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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

M. SCOT ROBERTS,[1] ANNE BOUNDY,[2] PETER O'HARE,[2] MARIE C. PIZZORNO,[1] DOLORES M. CIUFO,[1] AND GARY S. HAYWARD[1]*

The Virology Laboratories, Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205,[1] and Marie Curie Memorial Foundation Research Institute, Oxted, Surrey RH8 0TL, United Kingdom[2]

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In transient-expression assays, the IE175 (α4) promoter region of herpes simple virus is down-regulated after cotransfection with DNA encoding its own protein product (IE175 or ICP4). The inhibition by IE175 proved to be highly specific for its own promoter region and did not act on either the herpes simplex virus type 1 IE110 (α0) or human cytomegalovirus major immediate-early promoters. Furthermore, the inhibition was still exhibited by IE175 effector plasmids driven by strong heterologous promoters and therefore must be a direct autoregulatory response that cannot be explained by promoter competition effects. In gel mobility retardation assays with infected-cell nuclear extracts, a prominent and specific DNA-protein complex was formed with DNA fragments containing sequences from −108 to +30 in the IE175 promoter region. This activity was not present in mock-infected samples. Even stronger binding occurred with a fragment containing sequences from −128 to +120 in the IE110 promoter, but this second locus was not associated with any detectable response phenotype in cotransfection assays. Supershift experiments with an anti-IE175 monoclonal antibody confirmed the presence of the IE175 protein in both DNA-protein complexes. In the IE175 promoter, specific binding correlated closely with the presence of an intact autoregulatory signal near the cap site as judged by the loss of both activities in a 3′-deleted promoter fragment lacking sequences from −7 to +30. Insertion of a cloned 30-mer synthetic oligonucleotide sequence from positions −8 to +18 in IE175 restored both IE175 binding activity and the down-regulation phenotype. Direct shift-up assays with a similar 30-base-pair (bp) oligonucleotide containing 21 bp from positions −75 to −55 of IE110 (which encompasses a consensus ATCGTC motif) also produced a specific DNA-protein complex containing the IE175 protein. This ATCGTC motif proved to be a necessary component of both the IE110 and IE175 binding sites, but was insufficient on its own for complex formation. Finally, deletion of 2 bp from positions −3 and −4 within the ATCGTC sequence in the IE175 cap site region abolished both binding activity and the IE175-dependent autoregulation phenotype.

At least five immediate-early (IE) genes have been defined in herpes simplex virus (HSV) DNA on the basis of synthesis of new mRNA species after infection in the absence of de novo protein synthesis. The HSV IE genes all respond specifically to transcriptional stimulation by a 65-kilodalton virion factor ($V_{MW}65$, VF65, or αTIF) that is active in infected cells even in the presence of protein synthesis inhibitors (2, 3, 7, 49). Expression of the IE genes is controlled by four distinct IE promoter-regulatory domains (32). Two of them occupy a 700-base-pair (bp) divergent region surrounding the *ori*-S replication origin within the S segment inverted repeats (33, 37, 61). One drives the expression of the 4.2-kilobase (kb) mRNA for the IE175 (ICP4) protein, and the other drives that of two distinct 1.8-kb mRNA species encoding either the IE68 (ICP22) or IE12 (ICP47) protein. The other two recognized IE control regions contain unidirectional promoters of 300 to 600 bp in length, driving the expression of a spliced 3.2-kb mRNA encoding the IE110 (ICP0) protein mapping within the L-segment inverted repeats (5, 45) or of a 1.8-kb mRNA species encoding the IE63 (ICP27) protein mapping in U_L (63). All of the IE promoters have in common multiple copies of a consensus response element commonly referred to as the TAATGARAT-box (26, 29, 33, 51, 63), plus, in some cases, an overlapping consensus octamer element, ATGCTAAT (39; C. apRhys, D. M. Ciufo, E. A. O'Neill, T. J. Kelly, and G. S. Hayward, submitted for publication). In addition, 11 CCCGCCC elements, which appear to be associated with basal expression properties of the IE175-IE68 region, have been shown by DNA footprinting studies to bind to the purified cellular Sp-1 factor (25).

During HSV infection, the IE175 gene product is essential for progression of the lytic cycle (8, 50, 60) and for activation of the transcription of the delayed-early (DE) class of viral genes (30, 31, 46, 50). The isolated viral DE and late promoters, whether associated with their own genes (11, 55) or as hybrid HSV promoter-driven reporter genes, also respond to virus superinfection in an IE175-dependent fashion, both when in an integrated state in permanent DNA-transfected cell lines (6, 9, 11, 12, 35, 53) and in transient-expression assays with superinfecting virus (14, 40). In DNA cotransfection assays, the isolated IE175 gene encodes a *trans*-acting factor that stimulates expression of HSV DE promoter targets (13, 21, 41, 52) and inhibits expression from its own promoter in IE175-CAT (9, 22, 42, 43). Although the HSV IE110 gene product was shown to stimulate expression

* Corresponding author.

from both DE promoters and the IE175 promoter in transient assays, this effect is nonspecific and also acts on heterologous viral and cellular promoters (44). Neither the functional significance nor the precise mechanism and target site for the activity of the IE110 protein is understood at present. The IE110 gene is apparently not essential for lytic-cycle growth at high multiplicities of infection (54, 58), and its physiological role may be more significant for reactivation from the latent state (57) or for initiating infection at low multiplicities of infection.

We showed previously that inhibition of expression from the IE175 promoter by cotransfection with an intact IE175 effector gene dominates over VF65 or IE110 *trans*-activation and requires only the basal region (−105 to +30) of the IE175 promoter (42, 43). However, those studies did not establish whether the other IE promoters might also be subject to down-regulation, nor did they directly exclude promoter-specific competition effects when measuring basal-level shut-off. The IE175 protein itself has been reported by Kristie and Roizman (27, 28) to be present in DNA-protein complexes formed between extracts of infected HeLa cells and the 5′ upstream promoter regions of several HSV genes including IE175, IE110, IE63, gpC, and TK. Furthermore, Faber and Wilcox (15) showed by McKay immunoprecipitation and DNA footprinting studies that the partially purified IE175 protein protects specific DNA sequences in the HSV gD promoter regions and in the pBR322 tetracycline resistence gene, although they failed to detect binding to the TK and IE63 promoters. More recently, Muller (36) and Faber and Wilcox (16) have described an IE175 binding site near the cap site in the IE175 promoter, but they dispute the claims by Kristie and Roizman for a far-upstream site. No clear consensus has yet emerged on whether IE175 binds directly to specific DNA target sites or requires intermediate cellular DNA-binding factors (18).

In the studies reported here, we set out to (i) define whether the IE110 promoter-regulatory region also contains negative response signals for the IE110 or IE175 *trans*-acting factors; (ii) examine further the target promoter specificity for IE175 inhibition and determine whether the down-regulation of its own promoter represents a true autoregulation effect; (iii) identify the target DNA sequence required for IE175-mediated down-regulation; and (iv) search for evidence for DNA-binding activities in infected and uninfected Vero cell nuclear extracts that may be associated with the autoregulatory response.

MATERIALS AND METHODS

Construction of IE110-CAT and IE68-CAT gene plasmids. The hybrid HSV-1 IE target genes IE175(−1900/+30)-CAT and IE175(−380/+30)-CAT, HSV-2 DE38(−420/+50)-CAT, and human cytomegalovirus (HCMV) IE68(−760/+10)-CAT in plasmids pPOH2, pPOH13, pPOH1, and pCAT760wt, respectively, have been described previously (40, 43, 48). To make an HSV-1 IE110-CAT hybrid test gene containing the intact HSV-1(KOS) IE110 promoter, the pIGA15 effector plasmid was digested with BAL 31 from the *Bam*HI site in the large intron in the IE110 coding region, and *Bam*HI linkers were added. A deleted clone lacking the *Nco*I site at +150 was selected and sequenced, and the resulting fragment, which encompassed sequences between the *Bam*HI linker at +120 in the leader sequence and an upstream *Hin*dIII linker outside the original *Sac*I site at −800, was inserted into pCATB′ to give a functional IE110-CAT gene (pGH83). A second version of IE110-CAT contained all of the leader sequences up to the *Nco*I site at +150 fused to a *Sal*I linker and inserted in front of a CAT coding-region cassette (pPOH49). To construct the HSV-1 IE68-CAT hybrid gene a 540-bp *Sau*3A fragment from within the *Hin*dIII-to-*Bam*HI sequence of HSV-1(MP) DNA in plasmid pGH12 was inserted into the *Bam*HI site in pKP54 to make pKR46 (a gift from Dan Rawlins). This fragment was then moved as a *Sal*I-*Xba*I fragment into pCATB′. The resulting plasmids, pGH77a and pGH77b, contain the IE68 upstream promoter-leader region sequences from −420 to +120 placed either in the forward or backward orientation in front of the CAT coding region.

Variant IE175-CAT target reporter genes. To prepare an IE175-CAT target plasmid that included most of the long IE175 leader sequence, an HSV-1(KOS) DNA fragment from p*Xho*I-C containing the sequence between *Eco*RI at −108 and *Sal*I at +178 was inserted first into a version of pUC18 with an additional *Bgl*II site between two *Hin*dIII sites in the polylinker (pGH56). The 150-bp region from *Bam*HI at +30 to *Bgl*II at +178 was then inserted in both orientations at the *Bam*HI site inside the leader sequence in the IE175(5′ −380/+30)-CAT gene in pPOH13. The resulting plasmid, pGH106a, contains all IE175 sequences from −380 to +178, whereas pGH106b contains all of the same sequences but with the leader region between +30 and +178 inverted.

