JOURNAL OF VIROLOGY, Feb. 1992, p. 623–631
0022-538X/92/020623-09$02.00/0

Vol. 66, No. 2

The ICP4 Binding Sites in the Herpes Simplex Virus Type 1 Glycoprotein D (gD) Promoter Are Not Essential for Efficient gD Transcription during Virus Infection

J. R. SMILEY,[1]* D. C. JOHNSON,[1] L. I. PIZER,[2] AND R. D. EVERETT[3]

Molecular Virology and Immunology Program, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5[1]; Department of Microbiology/Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262[2]; and MRC Virology Unit, Glasgow G11 5JR, Scotland, United Kingdom[3]

Received 4 September 1991/Accepted 21 October 1991

Activation of the early and late genes of herpes simplex virus type 1 during infection in tissue culture requires functional immediate-early regulatory protein ICP4. ICP4 is a specific DNA-binding protein which recognizes a variety of DNA sequences, many of which contain the consensus ATCGTC. In general, mutations which impair the ability of ICP4 to bind to DNA also eliminate its ability to activate viral early and late promoters both in transfection assays and in the infected cell. However, the role of ICP4 binding sites in the viral genome is unclear; many early and late promoters do not contain consensus binding sites in their vicinity. The glycoprotein D (gD) gene contains two well-characterized ICP4 binding sites upstream of its promoter and a third downstream of the transcription start site. Multimerization of one of these sites has been shown to increase the response of the gD promoter to ICP4 in transfection assays, while their removal reduces stimulation of the gD promoter by ICP4 in vitro. To assess the role of these binding sites during virus infection, we have constructed a recombinant viral genome which has mutations affecting all three. Comparison of the amounts of gD RNA synthesized by the recombinant and wild-type viruses indicated that the mutations had little or no effect on the activity of the gD promoter. Therefore, either the sites have no essential role in gD promoter regulation in the presence of all of the herpes simplex virus type 1 IE polypeptides during a normal infection or they can be functionally substituted by other ICP4 binding sites elsewhere in the genome.

Analysis of the complete sequence of the genome of herpes simplex virus type 1 (HSV-1) has revealed at least 70 distinct genes (25). During infection in tissue culture, these genes can be divided into three main classes, based on the kinetics and characteristics of their expression. The immediate-early (IE) (or α) genes are defined as those which can be transcribed in the absence of de novo viral protein synthesis, while the early (or β) genes require functional IE polypeptides for their transcription. The late (or γ) genes are expressed efficiently only after the onset of viral DNA replication (for reviews, see references 12, 39, and 49). The IE polypeptides are therefore crucial for the normal lytic viral replicative cycle; of these, ICP4 is perhaps the most important since its inactivation by mutation leads to a complete failure to activate early or late gene expression (5, 37). Therefore, ICP4 has been the subject of intensive research.

A prominent property of ICP4 is its ability to bind to selected DNA sequences. Many of these sequences include the consensus ATCGTC (15, 22, 30), although this sequence is not sufficient for binding (36, 38) and other sites which appear not to include the consensus have been defined (1, 23, 28, 29, 46). The ability of ICP4 to bind to DNA appears to be crucial for its functions of activation of early and late gene expression and the apparent repression of IE gene transcription, at least in transfection assays (6, 33, 34, 41). However, the role of individual ICP4 binding sites in transactivation by ICP4 is paradoxically not so clear-cut. Consensus ICP4 binding sites are not commonly found in the promoter regions of HSV-1 early and late genes (24b), and while weaker (nonconsensus) sites may be present in some promoters, for example, the *tk* (20) and glycoprotein C (gC) (our unpublished data) promoters, recent evidence suggests that transactivation of the *tk* promoter does not require ICP4 to bind to these sites (20, 40).

The gD promoter is one of the few well-characterized HSV-1 early promoters which contains both consensus and nonconsensus ICP4 binding sites in its vicinity. Two sites were located just 5′ to the gD promoter region, and a third was found downstream of the transcription start site (1, 46). The role of these sites in the mechanism of transactivation by ICP4 has been investigated by a variety of transfection and in vitro transcription experiments. Early studies found that the two sites upstream of the promoter were not essential for transactivation of the gD promoter in transfected cells when all of the IE polypeptides were provided by virus infection (9, 10). However, the binding sites contributed to the activation of the gD promoter by ICP4 in vitro (46), and multimerization of one of the sites increased activation by ICP4 in transfected cells (47). Since the results of transfection experiments of this type are, to a certain extent, operator controlled (13; our unpublished results), the key question is what role the ICP4 binding sites play in the context of the viral genome during a normal infection. To this end, we have constructed a variant of HSV-1 with mutations in the three previously characterized ICP4 binding sites in the gD promoter region. We found that mutation of these sites had no significant effect on the efficiency of gD transcription during infection.

* Corresponding author.

