Promoter Domains Required for Expression of Plasmid-Borne Copies of the Herpes Simplex Virus Thymidine Kinase Gene in Virus-Infected Mouse Fibroblasts and Microinjected Frog Oocytes

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A transient expression assay was used to measure the relative template activities of mutated *tk* genes in mouse L cells induced in *trans* by herpes simplex virus (HSV). In this assay, expression of the wild-type HSV type 1 *tk* gene is induced at least 200-fold by the superinfecting virus. Genetic lesions that were assayed include 5' deletions, clustered base substitutions, single base substitutions, intrapromoter inversions, and intrapromoter recombinants with the HSV type 2 *tk* gene. Roughly half of the mutations that were tested were found to weaken *tk* expression efficiency, and the remaining mutations did not alter expression. The spatial distribution of mutations that reduce expression efficiency in *trans*-induced mouse fibroblasts facilitated the construction of a map of promoter domains. The most gene-proximal promoter domain is located between 16 and 32 base pairs (bp) upstream of the *tk* mRNA cap site and contains a TATA homology. Two more distally located promoter domains were mapped to discrete locations upstream from the TATA homology. One of these distal domains is located between 47 and 79 bp upstream from the mRNA cap site, and the other is located between 84 and 105 bp upstream from the *tk* gene. The boundaries of these three promoter domains, with one exception, coincided with the set of domains delineated previously in a frog oocyte microinjection assay. The concordant behavior of *tk* promoter mutants in microinjected frog oocytes and *trans*-induced mouse fibroblasts leads us to propose that recognition and activation of the HSV *tk* promoter is mediated by cellular transcription factors that are common to frogs and mice.

When cultured mammalian cells are infected with herpes simplex virus (HSV), an ordered cascade of viral gene expression is initiated (9, 19, 20). During the first several hours postinfection, a set of immediate-early (alpha) genes are expressed. The accumulation of alpha gene products elicits a shift in the pattern of viral transcription resulting in the expression of a set of delayed-early (beta) genes. The beta gene products are required for viral DNA replication and include the viral DNA polymerase, several DNA-binding proteins, an alkaline exonuclease, and a deoxy-pyrimidine kinase better known as thymidine kinase (tk).

The focus of the work presented in this report concerns the mode by which immediate-early HSV gene products induce the expression of delayed-early genes. Although as yet formally untested, it is thought that this shift in viral gene expression involves a specific increase in the transcription rate of delayed-early genes. Should such a mechanism prove to be correct, obvious candidate sites for induction would be the transcriptional promoters of delayed-early genes. During the past several years we have carried out a series of experiments that were designed to identify DNA sequences of the HSV *tk* gene that behave as *cis*-acting transcriptional control signals in microinjected frog oocytes. On the one hand, we reasoned that the oocyte assay was of some value because it proved capable of accurate transcription of the *tk* gene (15). On the other hand, since we introduced the gene into oocytes as a recombinant plasmid in the absence of any

immediate-early regulatory genes or gene products, we judged the oocyte environment to be of a constitutive nature.

Having demonstrated the capacity of frog oocytes to express the HSV *tk* gene accurately, we attempted to identify DNA sequence signals that are required for accurate and efficient *tk* mRNA expression. To this end, we used *in vitro* methods of mutagenesis that were separated into three phases. First, we established rough boundaries of a promoter region by deletion mapping (15). We then prepared and assayed a library of promoter mutations that carry clustered base substitutions. These experiments facilitated the development of a refined map of the HSV *tk* promoter (16). This map showed the promoter to consist of three distinct domains housed within the 105 base pairs (bp) immediately flanking the *tk* mRNA cap site. Finally, we introduced single point mutations in two of the three promoter domains. The expression behavior of these point-mutated *tk* promoters indicated that the two more distally located promoter domains might be recognized by the same transcription factor (17).

The goals of the experiments included in this study were to establish a facile, reproducible assay for *tk* gene expression that is dependent on the viral regulatory gene products that normally induce *tk* gene expression. Once such an assay was established, we then sought to assess the expression phenotypes of all of the *tk* promoter mutations that had been characterized in the constitutive oocyte environment. In comparing the behavior of each mutation in constitutive and regulated environments, we expected to observe one of two results. Either the promoter mutations should behave in a concordant manner in the two assays or they should not. The former result would indicate that the delayed-early induction of *tk* gene expression does not involve a qualitative alteration in promoter recognition, and the latter would indicate

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that the induction might employ such a mechanism. The validity of interpretations based on this approach is clearly dependent upon the level of saturation covered by the library of promoter mutations. In that we have delineated the *tk* promoter to a 105-bp area by deletion mapping and further substituted 76 of these residues, we believe it reasonable to proceed with this comparison.

