Herpes Simplex Virus Type 2 Mutants with Deletions in the Intergenic Region between ICP4 and ICP22/47: Identification of Nonessential *cis*-Acting Elements in the Context of the Viral Genome

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In herpes simplex virus type 2, the mRNAs of ICP4 and ICP22/47 are divergently transcribed and their transcription initiation sites are separated by 750 base pairs (L. J. Whitton and J. B. Clements, *Nucleic Acids Res.* 12:2061-2078, 1984). This 750-base-pair region contains many recognized *cis*-acting elements, including two TATA boxes, numerous Sp1-binding sites, four TAATGARAT motifs, at least one ICP4-binding site, and two origins of replication (*oriS*) linked in tandem. In this report, we describe the construction of mutant viruses with defined deletions that eliminate these elements either singly or in combination. The phenotypic properties of these mutants indicate that (i) the TAATGARAT motifs and their neighboring elements affect the levels of transcription of both ICP4 and ICP22/47 similarly, (ii) the TATA box serving ICP4 is required for efficient ICP4 mRNA synthesis and for determining the initiation site of transcription, (iii) the ICP4-binding site located at the start of ICP4 transcription is at least partially responsible for the decreased levels of ICP4 mRNA observed in the presence of immediate-early and early gene products, and (iv) mutants bearing deletions that eliminate the entire conventionally recognized ICP4 promoter generate sufficient ICP4 mRNA to maintain viability in cells not expressing ICP4. Additionally, our inability to generate viable deletion mutants lacking all copies of *oriS* suggests that at least one copy of *oriS* may be essential for virus replication.

The immediate-early genes of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) encode phosphoproteins ICP0, ICP4, ICP22, ICP27, and ICP47. Immediate-early genes are the first viral genes to be transcribed during the course of infection and are maximally expressed in the absence of prior viral protein synthesis. Of the five immediate-early proteins, only ICP4 and ICP27 are essential for virus replication in cell culture (13, 47, 50). Expression of immediate-early proteins is required for expression of the early and late classes of proteins which function in viral DNA replication and are virion structural components, respectively.

ICP4 is a regulatory protein that acts to repress transcription of immediate-early genes and activate transcription of early and late genes (9, 12-14, 47, 48, 52). ICP4 binds specifically to sequences in the promoters of immediate-early, early, and late genes (12, 15, 16, 24, 25, 36, 37; N. DeLuca, personal communication), suggesting that DNA binding may be necessary for ICP4 to mediate its regulatory effects. In support of this suggestion, DeLuca and Schaffer (12) have shown that truncated ICP4 proteins which fail to bind the ICP4 promoter also fail to down regulate ICP4 transcription.

Like ICP4, ICP27 is an essential regulatory protein. ICP27 deletion mutants are characterized by overexpression of early genes, less than wild-type levels of late-gene transcription, and deficiencies in viral DNA synthesis (34). The role of ICP27 in viral DNA synthesis is apparently distinct from its role in late-gene expression, as demonstrated by the existence of an ICP27 temperature-sensitive mutant (*tsY46*)

that fails to induce wild-type levels of late-gene expression but permits synthesis of wild-type levels of viral DNA (50).

ICP0 is a potent transcriptional activator of all three classes of HSV genes, as demonstrated in transient expression assays (2, 10, 18, 33, 39, 48, 51, 52). Interestingly, however, phenotypic analysis of deletion mutants has shown that ICP0 is not absolutely required for virus replication *in vitro* or *in vivo* (51, 54). Moreover, despite its demonstrated transactivating ability, ICP0 is apparently unable to transactivate genes in the context of the viral genome in the absence of ICP4. This was shown by the demonstration that HSV mutants with deletions of both copies of the ICP4 gene do not express early or late genes, despite the fact that these mutants retain both intact copies of the ICP0 gene (9). At present it is unclear whether ICP0 and ICP27 mediate their regulatory activities directly through binding to *cis*-acting elements in viral promoters as ICP4 does or indirectly through binding to other viral and cellular proteins. The roles of the other two immediate-early proteins, ICP22 and ICP47, have not yet been determined, although deletion mutants in both genes are replication competent in cell culture (28, 32, 42; C. Bogard, personal communication).

Although much has been learned about the roles of immediate-early *trans*-acting proteins in the regulation of HSV gene expression, much remains to be established about the functions of individual *cis*-acting elements in immediate-early promoters in this process. To assess the roles of specific DNA sequences in regulation in the background of the viral genome, we have undertaken deletion analysis of the intergenic region between ICP4 and ICP22/47 of HSV-2 (Fig. 1). The analogous region of the HSV-1 genome is organized in a similar manner (61), and much of what we know about HSV *cis*-acting elements comes from studies of

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HSV-1. The region analyzed contains several *cis*-acting elements which have been identified and characterized by using transient expression assays. First, the region contains two tandem copies of an origin of viral DNA replication, designated *oriS*. *oriS* drives the replication of viral DNA in *cis* when the appropriate HSV-encoded *trans*-acting proteins are present (62). Second, this region contains four homologs of the consensus sequence TAATGARAT (R = purine). One or more copies of this sequence are found exclusively in the promoters of all immediate-early genes (61). They function to upregulate transcription in response to a constituent of the virus particle variously designated VP16, ICP25, Vmw65, or α -TIF (35, 41, 61). The TAATGARAT elements bind VP16 in association with host proteins and thus mediate a stimulatory effect on transcription in a distance- and orientation-independent manner (6, 8, 17, 23, 26, 30, 31, 38, 40, 44–46, 55, 56). Third, Sp1-binding GC boxes (4, 21, 61) are also found throughout this region. In addition to their ability to confer higher levels of constitutive transcription, these GC boxes appear to augment the ability of certain TAATGARAT elements to respond to VP16 (44, 56). Fourth, the ICP4 protein itself binds strongly to the initiation site of ICP4 transcription (12, 16, 24, 25, 37). It also binds upstream sequences but with less affinity (16, 24, 25).

