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Cells that Constitutively Express the Herpes Simplex Virus Immediate-Early Protein ICP4 Allow Efficient Activation of Viral Delayed-Early Genes in *trans*

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To study the role of herpes simplex virus type 1 immediate-early proteins in the transcriptional activation of herpes simplex virus genes, we isolated stably transformed cells expressing herpes simplex virus type 1 ICP4, an immediate-early protein known from previous studies to be necessary for delayed-early and late transcription. These cells efficiently expressed six delayed-early herpes simplex virus genes introduced by viral superinfection, in the absence of de novo viral protein synthesis. In contrast, the delayed-early gene encoding alkaline exonuclease and the late gene encoding the capsid protein VP5 were expressed at much lower levels. Expression of a second late gene, that for glycoprotein C, was undetectable under the same experimental conditions. These results suggest that many, but not all, delayed-early genes are efficiently activated by ICP4; in addition, they demonstrate that although the late gene for VP5 is detectably activated by ICP4, its full expression requires additional factors.

Herpes simplex virus (HSV) genes are expressed in three sequential waves during lytic infections, largely as a result of regulation at the level of transcription (20). Although viral genes are transcribed in the nucleus of the infected cell by the host RNA polymerase II (14), virally coded proteins are known to play a major role in controlling the transition between transcriptional phases. Because most of the HSV genes are controlled by separate promoters (for a review, see E. K. Wagner *in* B. Roizman, ed., *The Herpes Viruses, Vol. 3*, in press), this transcriptional control is presumably mediated by the differential interactions of a limited number of regulators with a large number of *cis*-acting sites. The five immediate-early (or α) genes are the first HSV genes to be expressed; they are also the only ones transcribed when viral protein synthesis is prevented (1, 10, 27, 34, 35, 42–44). The product of one of these genes (ICP4) is necessary for both the initial activation and the continued transcription of the delayed-early (or β) and late (or γ) viral genes (34, 41). Indeed, ICP4 is the only HSV protein known at present to be involved in the positive control of transcription. The role, if any, of the remaining four immediate-early proteins in viral gene activation remains unknown.

The results of Honess and Roizman (20) and Watson and Clements (41) are consistent with the idea that expression of one or more of the five immediate-early proteins suffices for the full activation of delayed-early genes and that ICP4 is necessary for this activation. The scheme of Honess and Roizman (20) also suggests that the efficient expression of late genes requires the action of delayed-early proteins in addition to immediate-early proteins. Results of more recent studies have suggested that the late genes are heterogeneous in their transcriptional requirements, with some being expressed at low levels at early times and when viral DNA replication is blocked (for example, the gene encoding the major capsid protein VP5 [15; Wagner, in press]), whereas others are expressed only after the onset of DNA replication (for example, glycoprotein C [gC; 12; Wagner, in press]). Because the former class of late genes (the "leaky late" or β-γ) are first expressed at early times, it seems possible that they are directly activated by immediate-early proteins.

To examine the role of ICP4 in the activation of delayed-early and late genes, we transfected a cloned HSV type 1 (HSV-1) DNA fragment encoding ICP4 and possibly ICP47 into mouse cells and report here on the isolation of stably transformed cell lines that constitutively express these immediate-early proteins. These cells supported efficient activation of a variety of delayed-early genes under conditions that preclude de novo viral protein synthesis. An exception within this class was the gene for alkaline exonuclease. We conclude that, at most, only two of the five immediate-early proteins are required for the activation of the majority of delayed-early genes. By contrast, the leaky late gene encoding the capsid protein VP5 was expressed only at a low level, and expression of the late gene for gC was not detected. These results suggest that although immediate-early proteins suffice for the detectable activation of some leaky late genes, their efficient expression requires additional factors. The same appears to be true for the activation of tight late genes.

MATERIALS AND METHODS

Cells and virus. Vero cells and thymidine kinase-deficient (tk^-), adenine phosphoribosyl transferase-deficient ($aprt^-$) LTA cells were maintained in α-minimal essential medium (GIBCO Laboratories) containing 10% fetal bovine serum. Cell lines cotransformed by pRHP6 and PN17BS3 were isolated by transfecting subconfluent 60-mm plates of LTA cells with 100 ng of each plasmid DNA and 10 μg of high-molecular-weight LTA cell carrier DNA per ml by the calcium phosphate coprecipitation technique (18) as described previously (15). After HAT selection (40), individual colonies were picked with a cloning cylinder and expanded into cell lines. HSV-1 (strain KOS) was propagated and titrated on Vero cells.

