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DNA Binding by the Herpes Simplex Virus Type 1 ICP4 Protein Is Necessary for Efficient Down Regulation of the ICP0 Promoter

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The herpes simplex virus type 1 ICP4 and ICP0 polypeptides are immediate-early proteins that positively and negatively regulate expression of other viral genes in *trans*. ICP4 has recently been shown to bind DNA bearing the consensus sequence 5′-ATCGTCNNNN(T/C)CG(A/G)C-3′, present upstream of a number of viral genes. To test the hypothesis that this DNA-binding activity is involved in ICP4-mediated gene regulation, site-specific mutagenesis was employed to mutate the version of this sequence in the promoter of the ICP0 gene. The mutation eliminated detectable binding of ICP4 to the promoter as measured in vitro by a gel electrophoresis band shift assay. The ability of the mutated ICP0 promoter to direct synthesis of a reporter gene was also investigated in a transient transfection assay. Whereas ICP4 was found to transactivate the wild-type ICP0 promoter two- to threefold, the mutated promoter was transactivated seven- to ninefold. In assays containing the ICP0 transactivator gene, ICP4 down regulated the wild-type promoter far more efficiently than the mutated promoter. Finally, both the wild-type and mutated ICP0 promoters exhibited a similar response to ICP4 in transfections that included a vector expressing the viral transactivator protein VP16. These experiments suggest that the sequence-specific DNA-binding activity of ICP4 is an essential element of its role as a negative regulator of gene expression.

Studies on the replication cycle of herpes simplex viruses have revealed a temporally regulated cascade of viral gene expression (6, 20). Of the many proteins produced during infection, four have been shown to directly effect viral gene expression. Three of these proteins, ICP4, ICP0, and ICP27, are members of the immediate-early class, whereas VP16 is a late viral gene product. The VP16 polypeptide (α-TIF, Vmw65), contained in the virion, is carried into the newly infected cell, associates with host proteins, and mediates transactivation of the viral immediate-early genes (1, 5, 32, 44). ICP4 (Vmw175) is essential for productive infection and appears to be the major viral regulatory protein (8, 10, 42, 57). ICP0 (Vmw110) is a potent transactivator of genes from a wide variety of sources (13, 15, 37, 38, 45, 49, 55). ICP27 (Vmw63) has been shown to be involved in both positive and negative gene regulation of viral early and late genes (3, 12, 31, 46, 48, 51).

Mutational analysis of the ICP4 gene has demonstrated the central role of this polypeptide in the viral growth cycle. Temperature-sensitive and deletion mutants in ICP4 usually fail to adequately express early and late functions while overexpressing immediately early functions (7–9, 42, 50, 57). Results from a number of laboratories have demonstrated that ICP4 is capable of both positive and negative transcriptional regulation in transient expression assays, dependent on both the nature of the target promoter and the dose of ICP4 effector gene used (11, 15, 38, 45). Although the exact mechanisms underlying ICP4-mediated gene regulation are not yet understood, ICP4 protein can bind to a conserved sequence present in the 5′ flank of a number of viral genes, including the ICP0 promoter (2, 14, 23, 24, 35, 36).

To better define the biological role of the ICP4 DNA-binding activity, we created a mutation in the ICP4 recognized sequence, the "ICP4 box," in the ICP0 promoter. This mutation abolished detectable DNA binding in vitro. We found that, relative to the wild type, the mutated promoter was more strongly transactivated by ICP4 in transient assays. Furthermore, the negative regulatory effect of ICP4 DNA recognition was epistatic to the positive effect of ICP0 on the ICP0 promoter.

MATERIALS AND METHODS

Mutagenesis. All plasmids bearing a "68" designation bear two point mutations in the ICP0 promoter created by oligonucleotide-directed mutagenesis (25) with the synthetic 20-mer 5′-GCGGCAGTGAAGCTTCCCCC-3′ complimentary to the sense strand of ICP0 DNA positions 1639 through 1658 (numbering system of Perry et al. [41]). The two bases specifying mutations are underlined. Mutagenesis was performed on a fragment of the herpes simplex virus type 1 KOS strain ICP0 gene spanning positions 905 to 2508 cloned into M13mp18 between the *Sst*I and *Bam*HI sites. After mutagenesis, progeny phage were screened for the presence of a novel *Hin*dIII site, and the mutation was confirmed by direct DNA sequencing.