Because the roles of these *cis*-acting elements have been investigated primarily in transient expression assays, we undertook the task of assessing their functions in the context of the viral chromosome. We were motivated by the concern that transient expression assays have several shortcomings, especially when used to study complex *cis*-acting regulatory units in viruses. First, they do not tell us whether a particular element is essential for virus replication. Second, it is often difficult to determine physiologically relevant ratios of the effector molecule to target gene when using transient expression assays. This issue was of special concern in the study of VP16-mediated induction of immediate-early genes. Thus, Gelman and Silverstein (19) noted that a TAATGARAT-less test gene was *trans*-activated in the presence of high intracellular concentrations of VP16. It is important to determine whether such a substrate is *trans*-activated in the context of the viral genome during natural infection, i.e., under conditions where the stoichiometry of template to VP16 is physiological. Third, transient assays do not reflect gene expression in the presence of the full complement of viral *trans*-activating factors which likely modify both the viral genome and the intracellular environment. Finally, viral chromosomes exhibit unique features not characteristic of DNA introduced into cells by transfection. For instance, although transfected plasmid DNA is assembled into nucleosomes, HSV chromosomes are not (7, 27).

Accordingly, we have generated HSV-2 deletion mutant viruses which lack specific *cis*-acting landmarks in the intergenic region between ICP4 and ICP22/47. We constructed a series of deletions in a cloned copy of this region as the first step in generating these mutants. We next determined which of these deletions could be transferred correctly into the HSV-2 genome without abrogating ICP4 expression or other aspects of the viral replicative cycle essential for viability. Mutants that had correctly acquired the intended deletion were then characterized with respect to the levels of ICP4 and ICP47 mRNAs they induced. In addition, the 5' ends of the ICP4 mRNAs generated by the mutants were mapped to determine whether the deletions affected the site of transcription initiation.

MATERIALS AND METHODS

Viruses and cells. The wild-type strain of HSV-2 used in this study was strain 186 (49). Mutant *hr259* is an HSV-2 host range mutant containing a 0.6-kilobase deletion in both copies of the ICP4 gene (53). *hr259* was propagated and assayed in ICP4-expressing n-33 cells (53). Vero cells and n-33 cells were grown and maintained as previously described (58).

Plasmids. The plasmids used in this study are illustrated in Fig. 1. Plasmids p Δ 1 through p Δ 11 were generated by BAL 31 nuclease digestion. pBal4 (53; Fig. 1) was cleaved with *Hind*III, digested with BAL 31 nuclease, and ligated with the *Hind*III linker 5'-CCAAGCTTGG-3'. pBal2-BglII, a derivative of pPst (53) whose *Hind*III site was modified to form a *Bgl*II site, was cleaved with *Nru*I, digested with BAL 31 nuclease, and ligated with the *Hind*III linker described above. Sequencing was then carried out on the resulting products to determine their associated deletion endpoints. To generate p Δ 1, p Δ 2, p Δ 3, p Δ 4, p Δ 6, p Δ 7, and p Δ 11, appropriate *Hind*III-*Pst*I fragments from derivatives of the two BAL 31 digestions were ligated together. For p Δ 5, p Δ 8, p Δ 9, and p Δ 10, this could not be accomplished because BAL 31 nuclease digestion had removed the *Pst*I site in the pBal2-BglII derivatives with the desired right-hand endpoints (relative to the expanded portion of Fig. 1). In these cases, the pBal2-BglII derivatives were cleaved with *Bgl*II and *Hind*III, and the desired *Bgl*II-*Hind*III fragments were ligated to pUC8 that had been digested with *Bam*HI and *Pst*I together with the indicated *Hind*III-*Pst*I fragments taken from pBal4 derivatives.

To generate p Δ 12, the 730-base-pair (bp) *Pst*I-*Bam*HI fragment of *Bam*HI a' (60) was modified to generate a *Hind*III-*Bam*HI fragment which was ligated to the indicated *Hind*III-*Pst*I fragment of p Δ 8 together with pUC8 which had been digested with *Bam*HI and *Pst*I.

p Δ 13 was generated in the following way. The 670-bp *Bst*EII fragment of *Bam*HI a' (60) was modified with *Pst*I linkers and cloned into the *Pst*I site of pUC8 in the appropriate orientation. The fragment was then excised as a *Bam*HI-*Hind*III piece and ligated, together with the indicated *Hind*III-*Pst*I fragment of p Δ 8, to pUC8 which had been doubly digested with *Bam*HI and *Pst*I.

Sequencing. DNA sequencing was carried out by the Sequenase system (United States Biochemical Corporation, Cleveland, Ohio). To sequence the right-hand deletion endpoints of the pBal2-BglII derivatives, the appropriate *Hind*III-BglII fragments were cloned into M13mp18 and sequenced starting from the *Hind*III site. To sequence the left-hand deletion endpoints of the pBal4 derivatives, the appropriate *Hind*III-*Bam*HI fragments were cloned into M13mp18 and sequenced starting from the *Hind*III site. To sequence the right-hand deletion endpoint of *hr259*, the appropriate *Bgl*II-*Hind*III fragment from p Δ 1Bal-4 (53) was cloned into M13mp19 and sequenced starting from the *Bgl*II site.

The clones used in this study were derived from HSV-2 strain 186 DNA. These clones exhibited only minor strain-specific sequence polymorphisms relative to HSV-2 strain HG52 DNA (61). These differences were detected primarily in sequences specifying the 5'-untranslated leader of the ICP4 mRNA and consist exclusively of minor deletions and insertions.

