
Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides

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Received February 11, 1987; Revised and Accepted May 8, 1987

ABSTRACT

Synthetic oligonucleotide linkers containing translational termination codons in all possible reading frames were inserted at various positions in the cloned gene encoding the herpes simplex virus type 1 (HSV-1) immediate-early regulatory protein, ICP4. It was determined that the amino-terminal 60 percent of the ICP4 gene was sufficient for trans-induction of a thymidine kinase promoter-CAT chimera (pTKCAT) and negative regulation of an ICP4 promoter-CAT chimera (pIE3CAT); however, it was relatively inefficient in complementing an ICP4 deletion mutant. The amino-terminal ninety amino acids do not appear to be required for infectivity as reflected by the replication competence of a mutant virus containing a linker insertion at amino acid 12. The size of the ICP4 molecule expressed from the mutant virus was consistent with translational restart at the next methionine codon corresponding to amino acid 90 of the deduced ICP4 amino acid sequence.

INTRODUCTION

The sequential expression of HSV-1 genes (1) is, in part, due to the requirement for functional immediate-early proteins. The requirement for immediate-early proteins for the efficient and regulated expression of early and late viral genes initially provided evidence for the essential role of one or more of these proteins in the productive expression of the HSV-1 genome (2). Phenotypic analysis of temperature-sensitive (3-5) and deletion mutants (6) in the gene for the immediate-early protein, ICP4, have implicated this molecule as an important eukaryotic regulatory protein.

ICP4 migrates with an apparent molecular weight of 175 Kd on SDS-polyacrylamide gels (7-9), although the size of the primary translation product as predicted by the nucleotide sequence is 132,835 d (10). This discrepancy is most likely due to the

atypical amino acid composition and post-translational processing of ICP4. The ICP4 molecule is phosphorylated in a complex pattern (11) - possibly involving several mechanisms (12) - to yield at least three phosphorylated species as observed by one-dimensional SDS-polyacrylamide gel electrophoresis. ICP4 is found predominantly in the nucleus shortly after infection (7,13,14). In addition, it has been shown that ICP4 extracted from infected cells can bind specifically (15,16) and nonspecifically (17,18) to added target DNA. The functional significance of the DNA-binding properties of ICP4 have yet to be determined.

Much of what is known of the transcriptional regulatory function of ICP4 was provided by the study of mutants in this gene, and by transient expression assays using cloned ICP4 and a suitable test gene. Experiments with temperature-sensitive (3-5) and deletion (6) mutants have shown that ICP4 is absolutely required for transcriptional activation of early and late genes and the repression of expression of immediate-early genes. It has also been shown that some temperature-sensitive forms of ICP4 exhibit a trans-dominant effect on the wild-type polypeptide (19,20). In transient expression assays it has been shown that ICP4 alone is sufficient to trans-activate early and late promoters (19-25) and repress expression from its own promoter (19,20,26). Recently, it has been demonstrated that partially purified ICP4 when added to an in vitro transcription extract prepared from uninfected cells can stimulate early gene transcription and repress transcription from the immediate-early ICP4 promoter (27).

The known functions of ICP4 include the activation of transcription from a variety of promoters and the repression of transcription from its own and possibly other immediate-early promoters. How these functions relate to the known physical parameters of the ICP4 protein is for the most part unknown. In the present study we have excluded various portions of the ICP4 primary amino acid sequence by the introduction of nonsense mutations within the coding region of the ICP4 gene. The cloned ICP4 genes containing nonsense mutations were then compared to the wild-type gene for the ability to repress expression from

the ICP4 promoter (immediate-early) and stimulate expression from the tk promoter (early) in transient expression assays. In addition, the same plasmids were used in transient complementation assays with an ICP4 deletion mutant to assess the ability of the truncated protein to function in the viral life cycle. From the data presented in this study it is possible to assign specific functional activities to regions of the ICP4 molecule.

MATERIALS AND METHODS

Cells and viruses

The procedures used for the growth and maintenance of African green monkey kidney cells (CV-1, Vero and E5 cells) have been previously described (6,19,28). E5 cells are biochemically transformed Vero cells containing the plasmids pK1-2 (see below) and pSV2neo (manuscript in preparation). E5 cells were derived in a manner identical to that used to generate other ICP4-containing cells (6) and are efficient hosts for mutants defective in the gene for ICP4.

The ICP4 deletion mutants, d120 and d202 (6), were propagated and assayed on E5 cells. The yields of d120 and d202 propagated on E5 cells are between 200 and 500 PFU/cell compared with 500-1000 PFU/cell for wild-type virus in Vero cells. Since E5 cells do not contain HSV-1 information which flanks the d120 deletion, wild-type recombinants are rarely seen.

Recombinant plasmids

The HSV-1 inserts in pK1-2 and pW3- Δ H58 are shown relative to the HSV-1 genome in Fig. 1. The plasmid, pK1-2, was assembled in the plasmid pUC8 from subclones containing ICP4 gene sequences and contains the HSV-1 information from -330 bp (SmaI site) of the ICP4 mRNA start site to approximately 400 bp downstream of the 3' end of the ICP4 mRNA (a Sau3A site). The HSV-1 information in pK1-2 includes all the identified cis-acting elements required for ICP4 expression as well as the protein coding sequence itself. The plasmid pW3- Δ H58 containing the gene for the immediate-early protein, ICP0, was kindly provided by Wendy Sacks (unpublished observation). This plasmid, as has previously been shown for ICP0-containing plasmids (21-23,25), will trans-activate both HSV-1 immediate-early and early genes

in transient, cotransfection assays. A plasmid encoding a human amber suppressor tRNA was obtained from John Sedivy (Massachusetts Institute of Technology). Except where noted, all DNA-modifying enzymes were used as prescribed by the manufacturer (New England Biolabs, Beverly, MA).

