The Products of Herpes Simplex Virus Type 1 (HSV-1) Immediate Early Genes 1, 2 and 3 Can Activate HSV-1 Gene Expression in trans

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SUMMARY

Expression of the early and late genes of herpes simplex virus type 1 (HSV-1) during infection of tissue culture cells requires the prior expression of the immediate early (IE) genes. The requirement for the product of IE gene 3, Vmw175, for the activation of early promoters has been revealed by studies with temperature-sensitive virus mutants. Recent experiments using transfection assays have shown that both Vmw175 and the product of IE gene 1, Vmw110, are involved in the transactivation of a variety of HSV-1 early promoters. This paper describes experiments which compared the activation of two early promoters [those of the glycoprotein gD and thymidine kinase (tk) genes] with that of a member of a later class of genes (the major capsid protein, VP5). Plasmids containing these promoters linked to the chloramphenicol acetyltransferase (CAT) gene were transfected into HeLa cells with plasmids containing one or more HSV-1 IE genes. Promoter activity was estimated by measurement of CAT activity in extracts of transfected cells. The gD and tk promoters were activated by both Vmw175 and Vmw110, and the combination of these two IE gene products resulted in very high levels of activation. Addition of further IE gene products did not result in any significant increase in the activation seen with the combination of Vmw175 and Vmw110. In contrast, the activation of the VP5 promoter brought about by the combination of Vmw175 and Vmw110 was relatively slight, but was increased further when plasmids containing IE gene 2, encoding Vmw63, were included in the transfection. These data suggest that Vmw63, like Vmw175 and Vmw110, is also involved in the activation of transcription from HSV-1 promoters. The effect of Vmw63 may be limited to the activation of a subset of HSV-1 genes.

The genome of herpes simplex virus type 1 (HSV-1) consists of a double-stranded 150 kb DNA duplex containing at least 50 genes. These genes have been classified into at least three groups according to their time of expression during growth of the virus in tissue culture (for review, see Wagner, 1985). The immediate early (IE) genes are transcribed first by the host RNA polymerase (Costanzo et al., 1977). The control of expression of the IE genes involves both cis-acting enhancer elements (Lang et al., 1984; Preston & Tannahill, 1984) and activation in trans by a component of the virus particle (Post et al., 1981) in a process that requires the presence in the promoter of a specific DNA sequence (Campbell et al., 1984; Kristie & Roizman, 1984; Gaffney et al., 1985). All IE gene promoters contain one or more copies of this consensus sequence (Mackem & Roizman, 1982; Whitton & Clements, 1984).

Early genes are not transcribed before functional IE gene products have been produced (Clements et al., 1977; Watson & Clements, 1980). Studies with virus mutants have shown that the product of IE gene 3, Vmw175, is essential for transactivation of early promoters (Preston, 1979). Recently, it has been demonstrated in transfection assays that the product of IE gene 1, Vmw110, is also involved in this process (Everett, 1984a; O'Hare & Hayward, 1985; Quinlan & Knipe, 1985; Gelman & Silverstein, 1985). Early promoters do not appear to contain a virus-specific DNA sequence regulatory element (Everett, 1984b; Eisenberg et al., 1985), and this is...
consistent with observations that HSV-1 IE polypeptides can activate cellular promoters (Everett, 1984a, 1985) and that the IE gene products of other herpesviruses can activate an HSV-1 early promoter (Everett & Dunlop, 1984). The control of expression of the late genes has not been extensively studied. True late transcripts do not become abundant until comparatively late in infection, and their expression is most severely reduced, compared to all other groups of genes, under conditions of severely inhibited DNA replication (Johnson et al., 1986).