Marker transfer. *hr259* lacks the transcriptional start site and 5'-coding sequences of both copies of ICP4 and synthesizes no detectable ICP4 (53). Introduction of plasmid-borne

FIG. 1. Intergenic region between ICP4 and ICP22/47 of HSV-2. A diagram of the structural organization of HSV-2 DNA is shown at the top of the figure. The locations of the transcripts specifying the five immediate-early proteins (ICP0, ICP4, ICP22, ICP27, and ICP47) are shown in this diagram, as are the limits of the *HindIII* K, L, and M fragments and the *BamHI* m', a', and z fragments. Beneath the diagram of the genome is an expanded diagram of the intergenic region under study showing the locations of pertinent restriction sites, Sp1-binding sites (61), the ICP4 in-phase start codons (ATG), TATA boxes, TAATGARAT elements, and two copies of *oriS*. The elements designated *oriS* in this figure correspond to the direct repeats, DR1 and DR2, of Whitton and Clements (61). The locations of the start sites for the ICP4 and ICP22/47 transcripts are also shown. The HSV DNA sequences in each plasmid used in these studies are shown in the lower portion of the figure. The numbers represent nucleotide positions relative to the *BamHI* site common to the *BamHI* m' and a' fragments (61) shown on the far left of the diagram. Although not shown, note that a *HindIII* linker exists between the deletion endpoints of plasmids pΔ3 through pΔ13.

mutations into the viral genome was accomplished by a procedure involving simultaneous rescue of the host range phenotype of *hr259* and transfer of the engineered deletion into the viral chromosome. This procedure was performed essentially as described by DeLuca and Schaffer (11). Plasmids pΔ1 through pΔ13 were linearized with *PstI* and individually coprecipitated with infectious *hr259* DNA. The coprecipitate was added to n-33 cells, and the monolayers were then treated with glycerol (10) and harvested when high levels of cytopathic effect were observed. Recombinant viruses able to produce plaques in Vero cells were picked and amplified.

Southern blot analysis. Southern blot hybridization was carried out as described previously (53). The probes used in these studies are described in the figure legends.

S1 analysis. S1 analysis was carried out essentially as described by Whitton and Clements (61). To map the 5' ends of the ICP4 mRNAs produced by the deletion mutants, the corresponding deletion plasmids were cleaved with *BamHI*, end labeled with T4 polynucleotide kinase and [γ - 32 P]ATP, and digested with either *BglII* or *EcoRI*, depending on the manner in which the plasmid was constructed. The appropriate *BamHI*-*BglII* or *BamHI*-*EcoRI* fragments were then isolated and used as probes. To map the 5' ends of the ICP4 mRNAs produced by wild-type HSV-2, the 715-bp *BamHI*-*HindIII* fragment of pBal4 was used (53). To generate a probe for the quantitation of ICP4 mRNA, the 330-bp

BamHI-*BglII* fragment of pΔ14 (53) was modified to form a *BamHI*-*HindIII* fragment and cloned into pUC18. This fragment lies within *BamHI* m' on the viral genome and therefore lies totally within the coding sequences of ICP4. This construct was cleaved with *BamHI*, end labeled, and digested with *PvuII*, which cleaves outside HSV DNA sequences. The resulting 420-bp *BamHI*-*PvuII* fragment, containing both HSV and vector sequences was used as probe (see Fig. 3A).

To generate a probe for quantitation of ICP27 mRNA, the 280-bp *AvaII*-*AvaI* fragment from a plasmid analogous to pGZ72 (59) was modified to form a *HindIII*-*AvaI* fragment such that the original *AvaII* site was reconstructed. This fragment, which lies entirely within the first 50% of the coding sequences of ICP27, was then ligated into pUC18 which had been doubly digested with *HindIII* and *AvaI*. This construct was digested with *AvaII*, end labeled, and then cleaved with *PvuII*, which cleaves exclusively outside HSV sequences. The resulting 461-bp *AvaII*-*PvuII* fragment was used as a probe.

To generate a probe for quantitation of ICP47 mRNA, the 450-bp *BamHI*-*EcoRI* fragment of pB6 (53) was ligated into pUC18, which had been doubly digested with *BamHI* and *EcoRI*. This fragment lies completely within *U_S* and encompasses sequences entirely within the coding sequences for ICP47. This construct was digested with *EcoRI*, end labeled, and then cleaved with *PvuII*, which cuts outside the HSV

fragment. The resulting 540-bp fragment was used as probe (see Fig. 3A).

RESULTS

cis-acting elements deleted in mutant plasmids. In this study, it was our ultimate goal to generate HSV-2 mutant viruses with deletions that eliminate specific *cis*-acting elements in the intergenic region between ICP4 and ICP22/47. As the first step in this process, we constructed the plasmid-borne deletions in HSV DNA sequences diagrammed in Fig. 1. The endpoints of the deletions were confirmed by DNA sequence analysis. Plasmids p Δ 7, p Δ 6, p Δ 1, and p Δ 3 lack one, two, three, and four of the TAATGARAT motifs, respectively. Plasmid p Δ 2 lacks both copies of *oriS*, plasmid p Δ 4 lacks the ICP4 TATA box, and plasmid p Δ 5 lacks the sequences in the immediate vicinity of the ICP4 mRNA start site. This is the region to which HSV-1 ICP4 specifically binds (12, 16, 24, 25, 37). Although not tested directly, it is likely that the analogous site in the HSV-2 ICP4 gene binds HSV-2 ICP4, as evidenced by the fact that the region surrounding the HSV-2 ICP4 mRNA start site is nearly identical to its HSV-1 counterpart (61). The deletion in plasmid p Δ 8 eliminates most of the DNA encoding the 5'-untranslated leader of the ICP4 mRNA, p Δ 9 lacks the ICP4 TATA box as well as flanking sequences that putatively bind Sp1, p Δ 10 lacks the first in-phase start codon of the ICP4 open reading frame, and p Δ 11 lacks sequences located between the first ICP4 start codon and a site immediately to the right of the most distal TAATGARAT element. Plasmids p Δ 12 and p Δ 13 lack the entire recognized ICP4 promoter, both copies of *oriS*, the TATA box, and Sp1-binding sites within the ICP22/47 promoter, as well as various amounts of the 5'-untranslated region of the ICP22/47 genes.

