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Genetics

Repression of the herpes simplex virus 1 α4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites

(infected cell protein 4/DNA-binding sites/mutagenesis/additive repression)

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ABSTRACT **The infected cell protein 4 (ICP-4), the major regulatory protein encoded by the a4 gene of the herpes simplex virus 1, binds two sites (α4-1 proximal, α4-1 distal) at the 5′-untranscribed domain and at the transcription initiation site (α4-2) of the α4 gene. Chimeric genes consisting of the 5′-untranscribed and transcribed noncoding domains of the α4 gene fused to the coding sequences of the thymidine kinase gene were mutagenized to abolish binding of ICP-4 by substitution of bases, including the guanines whose methylation interferes with binding of the protein, and recombined into the viral genome. The cytoplasmic RNAs extracted from infected cells treated with cycloheximide, from untreated infected cells maintained for 4 or 8 hr, and from cells infected first with a virus deleted in the α22 gene and 3 hr later with the test viruses were tested in RNase protection assays for amounts of the chimeric gene RNA relative to amounts of α22 gene RNA. We report the following: (*i*) Mutation of the α4-2 binding site resulted in a 5- to 6-fold higher accumulation of chimeric gene RNA at 4 hr and as much as 15-fold higher accumulation by 8 hr after infection. (*ii*) Mutations of α4-1 sites by themselves had no effect on RNA accumulation. However, mutagenesis of all three sites significantly increased mRNA amounts above the levels seen in cells infected with α4-2 site mutants. (*iii*) The mutations have no effect on accumulation of α4 mRNA in the absence of ICP-4 synthesis and, therefore, the mutations had no effect on RNA stability or transcription rate. (*iv*) Accumulation of α4 mRNA relative to that of α22 mRNA is highest in the presence of cycloheximide and decreases with time after infection. We conclude that ICP-4 autoregulates the transcription of its own gene in infected cells and that binding of ICP-4 to three sites in its promoter is additive in its effects on this process.**

This report concerns infected cell protein 4 (ICP-4), the major regulatory protein encoded by herpes simplex virus 1 (HSV-1) and α4, the gene encoding this protein. The background and fundamental issues dealt with in this report are as follows.

(*i*) The α genes—α4, α0, α22, α27, and α47—are induced by a virion protein (VP16 or α gene trans-inducing factor) and are the first genes expressed after infection (for review, see ref. 1). Functional products of the α genes, particularly those of the α4 gene, are required for the expression of β and γ genes, which are expressed later in infection (2). ICP-4 has been shown to bind directly to DNA (3, 4); it binds with high affinity to the sequences with the consensus ATCGTCNNNNYCGRC (5) and with a lower affinity to a variety of sequences that are not represented by a single consensus (6, 7).

(*ii*) The ICP-4 DNA-binding sites have been thoroughly mapped in several genes. In α0, a single site conforming to the consensus is upstream of the cap site (8). In α4, three sites have been mapped. One site that has a sequence conforming with the consensus spans the transcription initiation (cap or α4-2) site (9–12), whereas two nonconsensus sites α4-1P (proximal) and α4-1D (distal) are located upstream of the cap site (6, 13, 14). In late (γ) genes, binding sites have been found both in 5′-untranscribed and in 5′-transcribed noncoding domains (3, 15, 16).

(*iii*) α4 gene expression reaches peak rates between 2 and 4 hr after infection and then declines (17, 18). The initial decrease in α4 gene expression has been suggested to be due to ICP-4 (19–20) and later in infection a more general shutoff of α gene expression takes place (2, 18, 21, 22). The hypothesis that ICP-4 represses itself by binding to its cap site is obvious and has been investigated in numerous laboratories with transient expression assays in which domains of wild-type or mutated α4 gene sequences were fused to a reporter gene and transfected into cells. In nearly all studies repression of reporter-gene expression was alleviated by mutations in or around the cap site (12, 23–31). However, viral or cellular genes introduced into cells by transfection and transactivated by superinfection of virus are frequently regulated improperly as β genes (32–34) and, therefore, transient expression systems are not a reliable indicator of the regulation to which the gene is subjected as a natural constituent of the viral genome during productive infection.

