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Differentiation between α promoter and regulator regions of herpes simplex virus 1: The functional domains and sequence of a movable α regulator

(induction/cell conversion/transcription-initiation/sequence analysis/chimeric thymidine kinase genes)

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ABSTRACT **The herpes simplex virus genome consists of at least three groups of genes—α, β, and γ—whose expression is coordinately regulated and sequentially ordered in a cascade fashion. We have established that the elements involved in regulation of α genes are a sequence that promotes gene expression and a sequence that confers α regulation on the gene by responding to *trans*-acting regulatory signals. The domains of these sequences were mapped by determining the regulation of thymidine kinase (TK) in L cells converted to TK$^+$ phenotype by chimeric *TK* indicator genes. The chimeric genes were constructed from appropriate portions of the *TK* gene fused to donor sequences derived from the 5′ nontranscribed and nontranslated leader portions of the viral α gene *4*. The results were as follows. (*i*) The natural β *TK* indicator extending 5′ up to −80 and the chimeric α *TK* extending 5′ up to −110 both converted cells to TK$^+$ phenotype but were not regulated. (*ii*) A segment of the regulator region of the α gene *4*, extending 5′ from position −110, confers inducible α-type regulation when fused to the nonregulated but expressible β *TK* indicator described above. (*iii*) The extent of gene induction appears to hinge on the size of the regulatory region inserted into the chimeric gene and correlates with the presence of repeated consensus sequences and G+C-rich inverted repeats in the regulatory region of the α gene *4* and other α genes.**

The herpes simplex virus (HSV) genome consists of at least three groups of genes—α, β, and γ—whose expression is coordinately regulated and sequentially ordered in a cascade fashion (1). This cascade regulation is determined, at least in part, at the transcriptional level, inasmuch as the transcription of the five known α genes (*4*, *0*, *22*, *27*, and *47*) (2–5) does not require *de novo* protein synthesis (1, 6), whereas functional α gene products, and especially α gene *4*, are required for the expression of β and γ genes (7–9). One key, unresolved question is the basis for differentiation of α genes from β and γ genes by host RNA polymerase II, which transcribes the HSV genome (10).

In an attempt to identify the regulatory and promoter regions of α genes, we took advantage of the known properties of the viral thymidine kinase (*TK*) gene. The *TK* gene is regulated as a β gene both during productive infection of normal cells with wild-type virus (11) and in cells converted to TK$^+$ phenotype by an isolated DNA fragment carrying the gene (12–14). Specifically, the induction of TK activity in productive infection and in converted cells requires a functional α gene *4* product. Whereas infection of converted TK$^+$ cells with a TK$^-$ temperature-sensitive (ts) mutant in α gene *4* will induce the enzyme multifold at the permissive temperature, no increase above baseline levels is observed in cells infected and maintained at the nonpermissive temperature.

In experiments reported earlier (14), we identified the sequences conferring α regulation to genes by replacing part of the nontranslated leader and sequences upstream from the transcription initiation site of the *TK* gene with the corresponding sequences from α gene *4*. This chimeric *TK* gene was expressed as an α gene during productive infection, when it was recombined into the viral genome. When introduced into cells, this chimeric *TK* gene was inducible by infection with a TK$^-$ virus, but the induction was independent of *de novo* protein synthesis and of the presence of functional α gene *4* product. Subsequent studies (15) with α gene *27*–*TK* chimeras established that the domain of the α *27* promoter–regulator region was contained within 270 base pairs (bp) upstream from the transcription initiation site.

In this paper we report (*i*) the ability to express, but not to regulate, is conserved in β *TK* genes and α–*TK* chimera that contain sequences extending −80 and −110 bp upstream, respectively, from the transcription initiation sites; (*ii*) the identification of a movable α gene regulator sequence that converts an expressible but nonregulated *TK* gene into a regulated α gene; and (*iii*) suggestive evidence that α gene *4* regulator regions may consist of multiple regulatory units acting cumulatively.

MATERIALS AND METHODS

Viruses. HSV-1(C101)B2006, a TK$^-$ mutant (16), was obtained from S. Kit. HSV-1 ts502Δ305 is temperature sensitive in α gene *4* and contains a 700-bp deletion in the *TK* gene (14).

Cloning of DNA. The procedures for construction, cloning, screening, and purification of recombinant plasmids were as described (15, 17). The vectors used for cloning were pBR322 (18), pACYC184 (19), and PKC7 (20).