Isolation of mutant viruses. After we generated the desired mutant plasmids, we attempted to introduce the deletion mutations into the viral chromosome. To accomplish this, the deletion plasmids were used individually to rescue the host range phenotype of *hr259* via homologous recombination. We reasoned that for each plasmid, recombinant viruses bearing the corresponding engineered deletion would be recovered, provided incorporation of the deletion did not abrogate ICP4 expression or adversely affect some other element essential for virus replication. We further reasoned that if a particular deletion did interfere with the function of an essential *cis*- or *trans*-acting element, the corresponding plasmid would fail to rescue the host range phenotype of *hr259* or it would do so at a low frequency. Briefly, the rescue procedure involved (i) transfecting the ICP4-expressing cell line, n-33, with infectious *hr259* DNA together with individual deletion plasmids linearized with *Pst*I to allow for homologous recombination, (ii) harvesting the resulting viral progeny, (iii) determining the titer of the progeny on n-33 cells and Vero cells, (iv) picking the rescued recombinants from Vero cells, and (v) determining whether these recombinants had acquired the engineered deletion in the plasmid.

All 13 deletion plasmids (p Δ 1 through p Δ 13) rescued the host range phenotype of *hr259*. It was observed that p Δ 1 through p Δ 11 yielded recombinants at frequencies between 0.2 to 7.2%, a range quite similar to that observed in marker rescue experiments involving a single defined mutation in HSV-2 (53). In contrast, rescue frequencies with plasmids p Δ 12 and p Δ 13 were 10 to 100 times lower (0.01%, p Δ 12; 0.002%, p Δ 13), suggesting that the deletions in these plas-

FIG. 2. Restriction patterns of deletion mutant DNAs cleaved with *Hind*III. Ethidium bromide was used to stain an agarose gel of the indicated mutant viral DNAs digested with *Hind*III. The *Bam*HI m fragment (m) of the host range mutant, *hr259* (53), is shown. Note that the deletion mutant DNAs lack the m fragment and instead each contains three fragments not found in *hr259* DNA.

mids affected either viability or the recombination process in some manner.

Analysis of mutant viral DNAs. Restriction enzyme analysis was conducted on the isolated DNAs of recombinant viruses generated with plasmids p Δ 1, p Δ 2, p Δ 3, p Δ 4, p Δ 5, p Δ 6, p Δ 7, p Δ 8, p Δ 11, p Δ 12, and p Δ 13. The recombinants generated with p Δ 1, p Δ 3, p Δ 4, p Δ 5, p Δ 6, p Δ 7, p Δ 8, and p Δ 11 had acquired the engineered deletions in both copies of the intergenic region between ICP4 and ICP22/47 (Fig. 2 and 3). Figure 2 is an ethidium bromide-stained agarose gel of the indicated viral DNAs digested with *Hind*III. As shown, the recombinant DNAs lacked the *Hind*III terminal fragment m (and presumably k [Fig. 1], although it cannot be discerned in this gel) and instead exhibited three novel fragments not seen in *hr259*. Since k and m each contained one copy of the ICP4 gene and since the deletion plasmids each contained a unique *Hind*III site, it is reasonable to conclude that the recombinants contain the constructed deletions in both IR_S and TR_S. Results of restriction analysis with V Δ 11 are not shown but indicate that this mutant, like the others just mentioned, possesses a unique *Hind*III site in both copies of the ICP4 gene.

Southern blot hybridization of mutant viral DNAs and of the mutant plasmids from which they were derived are shown in Fig. 3B and C. The indicated viral and plasmid DNAs were first cleaved with *Bam*HI and *Hind*III and probed with an isolated fragment spanning nucleotides 0 to 144 (Fig. 3B) (numbers are relative to the *Bam*HI site shared by *Bam*HI a' and *Bam*HI m'; see Fig. 1). As shown, the *Bam*HI-*Hind*III fragments detected in the viral DNAs comigrated with the corresponding fragments from the parental plasmid DNAs, indicating that the deletion plasmids and the corresponding viruses share the same left-hand endpoints (relative to the expanded portion of Fig. 1). The indicated

FIG. 3. Southern blot analysis of VΔ1, VΔ3, VΔ4, VΔ5, VΔ6, VΔ7, VΔ8, and VΔ11 and the mutant plasmids from which they were derived. (A) Locations of the probes used in the Southern blots presented in panels B and C. The pertinent *Bam*HI and *Nru*I restriction sites are shown. Relevant *Hind*III sites are shown in Fig. 1. The numbers beneath the probes indicate nucleotide numbers to the right of the leftmost *Bam*HI site and correspond to the nucleotide numbers that mark the limits of deletions shown in Fig. 1. (B) The indicated plasmids and viral DNAs were cleaved with *Bam*HI and *Hind*III and probed with a fragment spanning nucleotides 0 through 144. (C) Viral DNAs were cleaved with *Hind*III and *Nru*I and probed with a fragment spanning nucleotides 655 through 1042. *Hae*III-digested φX174 DNA was used as markers (numbers on the left).

viral DNAs were cleaved with *Hind*III and *Nru*I and probed with an isolated fragment spanning nucleotides 655 to 1042 (Fig. 3C). As shown, the *Hind*III-*Nru*I fragments detected in the viral DNAs were the sizes expected if the viruses had acquired the engineered deletions. This result suggests that the indicated viral and plasmid DNAs share the same right-hand endpoints. Therefore, as determined by restriction enzyme analysis, the recombinant viruses generated with pΔ1, pΔ3, pΔ4, pΔ5, pΔ6, pΔ7, pΔ8, and pΔ11 had correctly acquired the intended deletions. Confirmation that the deletions contained in mutant viruses are identical to those found in plasmids will necessitate recloning and resequencing of the mutated genes from mutant viruses. Considering the high

FIG. 4. Southern blot analysis of VΔ12 and VΔ13 DNAs. The indicated viral DNAs were cleaved with *Bam*HI and blotted with an isolated fragment spanning nucleotides 928 to 1042, a sequence lying totally within the deletions in pΔ12 and pΔ13 (see Fig. 1). *Bam*HI z and *Bam*HI a' refer to the bands in the lane in which wild-type HSV-2 strain 186 DNA was run.

frequency with which these viruses were derived, however (see above), it is unlikely that they have suffered secondary mutations.