(*iv*) With few exceptions (16), mutations of ICP-4-binding sites made to date in genes of the viral genome have little or no effect on the expression of viral genes tested (35, 36). To account for the failure to affect gene expression by mutagenesis of the binding sites, ICP-4 has been postulated to act globally rather than locally by binding to the viral genome and that individual sites contribute, but do not solely determine, expression. A more testable hypothesis is that regulation of viral gene expression is affected by a multiplicity of mechanisms that fine tune and ultimately ensure the appropriate expression level. A mechanism consistent with the latter view was apparent for mutations in the VP16 response element for expression of an α gene (37, 38).

To test the role of the ICP-4-binding sites in the α4 gene we constructed a chimeric gene consisting of a minimal α4 promoter-regulatory domain fused to the coding sequences of the viral thymidine kinase gene (*tk*). The ICP-4-binding sites were abolished by mutagenesis, and *tk* in the viral genome was replaced with the corresponding sequences of the parent and mutated chimeric genes. We note that there was no agreement on the exact location of the α4-2 binding site. Thus, guanines whose methylation blocks binding of ICP-4 to the α4-2 site in this laboratory (11) overlap only in part with the site reported by DiDonato and Muller (9, 39). Although

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Abbreviations: HSV-1, herpes simplex virus 1; ICP-4, infected cell protein 4.

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Everett *et al.* (25) confirmed our results, it was thought prudent to mutagenize both sets of reported binding sites.

MATERIALS AND METHODS

Viruses. HSV-1(F) is the prototype strain of HSV-1 used in this laboratory. HSV-1(F)Δ305 has a 501-bp deletion in the 5′-transcribed noncoding and coding domains of *tk* (40) and served as the parent of all other mutants generated in this study. R325 was derived from HSV-1(F)Δ305 by deletion of 821 bp of the α22 coding sequences (40).

Genetic Engineering of α4–tk Recombinant Viruses. The recombinant viruses R4018 and R4575–R4581 were made by cotransfecting the plasmid DNA of the corresponding designation with HSV-1(F)Δ305 DNA into rabbit skin cells. tk^+ progeny were selected on 143TK$^-$ cells, as described (40). The recombinant viruses were plaque-purified at least twice and checked for purity by hybridization with probes specific for the chimeric gene.

RESULTS

Experimental Design. The sequence arrangement of the HSV-1 genome and the location of the viral genes relevant to these studies are shown in Fig. 1. Fig. 2*B* shows the nucleotide sequence of the α4-1 and α4-2 binding sites for ICP-4 in the α4 gene (41). The experimental design used in these studies was as follows. (*i*) Sequence −332 to +311 of the α4 gene was fused to *tk* at position +117. In the resulting chimeric gene, codons 1–3 of the α4 gene were fused to codon 4 of *tk*, converting codon 4 from tyrosine to aspartate. (*ii*) A series of chimeric genes with mutations in the ICP-4 binding sites in sequences from the α4 gene were constructed (Fig. 2). Mutagenesis involved the substitution of guanines for which methylation was previously shown to interfere with the binding of ICP-4 (6, 9, 11, 39). (*iii*) The mutated chimeric genes were recombined into the viral genome at the *tk* locus, such that the α4 5′-untranscribed and -transcribed noncoding domains replaced the corresponding domains of *tk*. (*iv*) Once the presence of the mutated sequences in the viral genomes was confirmed, studies on the role of the ICP-4 binding sites were initiated.