MATERIALS AND METHODS

Plasmids, cell lines, and viruses. The construction of the following recombinants was described previously: 5' deletion mutants (15); linker scanning (LS) mutants and the pseudo-wild-type *tk* gene (16); single point mutants, double LS mutant LS -119/-109// -42/-32, and distal inversion mutants (17). Intertypic recombinants between the HSV type 1 (HSV-1) (strain MP) and HSV-2 (strain 333) *tk* promoters were prepared as follows. A fragment of the HSV-2 *tk* gene progressing from a unique *Bgl*III restriction site 0.6 kilobase pairs upstream from the *tk* mRNA cap site to a *Pst*I restriction site 17 bp downstream from the cap site was isolated. This HSV-2 promoter fragment was used to replace the HSV-1 analog. Recombinants consisting of an HSV-2 second distal signal and an HSV-1 first distal signal, as well as the reciprocal, were prepared by *in vitro* recombination at the unique *Eco*RI site that occurs between the first and second distal signals in each viral *tk* gene (see Fig. 5A for a diagram of the structures of these recombinants). Plasmids were propagated in *Escherichia coli* HB101 and purified as described previously (1).

The mouse LM *tk*⁻ *aprt*⁻ cell line was provided by H. Weintraub. The HSV-1 mutant Δ35, derived from the KOS strain (8), was generously provided by J. Smiley. The Δ35 mutant virus carries a 170-bp deletion at the viral *tk* locus and produces no detectable *tk* mRNA when assayed by primer extension. Viral stocks were propagated on baby hamster kidney cells, and titers were determined on African green monkey kidney (Vero) cells. The HSV-1 mutant ACG⁴ (KOS) induces no detectable *tk* enzyme activity (2) or full-length *tk* polypeptide (D. Coen, unpublished data) but does produce full-length *tk* mRNA. All cell lines were grown in Dulbecco modified Eagle medium (DME) supplemented with 1/10 volume of fetal calf serum (FCS; Hyclone-defined sera) and 100 U each of penicillin and streptomycin per ml.

Transient expression assay. LM *tk*⁻ *aprt*⁻ cells growing in log phase were plated at a density of 10⁶ cells per 60-mm plastic culture dish. After 30 h of growth, cells were washed twice with 1 ml of sterile TBS (25 mM Tris hydrochloride [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.05 mM MgCl₂, 0.7 mM CaCl₂) and transfected with 2 μg of supercoiled DNA (1 μg of pseudo-wild-type *tk* plasmid and 1 μg of mutant *tk* plasmid). Transfections were carried out in 0.6 ml of DME containing 400 μg of DEAE-dextran (Pharmacia Fine Chemicals) per ml and 50 mM Tris hydrochloride (pH 7.3) (14, 22). After incubation at 32°C for 1 h, cells were washed with TBS and incubated for 3 h at 37°C in 4 ml of DME-10% FCS containing 90 μM chloroquine (13). Cells were then washed with TBS and incubated for 36 h in DME-10% FCS at 37°C. At the time of infection with HSV, the number of cells in a parallel dish of transfected cells was determined. The cells to be infected were washed with TBS and infected with 10 PFU of HSV-1 per cell in 0.5 ml of DME-2% FCS. Dishes were incubated at 37°C with periodic rocking to prevent the cells from drying and to ensure uniform distribution of the virus. After 1 h, the virus inoculum was removed and replaced with 4 ml of DME-5%

FCS. After an additional 7 h of incubation at 37°C, the cells were washed twice with TBS and then lysed by the addition of 1 ml of 10 mM Tris hydrochloride (pH 7.5)-5 mM EDTA-1% Sodium dodecyl sulfate-200 μg of proteinase K per ml.

RNA preparation and transcript mapping. Lysates of *trans*-induced L cells were scraped into polypropylene tubes, frozen, thawed, and incubated at 50°C for 2 h to permit proteinase K digestion. One-tenth volume of 3 M sodium acetate (pH 5.2) was added to the digested lysates, and nucleic acids were extracted twice with 0.7 volume phenol and 0.7 volume chloroform. After the second phenol-chloroform extraction, the nucleic acids were ethanol precipitated, suspended in 200 μl of 10 mM Tris hydrochloride (pH 7.4)-1 mM EDTA, and reprecipitated. The pelleted nucleic acids were then dissolved in water and treated for 2 h at 37°C with 5 μg of RNase-free DNase I (grade DPRF; Worthington Diagnostics) in a 200-μl reaction containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; pH 7.0), 1 mM CaCl₂, 5 mM MgCl₂, and 250 μg of heparin per ml. DNase I reactions were extracted twice with phenol-chloroform. RNA was recovered by ethanol precipitation and suspended in 10 μl of water. *tk* mRNA levels were measured by a primer extension assay (15). Purified RNA (10 μl) was mixed with 10⁴ cpm of primer and annealed at 60°C in a 20-μl reaction consisting of 0.3 M KCl, 10 mM Tris hydrochloride (pH 7.5), and 1 mM EDTA. Annealed samples were diluted directly into 40 μl of primer extension reaction buffer, yielding final concentrations of 100 mM KCl, 20 mM Tris hydrochloride (pH 8.1), 8 mM MgCl₂, 0.33 mM EDTA, 250 μM deoxynucleoside triphosphate, 50 μg of actinomycin D per ml, and 50 U of avian myeloblastosis virus reverse transcriptase per ml. Primer extension reactions were incubated at 37°C for 1 h, ethanol precipitated, suspended in 20 μl of 99% formamide-0.1% bromophenol blue-0.1% xylene cyanol, and boiled for 5 min. Samples were electrophoresed on 9% polyacrylamide-8 M urea sequencing gels. Gels were dried and exposed to X-ray film. Radioactive primer extension products were quantitated by densitometric scanning of gel exposures obtained without intensifying screens.