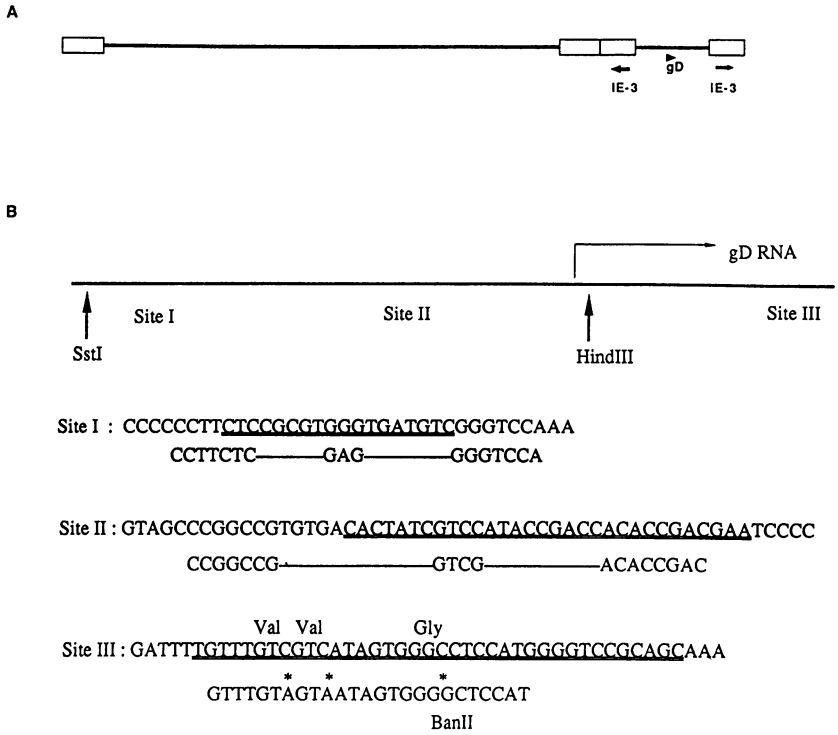

FIG. 1. Map of the HSV-1 gD region and the mutations removing the ICP4 binding sites. (A) The HSV-1 genome. The gD gene (US6) is marked in the short unique section, and the ICP4 gene (IE3) is indicated in the adjacent inverted repeats. A more detailed map of the gD region in plasmid pJB39 is shown in Fig. 3B. (B) The gD promoter and flanking region with the three ICP4 binding sites investigated in this study. The *Sst*I and *Hin*dIII sites are those at nucleotides −392 and +11 relative to the start site of the gD RNA. The sequence in the vicinity of binding sites I, II, and III is shown, with the extent of the ICP4 footprint double underlined. The sequence of each oligonucleotide used for mutagenesis is shown underneath. Nucleotides −301 to −287 (relative to the 5′ end of gD RNA) have been deleted and a *Xho*I site has been created at site I, nucleotides −116 to −92 have been deleted and a *Sal*I site has been created at site II, and nucleotides +130, +133, and +142 have been mutated to alter the ATCGTC consensus and create a *Ban*II site at site III. The conservative nucleotide changes are indicated in site III by asterisks.

MATERIALS AND METHODS

Plasmids, bacteriophage, and bacteria. The promoter and coding region of the gD gene were derived from plasmid pJB39, which contains the 3.4-kb *Sma*I fragment encompassing the entire gD region of HSV-1 strain KOS cloned into the Bluescribe vector (3). A map of this *Sma*I fragment is shown in Fig. 3B; the orientation of the clone is such that the vector polylinker *Bam*HI site is at the left end of the clone (5′ to gD) and the polylinker *Eco*RI site is to the right. The *Bam*HI (polylinker)-to-*Hin*dIII promoter region and the *Hin*dIII-to-*Eco*RI (polylinker) coding region fragments of pJB39 were recloned into M13 mp18 and mp19, respectively, for oligonucleotide-directed mutagenesis. After mutagenesis, the mutated fragments were purified and ligated into pUC9 which had been cut by *Bam*HI and *Eco*RI to rebuild a complete gD gene with all three mutations in plasmid pgD123mut. All plasmids and bacteriophage were maintained in *Escherichia coli* HB101 and JM101, respectively.

Viruses and cells. The gD-deficient HSV-1 strain F-gDβ carries the *E. coli* β-galactosidase gene inserted across a deletion which removes the entire gD gene and part of the neighboring gI gene (24). Strains wt61 and mu31 were derived from F-gDβ by rescuing gD coding sequences from plasmids pJB39 and pgD123mut, respectively, as described below. F-gDβ was propagated on gD-producing VD60 cells (24), and wt61 and mu31 were grown on Vero cells. F-gDβ forms syncytial plaques on VD60 cells as the result of a *syn* mutation outside the region encoding gD; wt61 and mu31 retain this mutation and form syncytial plaques on both VD60 and Vero cells.

Oligonucleotide-directed mutagenesis. The ICP4 binding sites in the gD promoter (sites I, II, and III; Fig. 1) were mutated by using oligonucleotides 5′-TGGACCCCTCGAG AAGGG-3′, 5′-GTCGGTGTCGACCGGCCGG-3′, and 5′-A TGGAGCCCCACTATTACTACAAAC-3′, respectively, in separate mutagenesis experiments, using the M13 universal primer in a double-primer method loosely based on that described by Zoller and Smith (53). Briefly, after hybridization of M13 universal and mutagenic primers to the single-stranded template DNA and treatment with Klenow DNA polymerase in the presence of DNA ligase, the mutagenized M13 DNA was transfected into JM101. The plaques were screened initially by differential hybridization then by restriction enzyme digestion and dideoxy DNA sequence analysis. The first two oligonucleotides delete the footprinted regions of sites I and II (15, 46) without affecting the mapped minimal gD promoter (9) while introducing *Xho*I and *Sal*I restriction sites, respectively. The third mutant oligonucleotide introduces two point mutations into a sequence related to the ICP4 binding site consensus in site III (as defined by DNA footprinting; 46) and a third which creates a novel *Ban*II restriction site. None of the three site III mutations, which lie in coding sequences, affect the predicted translation product. Bacteriophage with the desired

restriction pattern and sequence were purified and resequenced. To create a fragment with both sites I and II mutated, the clone with the site I mutation was mutagenized with the second oligonucleotide, and the desired product was detected, purified, and analyzed as described above. A mutant fragment containing both site I and II deletions was ligated into pUC9 vector, with the fragment containing the site III mutations, to create plasmid pgD123mut, which contains the entire gD region but none of the previously defined ICP4 binding sites in the gD promoter.

Gel retardation assays. The band shift assay was used to compare binding of ICP4 to DNA fragments containing mutated or wild-type site I, II, or III. Fragments containing wild-type sites were excised from plasmid pDGT26 (46), and individual sites were subcloned into the Bluescribe vector. To obtain fragments with the mutated binding sites, replicative-form DNA from mutant M13 bacteriophage was purified and subcloned into Bluescribe. Radioactive wild-type and mutant probe DNA fragments were produced by cutting plasmid DNA at the *Hin*dIII site in the polylinker, labelling with Klenow polymerase and [α-^{32}P]dATP, and cutting with an appropriate second enzyme. To obtain wild-type and mutant fragment II, the second cut was made in the polylinker with *Eco*RI. For probe fragments I and III, the second cut was with *Sst*II. The *Sst*II site in fragment I is 30 bp from the 5′ end of the cloned fragment, while that in fragment III is 80 bp from its 3′ end and is located at the 3′ edge of the footprinted region (46). By cutting with *Sst*II, other ICP4 binding sites which had not been previously detected during the footprint experiments were removed from the DNA probes containing sites I and III. Probe fragments were prepared under identical conditions at the same time to ensure that their specific activities were as similar as possible. The probes were purified by gel electrophoresis.

