JOURNAL OF VIROLOGY, June 1989, p. 2773–2784
0022-538X/89/062773-12$02.00/0

Vol. 63, No. 6

Identification of Immediate-Early-Type *cis*-Response Elements in the Promoter for the Ribonucleotide Reductase Large Subunit from Herpes Simplex Virus Type 2

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Received 8 November 1988/Accepted 1 March 1989

Regulation of the expression of the herpes simplex virus (HSV) type 2 large subunit of ribonucleotide reductase (ICP10) gene was studied directly by immunofluorescence or by chloramphenicol acetyltransferase analysis with hybrid ICP10 promoter constructions. In Vero cells, cotransfection with DNA encoding HSV IE110 or Vmw65 proteins or HCMV IE2 enhanced expression at least 10-fold. In contrast, expression was minimally enhanced by DNA encoding IE175 at low doses and slightly reduced at high doses. IE110-mediated *trans*-activation was minimal in primary astrocytes and cells from line 293. However, Vmw65 enhanced expression 20-fold in all cell types. *cis*-Response elements in the ICP10 promoter include a TAATGARAT-like element and other sequences associated with regulation of IE gene expression and potential SP-1, consensus AP-1, and octamer transcription factor 1 binding elements. Factors that bind to the ICP10 promoter were identified in mock and HSV-infected cell extracts. DNA-protein complex formation, presumably involving Vmw65, was demonstrated by gel retardation analysis with mixtures of uninfected cell nuclear extracts and virion lysates. The octamer transcription factor 1 motif (ATGCAAAT) was necessary for optimal Vmw65 binding to the ICP10 promoter as evidenced by competition experiments with oligonucleotides overlapping the consensus IE110 promoter virion response element. The data suggest that ICP10 can be regulated as an immediate-early gene.

Ribonucleotide reductase provides a major pathway in the formation of DNA precursors, thereby playing a key role in DNA synthesis of eucaryotic and procaryotic cells. Mammalian and *Escherichia coli* ribonucleotide reductases consist of two subunits, and their activities are under stringent allosteric control (58). Like the mammalian and bacterial enzymes, the herpes simplex virus (HSV) ribonucleotide reductase also consists of two subunits. The large subunit (RR1, with a molecular weight of 140,000), designated ICP6 and ICP10 for HSV-1 and HSV-2, respectively, is encoded within the U_L region of the viral genome (map units [m.u.] equal 0.554 to 0.584). The small subunit (RR2, with a molecular weight of 38,000) is encoded by a 1.2-kilobase mRNA that overlaps the 3′ end of the 5.0-kilobase transcript of the large subunit (1, 2, 13, 35). Unlike the mammalian and bacterial enzymes, however, HSV ribonucleotide reductase is insensitive to dTTP or dATP inhibition and does not have an absolute Mg^{2+} requirement (29, 30). Furthermore, the large subunit contains an additional domain, localized to the amino terminus, which produces a 50% increase in molecular weight (38, 57).

Studies of the large subunit have led to an apparent paradox. HSV gene expression is regulated at the transcriptional level in a cascade fashion that includes three classes of genes: immediate early (IE; alpha), delayed early (DE; beta), and late (gamma) (for a review, see reference 21). Temporally, the large subunit behaves as a DE protein. However, factors which typically do not allow DE gene expression (for example, presence of cycloheximide and IE175 temperature-sensitive mutants) do not affect its expression (8, 22, 27, 36, 56). By using an HSV-1 temperature-sensitive mutant that maps within ICP6, ribonucleotide reductase activity was shown to be required for viral DNA synthesis and replication (11, 47). However, more recent studies with an ICP6 deletion mutant demonstrated that ICP6 is required for virus growth and DNA synthesis only in nondividing cells and in cells grown at 39.5°C (19). We reached similar conclusions recently by using a HSV-2 isolate defective in ICP10 synthesis at 39.5°C (C. Smith, M. Kulko, T. D. Chung, S. Yasumoto, and L. Aurelian, submitted for publication). Moreover, DNA sequences that encode the large subunit of the HSV-2 ribonucleotide reductase (mtrIII, 0.554 to 0.584 m.u.) have been shown to have neoplastic potential (20). The latter did not depend on enzymatic activity because it was mediated by subfragments that cover the promoter, the proximal one-third of the ICP10 coding region, or both (20, 23–25). These findings, as well as the observations that the transforming potential of HSV-1 DNA maps at a site distant from ICP6 (6) and that the amino-terminal portions of the ICP6 and ICP10 proteins show the least homology (38%) (38) and differ from the amino-terminal domains of the large subunits of other ribonucleotide reductases studied to date (Epstein-Barr virus, *E. coli*, mouse, and clam [57]), raise the interesting possibility that the amino-terminal region of the HSV large subunit represents a functionally different domain.