A set of 3′-deleted versions of the IE175 promoter region were constructed by BAL 31 digestion of pGH12 DNA after linearization at the *Bam*HI site at +30. *Bam*HI linkers (CCCGGATCCGGG) were added, and selected clones were sequenced in the vicinity of the deletions. Finally the 1.8-kb *Bam*HI-to-*Sal*I CAT coding-region cassette from pCATB′ was added at the *Bam*HI linker site. The six resulting plasmids in this set (pPOH24, pPOH26, pPOH25, pPOH30, pPOH32, and pPOH31) contain all IE175 5′ upstream sequences from position −1900 down toward the cap site. To increase basal expression, the HSV sequences between *Hin*dIII at −1900 and *Bss*HII at −380 (43) were deleted from each of the six 3′ variants to create plasmids pPOH40, pPOH39, pPOH38, pPOH51, pPOH37, and pPOH50, respectively. A summary diagram comparing the structure of the relevant portions of each these 3′ variant plasmids with those of the parent undeleted forms is presented in Fig. 5b. Two additional mutations in the IE175 promoter target plasmids were prepared by partial cleavage of pPOH13 DNA with *Pvu*I followed by incubation with T4 DNA polymerase in the presence of exogenous nucleoside triphosphates, to create blunt ends, and rejoining in the presence or absence of *Bgl*II linkers (CAGATCTG). The structure of the resulting clones was confirmed by dideoxynucleotide DNA sequence analysis with a synthetic 19-bp oligonucleotide primer from within the CAT gene leader sequence annealed to alkali-denatured double-stranded DNA. Thus, plasmid pGH129 contains a 2-bp deletion at the *Pvu*I site at positions −3 and −4 in the IE175 promoter, and pGH128 contains the same deletion plus a single-copy 8-bp insert of the *Bgl*II linker sequence (see Fig. 5b).

Synthetic oligonucleotide binding sites. Two 30-bp single-stranded oligonucleotides, 5′-GATCCCGATCGTCCACA CGGAGCGCGGCTA-3′ (no. 18) and 5′-GATCTAGCCGCG CTCCGTGTGGACGATCGG-3′ (no. 19), were synthesized by S. Morrow, Department of Biochemistry, Johns Hopkins School of Hygiene, Baltimore, Md., and purified by high-pressure liquid chromatography procedures. The single-stranded oligonucleotides were radiolabeled by end-labeling with polynucleotide kinase and [γ-^{32}P]ATP. The two com-

plementary oligonucleotides were annealed by heating to 65°C in 50 mM NaCl–10 mM $MgCl_2$–25 mM Tris hydrochloride (pH 8.0)–100 μg of bovine serum albumin per ml–2 mM β-mercaptoethanol followed by slow cooling. For cloning, the double-stranded oligonucleotides were ligated after phosphorylation with polynucleotide kinase to *Bam*HI-plus-*Bgl*II-cleaved pGH59 DNA. The resulting plasmids, pGH123a and pGH123b, contained sequences from −8 to +18 in the HSV-1 IE175 promoter inserted in either orientation in the polylinker region of pKP54 vector DNA. The same pair of oligonucleotides (no. 18 and 19) were also inserted in both orientations into the *Bam*HI linker in pPOH38 to produce pGH145a and pGH145b. Another pGH 59-based plasmid (pGH66a), containing a 30-mer synthetic oligonucleotide binding site for the EBNA-1 protein, was used as a negative control. Three additional pairs of annealed 30-bp oligonucleotides were used directly without cloning; these included 5′-GATCCATGGGGGAATCGTCA CTGCCGCTGA-3′ (no. 47) and 5′-GATCTCAGCGGCAGT GACGATTCCCCCATG-3′ (no. 48), containing the sequence from positions −75 to −55 in the HSV-1 IE110 promoter; 5′-GATCCGACGCCCCGATCGTCCACACGGA GA-3′ (no. 90) and 5′-GATCTCTCCGTGTGGACGATCGG GGCGTCG-3′ (no. 91), containing the sequence from −13 to +11 in the HSV-1 IE175 promoter; and 5′-GATCCTCTAG AGTCATCGTCAACTGACTGA-3′ (no. 92) and 5′-GATCT CAGTCAGTTGACGATGACTCTAGAG-3′ (no. 93), containing the viral consensus ATCGTC sequence only.

Construction of new HSV IE effector plasmids. The plasmids p*Xho*I-C, pIGA15, pGR135, pGR162, and pGR169, which retain intact the HSV-1(KOS) IE175 gene, the HSV-1(KOS) IE110 gene, the HSV-2(333) VF65 gene, the HSV-1(MP) IE63 gene, and the HSV-1(MP) IE68 gene, respectively, were all described previously (41). To replace the IE175 promoter region in p*Xho*I-C with those of heterologous promoters, a 7.7-kb *Sal*I-*Xho*I fragment, containing the entire IE175 coding sequence (plus 3′ transcription signals) downstream from position +178 in the leader region, was inserted into the *Sal*I site of pGH56 to create plasmid pGH68. To construct an SV2-IE175 hybrid effector gene containing the simian virus 40 340-bp early promoter-enhancer region, pSV2-CAT DNA was first cleaved with *Bam*HI and *Hin*dIII and then rejoined after incubated with Klenow DNA polymerase to produce blunt ends for insertion of *Sal*I linkers (pPOH34). Subsequently, the IE175 coding region from pGH68 bounded by *Sal*I and *Eco*RI sites was inserted between the *Sal*I and *Eco*RI sites of pPOH34. Therefore, plasmid pGH71 contains SV40 early-region sequences from −272 to +68 joined to the IE175 leader sequence at position +178. A similar construction containing the IE94 promoter-enhancer region from simian cytomegalovirus [SCMV(Colburn)] was formed by moving the 7.7-kb IE175 coding region from pGH68 as an *Eco*RI-to-*Bgl*II fragment into pTJ278 (24). The resulting plasmid, pGH78, contains an IE94-IE175 hybrid gene with IE94 sequences from −990 to +30 fused to the IE175 coding-region leader sequence at +178. A TK-IE175 hybrid gene was also prepared, by moving an 950-bp *Bam*HI-*Bgl*II fragment from pHSV106 into the *Bgl*II site of pGH68. Thus, plasmid pGH79 contains sequences from −800 to +50 of the HSV-1(MP) TK gene upstream from the same IE175 coding-region fragment used in the other two hybrid gene constructions. A plasmid containing the IE175 coding region without the additional internal L/S junction "a" sequence and the IE110 promoter region sequences (pGH108) was prepared from pGH68 by insertion of an *Xba*I linker after deletion of a 2.3-kb region between the *Aha*III site 1.2 kb beyond the IE175 poly(A) signal and the *Xba*I site in the 3′ polylinker region. The 990-bp IE94 promoter bounded by *Eco*RI and *Hin*dIII sites was then moved from pGH78 into pGH108 to create an identical IE94-IE175 hybrid gene to that in pGH78, except that it was contained within a 5.4-kb rather than a 7.7-kb viral insert (pGH114).

Preparation of nuclear extracts. Crude nuclear extracts were prepared from two to four roller bottle cultures of infected cells as described by Metzler and Wilcox (34), with the following modifications. HSV-1(KOS) was used to infect Vero cells at a multiplicity of infection of 20 for 8 h. The cells were washed with phosphate-buffered saline (PBS), swollen in hypotonic buffer, and then subjected to Dounce homogenization and pelleting of the nuclei. All of the buffers used after the phosphate-buffered saline wash of the cells contained 1 mM tosyl phenylalanyl chloromethyl ketone (TPCK), 1 mM tosyl lysyl chloromethyl ketone (TLCK) and 0.1 mM phenylmethylsulfonyl fluoride, and the addition of 3[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) buffer to the sonicated nuclei was omitted. After clarification of the sonicated nuclei, 0.14 g of ammonium sulfate was added per ml of extract, and the precipitate was collected by centrifugation. The supernatant was then supplemented with 0.21 g of ammonium sulfate per ml, and the precipitate was collected again. The precipitates were suspended in 300 mM ammonium sulfate–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–10 mM β-mercaptoethanol–1 mM TPCK–1 mM TLCK and termed the 33% and 65% ammonium sulfate fractions, respectively. The extract fractions were aliquoted and stored at −70°C. Protein concentrations ranged from 1 to 5 mg/ml for both the 33% and 65% ammonium sulfate fractions.

Gel electrophoresis DNA-binding assays. After digestion with appropriate restriction endonucleases, DNA fragments were size separated on a small polyacrylamide gel and isolated by electroelution after visualization by wet-gel autoradiography. Recovered fragment DNA concentrations were determined by comparison of ethidium bromide-stained bands with standards of known size and concentration. Radioactive labeling was accomplished by a fill-in reaction with Klenow DNA polymerase. Specific activities varied from 2,000 to 6,000 cpm/fmol of DNA fragment. The following HSV-1 DNA fragments were tested for binding activity: IE110 promoter, 285-bp *Sph*I-to-*Bam*HI and 147-bp *Sma*I-to-*Sma*I fragments from pGH83; IE175 promoter, 140-bp *Eco*RI-to-*Bam*HI and 192-bp *Sph*I-to-*Eco*RI fragments from pPOH2; 138-bp *Eco*RI-to-*Bam*HI fragment from pGH129; 103-bp *Eco*RI-to-*Bam*HI fragment from pPOH38 and 83-bp *Eco*RI-to-*Xba*I fragment from pGH123a and pGH123b; glycoprotein D (gD) promoter, 370-bp *Sac*I-to-*Hin*dIII fragment from pPOH17 (gD-CAT); and TK promoter, 250-bp *Pvu*II-to-*Bgl*II fragment from pHSV106.

Typical binding assays for gel retardation experiments (4, 19, 20) contained 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5); 50 mM KCl; 1 mM EDTA; 0.1 mM dithiothreitol; 1 mM TPCK; 1 mM TLCK; 0.1% Triton X-100; 5% glycerol; 1.25 μg of poly(dI-dC) · poly(dI-dC) alternating copolymer; 1 fmol of labeled probe; and 1, 2, or 4 μl of extract in 25 μl. Mouse monoclonal antibodies (56) were gifts from M. Zweig, and usually 1 μl of a 50-fold dilution was added to the binding assay at the beginning of the incubation. After 60 min at 23°C, 1 μl of 0.1% bromophenol blue was added and the samples were loaded on a 4.5% nondenaturing polyacrylamide gel. The gel and electrophoresis buffer contained 10 mM HEPES (pH

7.5), 1 mM EDTA, and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Electrophoresis was carried out at a constant voltage of 15 V/cm for 120 min. The gels were then dried and exposed to Kodak X-Omat AR film at −70°C.

