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Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene

(transient transcription/gene regulation)

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ABSTRACT **A HeLa cell transient-expression assay system was used to determine if isolated immediate early (α) genes from herpes simplex virus (HSV) could transcriptionally activate (transactivate) the type 1 (HSV-1) thymidine kinase (TK) gene [an early (β) gene]. Cells transfected with the TK gene alone transcribed very low levels of TK RNA. Cells cotransfected with plasmids bearing the sequences that encode the α-gene product infected cell protein 0 or 4 (ICP0 or ICP4) and the TK gene faithfully transcribed high levels of TK RNA. The plasmid containing the sequences encoding ICP0 was a more potent transactivator than the plasmid containing the sequences for ICP4.**

Herpes simplex virus (HSV) is a large double-stranded DNA virus that is replicated and transcribed in the nucleus of infected cells. The regulation of HSV gene expression is a model for coordinate transcriptional control in eukaryotic cells because there are at least three sequentially ordered gene families whose expression is temporally regulated in a cascade fashion (1, 2). During the course of lytic infection, transcription of the five members of the immediate early gene family (α genes) is activated by Vmw 65 (3), a virion-associated protein that is transcribed late in the infectious cycle (4, 5). These α gene sequences are transcribed in the presence of cycloheximide or anisomycin by the host RNA polymerase (2, 6–9). The α mRNAs are subsequently translated into five infected cell proteins (ICPs) designated ICP4, -0, -27, -22, and -47 (1, 2). Studies with temperature-sensitive mutants have shown that at least one of these gene products, ICP4, is required in an active form throughout the course of infection for expression of the second (β) gene family (10, 11). Two other α genes, those encoding ICP22 (12) and ICP27 (13, 45), have been examined in some detail. Interruption of the gene coding for ICP22 does not affect virus growth in cell culture (12), whereas the presence of ICP27 appears to be required for expression of a late (γ) gene (13, 45). Attempts to ascribe functions to the two other α genes (ICP0 and ICP47) have been hampered by an inability to isolate conditionally lethal mutants of these genes. Recently, short-term transfection expression systems have been used to demonstrate that isolated immediate early genes from both adenovirus and pseudorabies virus can transactivate virus and cell genes (14–16).

In this study we have employed short-term transfection assays of HeLa cells to investigate the transactivation potential of individual α genes on the thymidine kinase (TK) gene (a β gene) of HSV. Our results demonstrate that plasmids containing the sequences that encode either of the α gene products ICP4 or ICP0, from HSV type 1 (HSV-1), transactivate the TK gene in the absence of other α gene or virus factors. Similar findings have been reported recently by O'Hare and Hayward (17) and data that are compatible with, but not identical to, our findings have been described by Everett (18).

MATERIALS AND METHODS

Cells and Viruses. The viruses used in this study were D_2, a TK^- deletion mutant of HSV-1 (19), and F (20). All virus stocks were grown and titrated on Vero cells (21). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

Plasmids. The pseudogene-TK deletion mutant pRB316, pGR150b, and pRB114 plasmids have been described (4, 22–24). pRHP6 is a HSV-1 (KOS) *Xho* I–D junction clone that contains sequences coding for ICP4 and the 5′ ends of ICP0 and ICP47. pIGA-15 contains a HSV-1 (KOS) *Sst* I/*Eco*RV fragment that bears the sequence coding for ICP0. pIGA-30 contains the *Bam*HI–B fragment from HSV-1 (KOS) that codes for ICP27 and the 3′ end of ICP0. pIGA-31 contains the *Eco*RI/*Sal* I fragment from HSV-1 (KOS) that codes for ICP47. pIGAs −15, −30, and −31 are in pUC vectors (25). See Fig. 1 for map locations of each sequence.

Plasmid DNA was extracted from bacteria as described (26).

Transfection Procedure. HeLa cells were transfected with 15 μg of target DNA (pseudogene-TK) and a molar gene equivalent of IE-plasmid DNA as described (27). The medium was removed and exchanged at 12–24 hr posttransfection and the cells were allowed to incubate for an additional 24–36 hr. To normalize for the delivery of transfected DNA, 5 μg of pSV2CAT (28) per dish was added to the cotransfection mixtures and chloramphenicol acetyltransferase (CAT) activity was assayed (28).