Analysis of Mutant Fragments for Ability to Complex with ICP-4. DNA fragments containing the wild-type or mutant ICP-4-binding sites served as probes in a gel-retardation assay to determine whether the mutations abolished ICP-4

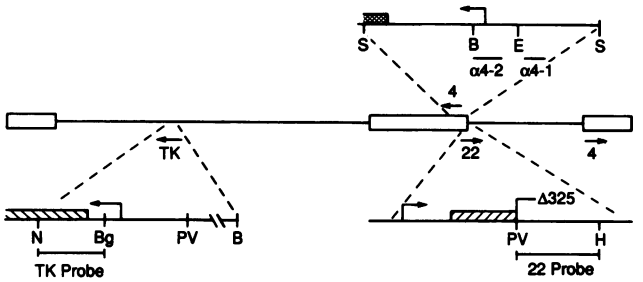

FIG. 1. Diagram of HSV-1 genome. The reiterated sequences (rectangles) flank the long and short components (line); relative location, direction of transcription (little arrows), and sequence arrangements of α4, βtk, and α22 genes are shown above or below. The bent arrow represents the start site of transcription, and the hatched box represents the coding domain of each gene. The α4-1 and α4-2 ICP-4-binding sites are shown. The 397-base TK probe includes 347 bp from +51 to +398 of *tk* gene (pRB4582) and hybridizes to 281 bases of the chimeric α4-tk mRNA (as shown in Fig. 2). The 459-base α22 probe includes 225 bp from +1117 to +1342 of the α22 gene plus 181 bp from pUC19 (pRB4583). Both probes were generated by Sp6 transcription from pRB4582 and pRB4583, linearized with *Eco*RI, in the presence of [α-^{32}P]CTP. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; N, *Nru* I; PV, *Pvu* II. Δ325, one boundary of the α22 deletion in R325 virus.