Transient DNA transfection and CAT assays. All short-term expression assays were carried out by the calcium phosphate precipitation procedure with a glycerol boost into subconfluent monolayers of 4×10^5 Vero cells in 35-mm-well culture dishes. The details have been described previously (40, 41). In most experiments all input DNA samples were brought up to a total of either 3 or 10 μg of DNA per 35-mm well by addition of pBR322 or pUC19 carrier DNA. The percent conversion of [^{14}C]chloramphenicol to 1′ and 3′-acetylchloramphenicol was measured by scintillation counting of the radioactivity in appropriate isolated spots from the silica gel plate.

RESULTS

The IE110 promoter is not a target for IE175-dependent down-regulation. We have previously described the stimulatory effects of IE110 and VF65 effector DNA and the inhibitory effects of IE175 effector DNA on the expression of IE175(−1900/+30)-CAT after cotransfection in transient assays in Vero cells (42). To examine the properties of other intact IE promoters, new hybrid plasmids with the HSV IE110 or IE68 promoter regions upstream from the CAT coding sequences were prepared, and the basal expression levels, together with their responses to cotransfection with various effector plasmids, were compared with those of the parent IE175-CAT gene. Both the IE110(−800/+120)-CAT and IE68(−420/+120)-CAT genes proved to give 5- to 10-fold-greater basal expression than the IE175(−1900/+30)-CAT gene and 2- to 3-fold greater expression than IE175(−380/+30)-CAT in Vero cells (data not shown). Part of the increased basal expression from the IE110 promoter compared with IE175 can be ascribed to longer leader region effects, because insertion of additional leader region sequences from +30 to +178 (in either orientation) boosted basal expression of the IE175(−1900/+178)-CAT target gene (plasmid pGH106a) two- to threefold but gave no other effects on its phenotype in terms of responses to cotransfection with the VF65, IE110, and IE175 effector plasmids (not shown).

In cotransfection studies, expresion from the IE110-CAT gene, as well as from a control IE175(−380/+30)-CAT target, was stimulated efficiently (8- to 12-fold in Fig. 1) by either the IE110(pIGA15) or VF65(pGH63) effector plasmids but not by those plasmid DNAs containing the IE175, IE68, or IE63 genes. Similarly, down-regulation of both basal IE175-CAT and IE110-activated expression of IE175-CAT by cotransfection with IE175(p*Xho*I-C) effector DNA occurred as expected (5- to 10-fold in Fig. 1), but, surprisingly, there were no negative effects on the IE110-CAT target DNA. Some inhibition of basal expression from both HSV IE promoter targets was observed after cotransfection with plasmid DNA encoding the HSV IE63 gene (pGR169); however, this proved to be a nonspecific effect that also occurred with several heterologous targets tested (data not shown). The HSV-1 IE68(−420/+30)-CAT target DNA gave essentially identical responses to those obtained with IE110-CAT (not shown).

To examine the possibility of IE175 dosage-dependant inhibition of IE110-CAT basal expression, we carried out a series of cotansfection experiments with various amounts of

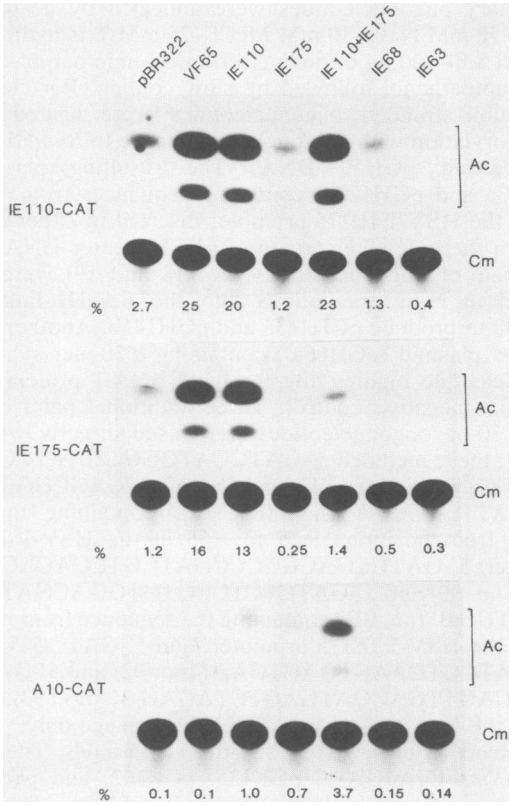

FIG. 1. Different responses of the HSV-1 IE110 and IE175 promoters to cotransfection with HSV IE effector genes. The autoradiographs show the results of standard CAT transient-expression assays in Vero cells with 1 μg of IE110(−800/+120)-CAT (pGH83), IE175(−1800/+30)-CAT (pPOH2), and SV2(−110/+58)-CAT (A10-CAT) target DNAs. The cotransfected effector DNAs consisted of 1 μg of the following plasmids, from left to right: pBR322, pGR212b (VF65), pIGA15 (IE110), p*Xho*I-C (IE175), pIGA15 plus p*Xho*I-C (IE110+IE175), pGR169 (IE68), and pGR162 (IE63). Measurements of percent [^{14}C]chloramphenicol (Cm) converted to the 3′-acetylated form (Ac) are listed below each lane.

input IE175 effector DNA. However, none of the conditions tested gave any evidence for direct down-regulation of the IE110 promoter. For example, in both experiments shown in Fig. 2, IE175(−380/+30)-CAT expression was inhibited a maximum of 8- to 10-fold at the highest ratios of effector to target DNA, but the level of IE110-CAT expression was either slightly activated or unaffected. Therefore, only the IE175 promoter, and not that of IE110, appears to contain a response signal for inhibition by the IE175 gene product.

Specificity of the down-regulation by IE175. We have recently described a similar phenomenon of specific inhibition of expression from the major IE68 promoter of HCMV by a powerful *trans*-activator protein encoded by the HCMV IE2 gene (48). This effect is not exerted on the simian virus 40 early promoter, but does act to inhibit the closely related IE94 promoter from SCMV(Colburn). As an additional measure of IE175 target specificity, we have examined whether the HSV and HCMV IE autoregulation responses either complement or cross-react with one another. The results of parallel cotransfections of HCMV IE68(−760/+10)-CAT and HSV IE175(−380/+30)-CAT reporter genes with p*Xho*I-C at several different effector-to-target DNA ratios revealed marked 25- to 40-fold inhibition of the homologous HSV

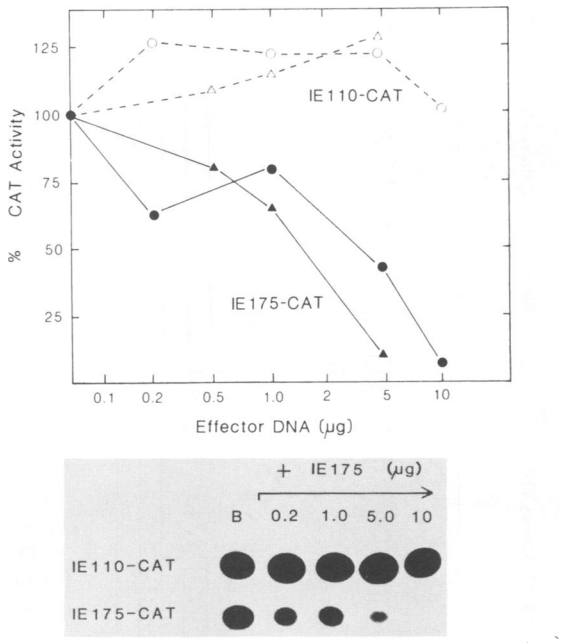

FIG. 2. Lack of down-regulation of basal IE110-CAT activity by cotransfected IE175 DNA. The graph shows the relative levels of CAT activity obtained from IE110(−800/+150)-CAT (pPOH49) target DNA (○, △) or from parallel samples of IE175(−1900/+30)-CAT (pPOH2) control DNA (●, ▲) in two experiments after cotransfection with increasing amounts of IE175 effector DNA (p*Xho*I-C). The input levels of target and control DNA were 4 μg in the first experiment (△, ▲) and 2 μg in the second (○, ●). The 3′-acetyl-[^{14}C]chloramphenicol spots from the autoradiograph of the second experiment are shown below the graph.

target promoter under conditions that had little or no effect on the heterologous HCMV promoter (Fig. 3). Since neither the HSV-1 IE175 nor the HCMV IE2 gene products have negative effects on SV2-CAT, and since both activate other promoter targets, such as TK-CAT (48), we conclude that the autoregulatory effects of the HSV IE175 and the HCMV IE2 gene products act quite specifically upon their own promoter regions only.

Lack of promoter-specific competition effects on basal expression. Previous dose-response experiments showed

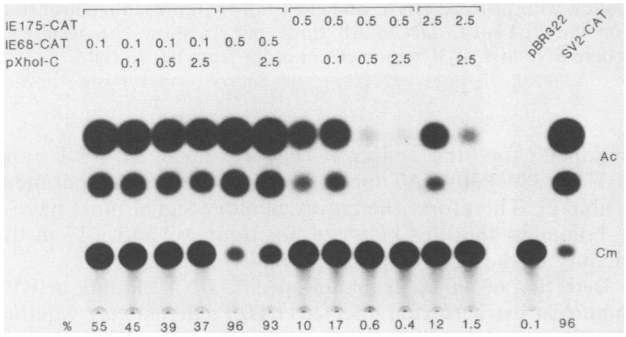

FIG. 3. Lack of response of HCMV IE68-CAT to inhibition by the HSV-1 IE175 gene product in transient assays. The autoradiograph shows the levels of expression from input IE68(−760/+10)-CAT and control IE175(−380/+30)-CAT target DNA obtained after cotransfection with HSV-1 IE175 effector DNA (p*Xho*I-C) at various effector-to-target-DNA ratios. Addition of pBR322 was used to bring the total DNA in all samples to 5 μg.

that inhibition of IE175-CAT expression by the IE175 effector plasmid is not only dominant over stimulation by either IE110 or VF65, but is also enhanced in the presence of an IE110 effector plasmid (42). For example, in a situation in which 2 μg of pIGA15 gave 50-fold stimulation of expression from 2 μg of IE175-CAT target DNA, the inclusion of just 0.01 μg of p*Xho*I-C DNA was sufficient to abolish the stimulation, whereas 2 μg of p*Xho*I-C DNA was needed to give 30-fold inhibition of basal IE175-CAT expression. This shutoff did not occur if IE175 promoter sequences only were introduced, suggesting that it did not involve promoter-specific competition effects. We originally anticipated that IE175 would also directly down-regulate IE110 expression (44), but since that did not happen, we now suspect that the IE110 protein stimulated higher levels of IE175 expression from the input effector gene, leading to inhibition of the IE175-CAT target promoter at a much lower dose of p*Xho*I-C DNA. Presumably, the same arguments would also hold true to explain the dominance of IE175 inhibition over VF65 stimulation. Nevertheless, there are also many hints that the introduction of two different viral *trans*-activators at the same time often leads to complicated interactions, which may include antagonizing one another (D. M. Ciufo, L. Sha, and G. S. Hayward, unpublished data; 17, 22, 59). Therefore, we consider that the only really valid test for autoregulation involves direct inhibition of basal expression or of *cis*-enhanced expression.