RNA Purification. RNA was purified by the guanidine thiocyanate/CsCl procedure as described (29).

Probe Preparation. mTP8, an M13mp8 recombinant phage, has been described (30). Single-stranded M13 template DNA was purified according to the procedure of Heidecker *et al.* (31). Uniformly labeled, single-stranded cDNA probes were synthesized as described by Hu and Messing (32).

S1 Nuclease Hybridization Assay. Twenty to 50 μg of total RNA was analyzed as described (30). When quantitation was required the bands were cut from the gel and radioactivity was counted by liquid scintillation spectrometry.

RESULTS

Activation of α and β Promoters. Previous results suggested that the promoter of the TK gene is composed of two overlapping transcription signals (22, 30). In this first experiment we demonstrate that promoters for α and β genes are

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Abbreviations: TK, thymidine kinase; HSV, herpes simplex virus; ICP, infected cell protein; HSV-1 and HSV-2, HSV types 1 and 2; nt, nucleotides.

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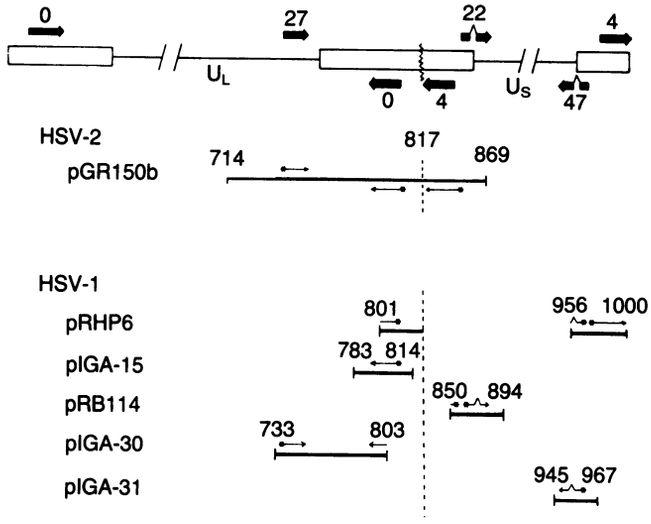

FIG. 1. Map location of the sequences containing the immediate early genes of HSV and the plasmids used in the analyses described in this study. The HSV genome is shown at the top; U_L and U_S refer to the unique sequences that are bounded by repeats (open rectangles). The location and direction of transcription of the α mRNAs are indicated by the arrows. The designation for the protein (ICP) encoded by each mRNA is shown above the arrows. The virus inserts in each of the plasmids used in this study are shown below the schematic of the virus genome, along with their map coordinates. A description of the contents of each plasmid is provided in the text.

recognized and appropriately transcribed in the HeLa cell transient transcription assay system. TK genes containing the natural (β) promoter or an α gene promoter [the promoter for ICP4, inserted between the TK gene promoter with 55 base pairs of TK coding sequence and the remaining TK coding region (4)] were transfected into HeLa cells. After 36-hr the transfected cells were infected with D_2 in the presence or absence of cycloheximide. At 6 hr postinfection, half of the drug-treated cultures were harvested. The other half was washed free of drug to restore protein synthesis and then incubated for an additional 6 hr prior to harvesting. RNA isolated from these cultures and from controls infected in the absence of drug for 6 and 12 hr was hybridized to uniformly ^{32}P-labeled, single-stranded DNA and then digested with S1 nuclease (S1 nuclease analysis). The results are shown in Fig. 2. Cells transfected with a β-TK gene and then infected with virus and maintained in the presence of cycloheximide (Fig. 2*a*, lane A) accumulate very little TK RNA when compared with cells incubated without the drug (Fig. 2*a*, lane C). In contrast, removal of the cycloheximide permits translation of the α mRNAs that accumulate during the block (2, 7). These translation products, the α ICPs, transactivate the β-TK gene promoter (33, 34). The level of the TK transcripts that accumulate after reversal is similar to the level found in the untreated culture at 12 hr postinfection (Fig. 2*a*, lanes B and D). Thus, transcription from a TK gene containing its natural promoter is dependent on the prior synthesis of virus-specified α gene polypeptides, as it is during lytic infection (2, 33, 34).