Plasmids. (i) p0WTCAT and p068CAT. A chloramphenicol acetyl transferase (CAT) expression cassette was prepared by digestion of pSV2CAT (18) with *Hin*dIII and *Bam*HI, repair of the staggered ends with T4 DNA polymerase, ligation of synthetic *Pst*I linkers, and cloning of the CAT gene fragment into the *Pst*I site of the pTZ19R polylinker (33). The *Sst*I (nucleotide 905)-to-*Nco*I (nucleotide 1862) fragment from either the wild-type promoter or the mutated ICP0 promoter was then cloned into this plasmid to yield p0WTCAT or p068CAT, respectively (Fig. 1B). During this cloning operation the ICP0 *Nco*I sites were converted to *Xba*I sites by the addition of synthetic linkers, and the fragments were cloned into the polylinker *Sst*I and *Xba*I sites upstream of the CAT gene. To ensure that the CAT gene translational start codon was the first encountered, the ICP0 *Nco*I sites were first rendered blunt ended by digestion with mung bean nuclease and repaired with T4 DNA poly-

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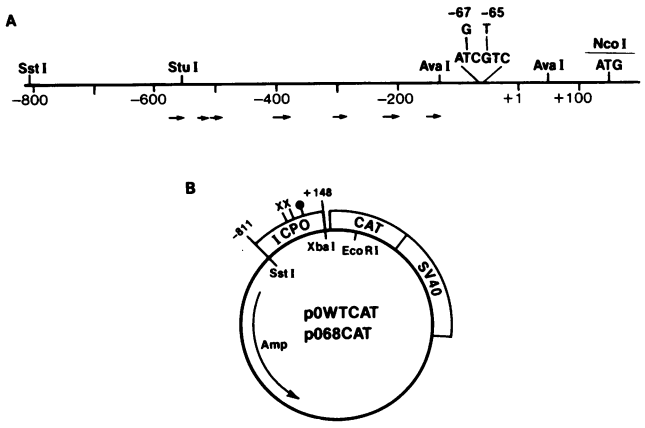

FIG. 1. (A) Physical map of the ICP0 upstream region. The numbers below the line indicate nucleotide positions relative to the 5′ cap site of ICP0 mRNA at position +1. The locations of various restriction endonuclease recognition sites referred to in the text are indicated above the line, as is the position of the ICP4 box and mutation created in this promoter. The arrows below the line indicate the locations and directions of the TAATGARAT homologies implicated in response to the viral transactivator protein VP16 (22, 26, 43, 59). (B) Representation of the plasmids p0WTCAT and p068CAT. These plasmids contain ICP0 promoter sequences from the *Sst*I site at position −805 to the *Nco*I site at +148 linked to the bacterial CAT gene and SV40-derived splice and polyadenylation signals. The relevant restriction endonuclease recognition sites, location of the mutation described here (X), and position of the 5′ end of ICP0 mRNA (●) are shown.

merase before attachment of the *Xba*I linkers. The *Nco*I-*Xba*I junctions were sequenced by the method of Maxam and Gilbert (30) from the nearby EcoO109 site at position 1824. Both p0WTCAT and p068CAT retained the first C of the *Nco*I overhang but had lost the ATG.

(ii) p68α0. The wild-type 6.2-kilobase genomic *Sst*I-*Pst*I fragment encoding the KOS ICP0 gene was cloned from viral DNA into the pUC18 *Sst*I and *Pst*I sites. The 1.6-kilobase *Sst*I-*Bam*HI fragment containing the ICP0 gene upstream region was then excised and replaced with the corresponding fragment from the M13 phage bearing the two-base mutation in the ICP0 promoter.

(iii) pMEX4. pGX58 (54) was digested with *Pst*I and *Dra*I, *Hin*dIII linkers were added to the *Dra*I site, and the 7.2-kilobase fragment encoding ICP4 was cloned into the *Pst*I and *Hin*dIII sites of pUC18. This plasmid encodes the herpes simplex virus type 1 strain 17 ICP4 gene.

(iv) pMSVP163′δβ58N. pMSVP163′δβ58N expresses the KOS VP16 gene under the regulatory sequences of the murine sarcoma virus long terminal repeat. This construct was the generous gift of S. Triezenberg and S. McKnight (Carnegie Institution).