Transfection

CV-1 cells were used for both transient expression assays and complementation tests. The transfection of CV-1 cells by the $\text{Ca}^{+2}\text{PO}_4^-$ technique (29) with supercoiled plasmid DNA was conducted as previously described (19). The amount and types of plasmids used in transfections are indicated in the figure legends.

The marker rescue of d202 with the cloned ICP4 gene was performed by cotransfection as previously described (30). CsCl banded KOS, d202, and n6 DNAs were prepared as previously described (6,31,32).

Electrophoresis

Polyacrylamide gel electrophoresis for the separation of viral and cellular polypeptides (33,34) and agarose gel electrophoresis for the separation of DNA restriction fragments (35) were performed as previously described.

Complementation

Complementation of the ICP4 deletion mutant, d120, by mutant forms of ICP4, was performed in a manner similar to that described previously (19) with the following modifications. One microgram of test plasmid per 10^6 cells was used instead of 2 μg . The transfected cells were infected with d120 4 h after glycerol shock treatment. At 18 h post-infection the harvested cultures were assayed on E5 cells for complementation and on Vero cells for recombinants. Recombinants between d120 and the plasmids used in this study were not detected.

CAT assays

The in vitro assay for chloramphenicol acetyltransferase (CAT) was conducted as previously described (19,36).

RESULTS

Construction of nonsense mutations in ICP4

The objective of this study was to identify regions of the ICP4 molecule which are important for the functional activities

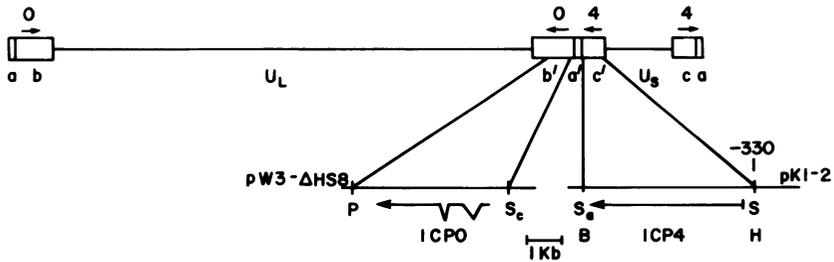


FIGURE 1. The locations of genes encoding ICP4 and ICP0 with respect to the HSV-1 genome. The HSV-1 inserts in the plasmids pK1-2 and pW3- Δ HS8 are described in the text. The HSV-1 *Sau*3a (Sa) to *Sma*I (S) fragment is contained in pK1-2 as a *Bam*HI (B) to *Hind*III (H) fragment. The relevant sites in pW3- Δ HS8 are *Sac*I (Sc) and *Pst*I (P). The *Pst*I site is provided by a synthetic linker (W. Sacks, unpublished).

of the protein. One strategy employed in this study was to introduce nonsense mutations at various distances from the initiator methionine codon. The source of the ICP4 gene used for this purpose was pK1-2. This plasmid and the ICP0-containing plasmid, pW3- Δ HS8, are shown relative to their corresponding sequences in the HSV-1 genome (Fig. 1).

Nonsense mutations were introduced into the ICP4 gene in the following manner: The plasmid pK1-2 contains 16 *Sma*I sites (Fig. 3) - fifteen in the ICP4 coding sequence and one in the polylinker of pUC8. pK1-2 was digested with *Sma*I in the presence of increasing concentrations of ethidium bromide. Between 40 and 60 μ g/ml inhibited cleavage by *Sma*I such that predominantly linear molecules were generated (Fig. 2A). Linear molecules generated by digestion with *Sma*I in the presence of 60 μ g/ml EtBr were eluted from the gel by electroelution. Analysis of this material on a separate agarose gel yielded a single band (Fig. 2B, lane b). Digestion of an aliquot of the eluted material with *Hind*III resulted in a ladder of bands descending from the mobility of the uncut material. *Hind*III cuts pK1-2 only once. Therefore, the appearance of this ladder indicated that the initial linearization with *Sma*I occurred at various, albeit, discrete loci. The oligonucleotide diagrammed in Fig. 2C was ligated to the linearized plasmid, digested with excess *Hpa*I and then recircularized. The salient features of this oligonucleo-