Labeling and immunoprecipitation of proteins. Infected (20 PFU per cell, unless indicated otherwise) or uninfected cells were labeled for the indicated times in methionine-free medium supplemented with 2% dialyzed fetal bovine serum and 25 to 40 μCi of [L-^{35}S]methionine (1100 to 1400 Ci/mmol;

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Amersham Corp.) per ml. Where indicated, cycloheximide (50 μg/ml) was added 45 min before infection, maintained until 6 h postinfection, and then replaced with actinomycin D (10 μg/ml). After labeling, the cells were scraped from the dishes, washed three times with phosphate-buffered saline, and lysed by sonication in RIPA buffer (50 mM Tris-hydrochloride [pH 7.2], 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100) plus 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 0.11 trypsin inhibitor unit of aproteinin (Sigma) per ml. The lysates were clarified by centrifugation (27,000 × *g* for 15 min). For immunoprecipitations, 0.5 ml of lysate and 10 μl of ascitic fluid containing the appropriate monoclonal antibody were incubated at 4°C on a rotating wheel for 2 to 8 h in the presence of protein A-Sepharose beads (Pharmacia Fine Chemicals). After the beads were washed three times with RIPA buffer, the precipitated proteins were released by heating at 100°C for 3 min in sample buffer and were electrophoresed overnight at 80 to 90 V in 9% polyacrylamide gels by the method of Laemmli (23). The gels were prepared for fluorography by the method of Bonner and Laskey (8). The following monoclonal antibodies were used to detect specific HSV proteins: 58S (ICP4) and 19S (gC) (38); 74S (p40) (M. Zweig, personal communication); Q1 (alkaline exonuclease) (5); II 481B-2 (gE) (25); 18 β B3 (gD) (3); A4-2 (ICP8), 13 α A5 (ribonucleotide reductase), B2 (gB) and α-E-10 (control) (M. J. Evelegh and S. Bacchetti, unpublished data). ICP47 was detected after immunoprecipitation with the IE12/76 antiserum (30).

S1 nuclease mapping. Total cytoplasmic RNA was prepared from infected (10 PFU per cell) or uninfected cells by the method of Berk and Sharp (7). Where indicated, cycloheximide (50 μg/ml) was added 45 min before infection and maintained continuously. Uniquely end-labeled, single-stranded hybridization probes were prepared from wild-type thymidine kinase and VP5 DNA fragments with [γ-^{32}P]ATP (New England Nuclear Corp.) and T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.), as described by Maxam and Gilbert (29). RNA (10 μg) was used for each hybridization reaction. Conditions for hybridization and S1 nuclease digestion were as previously described (15).

RESULTS

Isolation of cells expressing ICP4. An *Xho*I fragment of HSV-1 DNA present in plasmid pRHP6 was used in these studies as a source of the ICP4 gene (Fig. 1). Although this fragment bears only one complete transcription unit, that which encodes ICP4 mRNA, it also contains the complete protein coding sequence for ICP47 (43) and sequences encoding a portion of the amino terminus of ICP0 (27). To obtain cells which constitutively express ICP4, we cotransfected mouse Ltk$^-$ aprt$^-$ (LTA) cells (31) with pRHP6 and pN17BS3 (Fig. 1B). pN17BS3 bears a recombinant gene in which the HSV-1 thymidine kinase structural sequence is under the control of the promoter of the leaky-late gene encoding the major capsid protein VP5 (15). The rationale for using this recombinant gene as the selected marker was twofold. First, because the recombinant gene lacks part of the sequences present in the thymidine kinase and VP5 transcription units, we were subsequently able to discriminate between transcripts arising from pN17BS3 and the wild-type HSV genome in superinfected cells (see below). Second, we have previously found that the VP5 promoter of the VP5-thymidine kinase hybrid gene is not used in uninfected cells; instead, transcription initiates at a weak internal promoter within the thymidine kinase structural sequence