RESULTS

Development of a transient *trans*-induction assay. The assay we used to monitor virus-specific induction of *tk* gene expression follows the initial observation of Lin and Munyon (12). These authors found that when the HSV *tk* gene is stably acquired by mammalian cells, it retains the capacity to respond to *trans*-acting factors produced by superinfecting virus. The parameters of this *trans*-induction assay have been expanded and refined by a variety of different investigators (4, 10, 11, 21, 26). Several studies have established the involvement of a specific gene product, termed ICP4 (infected cell protein 4) in the alpha-to-beta transcriptional shift (3, 19, 24). Since efficient activation of stably acquired copies of the HSV *tk* gene does not occur when an ICP4^{ts} virus is used at the restrictive temperature (10, 11), it is believed that the *trans*-induction phenomenon observed in this assay reflects the appropriate biological activation of *tk* gene expression.

The primary objective of the present study was to assess the phenotypes of a wide variety of promoter mutations of the *tk* gene under *trans*-induced conditions. Since the construction and careful analysis of multiple independent cell lines carrying each promoter mutation represented an unre-

alistically laborious task, we modified earlier *trans*-induction procedures by using a transient inoculation procedure.

In our initial experiments, mouse L cells were transfected with the pseudo-wild-type *tk* gene. This derivative of the HSV-1 *tk* gene is missing 10 nucleotides between the mRNA cap site and the unique *Bgl*II restriction site. Thus, the mRNA molecule transcribed from the pseudo-wild-type gene is 10 ribonucleotides shorter than that produced by an intact *tk* gene. The shortened mRNA is easily distinguished from the authentic *tk* mRNA when assayed by primer extension (16). Thirty-six hours after introduction of the pseudo-wild-type *tk* gene, the transfected cells were either infected with 10 PFU of HSV-1 per cell or mock infected. Eight hours later, total RNA was purified and assayed for the presence of *tk* mRNA. Figure 1 is a schematic diagram showing the *trans*-induction protocol, and Fig. 2 shows an autoradiographic exposure of an electrophoresis gel that was used to map primer extension products derived from RNA isolated from *trans*-induced cells.

Expression of the pseudo-wild-type template was highly dependent on HSV-1 infection (Fig. 2). At the exposure level shown in Fig. 2, no primer extension product corresponding to the pseudo-wild-type transcript was observed in the mock-infected sample. However, the assay clearly shows that the HSV-1-infected sample contained mRNA synthesized from the pseudo-wild-type template. Densitometric scanning of an autoradiogram exposed for roughly 10 times the length of the exposure shown in Fig. 2 indicated that transcription of the pseudo-wild-type template was induced at least 200-fold by viral infection. The additional primer extension product evident in the HSV-1-infected sample

FIG. 1. Diagram of mouse L-cell *trans*-induction assay. One microgram of each *tk* template was transfected into washed L cells in the presence of DEAE-dextran. After a 3-h exposure to medium containing 90 μ M chloroquine and a 36-h incubation in fresh culture medium, the cells were infected with 10 PFU of HSV per cell. Incubation times and temperatures are indicated. See the text for details of transfection, infection, and mRNA mapping procedures.