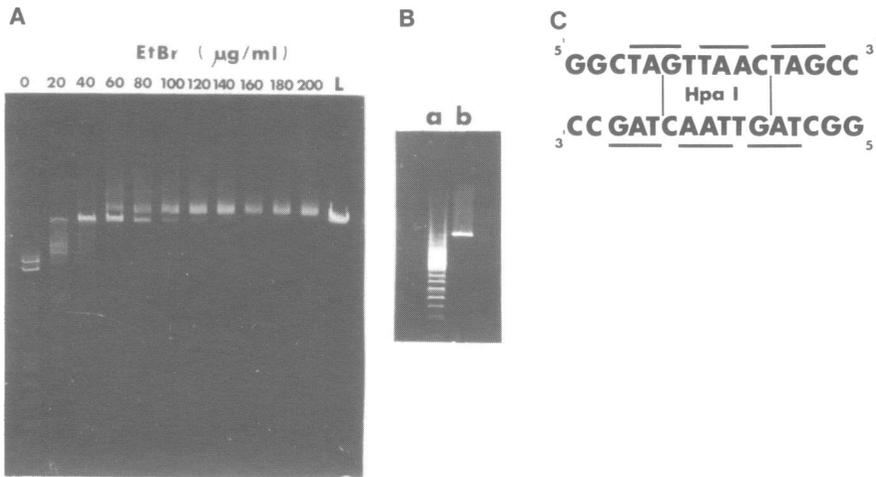


FIGURE 2. Linker insertion mutagenesis of ICP4. The plasmid pK1-2 was digested with 1 u of SmaI/ μ g of pK1-2 in the presence of indicated concentrations of ethidium bromide for 1 h at 37°C. An aliquot of the reaction was run on an agarose gel and compared with linearized (HindIII-digested) pK1-2 (A). As described in the text, linearized (SmaI-digested) molecules were extracted from a preparative gel by electroelution. This material ran as a single band when electrophoresed on a separate gel (B, lane b). Digestion of this material with HindIII resulted in a ladder of bands descending from the position of the full length linear (B, lane a). To the linearized population was ligated the indicated oligonucleotide linker containing nonsense codons (underlined) in all three reading frames (C). The properties of this oligonucleotide are summarized in the text.

tide are: 1) the sequence is self-complementary, therefore only one oligonucleotide need be synthesized to generate an intact double-strand linker, 2) the double-stranded linker contains a HpaI site which is a useful marker for analysis of plasmids containing the oligonucleotide, and 3) the linker contains translational termination codons in all three reading frames (solid lines). Plasmids from individual transformants were analyzed for the presence and location of the introduced HpaI site. A set of clones was chosen for further analysis. Each isolate, labelled pn1 through pn11, contains a nonsense insertion mutation at the indicated location (Fig. 3). pn1 through pn9 and pn11 contain the HpaI linker at the indicated locations. pn10 was constructed by inserting the linker (CTAG)₃ at the indicated

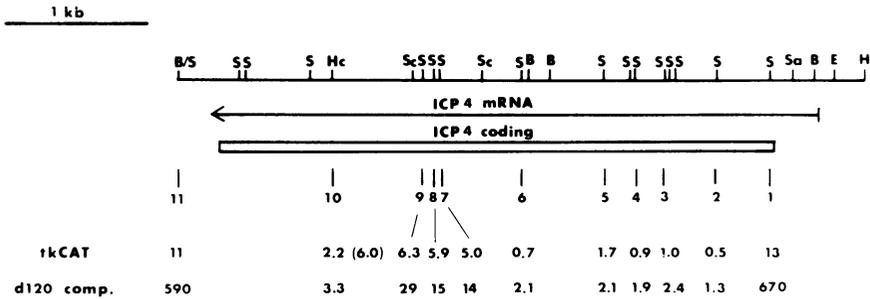


FIGURE 3. Trans-induction of ptkCAT and complementation of d120 by mutagenized pK1-2. Beneath the line depicting restriction sites in pK1-2 (B, BamHI; E, EcoRI, Hc, HincII; H, HindIII; Sc, SacI; Sa, Sau3a; S, SmaI), are shown the locations of the mRNA and coding sequences for ICP4. For cotransfections with ptkCAT and the mutated ICP4 genes, 10^6 CV-1 cells were transfected with 2 μ g of ptkCAT and 1 μ g of a mutated plasmid containing a linker insertion at the indicated position (1-11). Position 11 is outside HSV-1 DNA sequences such that the ICP4 gene in this plasmid is wild-type. To measure the uninduced level of tkCAT expression, 1 μ g of pUC8 was included in place of the ICP4-encoding plasmids. All transfections were brought up to 20 μ g with salmon testis DNA. Shown under each linker insertion position for tkCAT induction is the ratio of CAT activity resulting from transfection with the mutant ICP4 plasmid to the CAT activity resulting from transfection with pUC8. The number in parentheses next to the induction value for pn10 represents the induction level for pn10 when an amber suppressor tRNA was added to the transfection (see Fig. 4). For complementation of d120, 10^6 CV-1 cells were transfected with 1 μ g of the indicated plasmid as described in Materials and Methods. Shown is the ratio of d120 yield for the indicated plasmid to the d120 yield for pUC8.

HincII site in a similar manner. This dodecanucleotide is self-complementary, contains a single NheI site and codes for amber termination codons (UAG) in all three reading frames.

Transient activity of ICP4 linker insertion mutants

The activities specified by the linker insertion mutants were determined by two independent methods. First, each of the plasmids encoding a linker insertion mutation was cotransfected with a plasmid encoding the bacterial gene for CAT under the control of the HSV-1 thymidine kinase (tk) (37) promoter, ptkCAT (19). tk is an early gene encoded by HSV-1 and is readily trans-activated by cotransfection with wild-type ICP4 (19,22, 25). Therefore, this experiment assessed the ability of the

ICP4 nonsense peptides to trans-activate an HSV-1 early gene. Second, the ability of cells transfected with each of the mutant plasmids to elevate the yield of an HSV-1 ICP4 deletion mutant, d120 (6), was evaluated in transient complementation tests. These tests compared the relative abilities of the nonsense peptides and wild-type ICP4 to complement the defect in d120.