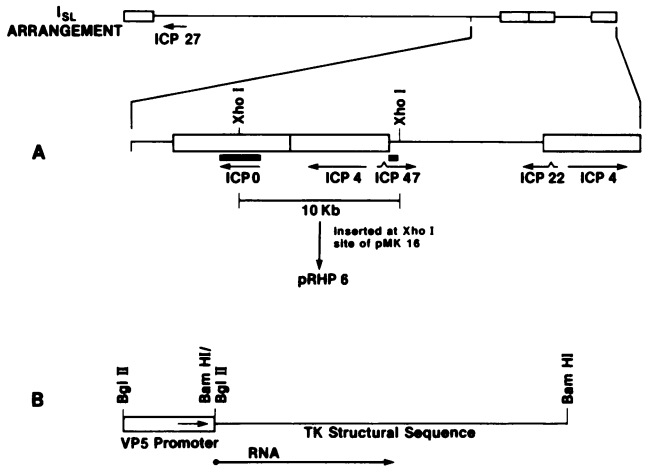

FIG. 1. Structure of plasmids used. (A) Plasmid containing the ICP4 gene. The figure diagrams the locations of the five HSV-1 immediate-early genes on the I_{SL} arrangement of the viral genome (see text for references). The map location of the 10-kilobase (kb) *Xho*I fragment used as a source of the ICP4 gene is indicated. Black bars indicate the location of the ICP47 coding sequences and the approximate location of the ICP0 coding sequences. pRHP6 was derived by cloning the *Xho*I fragment into the *Xho*I site of pMK16 (22). (B) Plasmid containing the VP5-thymidine kinase hybrid gene. pN17BS3 (15) bears the VP5 gene promoter fused to the thymidine kinase (TK) structural gene sequence. The fusion links the VP5 promoter elements upstream of −10 to the nontranslated leader of thymidine kinase at +56. After activation by HSV gene products, transcription driven from the VP5 promoter initiates just downstream from the fusion site in the thymidine kinase nontranslated leader sequences.

(15), resulting in an extremely low frequency of wild-type thymidine kinase-transformed colonies. However, the VP5 promoter present in the resulting transformants is activated after infection with HSV, with delayed-early kinetics. Therefore, we thought it possible that the presence of a cotransfected ICP4 gene would activate the VP5 promoter, resulting in an increased frequency of thymidine kinase transformants. The increase in the number of thymidine kinase-positive colonies obtained in cotransfections, as compared with that obtained with the VP5-thymidine kinase gene alone, varied in several trials from none at all to approximately 50-fold (data not shown). At present we are unable to explain this variability. Four thymidine kinase-positive colonies isolated from a trial showing a 50-fold elevation in the frequency of thymidine kinase transformants were screened for expression of ICP4 by immunoprecipitation of [^{35}S] methionine-labeled cell extracts with the 58S monoclonal antibody specific for ICP4 (38). All four colonies produced readily detectable quantities of the ICP4 protein (Fig. 2A). One of these cell lines, Z4, continued to produce ICP4 at a constant rate during 11 months of continuous culture. In addition, Z4 cells were found to express barely detectable levels of ICP47 (Fig. 2C). Because the pRHP6 plasmid does not contain the complete ICP47 transcription unit, expression of this protein evidently was made possible through the acquisition of a functional polyadenylation signal during transfection. Results of preliminary experiments, to be discussed below, however, have suggested that ICP47 per se does not play a role in the activation of the viral genes analyzed. Therefore, in the remainder of this report we assume that the transcriptional activation of HSV genes observed in Z4 cells is due to ICP4 alone.

