Evidence for a Direct Role for Both the 175,000- And 110,000-Molecular-Weight Immediate-Early Proteins of Herpes Simplex Virus in the Transactivation of Delayed-Early Promoters

PETER O’HARE* AND GARY S. HAYWARD

Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 11 October 1984/Accepted 28 November 1984

We reconstructed the regulated induction of delayed-early (DE) transcription that occurs during herpes simplex virus (HSV) infection by using a transient expression system in which recombinant target genes were cotransfected into Vero cells together with intact activating genes. Plasmids containing cloned HSV-1 or HSV-2 immediate-early (IE) genes stimulated by up to 100-fold the expression from recombinant constructs containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the DE promoter/regulatory region from the genes for an HSV-2 38,000-molecular-weight (38K) protein and the HSV-1 thymidine kinase. This activation was specific to hybrid genes containing DE regulatory regions since no significant increases in expression were observed in cotransfection experiments with the CAT gene without any promoter region or under the control of a number of other regulatory regions, including an HSV-1 IE regulatory region, the complete or enhancerless early regulatory region of simian virus 40, and an inducible cellular promoter/regulatory region. By using a variety of cotransfected plasmids containing individual or different combinations of HSV-1 or HSV-2 IE genes, we show that if the five known IE genes, two, those coding for the 175K and 110K polypeptides, each possessed the ability to stimulate expression from both DE promoters. Cleavage of the input plasmids within the known coding regions for the 175K and 110K proteins abolished stimulation of DE/CAT gene expression, whereas cleavage outside the coding regions had no effect on stimulation. We conclude that stimulation of CAT expression occurred exclusively by a transactivation mechanism in which the products encoded by these IE genes act on the DE hybrid constructs at the transcription level. No transcriptional stimulatory function was demonstrated for the IE 68K and 63K proteins, although our results indicate that the IE 12K protein may augment the DE stimulatory activity of the 175K and 110K proteins.

The genome of herpes simplex virus (HSV) consists of a large (150 kilobase pairs), linear, double-stranded DNA molecule which, considering the observation of symmetric transcription and overlapping transcription units (5, 8, 26, 35), encodes well over 50 polypeptide products (15). Although regulation of the synthesis of these gene products is known to involve complex processes at many levels (14, 16, 19), abundant evidence exists that transcriptional regulation is the main level of control of the coordinate induction of the differentially regulated immediate-early (IE), delayed-early (DE), and late groups of virus genes (1, 3, 22, 25). With the aim of elucidating both cis- and trans-acting functions involved in this transcriptional regulation, we developed a short-term transfection system, using recombinant constructs containing the promoter/regulatory regions of IE and DE genes from HSV linked to the coding region for the bacterial enzyme chloramphenicol acetyltransferase (CAT) (28). Eucaryotic cells do not synthesize this protein, and several previous studies have shown that measurements of enzyme levels expressed from hybrid genes containing the CAT region provide rapid and very sensitive estimates of promoter activity (12, 18, 39). We showed that expression of the HSV/CAT recombinant genes could be stimulated by virus infection of transfected cells with the patterns of stimulation correctly reflecting characteristics of expression of the parent virus genes from the IE and DE groups (28). Analysis of the factors modulating expression of these recombinant CAT genes therefore provides a valid assay system for dissecting the detailed requirements for induction of the different classes of virus genes at the transcriptional level.

The nature of the requirements for trans-acting factors involved in the induction of DE gene expression remains unclear. The host RNA polymerase II enzyme is thought to be involved in all stages of HSV transcription (6), and previous work has shown that functional IE polypeptides and de novo rounds of transcription are required for DE protein synthesis (16). In the absence of IE protein synthesis, transcription is limited to the IE genes themselves, and it is therefore proposed that IE products are involved in the induction of DE expression at the transcriptional level. Attempts to examine the identity and function of IE polypeptides involved in this induction have been largely limited to the use of metabolic inhibitors (16, 24, 30) and phenotypic analysis of conditional lethal mutants possessing lesions in IE genes (7, 33, 37). However, to date, mutations of this type have been demonstrated in only one of the five IE genes, that coding for the 175,000-molecular-weight (175K) polypeptide (ICP4). Studies with temperature-sensitive viruses possessing mutations in this gene have demonstrated a requirement for the 175K IE protein in transcriptional regulation at both early and late times in the virus growth cycle (33, 38). Nevertheless, this protein may not be the sole requirement from the IE group, and the mechanism of stimulation of DE expression could be either direct or indirect through some intermediate function. In addition,
three other IE polypeptides (110K, 68K, and 63K), like the 175K protein, are known to be phosphorylated and located predominantly on the surface of infected cells (40), but there are few data illuminating the function of these products.

