 on transcription of its own gene. Since the α4 gene cannot be deleted without affecting the ability of HSV to multiply and since it was not desirable to modify the amounts of ICP4 produced during infection, earlier studies employed a chimeric gene that consisted of the coding sequences of the *tk* gene fused to nucleotides −332 to +311 of the α4 gene. This region, comprising the 5′ untranscribed and transcribed sequences of the α4 gene, replaced the corresponding domains of the *tk* gene (Fig. 1B). To determine whether repression by ICP4 was determined by the position of the ICP4 binding site on DNA, a series of spacer sequences were inserted between the TATA box and the α4-2 site (Table 1). The mutated chimeric genes replaced the corresponding *tk* gene sequences in the viral genome by homologous recombination through flanking sequences. Electrophoretically separated restriction enzyme digests of recombinant DNAs transferred to a nitrocellulose sheet were hybridized with appropriate probes to verify the sequence arrangements and to exclude the presence of parental viruses (data not shown).

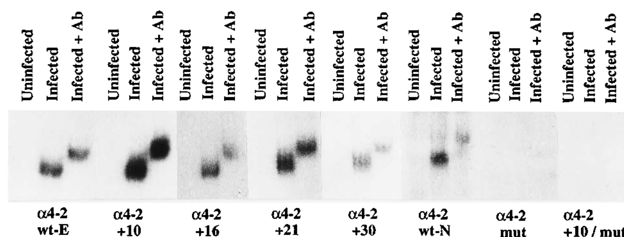

FIG. 2. Autoradiogram of a gel retardation assay with DNA probes that spanned a region from upstream of the TATA box to downstream of the α4-2 site in all constructs used in this study. For each probe, three lanes show the complex formed by the reaction of the labelled probe DNA with nuclear extracts of uninfected cells, HSV-1(F)-infected cells, and HSV-1(F)-infected cells plus monoclonal antibody (Ab) H943, which reacts with ICP4 but does not affect the actual binding of ICP4 to its cognate site. All probes were *Eco*RI-*Bam*HI fragments, with the exception of α4-2/mut and α4-2/+10/mut, which contain an additional *Eco*RI site in the mutagenized sequences. For these constructs, the probes consisted of *Nco*I-*Bam*HI fragments. Both *Eco*RI-*Bam*HI and *Nco*I-*Bam*HI DNA fragments of the α4-*tk* parental construct were tested for binding with ICP4 as indicated (α4-2/wt-E and -N, respectively).

Analysis of mutant fragments for ability to form complexes with ICP4. DNA fragments that contained wild-type or mutant sequences that spanned the region from upstream of the TATA box to downstream of the α4-2 binding site were used as probes in gel retardation assays to determine whether the insertion of spacer sequences altered the ability to form complexes with ICP4. All mutant DNA constructs in which an α4-2 site was present formed complexes similar to those formed by the wild-type DNA sequence; conversely, the corresponding fragments of pRB4579 and pRB4834, which contained the α4-2 site mutagenized to abolish binding, did not form any complexes (Fig. 2). We conclude that the insertion of intervening sequences between the TATA box and the α4-2 site did not interfere with the ability of ICP4 to bind.

Insertions between the TATA box and the ICP4 binding site do not affect accumulation of the chimeric α4-*tk* transcript in the absence of ICP4. In an earlier report, we noted that mutagenesis of transcribed sequences may affect RNA stability or the transcription rate of the chimeric gene independently of the effect of ICP4. To determine whether this is the case, we measured the accumulation of transcripts in the presence of cycloheximide added to the medium at 1 h before infection and for 4 h after exposure of cells to virus. At least three sets of test RNA were prepared according to this protocol. The amounts of chimeric α4-*tk* and α22 mRNA were determined in an RNase protection assay with excess ^{32}P-labelled probe RNA specific for each transcript. α22 mRNA served as an internal control as previously described (26). The amounts of probe RNA protected by the *tk* and α22 mRNAs were measured three times each in the Betagene counter by resetting the boundaries of the bands each time. The radioactivity of a corresponding space above the band of interest was also measured and subtracted as background from the radioactivity of the specific band of interest. The amounts of protected *tk* probe were normalized with respect to those of the protected α22 probe, and the ratios for each mutant were averaged and