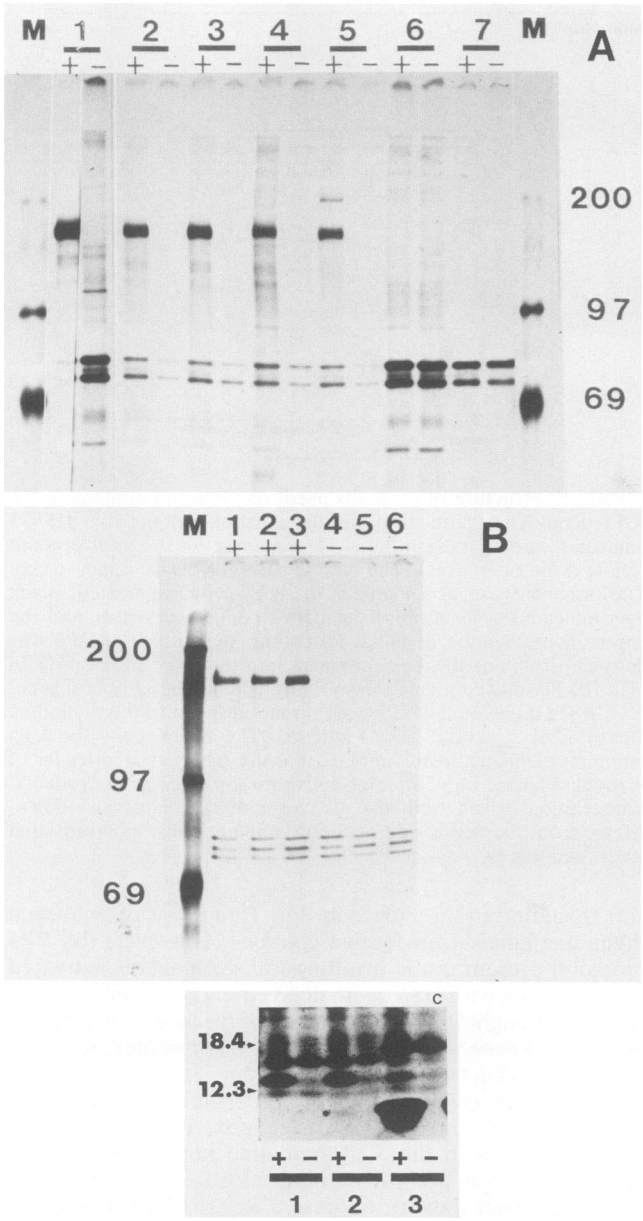

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine labeled proteins immunoprecipitated from lysates of transformed cells by anti-ICP4 or anti-ICP47 antibodies. (A) Screening of cell lines for ICP4 synthesis. Proteins immunoprecipitated from cell lysates by a monoclonal antibody specific for ICP4 were loaded in lanes marked +. Proteins precipitated nonspecifically from the same lysates by an inactive unrelated antibody were loaded in lanes marked −. Lysates 2 through 5 are from the four cell lines established by cotransfection. Lysates 1 and 6 are from HSV-infected and mock-infected cells, respectively. Lysate 7 is from control N17BS3 cells. The plate is a composite of the following different exposures of the same gel: 18 h (lysate 1); 6 days (M) [marker]; and 20 days (the remainder). Labeling was for 4 h (infected cells) or 16 h. The numbers on the sides refer to the molecular weights ($\times 10^{-3}$) of ^{14}C-labeled marker proteins (New England Nuclear) loaded in the lane marked M. (B) Stability of ICP4 in Z4 cells. Lanes 1 and 4 contain protein immunoprecipitated from Z4 cells at the end of a 3-h labeling period; lanes 2 and 5 contain protein from Z4 cells after a 3-h labeling and a subsequent 8-h chase; and lanes 3 and 6 contain protein from Z4 cells after a 3-h labeling and a subsequent 8-h chase in the presence of 50 μg of cycloheximide per ml. Symbols are as in (A). (C) Detection of ICP47 synthesis in Z4 cells. Proteins immunoprecipitated from cell lysates by a rabbit antiserum directed against the presumptive C-terminus of ICP47 (30) were loaded in lanes marked +. Proteins precipitated nonspecifically from the same lysates by normal rabbit serum were loaded in lanes marked −. Lysate 1, control LTA cells (31); lysate 2, Z4 cells; lysate 3, LTA cells infected with 40 PFU of HSV-1 (KOS) per cell and labeled 1 to 3 h postinfection. Labeling in all cases was for 2 h with 75 μCi of [^{35}S]methionine per ml. Numbers to the left are in kilobases. Gels in (A) and (B) were 9% polyacrylamide; the gel in (C) was a 5 to 12% gradient gel.

The initiation site of the thymidine kinase-related transcripts present in two of these cotransformed cell lines was located by S1 nuclease mapping with a probe derived from the wild-type thymidine kinase gene. The results (data not shown) demonstrate that both lines expressed thymidine kinase RNA driven from the VP5 promoter; this result is consistent with the activation of this promoter by ICP4. An alternative explanation is that the VP5 promoter was activated by linkage to the ICP4 enhancer element (11, 24, 28) during transfection. However, data presented in the next section more directly demonstrate that the ICP4 present in these cells is sufficient to partially activate the VP5 promoter in *trans*.

***trans*-activation of the thymidine kinase and VP5 genes in Z4 cells.** To determine whether the ICP4 in Z4 cells was sufficient to activate the thymidine kinase and VP5 genes in *trans*, we employed the following experimental design: Z4 and control nonexpressor N17BS3 cells (15) were superinfected with HSV-1 in the presence of cycloheximide to prevent viral protein synthesis. Under analogous conditions in cells not expressing ICP4, only immediate-early mRNAs are transcribed (1, 10, 26, 42). The accumulation of viral mRNA was then assessed by S1 nuclease analysis of infected cellular RNA with probes specific for transcripts arising from the viral genome. For these experiments to be meaningful, it was first necessary to show that the ICP4 protein present in Z4 cells was stable during the cycloheximide block. This was found to be the case. Prelabeled ICP4 was not detectably degraded during an 8-h chase in the presence of cycloheximide (Fig. 2B). This result is in accord with previous data on the stability of the ICP4 protein (45).