The values for the induction of tkCAT expression are shown in Fig. 3. The level of induction with isolate pn11 is the same as the level seen with pK1-2 and represents complete induction in this experiment (data not shown). Isolates pn2 through pn6 were unable to trans-activate tkCAT. Isolates pn7 through pn9 were able to induce tkCAT expression to one half the wild-type level. This indicates that the nucleotide sequence defined by the region between pn6 and pn7 encodes amino acids which contribute significantly to the trans-inducing activity of ICP4. Upon the addition of the amino acid sequence between pn9 and pn10, trans-inducing activity was reduced. Therefore, while pn10 contains the primary amino-acid sequence sufficient for tkCAT induction, the added sequence between pn9 and pn10 must interfere with the inducing activity.

An unexpected result was obtained from cotransfection with ptkCAT and isolate pn1. This transfection results in tkCAT induction similar to that of the wild-type gene. Several independent isolates of pn1 behaved in a similar manner (data not shown). pn1 contains a HpaI insertion mutation at amino acid 12 of the ICP4 nucleotide sequence of McGeoch et al. (10). One plausible explanation for the observed activity is translation restart at a suitable downstream methionine codon. The next methionine codon is 72 amino acids downstream of the introduced stop codon in pn1. Isolate pn2 contains the introduced stop codon after the second methionine codon in ICP4 and exhibits no activity. Experiments in the next section will deal with a more detailed description of the pn1 ICP4 polypeptide.

The polypeptide specified by pn1 is as efficient as pn11 (which encodes the wild-type ICP4 protein) in the complementation of d120. pn2 through pn6 fail to complement d120. This is consistent with the inability of the plasmids to trans-activate tkCAT. Cells transfected with pn7 through pn9 were able to com-

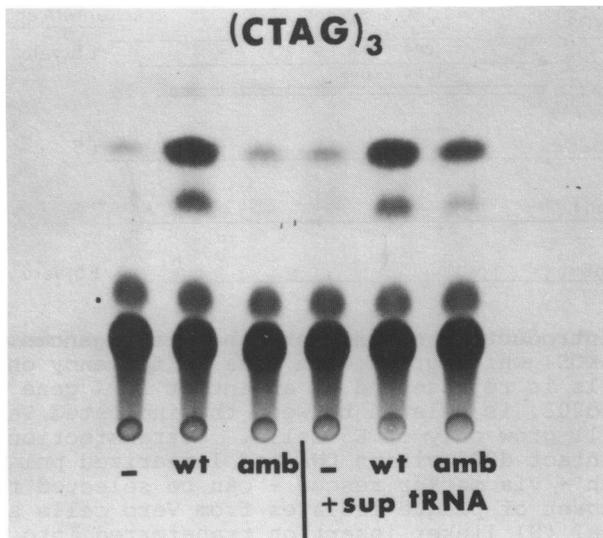


FIGURE 4. Nonsense suppression of pn10. The plasmid pn10 contains the synthetic oligonucleotide $(CTAG)_3$ at the position indicated in Fig. 3. CV-1 cells were transfected with ptkCAT and pUC8 (-), pn11 (wt), or pn10 (amb) in an identical manner to that described in Fig. 3 except that where indicated, 1 μ g of a plasmid encoding a human tRNA suppressor gene was included in the transfection. Shown are the ascending autoradiographic images of chromatographically separated input chloramphenicol (lower image) and the acetylated products formed by the CAT enzyme expressed from ptkCAT.

plement d120 to a small degree. The degree of complementation of d120 by pn7 through pn9 was only 2.3 to 4.9 per cent, respectively, that of pn11. pn7 through pn9, however, were able to trans-activate tkCAT to 45 to 57 per cent, respectively, that of pn11. Therefore, while pn7 through pn9 have substantial ptkCAT trans-inducing activity, the ability of these plasmids to complement d120 was marginal by comparison. The plasmid pn10 behaves in complementation tests in a manner similar to what one would expect from its ability to trans-activate tkCAT.

The ICP4 gene specified by pn10 contains the linker $(CTAG)_3$ - and thus amber codons in all three reading frames - at the position indicated in Fig. 3. If a plasmid specifying a human amber suppressor tRNA is included in the transfection with pn10 and tkCAT, suppression of the pn10 phenotype is observed (Fig.

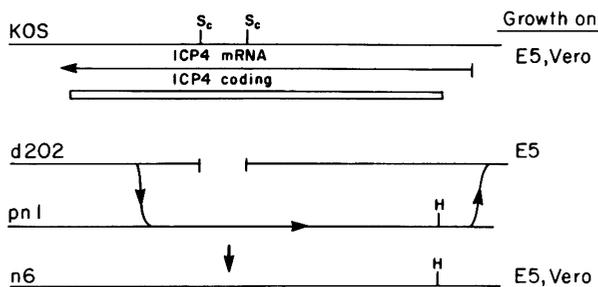


FIGURE 5. Introduction of *pnl* into the viral genome. The wild-type virus (KOS) which grows with equal efficiency on both E5 and Vero cells is represented by an intact ICP4 gene (top line). The mutant, d202, is deleted between the indicated SacI (*Sc*) sites and will grow only on E5 cells. Cotransfection of E5 cells with intact d202 virion DNA and linearized *pnl* results in progeny which - via marker rescue - can be selected for on Vero cells. A subset of plaque isolates from Vero cells also contains the HpaI (*H*) linker insertion transferred into the viral genome by *pnl*. One such virus isolate, n6, can be propagated on either E5 or Vero cells.