FIG. 2. Detection of *tk* mRNA synthesized in cells transfected with plasmid-borne *tk* genes. Left panel, Autoradiographic exposure of a polyacrylamide electrophoresis gel used to map primer extension products specific to *tk* mRNA. Gel lanes were loaded with primer extension products derived from the RNA isolated from a culture dish of transfected cells. In each case, cells were transfected with 1 μ g of the pseudo-wild-type *tk* gene. After transfection, cells were mock infected (Mock), infected with HSV-1 strain ACG'4 which synthesizes a full-length *tk* mRNA (HSV-1 inf), or infected with HSV strain Δ 35 that contains a deletion at the viral *tk* locus and thus produces no authentic *tk* mRNA (HSV-1 *tk*⁻ inf). Cells were harvested 8 h post infection, and total RNA was purified. *tk* mRNA was identified by a primer extension transcript mapping assay. The *tk* mRNA synthesized from the transfected pseudo-wild-type template is 10 ribonucleotides shorter than that synthesized from a wild-type *tk* gene. The locations of primer extension products derived from wild-type and pseudo-wild-type *tk* mRNA are designated at the right. Note that the pseudo-wild-type transcript is only detected in infected cells and that the authentic *tk* transcript (expressed from the incoming virus) is synthesized in cells infected with wild-type virus but not in cells infected with *tk*-deleted virus. Right panel, Primer extension assay of mRNA synthesized from 5' deletion mutants of the *tk* gene. Cells were cotransfected with 1 μ g of the pseudo-wild-type *tk* gene and 1 μ g of the 5' deletion mutant indicated above each gel lane. Cells were subsequently infected with the *tk*-deleted HSV-1 strain. The mRNA synthesized from 5' deletion mutants is equivalent in size to the authentic *tk* transcript and therefore maps at the same location on the polyacrylamide gel (designated WT signal). For details of transcript mapping procedure see the text.

corresponds to authentic *tk* mRNA that is expressed from the infecting virus.

The appearance of the pseudo-wild-type *tk* transcript in the HSV-infected sample apparently reflects a virus-mediated *trans*-induction event. When this experiment is repeated with a virus stock that carries a temperature-sensitive allele of the immediate early regulatory gene ICP4, efficient *trans* induction only occurs at the permissive temperature (6). Such a result does not imply that the ICP4 gene product acts directly on the plasmid-borne pseudo-wild-type *tk* gene. It does, however, indicate that *trans* induction is specifically dependent upon the expression of viral regulatory genes.

All of the *tk* promoter mutations that we constructed maintained an intact mRNA coding element. Thus, to assay these mutations in *trans*-induced L cells we used an HSV-1 strain that is deleted in a region surrounding the viral *tk* promoter (kindly provided by J. Smiley). When this *tk*-

FIG. 3. Expression levels of LS mutants in *trans*-induced mouse L cells. Cultured mouse L cells were transfected with equimolar amounts of the pseudo-wild-type *tk* gene and each LS mutant of the *tk* gene (15), induced by infection with *tk*-deleted HSV-1, and assayed by primer extension (see the text). The library of LS mutants was split into two groups that were assayed in separate trials. Six LS mutants were assayed in both trials (LS -80/-70, LS -79/-69, LS -70/-61, LS -59/-49, LS -56/-46, and LS -47/-37). (A) Autoradiographic exposures of electrophoresis gels that were used to map primer extension products. The LS mutant tested is identified above each gel lane. An autoradiographic exposure developed without the aid of an intensifying screen was scanned by densitometry to quantitate radioactive bands. The expression efficiency of each LS mutant was then calculated relative to the pseudo-wild-type internal control. (B) Histogram that compares LS mutant phenotypes in *trans*-induced L cells (hatched bars) with phenotypes in microinjected oocytes (open bars). Quantitation of LS mutants that were assayed twice by *trans* induction represents the arithmetic mean of the two trials. Oocyte quantitations are taken directly from the data of McKnight and Kingsbury (16). Areas of mutation sensitivity are diagrammed above the histogram.

deleted HSV-1 was used to *trans* induce mouse L cells that had been transfected with the pseudo-wild-type *tk* gene, expression from the pseudo-wild-type template was induced. However, no transcript detectable in the *tk* primer extension assay was produced by the incoming virus (Fig. 2). This set of reagents, a pseudo-wild-type template and a *tk*-deleted HSV-1 strain, allowed us to monitor the expression of the *tk* promoter mutations in a quantifiable manner. Equimolar amounts of mutant and pseudo-wild-type template were cotransfected into L cells, *trans* induced with *tk*-deleted HSV-1, and mapped by primer extension. The phenotype of a mutant was thereby judged by measuring its expression

relative to that of the internal control, pseudo-wild-type *tk* gene.

Phenotypes of 5' deletion mutants. In our first attempt to characterize transcription signals of the *tk* gene that are recognized in *trans*-induced cells we utilized a set of 5' deletion mutants. The structures of these deletions were described in a separate report (15). In brief, they remove progressively greater amounts of DNA flanking the 5' terminus of the *tk* gene and replace the missing HSV-1 sequences with pBR322 DNA. As shown in the right-hand panel of Fig. 2, deletions that retained 119 or more nucleotides of 5'-flanking DNA were expressed at a high level. Deletion mutants that retained only 95 and 85 residues upstream of the mRNA cap site were expressed at 7- and 20-fold lowered levels, respectively. This experiment established the 5' boundary of an element required for efficient expression of *tk* mRNA in *trans*-induced mouse L cells. The location of the 5' boundary is coincident with that established for constitutive expression of the HSV *tk* gene in microinjected frog oocytes and uninduced mouse L cells (15). Evidently, viral regulatory components do not require sequences upstream of nucleotide -119 to induce expression of *tk* mRNA.

