A Genetic Approach to Promoter Recognition During trans Induction of Viral Gene Expression

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Viral infection of mammalian cells entails the regulated induction of viral gene expression. The induction of many viral genes, including the herpes simplex virus gene encoding thymidine kinase (tk), depends on viral regulatory proteins that act in trans. Because recognition of the *tk* promoter by cellular transcription factors is well understood, its trans induction by viral regulatory proteins may serve as a useful model for the regulation of eukaryotic gene expression. A comprehensive set of mutations was therefore introduced into the chromosome of herpes simplex virus at the *tk* promoter to directly analyze the effects of promoter mutations on *tk* transcription. The promoter domains required for efficient *tk* expression under conditions of trans induction corresponded to those important for recognition by cellular transcription factors. Thus, trans induction of *tk* expression may be catalyzed initially by the interaction of viral regulatory proteins with cellular transcription factors.

THE SELECTIVE EXPRESSION OF MESSENGER RNA (MRNA) in eukaryotic cells results largely from events that control the transit of RNA polymerase II molecules across structural genes. This regulation is mediated in part by interactions between cellular transcription factors and cis-acting DNA sequences (*1*). One class of such DNA sequences is located in the vicinity of the transcription start site of eukaryotic structural genes. These sequence components have been termed promoters, in agreement with the nomenclature adopted by prokaryotic geneticists. Eukaryotic promoters have been analyzed principally by studies in which individual genes are removed from their genomes, altered in vitro by site-directed mutagenesis, and assayed for their ability to direct the accumulation of mRNA or protein products in various expression systems.

A eukaryotic promoter that has been analyzed in considerable detail is that of the herpes simplex virus (HSV) thymidine kinase (*tk*) gene. The *tk* gene can be excised from the viral chromosome and expressed at a "basal" level in the absence of other viral gene products. Analyses of the *tk* promoter in basal expression environments have been performed primarily by measuring mRNA accumulation in microinjected *Xenopus laevis* oocytes (*2–5*). Mutants of the *tk* promoter have also been scored for their ability to transform *tk*-deficient (tk^-) mouse L cells to the *tk*-positive (tk^+) phenotype (*2, 6*), as well as by their ability to direct accurate mRNA synthesis in soluble transcription extracts derived from uninfected HeLa cells (*7*). The cumulative results of these various experiments have shown that the *tk* promoter consists of at least four domains. These domains interact with at least three different sequence-specific DNA binding proteins that are probably common to many animal cell types (*5, 7*).

During the infectious cycle of HSV, the *tk* gene is expressed in a regulated fashion. In this setting, prior expression of other viral genes is stringently required for *tk* expression (*8*) and for expression of other "delayed early" HSV gene products (*9*). Analyses of the effects of temperature-sensitive and deletion mutations in the gene encoding the 175,000-dalton "immediate early" protein ICP4 have implicated ICP4 as a regulatory factor that is required for the induction of *tk* expression (*10, 11*). This result raises the question as to whether the *tk* promoter functions differently when excised from the HSV genome in a basal expression system than when it is embedded in the HSV genome and subject to inducers such as ICP4.

To address this question, we set out to establish a comparison of the promoter sequences required for *tk* mRNA expression in basal expression systems and during its regulated expression. To study *tk* promoter recognition under conditions of authentic regulation, we introduced a library of clustered point mutations into the *tk* locus of the HSV chromosome by homologous recombination. Because these same promoter mutations had been studied extensively in basal expression assays, their behavior under regulated conditions in an otherwise isogenic viral background provides a straightforward test of whether the *tk* promoter is recognized similarly, or differently, in the two expression systems. The viruses bearing promoter mutations at the *tk* locus also allow determination of whether mutations within a eukaryotic promoter directly affect transcription rates in cell nuclei. We have addressed this question by performing nuclear "run off" assays with each mutant virus.

Construction of HSV linker scanning mutants. We used 18 linker scanning (LS) mutations (*3*) that are a set of clustered point mutations that collectively alter approximately 70 percent of the residues within the *tk* promoter. To transfer the LS mutations to their analogous locations within the HSV genome, we employed a modification of marker transfer procedures developed by Smiley (*12*) and Post *et al.* (*11*). Because active tk enzyme is not essential for virus growth in cell culture, it is possible to replace the native allele of the HSV genome with a mutated *tk* gene. DNA fragments containing each LS mutation were joined to the protein-coding sequences of a mutant *tk* gene that specifies thermolabile tk activity

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(*13*) due to a truncated tk polypeptide that is unstable at 39°C (*14*). Viruses containing the mutant *tk* gene are fully resistant at 39°C to high concentrations of drugs such as acyclovir (ACV) that require tk for antiviral activity. We refer here to the mutant gene as temperature sensitive for *tk* (*tk*^{ts}).

We cotransfected Vero cells with individual chimeric LS-*tk*^{ts} plasmids and infectious *tk*$^{+}$ viral DNA purified from the HSV mutant, PAA^{r}5. We used PAA^{r}5 because it carries a mutation that increases fidelity of HSV DNA polymerase (*15*), thus yielding fewer spontaneous *tk*$^{-}$, ACV-resistant mutants. To select for virus bearing *tk*^{ts} alleles, progeny arising from the transfections were plated onto Vero cells in the presence of high concentrations of ACV at 39°C. Plaques resistant to ACV were picked, amplified, and screened by Southern blotting for the presence of the Bam HI linker sequence that marks the site of each LS mutation. The LS mutation was thereby introduced into the genome as an unselected marker linked to the *tk*^{ts} mutation (Fig. 1). An advantage of this procedure is that it permits the isolation of LS mutants regardless of the effect of LS lesions on *tk* gene expression. Isolates containing LS mutations were plaque-purified until no Bam HI fragments corresponding to the wild-type *tk* gene could be detected and then plaque-purified once more (a minimum of three times in each case). To ensure that adventitious mutations unrelated to the LS mutation were not introduced during the marker transfer procedure, we isolated from each chimeric LS-*tk*^{ts} plasmid at least two independent HSV derivatives that were indistinguishable in terms of *tk* expression measured by drug sensitivity at 34°C (see below).