In contrast to these recombinants, those generated with pΔ2, pΔ12, and pΔ13 did not contain the intended deletions. As determined by Southern blot analysis, the pΔ2 recombinants were either wild type or exhibited gross rearrangements or deletions in this region (data not shown). The wild-type recombinants probably arose from crossovers in the 263-bp sequence between the *hr*259 deletion at nucleotide 452 and the deletion in pΔ2 at nucleotide 715 (Fig. 1). The origin of recombinants exhibiting gross rearrangements or deletions is unclear but may reflect the need for at least one copy of HSV DNA sequences between nucleotides 715 and 996 to ensure viability. As for the pΔ2 recombinants, the pΔ12 and pΔ13 recombinants did not contain the intended deletions. These recombinants all contained deletions smaller than those intended and their DNAs hybridized to an isolated fragment spanning nucleotides 928 to 1042, a sequence lying totally within the deletions in pΔ12 and pΔ13 (Fig. 4).

The results obtained with pΔ2, pΔ12, and pΔ13 suggest several possibilities, the most straightforward of which is that they lack a sequence(s) required for virus replication. These plasmids all have in common the fact that the sequence from nucleotides 715 to 996 has been deleted. Therefore, it is reasonable to conclude that this sequence contains one or more elements essential for virus replication. In this regard, it should be noted that *oriS* is located within this sequence and that *oriS*-associated sequences may comprise part of the open reading frame of a newly identified viral gene (22). Alternatively however, pΔ2 has only 46 nucleotides flanking its right-hand deletion endpoint. Such a short region of homology may have decreased the probability of a second crossover event during recombination with *hr*259 DNA, resulting in our inability to generate viruses containing the Δ2 deletion. In contrast to pΔ2, plasmids pΔ12 and pΔ13 contain hundreds of homologous nucleotides flanking

TABLE 1. Growth properties of mutant viruses

Virus	Titer ^a on Vero cells	Titer on n-33 cells	EOP ^b	Yield ^c on Vero cells	Yield on n-33 cells	EOR ^d
186	6.2×10^8	6.5×10^8	0.95	1.0×10^8	1.2×10^8	0.83
hr259	$<1.0 \times 10^3$	1.0×10^8	$<1.0 \times 10^{-5}$	$<1.0 \times 10^4$	2.4×10^7	$<4.2 \times 10^{-4}$
VΔ1	9.0×10^7	1.0×10^8	0.90	5.3×10^7	2.8×10^7	1.90
VΔ3	3.1×10^8	5.0×10^8	0.62	2.1×10^7	3.2×10^7	0.66
VΔ4	2.0×10^8	3.8×10^8	0.53	6.5×10^6	1.2×10^8	0.05
VΔ5	2.6×10^8	3.6×10^8	0.72	6.3×10^7	4.8×10^7	1.31
VΔ6	2.8×10^8	2.1×10^8	1.33	8.7×10^7	1.56×10^8	0.56
VΔ7	3.5×10^8	2.7×10^8	1.30	5.7×10^7	7.8×10^7	0.73
VΔ8	1.0×10^8	1.1×10^8	0.91	5.0×10^7	1.7×10^8	0.29
VΔ11	1.7×10^7	1.6×10^8	0.11	5.8×10^6	5.8×10^7	0.10

^a Titer = PFU of mutant virus stocks per milliliter.

^b EOP, Efficiency of plating = titer on Vero cells/titer on n-33 cells, with virus titer measured by PFU per milliliter.

^c Yield = PFU/milliliter; cells were infected at a multiplicity of infection of 2.5 PFU per cell and harvested at 18 h postinfection.

^d EOR, Efficiency of replication = yield on Vero cells/yield on n-33 cells.

their right-hand deletion endpoints. Consequently, it is unlikely that our inability to generate viruses with the Δ12 or Δ13 deletions reflects infrequent crossover events.

Growth properties of mutants. Although we had selected our mutants for viability, we assessed their growth properties and plating efficiencies in Vero cells relative to those in n-33 cells to determine whether any of the deletions affected the efficiency of virus replication. As expected, none of the mutants exhibited the dramatic host range phenotype of hr259 (Table 1; 53). With regard to plating efficiency, mutants VΔ4 and VΔ11 plated least well on Vero cells relative to n-33 cells of the eight deletion mutants tested. This pattern was also evident in one-step growth curves in which VΔ4, VΔ11, and VΔ8, in that order, replicated less efficiently in Vero cells relative to n-33 cells than the other five mutants. These observations suggest that sequences deleted in VΔ4 and VΔ11 (and perhaps in VΔ8) affect the replication competence of the virus.

Characterization of ICP4 mRNAs generated by mutants. The 5' ends of the ICP4 mRNAs generated by the mutant viruses were then mapped to assess the effects of the deletions on the site of ICP4 mRNA initiation. Vero cells were infected at a multiplicity of 20 PFU per cell, and cytoplasmic RNA was harvested at 6 h postinfection. Cycloheximide (75 μg/ml) was used to prevent protein synthesis and to augment the accumulation of immediate-early transcripts (29).

HSV-2 strain 186 generated transcripts whose 5' ends clustered within a region of about 10 nucleotides between 310 and 320 relative to the BamHI site in Fig. 1 (Fig. 5). It is unclear whether this heterogeneity results from a technical artifact or truly represents individual initiation sites. Strain 186 also appears to generate 5' ends which may map between nucleotides 70 to 73 (Fig. 5). Many of the mutants generate these ends as well. We believe this to be an artifact, since the region immediately surrounding this area is unusually AT-rich (61), a characteristic which may result in transient melting of DNA-RNA hybrids.