4). Suppression results in CAT activity on the order of 50 to 60 per cent that of wild-type. The less than full activity can either be due to incomplete suppression by the tRNA, and/or the insertion of the sequence leu-ala-ser-ser due to suppression and translation of the nucleotide sequence (CTAG)₃. There is no effect of the suppressor on either uninduced or wild-type ICP4-induced CAT expression (Fig. 4).

Transfer of *pnl* to the viral genome

pnl contains a HpaI insertion and therefore a nonsense mutation at amino acid 12 of the ICP4 polypeptide. In order to gain more insight into the underlying mechanism by which *pnl* specifies a completely active ICP4 polypeptide, the mutation in *pnl* was transferred into the genome of the virus. Because *pnl* was as active as *pnl1* in the assays described in Fig. 3, it was assumed that incorporation of the *pnl* allele into the viral genome would result in a viable virus. The strategy for transfer of the *pnl* mutation is depicted in Fig. 5. Linearized *pnl* was coprecipitated with intact d202 DNA. d202 is an HSV-1 mutant with a 0.5 kb deletion in both copies of the ICP4 gene. Because d202 can grow only on ICP4-transformed cells, the precipitate

Table 1. Growth and Plating Efficiency of KOS and Mutant n6 on Vero and ICP4-Transformed Cells^a

	PFU/ml when grown in					
	E5			Vero		
	E5	Vero	EOP (Vero/E5)	E5	Vero	EOP (Vero/E5)
KOS	3.1×10^8	2.8×10^8	(0.9)	2.4×10^8	2.5×10^8	(1.1)
n6	1.1×10^8	7.9×10^7	(0.7)	5.2×10^7	3.6×10^7	(0.7)

^a E5 or Vero cells were infected with the indicated virus at an m.o.i. of 5 PFU/cell. At 18 h post-infection, infected monolayers were harvested and assayed for virus able to produce plaques on E5 and Vero cells.

was applied to E5 cells. Thirty-six hours after infection, the progeny of transfection were harvested and plated on both E5 cells and Vero cells. The titer on Vero cells was approximately 5% that of the titer on E5 cells. Isolates picked from Vero cell monolayers were able to plaque on both cell types with equal efficiency. A number of plaques isolated from Vero cells were plaque-purified, amplified in E5 cells, and analyzed by restriction enzyme analysis. Some isolates contained the introduced HpaI site near the 5' ends of both ICP4 genes and also lacked the d202 deletions. One such isolate designated n6 was retained for further analysis. n6 grows and plaques nearly as efficiently on Vero cells as it does on E5 cells (Table 1). The presence of the HpaI site in n6 DNA is shown in Fig. 6A. The HpaI terminal C (15.8 kb) and G (10.5) fragments are interrupted by the inserted HpaI linker giving a 5 kb one-molar terminal fragment and two unique fragments of 5.5 kb and 10.8 kb.

To examine the immediate-early polypeptides synthesized by n6, cycloheximide-treated cells were infected with either KOS or n6 in the presence of cycloheximide. At 6 hr post-infection the cycloheximide was removed and incubation was continued in the presence of actinomycin D and ³⁵S-methionine. Under these conditions only immediate-early polypeptides and ICP6 are synthesized (Fig. 6B). The ICP4 synthesized in KOS-infected cells has an apparent molecular weight of 170 kd under these conditions. Although it contains a nonsense mutation at amino acid 12, n6 synthesized an ICP4 polypeptide with an apparent molecu-

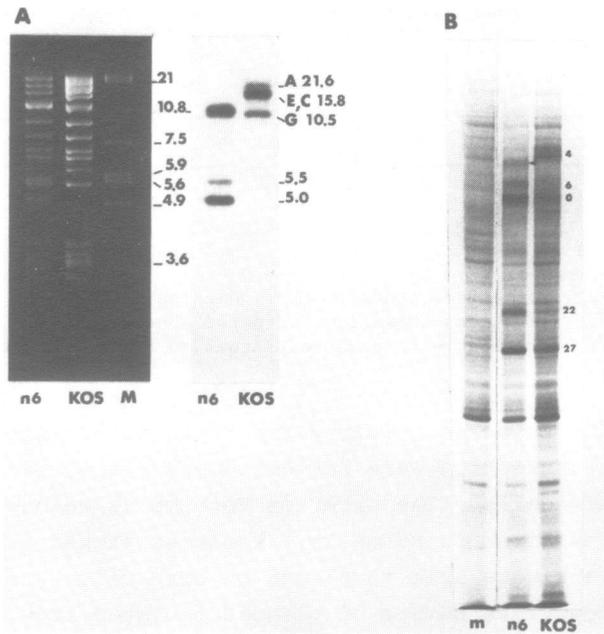


FIGURE 6. Location of the HpaI linker insertion in n6 (A) and consequence of this mutation on immediate-early polypeptide synthesis (B). KOS and n6 DNAs were digested with HpaI and electrophoresed on a 0.7% agarose gel adjacent to EcoRI digested λ DNA as marker (M). The ethidium bromide-stained fragments (left panel) were transferred to nitrocellulose and probed with ³²P labelled pK1-2 (see Fig. 1, right panel). Relevant sizes of DNA fragments in kilobases are indicated. The electrophoretically separated immediate-early polypeptides synthesized in n6 and KOS infected cells (B) were visualized as described in the text. The mobilities of the immediate-early polypeptides on a 9% polyacrylamide gel are indicated by ICP number.