Binding reactions with labelled probe were carried out either with nuclear extracts of HeLa cells prepared 6 h after infection with HSV-1 strain KOS or with partially purified ICP4 fraction VIII (27; a generous gift from K. Wilcox). Incubation conditions were as previously described, with 3 μg of salmon sperm DNA as a nonspecific competitor (46). The presence of ICP4 in binding complexes was demonstrated by a further shift in mobility after the addition of monoclonal antibody H944 against ICP4. The specificity of binding was tested by using an unlabelled 47-bp DNA fragment which contains the gD site II sequence and acts as a specific competitor (36). Gels were run until the free DNA had migrated approximately 10 cm, and exposure times of dried autoradiographs were adjusted according to the specific activity of the probes. To determine the fraction of DNA probe in the ICP4 complex, bands were excised from the dried gels and the radioactivity present was counted in a scintillation counter.

Transfer of the mutations into the viral genome. The ICP4 binding site mutations present in pgD123mut were transferred into the gD locus of F-gDβ by DNA-mediated marker rescue. F-gDβ DNA was extracted from cytoplasmic nucleocapsids as previously described (43). Semiconfluent VD60 cell monolayers in 60-mm plastic dishes were transfected with infectious F-gDβ DNA and 10 μg of circular pgD123mut or pJB39, using the calcium phosphate coprecipitation technique (17) followed by a glycerol shock (24). The resulting progeny were harvested after 5 days and titered on VD60 and Vero cell monolayers. Recombinants capable of growth on Vero cells were plaque purified, and viral DNA prepared from Hirt extracts (19) of infected cells was analyzed by Southern blot hybridization (45) as previously described (42). Isolates mu31 and wt61, bearing the mutant sequences derived from pgD123mut and the wild-type sequences derived from pJB39, respectively, were plaque purified two additional times on Vero cells. Virus stocks were prepared, and Southern blot analysis was performed to confirm the structure of viral DNAs.

Primer extension analysis and quantification of gD RNA. Cytoplasmic RNA prepared by the method of Berk and Sharp (2) was scored for the levels of gD and gB transcripts by primer extension analysis as previously described (44). For quantification, gels were exposed to Kodak XAR5 film without an intensifier, and suitably exposed autoradiographs were scanned with an LKB laser microdensitometer. The following 5′-^{32}P-labelled oligonucleotides were used as primers: gD, 5′-CCCCATACCGGAACGCACCACACAA-3′ (32), predicted extension products of about 80 to 90 nucleotides (50); and gB, 5′-CGGCTGCAGTCCGGGCGGCGCAACT-3′, predicted extension product of 60 nucleotides (21).

RESULTS

Mutation of the ICP4 binding sites in the gD promoter greatly reduces binding by ICP4. The ICP4 binding sites in the gD promoter have been previously characterized by footprint and gel retardation analysis (46) and are shown in Fig. 1. To mutate these sites, those in the noncoding region (sites I and II) were eliminated by oligonucleotide-directed deletion. Site III in the gD coding region was mutated at several positions so as to alter the binding consensus while leaving the coding potential unchanged (Fig. 1). The mutated fragments from the appropriate M13 clones were used to rebuild a complete gD gene in plasmid pgD123mut with mutations in all three of the ICP4 binding sites (Fig. 1; see Materials and Methods).

Gel retardation assays (Fig. 2) showed that DNA fragments containing the deletion and point mutations in ICP4 binding sites I, II, and III were severely impaired in their ability to form DNA-protein complexes. DNA fragments containing wild-type or mutant forms of sites I, II, and III (Fig. 2A) were incubated with nuclear extract from infected cells (Fig. 2B) or purified ICP4 fraction VIII (Fig. 2C). Nuclear extract formed specific complexes with wild-type fragments, as shown by distinct bands with reduced mobility (lanes 1, 5, and 9; indicated by an arrow). Addition of an anti-ICP4 monoclonal antibody to the binding reaction further retarded the migration of this band (lanes 2, 6, and 10). This result demonstrates the presence of ICP4 in the complexes, which were not produced when uninfected cell extract was used or when 200 ng of a specific 47-bp competitor fragment (which contains site II) was added (data not shown). With fragments containing the mutant forms of sites I and II, ICP4 complex formation was very poor or undetectable (compare lanes 1 and 3 and lanes 9 and 11). Probe fragment III gave a weaker ICP4-specific complex. Following determination of the radioactivity in the free and bound DNA, we found that binding to the mutant probe fragment III was no more than 15% of that obtained with the wild-type fragment (compare lanes 5 and 7 or lanes 6 and 8).

The results of an analogous experiment using partially purified ICP4 fraction VIII are shown in Fig. 2C. While the proportion of probe in the ICP4 complexes was higher than with the nuclear extract, binding to the mutant probes (lanes 14, 16, and 18) was also very greatly diminished compared with the levels observed with the wild-type probes (lanes 13, 15, and 17). We conclude that, at least in terms of the previously characterized ICP4 binding sites, the gD gene in