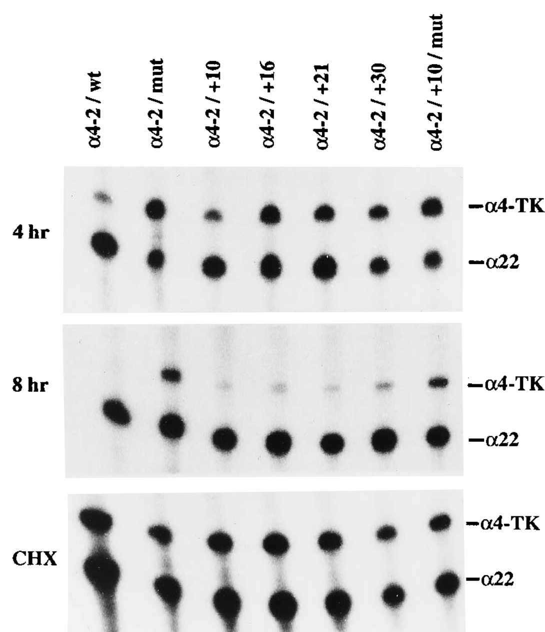

FIG. 3. Autoradiogram of an RNase protection assay. Bands represent the 281 nt of the *tk* gene and the 225 nt of α22 protected by cellular RNA after digestion with RNase. The results are representative of the following three experimental conditions: cells infected for 4 or 8 h or cells pretreated for 1 h with cycloheximide (CHX) prior to infection and for 4 h after exposure to appropriate viruses.

TABLE 2. RNase protection analyses of α4-*tk* RNA transcribed by recombinant viruses in the presence of cycloheximide[a]

Virus	Mutant/wt ratio			
	RNA batch			Mean
	1	2	3	
R4018	1.0	1.0	1.0	1.0
R4579	0.93 ± 0.07	1.03	1.09	1.02 ± 0.07
R4711	1.07 ± 0.06	0.90	1.17	1.05 ± 0.13
R4712	1.01 ± 0.06	1.10	1.18	1.10 ± 0.08
R4713	1.10 ± 0.02	1.13	1.05	1.09 ± 0.03
R4833	0.92 ± 0.04	1.06	1.19	1.06 ± 0.13
R4834	0.9 ± 0.01	1.09	1.22	1.07 ± 0.16

[a] RNase protection assays were done on three independently derived batches of RNA extracted from cells infected and maintained in the presence of cycloheximide. Each result shown is a mean ± 1 standard deviation. Batch no. 1 was analyzed twice. Batches 2 and 3 were analyzed once. α22 mRNA served as the internal control. ICP4-*tk* RNA levels were normalized with respect to α22 mRNA levels. Data are the amounts of mRNA detected by RNase protection assay and expressed as ratios with respect to the levels of RNA measured in wild-type (R4018)-infected cells.

normalized with respect to the parental α4-*tk*. An autoradiogram of representative gels that contained electrophoretically separated fragments of labelled RNA protected by mRNA from RNase degradation in one experiment is shown in Fig. 3. As shown in Table 2, the results obtained in three experiments are in close agreement and indicate that the insertion of sequences between the TATA box and the α4-2 site did not affect accumulation of α4-*tk* RNA and that, therefore, the mutagenesis done on these sequences affected neither the stability of transcripts nor the rate of their transcription.

Effects of the distance from the α4-2 binding site to the TATA box on the level of repression of α4 gene transcription. The effects of α4-2 site displacement on the relative accumulation of α4-*tk* RNA during infection was tested as follows. Total RNA was extracted from Vero cells at 4 and 8 h after infection with the test viruses listed in Table 1. Three different RNA preparations were tested. RNA preparation no. 1 was tested three times, whereas RNA preparations no. 2 and 3 were tested twice. In each case, the procedures used were exactly those described above. A representative autoradiogram of the results is shown in Fig. 3. The results of the RNase protection assay (Table 3 and Fig. 4) were as follows.

(i) At 4 h postinfection, α4-*tk* RNA that contained wild-type α4 sequences was present in an amount that was 10-fold lower than that of mutant R4579, in which the α4-2 site was mutated to abolish the binding of ICP4. Shifting the α4-2 site 10, 21, and 30 nt downstream of its natural position increased transcription 2.5-, 3.5-, and 5.5-fold, respectively. If the assumption is made that the 10-fold increase represents the maximum level of expression (100%) at 4 h for the model employed, the relative percentage of expression was 10, 25, 35, and 55% when the distance from the α4-2 site to the TATA box was increased by 0, 10, 21, and 30 nt, respectively.