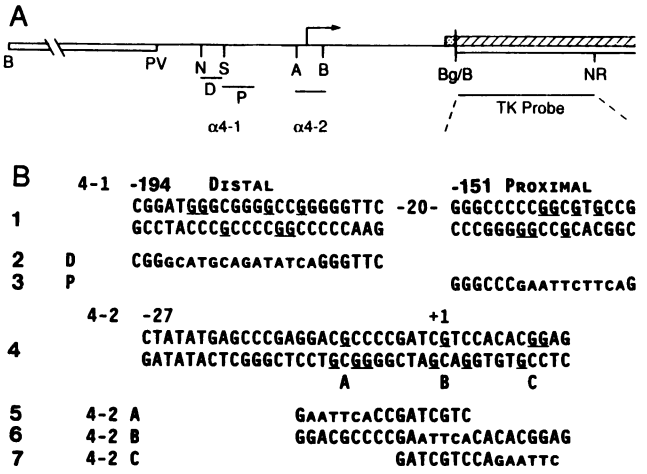

FIG. 2. Diagram showing structure of the chimeric α4–*tk* gene. (*A*) The open bar represents the −725 to −200 sequence of *tk*. The single hatched bar represents coding sequences of *tk* starting with nt +117. The short double-hatched bar represents the three codons of the α4 gene fused to the modified fourth codon of *tk*. The α4–*tk* gene was assembled as follows. The 667-bp *Sma* I fragment, −332 to +335 of the α4 gene, cloned into the *Hin*cII site of pGEM3Zf (pRB4011), was mutagenized to create a *Bgl* II site at nt +311 (pRB8059). The *Hin*dIII–*Bgl* II fragment from pRB8059 contains the α4 gene sequences from nt −332, the transcribed noncoding sequences, and the first 9 nt of the coding sequences. pRB3838 was mutagenized to create a unique *Bam*HI site at nt +117 in *tk* (pRB8053). The *Bam*HI–*Hin*dIII fragment from pRB8053 was cloned into the *Bam*HI–*Hin*dIII site of pRB4050, creating plasmid pRB4016, which has *tk* sequences from −725 to −200, polylinker sequences, and then *tk* coding sequences from the new *Bam*HI site at codon 4 through the end of the gene. The f1 origin of replication, a 565-bp fragment from pUCf1 (Pharmacia 27-4934)-made *Bam*HI$^-$, was cloned into the *Hin*cII–*Hin*dIII site of this plasmid (pRB4017). The *Hin*dIII–*Bgl* II fragment from pRB8059 was then cloned into the *Eco*RI–*Bam*HI site of pRB4017, effecting the insertion of the α4 promoter-leader sequences into the polylinker, completing assembly of the chimeric α4–*tk* gene (pRB4018). The thymidine kinase (TK) probe was described in the legend for Fig. 1. DNA probes used in gel-retardation assay were α4-1D, the *Nco* I–*Sac* II (−209 to −161) fragment; α4-2, *Ava* I–*Bam*HI (−18 to +27); α4-1P (−160 to −125) double-stranded fragment made by hybridizing oligonucleotides synthesized to contain parental or mutant sequences. A, *Ava* I; B, *Bam*HI; Bg, *Bgl* II; N, *Nco* I; NR, *Nru* I; PV, *Pvu* II. (*B*) DNA sequence of wild-type and mutant ICP-4-binding sites. Lines: 1, wild-type double-stranded DNA sequence of distal and proximal α4-1 site, starting at nt −194 and −151, respectively; 2 and 3, sequence of D and P mutations; 4, wild-type double-stranded DNA sequence of α4-2 site starting at nt −27; 5–7, sequence of A–C mutations, respectively. The guanines whose methylation interferes with ICP-4 binding are underlined. Mutated sequences are shown as single strands with lowercase letters identifying the substituted nucleotides. The mutants α4-1P, α4-1D, α4-1PD, α4-2A, α4-2B, α4-2C, α4-(1PD, 2B), α4-(1D, 2B) were recovered as plasmids pRB4575–pRB4582. All mutations were made by site-directed mutagenesis of the appropriate parental plasmid with oligonucleotides synthesized as 45-mers on an Applied Biosystems model 380B DNA synthesizer using reagents from Bio-Rad, MutaGene m13 *in vitro* mutagenesis kit 170-3571, as reported (37).

binding. The results (Fig. 3 *A–C*) show the following: (*i*) The A mutation did not abolish the binding of ICP-4 to the α4-2 site. In contrast, fragments bearing either the B or C mutation did not form detectable complexes with ICP-4. (*ii*) The affinities of the wild-type α4-1 proximal (P) and distal (D) binding sites for ICP-4 are considerably lower than that of α4-2 site. Both proximal and distal mutant (M) sequences failed to form demonstrable complexes with ICP-4.

Effect of Mutations in the α4 Binding Sites on the Relative Accumulation of α4–tk RNA During Infection. The experimental design was as follows. (*i*) Cytoplasmic RNA was