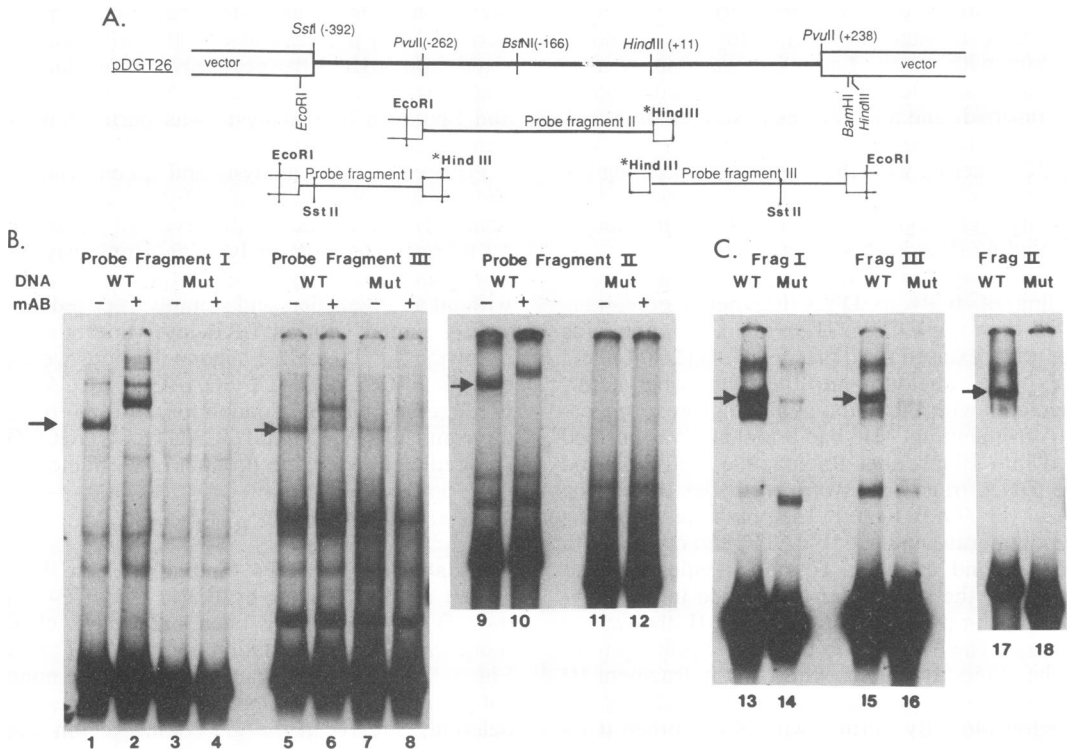

FIG. 2. (A) Simplified representation of the promoter region of the gD gene showing the subcloned probe fragments used to measure complex formation between ICP4 and sites I, II, and III. (B) Band shift assays comparing wild type (WT) and mutant (Mut) DNA probes containing the previously identified binding sites in fragments I, II, and III. Approximately 5 ng of each labelled fragment was incubated with infected-cell nuclear extract containing 15 μg of protein; 2 μg of anti-ICP4 monoclonal antibody (mAB) was present in lanes 2, 4, 6, 8, 10, and 12. Radioautographs were exposed against the dried gel for 48 h at room temperature. DNA-protein complexes containing ICP4 are indicated with arrows. (C) Band shift assays using ICP4 fraction VIII. The assay conditions were as described above except that 5 μg of ICP4 fraction VIII was used. The radioautograph was exposed for 16 h at −70°C with an intensifying screen. DNA-protein complexes containing ICP4 are indicated with arrows.

plasmid pgD123mut is severely deficient in its ability to bind ICP4.

A virus bearing the mutations in the ICP4 binding sites in the gD promoter is viable in cultured cells. The ICP4 binding site mutations in pgD123mut were transferred into the gD locus of the HSV-1 genome by DNA-mediated marker rescue. To facilitate the identification of recombinants, the gD-deficient HSV-1 strain F-gDβ (24) was used as the recipient. F-gDβ bears a deletion/substitution mutation that replaces the gD coding region and part of the flanking gI gene with *E. coli* β-galactosidase sequences (Fig. 3). Since gD is essential for virus entry into cells, F-gDβ can be propagated only on VD60 cells that provide gD in *trans* (24). The HSV-1 DNA insert in pgD123mut overlaps the F-gDβ deletion endpoints and is therefore able to restore gD coding sequences through crossover events in the flanking homologous sequences (Fig. 3). Because ICP4 site III is located within sequences that are deleted from F-gDβ, all such recombinants should acquire the site III mutation from the rescuing plasmid. Sites I and II are located within 450 nucleotides of the 5′ deletion endpoint and were likely to be cotransferred at high frequency.

F-gDβ DNA was cotransfected with either pgD123mut or control pJB39 plasmid DNA onto complementing VD60 cell monolayers, and the resulting virus stocks were titered on VD60 and Vero cells to determine the frequency of recombinants capable of growth on noncomplementing cells. Both plasmids gave rise to rescue products at similar frequencies (pgD123mut, 1.1%; pJB39, 1.6% [averages of two experiments]). Recombinant viruses arising from pgD123mut were plaque purified on Vero cells and then examined for the mutations of the ICP4 binding sites by Southern blot hybridization. We identified three recombinants that arose via a double-crossover event between pgD123mut and viral DNA. These isolates lacked β-galactosidase sequences and contained intact gD and gI genes derived from the rescuing plasmid. All three acquired the *Xho*I and *Sal*I cleavage sites that delineate the site I and II mutations, respectively. One of these recombinants, designated mu31, was plaque purified, expanded, and examined in more detail (see below). Eight additional pgD123mut rescue products displayed more complex restriction patterns that most likely reflect integration of the entire rescuing plasmid into F-gDβ via a single-crossover event. These isolates were not examined further. A similar screen of six recombinants derived from the control pJB39 transfection yielded two double-crossover products. One of these, wt61, was selected for further study.

We examined mu31 DNA for the novel restriction endonuclease cleavage sites that mark the mutations of the ICP4 binding sites (Fig. 4). The site I mutation introduces an *Xho*I site into the 3.4-kb *Sma*I fragment that contains the gD gene. To test for the presence of this mutation, viral DNA was cleaved with a mixture of *Sma*I and *Xho*I and analyzed by Southern blot hybridization using radiolabelled pJB39 as a

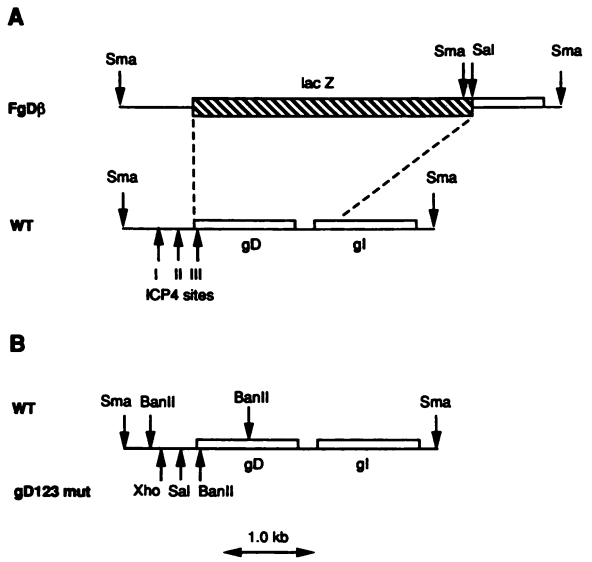

FIG. 3. Map of the gD region of F-gDβ and wild-type HSV-1. (A) F-gDβ contains a lethal deletion/substitution that removes the gD coding region and part of the gI gene and places the *E. coli lacZ* gene under the control of the gD promoter. ICP4 binding site III is located within the sequences that are deleted from F-gDβ; sites I and II are located in the upstream flanking sequences. (B) Comparison of the restriction endonuclease cleavage maps of the 3.4-kb *Sma*I fragments present in pJB39 and pgD123mut. Only the *Ban*II sites that flank the ICP4 binding sites are displayed.