Conversion of TK$^-$ Cells to TK$^+$ and Assay of TK Activity in Converted Cells. The conversion of LTK$^-$ cells (obtained from S. Kit) or adenine phosphoribosyltransferase negative (APRT$^-$) TK$^-$ L cells (obtained from S. Silverstein) to TK$^+$ phenotype with plasmid DNAs, for selection of the converted TK$^+$ cells, and the procedures for infection of TK$^+$ cells and for the extraction and assay of TK activity, by using either [^{3}H]thymidine or [^{14}C]thymidine, were as described (14, 15).

Sequence Analysis of DNA. The procedures for purification of DNA fragments labeled at one terminus and for base-specific chemical sequence analysis were all according to the protocol of Maxam and Gilbert (21). The entire sequence was verified by sequence analysis of both strands of the DNA.

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Abbreviations: TK, thymidine kinase; HSV, herpes simplex virus; bp, base pair(s); ts, temperature sensitive.

5'-End Analysis of RNA. The procedures for isolation of cytoplasmic RNA from HSV-1 F-infected cells, for hybridizations to single-stranded end-labeled DNA, and for analysis of hybrids by S1 nuclease and by exonuclease VII digestion were as described (3, 15).

RESULTS

The Site of Initiation of Transcription of α Gene 4. The site of initiation of α gene 4 transcription was previously localized within a 35-bp sequence in the *Bam*HI N fragment (Fig. 1) between the left *Bam*HI and the first *Pvu* I cleavage site (3). To localize the initiation site more precisely, the 290-bp *Sal* I/*Eco*RI fragment spanning the junction between *Bam*HI Y and N fragments (Fig. 1) was labeled with [γ-^{32}P]ATP and polynucleotide kinase. The purified single strand labeled at the *Sal* I site was hybridized to poly(A)$^+$ RNA from infected cells and was then digested with either S1 nuclease or exonuclease VII. As shown in Fig. 2, the major protected products of both S1 nuclease and exonuclease VII digestions of the RNA·DNA hybrids comigrated at positions approximately 180 to 184 bases from the labeled *Sal* I site. The observed heterogeneity probably reflects incomplete digestion but we cannot exclude slight imprecision in the transcription initiation site. The major band at position 182 is within the *Pvu* I cleavage site of *Bam*HI N. These results place the initiation site 33 bp to the right of *Bam*HI Y–N cleavage site in agreement with previous results (3).

Construction and Regulation of the *TK* Chimeric Genes in Converted TK$^+$ Cells. Two series of experiments were done. In the first series—designed to define the limits of the α gene 4 regulator region—the *TK* indicator gene *407* (Fig. 1) was fused in the proper transcriptional orientation to α gene 4 donor frag-

