JOURNAL OF VIROLOGY, Sept. 1989, p. 4088–4092
0022-538X/89/094088-05$02.00/0

Vol. 63, No. 9

Examination of the Roles of Transcription Factor Sp1-Binding Sites and an Octamer Motif in *trans* Induction of the Herpes Simplex Virus Thymidine Kinase Gene

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Received 4 January 1989/Accepted 17 May 1989

Herpes simplex virus mutants with both Sp1-binding sites in the thymidine kinase (*tk*) promoter inactivated or an octamer motif deleted were at most modestly impaired for *tk* expression. Thus, no cellular transcription factor that binds upstream of the *tk* TATA box is solely required for *trans* induction of this gene.

The promoter of the thymidine kinase (*tk*) gene of herpes simplex virus (HSV) consists of a TATA box and distal signals that include two G+C-rich sequences that bind transcription factor Sp1 and a CCAAT motif that is able to bind several different factors (reviewed in references 18 and 26). Additionally, Parslow et al. (27) have identified an octamer motif, ATTGCAT, that was required for full *tk* promoter function in transfected rodent cells. This motif also binds cellular transcription factors (18). The locations of these promoter domains are shown in Fig. 1.

During HSV infection, prior expression of viral immediate-early genes is stringently required for *tk* expression (8, 11, 20, 28, 29) and for expression of other "delayed-early" genes (15). In this setting, a mutation that interferes specifically with HSV *trans* induction should decrease *tk* expression drastically, to an extent comparable to a cycloheximide block of immediate-early protein expression. When linker-scanning (LS) mutations—clustered point mutations that substitute wild-type sequence with restriction enzyme linkers—were introduced into the *tk* promoter present in the HSV genome (7), the only mutation that decreased *tk* expression more than 10-fold was LS −29/−18, which replaces the *tk* TATA box with a *Bam*HI linker. Mutations in the Sp1 and CCAAT motifs and a mutation just downstream from the *tk* mRNA start site exerted more modest effects on *tk* expression. Thus, no new promoter domain that could fully account for *trans* induction was identified. These results suggested that *trans* induction of *tk* expression is mediated via interactions of immediate-early regulatory proteins with cellular transcription factors.

Several observations made attractive the hypothesis that increases in the activity or availability of Sp1 mediate *trans* induction: (i) experiments in which *tk* promoter sequences were fused to the "true late" *gC* gene suggested that the *tk* TATA box is not sufficient to mediate *trans* induction and that *tk* distal signals are required for early *tk* expression (14); (ii) sequences corresponding to the Sp1 consensus sequence (2) can be found upstream of many HSV delayed-early genes, whereas CCAAT motifs are missing from several of these (34, 38), suggesting an important role for Sp1; (iii) LS mutations that eliminated the CCAAT motif decreased *tk* expression only modestly during HSV infection (7), arguing against an essential role for factors binding this site; and (iv) there are two Sp1 consensus sequences in the *tk* promoter. Thus, if Sp1 activity or availability is increased during HSV infection, a single site might be enough to induce transcription of *tk*. This hypothesis predicts that inactivation of both Sp1 consensus sequences would reduce *tk* transcription drastically.

In addition, none of the original set of LS mutations introduced into HSV (7) altered the octamer motif that was reported to be important for *tk* promoter function in transfected rodent cells (27). We wished to test the role of this and other sequences between positions −116 and −197 upstream of the *tk* gene.

Construction of mutants. As a first step in constructing virus mutants with both Sp1 sequences inactivated, plasmids carrying a single LS mutation in the more distal Sp1 site in the *tk* promoter (LS −119/−109 and LS −111/−101) were recombined with similar plasmids containing a single LS mutation in the more proximal Sp1 site (LS −59/−49 and LS −56/−46) linked to a *tk* gene carrying a temperature-dependent drug resistance marker (tk^{ts}) (7), by a modification of the strategy of McKnight (24). Plasmid pdl −197/−116, which lacks the octamer motif, was constructed from plasmids pKG3.6 (16), which contain the tk^{ts} gene cloned into pXf3 (13), and pLS/ts −119/−109 (7). pLS/ts −119/−109 was digested with *Bam*HI, and the ends were filled in with the Klenow fragment of DNA polymerase I. The DNA was then digested with *Pvu*II, and a 2-kilobase-pair (kbp) *Pvu*II-*Bam*HI blunt-ended fragment was then ligated into *Pvu*II-cleaved pKG3.6. After transformation of *Escherichia coli*, colonies containing plasmids with the mutations were identified by restriction enzyme analysis.