Virion DNA from each HSV LS mutant was digested with Bam HI and the digestion products were separated on agarose gels, transferred to nitrocellulose, and probed with ^{32}P-labeled *tk* DNA. All of the mutants lacked the 3.6-kbp Bam HI *tk* fragment that is generated by digestion of wild-type HSV at the Bam HI sites at map

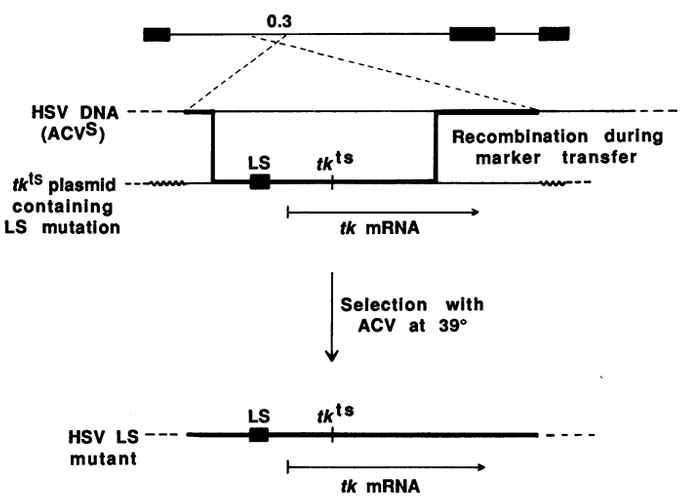

Fig. 1. Strategy for the introduction of LS mutations into the HSV chromosome. The top line is a schematic representation of an ACV-sensitive (ACV^{s}) HSV genome in the prototype arrangement with the major repeat sequences shown as black boxes. The location of the *tk* gene is indicated at map coordinate 0.3; the dashed lines connect to an expanded view of this region on the second line, presented by means of a bold line as participating in a recombination event during marker transfer with a chimeric LS-*tk*^{ts} plasmid indicated on the third line. The LS mutation is shown as a box and the location of the *tk*^{ts} mutation is indicated with a vertical line. The wavy lines indicate vector sequences. Below is an arrow indicating the location and orientation of *tk* mRNA coding sequences. Because the LS and *tk*^{ts} mutations are closely linked [<0.4 kb apart (*14*)], selection for recombination of the *tk*^{ts} mutation into HSV frequently leads to formation of virus containing both markers. The bottom line shows the HSV LS mutant that is the product of such a recombination event after selection for the *tk*^{ts} mutation with ACV at 39°C (*37*).

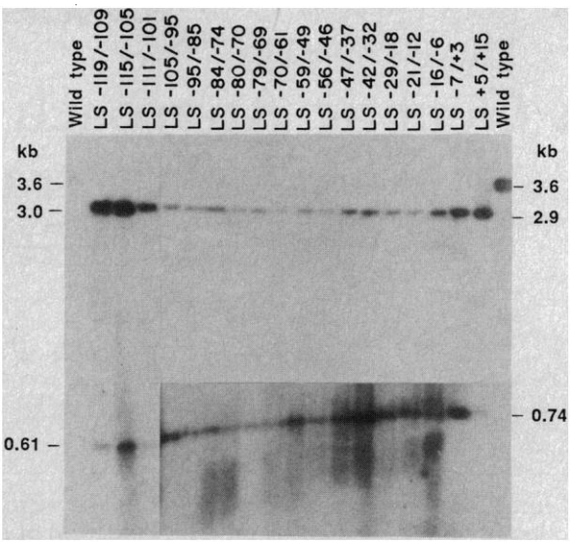

Fig. 2. Southern blot analysis of virion DNA of HSV LS mutants. HSV DNA was purified from virions prepared from stocks of each LS mutant and from wild-type strain KOS, digested with Bam HI, separated on a 1.4 percent agarose gel, and transferred to nitrocellulose (*38*). The filter was hybridized to ^{32}P-labeled *tk* DNA, washed, and exposed to x-ray film. The figure represents a composite of separate blots and exposures to allow visualization of both Bam HI fragments generated from the HSV LS DNA samples. The approximate molecular weights of the different DNA fragments are indicated according to molecular size markers.

coordinates 0.298 and 0.319 on the HSV genome (Fig. 2). Instead, each mutant generated two *tk* fragments, one ranging from 2.9 to 3.0 kbp and another smaller fragment ranging from 610 to 735 bp. The sequential decrease in size of the larger fragment and the concomitant increase in size of the smaller fragment (Fig. 2) is consistent with the location of the Bam HI linker as it progresses across the *tk* promoter in the library of LS mutants (*3*). The data indicate that each HSV LS mutant contains a new Bam HI site in the *tk* promoter, as well as the native Bam HI sites that normally flank the termini of the *tk* gene.

Analysis of *tk* expression in intact cells by HSV LS mutants. The effects of the HSV LS mutations on tk expression during viral infection were measured first in intact cells by a simple pharmacological approach. Decreases in tk activity usually render HSV correspondingly less sensitive to antiviral drugs that require tk for their activity (*14, 16*). Because each LS mutation was linked to a *tk*^{ts} gene, we expected LS mutations that reduced *tk* expression to confer reduced drug sensitivity at low temperature (34°C). To assay the LS mutants, we used the drug BW B759U (*17*) in plaque reduction assays. Typical data (Fig. 3) clearly indicate that mutant HSV LS −29/−18 (linker scanning mutation extending from 29 bp upstream of the mRNA cap site to 18 bp upstream) was less sensitive to BW B759U than mutants HSV LS −105/−95 and −84/−74, which were in turn less sensitive than mutant HSV LS −80/−70.