When RNA extracted from cells transfected with the α-TK chimera was examined by S1 nuclease analysis we demonstrated that (*i*) the α promoter is activated in the presence of cycloheximide (Fig. 2*b*, lane A) and (*ii*) transcripts originating from the α and β promoters are detected when transfected cells are infected in the absence of drug (Fig. 2*b*, lane B). Thus, RNA from drug-treated cells protects only that portion of the probe that corresponds to the TK body present in the construction downstream from the α promoter (55 nt), whereas RNA from untreated cells protects this fragment of

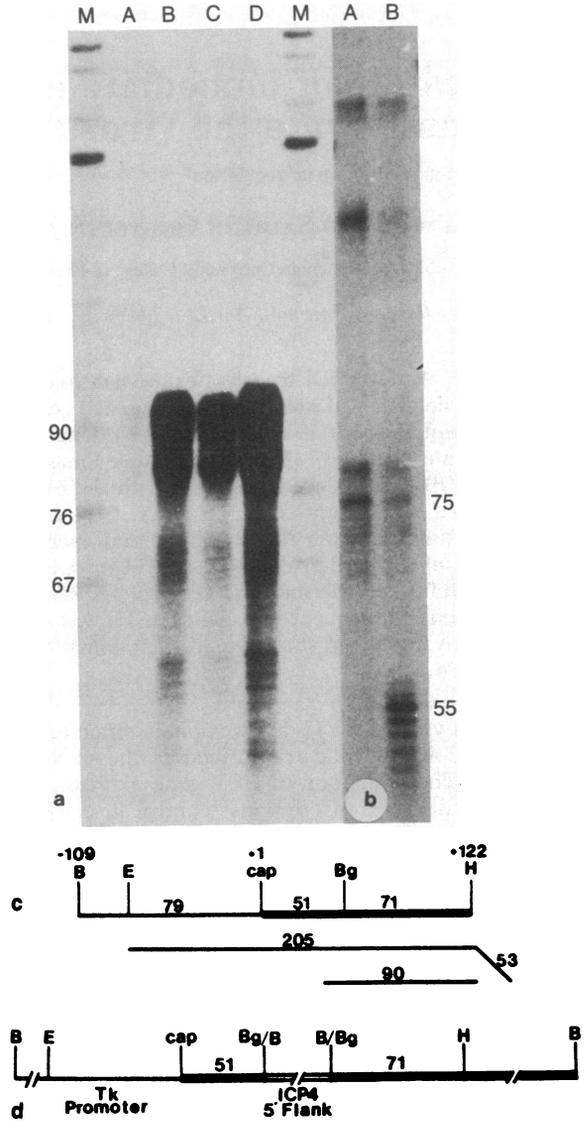

FIG. 2. Transient transcription of TK and chimeric α-TK genes. (*a*) Lanes A–D: fragments of the probe protected from S1 nuclease by 25 μg of RNA isolated from D_2-infected cells maintained in the presence of 50 μg of cycloheximide per ml for 6 hr (lane A), infected cells maintained in the presence of cycloheximide for 6 hr and then washed free of drug and incubated for an additional 6 hr (lane B), and untreated infected cells after 6 hr (lane C) or 12 hr (lane D) of incubation. (*b*) Lanes A and B: S1 nuclease-resistant probe protected by 25 μg of RNA extracted from cells transfected with an α-TK chimera [pRB316 (4)] and then infected with D_2 in the presence (lane A) or absence (lane B) of cycloheximide. The probe used for the experiments in *a* and *b* was uniformly ^{32}P-labeled, single-stranded DNA that was 258 nucleotides (nt) long. (*c*) Transcripts from the β-TK gene can protect 90 nt. (*d*) Transcripts from the α-TK chimera can protect two regions of the probe that are 4 nt longer than the distances shown either because of splicing of restriction sites or because of fortuitous protection of a small portion of the M13 tail. Protected fragments were analyzed on a 10% denaturing polyacrylamide gel. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; Bg, *Bgl* II; H, *Hpa* II. DNA size markers (lane M) are *Hpa* II fragments of pBR322.

the probe and a region of the probe corresponding to the TK body that is present upstream from the α promoter and fused to the natural β promoter (75 nt). These results demonstrate that under these assay conditions an α promoter is recognized and transcribed in the absence of *de novo* virus-specified protein synthesis. This is similar to the way that α promoters are regulated during the course of infection (4).