probe. As expected, wt61 DNA displayed a single fragment that comigrated with the 3.4-kb *Sma*I fragment of strain F. In contrast, mu31 lacked the wild-type fragment and displayed novel fragments of about 3.0 and 0.4 kb that comigrated with the *Sma*I/*Xho*I digestion products of pgD123mut. Thus, mu31 DNA contains the novel *Xho*I site that marks the site I mutation. A similar analysis of *Sma*I/*Sal*I digests showed that the mu31 3.4-kb *Sma*I fragment was cleaved by *Sal*I to give the same approximately 2.8- and 0.6-kb fragments as pgD123mut, indicating the presence of the novel *Sal*I site diagnostic of the site II mutation. ICP4 site III is located within a 1.1-kb *Ban*II fragment of wild-type DNA. As predicted, mu31 DNA lacked this fragment and displayed novel bands of about 570 and 530 bp that comigrate with the corresponding *Ban*II fragments of pgD123mut DNA. These data demonstrate that mu31 DNA contains all three ICP4 binding site mutations.

The titers of mu31 virus stocks derived by infecting noncomplementing Vero cells at a multiplicity of 0.01 averaged 68% of those of wt61 stocks prepared in parallel (average of three experiments). Therefore, mutation of all three ICP4 binding sites in the gD promoter did not dramatically impair virus growth in tissue culture. While the levels of gD required for normal virus growth are unknown, virus yields are reduced dramatically (>100-fold) when F-gDβ is propagated on cells that provide 10 to 25% of the amount of gD as do VD60 cells (24a). These results suggest that the mutations of the ICP4 binding sites do not have a major effect on gD expression.

Mutation of the ICP4 binding sites in the gD promoter does not markedly affect the efficiency of gD transcription. We used a primer extension assay to assess the effects of the mutations in the ICP4 binding sites on the accumulation of gD mRNA during lytic infection of Vero cells. To control for experimental variation, a primer for gB mRNA was included as an internal standard in each assay. Control experiments with wt61 RNA demonstrated that the gD and gB primers gave rise to the predicted extension products, that both transcripts could be detected in a single extension reaction, and that gD and gB signal intensities increased linearly with RNA concentration (Fig. 5). We then compared gD mRNA levels during infection with mu31 and wt61 by analyzing RNA samples harvested at 6 and 12 h postinfection with a mixture of the gD and gB primers (Fig. 6). Following electrophoresis and autoradiography, signal intensities were quantitated by microdensitometry, and the gD signals were

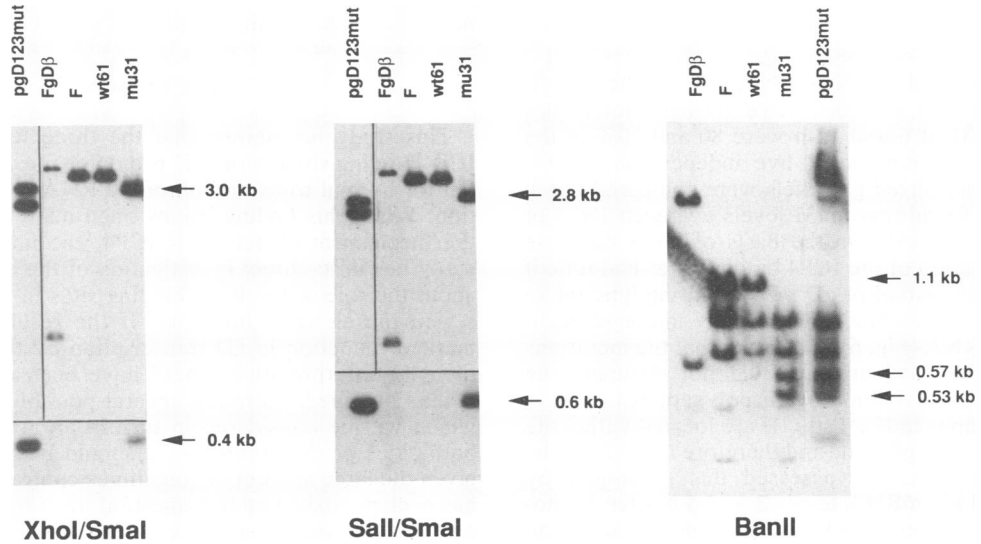

FIG. 4. Southern blot analysis of mu31 and wt61 DNA. Viral DNA prepared from Hirt extracts of infected cells was cleaved with the indicated restriction endonucleases, and the resulting fragments were separated by electrophoresis through a 1.4% agarose gel. Following transfer to nitrocellulose, gD-related fragments were detected by hybridization with ^{32}P-labelled pJB39. The panels displaying the *Xho*I/*Sma*I and *Sal*I/*Sma*I digests are composites made from three different exposures of the autoradiogram.