The relative drug sensitivities of all of the mutants are compiled in Table 1. Using previously established criteria (*18*), we were able to discern significant differences between a given mutant and an index mutant (Table 1) when the doses required to reduce plaque formation by 50 percent (ED_{50}) differed by more than 30 percent. The seven mutants that showed such differences also showed meaningful differences in other measures of *tk* expression.

Effects of LS mutations on accumulation of *tk* mRNA. We examined the HSV LS mutants for the accumulation of *tk* transcripts by Northern blot hybridization and by primer extension. To control for possible variations in multiplicity of infection, time

course of infection, and recovery of RNA, we measured the accumulation of a second virus-encoded RNA as an internal reference. In the Northern blot hybridization procedure, the internal reference was a viral transcript encoding alkaline exonuclease (*exo*); in the primer extension analyses, the reference was a transcript from the HSV DNA polymerase (*pol*) gene. Each of these genes, *tk*, *exo*, and *pol*, is transcribed during the same temporal phase of the viral infectious cycle (*19*). Their mRNA products accumulate efficiently in the absence of HSV DNA replication, exhibit similar kinetics of accumulation and decay during HSV infection, and are members of the delayed early class of transcripts (*19–21*).

Northern blot hybridizations were performed with RNA prepared 5 hours after infection (when *tk* mRNA accumulation is maximal) from cells infected with each of the HSV LS mutants (Fig. 4A). The blots were probed with a mixture of two ^{32}P-labeled probes, one derived from the *tk* structural gene and, as the internal control, one from the *exo* gene (*20*). Neither probe hybridized detectably with RNA prepared from mock-infected cells (Fig. 4A, lane 3). The *tk* probe hybridized principally to the 1.5-kb *tk* mRNA and slightly to a 4.1-kb species (*21*), while the *exo* probe hybridized to the 2.5-kb *exo* mRNA as well as two larger species (*21*), only one of which is shown (Fig. 4A). The hybridization to RNA from cells infected with a control virus, PKG7, which contains the *tk*^{ts} mutation in a PAA^{r}5 background (*14*) is shown in Fig. 4A, lane 2. The HSV LS mutants accumulated varying amounts of the 1.5-kb *tk* mRNA; none of the mutants generated transcripts of a novel size. Only HSV LS −29/−18 showed an obvious reduction in *tk* mRNA levels.

Similar data were obtained from primer extension analyses of the same RNA samples (Fig. 4B). These assays were again conducted with a mixture of two ^{32}P-labeled probes, one derived from the *tk* gene and, as an internal control, one derived from the *pol* gene (*22*). No detectable extension products were seen with RNA prepared from mock-infected cells (Fig. 4B, lane 2). Visual inspection of Fig. 4, along with the quantitations compiled in Table 1, shows that ten

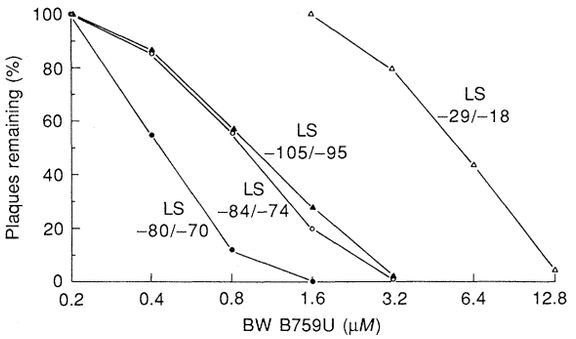

Fig. 3. Effect of various concentrations of BW B759U on HSV LS mutants. Plaque reduction assays were performed at 34°C as described by Coen *et al.* (*39*) under methylcellulose containing the indicated concentration of BW B759U. Dose response curves for mutants HSV LS −80/−70, −105/−95, −84/−74, and −29/−18 are presented.

mutants accumulate reduced levels of *tk* mRNA. This set of ten includes the seven that showed decreases in *tk* expression measured by drug sensitivity.

Effects of LS mutations on transcription rate. We examined the transcription of the viral *tk* gene in infected cell nuclei using an RNA polymerase II run off assay (*23, 24*). This assay allows incorporation of ^{32}P-labeled guanosine triphosphate into nascent RNA in the absence of further transcription initiation. Thus, the amount of incorporation is a measure of the density of RNA polymerase II molecules on the template DNA. Nuclei were isolated from HSV-infected cells 4.5 hours after infection, a time when *tk* transcription is maximal (*19*), and allowed to incorporate label (*24*). Labeled RNA was recovered and hybridized with nitrocellulose filters, each containing sequential dilutions at half-log intervals of six single-stranded DNA probes. The probes included intragenic segments derived from two delayed early genes of HSV (*tk* and *pol*) as well as viral genes of other temporal expression classes: (i) the immediate

Table 1. Relative levels of *tk* expression of LS mutants in four different expression systems. Values for RNA accumulation from plasmids in microinjected *Xenopus* oocytes are from McKnight and Kingsbury (*3*), values for RNA synthesis from plasmids in in vitro transcription extracts of HeLa cells are from Jones, Yamamoto, and Tjian (*7*), and values for expression from HSV in HSV-infected Vero cells as measured by drug sensitivity, RNA accumulation (Northern blot and primer extension analyses) and by nuclear transcription rate measurements are from our report. Values in boldface italics indicate reductions in expression regarded as meaningful by the authors of each report.