Transactivation by a Plasmid Containing Four α Genes. To examine if any of the α genes from HSV would transactivate a β gene in a transient expression assay we first cotransfected HeLa cells with an equimolar amount of pGR150b, a plasmid containing the *Bgl* II HM fragment from HSV-2 333 (23), and a plasmid containing the sequences that encode the TK gene. Although pGR150b contains sequences exclusively from HSV-2, it was used in this first experiment because it carries the genes encoding the HSV-2 analogues of ICPs -4, -0, -27, and -22. Moreover, genetic experiments have established that, for the most part, the maps of HSV-1 and HSV-2 are colinear and that mutations in one of these viruses are readily complemented or rescued by sequences from the other (35, 36). At 48 hr posttransfection, cells were harvested and total cellular RNA was extracted and analyzed for the presence of TK RNA sequences by S1 nuclease analysis. In the absence of transacting transcription factors, the TK gene is only poorly transcribed under the conditions of this assay (30). Fig. 3 reveals that the constitutive level of TK transcripts is near the lower limit of sensitivity of the S1 nuclease assay. In addition, we note that in the presence of pGR150b there is >1000-fold more TK RNA than in its absence (determined by excising the relevant bands from the gel and measuring the amount of ^{32}P in S1 nuclease-resistant probe by liquid scintillation spectrometry). Two conclusions are drawn from this result: (*i*) α genes can be expressed in HeLa cells in the absence of the virion-associated factor and (*ii*) the product of at least one of these α genes can transactivate the TK gene, resulting in the stable accumulation of TK RNA.

Titration of Transactivation Activity. When HSV infects a cell each genome brings in two gene equivalents of the sequences that code for both ICP0 and ICP4 but only a single copy of TK. Therefore, we asked if altering the ratio of effector gene to target sequence would change the level of TK transcripts that accumulate during the short-term transfection assay. Cells were cotransfected with TK and different molar equivalents of pGR150b. RNA was isolated after 48 hr and analyzed for the presence of TK transcripts by S1 nuclease analysis. The bands corresponding to probe hybridized to RNA were excised from the gel and radioactivity was counted. Altering the ratio of α gene template (the effector molecule) to target (TK gene) results in the accumulation of increasing levels of TK mRNA (Fig. 4). Thus, the transactivator(s) specified by the gene(s) on pGR150b appears to act as a positive regulator in a stoichiometric manner.

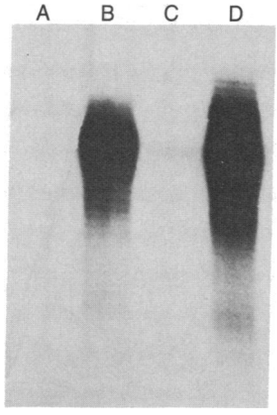

FIG. 3. Cotransfection of the TK gene from HSV-1 and a cluster of immediate early genes from HSV-2. HeLa cells (3×10^6) were cotransfected with a calcium phosphate precipitate containing 15 μg of pseudogene-TK plasmid or with 15 μg of TK DNA and an equimolar amount (75 μg) of pGR150b. Total cellular RNA was isolated at 48 hr posttransfection and the relative amount of TK transcripts was determined by S1 nuclease protection after hybridization of either 20 or 50 μg of total cellular RNA with an excess of uniformly ^{32}P-labeled, single-stranded probe. Protected fragments were electrophoresed on a 6% denaturing polyacrylamide gel. Lanes A and B, probe protected after hybridization with 20 μg of RNA from cells transfected with TK plasmid alone or cotransfected with TK and pGR150b, respectively. Lanes C and D, as above except that 50 μg of RNA was used in the hybridization reaction.