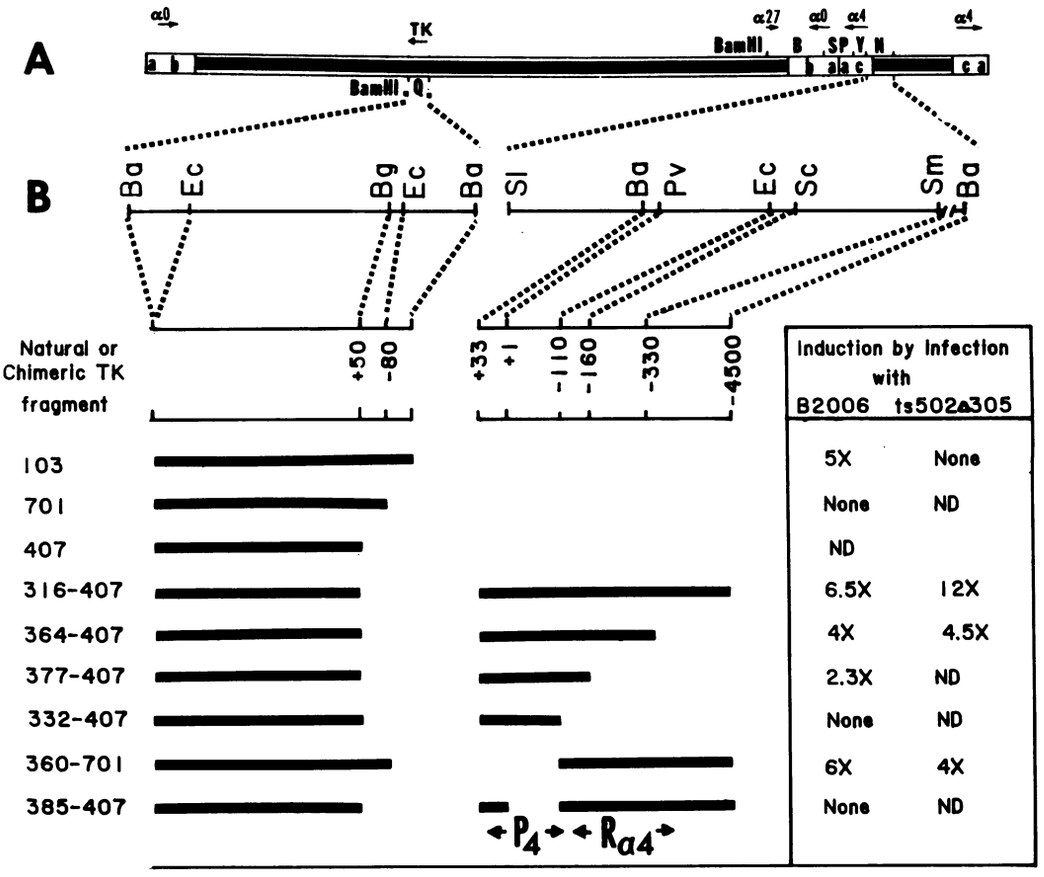

FIG. 1. Sequence arrangement of HSV-1 DNA and of natural and chimeric *TK* fragments and summary of the regulation of natural and chimeric *TK* genes. (*A*) A schematic diagram of the HSV-1 genome (prototype arrangement) showing the positions of reiterated sequences ab and ca (22), the locations and orientations of the *TK* gene and of α genes *27*, *0*, and *4* (2–4), and the locations of several *Bam*HI fragments (23). (*B*) Restriction enzyme cleavage sites in the *Bam*HI Q fragment (24) and the region spanning the *Bam*HI Y–N junction. The cleavage sites were abbreviated as follows: Ba, *Bam*HI; Ec, *Eco*RI; Bg, *Bgl* II; Sl, *Sal* I; Pv, *Pvu* I; Sc, *Sac* II; Sm, *Sma* I. The *TK* gene is transcribed within *Bam*HI Q from position +1 and translated from +110; it terminates at about +1,300, before the *Eco*RI site (25, 26). The domains and orientations of the components of the chimeric *TK* fragments are as shown. The inset box summarizes the induction of these genes by B2006 and by ts502Δ305 at 39°C, from the results in Fig. 3, as the maximum-fold increase in TK activity above the baseline level. (ND, not done.) P_4 and $R_{\alpha 4}$ refer to promoter-competent and regulatory regions of α gene *4* deduced from this study. The following describes the plasmid constructions of the genes used in this study: *103* [previously cloned as pRB103 (17)], the HSV-1 *Bam*HI Q fragment in the *Bam*HI site of pBR322; *701* (cloned as pRB701), the *Eco*RI fragment from *Bam*HI Q in the *Eco*RI site of pACYC184; *407* (cloned as pRB407), the *Bgl* II/*Bam*HI fragment from *Bam*HI Q in the *Bgl* II/*Bam*HI sites of pKC7; *316–407* [previously cloned as pRB316 (14)], the *Bam*HI N fragment in the *Bgl* II site of pRB103 (*Bam*HI Q); *364–407* (cloned as pRB364), the *Bgl* II/*Bam*HI fragment from pRB407 fused to the *Bam*HI/*Sma* I fragment of *Bam*HI N-containing nucleotides +33 to −330 of α gene *4*, in pBR322. This construction necessitated that the plasmid contains, in addition, 100 bp from adjacent to the unique *Pvu* II site of *Bam*HI N—which is within the coding sequence of α gene *22* (5); *377–407* (cloned as pRB377), the −160-bp to +33-bp (*Sac* II/*Bam*HI) fragment of α gene *4* (from *Bam*HI N) in the *Bgl* II site of pRB407; *332–407* (cloned as pRB332), the *Eco*RI (chimeric *TK*) fragment from pRB316 in the *Eco*RI site of pACYC184. This includes α gene *4* sequences up to −110 bp; *360–701* (cloned as pRB360), the *Eco*RI fragment from pRB701 fused to the large (−110 to −4,500) *Eco*RI/*Bam*HI fragment from *Bam*HI N, in pBR322; *385–407*, the *Pvu* I/*Eco*RI (chimeric *TK*) fragment from pRB332 fused to the large (−110 to −4,500) *Eco*RI/*Bam*HI fragment from *Bam*HI N, in pBR322.