(ii) In recombinant R4834, the α4-2 site was shifted 10 nt (as in R4711). In addition, the α4-2 site was mutagenized, as in R4579; that is, the site no longer bound ICP4. This recombinant was made to determine whether the spacer sequence affected expression of the chimeric gene in some unpredictable manner. In all assays with RNAs extracted at 4 and 8 h from infected cells, however, the results obtained with this recombinant mirrored those obtained with recombinant R4579 (α4-2/mut). We conclude that spacer sequences did not affect expression of the α4-*tk* gene in any demonstrable fashion.

(iii) In recombinant R4712, the α4-2 site was shifted 16 nt downstream of its natural position. This shift placed the α4-2 site approximately four DNA helical turns away from the TATA box. The stereoaxial alignment of ICP4 was therefore opposite to that of its natural position (approximately 2.5 turns) and its position in all other recombinants (approximately 3.5, 4.5, and 5.5 turns). At 4 h postinfection, cells infected with this recombinant accumulated as much RNA as did cells infected with the recombinant with the α4-2 site shifted 30 nt, that is, 50% more RNA than that accumulated by cells infected with the recombinant with the α4-2 site shifted 21 nt. It should be stressed that while the three RNA preparations tested showed slight differences in the amounts of probe RNA protected by mRNA, the relative amounts of probe RNA protected by mRNA extracted from cells infected with this recombinant relative to those of other recombinants were highly reproducible (Table 3). We take these results to mean that both stereospecificity and distance from a specific site on DNA (e.g., TATA box or transcription initiation site) determine the extent of repression.

(iv) At 8 h postinfection, the relative accumulation of α4-*tk* RNA (vis-à-vis α22 mRNA) in cells infected with R4579 (α4-2/mut) or R4834 (α4-2/+10/mut) was higher than that observed at 4 h postinfection (Table 3). In both of these recombinants, the α4-2 site was destroyed by mutagenesis and the two recombinants differed solely with respect to the presence of a spacer between the TATA box and the original position of the α4-2 site in R4834. However, the relative accumulation of α4-*tk* mRNA in cells infected with any of the recombinants that contained a shifted α4-2 site decreased. In percentage terms, shifts of 10, 21, and 30 nt corresponded to 13, 17, and 32%, respectively, of the derepression seen in cells infected with mutants that lacked the α4-2 site. These experiments indicate that at later times in infection, the mere presence of

TABLE 3. Quantitative analyses of α4-*tk* RNA transcribed by recombinant viruses[a]

Virus	Fold increase in RNA extracted from infected cells ± SD at:							
	4 h				8 h			
	RNA batch			Mean	RNA batch			Mean
	1	2	3		1	2	3	
R4018	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
R4579	7.1 ± 1.5	11.0 ± 1.2	11.5 ± 1.5	9.9 ± 2.4	16.4 ± 2.7	11.9 ± 0.8	12.7 ± 0.7	13.6 ± 2.4
R4711	2.0 ± 0.1	2.9 ± 0.1	2.3 ± 0.3	2.4 ± 0.4	1.8 ± 0.6	2.0 ± 0.6	1.7 ± 0.2	1.8 ± 0.1
R4712	4.2 ± 0.6	6.5 ± 0.6	5.3 ± 0.1	5.3 ± 1.2	3.0 ± 0.5	3.0 ± 0.5	2.7 ± 0.2	2.9 ± 0.1
R4713	2.9 ± 0.6	4.0 ± 0.5	3.7 ± 0.6	3.5 ± 0.5	2.6 ± 0.5	2.4 ± 0.1	2.0 ± 0.6	2.3 ± 0.3
R4833	4.4 ± 1.2	7.3 ± 1.1	5.8 ± 0.4	5.8 ± 1.4	5.7 ± 1.9	4.0 ± 0.3	3.7 ± 1.3	4.4 ± 1.1
R4834	6.5 ± 1.3	13.5 ± 3.3	10.9 ± 0.1	10.3 ± 3.5	17.1 ± 2.7	11.0 ± 0.1	13.2 ± 1.2	13.8 ± 3.1

[a] Each sample was analyzed independently two to three times by RNase protection. For each sample, the fold increase in RNA amount ± standard deviation with respect to that of the parental R4018 is shown. Data were calculated by using the mean for each of three RNA samples as an independent measurement.

an ICP4 binding site, regardless of position, enables repression by an alternate or additive mechanism.

DISCUSSION

In an earlier article, this laboratory reported that mutagenesis that resulted in destruction of the ICP4 binding site (α4-2) at the transcription initiation site of the α4 gene resulted in increased accumulation of α4 transcripts at 4 and 8 h postinfection (26). The experimental design of those studies involved analyses, within the context of the viral genome, of the transcription of a chimeric gene that consisted of the α4 promoter which contained two α-transinducing factor (virion protein no. 16) response elements, the α4 transcribed noncoding domain, and the coding domain of the *tk* gene. This report is an extension of those earlier studies. The reproducibility of the quantitative data presented in that earlier report in an entirely different set of hands in this study emboldened us to measure the effects of small displacements of the ICP4 binding site along the transcribed domain of the α4 sequences.