Although we have previously demonstrated down-regulation of both basal and *cis*-enhanced expression on the IE175-CAT and IE175(SVL)-CAT targets (43), the promoter sequence controls were not included in those experiments, and it could still be argued that the relatively high input levels of p*Xho*I-C DNA often required for 20- to 30-fold inhibition (e.g., molar ratios of effector to target DNA between 3:1 and 10:1) could permit specific promoter competition effects between the input IE175 promoter sequence in p*Xho*I-C DNA and the target promoter sequences in IE175-CAT. Consequently, we prepared a new series of hybrid effector plasmids in which the IE175 coding region from position +30 in the leader sequence was placed adjacent to promoters that would neither provide such competitor effects nor themselves be subject to IE175 inhibition (SV2, IE94, and TK). In all cases, cotransfection experiments gave 10- to 20-fold stimulation of expression from the HSV-2 DE promoter in DE38-CAT with 0.1 μg of effector DNA, showing that the *trans*-activation properties were unaltered by these changes. More significantly, the SV2-IE175(pGH71), IE94-IE175(pGH78), and TK-IE175(pGH79) effector genes all still down-regulated basal expression from IE175-CAT between 7- and 25-fold at a 1:1 molar ratio of effector to target DNA (Table 1).

Although the inserted HSV DNA sequences present in the IE175 effector plasmids were reduced from 10.8 kb in p*Xho*I-C to 7.7 kb in pGH71, pGH78, and pGH79, they still included both the IE110 upstream promoter region and a newly identified gene mapping across the L/S junction region between the 3′ end of IE175 and the 5′ end of IE110 (5). To eliminate the possibility that this new gene product is necessary for the inhibition (rather than IE175 acting directly), an additional 3.3 kb was removed from the 3′ end of the insert in pGH78 (up to the *Aha*III site), leaving only 5.4 kb of viral DNA, with no more than 1.2 kb from beyond the 3′ end of the IE175 coding region. This version of the IE94-IE175 gene (in plasmid pGH114) produced high levels of the IE175 protein in the nuclei of transfected cells as judged by immunofluorescence with the 58S monoclonal antibody (56)

TABLE 1. *trans*-Activation and autoregulation properties of IE175 effector plasmids driven by heterologous promoters

Effector plasmid	Hybrid gene	Target DNA (CAT activity)			
		DE38-CAT[a] (pPOH1, 1 μg)		IE175-CAT[b] (pPOH2, 1 μg)	
		% Conversion[c]	Fold change	% Conversion[c]	Fold change
pBR322		0.06	1	2.3	1
p*Xho*I-C	Intact IE175	0.6	10	0.18	0.08
pGH71	SV2-IE175	1.0	17	0.18	0.08
pGH78	IE94-IE175	1.4	23	0.08	0.03
pGH79	TK-IE175	0.58	9.5	0.35	0.15

[a] 0.1 μg of effector DNA.
[b] 1.0 μg of effector DNA.
[c] 3′-Acetyl[^{14}C]chloramphenicol levels produced in 1 h by extracts from Vero cells at 48 h after DNA transfection.

and gave normal *trans*-activation and down-regulation phenotypes in cotransfection experiments (not shown).

Therefore, most possibilities for promoter-specific competition effects have been eliminated, and the selective down-regulation of basal IE175-CAT expression by the IE175 effector DNA must be considered a true *trans*-acting autoregulatory effect, which is manifested only in the presence of both the IE175 gene product and a specific *cis*-acting signal sequence in the target DNA.

Removal of cap site sequences in the IE175-CAT target DNA abolishes autoregulation. We demonstrated previously that removal of 5′ upstream sequences from IE175(−1900/+30)-CAT as far as the *Eco*RI site at −108, although eliminating the VF65 *trans*-activation response, failed to eliminate the down-regulation obtained by cotransfection with IE175 effector DNA (43). To define further the limits of the putative negative autoregulation signal, we prepared a series of BAL 31 deletions from the *Bam*HI site at the 3′ end of the IE175 sequences and reinserted 12-bp *Bam*HI linkers to permit rejoining to the CAT coding cassette. Six new deleted constructions based on the IE175(−1900/+30)-CAT parent plasmid (pPOH2) were isolated and sequenced (for a summary of their structures, see Fig. 5b). However, in all cases, basal expression fell 5- to 10-fold relative to the parent DNA, and, consequently, all six deletions were also moved into an IE175(−380/+30)-CAT background (pPOH13) to boost the levels of basal expression (43). Both sets of targets were cotransfected with either pBR322 DNA or an equal amount of p*Xho*I-C DNA at a 1:1 molar ratio of effector to target DNA (Fig. 4; Table 2). These experiments revealed a dramatic difference between parent and deleted target DNAs that ended at +30, +19, +18, and +17 and those that terminated at −7, −17, and −18. The levels of expression from each of the first group of 3′-deleted targets were all inhibited between 8- and 12-fold in the −1900 series and between 10- and 30-fold in the −380 series when cotransfected with p*Xho*I-C DNA compared with pBR322 DNA. In contrast, the second group of 3′-deleted targets gave between 1.2- and 2.0-fold activation in both sets of plasmids.

To reduce possible concerns about having damaged the ability of the 3′-deleted target DNAs to act as promoters, we also investigated whether the −7, −17, and −18 constructions could still respond to *trans*-activation by the virion factor encoded by the HSV-2 VF65 gene (3, 42, 43). The results showed that both sets of 3′-deleted targets were still activated between 40- and 250-fold by cotransfection with pGR135 DNA, similar to the 60- to 90-fold activation levels

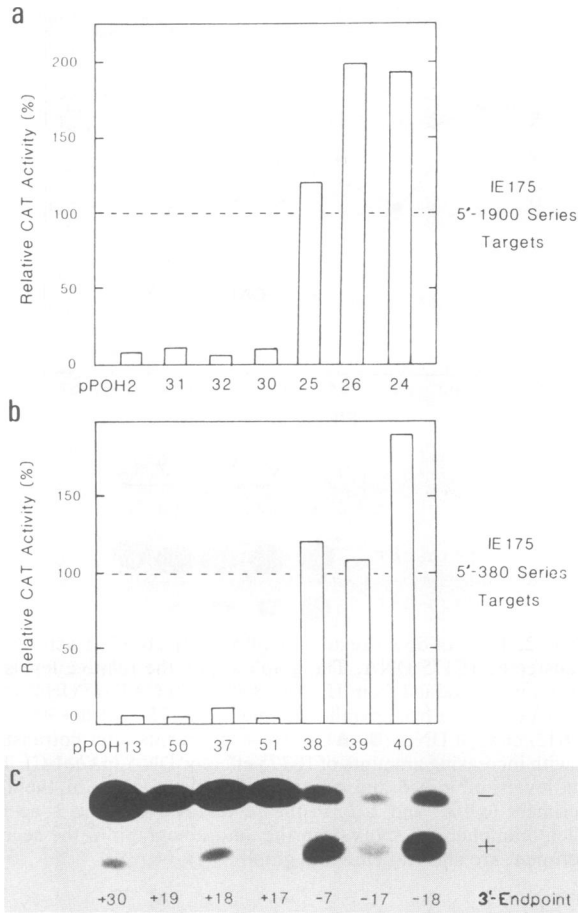

FIG. 4. Autoregulation responses of IE175-CAT targets with deletions near the cap site. The diagram illustrates the relative change from basal CAT activity for two series of 3′ promoter deletion target plasmids after cotransfection with IE175 effector DNA. The basal levels for each plasmid are set at 100% in the histograms, and the measured percent conversion levels and inhibition values for both experiments are listed in Table 2. (a) 5′ −1900 series: 1-μg samples of various target DNA samples were cotransfected with either 2.5 μg of pBR322 DNA (basal activity) or 2.5 μg of p*Xho*I-C DNA (bars). (b) 5′ −380 series: 4-μg samples of target DNA were cotransfected with 10 μg of pBR322 or 10 μg of effector DNA. (c) Autoradiograph of the 3′-acetyl-[^{14}C]chloramphenicol spots only from the 5′ −380 series. The (−) row represents cotransfection with pBR322 DNA, and the (+) row represents cotransfection with p*Xho*I-C DNA. All three panels show the results of progressive loss of 3′ sequences in order from left to right.

obtained for the parent IE175(−1900/+30)-CAT and IE175(−380/+30)-CAT target genes in the same experiment (Table 2). Therefore, the autoregulatory signal must have a 3′ boundary that lies between positions −7 and +17 in the vicinity of the IE175 transcriptional start site.