Identification of Transactivating α Genes. To determine if any of these four α gene products was independently able to transactivate the TK gene, recombinant DNA plasmids containing each of the five α gene sequences were isolated, or constructed, from a bank of HSV-1 *Eco*RI fragments cloned in pBR325 (37). Plasmids containing the promoter and coding sequences for each of these five α genes, along with flanking virus DNA (see Fig. 1), were cotransfected with a plasmid containing the TK gene. After 48 hr, RNA was isolated from the transfected cultures and analyzed for the presence of TK transcripts as described in the preceding experiment. Fig. 5 demonstrates that there is no increase above the constitutive level of stable TK transcripts when plasmids containing the sequences that code for either ICP22, ICP47, or ICP27 are cotransfected with TK DNA. In contrast, significant levels of stable TK RNA accumulate when HeLa cells are cotransfected with plasmids containing the sequences encoding either ICP4 or ICP0. Comparison of the level of TK RNA that accumulates in cells cotransfected with these two α genes reveals that the plasmid containing ICP0 is a more potent transactivator of the TK gene (7- to 10-fold greater) than is the ICP4-containing sequence. Neither of these two sequences transactivates the TK gene as effectively as pGR150b. Moreover, we have consistently noted the appearance of abundant, TK-containing transcripts that initiate at upstream sites when cells are cotransfected with pIGA-15 and a plasmid containing a β-TK gene. From this experiment we conclude that the sequences encoding either ICP4 or ICP0 are each capable of transactivating the TK gene from HSV-1 in the absence of other virus gene products.

DISCUSSION

The experiments described in this manuscript suggest a role for specific α genes in transactivation of HSV genes. Our results demonstrate that cotransfection of the HSV-1 genes coding for either ICP0 or ICP4 with TK DNA results in the transactivation of the TK gene in the absence of other virus-specified gene products. This conclusion is based on experiments that demonstrate that increased levels of TK RNA (above the constitutive level) accumulate under the

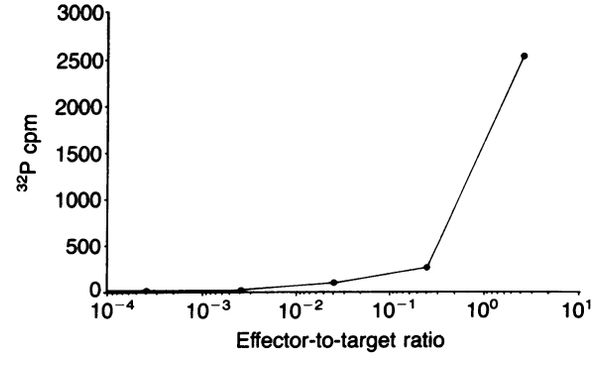

FIG. 4. Influence of altering the effector-to-target ratio. RNA was isolated from cells cotransfected with varying ratios of pGR150b to TK [ranging from 4:1 to 4×10^{-4} (molar gene equivalents)] in 10-fold increments. The total amounts of plasmid DNA were kept constant by adding varying amounts of pUC9 after 48 hr of incubation and analyzed for the presence of TK mRNA by S1 nuclease analysis. Bands corresponding to probe protected by TK mRNA were excised from the gel and the amount of ^{32}P was determined.