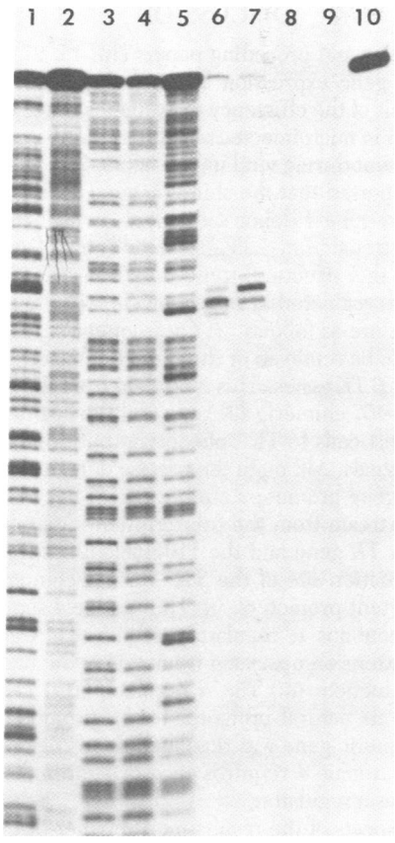

FIG. 2. Autoradiogram of the products of S1 nuclease and exonuclease VII digestion of $\alpha 4$ mRNA·HSV-1 DNA hybrids. Poly(A)$^+$ RNA from infected cells was hybridized to the appropriate strand of 5′-^{32}P-end-labeled *Sal* I/*Eco*RI DNA, which spans the *Bam*HI Y–N junction as shown in Fig. 1*B*. The $\alpha 4$ mRNA·DNA hybrid and the control DNA hybridized by itself were digested with either S1 nuclease or exonuclease VII. The products were subjected to electrophoresis on an 8% polyacrylamide/8.3 M urea gel. The products of base-specific chemical sequence analysis reactions from the same DNA that was used for hybridizations were also run on the gel to serve as size markers (lanes 1–5), along with the intact, untreated DNA that was used. Lane 1, G reaction; Lane 2, A+G reaction; Lane 3, C+T reaction; Lane 4, C reaction; Lane 5, A>C reaction; Lane 6, S1 nuclease digest of RNA·DNA hybrids; Lane 7, exonuclease VII digest of RNA·DNA hybrids; Lane 8, S1 nuclease digest of DNA hybridized in the absence of RNA; Lane 9, exonuclease VII digest of DNA hybridized in the absence of RNA; Lane 10, intact, untreated DNA.