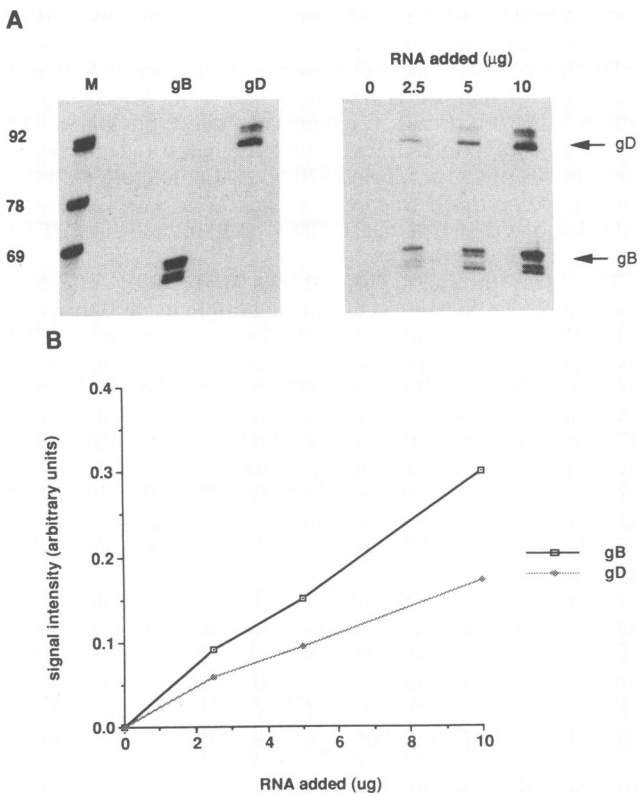

FIG. 5. Primer extension assay for gD mRNA. (A) Vero cells were infected with 10 PFU of wt61 per cell, and cytoplasmic RNA was harvested at 6 h postinfection. RNA samples were then analyzed for gD and gB mRNA by primer extension using 5′-^{32}P-labelled 25-mer oligonucleotides as primers as described in Materials and Methods. Following treatment with reverse transcriptase, extension products were resolved on an 8% sequencing gel. In the left panel, 10-μg aliquots of RNA were separately scored for gB and gD transcripts. In the right panel, the indicated amounts of RNA were analyzed with both primers simultaneously. Markers (M) were 3′-labelled *Hpa*II fragments of pBR322 DNA. (B) Following autoradiography without an intensifier screen, signals were quantified by microdensitometry.

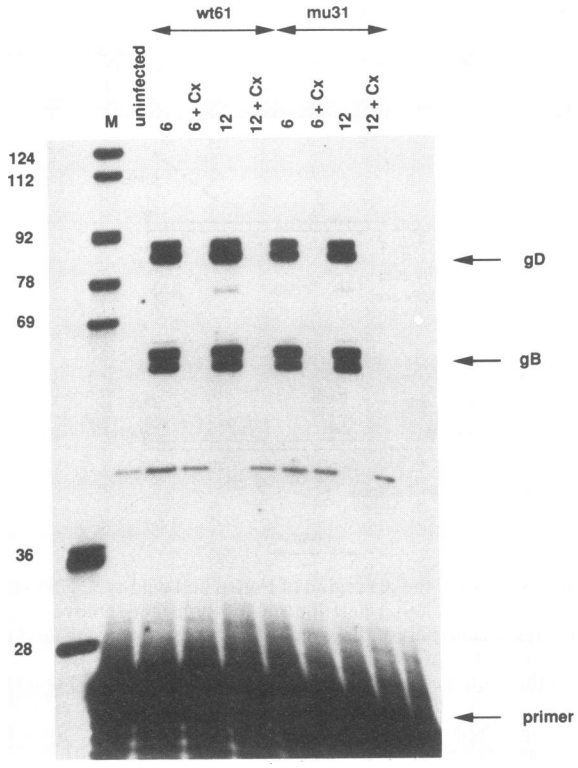

FIG. 6. Comparison of gD and gB RNA levels during infection with wt61 and mu31. Vero cells were infected with 10 PFU of the indicated virus per cell, and cytoplasmic RNA was harvested 6 and 12 h postinfection; 10-μg aliquots of RNA were then analyzed by primer extension with a mixture of gD and gB primers as described in the legend to Fig. 5. Where indicated, cycloheximide (Cx; 100 μg/ml) was added at the time of infection and maintained continuously. Markers (M) were 3′-labelled *Hpa*II fragments of pBR322 DNA. Sizes of marker fragments (in nucleotides) are indicated.

normalized to the corresponding gB signals. In the experiment depicted in Fig. 6, the normalized gD signal intensities obtained with mu31 at 6 and 12 h were 80 and 70% of the values for wt61. We conducted five independent experiments in which normalized gD levels were compared at 6 h postinfection and found that mu31 levels averaged 106% of those obtained with wt61. On the basis of these data, we conclude that mutation of the ICP4 binding sites had little if any effect on accumulation of gD mRNA during lytic infection of Vero cells. Cycloheximide strongly inhibited accumulation of gD mRNA (Fig. 6), indicating that the mutations in the ICP4 binding sites in mu31 did not eliminate the requirement for transactivation by IE polypeptides.

The point mutations in ICP4 site III are located within the transcribed body of the gD gene and therefore might alter the stability of gD mRNA. We assessed this possibility by examining gB and gD mRNA levels 3 and 6 h after actinomycin D had been added at 6 h postinfection. Comparison with the results from a parallel experiment with wt61 showed that the stabilities of the gD mRNAs of both viruses varied by not more than 10% from those of the gB mRNAs. Within the 6-h period of the experiment, the rates of decline of the mRNAs of mu31 were within 15% of those of wt61 (data not shown). We conclude that the mutations in mu31 have a negligible effect on the stability of gD mRNA.

DISCUSSION

This study has shown that the three well-characterized ICP4 binding sites in the gD promoter region are not essential for normal transcription of gD RNA during virus infection. While this finding leaves open many questions about the mechanism of action of ICP4, the importance of this study lies in its direct investigation of the obvious question about the role of the ICP4 binding sites in the gD promoter region during virus infection. If the results had shown a marked reduction in gD transcription by the mutant virus, then the interpretation would have been clear. Given the results obtained, there are several possible interpretations, which will be considered in turn in an attempt to provide both sides of the argument. It should be appreciated that, given the large amount of sometimes contradictory data that has been published on this question, the arguments are quite complex and inconsistencies are inevitable.

One interpretation is that although binding to the three sites by ICP4 has been very significantly reduced, sufficient binding still occurs for normal levels of gD transcription to be achieved. This interpretation requires the condition that if

activation by ICP4 results from DNA binding, then the level of activation is not proportional to the degree of binding. It is not yet possible to determine the validity of this hypothesis. However, the idea of activation through weak residual binding is related to the concept that ICP4 binding to other, perhaps weaker, sites in the vicinity of the target promoter may substitute for the loss of the sites mutated in this study. This suggestion is considered in more detail below.