We attempted to analyze the specific requirements for IE gene products in DE expression by cotransfecting isolated defined IE genes with target CAT constructs containing DE regulatory regions. Our results lead to the identification of a subset of the IE genes, which individually are sufficient for activation of expression of the DE/CAT genes, and implicate additional IE genes in the modulation of this activation. This approach provides a useful alternative to reconstruction in vitro for the direct identification of factors involved in transcriptional regulation of virus genes.

MATERIALS AND METHODS

Cells and transfection procedures. Vero cells obtained from the American Tissue Culture Collection, Rockville, Md., were grown in 32-oz. prescription bottles at 37°C in Dulbecco modified Eagle minimal essential medium containing 10% fetal calf serum. Cells were plated the day before transfection into cluster dishes (6 by 35 mm; Costar, Cambridge, Mass.) at 5 × 10^3 cells per well. At 3 to 4 h before transfection, the medium was aspirated and replaced with 2.5 ml of fresh medium. DNAtransfection was performed as described previously (28), using the calcium phosphate precipitation method (13) and glycerol boost (29). Routinely, 2-μg amounts of the recombinant CAT target genes and 2 μg of the cotransfecting plasmids containing IE genes were employed. Parallel cotransfections employed 2 μg of pBR322 or isogenic Vero cell DNA to control for the overall DNA concentrations in the transfection procedure.

Plasmids. The plasmid p38KCAT (28) contains a recombinant gene in which the CAT coding region was placed under the control of the DE promoter/regulatory region of the gene for the 38K protein contained within the BglII N fragment (0.582 to 0.628) of HSV-2[333]. The virus sequences in this recombinant gene extend from −400 to +100 relative to the mRNA start site of the parent gene, with the first translation start codon being that for the CAT protein. A second DE promoter/regulatory region, that of the HSV-1 thymidine kinase gene, was inserted upstream of the CAT gene and is contained in the plasmid pTKCAT. This recombinant gene was constructed by using the BamHI-BglII segment of the BamHI O fragment of HSV-1[MP] and contains sequences extending from −800 to approximately +60 relative to the mRNA start site for the thymidine kinase gene. In constructing the IE/CAT recombinants, we used the 1.9-kb BamHI-HindIII fragment (0.865 to 0.876) of HSV-1[MP]. The BamHI site at position 0.865 is located approximately 30 base pairs (bp) downstream from the transcriptional initiation site and before the translational initiation site of the gene coding for the IE 175K protein. Sequences located within this fragment upstream of the BamHI site have been shown to contain all the transcriptional control signals necessary to confer IE-type regulation on selected target genes (4, 28, 31). A detailed account of the construction of p38KCAT and p175KCAT has been given elsewhere (28). It should be noted that the pTKCAT construct (pPOH3) used in this work contains additional sequences 5' of the thymidine kinase RNA start site (−800) and exhibits higher basal and induced CAT enzyme levels compared with a different pTKCAT plasmid (−400) used in our previous study.

A recombinant construct (pSAACAT) containing the CAT gene under the control of a eucaryotic cellular promoter, that of the gene for the serum amyloid A protein, was kindly provided by C. Lowell and J. Morrow, Johns Hopkins University School of Medicine, Baltimore. This recombinant construct exhibits relatively weak constitutive promoter activity in short-term transfection but can be stimulated 20-fold by treatment of the transfection cells with a hormone (C. Lowell and J. Morrow, personal communication). Two additional plasmids used in comparative analyses in this work, which contain the CAT gene under the control of the simian virus 40 (SV40) early regulatory region, either with the 72-bp repeat enhancer region (pSV2CAT) or without the enhancer region (pA10CAT), were obtained from G. Khoury, National Institutes of Health, Bethesda, Md. Plasmid pCATB containing the CAT structural gene without any eucaryotic promoter/regulatory sequences was obtained from O. Andrisani, Purdue University, West Lafayette, Ind.

Relevant members of our series of plasmids containing sequences coding for various IE genes of HSV-1 and HSV-2 are illustrated below (see Fig. 1). A detailed account of the construction and physical map of a number of these plasmids together with evidence for the synthesis of an IE protein(s) after their short-term transfection has been published (27). Additional plasmids (see Fig. 1) containing only single intact genes for the 175K IE protein (pXhol-C) and the 110K IE protein (pIga-15 and pIga-6) were kindly provided by I. Gelman and S. Silverstein, Columbia University, New York, N.Y.