The ICP4 mRNAs generated by mutants VΔ1, VΔ3, VΔ6, VΔ7, and VΔ8 produced essentially the same S1 pattern as did the wild-type message. This was not the case for mutants VΔ5 or VΔ4, however. The ICP4 mRNA of VΔ5 initiates just inside the HindIII linker at position 316, and that of mutant VΔ4 lies well downstream of the beginning of the wild-type message, initiating at positions 242 and 244. The result with VΔ4 was predictable in that the deletion in this mutant eliminates the TATA box, a *cis*-acting element which in

many systems appears to determine the location of transcriptional initiation (3).

By using the 531-bp BamHI-BglII fragment of pΔ11 (Fig. 1) as the probe, no 5' ends were detected in RNA preparations from cells infected with VΔ11, the mutant lacking the entire conventionally recognized ICP4 promoter (data not shown). If transcription of ICP4 initiates within the region tested in VΔ11, it does so below our limits of detection. This mutant is nonetheless viable, implying that some ICP4 mRNA and protein must be synthesized.

Quantitation of ICP4 and ICP47 mRNAs induced by mutant viruses. The mutant viruses were then characterized with respect to the amounts of ICP4 and ICP47 mRNAs they induce by S1 analysis. It should be noted that the ICP4, ICP47, and control ICP27 probes used in these experiments contain HSV DNA sequences lying totally within the open reading frames of their respective genes. One consequence of the use of the 330-bp ICP4-specific probe is that it is unable to differentiate between ICP4 mRNAs that initiate in the conventionally recognized ICP4 promoter as opposed to those that may initiate upstream of this site. Despite this disadvantage, this probe and the ICP47 and ICP27 probes were chosen because their HSV DNA sequences do not overlap any of the deletions in the mutant viruses.

Results of a typical assay are shown in Fig. 6, and Table 2 shows the amounts of ICP4 mRNA the mutants induced relative to wild-type virus in the presence of cycloheximide. These results indicate that all of the mutants induced reduced amounts of ICP4 mRNA relative to wild-type virus in the presence of cycloheximide. VΔ11 and VΔ4 showed the greatest reduction. Since the deletion in VΔ11 eliminated the entire conventionally recognized ICP4 promoter, it is perhaps not surprising that this mutant was deficient in ICP4 mRNA synthesis. The deletion in VΔ4 eliminated the ICP4 TATA box and resulted in a translocated ICP4 transcriptional start site (Fig. 5). At this time, it is uncertain whether the deficiency in ICP4 mRNA in VΔ4-infected cells is due to a reduction in transcriptional initiation or to altered mRNA stability caused by the truncation of the 5'-untranslated leader. Mutants VΔ1 and VΔ3 generated approximately equal amounts of ICP4 mRNA, about 30% of the wild-type level. The deletions in these two mutants were nearly identical, the only difference being that VΔ1 retained the TAATGARAT element most proximal to the ICP4 mRNA start site whereas VΔ3 lacked it (Fig. 1). Since both VΔ1 and VΔ3 specify ICP4 mRNAs with wild-type 5' termini, it is likely that their reduced ability to generate ICP4 mRNA is

The results presented in Table 3 indicate that ICP47

The deletion in VΔ5 eliminates the ICP4-binding site located at the ICP4 mRNA start site. The representative experiment shown in Fig. 7 and Table 4 was designed to test whether deletion of this site alleviates the repression exerted on ICP4 transcription when viral protein synthesis is allowed to occur (12, 20, 57). Table 4 shows the amounts of ICP4

FIG. 6. Quantitative S1 analysis of ICP4 and ICP27 mRNAs generated by the mutant viruses. Vero cells were incubated in the presence of 75 μ g of cycloheximide per ml beginning 1 h before infection and maintained in this concentration of drug until harvested. Cells were infected at a multiplicity of infection of 20 PFU per cell, and cytoplasmic RNA was harvested at 6 h postinfection. S1 analysis was conducted as previously described (61). (A) RNA was probed simultaneously for ICP47 and ICP27 messages. Protected fragments are shown. The ICP27 signal was used to normalize the data to control for experimental variation in infection efficiency and RNA recovery. (B) RNA was probed simultaneously for ICP4 and ICP27 messages. The protected fragments are shown. The ICP27 signal was used to normalize ICP4 signals. The results shown here were quantified to generate the data presented in experiment 2 of Table 1 and in Table 2. Lane M, Mock infected.

mRNA induced by V Δ 5 relative to HSV-2 strain 186 in the presence of cycloheximide or phosphonoacetic acid (PAA), an inhibitor of HSV DNA synthesis. PAA was added to ensure equal genome copy numbers of the two viruses and to allow for immediate-early and early protein synthesis. As for the wild-type virus, the ratio of ICP4 mRNA to ICP27 mRNA did not change regardless of which drug was present. For V Δ 5 in the presence of PAA, however, the ratio increased at least threefold. This result indicates that in the absence of the ICP4-binding site at the ICP4 transcriptional start site, ICP4 and ICP27 mRNA levels are no longer coordinately regulated but rather, ICP4 mRNA accumulates to higher levels.