Phenotypes of LS mutants. Promoter components required for transcription of the *tk* gene in frog oocytes have been delineated by the use of a set of alleles that carry clustered base substitutions. These alleles, termed LS mutants, each contain between 5 and 10 base substitutions clustered in overlapping segments between residues -119 and +15 (relative to the *tk* mRNA cap site). A description of the construction and characterization of the series of LS mutants is in reference 16.

The *trans*-induced phenotypes of LS mutants were assessed by cotransfecting each mutant into L cells with an equimolar amount of the pseudo-wild-type *tk* gene and then superinfecting the cells with the *tk*-deleted HSV-1 strain. RNA was isolated and mapped by primer extension. Figure 3A shows autoradiographic exposures of the sequencing gels used to map extension products, and Fig. 3B shows quantitation of these data compared with data derived from a series of oocyte microinjection assays.

A strikingly similar pattern was observed when the *trans*-induced L cell results were compared with the frog oocyte results. Both studies reveal three mutation-sensitive domains that occur at coincident locations. We assume that these mutation-sensitive areas indicate the presence of discrete transcription signals. The mutation-sensitive area located closest to the *tk* mRNA cap site was revealed by the phenotypes of LS -21/-12 and LS -29/-18. This 17-nucleotide region contains the TATA homology of the *tk* gene. Upstream of this proximal transcription signal we observed two additional areas that when mutated substantially reduce expression efficiency in both *trans*-induced L cells and microinjected oocytes. One of these two distal transcription signals was located between residues -105 and -80, according to both transcription assays. We termed this the second distal signal (dsII). The other mutation-sensitive area, termed the first distal signal (dsI), was delimited between residues -59 and -46 in the oocyte assay. The mutation-sensitive domain which includes dsI appears to encompass a larger area in *trans*-induced L cells than in oocytes. LS -79/-69 and LS -70/-61 exhibited reduced transcription efficiencies relative to the pseudo-wild-type control template in *trans*-induced L cells but not in microinjected oocytes. The implications of this extended domain that includes dsI will be discussed subsequently.

Phenotypes of single point mutants. The two distal tran-

scription signals of the viral *tk* gene share an inverted hexanucleotide repeat. The sequence 5'-CCGCCC-3' is located between residues -103 and -98 of dsII, and the inverted complement 5'-GGGCGG-3' is located between residues -55 and -50 of dsI. Evidence has been presented elsewhere (16, 17) which indicates that these hexanucleotides constitute important components of each distal transcription signal. In comparing the phenotypes of LS mutations in *trans*-induced L cells and micro-injected oocytes, we noted a quantitative difference that may be of interest. In the oocyte assay, mutation of the 5'-CCGCCC-3' hexanucleotide of dsII (LS -111/-101 and LS -105/-95) causes a more severe phenotypic effect than does mutation of the 5'-GGGCGG-3' hexanucleotide of dsI (LS -59/-49 and LS -56/-46). This inequivalence is not the case in *trans*-induced L cells. LS mutations that disrupt either hexanucleotide reduce transcription efficiency to about the same level (Fig. 3B).

We investigated this issue in more detail with a set of six single point mutations (17). Three of the point mutations create C-to-G transversions in the hexanucleotide of dsII. The three C-to-G transversions in the hexanucleotide of dsII (positioned at residues -103, -102, and -100) create, within the context of the hexanucleotide inverted repeat, lesions that are identical to the three G-to-C transversions in the hexanucleotide of dsI (positioned at residues -53, -51, and -50). Each single point mutation was assayed for transcription efficiency in *trans*-induced L cells (Fig. 4A). The phenotypes observed in *trans*-induced L cells are compared with those seen in the frog oocyte assay in Fig. 4B. Two aspects of these data should be noted. First, in both transcription assays a similar hierarchy of phenotypes was observed. Considering the hexanucleotide of dsII, the C-to-G transversion at residue -103 caused a less severe reduction in transcription efficiency than did the transversions at residues -102 and -100. Likewise, the G-to-C transversion at residue -50 of the hexanucleotide of dsI was less detrimental than the transversions at residues -53 and -51. Since the C-to-G transversions in the hexanucleotide of dsII represent the same lesions as the G-to-C transversions of the hexanucleotide of dsI and since they create a matching pattern of phenotypic effects in both expression assays, we conclude that the two hexanucleotides represent functionally analogous components.