CAT assays. Cell harvesting and CAT assays were performed as described previously (12, 28). Under our routine conditions with 0.2 μCi of [3H]chloramphenicol, the assay was linear with respect to enzyme concentration until at least 10^6 cpm appeared as the chloramphenicol-3-acetate (Cm-3-Ac) product; with standard enzyme concentrations, the assay was linear with respect to time for at least 60 min. For quantitative estimates of CAT activity and fold inductions, the appropriate sections were cut from the thin-layer chromatography plate and placed in toluene PPO (2,5-diphenyloxazole)-POPOP [1,4-bis(5-phenyloxazole)benzene] scintillation fluid, and the radioactivity was counted in an LKB 216 liquid scintillation counter. Shaw (36) has shown that analyses of the Cm-3-Ac product provide valid estimates of CAT activity. All quantitative comparisons were therefore made by measuring the amounts of the Cm-3-Ac product by the above method which was confirmed to be on the linear part of the curves of product formation versus enzyme concentration and time. Within individual experiments, enzyme levels expressed from the various CAT constructs were highly consistent, although some variation, perhaps due to differences in cell passage or plating efficiency, was observed in absolute enzyme levels between different experiments.

RESULTS

 Cotransfection assays for activation of CAT constructs containing DE promoter/regulatory regions. In the first series of experiments, we wished to determine whether expression from DE/CAT recombinant genes could be stimulated by cotransfection with isolated IE genes and, if so, whether we could demonstrate, as for stimulation by virus infection, specificity in this induction. Recombinant CAT genes containing various virus and cell promoter/regulatory regions were cotransfected with plasmids containing different combinations of HSV-2 IE genes. Each of these latter plasmids contains the genes coding for the 185K (equivalent to HSV-1 175K) and 110K proteins and in addition genes for the 63K (pGR151), 68K (pGR91), or 12K (pGR90) IE proteins. The map coordinates of the inserts and locations of the IE genes
are shown in Fig. 1. Previous results with immunofluorescence assays have demonstrated that an IE polypeptide(s) is expressed after short-term transfection of these plasmids (27). The effects of transfection of these plasmids on expression of the recombinant CAT gene under the control of the HSV-2 DE regulatory region, p38KCAT, are shown in Fig. 2. Quantitative results from this and additional experiments, obtained by scintillation counting of radioactivity from the products of the CAT enzyme, are summarized in Table 1. Enzyme levels expressed from p38KCAT were increased by cotransfection with each of the three plasmids containing the IE genes, the resulting increases in activity being 20-fold for pGR151, 20-fold for pGR91, and 40-fold for pGR90. Similar analyses demonstrated that expression from another DE regulatory region, that of the HSV-1 thymidine kinase gene contained in pTKCAT, could also be stimulated 20- to 40-fold by cotransfection with plasmids containing the HSV-2 IE genes (see, for example, Fig. 4). Throughout the course of this work, expression from the DE/CAT constructs was stimulated to a significantly greater extent (40- to 100-fold) by cotransfection with plasmid pGR90 compared with that obtained with other plasmids containing IE genes (see below). By comparison, cotransfection of DE/CAT constructs with pBR322 lacking IE genes or with cell DNA resulted in only up to threefold increases in enzyme activity.

Transactivation by IE genes is specific for CAT constructs containing DE promoter regions. In contrast to the dramatic stimulation of expression from CAT target genes under the control of DE promoter/regulatory regions, no significant increases in expression were observed in cotransfection experiments with CAT genes under the control of a number of other regulatory regions. Thus, Fig. 3 shows results from
a series of transfections performed in parallel with those illustrated in Fig. 2, which demonstrate that cotransfection with pGR151 or with other plasmids containing combinations of IE genes (see Fig. 5) had no significant effect on expression from the HSV IE promoter contained in p175KCAT or from the SV40 early regulatory region in pSV2CAT. Further analysis comparing the effects of cotransfecting IE genes with CAT constructs under the control of either HSV DE regulatory regions (p38KCAT and pTKCAT), a cellular regulatory region (pSAACAT), SV40 early region lacking the 72-bp repeat enhancer region (pA10CAT), or a construct lacking any eucaryotic promoter sequences (pCATB′) is shown in Fig. 4 (also Table 1). Again, significant induction of CAT expression occurred only with the HSV DE constructs. Therefore, the specific requirement for DE promoter/regulatory regions in the induction of expression indicates that activation is not due to a generalized stimulatory effect of the IE products on expression of cotransfecting genes and is also unlikely to be due to a recombinational process providing promoter or enhancer functions or both for a cis-type activation. Further data confirming that activation of expression from the DE regions occurs by a trans-acting mechanism is detailed below. These results represent the first report of such transactivation by using defined regulatory regions and cotransfected intact IE genes in the analysis of herpesvirus gene expression.