Cells, transfections, and CAT assays. Vero cells were routinely grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. Freshly confluent cells were split 1 to 4 the day before transfection. For CAT assays, transfections were performed on 60-mm Nunc plates by the calcium phosphate procedure (19) without an osmotic "boost." Transfection mixtures contained 0.25 μg of CAT plasmid DNA and additional plasmids as indicated. All transfection mixtures were brought to a constant amount of plasmid DNA per experiment by the addition of pUC18. Cells were harvested 44 or 48 h after transfection. CAT activity was determined by thin-layer chromatography as described previously (18) and was normalized to the protein content of each extract (4). Percent acetylation was determined by excision of the monoacetylated spot from the chromatograph and subsequent scintillation counting. In general, acetylation reactions were run for 30 min and always resulted in modification of less than 20% of the input [^{14}C]chloramphenicol. Each point represents the average of two independent determinations.

Band mobility shift assays. An extract enriched for ICP4, termed fraction VI, was prepared from 14-h herpes simplex virus type 1 strain KOS (obtained from Y. C. Cheng, Chapel Hill, N.C.)-infected Vero cells (multiplicity of infection, 5) exactly as described by Metzler and Wilcox (34). Probe DNA was prepared by excision of a 180-base-pair *Ava*I fragment bearing ICP0 promoter sequences from plasmid p0WTCAT or p068CAT. The DNA probe was end labeled with polynucleotide kinase and [γ-^{32}P]ATP. Binding reactions (20 μl) contained 1.5 ng of probe DNA (approximately 10,000 cpm for each probe) and 39 ng of protein extract in the buffer described by Kristie and Roizman (23). Reactions were analyzed on 6% polyacrylamide–0.2% bisacrylamide gels equilibrated in TBE buffer (28) and electrophoresed at room temperature.

RESULTS

Construction of a dual point mutation in the ICP4 box of the ICP0 promoter. Faber and Wilcox (14) have shown that DNA bearing a version of the consensus sequence ATCGTCNNNN(T/C)CG(A/G)C can form stable complexes with ICP4 in vitro (also see reference 35). Figure 1A presents landmarks in the ICP0 regulatory region and illustrates that an ICP4 box is located at position −68 (+1 taken as nucleotide 1716 in the numbering system of Perry et al. [41]). To create a mutation in this promoter which would abolish the DNA-binding activity of ICP4, two bases in the ATCGTC core recognition region were mutated by oligonucleotide-directed mutagenesis, converting this sequence to AGCTTC (see Materials and Methods). This region of the core sequence was chosen to avoid disruption of a putative SP1-binding site (21), which overlaps the downstream portion of the ICP4 box in this promoter. After mutagenesis, the promoter was sequenced from positions −310 to +150 to both confirm the mutation and ensure the absence of untargeted mutations. Although no untargeted mutations were detected, the KOS strain ICP0 promoter did exhibit a single nucleotide polymorphism with the published strain F sequence (27), a G/C-to-A/T transition at position +122.

ICP4 does not form a stable complex with the mutated ICP0 promoter in vitro. ICP4 binding to DNA was tested in a band mobility shift assay. A nuclear extract of 14-h infected Vero cells was incubated with a ^{32}P-end-labeled 180-base-pair *Ava*I restriction fragment (Fig. 2A) bearing the ICP4 recognized sequence from the ICP0 promoter (positions −128 to +54). As increasing amounts of unlabeled poly(dI)-poly(dC) competitor DNA were added to reaction mixtures containing the wild-type probe, a single stable complex was detected (Fig. 2A). No complexes were detected when the analogous fragment from the mutated ICP0 promoter was substituted in the assay. Evidence supporting the contention that ICP4 participates in the complex formed with the wild-type DNA probe is twofold. First, a similar extract of uninfected cells failed to produce this band with either the wild type or mutant probe (Fig. 2B). Second, the addition of the ICP4-specific 58S monoclonal antibody (53) to a binding reaction led to a further reduction in the electrophoretic migration of the complex. No change in complex migration was detected