The second noteworthy aspect of the data presented in Fig. 4 concerns a discrepancy between the L-cell and oocyte assays. In microinjected oocytes, each point mutation in the hexanucleotide of dsII exerted a more severe phenotypic effect than its corresponding point mutation in the hexanucleotide of dsI. In *trans*-induced L cells, this was not the case. Instead, we found that corresponding single point mutations in the two hexanucleotides led to roughly equivalent reductions in transcription efficiency. Recall that we noted this same difference when comparing the effects of LS mutations in the two assays. These results, coupled with the observation that the domain including dsI encompasses a larger area as deduced from the *trans*-induced L-cell assay (Fig. 3), suggest that this domain somehow plays a more critical role in *trans*-induced L cells than in microinjected oocytes.

Phenotypes of intertypic recombinant promoters. A comparison of the DNA sequences of the HSV-1 (strain MP) and HSV-2 (strain 333) *tk* promoters reveals five single base differences (23). Of the base changes, four map in dsII, and the remaining one maps in dsI. To assess whether any of these sequence differences alters transcriptional function,

FIG. 4. Expression levels of single point mutants. (A) Autoradiographic exposure of an electrophoresis gel used to map *tk*-specific primer extension products. Assay conditions are as described in the legends to Fig. 2 and 3. The six single point mutation that were assayed are identified above each gel lane. (B) Histogram that compares single point mutation phenotypes in *trans*-induced L cells (hatched bars) with phenotypes in microinjected oocytes (open bars). Oocyte quantitations are taken directly from the data of McKnight et al. (17). Quantitations were determined relative to the pseudo-wild-type internal control template as described in the legend to Fig. 3.

we compared the expression efficiency of each promoter in *trans*-induced L cells. The HSV-2 *tk* promoter is measurably weaker than the HSV-1 *tk* promoter (Fig. 5). By constructing and assaying the intrapromoter recombinants shown in Fig. 5A, we mapped sequences conferring the functional difference between the two promoters to dsII. Since there were 4-bp differences between the second distal signals of the two *tk* promoters, we were unable to distinguish the change(s) within dsII that account for the difference in expression efficiency. We noted however, that one of the base changes (the G-to-T transversion at residue -101) occurs within the hexanucleotide repeat (5'-CCGCCC-3' in HSV-1 versus 5'-CCTCCC-3' in HSV-2).

Phenotypes of distal signal inversion mutants. Positionally equivalent mutations in the two distal signals of the *tk* gene showed quantitatively similar phenotypes in *trans*-induced L cells but measurably different phenotypes in microinjected frog oocytes. What is the basis for this difference? As will be discussed in more detail later, the transcriptional apparatus of the frog oocyte is capable of using dsII in a manner that is independent of dsI (also see reference 17). In other words, the oocyte detects an intrinsic difference between the two distal signals. In *trans*-induced L cells, where equivalent mutations in the hexanucleotides of the two distal signals lead to equivalent phenotypic effects, we suspected that the dsI and dsII might be recognized and utilized in an equivalent manner.

We tested the respective roles of the two distal signals by using a distal signal inversion mutant. Although a detailed description of this mutant has already been published (17), we briefly recount its two-step construction. First, a double LS mutant was prepared by recombining LS -119/-109 and LS -42/-32. In this double LS mutant (dLS-co), synthetic *Bam*HI linkers bracket the distal transcription signals. One *Bam*HI site is located six residues upstream of the 5'-

FIG. 5. Structures and expression levels of intrapromoter recombinants between the HSV-1 and HSV-2 *tk* genes. (A) Structures of HSV-1 (open bars) and HSV-2 (hatched bars) *tk* promoters and intrapromoter recombinants. Hybrid promoters were generated *in vitro* by cross-ligation at the *Eco*RI restriction site that lies between the first and second distal signals of both *tk* genes. Derivatives of each promoter that retain only the first distal signal replace the second distal signal with pBR322 DNA. Each mutant was assayed for transcriptional efficiency in *trans*-induced L cells as described in the legends to Fig. 1 and 2. An autoradiographic exposure developed without the aid of an intensifying screen (B) was scanned by densitometry to quantitate the relative expression efficiency of each recombinant. These data are shown in the right-hand column of panel A.

CCGCCC-3' hexanucleotide of dsII, and another *Bam*HI site is located seven residues downstream of the 5'-GGGCGG-3' hexanucleotide of dsI. The distal inversion mutant (dLS-io) was prepared by digesting dLS-co with *Bam*HI and replacing the 77-bp distal signal fragment in the inverted orientation. dLS-co and dLS-io were tested in the *trans*-induced L-cell assay, and both were found to be approximately equivalent in expression efficiency to a wild-type *tk* promoter (Fig. 6).