We have shown previously that expression from transfected p175KCAT could be induced by virus infection, although at much earlier times than from p38KCAT. Since in the cotransfection experiments presented above enzyme levels were assayed only at 40 to 50 h after transfection, it seemed possible that the intact IE genes may have had an autoregulatory effect on expression from p175KCAT, which would be detectable only at earlier times after transfection. Therefore, an experiment was performed to measure and compare the effect of intact IE genes on expression from p38KCAT and 175KCAT at different times after cotransfected intact IE genes were transfected with 2 μg of p38KCAT alone (−) or together with 2 μg of isogenic Vero cell, pBR322, pGR151, pGR191, or pGR90 DNA as indicated. Cells were harvested 44 h after transfection, and equal proportions of the extracts were assayed for CAT activity. The lane labeled CON represents a control for background CAT activity in cells transfected with Vero cell DNA only. The lane labeled CAT represents the activity obtained with 0.6 U of commercially prepared enzyme.

![Figure 2](JaV.png)

**FIG. 2.** Induction of p38KCAT expression by cotransfection with plasmids containing HSV-2 IE genes. Monolayers of Vero cells were transfected with 2 μg of p38KCAT alone (−) or together with 2 μg of isogenic Vero cell, pBR322, pGR151, pGR191, or pGR90 DNA as indicated. Cells were harvested 44 h after transfection, and equal proportions of the extracts were assayed for CAT activity. The lane labeled CON represents a control for background CAT activity in cells transfected with Vero cell DNA only. The lane labeled CAT represents the activity obtained with 0.6 U of commercially prepared enzyme.

![Figure 3](JaV.png)

**FIG. 3.** Absence of induction from the HSV IE or SV40 early promoter/regulatory regions. A 2-μg amount of p175KCAT or pSV2CAT DNA was transfected alone (−) or together with 2 μg of Vero cell DNA or pGR151 DNA. Assays were performed on cells harvested 44 h after transfection. The lane marked CAT represents the level of activity in 0.6 U of purified enzyme.

**TABLE 1.** Quantitative estimates of trans activation by cotransfection with plasmids containing IE genes

<table>
<thead>
<tr>
<th>Expt</th>
<th>Hybrid test gene</th>
<th>Cpm in Cm-3-Ac with cotransfected plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBR322</td>
<td>pGR90</td>
</tr>
<tr>
<td>1*</td>
<td>p38KCAT</td>
<td>1,100</td>
</tr>
<tr>
<td>2*</td>
<td>pTKCAT</td>
<td>1,954</td>
</tr>
<tr>
<td></td>
<td>pA10CAT</td>
<td>216</td>
</tr>
<tr>
<td>3*</td>
<td>p38KCAT</td>
<td>504</td>
</tr>
<tr>
<td></td>
<td>pTKCAT</td>
<td>1,600</td>
</tr>
<tr>
<td>4*</td>
<td>p38KCAT</td>
<td>4,800</td>
</tr>
<tr>
<td></td>
<td>pTKCAT</td>
<td>1,340</td>
</tr>
</tbody>
</table>

* Fig. 2.
* Fig. 4.
* Fig. 6.
* Fig. 7.
infection (Fig. 5). The results show that even by 18 h, a relatively early time after transfection (23, 28), expression from p38KCAT was stimulated by 20-fold, whereas no significant effect was observed on levels expressed from p175KCAT. Enzyme levels expressed from p38KCAT were stimulated to the maximum extent (70-fold) by 30 h after transfection, and no stimulation from p175KCAT was observed at any time after transfection. These findings are consistent with our previous data demonstrating differential induction of the two hybrid constructs by ts mutants of HSV-1 (28) and confirm that induction of expression from p175KCAT and p38KCAT after virus infection proceeds via two qualitatively different pathways with stimulation of p175KCAT being mediated by virion component(s) and that of p38KCAT requiring functional IE polypeptides. Our results also indicate that, at least in this system, there is no absolute requirement for virion components or virion induced factors for the expression of IE proteins from transfected genes nor for their subsequent activation of expression from DE regulatory regions.