DISCUSSION

This study had several primary objectives. The first was to generate a series of mutant plasmids lacking one or more of the recognized *cis*-acting elements located in the intergenic regulatory region between the immediate-early genes specifying ICP4 and ICP22/47. The second was to determine whether the absence of any of these elements in the context of the viral genome affected the ability of the virus to replicate. Presumably, the virus would not replicate if a deletion destroyed the activity of any essential *cis*- or

TABLE 2. Quantitation of ICP4 mRNA generated by mutant viruses

Virus	Ratio ^a of mutant ICP4 mRNA to wild-type ICP4 mRNA		
	Expt 1	Expt 2	Expt 3
186	1.00	1.00	1.00
V Δ 1	0.28	0.16	0.29
V Δ 3	0.28	0.29	0.32
V Δ 4	<0.28	<0.16	<0.16
V Δ 5	0.71	0.61	0.65
V Δ 6	0.34	0.34	0.51
V Δ 7	0.79	0.74	0.66
V Δ 8	ND ^b	0.58	0.76
V Δ 11	<0.28	<0.16	<0.16

^a Autoradiograms like those shown in Fig. 6 were scanned by using an LKB densitometer (Pharmacia, Inc., Piscataway, N.J.), and peaks were quantified by weighing. For mutants V Δ 4 and V Δ 11, ICP4 mRNA was not detected. In experiments 2 and 3, the ICP4 probe described in Materials and Methods was used. In experiment 1, a probe containing the 144-bp *Bam*HI-*Hind*III fragment of p Δ 8 (Fig. 1) was used. The ratio of mutant ICP4 mRNA to wild-type ICP4 mRNA was calculated by using the following formula: [(weight of ICP4 mRNA peak/weight of ICP27 mRNA peak) for a given mutant]/[(weight of ICP4 mRNA peak/weight of ICP27 mRNA peak) for wild-type virus]. This ratio is equal to 1.00 when wild-type values are used in the denominator as well as the numerator.

^b ND, Not determined.

trans-acting element. The third objective was to determine which *cis*-acting elements affect the level of ICP4 transcription and which affect the location of the transcriptional start site.

Little difficulty was encountered in generating the desired deletions in the cloned intergenic region between ICP4 and ICP22/47. DNA sequence analysis confirmed that each of the plasmids lacked the intended *cis*-acting element(s).

The absolute requirement for each of these elements in virus replication first became apparent in efforts to isolate viable deletion mutant viruses by marker transfer. Our ability to generate mutants lacking one or all four TAATGARAT elements (V Δ 1, V Δ 3, V Δ 6, and V Δ 7), the ICP4 transcriptional start site (V Δ 5), the ICP4 5'-untranslated leader (V Δ 8), as well as sequences lying between the first ATG and a site immediately to the right of the most upstream TAATGARAT element (V Δ 11), indicate that none of these sequences contains an essential *cis*- or *trans*-acting element. The viability of V Δ 11 in cells that do not express ICP4 was especially surprising, given that this mutant lacks the entire recognized ICP4 promoter. In view of the demonstration that ICP4 is essential for the growth of HSV-2 (53), it is clear that V Δ 11 must somehow generate mRNA containing the

TABLE 3. Quantitation of ICP47 mRNA generated by the mutant viruses

Virus	Ratio ^a of mutant ICP47 mRNA to wild-type ICP47 mRNA
186	1.00
V Δ 1	0.13
V Δ 3	0.17
V Δ 4	0.97
V Δ 5	0.90
V Δ 6	0.23
V Δ 7	0.46
V Δ 8	0.94
V Δ 11	0.12

^a The procedures outlined in footnote a of Table 2 were used to generate the data presented here.

FIG. 7. S1 analysis of ICP4 and ICP27 mRNAs generated by HSV-2 wild-type strains 186 and VΔ5. Vero cells were infected with 20 PFU of either virus per cell. Where indicated, 75 μ g of cycloheximide (cyclo.) per ml or 300 mg of PAA per ml was present throughout the course of infection. PAA was added to ensure equal genome copy numbers of the two viruses and to allow for immediate-early and early protein synthesis. Cells were harvested and processed for S1 analysis at 6 h postinfection. The 420-bp *Bam*HI-*Pvu*II ICP4 probe described in Materials and Methods was used in these tests.

coding sequences for ICP4. Relevant to this point is the existence of an HSV-1 mRNA (*oriS* mRNA₂) that begins in the noncoding sequences of the ICP22/47 gene and contains the entire ICP4 open reading frame (22). Conceivably, such an mRNA, if one exists in HSV-2, could provide sufficient ICP4 to maintain viability of VΔ11. Alternatively, the deletion in VΔ11 may eliminate a transcriptional termination signal from yet another transcript that initiates to the right of ICP4. Elimination of this signal could allow the synthesis of an mRNA containing the ICP4 open reading frame under control of a distant rightward promoter. It should be noted that we were unable to demonstrate any ICP4 mRNA in VΔ11-infected cells in the presence of cycloheximide (Table 2). This would argue either that the putative alternative rightward promoter is not of the immediate-early class (since early and late transcripts are not synthesized in the presence of cycloheximide and hence we would not detect them) or that very little ICP4-specific mRNA was synthesized.

Our inability to transfer the desired deletions in pΔ2, pΔ12, and pΔ13 correctly into the viral genome is of interest. All three plasmids lacked the sequence 715 through 996, a fact which may or may not be coincidental. However, all plasmids that contain these sequences (pΔ1, pΔ3, pΔ4, pΔ5, pΔ6, pΔ7, pΔ8, pΔ9, pΔ10, and pΔ11) were transferred correctly into the viral genome. Although, as mentioned above, the short rightmost flanking sequence in pΔ2 may have discouraged crossover events in this region, this is not

likely the case for pΔ12 and pΔ13, which contain lengthy rightward flanking sequences. Of special note is the fact that recombinant viruses generated with VΔ12 and VΔ13 contained specifically those sequences whose deletion was sought (Fig. 4). (The VΔ2 recombinants have not been tested for the presence of these sequences.) We conclude from these experiments that sequences lying roughly between nucleotides 715 and 996 contain one or more essential *cis*- or *trans*-acting elements. Whether the essential element is the *cis*-acting *oriS* or coding sequences for an as yet unidentified essential viral gene remains to be determined. If, on the other hand, the failure to obtain viable deletion mutants lacking these sequences is a consequence of the failure to express ICP4, it should be possible to obtain the desired mutants in ICP4-expressing n-33 cells. These tests are currently in progress.