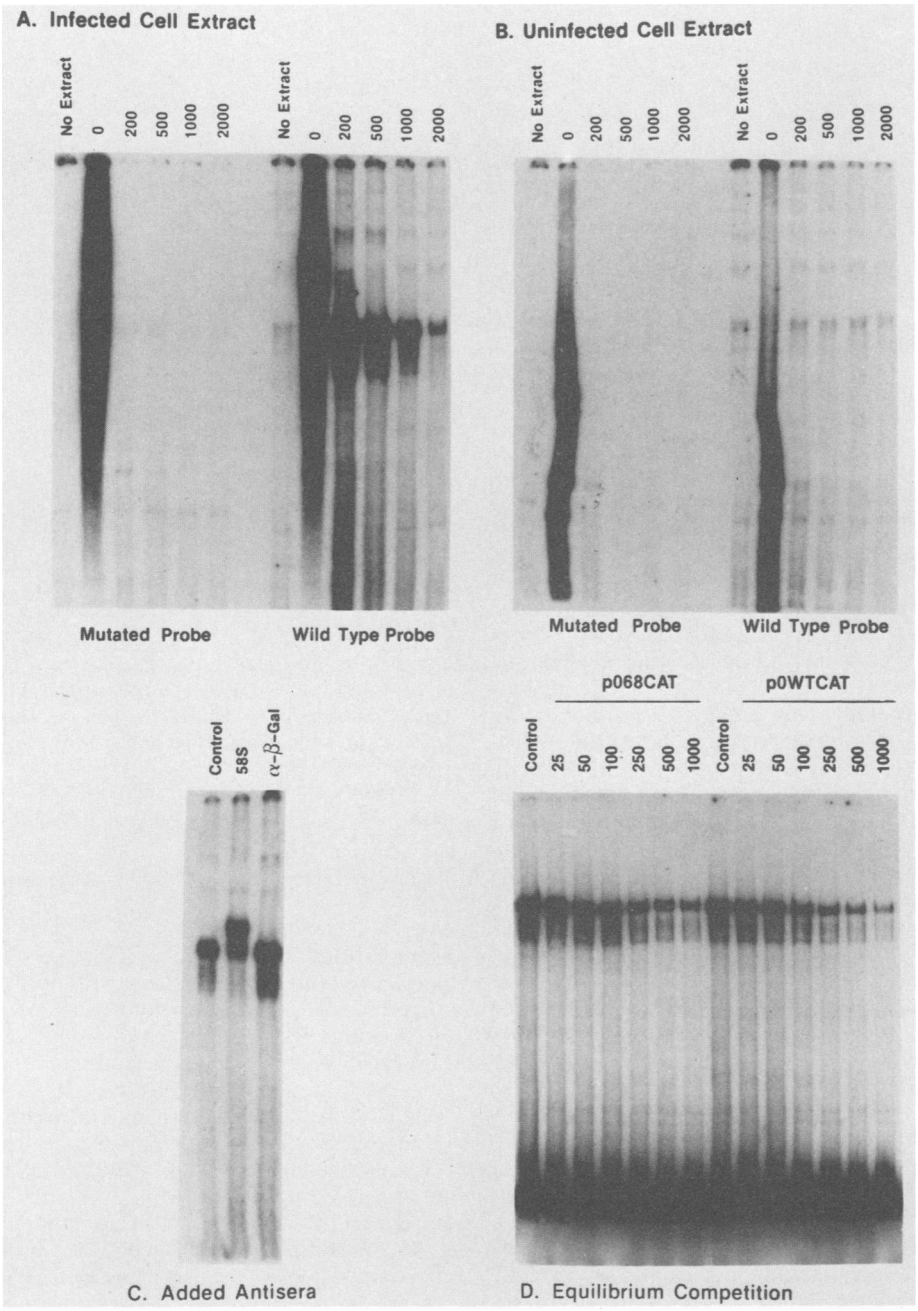

FIG. 2. Characterization of DNA-binding activities recognizing positions −128 to +54 of the ICP0 promoter by DNA band mobility shift assay. (A) Binding reactions contained ^{32}P-labeled DNA derived from either the wild-type or mutated promoter, infected-cell extract, and the indicated weight excess of poly(dI)-poly(dC) competitor DNA. (B) The experiment is identical to that in A, but with substitution of mock-infected cell extract. (C) Band shift assays contained infected cell extract, wild-type probe DNA, a 1,000-fold weight excess of poly(dI)-poly(dC), and 7 ng of purified immunoglobulin G monoclonal antibody 58S or anti-β-galactosidase (Promega Biotec Co.) as indicated. (D) Reactions contained wild-type probe DNA, a 1,000-fold weight excess of poly(dI)-poly(dC), and the indicated amounts (nanograms) of *Sst*I-linearized plasmid p0WTCAT or p068CAT. The infected-cell extract was added last.

by inclusion of an irrelevant antibody (Fig. 2C). These results suggest that the double mutation in the ICP0 promoter strongly diminishes interaction of ICP4 with this sequence.