We found that blocking de novo viral protein synthesis with cycloheximide completely suppressed the accumulation of viral thymidine kinase transcripts in N17BS3 cells (Fig. 3A). In contrast, ICP4$^+$ Z4 cells supported the accumulation of substantial quantities of thymidine kinase RNA under the same experimental conditions. Indeed, the level of thymidine kinase RNA found in cycloheximide-blocked Z4 cells was similar to that observed in unblocked N17BS3 cells and was about one-half of that observed in unblocked Z4 cells. Results of this experiment suggest that the ICP4 present in Z4 cells is sufficient to activate the expression of a thymidine kinase gene present on a superinfecting viral genome to levels of expression approximating those observed in a normal infection. In marked contrast to the results with thymidine kinase, only marginal activation of the leaky-late VP5 gene was observed in similar experiments (Fig. 3B). Infected Z4 cells accumulated detectable, but low, levels of VP5 RNA in the presence of the protein synthetic block, whereas no VP5 transcripts were detected in the control N17BS3 cells. We estimate that cycloheximide-blocked Z4 cells accumulated at most 1/20th of the levels of VP5 RNA found in unblocked cells. As described in detail below, these results support our previous suggestion that the VP5 promoter is first activated by immediate-early proteins and that

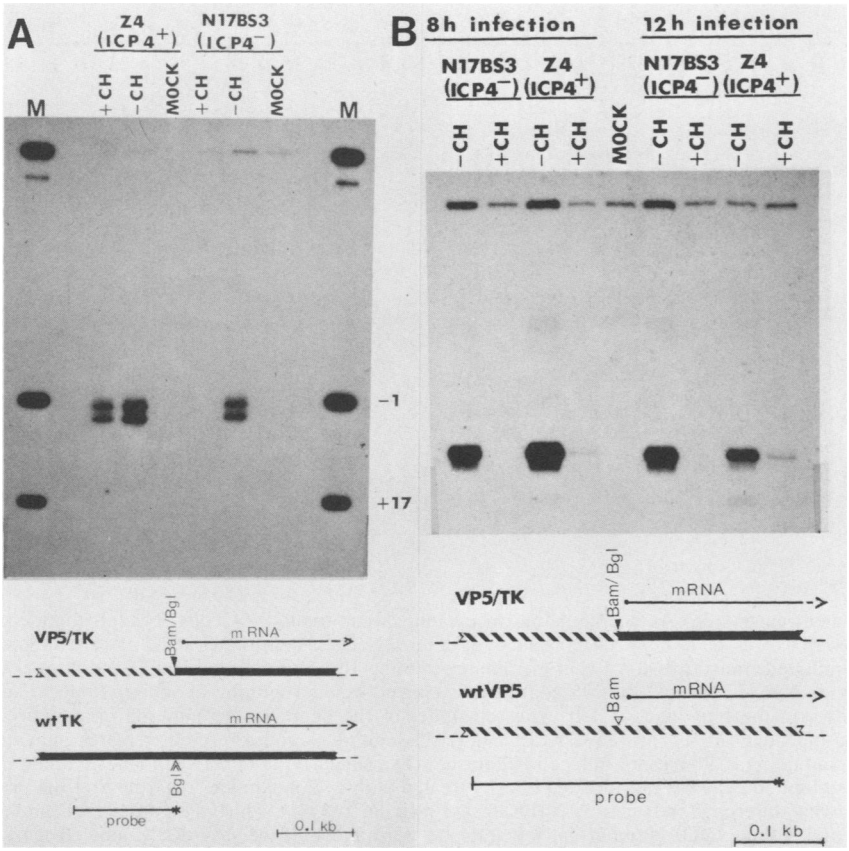

FIG. 3. S1 nuclease mapping of viral thymidine kinase (TK) and VP5 transcripts induced by ICP4. (A) Thymidine kinase RNA. A single-stranded probe labeled at the *Bgl*II site at +56 in the wild-type thymidine kinase gene and extending to the *Eco*RI site at −80 was hybridized to cytoplasmic RNA prepared from Z4 and N17BS3 cells 8 h after infection with 10 PFU of HSV-1 per cell. Where indicated, cycloheximide (CH; 50 μg/ml) was added 45 min before infection and maintained continuously. RNA prepared from mock-infected cells (MOCK) was used as a control. After S1 nuclease treatment, the digestion products were sized on an 8% polyacrylamide sequencing gel. Markers (M) were generated by cleaving the probe fragment with *Taq*I (−1) and *Pst*I (+16). (B) VP5 RNA. A single-stranded probe labeled at an *Rsa*I site at +158 in the wild-type VP5 gene and extending to a *Hha*I site at −159 was hybridized to cytoplasmic RNA prepared either 8 or 12 h after infection with HSV-1. Labels are as in (A). S1 nuclease digestion products were sized on a 6% polyacrylamide sequencing gel. Kb, kilobases.

its expression is subsequently boosted by the increased template copy number resulting from viral DNA replication, a process that requires delayed-early gene products (15).

Effects of ICP4 on expression of other HSV genes. The results presented above suggest that a delayed-early gene was efficiently activated by ICP4, whereas a late gene was activated only marginally. To test the generality of this finding, we surveyed the effects of ICP4 on the expression of a variety of viral genes. Insted of directly scoring for the RNAs derived from these genes, we used an indirect translational assay. After allowing mRNA to accumulate in the presence of cycloheximide for 6 h, the protein synthetic block was removed. Further transcription of viral mRNA was prevented by the addition of actinomycin D, and the accumulated mRNAs were allowed to be translated in vivo in the presence of [^{35}S]methionine. After 5 h, the resulting protein products were analyzed by gel electrophoresis (Fig. 4). The total complement of virally induced proteins was examined by directly analyzing the labeled extracts, and a number of specific proteins were detected by immunoprecipitation with monoclonal antibodies.