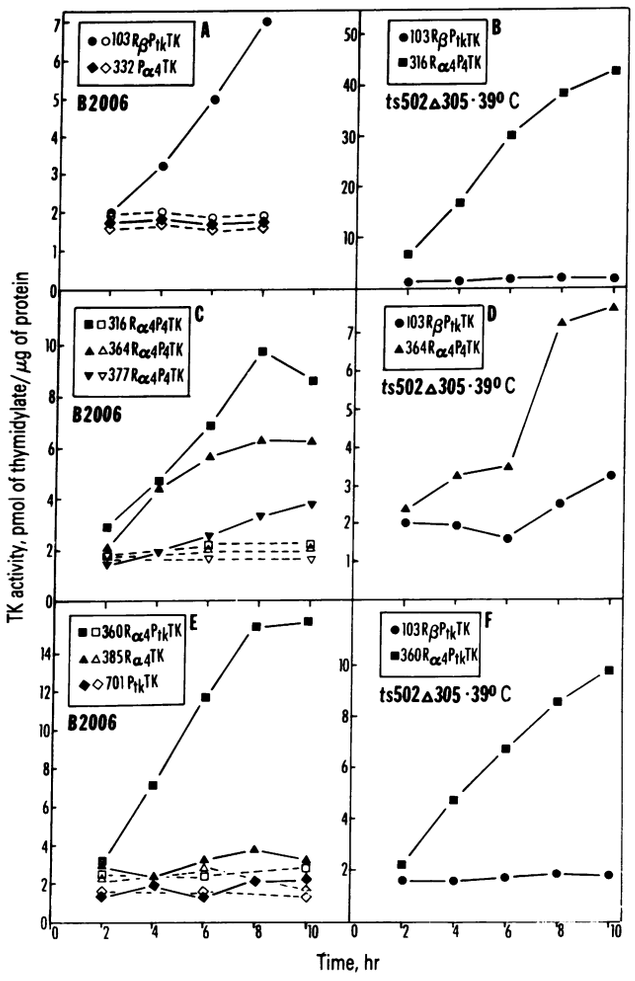

FIG. 3. Induction of natural and chimeric *TK* genes after infection with the TK$^-$ viruses HSV-1(C101)B2006 (*A*, *C*, and *E*) or ts502Δ305 (*B*, *D*, and *F*). Cells converted to TK$^+$ phenotype with the plasmid DNAs (indicated in the inset boxes) were superinfected with five B2006 or ts502Δ305 virus plaque-forming units per cell. Extracts from cells harvested at the times shown after infection were assayed for TK activity by conversion of either [^{3}H]thymidine or [^{14}C]thymidine into thymidylate. In the experiments illustrated in *B*, *D*, and *F*, the cells were exposed to virus for 1 hr at 10°C and then were transferred to 39°C (zero time). The level of TK activity present in cells at different times after infection is expressed as the number of pmol of thymidine phosphorylated per μg of protein in 30 min at 37°C. Closed symbols designate time points for infected cell lines and open symbols designate time points for mock-infected cell lines. The cell lines (inset boxes) were designated according to the numerical designations of the natural or chimeric *TK* fragment shown in Fig. 1, followed by a descriptive designation of the component of the *TK* gene: $R_{\alpha 4}$, α regulator region; R_β, β regulator; $P_{\alpha 4}$, $\alpha 4$ promoter region; and P_{TK}, β TK promoter region. The induction in enzymatic activity by infection with B2006 indicates that the resident *TK* gene responds to viral regulatory signals; induction by ts502Δ305 indicates that the *TK* gene is regulated as an α gene.

ment *332*, *377*, *364*, or *316*. The donor fragment (Fig. 1) extends from nucleotide +33 to nucleotide −110 (*332*), or −160 (*377*), or −330 (*364*), or −4,500 (*316*). The cell lines that converted to TK$^+$ phenotype by the chimeric genes were infected with B2006 to determine whether the resident *TK* gene responded to viral regulatory signals and with ts502Δ305 (a TK$^-$, ts α gene *4* virus) at 39°C to differentiate between α and β regulation. Cells that contained the wild-type β *TK* gene (*103*) served as a control for differentiation between α- and β-regulated TK. The results (Fig. 3) summarized in Fig. 1 show the following. (*i*) The α gene *4* regulatory region was apparently inactivated or removed from the *332–407* chimera because the TK activity did not increase upon infection with TK$^-$ virus (Fig. 3*A*). (*ii*) The regulatory regions in the TK$^+$ cell lines converted by all other chimeras in this series were functional in that the resident TK was induced after infection with TK$^-$ virus. In the instances tested (*364–407*, Fig. 3*D*, and *316–407*, ref. 14) the chimeric *TK* indicator genes were regulated as α genes. (*iii*) As shown in Fig. 3*C* and Fig. 1, the extent of induction was proportional to the number of nucleotides extending upstream from nucleotide −110.

The second series of experiments was designed to test whether the putative α regulatory region (upstream of position −110) was physically separable from the α promoter region. The design was based on the observation that *701* (Fig. 1) converted TK$^-$ cells to TK$^+$ phenotype and therefore retained some promoter function but did not respond to viral regulatory signals (Fig. 3*E*). The *360–701* chimeric *TK* gene (Fig. 1) responded to regulatory signals as an α gene (Fig. 3 *E* and *F*) in converted TK$^+$ cells. The *385–407* chimeric *TK* gene (Fig. 1) was con-

structed to test whether the α-regulatory sequence required an exogenous promoter region for its function. This chimeric gene contains the α sequences extending from +33 to −4,500 fused to the *407* indicator TK, but the putative α promoter region (−1 to −110) has been deleted. Although this chimera converted cells from TK^- to TK^+ phenotype, it did not respond to viral regulatory signals (Fig. 3*E*).