The second simple interpretation is that DNA binding by ICP4 is not important for its ability to activate transcription. There is considerable evidence to suggest that this is probably not the case. Mutations which destroy the ability of ICP4 to bind to DNA also, almost without exception, eliminate transactivation in transfection assays (7, 33, 41). The well-studied temperature-sensitive mutant *ts*K has a lesion in the DNA binding domain of ICP4 which results in failure to bind to DNA and to activate early gene expression at the nonpermissive temperature (35, 37). While a mutant ICP4 which fails to bind to weaker binding sites is still able to transactivate, it retains the ability to bind to sites of higher affinity (20). However, it is possible that the mutations which destroy both DNA binding and transactivation do so through indirect means, perhaps by gross structural inactivation of the whole protein.

On the other hand, the hypothesis that DNA binding is not important for transactivation by ICP4 is supported by the observation that certain temperature-sensitive mutants appear to have lost the ability to bind to a strong binding site in vitro yet still activate limited early gene expression during virus infection at the nonpermissive temperature (35). However, the mutations causing this phenotype are not in the DNA binding domain (14, 51), and it is possible that the large amounts of the mutant form of ICP4 expressed by these viruses at the nonpermissive temperature are able to bind to DNA sufficiently in vivo to activate some early gene transcription. A recent study has described the isolation of a virus with two point mutations in the proximal part of the DNA binding domain of ICP4 which greatly reduce the ability of ICP4 to complex DNA in gel shift assays yet do not affect the ability of the protein to regulate gene expression (40). This apparently conclusive finding cannot yet be considered definitive for the following reasons. First, the protein may still bind to DNA in vivo. Second, the mutations may have affected the ability of ICP4 to bind to DNA in the conditions enforced by the gel retardation assay; for example, it may still bind in a DNA footprinting assay. It may be pertinent to point out that the DNA binding ability of the varicella-zoster virus protein that is equivalent to ICP4 seems to be demonstrable (for technical reasons) only after expression of its DNA binding domain in isolation (8a, 52). Any hypothesis in which DNA binding plays no role in transcriptional activation by ICP4 must explain why the portion of the protein which is essential for transactivation, and which is exceptionally well conserved in all related proteins so far sequenced (4, 18, 26, 48), has the ability to bind to DNA.

The third possibility is that activation of the gD promoter is normally accomplished by interactions of ICP4 with the binding sites, but in their absence other viral IE polypeptides activate the promoter. Although the gD and other promoters can be activated by ICP0 in transfection assays (11, 16, 31), there is no evidence that ICP0 alone (or any other viral transactivator) can activate viral early promoters in the absence of ICP4 during virus infection (for example, see reference 5). If the gD promoter in the recombinant virus without the ICP4 binding sites was activated by other IE polypeptides, then inactivation of ICP4 in (for example) ICP4 deletion viruses should not eliminate gD transcription; experiments using such a deletion mutant have shown this not to be the case (41a).

The fourth alternative is that ICP4 is able to activate transcription from the same promoter by alternative means, only one of which requires sequence-specific DNA binding. This is an attractive possibility, given the size and complexity of the ICP4 molecule and the observation that many promoters that respond to ICP4 do not appear to have ICP4 binding sites in their vicinity. Further support of this compromise option requires greater understanding of how ICP4 interacts both with DNA and the cellular transcriptional machinery.

Another hypothesis to explain the lack of effect of removal of the ICP4 binding sites I, II, and III is that a cellular inhibitor is normally located on these sites and is displaced by ICP4 (that is, in the absence of the inhibitor binding sites, ICP4 is not required). If this were the case, then the gD promoter without the ICP4 binding sites should be constitutively active, a prediction contradicted by the observation that virus mu31 does not transcribe gD in the presence of cycloheximide.

These arguments illustrate that the mode of action of ICP4 is still unknown. However, our current working model is that DNA binding by ICP4 is required for transcriptional activation, but that the orientation of the binding site and its precise distance from the promoter are not critical. This model essentially suggests that ICP4 acts as a *trans*-acting enhancer, perhaps associating with one or several host transcription factors and then bringing them to the template via its affinity for DNA. Like that of a *cis*-acting enhancer, the function of such complexes might be expected to be position and orientation independent. Since relatively weak ICP4 binding sites appear to occur quite frequently in viral DNA (see, for example, references 8, 20, 28, and 29), a consequence of this model is that removal of any one site would have little or no effect since its function would be replaced by another. Indeed, we have evidence which identifies previously undetected ICP4 binding sites in the gD gene, which might substitute for those removed. However, given the frequency of occurrence of ICP4 binding sites, it would be impossible to remove all of them from the genome (or from the indicator plasmids used in transfection assays) to test this model directly.

Given the complexities in the arguments presented above, further elucidation of the mechanism of transcriptional activation by ICP4 will probably require an efficient in vitro system using purified components.

ACKNOWLEDGMENTS

We are grateful to John McLaughlan for oligonucleotide synthesis, Joan Betz for plasmid pJB39, Kent Wilcox for ICP4 fraction VIII, and Stuart Welsh, Betsy Litman, Veronica Feenstra, Margaret Howes, and Joanne Duncan for excellent technical assistance.

This work was supported by the Medical Research Council Virology Unit (R.D.E.), Public Health Service grants AI21402 and AG07347 (L.I.P.), the Medical Research Council of Canada (J.R.S.), and the National Cancer Institute of Canada (D.C.J.). D.C.J. is a Scholar and J.R.S. is a Terry Fox Senior Scientist of the National Cancer Institute of Canada.

REFERENCES

1. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83**:4016–4020.

2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell **12**:721–732.
3. **Betz, J. L., T. M. Hill, L. I. Pizer, M. L. Peake, and J. R. Sadler.** 1983. Transcription from the *Bam*HI J fragment of herpes simplex virus type 1 (KOS). J. Virol. **47**:238–243.
4. **Cheung, A. K.** 1989. DNA nucleotide sequence of the immediate-early gene of pseudorabies virus. Nucleic Acids Res. **17**: 4637–4646.
5. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56**:558–570.
6. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense polypeptides. Nucleic Acids Res. **15**:4491–4511.
7. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62**:732–743.
8. **DiDonato, J. A., J. R. Spitzner, and M. T. Muller.** 1991. A predictive model for DNA recognition by the herpes simplex virus protein ICP4. J. Mol. Biol. **219**:451–470.