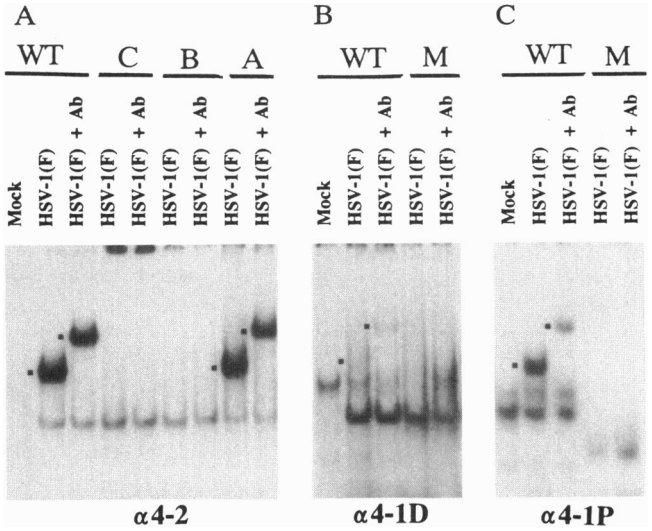

FIG. 3. Autoradiogram of a gel-retardation assay with DNA probes containing the wild-type (WT) and mutant (M) α4-1D (*B*), α4-1P (*C*), and α4-2 A–C (*A*) ICP-4-binding sites. The gel-retardation assay was as described (6, 8, 13); competitor DNA was 0.5 μg of poly(dI·dC) (Pharmacia 27-7875-02) for α4-2 and α4-1D reactions and was 0.5 μg of poly(dA·dT) (Pharmacia 27-7860) for α4-1D. ^{32}P-labeled DNA probes were incubated with proteins extracted from mock-infected (M) or HSV-1(F)-infected cells and electrophoretically separated on a nondenaturing gel. HSV-1(F) + Ab indicates that monoclonal antibody H943 to ICP-4 was included in the reaction mixture. DNA fragments identified in the legend for Fig. 2 were purified from restriction-enzyme digests of the wild-type (pRB4018) and mutant (pRB4575-4582) plasmids, treated with calf intestine alkaline phosphatase and 5′-end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The position of the ICP-4-containing band is marked by a square.

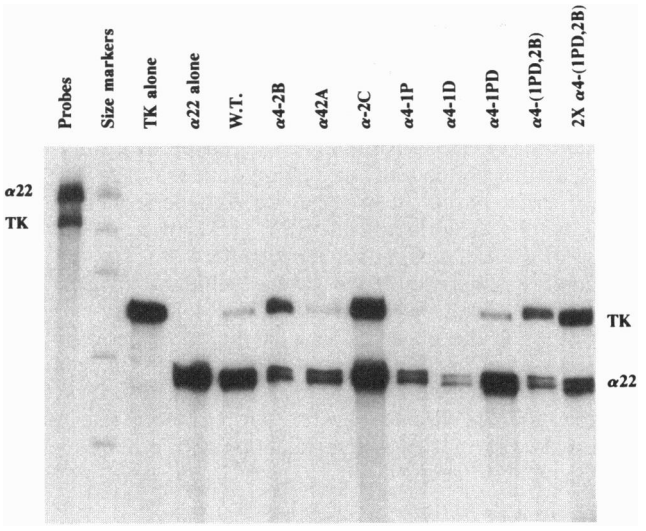

FIG. 4. Autoradiographic image of a RNase protection assay with test RNAs extracted from cells 8 hr after infection. Ten micrograms of test RNA, extracted from the cytoplasm of Vero cells infected with 10 plaque-forming units of test virus and harvested as described in *Results*, was ethanol-precipitated, resuspended in 1.0 μl of H_2O, and mixed with 30 μl of hybridization buffer containing an excess of *in vitro*-synthesized ^{32}P-labeled TK and ^{32}P-labeled α22 RNA probes (described in Fig. 1). Mixtures were incubated overnight at 53°C, digested with RNase A and RNase T1, and electrophoretically separated in a 8% polyacrylamide/urea gel as described (37, 38). The designations TK and α22 refer to the 281 bases of the TK probe and the 225 bases of the α22 probe protected from digestion by the chimeric α4–tk and α22 mRNAs, respectively. The wild-type or mutant binding site(s) present in the infecting virus is indicated above each lane. Lanes: Probes, aliquot of undigested α22 and TK probes; Size markers, ^{32}P-labeled fragments of 501/489, 404, 320, 242, and 190 bases; TK alone, TK probe only was present in reaction mixture; α22 alone, α22 probe only was present in reaction mixture; 2X α4-(1PD,2B), two times the amount of test mRNA was present in the reaction mixture to verify that labeled RNA probes were present in excess.