Plasmids containing either the IE 175K gene or the IE 110K gene can independently activate DE regulatory regions. Comparison of the common virus sequences in the plasmids used in the previous experiments indicated that the HSV-2 IE coding for the 185K and for the 110K polypeptides may be sufficient for activation of the two DE regulatory regions. Therefore, we wished to determine whether either of these or other IE genes individually could induce DE transcription. In the first set of experiments (Fig. 6), we used a series of plasmids which contain only one intact HSV-1 gene: pIGA-15 and pIGA-6, which contain the gene for the 110K protein, and pXhoI-C, which contains the gene for the 175K protein (equivalent to the 185K protein of HSV-2). The results show that the plasmid containing the gene for the 175K protein (pXhoI-C) could in the absence of other IE genes stimulate expression from both the 38K and thymidine kinase promoter regions with resulting increases in CAT activity of 20- to 40-fold (Fig. 6 and Table 1). Unexpectedly, we found that plasmids containing the gene for the 110K protein (pIGA-15 and pIGA-6) could also independently stimulate expression from the DE regulatory regions, albeit with lower overall increases in enzyme levels compared with those obtained with pXhoI-C. Similar analyses with two additional plasmids, pGR195 (Fig. 7) and pGH3 (data not shown), whose only intact IE genes are those for the HSV-1 or HSV-2 110K proteins (Fig. 1), confirmed that this gene product could independently activate expression from DE regulatory regions. None of these plasmids was as efficient in stimulating expression from the DE regulatory regions as was pGR90, which in parallel cotransfection experiments increased expression by 50- to 100-fold (Table 1). Parenthetically, our results show that an HSV-2 DE promoter/regulatory region could be stimulated by isolated individual HSV-1 IE genes and that an HSV-1 DE region could be activated by the HSV-2 IE genes contained in pGR90.

Results from further experiments indicated that the ability to stimulate DE expression may reside exclusively in this subset of IE genes, i.e., those for the 110K and 175K proteins. Thus, Fig. 7 shows results from an experiment comparing the effects of cotransfection of p38KCAT or pTKCAT with either pGR195, pGR162 (containing the gene for the HSV-2 63K protein), or pGR169 (containing the gene for the HSV-1 68K protein). Transfection with pGR195
resulted in high levels of expression from both target genes with increases in enzyme levels of approximately 12-fold for p38KCAT and 30-fold for pTKCAT. In contrast, cotransfection with pGR162 (or pGH18, which also contains the gene for the HSV-2 68K protein; Fig. 1 and data not shown) or pGR169 had no significant effect on expression from either DE/CAT recombinant (Fig. 7 and Table 1). These results indicated that, at least in our system, the individual IE genes coding for the 63K and 68K proteins were not sufficient for activation of expression from DE control regions. In an attempt to determine whether the 68K protein could augment activation by the 110K or 175K polypeptides, cells were transfected with the recombinant target genes and appropriate combinations of plasmids containing the IE genes. We could demonstrate no increased stimulation when the gene for the 68K protein was included, and on the contrary, a small but reproducible reduction (at least two-fold) in target gene activity was observed when this plasmid was cotransfected with either the plasmid encoding the 110K protein (Fig. 7) or that encoding the 175K protein (data not shown).

The gene coding for the 12K IE protein enhances stimulation of expression from DE control regions. Throughout this work comparative analyses of the levels of induction of expression from DE regulatory regions showed that cotransfection with plasmid pGR90 resulted in a 40- to 100-fold increase in activity, significantly higher (up to 5-fold) than that resulting from cotransfection with the individual or combined genes for the 110K, 175K, 68K, and 63K proteins. Inspection of the sequences contained within pGR90 (Fig. 1) led us to propose that the IE gene coding for a 12K protein may be responsible for the increased levels of stimulation. Results from an experiment attempting to demonstrate an involvement of this latter gene are shown in Fig. 8. Because no constructs containing only the intact gene for the 12K protein were available, the approach adopted was to selectively inactivate different genes within pGR90 before cotransfection with the DE/CAT target genes and then assay the effect on induction. Mapping of restriction enzyme sites in pGR90 in relation to the known gene locations indicated that digestion with SalI should cleave within the genes for the 185K and 12K proteins, leaving intact the gene for the 110K protein, whereas digestion of the plasmid with EcoRI should cleave only within the gene for the 12K protein, leaving intact the genes for the 185K and 110K proteins (Fig. 8b). The results show that prior digestion of pGR90 with SalI and EcoRI reduced the extent of stimulation of p38KCAT from approximately 50-fold to 10- and 14-fold, respectively. Since the genes for the 185K and 110K proteins could individually stimulate expression from the DE regulatory regions, the result with SalI cleavage is consistent with the interpretation that reduction in expression is due to inactivation of the 185K gene (or the remaining stimulation is due to the gene for the 110K). Cleavage with EcoRI, which inactivates only the gene for the 12K protein while leaving intact those for the 110K and 185K proteins, also significantly decreased the extent of stimulation of p38KCAT expression. Therefore, the gene coding for the 12K IE protein, although not absolutely necessary, may augment activation of the DE regulatory region by the 185K or the 110K protein or both.