Detection of an IE promoter-specific DNA-binding activity in nuclear extracts from infected cells. To investigate whether IE175 autoregulation correlated with a specific DNA-protein interaction, we examined crude extracts prepared from the nuclei of infected cells for IE110 or IE175 promoter-specific DNA-binding activities that differed from those of mock-infected cells. A variety of isolated [^{32}P]DNA fragments covering recognized consensus motifs in these two promoters were used as probes (Fig. 5a). Initial mobility shift-up

TABLE 2. Responses of IE175-CAT 3′ deletions to IE175 autoregulation and VF65 *trans*-activation

3′- End point	Target (−1900 series) (1 μg)	Effector (CAT activity) % Conversion pBR322 (2.5 μg)	Effector (CAT activity) % Conversion pXhoI-C (2.5 μg)	Fold change	Target (−380 series) (4 μg)	Effector (CAT activity) % Conversion pBR322 (10 μg)	Effector (CAT activity) % Conversion pXhoI-C (10 μg)	Fold change
+30	pPOH2	5.8	0.5	0.08	pPOH13	32	1.3	0.04
+19	pPOH31	0.9	0.1	0.11	pPOH50	9	0.35	0.04
+18	pPOH32	0.6	0.04	0.07	pPOH37	20	1.8	0.09
+17	pPOH30	1.0	0.1	0.1	pPOH51	11	0.25	0.02
−7	pPOH25	0.5	0.6	1.2	pPOH38	2.5	3.1	1.2
−17	pPOH26	0.2	0.4	2.0	pPOH39	1.2	1.3	1.1
−18	pPOH24	0.3	0.6	2.0	pPOH40	2.0	3.8	1.9
	Target (1 μg)	pBR322 (3 μg)	pGR135 (3 μg)		Target (1 μg)	pBR322 (3 μg)	pGR135 (3 μg)	
+30	pPOH2	0.7	64	91	pPOH13	1.0	67	67
+19					pPOH50	0.3	57	190
−7	pPOH25	0.6	20	33				
−17	pPOH26	0.4	19	24	pPOH39	0.2	49	240
−18	pPOH24	0.15	21	140	pPOH40	0.45	59	130

experiments (4, 19, 20) with a 285-bp *Sph*I-*Bam*HI fragment containing sequences from −165 to +120 in the IE110 promoter produced a very prominent, highly retarded complex (referred to as B) that was observed only after incubation with a 33% ammonium sulfate fraction from a nuclear extract of HSV-1 KOS-infected Vero cells (Fig. 6a, lanes 1 to 5). A 147-bp *Sma*I-*Sma*I fragment, which contained two consensus VF65 octamer-TAATGARAT response elements (ATGCTAATGARAT) mapping between −275 and −127 in the IE110 promoter, did not form this complex (data not shown). Increasing amounts of the infected-cell extract produced more of the B complex with the *Sph*I-*Bam*HI fragment, but similar amounts of an extract from mock-infected Vero cells prepared in parallel failed to produce any similar shift-up activity (Fig. 6a, lanes 6 to 8). The discrete B DNA-protein complex was detectable only in the presence of poly(dI-dC) competitor DNA and remained stable over a range from 50 μg to 1 mg of poly(dI-dC) per ml (Fig. 6b).

A similar assay with a 140-bp *Eco*RI-to-*Bam*HI DNA fragment from positions −108 to +30 in the IE175 promoter (Fig. 5a) again produced a sharply defined and highly retarded DNA-protein complex of the B type with the 33% ammonium sulfate fraction of the infected Vero cell nuclear extract (Fig. 7a, lanes 4 to 7). Importantly, an equivalent 103-bp DNA fragment containing bp −108 to −7 from one of the 3′ deletion plasmids that failed to autoregulate (pPOH38) also failed to participate in complex formation (Fig. 7a, lanes 13 to 16), suggesting that a binding site within the deleted cap region from −7 to +30 must be involved. An equivalent DNA-binding activity was not detected in a 65% ammonium sulfate fraction from the infected-cell nuclear extract, although that fraction contained a nonspecific binding activity that formed a faster-migrating complex with both the parent and deleted DNA species (Fig. 7a, lanes 8 to 11 and 17 to 20).

A 192-bp *Sph*I-to-*Eco*RI fragment covering the farther upstream region from −300 to −108 in the IE175 promoter was reported by Kristie and Roizman (27, 28) to contain IE175-binding activity, but this fragment failed to give specific complex formation in our experiments (Fig. 8a, lanes 8 and 9). Similarly, a 250-bp *Pvu*II-to-*Bgl*II fragment from positions −200 to +50 in the DE class thymidine kinase gene promoter failed to bind (Fig. 8a, lanes 11 and 12); however, a 370-bp *Sac*I-to-*Hin*dIII fragment from positions −340 to +30 in the glycoprotein D (gD) gene promoter did form a similar high-affinity B complex (Fig. 8a, lane 2).

Confirmation that both IE promoter complexes contain the IE175 protein. To show that the IE110 upstream promoter DNA-protein complex contained the viral IE175 protein, we performed double shift-up assays with monoclonal antibodies directed against several known HSV-1 proteins (Fig. 6c). Only the monoclonal antibody against IE175 (58S), and not that against the 132,000-dalton major DNA-binding protein (39S), gave a new, slower-migrating, super-shifted C complex of the type described previously by Kristie and Roizman (27). Titration of the ability of the antibody to interact with the DNA-protein complex revealed that the original B complex started to move into the supershifted C complex with 1 μl of a 250-fold dilution of the antibody (Fig. 6d). Similarly, in the presence of the 58S monoclonal antibody, the B complex that was formed with the 140-bp wild-type DNA fragment covering the IE175 minimal promoter and cap site also gave a supershifted species analogous to that observed with the IE110 upstream fragment (Fig. 7b, lane 3). Neither the 39S antibody against the major DNA-binding protein (Fig. 7b, lane 4) nor equivalent concentrations of bovine serum albumin (data not shown) had any effect on the mobility of the complex. The strong B complex formed with the gpD promoter fragment also produced a new supershifted form in the presence of 58S antibody (Fig. 8a, lane 3).

Examination of a parallel 33% fraction of the nuclear extract from mock-infected Vero cells revealed no trace of an activity producing a shifted protein-DNA complex of the B type with the IE175 probe, although a discrete slower-migrating complex was often seen in the mock-infected cell extracts but never in the infected cell extracts (compare, e.g., Fig. 7b, lanes 2 and 5). This A complex was not supershifted with the 58S monoclonal antibody (Fig. 7b, lane 6).

Localization of the IE175 binding site to a 26-bp sequence encompassing the mRNA cap site. Since a partially purified preparation of the IE175 protein had been shown to protect sequences over a consensus ATCGTC sequence near the cap site in the IE175 promoter (16; K. Wilcox, personal communication), we wished to determine directly whether this site was also involved in binding to the factor found in crude infected Vero cell extracts. We first prepared a 2-bp

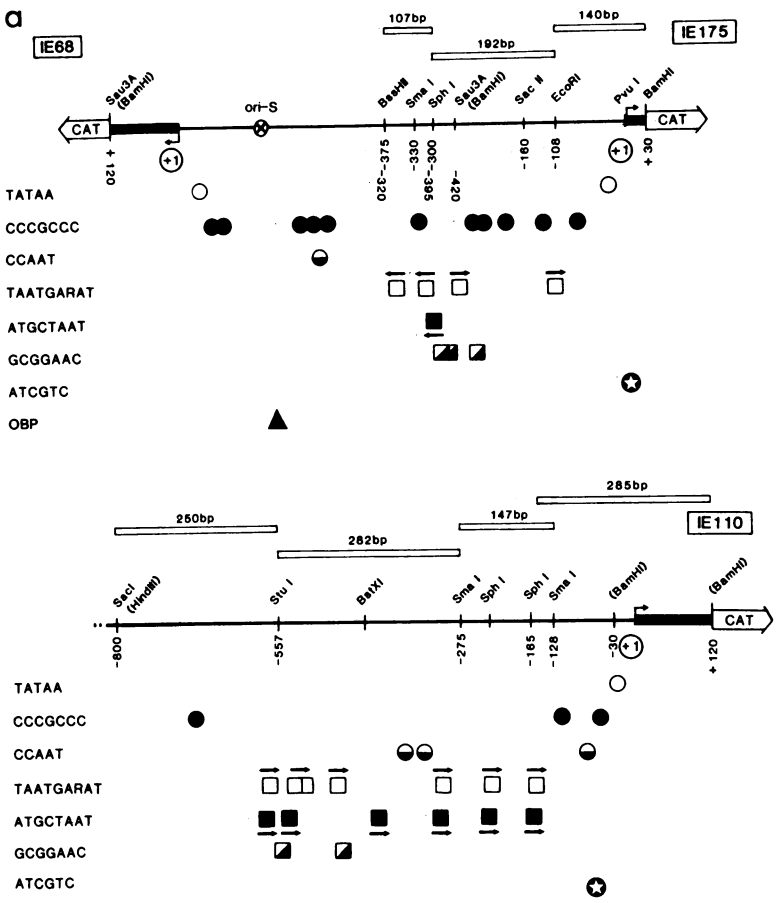

FIG. 5. (a) Summary of the arrangement of consensus elements and transcription factor-binding sites in the HSV-1 IE175 and IE110 upstream promoter domains. The upper portion of the diagram shows the 700-bp divergent IE68/*ori*-S/IE175 promoter, and the lower portion shows the unidirectional IE110 promoter. ▬, Untranslated leader sequences included in the various CAT reporter gene constructions. Restriction sites introduced as linkers are enclosed in parentheses. Symbols denote the locations of the indicated consensus motifs. The CCCGCCC motifs shown represent demonstrated Sp-1 factor-binding sites in the IE68/IE175 promoter region (25). TAATGARAT, ATGCTAAT, and GCG GAAC motifs represent binding sites for cellular factors that may be intermediaries for virion factor responses (39; apRhys et al., submitted; S. Triezenberg and S. McKnight, personal communication). The relative orientations of TAATGARAT and ATGCTAAT motifs are indicated by arrows. OBP, Origin-binding protein. ▭, Sizes and boundaries of DNA fragment probes used in the binding assays. (b) Comparison of DNA sequences in the cap site region of various deletion and oligonucleotide forms of the IE175 promoter and around the ATCGTC consensus in the IE110 promoter. The position numbers given relate to the assigned transcription start sites. pGH128 and pGH129 contain 2-bp deletions at the *Pvu*I site with and without insertion of an 8-bp *Bgl*II linker. pGH123a and pGH123b represent the two orientiations of the IE175 30-mer oligonucleotide in pUC19. Lower-case bases represent nonmatching sequences introduced as linkers. The ATCGTC consensus is shown in bold type.