The role of the domain including dsI in the distal inversion mutant was then assessed in two ways. First, we deleted it by removing all sequences upstream of the unique *Eco*RI site that occurs between the two distal signals. As a control to this experiment, we also truncated dLS-co to the *Eco*RI site (which removes the second distal signal). Removal of dsII from dLS-co resulted in a transcriptionally inactive gene as assayed in *trans*-induced L cells (Fig. 6). This result was expected because we already knew that removal of dsII from an otherwise native *tk* promoter results in inactivation (see phenotypes of Δ5' -95 and Δ5' -85, Fig. 2). In sharp contrast, *Eco*RI deletion of dsI from dLS-io caused less than a twofold reduction in transcription efficiency (Fig. 6). Thus, sequences within the domain including dsI are not, *per se*, essential for *trans* induction.

The second approach we used to assess the importance of dsI in the distal inversion mutant involved the transfer of the dsI single point mutations into the genetic background of the distal inversion mutant. The construction of these mutants

has been described elsewhere (17). Each of the three inverted point mutants of dsI (G-to-C/-53io, G-to-C/-51io, and G-to-C/-50io) exhibits a wild-type phenotype in *trans*-induced L cells (Fig. 6). These results reiterate the interpretation that dsII can act as an independent transcriptional activator when it occupies the position normally occupied by dsI.

One possible explanation for the activity of the distal inversion mutant and its derivatives that lack a functional dsI is that placement of dsII close to the TATA homology produces a strong constitutive promoter, a promoter capable of being actively expressed in uninfected L cells. This potential interpretation is eliminated by an experiment also shown in Fig. 6 in which the transcriptional activity of dLS-io was measured in mock-infected cells. In this case, no *tk* mRNA expression was observed.

The experiments outlined in this section indicated that *trans*-induced L cells recognize dsII in a manner that does not depend on the integrity of the domain including dsI. Such a result came as no surprise in the case of the oocyte assay, because we already knew that mutational inactivation of dsII led to more severe reduction in transcription efficiency than

FIG. 6. Structures and expression levels of distal signal inversion mutants. Top diagram shows structures of double LS mutant -119/-109/-42/-32 (dLS-co) and the derivative which inverts the positions of the two distal transcription signals (dLS-io). Both of these recombinants were truncated to the *Eco*RI restriction site, resulting in dLS-co ΔRI and dLS-io ΔRI. *Eco*RI truncation of dLS-co removes the second distal signal; truncation of dLS-io removes the first distal signal. The three single point mutations of the first distal signal (G to C/-53, G to C/-51, G to C/-50) were recombined into dLS-io as described previously (17). Each mutant was assayed for transcription efficiency in *trans*-induced L cells as described in the legends to Fig. 1 and 2. One recombinant, dLS-io, was also assayed in mock-infected cells. The autoradiographic exposure presented in the lower part of the figure shows the phenotype of each recombinant diagrammed in the upper part. An exposure developed without the aid of an intensifying screen was scanned by densitometry to quantitate the relative expression efficiency of each recombinant. These data are shown in the right-hand column of the upper part of the figure.

inactivation of dsI. In the *trans*-induced L-cell assay, however, the sequence domain including dsI appeared to play a relatively more important role. In light of these observations, we propose two conclusions. (i) Both *trans*-induced L cells and frog oocytes recognize dsII as an intrinsically stronger element than dsI. (ii) However, the position occupied by dsI in the native *tk* promoter is more important in *trans*-induced L cells than in microinjected frog oocytes.

DISCUSSION

The results of our *trans*-induction assays with *tk* promoter mutations can be summarized as follows. Three mutation-sensitive areas were observed. The area located most proximal to the mRNA cap site contains the TATA homology. Two areas upstream of the TATA homology were also observed. These distal signals share an inverted hexanucleotide repeat sequence, and the phenotypes of corresponding point mutations in each hexanucleotide repeat are concordant. With two exceptions, *trans*-induced mouse L cells recognize the *tk* promoter in the same way as microinjected frog oocytes. Both of these exceptions concern the first distal transcription signal (dsI). Compared with frog oocytes, *trans*-induced L cells depend more heavily on an intact dsI. Furthermore, assays of LS mutants in *trans*-induced L cells reveal a new component that is located immediately upstream of the dsI signal defined originally in the frog oocyte expression assay.

We initially wondered whether this new component, which is located between 79 and 61 residues upstream of the *tk* mRNA cap site, might contain a qualitatively distinct component of the *tk* promoter. Since transcriptional expression in the *trans*-induction assay is almost completely dependent on regulatory proteins synthesized by the incoming virus, it was tempting to speculate that the sequences between residues -79 and -61 represent a viral-specific transcriptional activator signal. However, *EcoRI* truncation of the distal inversion mutant completely removes these putative activator sequences without affecting *trans* induction. Upon further consideration, we suggest that the differences between *tk* promoter recognition in oocytes and *trans*-induced L cells are of a quantitative rather than qualitative nature. Although it is clear that *trans*-induced L cells depend more heavily on an intact dsI than do oocytes, we find no evidence indicating that the viral induction process depends upon it as a unique activator component.