Mutant	HSV-infected Vero cells				Micro-injected frog oocytes	In vitro HeLa extracts
	Drug sensitivity*	Northern blot†	Primer extension‡	Transcription§		
−119/−109	1.00	1.06	0.72	0.91	1.20	
−115/−105	1.09	0.68	0.71	0.96	1.40	0.95
−111/−101	0.76	1.17	0.87	0.70	***0.04***	***0.07***
−105/−95	***0.58***	1.17	***0.65***	***0.21***	***0.03***	***0.06***
−95/−85	***0.48***	1.10	***0.50***	***0.22***	***0.12***	***0.32***
−84/−74	***0.48***	***0.44***	***0.30***	***0.26***	***0.09***	***0.30***
−80/−70	1.18	0.77	0.71	0.98	0.87	0.82
−79/−69	0.72	***0.53***	***0.58***	***0.35***	0.70	***0.56***
−70/−61	0.88	1.02	0.60	0.68	0.94	***0.65***
−59/−49	0.70	0.84	***0.50***	0.68	***0.08***	***0.16***
−56/−46	***0.67***	***0.48***	***0.39***	***0.24***	***0.11***	***0.15***
−47/−37	0.84	1.26	0.76	1.45	1.30	1.40
−42/−32	0.78	1.17	0.80	1.50	0.68	1.08
−29/−18	***0.06***	***0.02***	***0.09***	***0.05***	***0.06***	***0.19***
−21/−12	***0.63***	0.85	***0.53***	***0.33***	***0.07***	
−16/−6	1.00	1.00	1.00	1.00	0.96	
−7/+3	0.76	1.14	0.79	0.68	1.00	
+5/+15	***0.43***	***0.56***	***0.56***	***0.17***	1.20	

*Tabulated as the ED_{50} of index mutant HSV LS −16/−6 divided by the ED_{50} of each indicated mutant. HSV LS −16/−6 was chosen as an index mutant because it accumulated levels of *tk* RNA indistinguishable from control virus PKG7 in Northern blot and primer extension analyses (Fig. 4). †Tabulated from densitometric scans using a Joyce-Loebl microdensitometer of Northern blot hybridization data (for example, Fig. 4A). Data were normalized as the ratio of the amount of *tk* RNA to the amount of *exo* RNA and then compiled relative to the value obtained from mutant HSV LS −16/−6, which was given the value 1.00. ‡Tabulated from densitometry of primer extension data (for example, Fig. 4B). Data were normalized as the ratio of the amount of *tk* RNA to the amount of *pol* RNA and then compiled relative to the value obtained from mutant HSV LS −16/−6, which was given the value 1.00. §Tabulated from densitometry of nuclear run off data (for example, Figs. 5 and 6). Data were normalized and compiled as described in text.

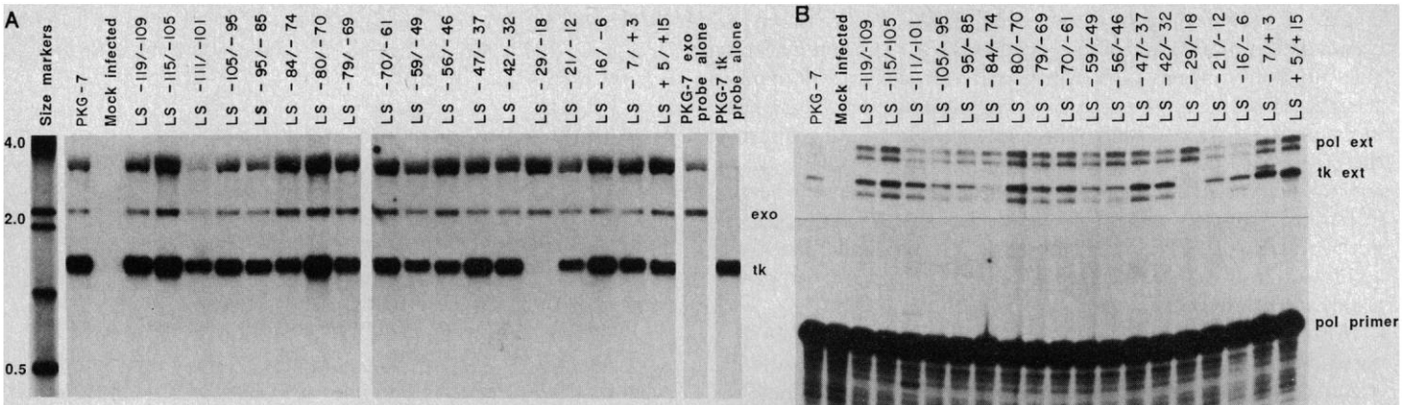

Fig. 4. Analysis of RNA's specified by HSV LS mutants. (A) Northern blot analysis. RNA samples (*40*) from cells mock infected or infected with each of the indicated mutants were separated on agarose gels, transferred to nitrocellulose, and probed with ^{32}P-labeled fragments of the *tk* and *exo* genes (*41*, *42*). Preliminary experiments allowed approximation of the relative abundance of viral specified mRNA as reflected by *exo* mRNA abundance within each sample. HSV LS RNA samples containing roughly equivalent amounts of *exo* mRNA were supplemented with RNA from mock infected cells to total 5 μg. Additional lanes contain size markers (left) and the autoradiographic images obtained when RNA from PKG7-infected cells was hybridized individually with either the *tk* or the *exo* probe (right). (B) Primer extension analysis. The same RNA samples were annealed with a mixture of two ^{32}P-labeled single-stranded primers from the *tk* and *pol* genes (*43*) and analyzed by extension of the primers with reverse transcriptase (*2*). Two autoradiographic exposures were taken and spliced together at a point between the two indicated unreacted primers (bottom portion) and the two extension products (ext) (upper portion). The indicated extension products were the predominant products seen when either the *tk* or *pol* primer was used alone (*34*).

early gene encoding ICP0, (ii) the "leaky late" gene encoding the major capsid protein, and (iii) the "true late" gene encoding glycoprotein gC (see Fig. 5). Because the HSV genome is particularly GC-rich (*25*), the GC-rich chicken *tk* gene (*26*) was included as a control for nonspecific hybridization.