The results support the general conclusions made above. In control N17BS3 cells, reversal of the cycloheximide block was followed by detectable translation of only a limited number of viral-specific proteins, as expected for cells blocked in the immediate-early phase of infection. The proteins that we could confidently identify include ICP4 and ICP0, both belonging to the immediate-early class. In marked contrast, reversal of the cycloheximide block in Z4 cells resulted in the translation of at least 5 additional polypeptides that were easily detectable in total cell extracts (arrowheads, Fig. 4). Among these five polypeptides, ICP6 (a delayed-early protein associated with ribonucleotide reductase activity [2]) and ICP8 (the major viral DNA binding protein [33]) could be identified on the basis of their electrophoretic mobility. Immunoprecipitations confirmed the presence of ICP6 and ICP8 and demonstrated the accumulation of readily detectable quantities of gB, gD, and gE as well. All of these proteins previously have been classified as belonging to the delayed-early class (Wagner, in press). We consistently found that the levels of ICP6 obtained after reversal of the cycloheximide block were similar to those obtained in unblocked cells, whereas the levels of ICP8, gB, gD, and gE were lower. Even with this reduced level of translation, ICP8 remained one of the most intensely labeled proteins visible in the total cell extracts. These data suggest that many delayed-early genes are efficiently activated by the ICP4 present in Z4 cells.

Unblocked Z4 and N17BS3 cells contained at least five easily detectable viral proteins that did not accumulate to