Regulation of gene expression is now commonly studied using vectors encoding the chloramphenicol acetyltransferase (CAT) gene linked to the promoter of interest. Such constructions are transfected into tissue culture cells and subsequently extracts are made which are assayed for CAT activity. The level of CAT activity obtained is taken as an indirect measure of the amount of transcription produced from the promoter. Increased levels of CAT activity correlate well with increased levels of cytoplasmic RNA in studies using the HSV-2 IE-4/5 promoter (McLauchlan et al., 1985; Gaffney et al., 1985) and the HSV-1 glycoprotein D (gD) promoter (Everett, 1984a and comparison with this work). The advantages of this method are that it is very sensitive (much more so than an RNA assay), and the background CAT activity of untransfected cells is zero. The experiments described in this paper have used this method to investigate the transactivation of HSV promoters by HSV-1 IE gene products. The CAT vector used was pBLW2 [derived from pLW2 (Gaffney et al., 1985)] which contains the CAT gene with
an HSV-2 polyadenylation signal at its 3' end, and several convenient restriction sites at its 5' end (Fig. 1). The promoter regions of the HSV-1 gD, thymidine kinase (tk) and major capsid protein VP5 genes were cloned in the correct orientation 5' to the CAT gene to give pgDCAT, pTKCAT and pVP5CAT (Fig. 1a). The tk gene has been classified as early because it is activated by IE products but is unaffected by inhibition of DNA replication. Glycoprotein D is expressed early in infection (Watson et al., 1983) but, in some studies, it is expressed in reduced amounts when DNA replication is inhibited (Johnson & Spear, 1984; Johnson et al., 1986); thus, gD has been referred to as being delayed early (Johnson & Spear, 1984; Wagner, 1985). The gene encoding the major capsid protein VP5 (ICP5 or Vmw155) has been described as late or leaky-late since its synthesis is markedly affected (although not as severely as a true late gene) by inhibition of DNA replication (Dennis & Smiley, 1984; DeLuca & Schaffer, 1985; Costa et al., 1985). Thus, the patterns of expression of these three promoters are distinguishable during HSV-1 infection of tissue culture cells. The regulation of these HSV-1 promoters by HSV-1 gene products was studied by co-transfection of one or more plasmids containing one or more IE genes; the structures of these activating plasmids are shown in Fig. 1(b).

The interpretation of the data presented in this paper requires an understanding of the limitations of transfections and CAT assays and the methods of calculation used. First, the data were normalized to the level of CAT activity obtained with a CAT vector plasmid in the absence of co-transfected activator plasmids. In these experiments, this basal level of activity was low, which could lead to relatively high variations in the relative activities of more active extracts. To overcome this problem, the experiments were repeated several times and the values of the standard errors of the mean are presented. Second, the actual values of stimulation observed can vary according to the efficiency of the transfection, which is affected by cell type, state of the cells and media, and even the incubator used. These variables have been standardized as far as possible in these experiments. These considerations mean that it is not possible to be sure of the significance of small (less than twofold) effects. What is important here is the relative effects of the different activators on a particular promoter and comparison of these relative effects between promoters.

Transfection of pTKCAT into HeLa cells in the absence of any activating plasmids resulted in a low level of CAT activity. This low level was increased when plasmids containing either the IE-3 gene (encoding Vmw175; pGX58) or the IE-1 gene (encoding Vmw110; pJR3) were included in the transfection. When both Vmw175 and Vmw110 were present in the transfection, the CAT activity obtained from pTKCAT was always several-fold more than the sum of that with Vmw175 or Vmw110 alone (Table 1). The activation of the tk promoter by Vmw175 alone (O'Hare & Hayward, 1985; Persson et al., 1985) and by Vmw110 alone (O'Hare & Hayward, 1985) have been reported before, but the synergistic effect of the two was not noted.

The response of the gD promoter to co-transfection activation by plasmids containing HSV IE genes has already been studied by direct quantification of the RNA (Everett, 1984a). This study was not able to detect activation of the gD promoter by Vmw110 alone. The activation of the gD promoter was re-investigated by the CAT assay method. The results indicated that the increase in correctly initiated gD RNA induced by Vmw110 alone (Everett, 1984a) was mirrored in an increase in CAT activity (Table 1). In contrast to the RNA analysis, there was a small increase in CAT activity derived from pgDCAT when Vmw110 was present in the transfection. This effect was either too small to be apparent in the analysis of RNA (Everett, 1984a) or was brought about by initiation of RNA synthesis at sites distinct from the normal gD 5' ends. In agreement with the RNA analysis, the combination of both Vmw175 and Vmw110 was at least 20-fold more active than with either alone (Table 1). These data indicate that, although the tk promoter was activated more strongly on average than the gD promoter by Vmw175 or Vmw110 by themselves, there was little general difference in the response of the gD and tk promoters to activation by the HSV IE gene products.