Run off assays with nuclei prepared from cells infected with HSV LS mutants −80/−70 and −29/−18 showed that nuclei from cells infected with mutant HSV LS −80/−70 synthesized more *tk* RNA relative to the other viral standards than did nuclei from cells infected with mutant HSV LS −29/−18 (Fig. 5). Densitometric scans of the hybridization profile of each single-stranded DNA demonstrated that the amount of hybridization varied proportionally with the amount of DNA loaded onto each slot (Fig. 5, bottom). The densitometric peak heights from each slot were calculated and a linear regression plot was generated for hybridization to each DNA sample. In all cases, the correlation coefficient for the regression plot equaled or exceeded 0.92, permitting precise quantitation of the *tk* transcription rates for the 18 HSV LS mutants.

Nuclear run off assays were conducted with nuclei from cells infected with each of the HSV LS mutants. To simplify visual comparison, we show only the *tk* and *pol* hybridization patterns (Fig. 6). These data were quantitated as outlined above and normalized to the amount of *pol* transcription (Table 1), relative to index mutant HSV LS −16/−6. In this assay, alterations of more than 50 percent were deemed meaningful. Eight mutants showed at least a 50 percent decrease in *tk* transcription (Fig. 6 and Table 1). These eight mutants had been identified in previous assays as being defective in *tk* expression. We infer that the LS lesions present in these mutants selectively reduce the rate of *tk* transcription. Since little or no transcription from the template strand is detected upstream of the *tk* RNA cap site (*19*), the promoter mutations appear to affect transcription initiation directly, rather than altering the degree to which ongoing nonspecific transcription is redirected to correct initiation sites. The results of other studies with intact promoters and enhancers have been interpreted similarly (*27*).

Recognition of the region upstream of the *tk* mRNA start site is qualitatively similar under basal and regulated conditions. To evaluate the map of *tk* promoter domains established by our study, it is necessary to review the map established with expression assays that lack HSV-encoded regulatory proteins. In these basal assays, three regions of the *tk* promoter proved to be sensitive to the introduction of base substitutions (*3*, *7*) (Table 1). These include a proximal signal (*ps*), which lies between 16 and 32 bp upstream from the mRNA cap site, and two separable distal signals. The first distal signal (*dsI*) lies between 47 and 61 bp or 47 and 79 bp upstream from the mRNA cap site, depending on the basal assay used; the second distal signal (*dsII*) lies between 80 and 105 bp upstream from the mRNA cap site. Each of these signals represents the binding site for one or more cellular transcription factors. The region *ps* represents the binding site for a TATA-box binding factor (*28*). Both *dsI* and *dsII* contain GC-rich hexanucleotides that form part of the binding sites for the HeLa cell transcription factor Sp1 (*7*). Finally, *dsII* also contains an inverted CCAAT pentanucleotide that forms part of the binding site for a factor termed CCAAT transcription factor (CTF) (*7*) or CCAAT binding protein (CBP) (*5*). The distributions of these factor binding sites relative to the mutation-sensitive promoter domains are diagrammed schematically in Fig. 6.

The effects observed for the 18 LS mutants in three different expression systems are compared in Table 1. Two of these expression systems represent basal expression environments and the other contains the products of HSV immediate early regulatory genes. All of the expression systems reveal the same mutation-sensitive domains upstream of the RNA start site (*ps*, *dsI*, and *dsII*). We conclude that the region upstream of the mRNA cap site of the *tk* gene is recognized similarly under basal and regulated conditions. Implicit to this interpretation are two predictions: (i) The function of the *tk* promoter in HSV-infected cells is effected by the same cellular factors that interact with the promoter in either uninfected cells or soluble in vitro transcription systems. (ii) The protein products of HSV immediate early regulatory genes do not bind directly to an induction-specific domain of the *tk* promoter. A possibility that is less likely is that HSV encodes transcription factors with recognition properties very similar to those of cellular factors.

Our conclusion that *tk* promoter recognition is qualitatively similar in basal and regulated environments differs from the interpretation favored in other reports—that new promoter domains are utilized under regulated conditions (*6, 29*). Our conclusion is in agreement, however, with that of another study (*30*). These previous studies attempted to model *tk* regulation by inducing the expression of transfected *tk* genes by superinfection with *tk*⁻ HSV. Such approaches, of necessity, are less direct than studies of *tk* expression in the natural context of the HSV chromosome.

Quantitative differences in utilization of promoter domains in different expression systems. Although, in qualitative terms, utilization of *tk* promoter domains is similar among different expression systems, there are several differences in the relative importance of *ps*, *dsI*, and *dsII* in these systems (Table 1). For example, LS −111/−101 of *dsII* and LS −59/−49 of *dsI* only affect *tk* expression marginally in HSV-infected Vero cells, yet impair expression severely in two basal assay systems. The opposite behavior is displayed by LS −29/−18; in the soluble mammalian transcription extract it is no more impaired than four mutants with lesions in *dsI* and *dsII*, yet in HSV-infected Vero cells it is by far the most impaired of the LS mutants.

It is possible that these quantitative differences reflect technical inconsistencies in the various assays. For example, template competition in certain assays may lead to a skewed estimation of the relative importance of the different promoter domains. On the other hand, it is possible that the observed quantitative differences reflect changes in the transcriptional environment that result from viral regulatory proteins. The observation that a mutation that maps in *ps* (LS −29/−18) is the only mutation that severely decreases *tk* expression raises the possibility that, under regulated conditions, the factors that bind to *ps* become rate limiting. Indeed, it is possible that viral regulatory proteins induce *tk* mRNA expression by binding or modifying the cellular transcription factor or factors that bind to *ps* [for example, TATA-box binding factor (*28, 31*)]. Alternatively, increases in the activity or availability of cellular transcription factors required for the function of *dsI* and *dsII* (Sp1 and CBP) may result in the overabundance of these factors relative to ones that bind to *ps*.

A mutation downstream from the mRNA start site reduces *tk* transcription. In contrast to the qualitatively similar manner in which upstream elements of the *tk* promoter are recognized in the different expression systems, the single LS mutation that occurs downstream from the mRNA cap site (LS +5/+15) behaves differently when embedded in HSV compared to other settings. This mutant was expressed effectively in microinjected oocytes (Table 1). In contrast, HSV LS +5/+15 expressed the viral *tk* gene at a reduced level according to measurements of drug sensitivity, mRNA accumulation, and transcription rate (Table 1). These reductions in mRNA accumulation and transcription rate were measured relative to appropriate internal standards.