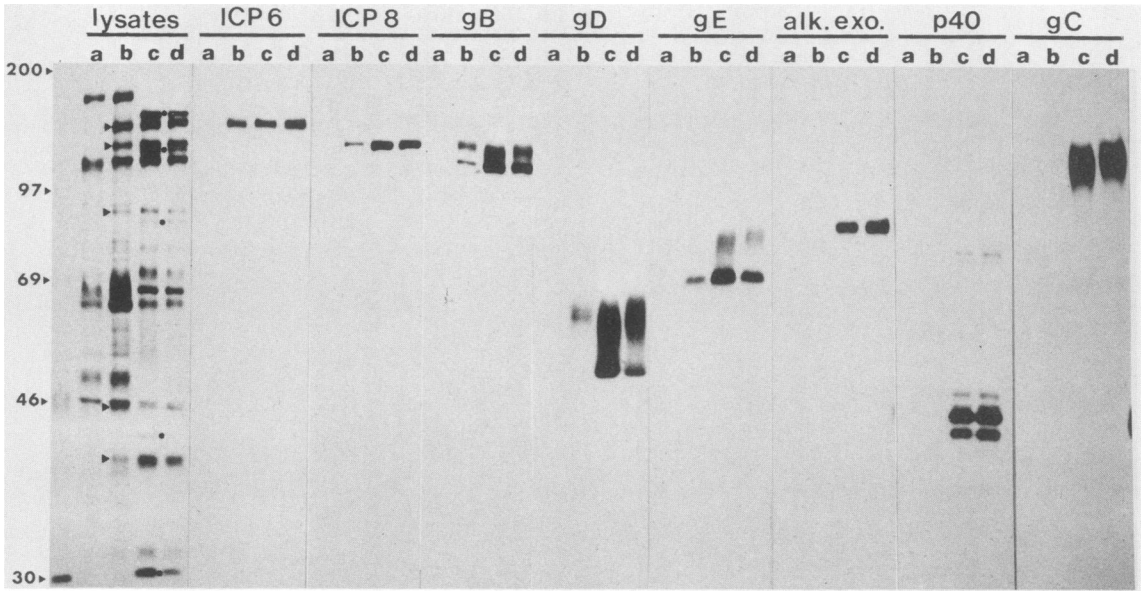

FIG. 4. Translational analysis of mRNAs accumulating in cycloheximide-treated Z4 cells. Cycloheximide-treated (lanes a and b) or untreated (lanes c and d) ICP4 expresser Z4 cells (lanes b and d) or control N17BS3 cells (lanes a and c) were infected for 6 h and subsequently allowed to translate accumulated transcripts for 5 h in medium containing 10 μg of actinomycin D and 40 μCi of [^{35}S]methionine per ml. Relative amounts of individual gene products synthesized were visualized by fluorography of sodium dodecyl sulfate-polyacrylamide gels. Total cell lysates are shown in the four lanes at left. The remainder of the figure shows material immunoprecipitated with monoclonal antibodies specific for the indicated HSV-1 proteins. Arrowheads between lanes a and b for the lysates indicate proteins translated from mRNAs induced in cells containing ICP4 but not induced in control cells. Similarly, dots between lanes b and c indicate proteins translated from mRNAs induced in untreated cells but not induced in cells treated with cycloheximide, regardless of the presence of ICP4. The figure is a composite of the following different exposures: 22 h (lysates), 1 h (ICP6 and gB), 4 h (ICP8), 4 days (gD and p40), 12 h (gE and alkaline exonuclease [alk. exo.]) and 3 days (gC). Numbers at left indicate the molecular weights of ^{14}C-labeled marker proteins (NewEngland Nuclear) ($\times 10^3$).

high levels in cycloheximide-blocked Z4 cells (Fig. 4). Presumably, these proteins are the products of viral genes that are not efficiently activated by ICP4. The largest and most abundant of these five proteins is the major capsid protein VP5, the mRNA of which was shown above to be only marginally activated in Z4 cells. Immunoprecipitations identified three other proteins that fall into this class: viral alkaline exonuclease, nucleocapsid protein p40, and gC. Both p40 and gC are late proteins, so it is not surprising that they behave similarly to VP5 in this assay. However, we were surprised to find that alkaline exonuclease did not accumulate in Z4 cells after reversal of the cycloheximide block, because it has been classified as a delayed-early protein (13). As discussed further below, this result may imply that the delayed-early genes fall into more than one class with respect to their transcriptional requirements.

DISCUSSION

In the experiments described here, we attempted to assess the role of a limited subset of the HSV-1 immediate-early proteins in the transcriptional activation of a variety of HSV genes. Our strategy was to first isolate stably transformed cell lines expressing ICP4 and then to examine expression of HSV genes introduced into these cells by viral superinfection under conditions preventing de novo viral protein synthesis. As mentioned above, Z4 cells also express the immediate-early protein ICP47. That this protein plays no major role in the activation of the viral genes studied, however, is suggested by preliminary, but essentially similar, results obtained with cells transformed by a plasmid encoding ICP4 and ICP22 (instead of ICP47). Although it can be argued that ICP22 and ICP47 might substitute for each other when in combination with ICP4, we consider this to be unlikely. Therefore, in the ensuing discussion we assumed that all of the observed effects result from the action of ICP4 alone. The specific questions that we have posed are as follows: (i) to what extent does ICP4 activate the transcription of delayed-early genes? and (ii) does ICP4 also directly activate leaky late genes?

Our experimental approach of using stably transformed lines expressing ICP4 was preferred for a number of reasons over the alternative strategy of cotransfecting cells with the ICP4 gene, along with suspected targets of ICP4 action, in a transient assay and scoring for target gene activation. First, even under the best conditions, only a small proportion of acutely transfected cells express the introduced markers, limiting the signal associated with a positive response. Second, it is not obvious a priori that HSV genes are controlled by a single regulatory event. In the present case we were able to quantify the response of the target genes to the suspected regulatory factor by comparing the signal with that obtained in viral infections. Based on this comparison, we conclude that ICP4 is capable of nearly full activation of at least two delayed-early genes, those encoding thymidine kinase and ICP6, but only marginally activates the late gene for VP5. It would have been difficult, if not impossible, to arrive at this conclusion by a transient expression assay. Third, during transfection, originally unlinked sequences become physically linked (31). Because a variety of viral and cellular genes have been shown to be associated with *cis*-acting DNA sequences that potentiate the activity of linked promoters in the absence of the *trans*-acting factors ordinarily required for their activity (4, 19, 21), the *trans* action of a suspected regulator can only be definitively

established when the possibility of recombination during transfection is excluded. Several studies have documented the fact that chromosomally integrated HSV sequences are not normally available for recombination with a superinfecting viral genome (9, 37). Consequently, we are confident that the observed effects are due to activation in *trans*.

Although our approach has a number of advantages, we relied on several assumptions that might limit the interpretation of our results. First, we assumed that all of the observed effects are due to the expression of the ICP4 protein in Z4 cells. Second, all of the assays for the effects of preexisting ICP4 were carried out by viral superinfection, a condition that unavoidably introduces virion structural proteins into the cells. Results of several studies have suggested that one or more virion components positively regulate HSV immediate-early promoters (6, 11, 28). Although there is no evidence that these virion components also directly contribute to the control of other HSV genes, this possibility has not yet been excluded. Consequently, at present we cannot eliminate a role for virion structural proteins in delayed-early gene expression. Third, our experiments have directly and indirectly measured the levels of viral mRNAs accumulating in the presence of cycloheximide. We assumed that these levels reflect the rate of transcription of the genes in question and that the rate of translation of the accumulated mRNAs reflects, at least grossly, their abundance. Finally, we assumed that the observed effects of cycloheximide on viral transcription result from its inhibition of viral protein synthesis, a view that seems well supported.