Each plasmid DNA was used in transfections of Vero cells as described by Chiou et al. (4), with infections HSV DNA from antimutator mutant PAA^r5 (12). Recombinant viruses were then selected for growth in the presence of acyclovir at 39°C (7). Viral DNAs were prepared and analyzed for the presence of the more distal LS mutations or the deletion mutation by Southern blotting and hybridization with radiolabeled *tk* probe (7). The presence of both LS mutations in the double LS mutants was confirmed by Southern blotting experiments in which virion DNA was double-digested with *Sst*I and *Bam*HI (unpublished results). Mutant viruses were plaque purified a minimum of three times. No wild-type *tk* genes could be detected by Southern blotting.

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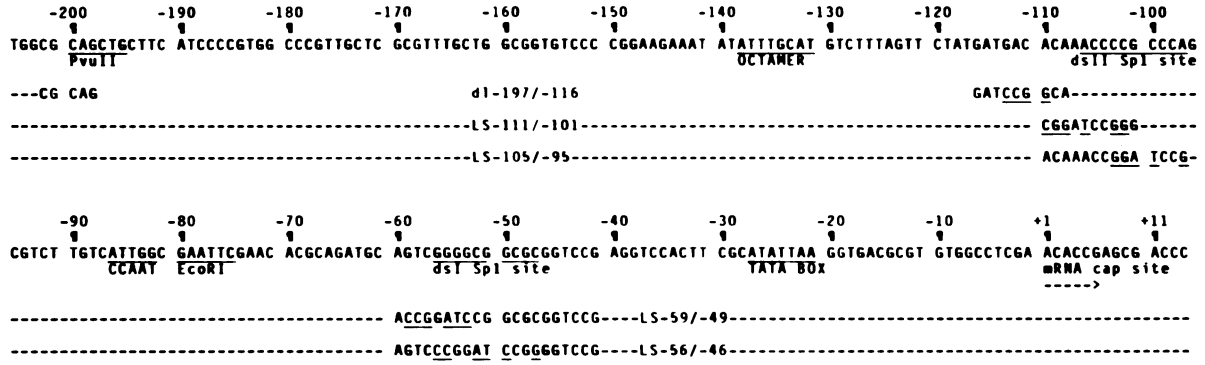

FIG. 1. Locations of LS mutations and promoter elements and transcription factor-binding sites in the *tk* promoter. The nucleotide sequence of HSV DNA in the region of the *tk* promoter is shown, with the nucleotide positions relative to the mRNA cap site at +1 listed above the sequence. Below the sequence are shown relevant restriction sites and the locations of the octamer, CCAAT, TATA box, mRNA cap site, and the two Sp1-binding sites, the more distal one in distal signal II (dsII), the more proximal in distal signal I (dsI). Along the dashed lines below the sequence are shown the locations of the mutations in *dl* −197/−116 and the individual LS mutations in the Sp1 sites.

To ensure that adventitious mutations affecting *tk* expression unrelated to the LS mutations or to the deletion mutation had not been introduced into the mutant viruses, two independent viral recombinants derived from each mutant plasmid were isolated and shown to be indistinguishable in terms of *tk* expression, as measured by drug sensitivity (see below).

Effects of double LS mutations on *tk* expression: pharmacological analysis in intact infected cells. The antiviral drug ganciclovir (DHPG) requires TK for its antiviral activity (3, 31, 32). With viruses that contain the tk^{ts} mutation, decreases in DHPG sensitivity at 34°C correlate roughly with decreases in levels of *tk* mRNA and polypeptides (6, 7). The double LS mutants all exhibited similar sensitivities to DHPG, with doses of DHPG that reduced plaque formation by 50% (ED_{50}s) only slightly higher than that of HSV LS −16/−6, which expresses *tk* mRNA at the wild-type level (7). None was as resistant as HSV LS −29/−18, which contains a mutation in the TATA box of the *tk* gene (Table 1). These results indicated that none of the four double LS mutations drastically affected TK enzyme expression.