Although we know of no other studies that have examined the effect of mutations downstream of the mRNA start site on transcription rate, two recent reports have suggested that novel transcription factors may bind to DNA around or within this region in other viral transcription units (*32*). The lesion within HSV LS +5/+15 could, perhaps, define a recognition site for a novel transcription factor, either encoded by HSV or induced by viral infection. However, two considerations favor a simpler interpretation. (i) It is known that cellular transcription factors that bind to TATA homologies, such as the sequence within *ps*, also interact closely with sequences internal to the mRNA cap site (*31*). Preliminary footprinting experiments on the *tk* promoter with rat liver nuclear extracts are consistent with such observations (*28*). (ii) Mutations in the TATA homology in *ps* of the *tk* promoter elicit particularly drastic reductions in *tk* RNA synthesis when tested in HSV-infected cells. Rather than invoke novel transcription factors or regulatory regions, we suggest that appropriate function of the TATA binding factor becomes sensitive to the LS +5/+15 lesion in the regulated environment, leading to decreases in *tk* transcription.

Our data do not absolutely exclude the possibility that HSV regulatory proteins could induce *tk* transcription by binding to specific domains upstream or downstream of the *tk* promoter. However, two HSV mutants, in which sequences downstream of the *tk* promoter (−12 to +189 and +53 to +554, respectively) are deleted, still induce substantial levels of *tk* mRNA (*33, 34*). In addition, transfection studies with *tk* plasmids indicated that se-

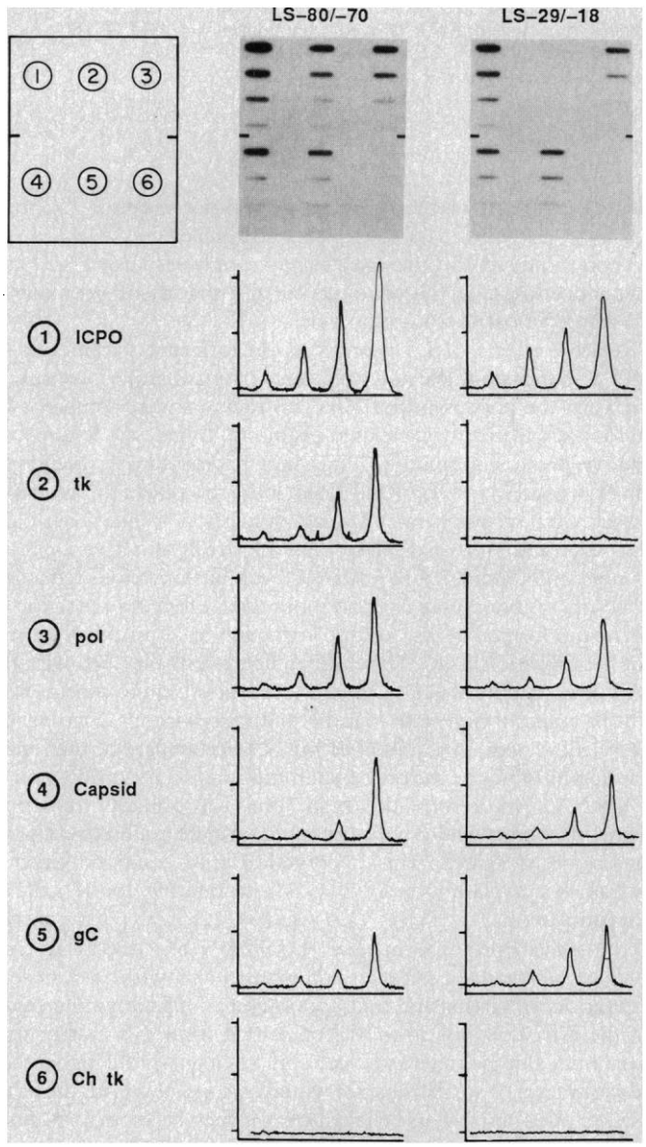

Fig. 5. Nuclear transcription from cells infected with two HSV LS mutants. Vero cells were infected with each of the indicated HSV LS mutants and used for in vitro transcription (*44*). The ^{32}P-labeled RNA was hybridized to the indicated single-stranded DNA probes immobilized on nitrocellulose filters in four amounts (10, 31.5, 100, and 315 ng) (*45*). The upper left panel is a schematic of the layout of the single-stranded DNA probes on each filter, the numbers refer to each set of four dilutions of the DNA probes listed next to the bottom panels; the upper center and upper right panels show the autoradiographic images of filters hybridized with RNA derived from nuclei infected with HSV LS −80/−70 and −29/−18, respectively. The lower panels show densitometric scans generated from these autoradiograms, with each probe indicated to the left.