Our results show that the promoter of the HSV *tk* gene is recognized and activated in a similar manner in both microinjected oocytes and *trans*-induced mouse L cells. If *tk* promoter recognition occurs similarly in both the constitutive (oocyte) and regulated (HSV-infected fibroblast) environments, it would appear that the virus-mediated induction process does not result from a qualitative alteration in promoter specificity. The major flaw in this deduction is the tentative identification of the oocyte as a constitutive environment. It is quite possible that frog oocytes contain transcription factors that are functionally equivalent to the immediate-early gene products of HSV.

Uninfected mammalian cells offer a more legitimate constitutive assay for *tk* promoter function. Unfortunately, the level of *tk* mRNA expression in uninfected cells is so low that we are unable to obtain reliable quantitative comparisons of promoter mutant phenotypes. This dilemma has recently been circumvented by the use of an *in vitro* transcription assay (K. Jones, K. Yamamoto, and R. Tjian, manuscript in preparation). Soluble extracts derived from HSV-infected and mock-infected HeLa cells have been

fractionated as a means of identifying transcription factors required for expression of the HSV *tk* gene. These studies have shown that the components required for transcription of the *tk* gene, in a manner that is dependent on all of the promoter domains described in the present study, occur in both infected and uninfected cell extracts. Furthermore, Jones, Yamamoto, and Tjian (personal communication) analyzed all of our HSV-1 promoter mutants in extracts prepared from uninfected cells and observed a map of promoter domains that coincides strikingly with the maps we have deduced from microinjected oocytes and *trans*-induced mouse fibroblasts. Taken together, these studies argue strongly against a mechanism of transcriptional induction whereby a viral regulatory gene product activates the *tk* promoter by binding to it directly. The possibility remains, however, that the induction of delayed-early HSV genes results from a promoter activation mechanism whereby immediate-early regulatory proteins facilitate the access of cellular transcription factors for delayed-early promoters.

The provisional conclusion we have drawn from the experiments outlined in the present study is that viral regulatory proteins do not induce *tk* gene expression by selectively altering promoter recognition. This interpretation is at odds with conclusions forwarded by at least three other laboratory groups. The various experiments of Zipser et al. (27), Smiley et al. (21), and El Karez et al. (3a, 4) have provided tentative assignments of *tk* promoter domains that are selectively necessary for expression under *trans*-induced conditions. Although we have attempted in the present study to conduct a well-controlled series of experiments on this topic, we wish to emphasize that our conclusions rest principally on studies of transiently inoculated genes. This method of examining the process of viral gene induction may harbor unanticipated artifacts. It is possible that these discrepant interpretations may be resolved by studies of gene expression from viral-borne genes. Such an approach is facilitated by the introduction of *tk* promoter mutations into the genome of HSV by homologous recombination (D. Coen and S. McKnight, unpublished data).

Our conclusion that viral *trans* induction of the HSV *tk* gene does not involve the selective alteration of promoter recognition is consistent with results published recently by Everett (5). In that study it was shown that promoter sequences of the glycoprotein-D gene of HSV do not uniquely specify its response to viral *trans* induction. Perhaps the most substantial argument against a promoter-specific model for *trans* induction is found in the work of Green and colleagues (7). The Green group showed that expression of an immediate-early regulatory gene of pseudorabies virus *trans* induces expression of the human beta-globin gene. The pseudorabies virus regulatory gene used in the aforementioned study appears to be analogous to an immediate-early regulatory gene of HSV. Elegant genetic studies have shown that the HSV regulatory gene (whose product is variously termed ICP4 or Vmw-175) is critically required for the expression of delayed-early viral gene products (3, 18, 19, 24).

If the HSV immediate-early regulatory proteins do not activate delayed-early promoters directly, how is the induction process achieved? In the case of the HSV *tk* gene it is clear that information capable of responding to *trans* induction is tightly linked to the gene. Recombinant copies carrying only 119 bp of DNA upstream from the *tk* mRNA cap site and only 294 bp of DNA downstream from the polyadenylation site are efficiently *trans* induced as both episomal (Fig. 2) and stably integrated templates (S. Eisenberg, unpub-

lished data; also see reference 4). Since we raise the possibility that the *tk* promoter may not house the *cis*-acting response element to *trans* induction, it would appear that either the transcribed portion of the gene or sequences immediately flanking its 3' terminus might carry such signals. Evidence supporting this conclusion is presented elsewhere (6). Graves and colleagues found that when the murine sarcoma virus long terminal repeat is fused to the HSV *tk* gene in place of its native promoter, the resulting recombinant is markedly *trans* induced by superinfecting HSV, whereas the parental MSV template is not. This observation raises the possibility that the transcribed segment of the HSV *tk* gene, rather than the promoter, facilitates its response to viral immediate-early regulatory factors.

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