Analysis of *tk* mRNA levels. We then measured *tk* expression in terms of *tk* mRNA levels 4.5 h after infection relative to those of another delayed-early mRNA, that encoding the major DNA-binding protein (ICP8; DBP), which served as an internal control. Vero cells were infected at a multiplicity of 2.5 and incubated at 37°C for 4.5 h. Total cellular (pooled cytoplasmic and nuclear) RNA was extracted after lysis of the cells with Nonidet P-40 in the presence of vanadyl ribonucleoside complexes essentially as described previously (21). To quantitate relative levels of *tk* and *dbp* mRNAs, S1 nuclease protection assays were performed as described previously (38) with an 0.8-kbp *Bam*HI-*Bgl*II *tk* probe, 5′-end labeled at the *Bgl*II site at position +57 in the *tk* gene (23, 35) combined with an 0.4-kbp *Bam*HI-*Bst*EII *dbp* probe, end labeled at the *Bst*EII site at position +89 in the *dbp* gene (34). Neither probe generated radiolabeled species after hybridization with mock-infected RNA and S1 digestion or species comigrating with species generated from the other after hybridization with infected-cell RNA and S1 digestion (Fig. 2A). Moreover, the conditions used to detect the *tk* and *dbp* mRNAs simultaneously yielded signals similar to those obtained when each probe was used separately, and hybridization signals were linear with the amount of RNA added (not shown). Levels of *tk* mRNA expressed by the double LS mutants were 5- to 10-fold lower than those expressed by HSV LS −16/−6. However, they were at least 10-fold higher than those expressed by HSV LS −29/−18, the TATA mutant (Fig. 2B, Table 1).

Analysis of *tk* transcription rates. The effects of the double LS mutations on *tk* transcription rates were determined in a nuclear run-off assay (19, 36) with nuclei prepared from Vero cells 4.5 h postinfection at a multiplicity of 2.5 PFU/cell.

TABLE 1. Relative levels of *tk* expression of HS mutants in different assays[a]

Mutant	Description[b]	Drug sensitivity[c]	S1 assay[d]	Nuclear run-off[d]
LS −16/−6	Wild-type control	1.0	1.0	1.0
LS −111/−101	LS in dsII Sp1	0.8	0.6	0.9
LS −105/−95	LS in dsII Sp1	**0.3**	**0.5**	**0.2**
LS −59/−49	LS in dsI Sp1	**0.5**	0.8	0.6
LS −56/−46	LS in dsI Sp1	**0.4**	**0.5**	**0.4**
LS −29/−18	LS in TATA box	**0.08**	**<0.01**	**0.06**
LS −111/−101, −59/−49	Double LS in both Sp1 sites	**0.4**	**0.1**	**0.2**
LS −111/−101, −56/−46	Double LS in both Sp1 sites	**0.4**	**0.1**	**0.2**
LS −105/−95, −59/−49	Double LS in both Sp1 sites	**0.5**	**0.2**	**0.3**
LS −105/−95, −56/−46	Double LS in both Sp1 sites	0.7	**0.2**	**0.3**
dl −197/−116	Deletion of octamer and other upstream sequences	1.0	0.7	0.8

[a] Values in boldface indicate reductions in *tk* expression that were regarded as meaningful.

[b] dsI and dsII, Distal signals I and II, respectively.

[c] Determined as dose required to reduce plaque formation by 50% (ED_{50}) of HSV LS −16/−6 divided by ED_{50} of mutant.

[d] Shown as *tk* expression of mutant divided by *tk* expression of HSV LS −16/−6, both normalized to an appropriate internal control. Preflashed X-ray film was exposed to filters or dried gels at −80°C with two enhancing screens. To ensure a signal in the linear range of the film, several exposures were taken. The films were then scanned on an LKB laser scanner, and the integrals of the peaks were computed with the scanner software. For mRNA levels, the ratio of the *tk* and *dbp* signals was calculated. Transcription rates were determined by linear regression analysis from the signals of the four dilutions of each probe. Correlation coefficients were equal to or greater than 0.96. To calculate relative rates, the ratio of the signals for *pol* and *tk* was determined, each at a theoretical value of 200 ng of probe. The mRNA level and transcription rate values were then standardized, with the values determined for HSV LS −16/−6 taken as 1.0 (7).