The mutated promoter exhibits increased transactivation by ICP4. To investigate the biological role of ICP4 recognition of the ICP0 promoter, wild-type and mutated promoter fragments were cloned adjacent to a reporter CAT gene cassette, creating vectors p0WTCAT and p068CAT, respectively (Fig. 1B). These constructs were then transiently introduced into Vero cells along with a plasmid that expresses ICP4 protein, and subsequent CAT gene activity was measured (Fig. 3). In the absence of additional factors, the wild-type and mutated ICP0 promoters directed synthesis of equivalent levels of CAT activity. As the gene dose of ICP4 was increased, the wild-type promoter exhibited a two- to threefold increase in activity, whereas the mutated promoter was elevated seven- to ninefold.

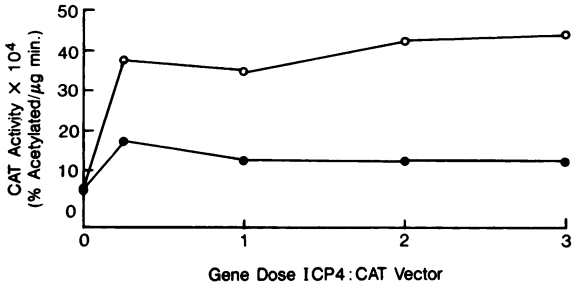

FIG. 3. Response of ICP0 promoter CAT vectors to ICP4. p0WTCAT (●) or p068CAT (○) plasmids were cotransfected with the indicated amounts of the ICP4 expression vector pMEX4, and the subsequent CAT activity of cell extracts was determined.

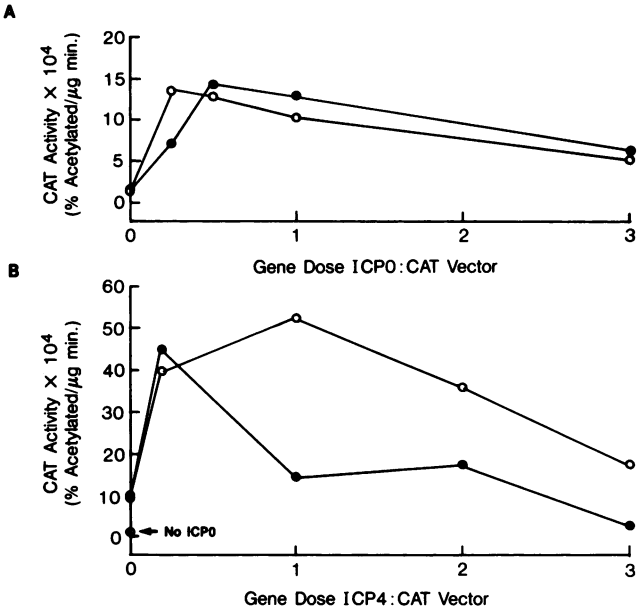

FIG. 4. Response of ICP0 promoter CAT vectors to ICP0 and ICP4. (A) p0WTCAT (●) or p068CAT (○) plasmids were cotransfected with the indicated amounts of ICP0 expression vector p68α0, and CAT activity of the cell extract was measured. (B) The same CAT expression plasmids were cotransfected with 0.25 gene equivalent of ICP0 expression vector p68α0 and various amounts of the ICP4 expression vector pMEX4. The two isolated points on the *y* axis indicate the activity derived from the CAT expression plasmids in the absence of ICP4 or ICP0.

Based on published sequences, the CAT reporter cassette, ICP0 promoter fragment, and pTZ19R bacterial cloning vector are all free of other ICP4 consensus sites. It is therefore unlikely that these experiments are complicated by the presence of sequences in the test plasmids which ICP4 recognizes with affinity equal to that of the established recognition site. Although sequences near the plasmid replication origin do contain the ATCGTC core, Faber and Wilcox (14) did not detect stable ICP4 complexes with a restriction fragment bearing this sequence. To more thoroughly investigate whether there might be strong ICP4-binding sites in the reporter plasmids, we performed equilibrium competition assays in which unlabeled p0WTCAT or p068CAT plasmid DNA was added to binding reactions containing the ^{32}P-labeled wild-type ICP0 promoter fragment probe. Although both plasmids were able to compete in complex formation, the plasmid bearing wild-type ICP0 sequences was the more effective competitor (Fig. 2D). This suggests that the mutant plasmid does not have a site of affinity equal to that of the ICP0 regulatory region. We conclude that ICP4 does not strongly recognize the mutated CAT expression vector, and that the absence of this interaction results in increased ICP0 promoter activity.