lar weight of 160 kd. As will be discussed later, this is consistent with translational restart at amino acid 90.

Regulation of immediate-early promoters by wild-type and non-sense ICP4 polypeptides

In transient assays and probably during the course of viral infection, immediate-early promoters are regulated in a complex manner. Expression from immediate-early promoters is stimulated by a virion component, Vmw65 (38-40), and subsequently repressed as infection proceeds. The repression of immediate-early gene expression, notably ICP4, is thought to be a direct func-

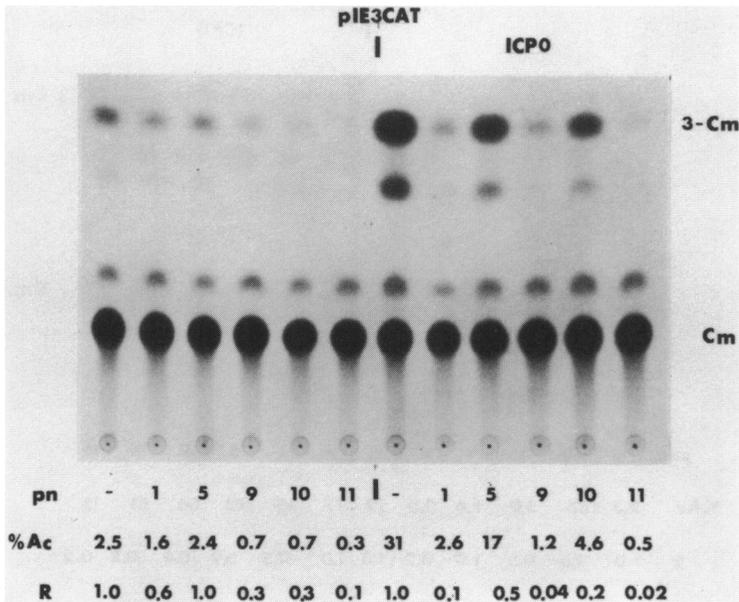


FIGURE 7. Effect of ICP4 nonsense peptides on IE3CAT expression. Two micrograms of pIE3CAT were coprecipitated with either 1 μ g of pUC8 (-) or 1 μ g of the indicated mutant ICP4 plasmid (see Fig. 3 for reference). Similar coprecipitates were also prepared in which 1 μ g of pW3- Δ H58 (ICP0) was included. All other manipulations are identical to those described in Fig. 3. Above the autoradiographic images is indicated the absence (left) or presence (right) of the ICP0 gene. Below the autoradiograph is shown the ICP4 nonsense plasmid (pn) included in the coprecipitation. The CAT activity as measured by the percent of the total 14 C present in the 3-Ac chloramphenicol spot (% Ac) and the ratio of the CAT activity for a given ICP4 plasmid to the CAT activity in the appropriate control (R) are shown at the bottom of the figure.

tion of the ICP4 polypeptide itself (4,5). In transient expression assays, cloned wild-type ICP4 is sufficient to marginally repress expression of a test gene under the control of the ICP4 regulatory sequences (19,26). ICP0, the product of another immediate-early gene, very efficiently stimulates expression from test genes fused to the ICP4 regulatory sequences (26). In transfection experiments with such test genes and both ICP4 and ICP0 simultaneously, the inhibitory effect of ICP4 is dominant to the stimulatory effect of ICP0 (26). Expression from the

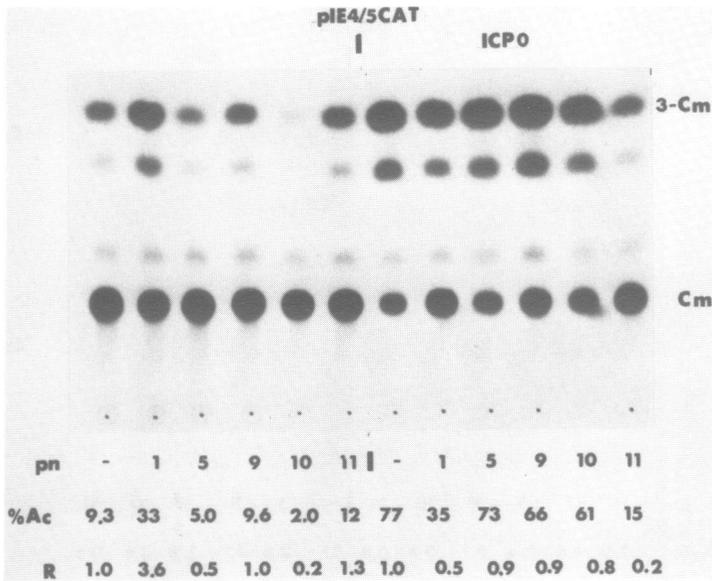


FIGURE 8. Effect of ICP4 nonsense peptides on IE4/5 CAT expression. This experiment was performed and represented in a manner identical to that described in Fig. 7 except pIE4/5CAT was substituted for pIE3CAT.

immediate-early regulatory sequences for ICP22/47 fused to a test gene can be stimulated by cotransfection with either ICP4 or ICP0 (19,38).