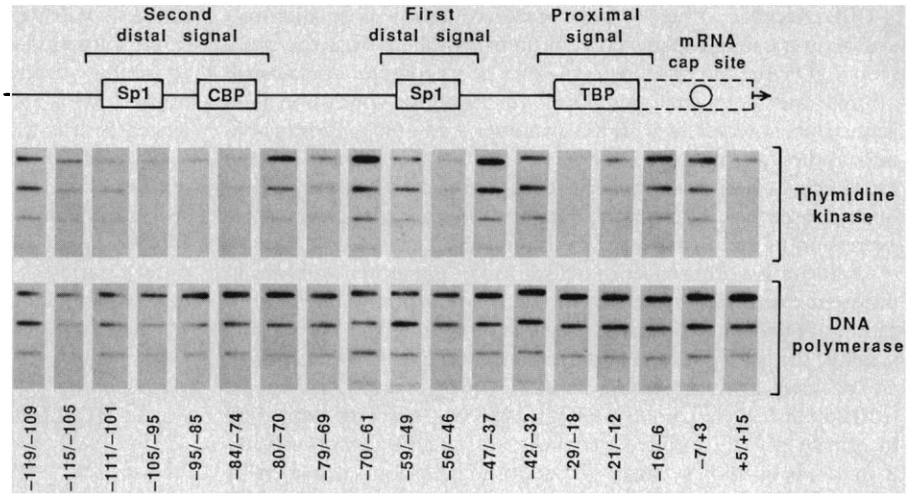

Fig. 6. Effects of HSV LS mutations on *tk* transcription in isolated nuclei. The diagram at the top of the figure shows the location of the mRNA cap site and brackets designating promoter domains established by analysis of LS mutants in frog oocytes (*3*). Labeled boxes identify the locations derived by DNA footprinting experiments of sequence-specific binding sites for transcription factors Sp1 (*7*), CBP (*5, 7*), and TATA binding protein (TBP) (*28*). The box formed by dotted lines adjacent to the TBP box represents sequences protected by TBP in footprinting experiments that lie outside the proximal signal established in oocyte assays. The panels below are autoradiographic images of filter hybridizations to single-stranded probes from the *tk* gene (upper set of panels) and the *pol* gene (lower set of panels) with labeled RNA derived from nuclei infected with the HSV LS mutants indicated at the bottom of each pair of panels.

quences upstream of the *tk* promoter are not required for trans induction (*29, 30*). Moreover, chimeric genes in which the *tk* promoter is fused to other genes are strongly responsive to HSV trans induction (*35*). These results suggest that the *tk* promoter is sufficient to mediate trans induction.

Relative effects of LS mutations on different parameters of HSV *tk* expression. We noted a pattern of quantitative inconsistencies when the phenotypes of HSV LS mutants were compared by the four assays that we have used (Table 1). In general, lesions that reduce expression meaningfully in assays that measure the accumulation of tk activity and *tk* mRNA do so more severely in the assay that measures transcription rate. Visual comparison of Northern blots (Fig. 4A) and run off assays (Fig. 6) reveals this trend. As an example, HSV mutant LS −105/−95 exhibits a fivefold reduction in the transcription assay, yet no more than a twofold reduction in any of the former assays (and no reduction at all in the Northern blot hybridization assay). Other differences are smaller, yet, with the possible exception of LS −29/−18 (see below), quite consistent.

If *tk* transcription rate can be reduced without eliciting an equivalent change in the level of mRNA accumulation, then transcription must not be the only rate limiting step in the production of *tk* mRNA. Apparently, the high levels of primary transcripts emanating from a wild-type *tk* promoter saturate a subsequent post-transcriptional "gate." This hypothetical gate could be either a nuclear or cytoplasmic event that is rate limiting for *tk* mRNA accumulation.

One of the *tk* promoter mutants (LS −29/−18) reduces transcription rate substantially, yet does not appear to show the discrepancy between accumulation and transcription rate. Difficulties in measuring the extremely low *tk* transcription rate from this mutant may have resulted in the value listed in Table 1 being twofold higher than the actual level. If so, its behavior would be consistent with the other mutants. Alternatively, its behavior may, in fact, be anomalous. Such anomalous behavior could be explained in one of two ways. It may be that the transcription level from HSV LS −29/−18 is so low that all of its primary transcripts are able to pass through the hypothetical posttranscriptional gate. An alternative interpretation holds that the effects of this mutation on transcription initiation might impede subsequent regulatory steps that act to control mRNA accumulation.

Mechanisms of HSV-mediated trans induction. Our results indicate that *tk* transcription rate is properly induced in response to immediate early regulatory proteins without a major qualitative change in promoter recognition. In other words, we have not identified an induction-specific domain of the *tk* promoter. This argues against a model in which viral proteins effect trans induction by binding directly to a domain of the *tk* promoter that is not bound by cellular transcription factors. If this interpretation is correct, it leaves unexplained how *tk* gene expression is induced by two to three orders of magnitude by viral regulatory proteins such as ICP4. We pay particular attention to two observations. (i) The transcription of *tk* in HSV-infected cells is exquisitely sensitive to mutations that affect binding of cellular factors to the TATA box. (ii) Accumulation of *tk* mRNA appears to be regulated at a step subsequent to transcription initiation. This latter observation may relate to evidence presented elsewhere that replacement of *tk* coding sequences with sequences derived from murine sarcoma virus can dampen the response to HSV trans induction (*36*). We suggest that trans induction is catalyzed initially by the activation of cellular transcription factors and speculate that this event leads not only to an increase in transcription rate, but also to an increased probability that primary transcripts will be converted to stable mRNA.