Intact IE genes are necessary for activation of the DE/CAT recombinants. In the cotransfection experiments described in this work, the formal possibility was raised that increased expression from the DE/CAT recombinants may not be due to transactivation by functional IE gene products but to cis activation, perhaps by a recombinational process with enhancer-type sequences proposed to exist within the 5'-upstream region of at least one IE gene (20). From our results showing that restriction enzyme cleavage of pGR90 within the gene for the 185K protein reduced but did not abolish activation of the DE/CAT construct, it was possible that linearization of the plasmid per se may reduce IE expression and subsequent transactivation or that the remaining activity was not due to other IE genes within the plasmid but to recombination and cis activation not requiring the intact 185K gene. To rule out these possibilities, we carried out a series of experiments in which plasmids were cleaved with enzymes cutting either within different IE coding regions or completely outside the IE genes. The results show that linearization of a plasmid containing IE gene(s) with enzymes which cut outside the gene(s) had no significant effect on activation of expression from the DE
region. Cleavage of a plasmid containing only one IE gene, i.e., the 110K or 175K gene, with an enzyme which cut within the gene abolished activation. In addition, digestion of a plasmid containing several IE genes, e.g., pGR90, with an enzyme which cleaved within each of them completely abolished activation.

Thus, Fig. 9 shows results from an experiment measuring the effect of prior cleavage of pGR90, which contains several IE genes, or of pIGA-15, which contains only one IE gene (110K protein), on the induction of expression from p38KCAT. Maps of the relevant cleavage sites in relation to the IE gene locations are shown in Fig. 8b and 9b. The 50-fold stimulation observed with uncut pGR90 was essentially unaffected by cleavage with HindIII, which cuts twice within the plasmid, whereas cleavage with BamHI, which cuts within each of the IE genes, completely abolished stimulation. With pIGA-15, a 20-fold increase in activity resulted from cotransfection with the uncut plasmid, and prior digestion with HindIII, which cuts twice, or EcoRV, which cuts once, each site being outside the 110K gene, had no significant effect on induction. However, digestion with BamHI, which cuts twice, once within the 110K gene, completely abolished activation. Similar results were obtained on analysis of induction by the gene for the IE 175K protein (Fig. 10). Digestion of pXhol-C with BamHI, which cuts within both the protein coding region and the leader region of the 175K gene, completely prevented activation of p38KCAT. Cleavage with Sall, which cuts only within the leader region, leaving the coding region and upstream regulatory region separate but otherwise intact, also abolished activation. Stimulation of DE/CAT expression was also prevented by cleavage of pXhol-C with PvuII, which cuts the plasmid once, within the protein-coding region of the 175K gene (data not shown). In contrast, digestion with HindIII, which cuts twice in pXhol-C but outside the 175K gene, had no significant effect on activation of the DE/CAT construct.

These results demonstrate that induction of expression of the DE/CAT recombinants by cotransfecting IE genes involved exclusively a transactivation mechanism mediated by the IE gene products. Furthermore, the reduction in DE activation by pGR90 after cleavage with EcoRI was not caused by linearizing the plasmid, which per se had no significant effect, but resulted directly from inactivation of the gene encoding the 12K protein.

FIG. 8. Effect of cleavage in the 12K gene region of pGR90 on activation of p38KCAT. (a) p38KCAT (2 μg) was cotransfected with 2 μg of pBR322 or 2 μg of pGR90 either uncut or after cleavage with the restriction enzyme Sall or EcoRI. After digestion or mock digestion, samples of pGR90 were precipitated with ethanol and suspended in water before cotransfection. Restriction enzyme cleavage was monitored by gel electrophoresis of samples of the resuspended plasmid DNA. (b) Restriction enzyme cleavage map in relation to the HSV-2 IE genes located in pGR90. B, BamHI; S, Sall; E, EcoRI; H, HindIII.

FIG. 9. An intact IE 110K gene is necessary for DE activation. (a) The p38KCAT target plasmid DNA (2 μg) was cotransfected with 2 μg of pGR90 or pIGA-15 DNA which had been cleaved within or outside the IE coding regions. BamHI cleaves within both the 175K and 110K genes in pGR90 and within the 110K gene in pIGA-6. HindIII and EcoRV cleave outside the IE coding regions in either plasmid. Cotransfection with uncut pGR90 and pIGA-15 DNA is included for comparison. Cells were harvested 45 h after transfection, and equal amounts were assayed for CAT activity. (b) Restriction enzyme cleavage map of pIGA-15 in relation to the IE gene coding for the 110K polypeptide. B, BamHI; H, HindIII; E, EcoRI; K, KpnI; S, Sall; V, EcoRV.
specific cis-acting regulatory domain is present in the inducible DE target regions. However, from analyses of the effects of virus infection on expression from a recombinant gene containing the glycoprotein D DE promoter region, Everett (9, 10) reached the conclusion that there may not be DE-specific cis-acting regions, with the only requirement for activation by IE proteins being a functional promoter. To resolve this question, we are now performing an analysis of constitutive and inducible expression of deletion mutants of the 38K promoter region and attempting to transfer DE-type responsiveness to previously unresponsive promoter regions.