harvested from replicate cultures of Vero cells infected with the test viruses under four different experimental protocols. The RNA harvested were from (*a*) cells infected with test viruses and maintained with cycloheximide for 4 hr, (*b*) cells infected and maintained for 4 hr, (*c*) cells infected and maintained for 8 hr, and (*d*) cells infected with an α22$^-$ virus (R325) for 3 hr and then infected with test viruses and maintained for an additional 4 hr. At least three sets of test RNA were prepared for each protocol. (*ii*) The amounts of chimeric α4–*tk* and α22 mRNA were determined in an RNase protection assay by using excess ^{32}P-labeled probes specific for each transcript. α22 mRNA served as an internal control; the selection of this gene was based on the observation that its expression is not affected by ICP-4 (J. Voss and B.R., unpublished work). (*iii*) The amounts of probe RNA protected by the α4–*tk* and α22 cytoplasmic RNAs were measured three times in the Betagen counter (Betagen, Waltham, MA) by resetting boundaries of the bands each time. A corresponding space above the band of interest was also measured for radioactivity and served as the determinant of the background radioactivity, which was subtracted from the radioactivity of the specific bands. (*iv*) The amounts of RNase-protected probe RNA for the parental and mutant α4–*tk* genes were normalized with respect to the α22 mRNA. To determine the effects of the mutation on expression of the α4–*tk* gene, the ratios for each mutant were averaged and normalized with respect to the ratio obtained for the parental, unmutagenized α4–*tk* gene.

Fig. 4 shows an autoradiographic image of electrophoretically separated probe RNA protected from RNase digestion by cytoplasmic RNA harvested 8 hr after infection with parent or test viruses. In the experiments reported here, bands like these were measured for the amount of RNA protected from RNase digestion. Results of the RNase protection assays (Tables 1 and 2) were as follows.

(*i*) The relative amounts of probe RNA protected by RNAs harvested from cells infected in the presence of cycloheximide with parent and test viruses did not differ significantly from each other (Table 1). These results indicate that the mutations introduced into the α4-1 and α4-2 sites had no significant effect on either the rate of transcription in the total absence of ICP-4 or the rate of decay of the RNAs.

(*ii*) In the presence of cycloheximide, the ratio of α4–*tk* RNA to α22 RNA was 0.56 ± 0.02 for the parental unmutated virus (Table 2). This ratio was 3- and 10-fold lower in the absence of the drug at 4 and 8 hr after infection, respectively. Assuming that the amount of α4 and α22 mRNAs that accumulate with cycloheximide are representative of the initial synthesis rate and that the mutations do not affect RNA turnover, as suggested by the data cited above, it follows that the reduction in synthesis of α4–*tk* RNA relative to α22 RNA was initiated before 4 hr after infection.

(*iii*) Mutation of the B and C sites increased the relative amount of probe RNA protected by RNAs harvested at 4 and 8 hr after infection (Table 1). The relative amounts of RNA protected were higher at 8 hr than at 4 hr after infection. The effect of these mutations was more pronounced in cells infected with the α22$^-$ virus before infection with the parent and test viruses. The mutation at the A site did not significantly change the relative amount of RNA probe protected from RNase digestion by RNAs harvested at either 4 or 8 hr after infection or after prior infection with α22$^-$ virus. This

Table 1. Relative amounts of probe RNAs protected by viral cytoplasmic RNAs

	Accumulating transcripts							
	4 hr		8 hr		After $\alpha 22^-$ virus		Cycloheximide	
Mutation	Ratio ± SE	Assays, no.	Ratio ± SE	Assays, no.	Ratio ± SE	Assays, no.	Ratio ± SE	Assays, no.
None	1.0	18	1.0	5	1.0	8	1.0	4
4-2A	1.5 ± 0.6	12	1.5 + 0.3	4	1.7 ± 0.5	8	1.1 ± 0.2	4
4-2B	6.6 ± 2.0	18	11.7 + 2.9	5	18.5 ± 3.1	7	1.0 ± 0.2	4
4-2C	5.1 ± 2.2	12	9.9 + 1.2	4	15.2 ± 3.5	7	0.9 ± 0.1	3
4-1P	1.3 ± 0.5	6	1.5 + 0.3	4	1.1 ± 0.3	3		
4-1D	1.2 ± 0.6	7	1.1 + 0.2	5	0.9 ± 0.6	4		
4-1PD	1.3 ± 0.5	7	0.9 + 0.2	4	1.2 ± 0.7	5	1.0	2
4-(1PD,2B)	4.8 ± 1.4	8	21.7 + 4.7	5	19.0 ± 5.7	5	0.9	1
4-(1P,2B)	5.8 ± 2.2	5	14.2 + 2.2	4	15.9 ± 1.7	4		

*Data were normalized with respect to ratio for the parental, nonmutated $\alpha 4$–*tk* gene for each experimental protocol.

observation was consistent with the observation that the mutated $\alpha 4$-2 A site bound ICP-4.