Here we report three observations that are fundamental to an understanding of the function of the α4 gene. First, displacement of the ICP4 binding site downstream from the transcription initiation site decreases the extent of repression. Second, the effect of displacement is nonlinear. Proportionality between the distance from the transcription initiation site and the amount of accumulated mRNA held only in the case of displacements that approximated full helical turns of DNA, i.e., 10, 21, and 30 nt from the original position at the transcription initiation site. A binding site displacement of approximately 1.5 helical turns (16 nt) from the transcription initiation site derepressed transcription of the α4 gene at 4 h to an extent comparable to that of the 30-nt displacement and exceeded that seen with a displacement of 21 nt. Lastly, whereas in the absence of the ICP4 binding site in both earlier and current studies relative derepression of the gene appeared to be greater at 8 h than at 4 h, the reverse was true when the binding site was present, albeit displaced 10 to 30 nt downstream of its normal position. It is convenient to view these observations as reflective of two distinct events that occur in infected cells.

ICP4 bound to DNA may block transcription by one of several mechanisms. Some of the nonmutually exclusive mechanisms currently in vogue are that ICP4 (i) physically blocks the assembly of transcriptional factors on DNA between the TATA box and the transcription initiation site, (ii) blocks the transcription of DNA by the assembled transcriptional factors, (iii) modifies the structure of DNA at the binding site, or (iv) interacts with transcriptional factors. The model for the hypothesis that ICP4 precludes the assembly of transcriptional factors rests on prokaryotic models and is also exemplified by simian virus 40 T antigen binding to its own promoter. This model is not consistent with the reported observation that ICP4 also reduces transcription of ICP0 (37); in this instance, the ICP4 binding site is upstream and some 35 to 40 nt from the TATA box. Parenthetically, Everett and Orr (14) reported that mutations of the ICP4 binding sites on both copies of the α0 gene in the context of the viral genome did not significantly alter the production of ICP0 protein. However, Koop et al. (20) inserted ICP4 binding sites into minimal HSV and non-

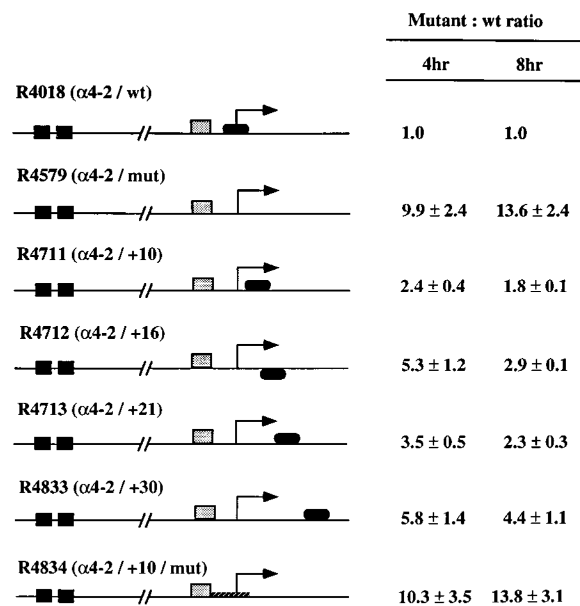

FIG. 4. Schematic representation of the promoter region of each recombinant virus used in this study and summary of results. The TATA element is represented by a stippled rectangle; a filled oval indicates the position of the α4-2 site with respect to its distance from the TATA box and the transcription initiation site as well as its stereospecific orientation with respect to its natural position (above or below the line). Filled rectangles represent the upstream α4-1P and α4-1D binding sites for ICP4. Bent arrows show transcription initiation sites. The hatched bar in R4834 highlights the presence of a 10-nt spacer sequence to distinguish it from R4579. The numbers on the right show the amounts of protected RNA (mean ± 1 standard deviation) normalized with respect to the amount of RNA protected from degradation by mRNA extracted from cells infected for 4 or 8 h, respectively, with the wild-type parent virus (R4018) as described in the text.

HSV promoters. With respect to the TATA box, these sites were located either in the natural position on the α4 promoter or in the natural position on the α0 promoter. In all instances, the presence of the binding site per se, independently of the type of construct used, was sufficient to downregulate gene transcription.