The isolation of mutants which are temperature sensitive because of lesions in the gene coding for the 175K protein obviously demonstrates an essential function for this protein in virus replication. The transcription patterns of most of these mutants at the restrictive temperature are similar to those observed under IE conditions (37); i.e., DE and late gene expression are virtually abolished. These observations imply that the temperature-sensitive defect is due to an inactive regulatory function(s) of the 175K protein that is normally required during infection to allow DE and late transcription. However, this does not necessarily mean that the 175K protein directly mediates DE and late expression since some other IE product(s) or complex, itself requiring the 175K protein for normal function, could have the direct regulatory effect. Our results now show that the 175K protein does stimulate expression from DE promoters in the absence of other IE genes, suggesting that during virus infection this protein may directly induce DE gene expression without the requirement for an intermediary function. However, our observation that the gene for the 110K IE protein can also independently activate DE expression raises some interesting new questions relating to a potential functional interaction between this protein and the 175K protein. Firstly, if the 110K protein possesses equivalent functions to the 175K protein, how can a temperature-sensitive mutant with a lesion only in the gene coding for this latter protein have been selected? Secondly, given the existence of temperature-sensitive mutants with lesions in the 175K gene, e.g., HSV-1[KOS]tsB2 and HSV-1[KOS]tsS9, how does the ability of the isolated individual 110K gene to stimulate DE expression be reconciled with the phenotype of these mutants which, although possessing wild-type genes for the 110K protein, express little, if any, DE and late genes at the nonpermissive temperature? One explanation to help answer both of these questions proposes that during virus infection the regulation of virus gene expression is mediated by a transcriptional complex in which, in addition to host cell functions, both the 175K and 110K IE polypeptides are sequestered. Thus, although these proteins individually possess a DE stimulation function, when they are present together in the infected cell the functional form is a complex containing both. Temperature-sensitive mutations in the 175K protein may then be dominant, conferring temperature sensitivity on the complex and thus also on the correct functioning of the 110K protein. An alternative related possibility is that a temperature-sensitive 175K protein may form an irreversible nonfunctional interaction with its regulatory target, e.g., DE gene templates or host transcriptional components, blocking the potential interaction of the 110K protein. The potential for a 175K/110K interaction is currently being tested in our system by cotransfecting DE target genes and the 110K gene together with a gene for a temperature-sensitive 175K protein. Since we already know that the 110K protein alone can activate DE expression, if this

We have previously shown that a short-term transfection system with recombinant constructs containing promoter/regulatory regions of IE and DE genes for HSV linked to the coding region for the bacterial enzyme CAT provides valid assays for the expression and regulation of HSV genes (28). The results presented here describe the use of this system for a more detailed examination of the features of HSV gene regulation and identification of those IE gene products required for the induction of DE gene expression. We demonstrate that the IE protein-mediated stimulation of DE promoter/CAT activity observed after virus infection of transfected cells (28) could also be achieved by cotransfecting isolated intact IE genes with the DE promoter/CAT target genes. Furthermore, our results show that the genes for both the IE 175K and 110K proteins can independently stimulate expression from two DE promoters, i.e., that of the gene for the HSV-2 38K protein and that of the HSV-1 thymidine kinase. The genes for two other IE proteins of 63K and 68K do not possess this property, although our results indicate that the 12K IE protein may augment activation of DE promoters by the 175K or 110K proteins. Recent results of other workers (I. Gelman and S. Silverstein, personal communication) have also shown that 110K protein can activate expression from the HSV-1 thymidine kinase promoter.

The specificity of trans activation in our system indicates that the presence of a promoter region per se is insufficient for response to the IE proteins and that, in addition, some
protein. However, to further explain the questions raised above, it is also necessary to propose that the functions of the 110K and 175K proteins do not completely overlap, with the 175K protein possessing additional properties compared with the 110K protein. Recent data indicate that the 175K polypeptide possesses multifunctional properties (6a). These workers have isolated mutants possessing lesions in the gene for the 175K protein which confer temperature sensitivity on virus growth but allow the normal expression of a number of DE genes. Furthermore, late gene products were absent or underrepresented although significant levels of DNA synthesis were observed. These findings indicate that the block in these mutants is in a regulatory event subsequent to that exhibited by other mutants possessing lesions in this gene and suggest a multifunctional role for the 175K polypeptide in virus gene regulation. Thus, the 110K protein may itself possess only a subset of these regulatory roles.