(*iv*) Mutations at the $\alpha 4$-1 sites, individually or together but in the absence of other mutations, had no significant effect on the relative amount of probe RNA protected from RNase digestion by test viruses in any experimental protocols tested (Table 1).

(*v*) Mutations in all three sites, $\alpha 4$(1PD,2B), increased the relative amount of probe RNA protected from RNase digestion by the test RNAs harvested at 8 hr after infection (Table 1).

DISCUSSION

The key findings we report are as follows.

(*i*) ICP-4 represses expression of the $\alpha 4$ gene promoter *in situ* by binding to the 5′ domains of the $\alpha 4$ gene. In as much as we compared expression of wild-type and mutated sequences in the same viral setting, we measured solely the effect of mutations at the binding sites of ICP-4; the shutoff of α gene expression by genes expressed later in infection would affect all constructs equally. Our data indicate that the $\alpha 4$ gene can express at least 15- to 20-fold more RNA in the absence of the repressive effect of ICP-4 binding.

(*ii*) The results indicate that $\alpha 4$-2, the high-affinity binding site, is the major site of autoregulation of $\alpha 4$ gene. The two $\alpha 4$-1 binding sites by themselves did not appear to affect significantly expression of the $\alpha 4$–*tk* chimeric gene. The low-affinity upstream binding sites may be saturated later in infection and augment repression of the $\alpha 4$ gene. Contribution of the $\alpha 4$-1 binding sites may be additive and potentially cooperative, only in conjunction with the $\alpha 4$-2 binding site and only late in infection. We should stress that "repression" does not signify shutoff of gene expression inasmuch as ICP-4 is expressed throughout the reproductive cycle, although at different rates (42).

Our results raise tantalizing questions regarding (*i*) the reported failure to detect repression of $\alpha 0$ or a decrease in the glycoprotein gene (gD) expression by mutations of the ICP-4-binding sites in those genes in the context of the viral genome and (*ii*) the need for autoregulation of $\alpha 4$ gene expression. With regard to the first, the published data suggest that the mutations did not completely abolish binding of ICP-4 to DNA, and conceivably residual ICP-4-binding activity was sufficient for transcriptional regulation. A nonmutually exclusive possibility is that the viral genome incorporates multiple alternative pathways to accomplish the same objective. This explanation is evident from the observation that the virus appears to have more than one cognate pathway for entry into cells (43), from the observation that many genes encode homologs of cellular functions (1), and from the observation that the abolition of the VP16 response element in an α gene had a minimal, cell-type-dependent effect on expression of the test gene: alternate mechanisms replaced VP16 function (37, 38).

The more interesting issue is the requirement for the autoregulation of $\alpha 4$ gene expression. If the $\alpha 4$-1 binding site enhances repression of the $\alpha 4$ gene, ICP-4-binding sites in other genes may also be negative rather than positive. Conceivably, overproduction of ICP-4 could have a negative effect on many genes, including genes for which expression is stimulated by ICP-4. Further studies may shed light on this issue.

Table 2. Ratios of probe RNAs protected by nonmutated, parental $\alpha 4$-tk RNA to that of $\alpha 22$ RNA under different experimental conditions

Conditions	Ratio of $\alpha 4$–tk/$\alpha 22$ RNA ± SE	Assays, no.
Cycloheximide	0.56 ± 0.02	4
4 hr after infection	0.19 ± 0.06	21
8 hr after infection	0.04 ± 0.03	4
Prior infection with $\alpha 22^-$ virus	0.07 ± 0.02	10

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