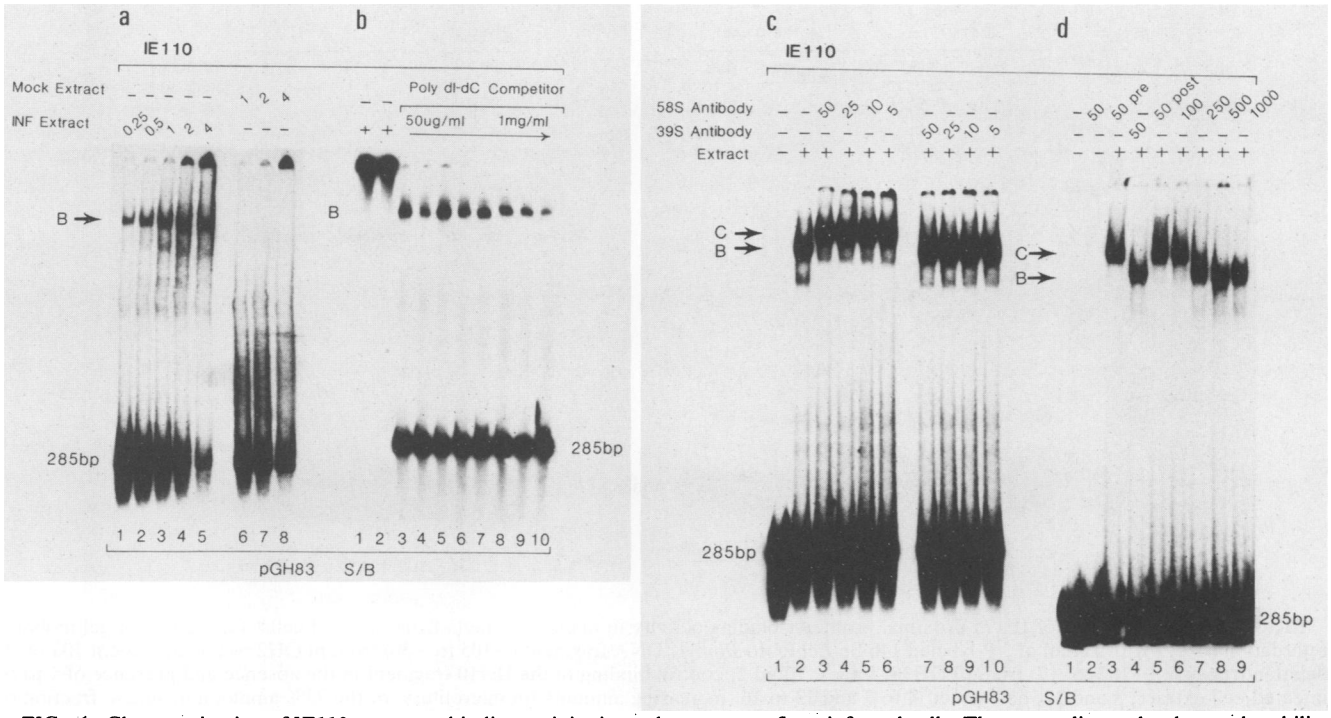

FIG. 6. Characterization of IE110 promoter-binding activity in nuclear extracts from infected cells. The autoradiographs show gel mobility retardation assays with the ^{32}P-labeled 285-bp *Sph*I-to-*Bam*HI DNA fragment (−165 to +120) from pGH83. (a) Formation of specific B complexes with infected but not mock-infected cell extracts. Lanes: 1 to 5, incubation with increasing volumes (in microliters) of the 33% ammonium sulfate fraction from 8-h HSV-1 KOS-infected Vero cells; 6 to 8, parallel samples from mock-infected Vero cells. (b) Stability of B complexes to the presence of nonspecific competitor DNA. Lanes: 1 and 2, nonspecific complex formed with 0.5 μl of extract in the absence of poly(dI-dC); 3 to 10, increasing concentration of poly(dI-dC). (c) Formation of supershifted C complexes with specific monoclonal antibody. Lanes: 1, control without extract; 2, incubation with 4 μl of infected-cell extract; 3 to 6, addition of 1 μl of 5- to 50-fold dilutions of 58S anti-IE175 monoclonal antibody; 7 to 10, addition of 1 μl of 5- to 50-fold dilutions of 39S anti-major DNA-binding protein monoclonal antibody. (d) Specificity of C complex formation. Lanes: 1, incubation of probe alone; 2, incubation with 1 μl of a 50-fold dilution of 58S antibody but no extract; 3, 1 μl of a 50-fold dilution of 58S antibody preincubated with the extract; 4, 1 μl of a 50-fold dilution of 39S antibody preincubated with the extract; 5, incubation with 1 μl of a 50-fold dilution of 58S antibody after addition of the extract; 6 to 9, titration with 1 μl of 58S antibody at dilutions ranging from 100-fold to 1,000-fold.

deletion variant (pGH129) of the 140-bp IE175 fragment, which lacked the central A and T bases (positions −3 and −4) of the *Pvu*I site that overlaps the consensus signal (Fig. 5b). This 138-bp mutant fragment failed to demonstrate binding activity (Fig. 9a, lanes 4 to 6). Second, we synthesized both strands of a 30-bp oligonucleotide containing 26 bp of the cap region from the IE175 promoter (positions −8 to +18). These oligonucleotides were annealed and used in both competition and direct DNA shift-up assays. The double-stranded oligonucleotide probe, but not a nonhomologous 30-bp probe (representing the binding site for EBNA-1 protein), competed for binding to the IE175 140-bp *Eco*RI-to-*Bam*HI fragment when present in a high molar excess (Fig. 7b, lanes 11 and 15). However, we were unable to find conditions under which a stable gel-retarded complex formed directly with this oligonucleotide in the presence of the 33% nuclear extract from infected cells. Some binding activity was detected in the 65% extract, but this proved to be present in both the infected and mock-infected samples and was nonspecific in that several other oligonucleotides also produced the same complex (not shown).

Surprisingly, after inserting the 30-bp cap site oligonucleotide between *Bam*HI and *Bgl*II sites in a pUC18-derived vector (pGH123b) and isolating an 83-bp fragment containing this sequence, we were able to demonstrate a stable DNA-protein interaction that again produced a discrete high-molecular-weight B type of complex with the 33% extract from infected cells but not from mock-infected cells (Fig. 9b, lane 3). This complex was also formed with the oligonucleotide when placed in the opposite orientation in pUC18 DNA (pGH123a; Fig. 9c, lane 2) and gave the typical supershifted C complex with the 58S but not the 39S monoclonal antibody (Fig. 9b, lanes 4 and 5; Fig. 9c, lane 3). The equivalent 83-bp region of plasmid pGH66, which contains the parent pUC18 linker region with an inserted irrelevant 30-mer EBNA-1 binding site, failed to produce any trace of the shifted B complex in the gel retardation assay (Fig. 9c, lanes 5 and 6). Both orientations of the 30-mer synthetic binding site also occasionally produced the slower-migrating A complex with the 33% mock-infected extract, but this species did not alter position in the 58S antibody supershift assay (data not shown). Removal of the ends of the 83-bp fragment by cleavage at other available restriction sites revealed that a 45-bp form appeared to be the smallest fragment capable of forming a stable shifted B complex with this oligonucleotide (data not shown). Therefore, formation of the specific protein-DNA complex with infected-cell extracts that contain the IE175 protein apparently requires a portion of the same 26-bp HSV DNA sequence that was essential for the autoregulation phenotype.

Demonstration that a synthetic oligonucleotide centered over the ATCGTC consensus from the IE110 promoter is sufficient for complex formation. Our shift-up experiments described above mapped the strong binding site with the