Before this report there have been no accounts of specific functions ascribed to the 110K polypeptide, and no viruses with conditionally lethal mutations in this gene have been isolated. However, a recent report from Brown et al. (2) indicates that the 110K polypeptide may have an important function in virus replication in cell culture. When isolating restriction enzyme site deletion mutants of HSV, these authors observed that a certain mutation in the terminal repeat region within the gene for the 110K protein was never found in the internal repeat region, suggesting that this mutation could not be tolerated in a homozygous condition. In addition, they reported the isolation of a mutant lacking the complete internal repeat region and containing a recombinant terminal repeat region. The replication of this mutant was significantly impaired compared with the wild-type virus. Both of these observations suggest an important, if not essential, role for the 110K polypeptide. It remains to be seen whether this role is the regulatory one which we have ascribed to the 110K protein in this report. The phenotypic characterization of mutants with specifically constructed lesions in the 110K gene should help illuminate this question and help identify the specific functions of 110K in the virus replicative cycle.

Overlapping functions among IE-type proteins have been observed in other systems. For example, two separate phase T4 genes, alt and mod, each code for enzymes which ADP ribosylate the Escherichia coli RNA polymerase at identical sites during modification of the host cell transcriptional machinery (11, 17). The purpose in having two separate genes to perform this function is unknown, but additional individual functions of either of these genes may exist. In the adenovirus system, some recent evidence suggests that products of both the 135 and 125 mRNAs of the E1a gene individually possess the ability to inactivate adenovirus early gene expression (21). Furthermore, Richardson and Westphal (34) have shown that both the adenovirus E1a and E1b gene products can each provide the function(s) required to activate the adenovirus helper function of other adenovirus early genes. Interestingly, in this latter work, it appeared that there were at least two distinct regulatory pathways leading to expression of adenovirus-associated virus and that adenovirus early products may enhance virus gene expression by several mechanisms which can operate independently but whose effect may be cumulative. These results are of obvious interest in view of our observations of independent proteins possessing functions for the stimulation of HSV early gene expression. We are now in the process of testing the relative ability of these two IE gene products to stimulate expression from additional DE promoters and whether either IE gene has a direct stimulatory effect on expression from late promoter(s), to provide evidence for functions unique to one or the other of these proteins.

Although we could demonstrate neither any significant effect of the 63K or 68K IE proteins individually on DE expression nor any modulating role of these proteins in DE activation by 110K and 175K, our results suggest that the 12K IE protein may play a role in virus gene regulation, augmenting the activation of DE expression by the 110K and 175K proteins. However, because EcoRI cleavage of PGR90, in addition to inactivating the transcript for the 12K protein, may also inactivate overlapping transcripts which code for 33K and 21K polypeptides (35), we cannot as yet exclude a possible contribution of these latter products in the effect of increased activation by IE 175K and IE 110K proteins. The absence of an effect of the 68K protein on DE transcription is consistent with previous results (32), which demonstrated that an intact 68K protein was dispensable for normal growth in cell culture. However, recent findings (P. Schaffer, personal communication) indicate that the 63K protein may possess an essential function for virus replication since mutations which appear to reside in this gene confer temperature sensitivity on virus growth. Our results suggest that this function may not involve a direct role in regulating the induction of DE gene expression. Analysis of transcription patterns in cells infected with these mutants should indicate at what stage virus replication is blocked and help illuminate the function of the IE 63K protein.

We are presently attempting to directly demonstrate our proposed role for the 12K protein, using cloned fragments containing only this gene in cotransfection experiments with the 110K and 175K genes. It will be of further interest to examine the spectrum of DE promoters which are responsive to the augmentary action of the 12K protein in an attempt to explain the observed heterogeneity in DE expression during virus infection.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants 2R01CA22130 and 2R01CA28473 from the National Institutes of Health to G.S.H. P.O'H. is supported by fellowship DRG714 from the Damon Runyon-Walter Winchell Cancer Fund, and G.S.H. is supported by American Cancer Society Faculty Research Award no. 247.

Technical assistance from Dolores Ciufio and Mabel Chiu and the typing skills of Nancy Standish and Paula Manzuk are gratefully acknowledged.

LITERATURE CITED


33. Preston, C. M. 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J. Virol. 29:275-284.