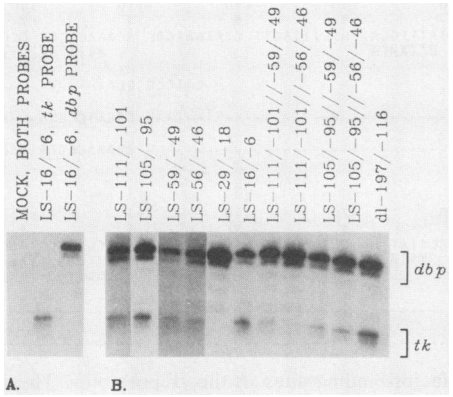

FIG. 2. Expression of *tk* mRNA by LS and deletion mutants. Shown are autoradiographs of polyacrylamide gels of S1 digestion products following hybridization with the RNA from cells infected with the indicated viruses. The locations of signals corresponding to *dbp* and *tk* mRNAs are indicated to the right. (A) Mock-infected-cell RNA was hybridized with both probes, while RNA from cells infected with HSV LS −16/−6 was hybridized individually with the *tk* or *dbp* probe, as indicated. (B) Infected-cell RNAs were hybridized with both probes.

These data were normalized to the amount of transcription from the viral DNA polymerase (*pol*) gene, another delayed-early gene. To verify the absence of nonspecific hybridization of labeled RNA to any G+C-rich DNA sequence, DNA of the chicken *tk* gene was included as a probe in all hybridization reaction mixes (36). Under the conditions of this assay, a *tk* TATA box mutation severely decreased *tk* transcription (Fig. 3), and no transcription was detected from the *tk* coding strand upstream of the *tk* mRNA cap site in any of the mutants (data not shown). Thus, *tk* transcription as measured was specific to the *tk* promoter. In this assay, nuclei from cells infected with the double LS mutants transcribed the *tk* gene at a rate similar to nuclei from cells infected with single LS mutant HSV LS −105/−95, while mutations in the TATA box reduced *tk* transcription much more dramatically (Fig. 3, Table 1). Together, the results of all three assay systems indicated that although the double LS mutations reduced *tk* expression, they did not do so drastically.

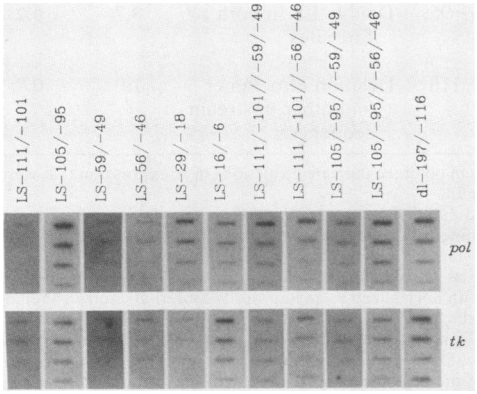

FIG. 3. *tk* transcription by HSV LS and deletion mutants. Nuclear run-off transcription assays were performed with nuclei from cells infected with the indicated mutants. The radiolabeled transcription products were hybridized to four different dilutions of single-stranded DNA from the *pol* gene (top panels) and the *tk* gene (bottom panels).

Construction and analysis of an upstream promoter deletion mutant. Mutant *dl* −197/−116, which lacks the octamer motif at position −135 and surrounding sequences, was analyzed for its *tk* expression as described for the double LS mutants. Its sensitivity to DHPG was indistinguishable from that of HSV LS −16/−6 (Table 1). *tk* mRNA levels 4.5 h postinfection were at most slightly reduced (Fig. 2, Table 1), and the *tk* transcription rate of *dl* −197/−116 was also only minimally affected (Fig. 3, Table 1). In summary, the elimination of nucleotides −117 to −197 did not meaningfully impair expression of the viral *tk* gene. These results are consistent with results derived from superinfection studies in mouse cells (9, 10).

Inactivation of the two Sp1 sites does not abolish *tk* induction. The double LS mutations we constructed should effectively inactivate both Sp1 sites. Each of the single LS mutations alters its Sp1 site so that it no longer fits even the relatively degenerate Sp1 consensus decanucleotide (2). Mutation LS −111/−101 abolishes Sp1 binding in a DNase footprint assay (17), while LS −59/−49 and LS −56/−46 drastically reduce Sp1 binding in band shift assays (N. DeLuca, personal communication). Yet none of the four double LS mutants exhibited drastically reduced *tk* expression. The greatest decrease observed was at least 10-fold less than that observed in the same assay with the TATA mutant HSV LS −29/−18 and is far less than that observed in cycloheximide reversal experiments (20, 28). These results argue strongly that *trans* induction does not require factors binding to the Sp1 sites.