ICP4 DNA recognition results in negative regulation of the ICP0 promoter in the presence of ICP0. The ICP0 promoter is responsive to a number of virus-encoded regulatory proteins, including ICP0 itself (16). It was therefore of interest to explore the role of ICP4 DNA recognition in the presence of ICP0. First, it was necessary to determine that both the wild-type and mutated ICP0 promoters responded comparably to ICP0. The wild-type and mutant ICP0 promoter CAT vectors were each cotransfected with a vector that expresses ICP0, p68α0. At low levels of the ICP0 effector gene, both the wild-type and mutant ICP0 promoters were strongly transactivated, whereas at higher ICP0 doses, a slight diminution of activity was detected (Fig. 4A). The relevant observations, however, are that both the wild-type and mutant promoters respond positively to ICP0 and are indistinguishable in this response.

Next, transfection mixtures were prepared with either the wild-type or mutant ICP0 promoter CAT vector, 0.25 gene equivalent of the ICP0 expression vector, and various concentrations of ICP4 expression vector. The p68α0 expression vector directs ICP0 synthesis from the promoter bearing the two-base mutation in the ICP4 box. This vector was chosen to avoid potential repression of ICP0 expression by ICP4. The addition of 0.25 gene equivalent of ICP4 expression vector resulted in a 4- to 5-fold increase in the CAT activity over the 6- to 8-fold increase mediated by the ICP0 vector alone, resulting in an overall 30- to 50-fold transactivation of both templates (Fig. 4B). Workers in a number of laboratories have previously documented synergy between ICP0 and ICP4 in transactivation of other promoters (11, 13, 15, 29, 38, 45, 51, 52). As the concentration of ICP4 gene was increased, however, the wild-type ICP0 promoter was down regulated while the mutant maintained high levels of CAT gene expression. These results suggest that in the presence of ICP0, increasing amounts of ICP4 lead to down regulation of the ICP0 promoter. Moreover, this down regulation is mediated by the ICP4 box in the ICP0 promoter.

ICP4 DNA recognition does not influence VP16-mediated transactivation of the ICP0 promoter. The ICP0 promoter responds positively to the virion transactivator protein VP16. To investigate whether ICP4 sequence-specific DNA-binding activity influences the activity of VP16, we first determined that both the wild-type and mutant ICP0 promoters responded similarly to VP16. The wild-type and mutant ICP0 promoter CAT plasmid were each cotransfected with various amounts of the VP16 expression vector pMSVP163′δβ58N. Both templates exhibited an equal positive response to VP16 (Fig. 5A). Transfection mixtures were then programmed with either the wild-type or mutant ICP0 promoter CAT vector, 0.4 gene equivalent of VP16 expression vector, and various levels of ICP4 expression vector. In contrast to cotransfection with the ICP0 expression plasmid, in the presence of VP16 both templates exhibited the same response to ICP4: a slight diminution of activity at high ICP4 gene concentrations (Fig. 5B). These data indicate that the ICP4 DNA-binding activity has little influence on VP16-mediated transactivation of the ICP0 promoter.