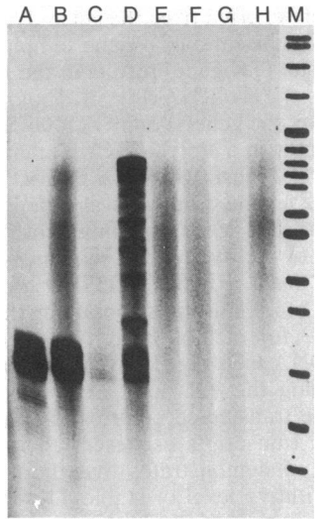

FIG. 5. Transaction of the TK gene by cotransfection with plasmids containing HSV-1 α genes. HeLa cells were cotransfected with a plasmid containing TK DNA and an equimolar equivalent of pGR150b (lane B), pRHP6 (lane C), pIGA-15 (lane D), pBR114 (lane E), pIGA30 (lane F), or pIGA31 (lane G). Cells were harvested at 48 hr posttransfection and analyzed for the presence of TK mRNA by S1 nuclease analysis. Lanes A and H contain probe protected by RNA isolated from HeLa cells transfected with TK DNA and, after 48 hr, infected for 6 hr (lane A) or mock-infected (lane H) with D_2. DNA size markers were *Hpa* II fragments of pBR322 (lane M).

conditions of this assay. O'Hare and Hayward reached a similar conclusion (17). This conclusion differs from that reached by Everett (18), who demonstrated transactivation of a gD-β globin chimera only when the sequences coding for both ICP0 and ICP4 were present. However, these results can be reconciled. The apparent discrepancy might result from subtle differences in the secondary structure of the promoters for these two β genes. Recently, we compared the sequences present in the promoters of a number of β genes and concluded that they shared a common structural element. Specifically, each promoter contains a hyphenated palindrome with a dyad of symmetry that is approximately equidistant from the ATA box (30). Although the promoter for gD is also composed in part of a hyphenated palindrome, subtle changes in structure, which result from the unique sequence of each promoter, might alter the specificity of the response to different transactivation signals. A recent analysis of the gD promoter failed to define a role for *cis*-acting control elements in its response to transactivation signals. The conclusion reached in this analysis suggested that the gD promoter, *per se*, was sufficient for transactivation (38). In this respect it differs from TK, in which there are two contiguous and partially overlapping sequences that control constitutive transcription and transactivation (30, 39–41). Thus, some HSV promoters might require a cooperative interaction between the α proteins specified by these two sequences (or perhaps other members of the α gene family), whereas other promoters (such as TK) might be transactivated, under the conditions of this assay, by either of these two α gene products.

The identification of transactivation functions for both ICP0 and ICP4 constitutes a paradox because cells infected with tsK [a virus whose defect has been localized to a single nucleotide change in the sequence coding for ICP4 (42)] at the restrictive temperature accumulate only α mRNAs and proteins (43). Genes of the β family (e.g., TK) are not transcribed under these conditions (43). Assuming the alleles for ICP0 are normal in tsK and having shown that ICP0 can independently transactivate the TK promoter in a short-term transfection assay, why do β transcripts not accumulate during a tsK infection at the nonpermissive temperature? To reconcile this paradox we suggest that there is a necessary cooperative interaction between these gene products that regulates transactivation of β genes during the course of a normal infection. Thus, wild-type ICP4 [a protein with multiple functional domains (44)], alone or in concert with other α gene products, might be required for ICP0 function. Therefore, a defective ICP4 might permit complex formation; however, the function of ICP0 could be inhibited in the presence of the defective ICP4 gene product synthesized in cells infected with tsK. In preliminary experiments we have demonstrated that HeLa cells transfected with a marked TK gene and infected at the nonpermissive temperature with tsK fail to accumulate TK-specific transcripts from either the virus or plasmid templates. This result provides support for a model requiring interaction between these immediate early gene products.

We cannot determine, from these experiments, what role ICP0 plays in regulation of the cascade of macromolecular synthesis during the course of a lytic infection by HSV-1. Nevertheless, it is clear from the data presented in this paper that HSV-1 possesses more than one immediate early gene product that has the capacity to transactivate at least one β gene. Moreover, the α gene battery present on pGR150b (from HSV-2) is more effective at transactivating the TK gene than plasmids containing either ICP4 or ICP0 from HSV-1. Whether this effect represents synergy between α genes, a greater transactivation potential by HSV-2 α genes, or enhanced transcription of the α genes present on pGR150b is not known. Ultimately, it will be necessary to determine if these α gene products bind to, or recognize, specific regions in the promoters of the β genes or if they exert their effect(s) by interacting with, or modifying, RNA polymerase II to form a unique transcription complex in virus-infected cells.

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