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37. Chimeric LS-*tk*^{ts} plasmids were constructed by replacing the 2.8-kb Bgl II–Bam HI DNA fragment that contains *tk* protein-coding sequences of each of the LS plasmids (*3*) with the corresponding fragment of DNA from mutant KG111 (*14*). Each chimeric plasmid was used in marker transfer experiments (*18*) with infectious viral DNA from mutant PAA^{r}5. Each suspension of transfected cells was plated into two 35-mm dishes to permit the isolation of two independent LS mutants per transfection. Progeny virus were plated at 39°C in the absence of drug or with ACV concentrations from 100 to 200 μ*M*. At these concentrations, the plating efficiency of progeny of transfections with PAA^{r}5 DNA and any LS-*tk*^{ts} plasmid was 10 to 1000 times higher than that of progeny of transfections with PAA^{r}5 DNA alone. Plaques resistant to ACV were picked and used to infect cells in suspension in culture tubes. The infected cells were allowed to attach overnight at 39°C. The following day the medium was decanted to remove any residual ACV, replaced with fresh medium, and incubated at 34°C. When generalized cytopathic effects were obtained, virus from each plaque was harvested (*39*). Viral DNA's were prepared by a virion "mini-prep" procedure: Crude virions from each plaque were prepared by centrifugation for 1 hour at 13,000*g* and then resuspended in 10 m*M* tris, 1 m*M* EDTA, *p*H 7.5 (TE). The suspensions were deproteinized once with phenol and extracted twice with chloroform. Aliquots of each final aqueous phase were digested with Bam HI, the digestion products were separated on agarose gels, transferred to nitrocellulose filters (*38*), and hybridized with ^{32}P-labeled *tk* DNA. LS mutants were identified by the presence of novel Bam HI fragments hybridizing with *tk* DNA (see text). Further rounds of plaque purification were performed in the absence of drug.
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40. For each preparation of RNA, cells were mock infected or infected at a multiplicity of infection of 1 to 2 plaque-forming units (PFU) per cell (confirmed by back titration) and incubated at 37°C. Five hours after infection total RNA was prepared [D. Kimelman, J. S. Miller, D. Porter, B. E. Roberts, *J. Virol.* **53**, 399 (1985)].
41. RNA samples (5 μg) were separated on 1.5 percent agarose gels containing 2.2*M* formaldehyde (*42*) and transferred to nitrocellulose filters. The filters were hybridized with viral genes labeled with ^{32}P. *tk* mRNA was detected with a 0.7-kb Sac I–Sma I fragment that lies within the *tk* protein coding sequences [S. L. McKnight, *Nucleic Acids Res.* **8**, 5949 1980)]. *exo* RNA was detected with a 0.5-kbp Pvu II fragment from the 5′ end of the *exo* gene (*19*). After hybridization, the filters were washed by a procedure that included three treatments at 70°C for 30 minutes each in 0.1× SET (15 m*M* NaCl, 5 m*M* tris-HCl, *p*H 8.0, 0.5 m*M* EDTA), and 0.1 percent SDS. The filters were exposed to preflashed x-ray film with an intensifying screen at −70°C [R. A. Laskey and A. D. Mills, *FEBS Lett.* **82**, 314 (1977)].
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43. One primer (*tk* primer) was a ^{32}P-labeled 27-base oligonucleotide that is complementary to a segment of *tk* mRNA 50 to 77 residues internal to its cap site. The other primer (*pol* primer) was a 38-base single-stranded Bam HI–Sma I restriction fragment derived from pKOS29.1 (*22*) labeled with ^{32}P at its 3′ Bam HI end with reverse transcriptase (*3*).
44. For each mutant, cells were infected at a multiplicity of infection of 2.5 PFU per cell and incubated at 37°C. At 4.5 hours after infection, nuclei were isolated and labeled by a modified procedure of Konieczny and Emerson (*24*) as modified (*19*).
45. DNA probes containing five viral genes and one control gene were cloned into bacteriophage M13: (i) ICP0, a 1.6-kb Sst I–Bam HI fragment [S. Mackem and B. Roizman, *J. Virol.* **44**, 939 (1982)]; (ii) *tk*, a 1.1-kb Bgl II–Hind III fragment from deletion mutant delta 3′ 1.6 [S. L. McKnight and E. R. Gavis, *Nucleic Acids Res.* **8**, 5931 (1980)]; (iii) *pol*, a 0.65-kb Bgl II–Eco RI fragment (*22*); (iv) capsid (ICP5), a 1.6-kb Bam HI–Hind III fragment [R. H. Costa, G. Cohen, R. Eisenberg, D. Long, E. Wagner, *J. Virol.* **49**, 287 (1984)]; (v) glycoprotein C (gC), a 1.6-kb Eco RI–Bam HI fragment [R. J. Frink, R. Eisenberg, G. Cohen, E. K. Wagner, *ibid.* **45**, 634 (1983)]; and (vi) as a control gene for nonspecific hybridization, a 1.5-kb Eco RI–Bgl II fragment of the chicken *tk* (ch *tk*) gene (*26*). Each phage DNA was applied to each filter with a slot blot apparatus in amounts of 315 ng, 100 ng, 31.5 ng, and 10 ng per slot. Filters were hybridized with the RNA products of nuclear transcription reactions. After washing, filters were treated with ribonuclease A, washed twice more, and exposed to preflashed x-ray film. Peak heights derived from densitometric scanning of autoradiograms were measured for each set of dilutions of each DNA probe, and plotted against an idealized set of half-logarithmic coordinates, generated by normalization to measurements in the linear range of detection for the wedge used in the densitometer. Linear regression analysis verified linearity of the observed hybridization data for *pol* and *tk*. In five cases (HSV LS −80/−70, −79/−69, −47/−37, −42/−32, and −21/−12), the correlation coefficient for the linearity of *pol* hybridization fell between 0.92 and 0.97. For the other *pol* hybridizations and all *tk* hybridizations, the correlation coefficient exceeded 0.98. The amount of ^{32}P-labeled RNA hybridized with immobilized DNA probes varies as a function of the amount of homologous RNA in the hybridization reaction (*19*).
46. We thank D. Barry (Burroughs Wellcome Co.) for providing BW B759U; R. Desrosiers for greatly improving the virion mini-prep procedure; H. Chiou for assistance with marker transfers; L. Bierut, M. Retondo, and R. Kingsbury for valuable technical assistance; J. Scales and J. Khorana for figure preparation; R. Kingston, S. Triezenberg, K. Yamamoto and B. Graves for helpful comments on the manuscript; and M. Manos for helping to arrange this collaboration and generously providing a preparation of cloned KG111 *tk* DNA. We also especially thank L. K. Leslie for assistance in plaque purification, preparation of high titer stocks and infected cell RNA's, and Southern blotting and drug sensitivity analyses. Supported by awards from the American Cancer Society (MV-242), the William F. Milton Fund, and the Charles A. King Trust (Boston, MA) (D.M.C.) and an NIH award (S.L.M.). This work represents partial fulfillment of the requirements for a Ph.D. degree at the University of Washington, Seattle, for S.P.W.

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