The successful transfer of the deletions in pΔ1, pΔ3, pΔ4, pΔ5, pΔ6, pΔ7, pΔ8, and pΔ11 afforded us the unique opportunity to study the effects of the corresponding deleted elements on transcription of ICP4 and ICP22/47 in the context of the viral chromosome. The absence of one, two, three, and all four TAATGARAT elements in VΔ7, VΔ6, VΔ1, and VΔ3, respectively, had similar effects on the expression of ICP4 and ICP47. The deletions in these viruses however, also eliminate other *cis*-acting sites such as Sp1-binding motifs. Consequently, it is uncertain as to which deleted elements are responsible for the observed bidirectional effects. It should be noted that transient expression experiments involving plasmid-borne substrates have shown that Sp1 sites and TAATGARAT elements affect transcription additively and in an orientation-independent manner (26, 30, 31, 40, 46). Confirmation of the roles of specific elements and sequences in the context of the viral genome will require the construction of mutants containing much smaller deletions than those harbored by VΔ1, VΔ3, VΔ6, and VΔ7. Despite this caveat, one further conclusion regarding VΔ1 and VΔ3 can be made. This concerns the fact that these two mutants shared the same right-hand deletion endpoints and differed by only 14 nucleotides with respect to their left-hand deletion endpoints (Fig. 1). The deletion in VΔ3 eliminated all four TAATGARAT motifs, whereas the deletion in VΔ1 retained the TAATGARAT most proximal to the start of ICP4 transcription (Fig. 1). Despite this difference, transcription driven by these two promoters in the presence of cycloheximide did not differ significantly (Tables 2 and 3). This result suggests either that the TAATGARAT element located between nucleotides 450 and 460 does not contribute to expression or that the deletion in VΔ1 eliminates some other *cis*-acting element which acts in conjunction with TAATGARAT to confer VP16 inducibility. The latter possibility appears more likely in that recent studies have shown that the TAATGARAT consensus is necessary but not sufficient for induction by VP16 (5, 23, 56). The sequence GCGGAA is also apparently critical (5, 23, 55). Both VΔ3 and VΔ1 lack two such elements located between the second and third TAATGARAT motifs (61). The absence of this sequence may therefore explain why the single TAATGARAT element retained by VΔ1 has no apparent activity.

In contrast to VΔ1, VΔ3, VΔ6, and VΔ7, the deletions in VΔ4, VΔ5 and VΔ8 did not appreciably affect ICP47 expression (Table 3), although they did reduce ICP4 expression (Table 2). This result was not unexpected, considering that the deletions in VΔ4, VΔ5, and VΔ8 eliminate what appear to be ICP4-specific landmarks: i.e., the ICP4 TATA box, the endogenous ICP4 mRNA start site, and the sequence encod-

TABLE 4. Quantitation of ICP4 mRNA generated by VΔ5 and HSV-2 strain 186 in the presence of two drugs

Virus	Drug	Ratio ^a of ICP4 mRNA to ICP27 mRNA	
		Expt 1	Expt 2
186	Cycloheximide	0.25	0.24
186	PAA	0.27	0.28
VΔ5	Cycloheximide	0.19	0.15
VΔ5	PAA	0.58	0.41

^a Autoradiograms like the one shown in Fig. 7 were scanned and the peaks were quantified by weighing. The ratio was calculated by using the following formula: (weight of ICP4 peak)/(weight of ICP27 mRNA peak).

ing most of the untranslated leader of the ICP4 mRNA, respectively.

As a consequence of the $\Delta 4$ deletion, ICP4 mRNA expression was reduced by 5- to 10-fold and the 5' end of ICP4 mRNA was translocated downstream of the normal site of initiation by approximately 80 nucleotides (Table 2 and Fig. 5). These results are in agreement with those of Cordingley et al. (8), who characterized a plasmid-borne HSV-1 ICP4 gene specifically lacking the TATA element. They found in transient expression assays that elimination of the TATA box caused a two- to threefold reduction in expression as well as a translocation of the ICP4 transcriptional start site.

As stated earlier, the deletion in V Δ 5 eliminates the ICP4-binding site located at the start site of ICP4 transcription. V Δ 5 generated modestly reduced levels of ICP4 mRNA in the presence of cycloheximide (Tables 2 and 4). However, the results shown in Table 4 demonstrate that V Δ 5 generated relatively more ICP4 mRNA than wild-type virus when immediate-early and early protein synthesis was permitted to occur (i.e., in the presence of PAA but not cycloheximide). The most straightforward interpretation of this latter result is that the lack of ICP4 binding at the start of ICP4 transcription results in higher levels of expression. While reproducible, these effects are not as dramatic as those obtained with ICP4 deletion or temperature-sensitive mutants under nonpermissive conditions (13, 43; N. DeLuca, personal communication). Therefore, it is reasonable to suggest that repression of ICP4 expression is mediated by more than one *cis*-acting signal.

The deletion in V Δ 8 eliminated most of the DNA encoding the 5'-untranslated leader of ICP4 mRNA. V Δ 8 exhibited a 20 to 40% decrease in ICP4 mRNA levels (Table 2). Whether this deficiency is caused by altered mRNA stability or reduced transcriptional efficiency is unclear from the data obtained in this study. Pertinent to this question is the report by Blair et al. (1) indicating that deletions in the sequence encoding the 5'-untranslated leader of VP16 cause not only reduced message stability but also lowered transcriptional efficiency during lytic infection. The ICP4 mRNA of Δ 8 may prove to have similar transcriptional and posttranscriptional properties.

This report describes preliminary studies designed to address the *in vitro* consequences of alterations in the intergenic region between ICP4 and ICP22/47; it does not, however, address the consequences of these alterations in the animal host. For this purpose, we are currently assaying the pathogenicity of our series of HSV-2 ICP4 promoter mutants as well as their ability to establish, maintain, and reactivate from latency in the mouse eye model.

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