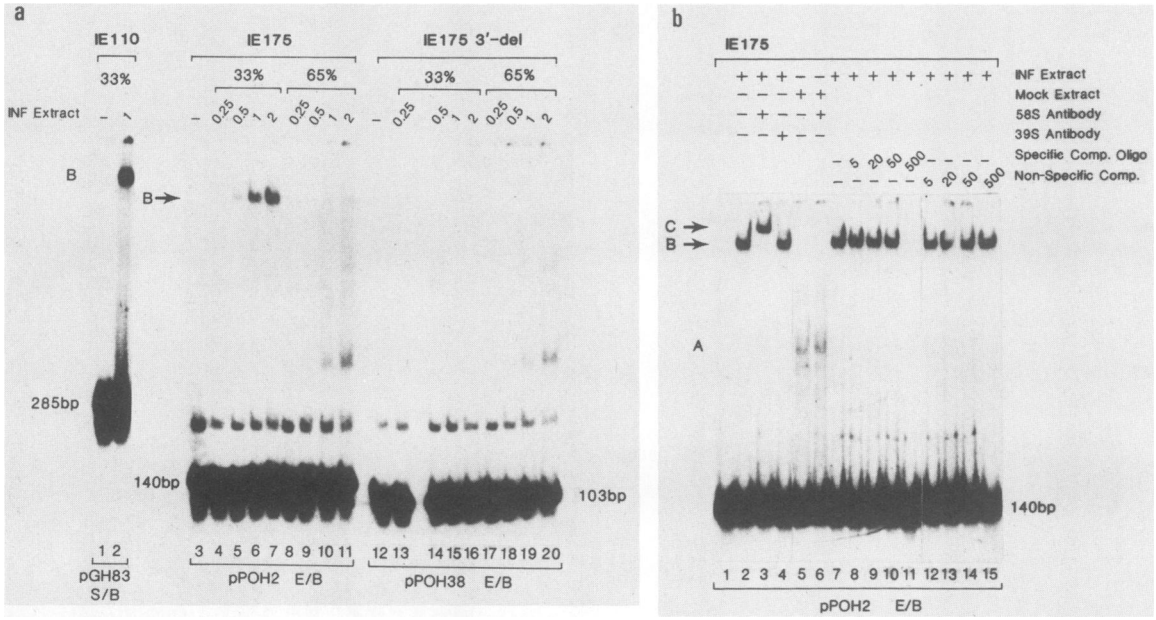

FIG. 7. Characterization of IE175 proximal promoter-binding activity in nuclear extracts from infected cells. (a) Results of gel mobility retardation assays with 1 fmol of ^{32}P-labeled 140-bp *Eco*RI-to-*Bam*HI DNA fragment (−105 to +30) from pPOH2 or the equivalent 103-bp 3′ deletion fragment (−105 to −7) from pPOH38. Lanes: 1 and 2, control binding to the IE110 fragment in the absence and presence of 1 μl of infected-cell extract; 3 and 12, no extract; 4 to 7 and 13 to 16, increasing amounts (in microliters) of the 33% ammonium sulfate fraction of the infected-cell extract; 8 to 11 and 17 to 20, similar amounts of the 65% ammonium sulfate fraction. (b) Results of antibody supershift and specific oligonucleotide competition assays with the 140-bp IE175 fragment. Lane 1 contains no extract. Lanes 2 to 4 and 7 to 15 contain 2 μl of infected-cell 33% extract fraction. Lanes 5 and 6 contain mock-infected 33% extract fraction. Lanes 3, 4, and 6 show the formation of the specific supershifted C complex with the infected-cell extract only plus 58S but not 39S antibody. Lanes 8 to 11 show incubation in the presence of 5 to 500 fmol of 30-mer IE175 cap site oligonucleotide. Lanes 12 to 15 contain 5 to 500 fmol of 30-mer EBNA-1 DNA-binding-site oligonucleotide.

IE110 promoter to within a 280-bp region between positions −165 and +120. To confirm that this interaction represented the same one observed by Kristie and Roizman (27), who used exonuclease III mapping, and by Faber and Wilcox (15), who used immunopreciptation studies with the partially purified IE175 protein, we prepared another pair of 30-bp oligonucleotides (Fig. 5b, no. 47 and 48) containing a 21-bp DNA sequence from positions −75 to −55 in the IE110 upstream region. Unlike the IE175 oligonucleotide, this synthetic binding site contained the consensus ATCGTC portion of the sequence (positions −68 to −63) located at the center of the 30-bp double-stranded structure. Moreover, in contrast to the original IE175 oligonucleotide described above, the uncloned 30-bp sequence alone proved to be sufficient to produce a stable and specific interaction with the binding activity in our 33% infected-cell extracts, and, again, the specific B protein-DNA complex that was formed could be supershifted in the presence of the IE175 monoclonal antibody (Fig. 8b, lanes 2 and 3). Additionally, a second IE175 oligonucleotide pair (Fig. 5b, no. 90 and 91) containing viral sequences from positions −13 to +11, and therefore with a more centrally located ATCGTC consensus, was also able to bind directly as an uncloned 30-mer (data not shown). Thus, no more than 21 bp of the IE110 region encompassing the consensus ATCGTC signal is necessary and sufficient for specific interaction with the IE175 protein complex. An additional 30-bp oligonucleotide pair (Fig. 5b, no. 92 and 93), containing the consensus ATCGTC portion of the viral sequences only, failed to bind (Fig. 8b, lanes 5 and 6).

Correlation between IE175 binding at the cap site and the autoregulation phenotype. Finally, to pinpoint the *cis*-acting element required for autoregulation, we investigated whether an IE175-CAT target gene with a 2-bp deletion in the binding site at positions −3 and −4 was still subject to IE175-mediated down-regulation. Cotransfection experiments with the wild-type and modified IE175(−380/+30)-CAT target genes and p*Xho*I-C effector DNA were carried out in Vero cells (Fig. 10). In comparison with an 11-fold shutoff of basal expression observed with the parent undeleted IE175(−380/+30)-CAT target gene in pPOH13 DNA and a complete lack of inhibition of the 3′-deleted IE175(−380/−7)-CAT target gene in pPOH38 DNA as a negative control, the 2-bp ATCGTC deletion mutant gene in pGH129 DNA exhibited only a very weak (40%) down-regulation response. Significantly, insertion of the IE175 30-mer oligonucleotide binding sequence (Fig. 5b, no. 18 and 19) from pGH123 DNA into the unresponsive 3′-deleted promoter in pPOH38 to create plasmid pGH145a restored most of the down-regulation phenotype (sixfold inhibition). Note that this construction results in placement of the ATCGTC consensus motif 10 bp farther downstream from the TATAA box than in the wild-type sequence (nominally at positions +6 to +11). Therefore, these results confirm that the down-regulation response correlates very closely with the presence of an IE175 binding site near the cap site and imply that the spacing requirements relative to neighboring features of the promoter may not be particularly stringent.

DISCUSSION

The results reported in this article extend our previous analyses of the properties of the IE175 and IE110 *trans*-acting regulatory proteins of HSV and of the *cis*-acting control elements governing their expression. Using transient

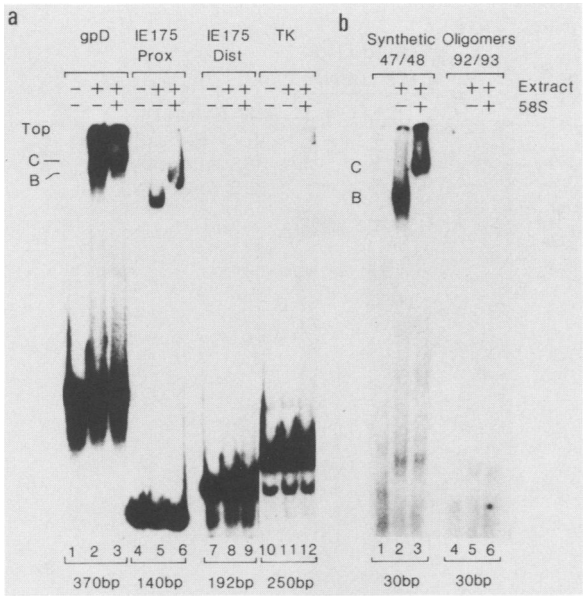

FIG. 8. Specificity of binding of IE175 protein in infected-cell extracts to various HSV promoter fragments. In all assays, 1 μl of an infected Vero cell extract (33% fraction) and 2 fmol of labeled DNA fragments were used. (a) Examination of binding to the following four isolated fragments from left to right: (i) 370-bp *Sac*I-*Hin*dIII fragment from pPOH17 (HSV-1 gpD −340 to +30); (ii) 140-bp *Eco*RI-*Bam*HI proximal species from pPOH2 (HSV-1 IE175 −108/+30); (iii) 192-bp *Sph*I-*Eco*RI distal fragment from pPOH2 (HSV-1 IE175 −300/−108); and (iv) 250-bp *Pvu*II-*Bgl*II fragment from pPOH3 (HSV-1 TK −200/+50). Lanes 1, 4, 7, and 10, no extract; lanes 2, 5, 8, and 11, extract only; lanes 3, 6, 9, and 12; extract plus 58S antibody. (b) Direct assay for IE175 binding to double-stranded 30-mer oligonucleotides containing sequences from −75 to −55 or the ATCGTC consensus only from the HSV-1 IE110 upstream promoter region (Fig. 5b, no. 42 and 43 and no. 92 and 93). Lanes 1 and 4, no extract; lanes 2 and 5, extract only; and lanes 3 and 6, extract plus 58S antibody.

cotransfection assays to examine the responses of various target reporter genes to the different effector plasmids, and using mobility retardation experiments to detect formation of specific DNA-protein complexes from nuclear extracts of HSV-infected cells, we have shown that (i) the down-regulation of the IE175 promoter by cotransfection with the IE175 gene is a specific autoregulatory response not caused by promoter competition effects; (ii) the target signal conveying an IE175 autoregulation phenotype maps between positions −7 and +17 surrounding the IE175 cap site region and is disrupted by deletion of 2 bp at positions −3 and −4; (iii) this signal correlates precisely with a 26-bp DNA sequence that is sufficient for formation of a DNA-protein complex containing the IE175 protein from infected-cell nuclear extracts; (iv) the consensus ATCGTC motif at −4 to +2 is a necessary component of the IE175-binding site but is not sufficient by itself for binding; and (v) the IE110 promoter is not subject to down-regulation by the IE175 protein (nor by its own gene product), although there is a strong IE175-binding site lying between positions −75 and −55 in its 5′ upstream regulatory region.

Since all of the HSV IE mRNAs are overproduced after infection in the presence of protein synthesis inhibitors such as cycloheximide or anisomycin, it had appeared plausible that each of the IE promoters may contain signals for IE175-mediated down-regulation. Gelman and Silverstein (22, 23) have produced a series of studies which appear to support the notion of IE175-directed down-regulation of both the complete and minimal promoter regions from the IE175, IE110, and IE63 promoters, especially when both the IE110 and IE175 effector plasmids are used together. However, we found that this was clearly not the case for direct effects on basal IE110 promoter activity when cotransfected with IE175 effector DNA under the conditions of transient-expression assays in Vero cells. Support for the validity of our result comes from recent runon transcription analysis of rates of IE mRNA synthesis in isolated nuclei of infected cells by Weinheimer and McKnight (62). In those studies, IE175 transcription was initiated at very early stages of infection but shut down at later stages (after 3 to 4 h), whereas that of IE110 remained at relatively high levels throughout the infection cycle. Of course, since both of these studies presumably measure transcription initiation effects only, they do not necessarily correlate with overall steady-state levels of the two IE mRNA species, nor do they address the levels of synthesis or accumulation of the protein products. For example, despite the shutoff of IE175 transcription and protein synthesis, an active IE175 protein product is believed to be required continuously throughout infection for transcriptional activation of most DE and late mRNA species (60).