Given these assumptions, the following conclusions are warranted. First, the ICP4 protein present in Z4 cells has a dramatic effect on the expression of many delayed-early HSV genes when these cells are superinfected with HSV-1. The synthesis of six delayed-early mRNAs was resistant to the effects of cycloheximide in these cells, yielding substantial levels of these mRNAs under conditions that otherwise prevent their detectable accumulation. At least two of these mRNAs, those encoding thymidine kinase and ICP6, were induced to levels close to those obtained in a normal infection. We conclude that ICP4 is sufficient for the efficient activation of the delayed-early genes tested, with the exception of the gene encoding alkaline exonuclease. This conclusion is reinforced by the fact that similar results were obtained regardless of the presence of ICP47 (or ICP22) in addition to ICP4 (unpublished data). As a corollary to this, we also conclude that the remaining HSV immediate-early proteins (ICP0 and ICP27) do not play an essential role in the activation of these delayed-early genes. However, it is possible that these proteins contribute to the expression of the alkaline exonuclease gene. Because the accumulation of alkaline exonuclease mRNA occurs with delayed-early kinetics (13) and is not inhibited when viral DNA replication is blocked, delayed-early proteins are unlikely to influence its expression. Our finding that the exonuclease gene was not efficiently activated under the same conditions in which six other delayed-early genes were activated provides evidence that delayed-early genes do not form a homogeneous class with respect to their transcriptional requirements, a conclusion that is reminiscent of the findings of Pereira et al. (32). It will be interesting to determine how many other delayed-early genes fall into the same class as the alkaline exonuclease gene and to identify the additional factor(s) required for their efficient expression.

The second major conclusion that we have reached in this study is that immediate-early proteins are sufficient for the detectable activation of the leaky-late gene encoding VP5. However, this gene is expressed at high levels only after the action of additional factors, so that the expression obtained in cycloheximide-blocked Z4 cells is only a small fraction of that obtained in unblocked cells. On the basis of the kinetics of activation of a VP5-thymidine kinase hybrid gene by the products of superinfecting HSV, we have previously argued (15) that immediate-early proteins alone suffice for the initial activation of the VP5 promoter and that the higher levels of expression obtained during the late phase of infection result from the effects of template amplification. The results obtained in this study support this hypothesis. We do not yet know whether the late genes for p40 and gC are also marginally activated by ICP4. The answer to this question will require a direct analysis of their transcripts.

The presence of ICP4 in Z4 cells supports the extraordinarily efficient expression of many HSV delayed-early genes, yet this striking alteration in the ability of the cells to utilize HSV promoters does not noticeably affect their viability. Presumably, then, the ordered and regulated expression of the cellular genes in Z4 cells is not dramatically perturbed. However, results of recent studies on an analogous protein encoded by pseudorabies virus (19, 21) suggest that such a protein is able to activate both transfected cellular β-globin genes and adenovirus genes. There is also evidence that HSV ICP4 itself activates a transfected cellular β-globin gene (16). The problem then, is to account for these seemingly nonspecific effects on non-HSV genes, while at the same time accounting for the lack of a lethal alteration in the expression of resident cellular genes. There are two alternative hypotheses to reconcile these data. The first, which has also been proposed by Everett (16), holds that ICP4 specifically activates extrachromosomal genes, regardless of sequence. This hypothesis accounts for the effects on acutely transfected adenovirus and β-globin genes and also for the activation of HSV genes borne on a superinfecting viral genome. However, it does not explain the fact that chromosomally integrated HSV genes are also efficiently activated by the immediate-early proteins supplied by a superinfecting viral genome (see, for example, references 37 and 39). The alternative extreme position holds that ICP4 acts in a sequence-specific fashion, activating only those genes bearing specific ICP4 recognition sequences. This hypothesis, although possible, is difficult to reconcile with the lack of obvious sequence homology among the promoters of delayed-early HSV genes (Wagner, in press) and with the effects of the pseudorabies ICP4 analog on adenovirus and cellular genes. Consequently, we are at present unable to arrive at a simple model to explain the effects of ICP4. Possibly, they involve both a generalized activation of the transcription of extrachromosomal genes and a more specific activation of specialized HSV promoters. Because ICP4 represents one of the proteins that has been most clearly demonstrated to be capable of modifying the transcriptional specificity of eucaryotic cells, clarification of its mechanism of action is likely to be of general interest.

We expect that Z4 cells will prove to be useful in this exercise, providing an HSV analog of 293 cells (17) (permissive cells expressing adenovirus E1A).

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