Plasmid pVP5CAT contains the major capsid protein gene promoter region, from positions approximately -800 to -11 relative to the RNA start sites, linked to the CAT gene. Therefore, the normal cap-site region of the promoter is not present, and the RNA produced from the VP5 promoter and TATA box is expected to initiate in vector sequences (Fig. 1). However, this
Table 1. Activation of HSV-1 genes

<table>
<thead>
<tr>
<th>Activating plasmid*</th>
<th>gD</th>
<th></th>
<th>s.E.M.</th>
<th>No.</th>
<th>Mean†</th>
<th>s.E.M.</th>
<th>No.</th>
<th>Mean†</th>
<th>s.E.M.</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>pGX58</td>
<td>2.5</td>
<td>0.6</td>
<td>9</td>
<td>1.7</td>
<td>5.6</td>
<td>1.7</td>
<td>11</td>
<td>1.8</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>pJR3</td>
<td>2.9</td>
<td>0.2</td>
<td>10</td>
<td>3.9</td>
<td>9.7</td>
<td>3.9</td>
<td>9</td>
<td>0.9</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>pGX58 + pJR3</td>
<td>61.4</td>
<td>9.0</td>
<td>11</td>
<td>12.7</td>
<td>72.1</td>
<td>12.7</td>
<td>14</td>
<td>2.6</td>
<td>0.5</td>
<td>11</td>
</tr>
<tr>
<td>pGX58 + pJR3 + pGX12</td>
<td>77.8</td>
<td>30.9</td>
<td>7</td>
<td>21.3</td>
<td>74.7</td>
<td>21.3</td>
<td>5</td>
<td>12.1</td>
<td>3.8</td>
<td>18</td>
</tr>
<tr>
<td>pGX12</td>
<td>47.3</td>
<td>10.7</td>
<td>3</td>
<td>38.2</td>
<td>111.1</td>
<td>38.2</td>
<td>4</td>
<td>2.6</td>
<td>0.4</td>
<td>10</td>
</tr>
<tr>
<td>pGX12 + pGX58</td>
<td>4.3</td>
<td>1.6</td>
<td>8</td>
<td>1.0</td>
<td>4.3</td>
<td>1.0</td>
<td>4</td>
<td>1.8</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>pGX12 + pJR3</td>
<td>1.3</td>
<td>0.1</td>
<td>2</td>
<td>0.3</td>
<td>6.0</td>
<td>0.3</td>
<td>2</td>
<td>1.5</td>
<td>0.6</td>
<td>2</td>
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</tbody>
</table>

* The activating plasmids are described in Fig. 1, and encode IE genes as follows: pGX58, Vmw175; pJR3, Vmw110; pGX12, Vmw63. pGX13 is derived from pGX12 and contains a frameshift mutation in the Vmw63 coding region.

† The mean values of activation above the CAT activity obtained in transfections with an equivalent amount of pBR322 are given with the standard errors of the mean. These values were obtained from the stated number of independent transfections. HeLa cells were seeded at 10⁶ cells per 50 mm Petri dish and transfected with 4 μg of each plasmid DNA by the calcium phosphate precipitation method (Corsalo & Pearson, 1981). pBR322 was used to equalize the amount of DNA in comparative experiments when different numbers of plasmids were used. After 24 h the cells were washed and fresh medium was added. After a further 24 h, extracts were prepared and CAT activities assayed as described by Gorman et al. (1982). The radioactivity in the substrate and monoacetylated product (position 3) spots was determined by scintillation counting to calculate the percentage conversion of substrate to product. The protein concentration of each extract was determined (Lowry et al., 1951) and the percentage conversion per mg protein calculated. This value was then expressed relative to that obtained in a control transfection which included the relevant CAT plasmid without any activating plasmids. Generally, one-third of the extract obtained from a 50 mm dish was incubated with 4 nmo1[14C]chloramphenicol for 1 h at 37 °C. In a typical experiment, approximately 25% acetylation of the substrate was obtained in a transfection with pTKCAT, pGX58 and pJR3, giving a specific activity of 13.3 pmol acetylated/μg protein in the extract/h. The basal levels of pTKCAT and pGD CAT were similar, and about three times that of pVP5CAT.

The involvement of Vmw63 in the activation of the VP5 promoter was confirmed by experiments using plasmid pGX13, which contains a frameshift mutation at the SalI site located in Vmw63 coding sequences (L. Perry & D. McGeoch, personal communication). Plasmid pGX13 was unable to increase the level of CAT activity derived from pVP5CAT above the level obtained with pGX58 plus pJR3 (Table 1). The stimulation effect of pGX12, and the lack of effect of pGX13, was reproducible over a large number of experiments (Table 1). The effect of Vmw63 on pVP5CAT expression was only observed when both Vmw175 and Vmw110 were included in the transfection; either by itself or in single combination with Vmw175 or Vmw110, Vmw63 did not stimulate pVP5CAT expression (Table 1). These results suggest that one function of Vmw63 is to increase the expression of at least one HSV gene, as measured in a short-term transfection assay. Consistent with the data presented here, Sacks et al. (1985) have observed that mutant viruses containing temperature-sensitive lesions in IE gene 2 express reduced amounts of many late genes, including VP5, at the non-permissive
temperature. To test whether Vmw63 had a general effect on increasing gene expression, pGX512 was included in co-transfection experiments with the gD and tk promoters. The results indicated that Vmw63 did not provide any additional stimulation over that obtained with Vmw175 and Vmw110 with either the gD or the tk promoter (Table 1). This result is again consistent with those of Sacks et al. (1985) and suggests that the stimulation effect of Vmw63 may be confined to later classes of HSV-1 genes.