In the following experiments, the effect of wild-type and nonsense ICP4 peptides on ICP4-CAT (pIE3CAT) and ICP22/47-CAT (pIE4/5CAT) expression was determined in the presence and absence of ICP0. The linker insertion mutants tested in this series of experiments were pn1, pn5, pn9, pn10 and pn11. pn11 represents the unaltered or wild-type ICP4. The results for pIE3CAT cotransfected with the various ICP4 alleles with and without ICP0 are shown in Fig. 7. The only nonsense peptides tested in this experiment which were inefficient in the repression of IE3CAT expression in the presence of ICP0 were pn5 and pn10. Interestingly, the pattern of repression of pIE3CAT in the presence of ICP0 follows the same pattern as that seen for the stimulation of pTKCAT (Fig. 3).

When pIE4/5 CAT was tested in a similar experiment, the

results shown in Fig. 8 were obtained. As demonstrated previously (19,38), expression from this immediate-early promoter element is marginally stimulated by the presence of wild-type ICP4 (pn11). The ICP4 expressed from pn1 was also capable of stimulating pIE4/5CAT. None of the other nonsense plasmids tested demonstrated significant stimulatory effects on pIE4/5CAT. On the contrary, pn5 and pn10 (the latter to a greater degree than the former), repressed pIE4/5CAT activity relative to the constitutive level. It is possible that the peptide produced by pn10 is inhibitory to IE4/5CAT expression. This may also explain the relatively low level of tkCAT expression with pn10 (Fig. 3). ICP0 increased the level of pIE4/5CAT expression 11-fold above the constitutive level. This increase was only marginally affected by any of the ICP4 alleles. Both pn1 (slightly) and pn11 (more dramatically) reduced the level of ICP0-induced pIE4/5CAT activity. Notably, pn9 did not behave similarly to pn1 and pn11 as it did in the previous experiment with pIE3Cat (Fig. 7). The results shown in Figs. 7 and 8 suggest that while ICP0 stimulates gene expression from both the ICP4 and ICP22/47 promoters, ICP4 may act differently on these promoter elements.

DISCUSSION

ICP4 is a complex, multifunctional protein. In order to define regions of ICP4 which contribute to the functions of the molecule, varying amounts of primary amino acid sequence were excluded from the carboxy-terminus by the introduction of translational termination codons. The nonsense mutations were generated by the insertion of synthetic oligonucleotide linkers containing nonsense codons in all three reading frames. The plasmids containing the linker insertions were tested in transient assays for the ability to stimulate expression from the early HSV-1 thymidine kinase promoter, affect expression from the immediate-early promoters from ICP4 and ICP22/47, and supply complementing levels and activities of ICP4 during the course of viral infection.

The amino-terminal 60% of the ICP4 molecule is sufficient for substantial induction of the early tk promoter (Fig. 3).

Other investigators have shown that a clone which contains a similar region of the ICP4 coding sequence can activate the early tk promoter (22). In that experiment, however, the termination of the peptide produced was not clearly defined by a translation termination codon. The experiments conducted in the present study extend this observation and also demonstrate that the amino terminal 60% of ICP4 is also sufficient for repression of expression from the ICP4 promoter (Fig. 7). The stimulation of early gene expression and the repression of ICP4 expression have been shown genetically, through the use of ts and deletion mutants in ICP4, to be activities of the ICP4 molecule which presumably are required for viral infection. The experiments in this study demonstrate that these activities of ICP4 alone may not be sufficient for infectivity. The insertion mutant plasmids pn7, pn8, and pn9 which exhibit both of these activities do not efficiently complement d120 (Fig. 3). Moreover, increasing or decreasing the quantity of plasmid DNA in the transfection did not appreciably alter the level of complementation seen with pn7-pn9 in comparison to pn11 (data not shown). The 3 to 5 percent complementing activity of these alleles, in fact, represents the highest level of complementation observed in these tests.

The contribution of the carboxy-terminal 40 per cent of the ICP4 molecule to its functions during productive infection cannot be determined from the experiments conducted in this study. This portion of the molecule, however, does appear to be required to decrease expression from the ICP22/47 promoter in the presence of ICP0. At present, the role of repression of immediate-early genes in the cascade of viral gene expression is not well understood. It may be that exaggerated expression of immediate-early genes may interfere with subsequent stages in the viral life cycle, hence the requirement that their expression be repressed.