The Nucleotide Sequence of the α Gene *4* Promoter–Regulator Region. To determine the structural characteristics of the α promoter and regulator regions, the nucleotide sequence of the promoter–regulator region that is contained between +33 and −330 in the domain of α gene *4* was determined according to the strategy shown in Fig. 4*B*. To emphasize features of potential interest, the nucleotide sequence of the nontemplate strand is shown in Fig. 4*A* as if it were able to form cruciform structures by intrastrand hybridization of nearby inverted repeated sequences.

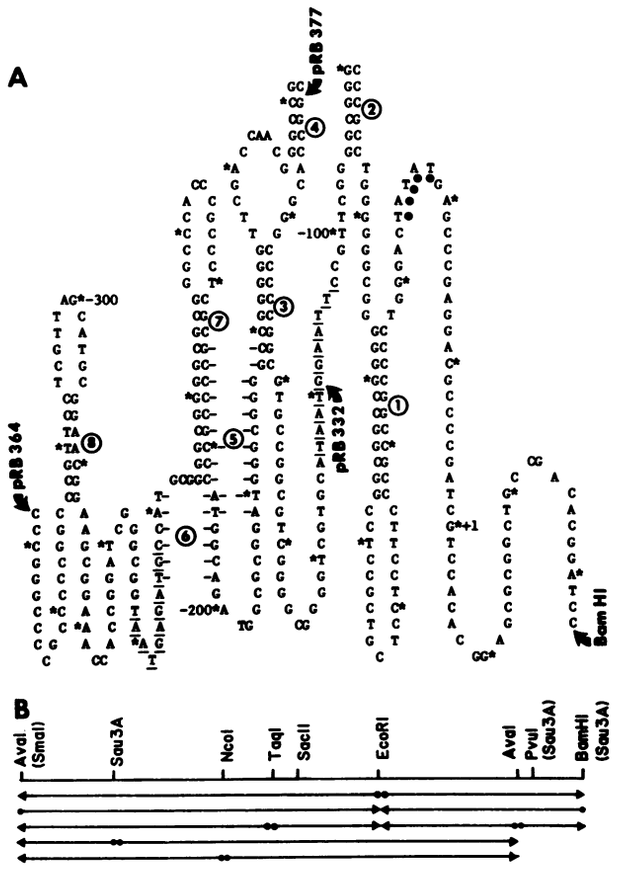

FIG. 4. Nucleotide sequence of the first 330 bp upstream from the transcription–initiation site of α gene *4*. (*A*) The sequence of the nontemplate strand, shown in serpentine form, extends from the *Sma* I site at −330 bp (far left) to the *Bam*HI site at +33 bp (far right). The position denoted as +1 identifies the most likely site of initiation of transcription of α gene *4* and is 26 bp downstream from T-A-T-A-T—a homologue of the T-A-T-A-A-A sequence (reviewed in ref. 25), denoted by large dots. The arrows indicate the 5′ terminus of α *4* sequences contained in the chimeric plasmids pRB332, pRB377, and pRB364. The major inverted repeated sequences are shown in cruciform structures designated as 1 through 8. The underscored, homologous, repeated sequence occurring at positions −103 to −113 and at positions −252 to −262 is discussed in the text. (*B*) Top line, restriction enzyme map of the sequence shown in *A*. The map is in the same orientation as the sequence presented in *A* but is in opposite orientation relative to the map shown in Fig. 1. Below the map, the sequence strategy used is shown schematically. The dots represent labeled termini that were sequenced in the direction of the arrows extending from them.