LS −111/−101 and LS −59/−49 exerted no more than minimal effects on *tk* expression from HSV (7; this report). This raised the possibility that a factor other than Sp1 recognized its binding sites during HSV infection. If this were true, one would predict that when these two mutations were combined, they would still exert only a minimal effect. Instead, they exerted a substantial effect (Table 1). Thus, the results are consistent with Sp1 playing a role in *tk* expression during HSV infection. It seems likely that Sp1 interacts with its binding sites primarily to increase promoter strength rather than as the principal mediator of *trans* induction.

The octamer motif is not important for *tk* induction in infected Vero cells. In their study reporting the presence of an octanucleotide motif at −135, Parslow et al. (27) recommended that its influence in various experimental systems be assessed. The deletion mutation in *dl* −197/−116 did not exert meaningful effects on *tk* expression from HSV, which argues that these sequences are not important for *tk* induction in this setting. The −117 endpoint of this deletion is identical to that of the deletion mutation reported by Parslow et al. (27) to decrease *tk* promoter function in transfected rodent cells. A search of HSV sequences upstream of −197 (22; J. Jacobson and D. Coen, unpublished results) found no new octanucleotide motif brought close to the *tk* promoter by the deletion. We conclude that the octamer motif is not important for *tk* induction during HSV infection of Vero cells in culture. On the other hand, sequences as A+T-rich as the octamer sequence are unlikely to occur by chance in the G+C-rich HSV genome. Perhaps the octamer motif plays another role in HSV gene expression, for example, late in infection or during infections of various tissues of mammalian hosts by HSV.

What sequences mediate *tk* induction? In the experiments reported here and previously (7), each of the binding sites for cellular transcription factors upstream of the *tk* TATA box—the two Sp1 sites, the CCAAT motif, and the octamer motif—has been inactivated. In each case, *tk* expression

continued at substantial levels, well above those obtained when *tk trans* induction by immediate-early proteins is prevented. We conclude that no single cellular transcription factor that recognizes sequences upstream of the TATA box is required for *tk trans* induction.

These results leave open the question of which sequences mediate *tk* induction. One interpretation of our data is that *tk*-distal signals do mediate *tk* induction and that either a CCAAT motif or an Sp1 site is sufficient. We cannot exclude this interpretation or more complex versions in which factors recognizing several different sites within, upstream, or downstream of the *tk* promoter mediate *trans* induction of *tk* expression.

We suggest, however, a simpler model—that *trans* induction of *tk* expression is mediated via the *tk* TATA box. An appealing aspect of this model is its similarity to the results of Homa et al. (14) on the expression of the HSV late gene, *gC*, in which a 15-bp sequence including the *gC* TATA box was required for expression of the *gC* gene, dependent upon viral DNA replication. Thus, the induction of both delayed-early genes like *tk* and late genes like *gC* would proceed via TATA boxes. Documented differences in the *tk* and *gC* TATA boxes (14) would contribute to the differences in regulation of the two genes. By this model, the ability of *tk*-distal signals linked to the *gC* TATA box to drive *gC* expression early in infection (14) could be due to the increased activity of a weak promoter that ordinarily requires DNA replication and/or additional *trans*-acting factors to activate it.

Homa et al. (14) failed to detect *gC* mRNA expressed from a virus in which the *gC* gene was placed downstream of sequences including the *tk* TATA box. They suggested that the *tk* TATA box is not sufficient to mediate *trans* induction. However, this virus construct also lacked sequences encoding the *gC* mRNA 5′ untranslated region that are required for efficient expression of *gC* (14). Removal of these sequences led to a fivefold decrease in *gC* expression. This effect plus the effect of removal of *tk*-distal signals could have reduced *gC* mRNA expression below the level of detection.

There are now several examples from other systems in which *trans* induction appears to be mediated via the TATA box (1, 30, 33, 37). These studies in other systems suggest experiments to test whether HSV *trans* induction of *tk* is mediated by factors that bind to its TATA box sequences.

We are indebted to S. Weinheimer for cheerful instruction and assistance in performing and analyzing nuclear run-off transcription assays, D. Yager for help with the S1 analyses, E. Zylstra and J. Scales for figure preparation, N. DeLuca for communicating unpublished results, and N. DeLuca and D. Yager for suggestions on the manuscript.

This work was supported by American Cancer Society research grant MV-242 and by Public Health Service grants AI26126 and BRSG 507 RR 05381-27 from the National Institutes of Health.

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