8a. **Disney, G. H., J. Tyler, and R. D. Everett.** Unpublished data.

9. **Everett, R. D.** 1983. DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lie within 83 bp of the RNA capsites. Nucleic Acids Res. **11**:6647–6666.
10. **Everett, R. D.** 1984. A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early gene products are not early-gene specific. Nucleic Acids Res. **12**:3037–3056.
11. **Everett, R. D.** 1986. The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate gene expression in trans. J. Gen. Virol. **67**:2507–2513.
12. **Everett, R. D.** 1987. The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. Anticancer Res. **7**:589–604.
13. **Everett, R. D.** 1988. Promoter sequence and cell type can dramatically affect the efficiency of transcriptional activation induced by herpes simplex type 1 and its immediate-early gene products Vmw175 and Vmw110. J. Mol. Biol. **203**:739–751.
14. **Everett, R. D., T. Paterson, and M. Elliott.** 1990. The major transcriptional regulatory protein of herpes simplex virus type 1 includes a protease resistant DNA binding domain. Nucleic Acids Res. **18**:4579–4585.
15. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **14**:6067–6083.
16. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
17. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
18. **Grundy, F. J., R. P. Baumann, and D. J. O'Callaghan.** 1989. DNA sequence and comparative analysis of the equine herpesvirus type 1 immediate-early gene. Virology **172**:223–236.
19. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. **26**:365–369.
20. **Imbalzano, A. N., A. A. Sheperd, and N. A. DeLuca.** 1990. Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. J. Virol. **64**:2620–2631.
21. **Johnson, D. C., G. Ghosh-Choudhury, J. R. Smiley, L. Fallis, and F. L. Graham.** 1988. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology **164**:1–14.
22. **Kattar-Cooley, P., and K. W. Wilcox.** 1989. Characterization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. J. Virol. **63**:696–704.
23. **Kristie, T. M., and B. Roizman.** 1986. α 4, the major regulatory protein of herpes simplex type 1, is stably and specifically associated with promoter-regulatory domains of α genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83**:3218–3222.
24. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.

24a. **Ligas, M. W., and D. C. Johnson.** Unpublished data.

24b. **McGeoch, D.** Personal communication.

25. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete nucleotide sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
26. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer.** 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res. **14**:1727–1764.
27. **Metzler, D. W., and K. W. Wilcox.** 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329–337.
28. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex virus major regulatory protein to viral DNA. Proc. Natl. Acad. Sci. USA **86**:9808–9812.
29. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein 4 of herpes simplex virus. Science **239**:1531–1534.
30. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. **61**:858–865.
31. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
32. **Panning, B., and J. R. Smiley.** 1989. Regulation of cellular genes transduced by herpes simplex virus. J. Virol. **63**:1929–1937.
33. **Paterson, T., and R. D. Everett.** 1988. The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Res. **16**: 11005–11025.
34. **Paterson, T., and R. D. Everett.** 1988. Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. Virology **166**:186–196.
35. **Paterson, T., V. G. Preston, and R. D. Everett.** 1989. A mutant of herpes simplex virus type 1 immediate-early polypeptide Vmw175 which binds to the capsite of its own promoter in vitro yet fails to autoregulate in vivo. J. Gen. Virol. **71**:851–861.
36. **Pizer, L. I., R. D. Everett, D. G. Tedder, M. Elliott, and B. Litman.** 1991. Nucleotides within both proximal and distal parts of the consensus sequence are important for specific DNA recognition by the herpes simplex virus regulatory protein ICP4. Nucleic Acids Res. **19**:477–483.
37. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *ts*K. J. Virol. **29**:275–284.
38. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62**:4307–4320.
39. **Roizman, B., and A. E. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
40. **Shepard, A. A., and N. A. DeLuca.** 1991. A second-site revertant of a defective herpes simplex virus ICP4 protein with restored regulatory activities and impaired DNA-binding properties. J. Virol. **65**:787–795.
41. **Shepard, A. A., A. N. Imbalzano, and N. A. DeLuca.** 1989. Separation of primary structural components conferring auto-

regulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **63**:3714–3728.

41a. **Smiley, J. R.** Unpublished data.

42. **Smiley, J. R., J. Duncan, and M. Howes.** 1990. Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS. J. Virol. **64**:5036–5050.

43. **Smiley, J. R., B. S. Fong, and W.-C. Leung.** 1981. Construction of a double-jointed herpes simplex viral DNA molecule: inverted repeats are required for segment inversion, and direct repeats promote deletions. Virology **113**:345–362.

44. **Smiley, J. R., C. Smibert, and R. D. Everett.** 1987. Expression of a cellular gene cloned in herpes simplex virus: rabbit beta-globin is regulated as an early viral gene in infected fibroblasts. J. Virol. **61**:2368–2377.

45. **Southern, E.** 1975. Detection of specific DNA sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

46. **Tedder, D. G., R. D. Everett, K. W. Wilcox, P. Beard, and L. I. Pizer.** 1989. ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. J. Virol. **63**:2510–2520.

47. **Tedder, D. G., and L. I. Pizer.** 1988. Role for DNA-protein interaction in activation of the herpes simplex virus glycoprotein D gene. J. Virol. **62**:4661–4672.

48. **Vlcek, C., V. Paces, and M. Schwyzer.** 1989. Nucleotide sequence of the pseudorabies virus immediate early gene, encoding a strong trans-activator protein. Virus Genes **2**:335–346.

49. **Wagner, E. K.** 1985. Individual HSV transcripts: characterisation of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Press, New York.

50. **Watson, R. G., A. M. Colberg-Poley, C. J. Marcus-Sekura, B. J. Carter, and L. W. Enquist.** 1983. Characterization of the herpes simplex virus type 1 glycoprotein D mRNA and expression of this protein in Xenopus oocytes. Nucleic Acids Res. **11**:1507–1522.

51. **Wu, C. L., and K. W. Wilcox.** 1990. Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA binding protein. Nucleic Acids Res. **18**:531–538.

52. **Wu, C. L., and K. W. Wilcox.** 1991. The conserved DNA-binding domains encoded by the herpes simplex virus type 1 ICP4, pseudorabies virus IE180, and varicella-zoster virus ORF62 genes recognize similar sites in the corresponding promoters. J. Virol. **65**:1149–1159.

53. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.