An interesting observation from these studies was that when the amino-terminal 80 per cent of the ICP4 molecule was expressed (pn10), both the ability to stimulate tkCAT and repress IE3-CAT expression were reduced relative to that of pn7, pn8, and pn9 (Figs. 3 and 7). Apparently, the addition of these amino

acids in the absence of the carboxy terminus interferes with the activities which are intrinsic to the rest of the molecule. That the defect in pn10 is due solely to the inserted nonsense mutation and not some other unseen alteration is evident from the ability to suppress the nonsense mutation with a human amber suppressor tRNA. In addition, temperature-sensitive mutations in this region of the molecule (5) interfere with the transport of ICP4 to the nucleus (7). Consistent with these observations is the striking failure of the great majority of the temperature-sensitive mutants which map to this region to induce early gene expression. A plausible explanation for these observations is that this portion of the ICP4 molecule is involved in modulating ICP4 activity by directing its own localization - and possibly that of other viral or cellular proteins - to specific sites of viral macromolecular synthesis during viral replication. In addition, because ICP4 and ICP8 co-localize during the infectious cycle (13), there exists the possibility that ICP4 may in some way be involved in viral DNA synthesis. ICP8 is the major DNA binding protein of HSV-1 and is absolutely required for DNA synthesis and infectivity (28,41). It has been shown that the transcriptional activator of adenovirus, E1A, may itself play a role in DNA synthesis (42). It is therefore of interest to note that ICP4 can complement the deficiency in adenovirus E1A⁻ mutants (43).

An unexpected observation made in this study is that insertion of nonsense mutations near the amino terminus, specifically at amino acid 12, did not inactivate ICP4. The available data shown in Fig. 6B are consistent with translational restart at the next in-frame methionine downstream from the site of linker insertion. The next in-frame methionine is 78 amino acids downstream from the site of linker insertion (10). This is consistent with the mobility of the n6 polypeptide seen in Fig. 6B, and with the fact that this peptide reacts with anti-ICP4 antibodies in Western blot analysis (data not shown). The nucleotide sequence surrounding the methionine codon at amino acid 90 (TCCATGG) differs from the optimal consensus sequence (ACCATGG) for ribosome utilization (44) by one nucleotide. The sequence surrounding the wild-type initiation methionine codon differs

from the optimal consensus by one nucleotide (GCCATGG). The virus n6 grows on both Vero cells and cells expressing complementing quantities of ICP4. Therefore, the first 90 amino acids of ICP4 are not required for viral growth. The viability of a previously described mutant containing a 96 amino acid deletion near the amino terminus of ICP4 is consistent with this conclusion (45).

The amino-terminal 60% of the ICP4 molecule is sufficient for the stimulation of expression from the tk promoter and possibly other early and late viral promoters. It is also sufficient for repression of expression from the ICP4 promoter. Shorter peptides (i.e., pn6) elicit neither of these activities. Several explanations for these observations are plausible: 1) that the nucleotide sequence between pn6 and pn7 encodes amino acids which directly contribute to both of these activities, assuming they are separate activities. However, it remains possible that the stimulation of early gene expression and the repression of ICP4 expression are events related to a single intrinsic activity of ICP4. Two previous observations argue against the one-activity hypothesis. Temperature-sensitive mutants of ICP4 exist which simultaneously overproduce ICP4 and express thymidine kinase and other early proteins (32). Specific DNA binding sites for ICP4 have been identified near the transcriptional start site of ICP4 mRNA (46). This site is not present near the start of the HSV-1 thymidine kinase mRNA, and ICP4 does not complex with the tk promoter (15). In addition there does not appear to be an induction-specific sequence in the tk promoter (47). Therefore, the DNA-binding activity observed in these studies may be related to the attenuation of ICP4 transcription rather than stimulation of early gene expression. To date, the relationship between DNA-binding at the ICP4 mRNA start site and autoregulation has yet to be demonstrated. 2) An amino acid sequence in the interval between pn6 and pn7 may be involved in a limiting step required for the function of ICP4. An example of such a step would be the transport of the molecule to its intracellular site of action. In the case of transcriptional regulation, this most probably would correspond to nuclear or subnuclear localization. It is notable that the

interval between amino acids 640 (pn6) and 773 (pn7) of HSV-1 contains very little homology with the analogous 140 kd VZV protein (10). There is a small region of homology, however, centered on amino acid 728 which is conserved in the HSV-1 and VZV proteins. This sequence is pro - arg⁺ - glu⁻ - gly - arg⁺ - lys⁺ - arg⁺ - lys⁺ - ser - pro (underlined amino acids in HSV sequence are conserved in the ICP4 analog of VZV), and is quite similar to the nuclear localization signal in SV40 large T antigen, pro - pro - lys⁺ - lys⁺ - arg⁺ - lys⁺ - val (48). The intracellular localization of selected mutant peptides is currently being examined in mutant viruses containing selected linker insertion mutations.

The availability of mutant viruses containing nonsense mutations in ICP4 will be useful in elucidating the biochemical basis for the observations made herein. Some of the functions of ICP4 which can easily be studied during viral infection include DNA-binding, phosphorylation and processing, transport of ICP4 to the nucleus, as well as expression of the viral genome in general.

ACKNOWLEDGEMENTS

We thank M. Polvino-Bodnar, W. Sacks, and C. Smith for helpful discussions and reviewing of the manuscript. We also thank M. Cook for manuscript preparation. This work was supported by Public Health Service Grant CA20260 from the National Cancer Institute. N.A.D. was the recipient of Public Health Service postdoctoral fellowship AI06790 from the National Institute of Allergy and Infectious Diseases.

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