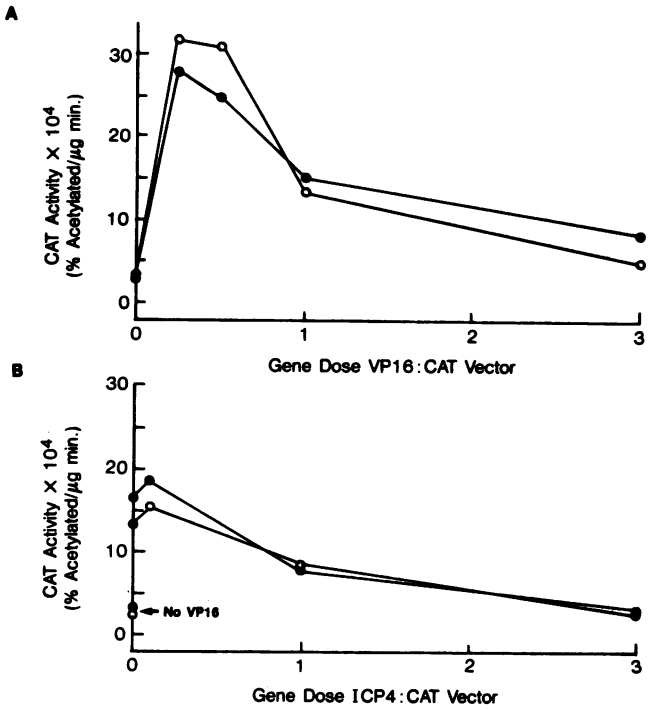

FIG. 5. Response of ICP0 promoter CAT vectors to VP16 and ICP4. (A) p0WTCAT (●) or p068CAT (○) plasmids were cotransfected with various amounts of plasmid pMSVP163'δβ58N, a vector which expresses the virion transactivator protein VP16, and the CAT activity of cell extracts was determined. (B) Cells were cotransfected with the same CAT expression vectors, 0.4 gene equivalent of pMSVP163'δβ58N, and the indicated amounts of the ICP4 expression vector pMEX4. The two isolated points on the *y* axis indicate the CAT activity in extracts of cells transfected with the ICP0 promoter CAT plasmids without VP16 or ICP4 expression vectors.

DISCUSSION

Recent research suggests that the domains of ICP4 involved in DNA binding are also important for transcriptional regulation. In a transient assay format, Paterson and Everett (40) found that small insertions into domains of ICP4 involved in DNA binding also disrupt the regulatory functions of the protein. DeLuca and Schaffer (9) reported that C-terminal truncations of ICP4 that eliminate sequence-specific DNA binding correlate with the ability to properly regulate ICP0 transcription. To test the premise that ICP4 binding to the ICP0 promoter is involved in regulation, we used site-specific mutagenesis to alter two bases in the ICP4 box of the ICP0 promoter. This mutation was sufficient to eliminate detectable DNA binding by ICP4 in vitro. In a transient assay system, both wild-type and mutated promoter sequences directed equivalent levels of transcription in the absence of additional factors. In the presence of an ICP4 expression vector, both promoters were transactivated, but the mutated promoter exhibited a two- to fivefold greater response (Fig. 3). Both promoters were stimulated by ICP0 alone. In the presence of ICP0, ICP4 DNA binding appeared necessary for efficient down regulation of the ICP0 promoter (Fig. 4), suggesting that the negative effect of ICP4 DNA binding is epistatic to the positive effect of ICP0. All of these effects are reflected in steady-state levels of correctly initiated transcripts (data not shown). Taken together, these experiments suggest that the DNA-binding activity of ICP4 is an essential element of its ability to down regulate transcription.

Over the first 3 to 4 h of infection, the transcription rate of the ICP0 promoter increases continuously, whereas the ICP4 promoter declines in activity (17, 58). This decline in ICP4 transcription rate is dependent on protein synthesis (58). The observations that, in the presence of the VP16, ICP4 effects only about a twofold reduction of ICP0 promoter activity (Fig. 5) and that DNA binding does not appear to influence this effect may relate to the dissimilar patterns of regulation of the ICP0 and ICP4 promoters. This difference in regulation could be explained if the ability of ICP4 to interfere with transactivation of the ICP4 promoter (39) were stronger than that observed here for the ICP0 promoter. Such a model may prove too simplistic, however, because other factors such as ICP27 may also influence immediate early gene regulation (3, 31, 46, 48, 51).

After completion of the present studies, Roberts et al. (47) reported that deletion of two bases in the ICP4 box of the ICP4 promoter reduced the ability of ICP4 to autoregulate this promoter, whereas Teddor and Pizer (56) found that deletion of an ICP4 consensus sequence from the glycoprotein D promoter reduced ICP4-mediated transactivation of this promoter by twofold. We have found a third pattern: ICP4 binding to its cognate sequence prevents full transactivation of the ICP0 promoter. Perhaps the alternative responses of these three promoters to DNA binding by ICP4 result from the different position of the ICP4 responsive sequences in these promoters, affinity of ICP4 for these versions of the consensus binding sequence, or promoter-specific interactions with other transcription factors.

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