Formation of a DNA-protein complex containing the IE175 protein at the cap site potentially provides a feedback mechanism to physically block initiation of transcription from its own promoter region. The mechanism of this negative autoregulation may resemble that of the simian virus 40 large T antigen (38), except that there is no evidence at present for the involvement of more than a single binding site in the HSV-1 IE175 system. On the basis of the abundance of the activity in infected-cell extracts and the complete lack of formation of the specific B type of DNA-protein complex with extracts from uninfected cells, together with the evidence for retention of DNA-binding activity through an extensive series of IE175 purification steps (15, 16), we expect that the IE175 protein itself binds directly to a target DNA sequence that includes the ATCGTC core consensus motif; however, the possibility of the involvement of an intermediary cellular factor has not yet been rigorously excluded.

In earlier studies with crude infected HeLa cell extracts, Kristie and Roizman (27, 28) claimed evidence for the formation of immunoprecipitable complexes containing the IE175 protein with upstream regions from the IE175, IE110, and IE63 promoter-regulatory regions, as well as with the gC promoter, and weak activity with the TK promoter. They did not describe the IE175 cap site but presented exonuclease III digestion studies showing protection at −71 to −46 in the IE110 promoter. The latter result correlates well with ours, but we found no evidence for formation of the same kind of IE175-containing complex with DNA sequences between positions −300 and −108 in the IE175 upstream promoter-regulatory region (nor between −200 and +50 in the TK gene promoter) under conditions favoring formation of complexes at the IE175 cap site, the IE110 upstream site, and the gD gene upstream site. In this regard, our studies agree much more with those reported by Muller (36), who defined a binding site at −10 to +3 in IE175 by gel shift-up and dimethysulfate protection studies, and also did not detect an IE175 binding site in the region farther upstream. Similarly, Faber and Wilcox (15, 16) used partially purified preparations of the IE175 protein in McKay assays, filter binding, and DNase I footprinting studies to demonstrate that binding of IE175 protects positions −111 to −81 in the gD promoter

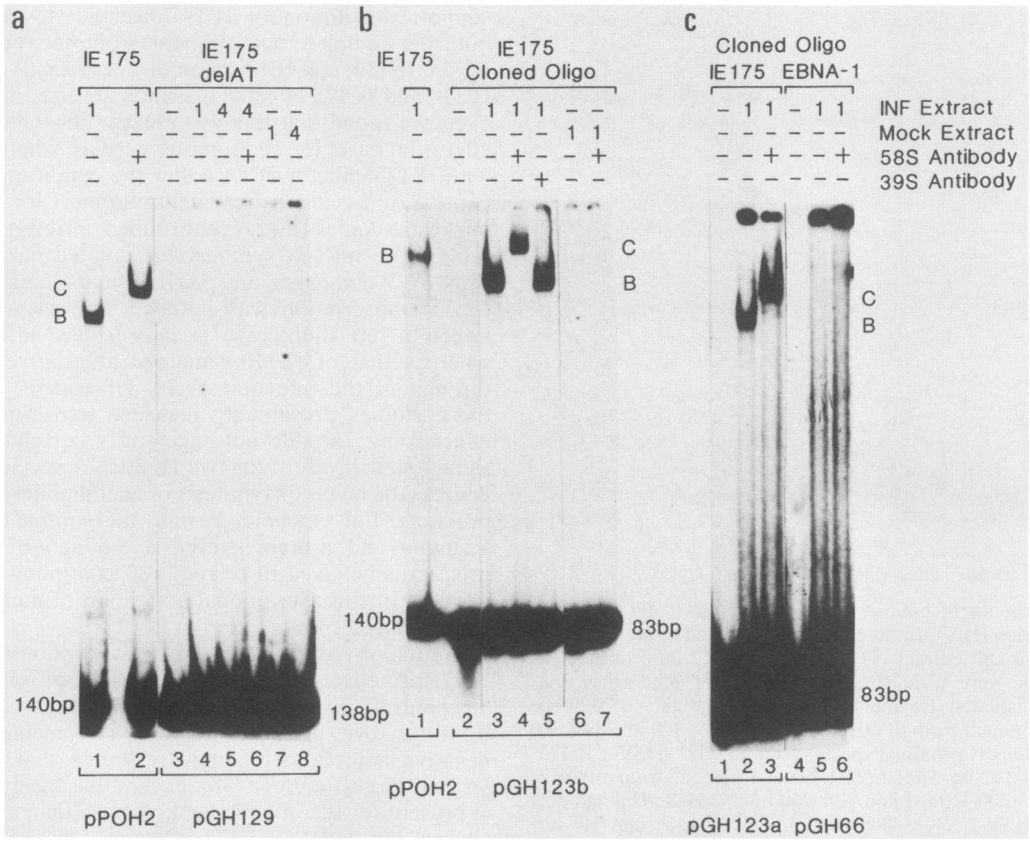

FIG. 9. Specific binding to the IE175 proximal promoter region requires the consensus ATCGTC sequence at the cap site. All panels represent autoradiographs of gel mobility retardation assays. (a) Lack of complex formation after deletion of 2 bp at the *Pvu*I site. Lanes: 1 and 2, positive controls with the 140-bp *Eco*RI-*Bam*HI fragment from pPOH2 plus 1 μl of infected-cell extract in the absence or presence of 58S antibody at a 1:100 dilution; 3 to 8, 138-bp *Eco*RI-*Bam*HI fragment from pGH129. (b) Binding with the cloned 30-mer IE175 cap site oligonucleotide (no. 18 and 19). Lanes: 1, positive 140-bp pPOH2 control; 2 to 7, 83-bp *Bam*HI-*Eco*RI fragment from pGH123b. (c) Demonstration of specificity of binding to the cloned oligonucleotide. Lanes: 1, 2, and 3, 83-bp *Bam*HI-*Eco*RI fragment from pGH123a containing the IE175 cap site 30-mer in the opposite orientation; 4, 5, and 6, 83-bp *Bam*HI-*Eco*RI fragment from pGH66a containing a 30-mer oligonucleotide EBNA-1 binding site.

and positions −7 to +20 in the IE175 promoter. Note that the three consistently observed IE175-binding sites in HSV promoter regions (IE175 cap site, IE110, and gD upstream sites) all contain the consensus ATCGTC signal, whereas the other putative loci do not. The lack of a detectable IE175-binding site or ATCGTC consensus signal in the TK and DE38-CAT delayed-early promoters, for example, which are well-defined targets for IE175-specific *trans*-activation, suggests that some other indirect mechanism that does not involve the specific DNA-binding properties of IE175 must be operative. Consequently, the significance of the binding site in the DE promoter for the gD gene is of some interest. Pizer et al. (47) have suggested that it has a positive effect from in vitro transcription studies, whereas Arsenakis et al. (1) argue that there may be negative effects as well. However, in transient cotransfection assays the IE175-binding site in the gD promoter is not required for IE175 activation of gD-CAT activity (P. O'Hare and A. Haig, unpublished data).

There are no hints available at present about the functional role of the IE175-binding site in the IE110 promoter. This locus produces a significantly more stable complex than that at the IE175 cap site in shift-up assays (and in footprinting studies; Wilcox, personal communication), but presumably its location at an upstream position, rather than at the mRNA start site, or its juxtaposition between potential CCAAT box and Sp-1 binding motifs, produces a different

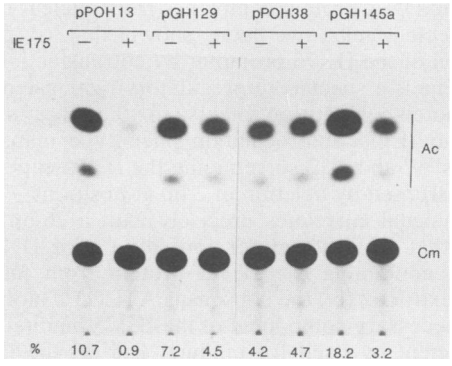

FIG. 10. Loss of the autoregulation response by deletion of 2 bp at the IE175 cap site. The autoradiograph shows levels of CAT expression in transient assays in Vero cells from four input IE175-CAT target DNAs (2 μg each) after cotransfection with either 8 μg of pBR322 DNA (lanes −) or 8 μg of p*Xho*I-C DNA (lanes +). The target gene constructions included the parent IE175(−380/+30)-CAT (pPOH13); the 2-bp deletion mutant IE175 (−380/+30del−3, −4)-CAT (pGH129), the 3′-deletion mutant IE175(−380/−7)-CAT (pPOH38), and pPOH38 with the 30-mer IE175-binding site oligonucleotides 18 and 19 inserted (pGH145a). Measurements of the percent [^{14}C]chloramphenicol (Cm) converted to the 3′-acetylated form (Ac) are listed below each lane.

type of functional response. In its natural context, this site produces neither a positive nor a negative phenotype in direct transient-cotransfection assays with IE175 effector DNA. Our demonstration that the 21 bp from positions −75 to −55 of IE110 (within a 30-bp synthetic oligonucleotide) is sufficient to give specific complex formation confirms the conclusions of Faber and Wilcox (15) and Kristie and Roizman (27) that a binding site encompasses the consensus ATCGTC sequence lying at positions −68 to −63. Furthermore, our studies demonstrate that the adjacent inverted CCAAT-box and CCGCCC (Sp-1) sequences, which are located one turn of the helix upstream and downstream of the ATCGTC motif in the viral sequence (but are absent in our oligonucleotide), are not necessary for binding, even in crude extracts. The higher-affinity binding of the IE110 sites relative to the IE175 cap site was reproduced with the synthetic oligonucleotides and therefore must be an intrinsic property of the different binding-site recognition sequences themselves rather than resulting from any influence of potential adjacent cellular factor binding sites. Although the only clear consensus among all the known binding sites for IE175 lies in the ATCGTC motif, this sequence occurs several times even within the coding regions for the IE175 and TK proteins, for example, and would not be expected to be sufficient by itself for sequence-specific binding. Consistent with this prediction, our studies with additional synthetic oligonucleotides revealed that inclusion of the ATCGTC motif alone at the center of a 30-mer oligonucleotide failed to give complex formation.

After our work was completed, DeLuca and Schaffer (10) reported studies with viruses containing deletion and nonsense mutations in IE175, showing that the DNA-binding domain maps with the NH_2-terminal half of the IE175 protein and that its absence correlates with increased transcription of IE175 mRNA as measured by nuclear runon assays in infected cells.

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