The hypothesis that ICP4 is a simple physical blocker of the RNA polymerase complex during transcription is untenable for several reasons. Foremost, however, we would expect transcription to terminate if the appropriate ICP4 binding site were present at any position within the transcribed domain of the gene. The studies presented in this report show that this expectation is not fulfilled since displacements of the same binding site decreased the extent of repression of transcription. Parenthetically, if ICP4 blocked transcription by simply derailing RNA polymerase, ICP4 synthesis would increase during virus DNA synthesis. In fact, the rate of ICP4 synthesis decreases during virus DNA synthesis (from 3 to approximately 15 h postinfection). It should be noted, however, that in the experiments of Koop et al. (20) described above, the expression of constructs that contained ICP4 binding sites was dependent on virus DNA synthesis. Dependence on virus DNA synthesis may reflect the presence of *cis*-acting elements that have been reported to be present in transcribed noncoding domains of late virus genes rather than simple denuding of DNA.

The hypothesis that transcription is terminated as a result of modification of the virus DNA structure rests on a report that ICP4 causes DNA to bend at the binding site (13). However, this report has ascribed to this modification a facilitation of transcription rather than inhibition. Could a bend in DNA both facilitate and block transcription? The data collected in this report do not readily lend themselves to this interpretation.

Interactions between ICP4 and transcription factors (e.g., TATA-binding protein and TFIIB) have been reported (44), and they have been suggested to enable transcription rather than to block it. The model which best fits our results, however, is one whereby the interaction between ICP4 and transcriptional factors assembled between the TATA box and transcription initiation site may both stimulate and inhibit transcription, depending on (i) the polarities of the interacting proteins (vis-à-vis the direction of transcription) and (ii) the strength of the interaction between the assembled factors and ICP4. For example, weak interactions as defined by distance from the binding site to the assembled transcriptional factors, strength of ICP4 binding to DNA, and polarity of the ICP4 binding site (vis-à-vis the assembled factors) may help stabilize the polymerase complex but not block its processivity on DNA. Alterations in the polarity of ICP4 (vis-à-vis the assembled factors), the strong binding of ICP4 to its binding site on DNA, and the juxtaposition of the ICP4 binding site close to the TATA box in a fashion that does not require either the folding of DNA on itself or both folding and partial uncoiling may block transcription by preventing the assembled polymerase transcription complex from initiating transcription. With respect to our data, displacement of the ICP4 binding site by one, two, or three helical turns of DNA would require a folding of DNA upon itself to sustain a complex between the bound ICP4 and the assembled transcriptional factors. The stability of such folded complexes may decrease with distance. In addition, positioning the binding site out of phase on the DNA helix may further detract from the stability of the interaction between bound ICP4 and the assembled complex.

A totally different issue arises from the observation that derepression of the α4-*tk* chimera was even more pronounced at 8 h postinfection for mutants that lacked ICP4 binding sites and much less pronounced in cells infected with mutants in which the binding site was merely displaced. In an earlier report (26), it was shown that the upstream ICP4 binding sites designated α4-1D and α4-1P play a role in the repression process, but that effect was minimal at 8 h and unobserved at 4 h. Thus, at 8 h postinfection, cells infected with mutants that lack all three binding sites yield twice as much protected RNA as those infected with mutants that lack the strong α4-2 binding site only. Consistent with our model, the weak upstream binding sites may add to the forces that freeze factors at the site of assembly between the TATA box and the transcription initiation site. This observation does not explain why the effect is detected at 8 h but not at 4 h.

One non-mutually exclusive alternative hypothesis rests on the observation that ICP4 is extensively posttranslationally modified. Current studies indicate that it is phosphorylated and poly(ADP-ribosyl)ated in infected cells (3, 32, 36, 47) and that it is capable of being nucleotidylylated in vitro (4). The function of posttranslational modifications is not known. It is conceivable that the ICP4 proteins that are bound at 8 h are more extensively processed than those bound at 4 h and that the nature of the interaction between ICP4 bound to the α4-2 site with the assembled factors and the ICP4 proteins bound at the upstream (α4-1D and α4-1P) sites differs from that which occurs earlier.

A central unresolved issue is whether ICP4 is inherently a repressor which transactivates or a transactivator which represses. In in vitro systems, ICP4 has been reported to be at best a modest transactivator, with far less potent activity than that ascribed to it in the context of infected cells. Dual functions as transactivator and repressor are not out of the question and rest on numerous precedents (46). It remains to be seen whether the multiple functions ascribed to ICP4 arise from its interactions with the same or different proteins.

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