In summary, this paper presents evidence that the product of HSV-1 IE gene 2, Vmw63, is a trans-acting transcriptional regulator. There is now strong evidence that HSV-1 encodes at least three transcriptional activators, namely Vmw175, Vmw110 and Vmw63. The function of these gene products is to switch on the expression of the bulk of the viral genome after the IE phase of infection. The mechanism of activation of transcription by trans-acting factors is unknown at present. HSV-1 early promoters do not contain detectable virus-specific regulatory sequence elements although the efficiency of activation depends on the integrity of promoter elements (such as the TATA box and 'upstream' sequences) which are commonly found in viral and cellular promoters (Everett, 1984b; Eisenberg et al., 1985; El Kareh et al., 1985). Mechanisms involving RNA stabilization or transport cannot be excluded, but appear less likely than a primary effect on transcription since increased levels of viral cytoplasmic RNA can be correlated with increased rates of transcription (Godowski & Knipe, 1986). Increased promoter usage could be brought about either by a direct interaction of IE products with general promoter element DNA sequences, or by an indirect association with the DNA through cellular factors that bind to these sequences.

An attractive hypothesis to explain the presence of multiple activators in HSV-1 is that they are required to regulate differentially the numerous early and late promoters. There have been some indications that this could occur. The tk and gD promoters were thought to respond differentially to Vmw110 (Everett, 1984a; O'Hare & Hayward, 1985), varied regulation of several early and late promoters by Vmw175 has been noted (Persson et al., 1985), virus mutants with lesions in Vmw175 coding sequences which express normal amounts of early but reduced levels of late gene products at the non-permissive temperature have been isolated (DeLuca et al., 1984; DeLuca & Schaffer, 1985), and mutations in Vmw63 which result in abnormal regulation of some delayed early and late viral genes (including VP5) have been described (Sacks et al., 1985). The results presented here support the suggestion that the IE gene requirement for activation of early promoters may differ from that required for the activation of later classes of genes. More work is required to identify other HSV promoters which are regulated in short-term transfection assays in a way similar to that of VP5. However, differences observed between the gD and tk promoters (Everett, 1984a; O'Hare & Hayward, 1985) may reflect differences in the assay systems or cell types used rather than any substantial differences between the mechanisms of activation of the two promoters.

Vmw175, Vmw110 and Vmw63 are phosphorylated nuclear proteins which bind DNA in crude cell extracts (Pereira et al., 1977; Hay & Hay, 1980). The functional relationships of these three polypeptides are not known; it is possible that they act in a complex, or singly at different stages of the same pathway of gene activation, or through alternative mechanisms of activation, or indirectly by stimulation of transcription of (for example) the IE-3 promoter to increase the intracellular levels of Vmw175. The additional role of Vmw63 in VP5 expression is unlikely to be due to activation of transcription by a pathway entirely different from that used by Vmw110/Vmw175 since Vmw63 shows no activation of any of the promoters in the absence of the other two IE polypeptides. It can also be argued that Vmw63 does not act in a complex with the other two IE products or by increasing the level of expression of Vmw175 or Vmw110 because, in these cases, it would be expected to affect the expression of all the promoters that Vmw110 and Vmw175 are known to stimulate. Since Vmw63 has an effect on VP5 expression both in the virus (Sacks et al., 1985) and in short-term transfection assays (with pVP5CAT), it is not likely to have a role in post-transcriptional events as the RNAs synthesized in these two circumstances are entirely different in sequence. Therefore, it follows that Vmw63 exerts its effect through sequences 5' to the RNA start sites. It is tempting to speculate that the herpesvirus IE gene products regulate transcription through cellular factors which recognize
promoter DNA sequences and that Vmw63 acts in conjunction with a cellular factor which interacts with sequences in the VP5 but not the gD or tk promoters.

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