DISCUSSION

The focus of this and preceding papers (14, 15, 27) was on the regulation of gene expression by viral signals, in contrast to determinations of the efficiency of promoter usage in TK^+-converted cells or in microinjected oocytes in the absence of signals normally present during viral infection (28, 29). This laboratory previously reported that the chimeric gene (*316–407*), created by fusion of α gene *4* donor sequences—which consisted of a sequence upstream from +33—to an indicator *TK* gene—which consisted of the structural gene and transcribed leader sequences—was regulated as an α gene (14). The salient features of our results are as follows. (*i*) The sequences responsible for regulation can be removed or their function inactivated in both α gene *4* and β *TK* genes. This is evident from the observations that the *332–407* chimeric *TK* gene and the *701* natural *TK* indicator convert cells to TK^+ phenotype but do not respond to regulatory signals. Although the precise domains of the α gene *4* and β *TK* gene promoters are uncertain, the residual 80 nucleotides upstream from the transcription initiation site of the *701* indicator *TK* gene and the 110 nucleotides from the transcription–initiation site of the *332–407 TK* chimera appear to act as competent promoters. (*ii*) The α gene *4* sequence −110 to −4,500 contains α regulatory sequences. Generally, the longer the extension upstream from −110, the higher was the observed induction. (*iii*) The α regulator in α gene *4* can be moved from its natural promoter to a β promoter. Thus, the *360–701* chimeric gene was regulated as an α gene. (*iv*) The α regulator in α gene *4* requires an α or β promoter region for its function as a regulator.

Several aspects of the results merit further discussion.

(*i*) Of the two apparent functions of the 5′ nontranscribed regions of viral genes, the regulator functions appear to be more specific and more readily operationally defined than is the promoter function. In some experiments the *407* fragment converted cells to TK^+ phenotype at low frequency and fusion of random fragments to the *407 TK* indicator gene also yielded several chimeric genes that converted cells. None of these gene fragments and chimeric genes responded to regulatory signals (unpublished results). At issue is the problem of differentiating true promoters from sequences that mimic but are not physiologic promoters. Conceivably the ability to respond to regulator sequences, as illustrated in this paper, is a suitable operational definition of a physiologic promoter.

However, we should note that though inactivation of the regulator function is readily accomplished, the ability to separate regulator and promoter domains may not be a general property of regulatory sequences. The work of other investigators (30) suggests that the β *TK* regulator–promoter domains may overlap.

(*ii*) The results of this and previous papers (14, 15, 27) suggest that α gene regulation involves three components: a *trans*-acting regulatory signal, a *cis*-acting regulator sequence, and a promoter sequence. The α gene *4* regulator sequence (located at or within the fragment −110 to −4,500), in the absence of the natural α or β promoter sequence (e.g., *385–407*, Fig. 3), allowed TK expression in converted cells. However, the chimeric gene did not respond to viral regulatory signals, suggesting that the promoter-like function encoded in that fragment was not adequate or sufficient for the expression of the *cis*-acting function of the regulatory sequence. In contrast, when the regulator segment is moved to an expressible but noninducible β *TK* gene, the resulting chimera (*360–701*) is regulated as an inducible α gene.

(*iii*) The observation that extending the sequences upstream beyond the −110 nucleotide of the α gene *4* promoter region

heightens the response to regulatory signals must be viewed in light of the nucleotide sequence of the promoter–regulator region of α gene *4* (Fig. 4) and of α genes *0* and *27* (unpublished results).

Thus, the only expected feature of the nucleotide sequence in Fig. 4 is the T-A-T-A-T sequence; the striking feature of the sequence, and of the other α gene sequences, is the high G+C content and the relatively large number of cruciform structures that could form (unpublished results). Comparison of the α nucleotide sequences contained in the *TK* chimeras *364–407*, *377–407*, and *332–407* suggests that the regulator sequence may consist of multiple quasi-equivalent regulator units and that the extent of induction may be proportional to the number of such units. Comparison of the nucleotide sequence in this and in other α gene promoter–regulator regions suggests—but does not yet prove—that the regulator unit may comprise a consensus sequence T-A-A-T-G-A-R-A-T-T-C (R = purine) and may also be related to the presence of G+C-rich inverted repeats. The promoter–regulatory region of α gene *4* contains two homologues of the consensus sequence between −110 and −330 (Fig. 4) and none is present in its entirety between −1 and −110. As noted earlier, the TK^+ cells converted by the *332–407* chimera that contains the +33 to −110 sequence of the α gene *4* donor were not inducible by infection with TK^- virus.

(*iv*) Homologues of the "consensus" sequence described above have been noted in single or in multiple copies in the promoter–regulator domains of other α genes, always upstream from inverted repeats capable of forming cruciform structures of the type shown in Fig. 4 (unpublished results). The stability of these cruciform structures is uncertain and may require the presence of proteins capable of modifying HSV DNA structure. Studies in progress on additional chimeras involving these sequences may elucidate their role, if any, in the regulation of α gene expression.

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