The studies described in this report sought to obtain a better understanding of the regulatory aspects of ICP10 synthesis. We used transient expression assays to examine the effects of herpesvirus *trans*-activating factors on expres-

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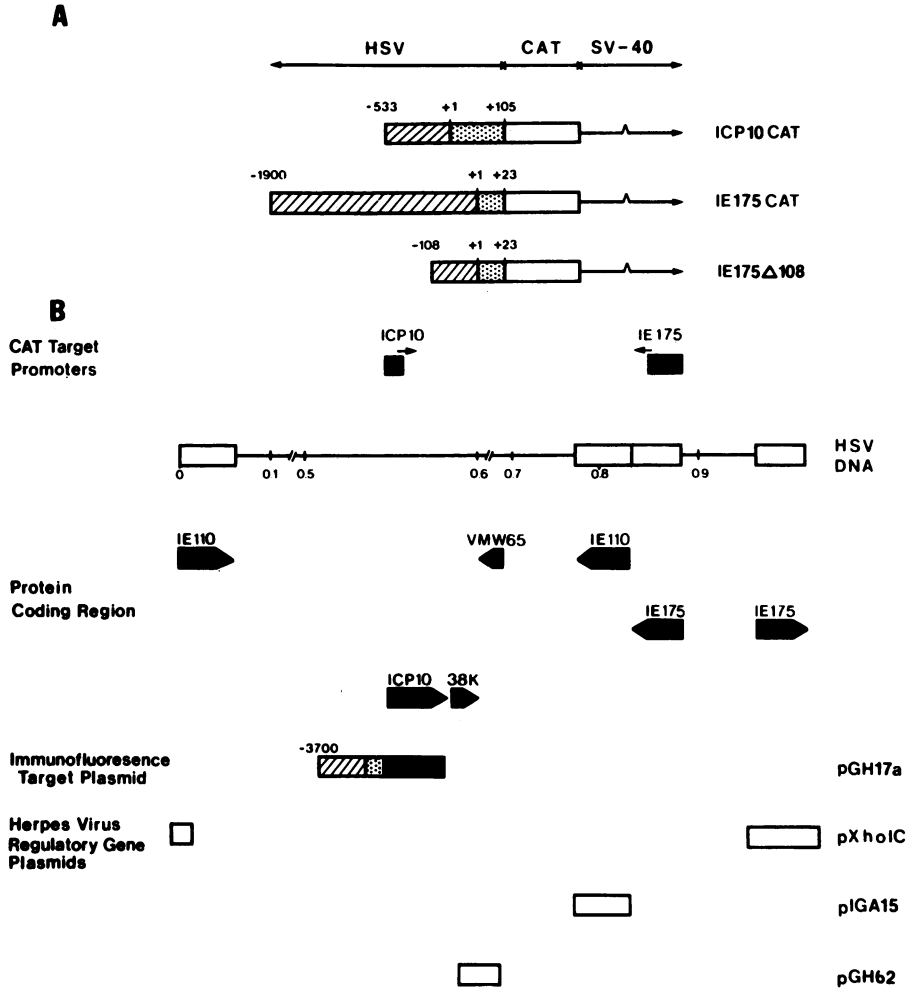


FIG. 1. Schematic representation of plasmids containing intact HSV *trans*-acting components and recombinant CAT target genes in relation to the HSV genomic map. (A) Summary of the structure of the recombinant CAT target genes containing ICP10-CAT, IE175-CAT, and IE175d108-CAT promoter regions. The HSV sequences in these recombinant constructions are indicated by the hatched and stippled boxes, with the numbers above them referring to sequences upstream (−) or downstream (+) from the cap site of the corresponding virus genes. The locations of these target promoters on the HSV genomic map are indicated by the solid boxes in B. Each CAT construct contained the virus mRNA cap sites and portions of the nontranslated leader sequences as indicated by the stippled boxes. In each case, the first translated codon was provided by the CAT coding region as indicated by open boxes. Each recombinant also contained the splice and polyadenylation signals from the SV40 early transcription unit. (B) Physical map of the locations of the cloned HSV DNA fragments containing IE genes or the late structural gene for Vmw65. The relative sizes and directions of transcription of the corresponding mRNAs are indicated by the solid arrowheads. The relative sizes and map locations of the virus DNA inserts in the series of plasmids used for cotransfection studies are indicated by the open boxes.

sion from the intact ICP10 gene and a chimeric ICP10 promoter-chloramphenicol acetyltransferase (CAT) gene construction. These studies, as well as sequence and gel retardation analyses, suggest that the ICP10 promoter contains response elements characteristic of IE genes.

MATERIALS AND METHODS

Cells and viruses. Vero (African green monkey) cells were grown in minimal essential medium containing 10% fetal calf serum, 150 U of penicillin G, and 150 μg of streptomycin sulfate per ml. HEp-2 cells were grown in medium 199 supplemented with 10% newborn calf serum and antibiotics. The cells from line 293 (adenovirus-type-5-transformed human embryonal kidney) were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and antibiotics. Primary rat astrocytes, obtained from M. Shin (University of Maryland at Baltimore, Baltimore, Md.), were prepared from dissociated neonatal rat brain and cleared of oligodendrocytes by consecutive cycles of shaking (100 rpm, 48 h, 37°C) in an orbital shaker (Bellco Glass, Inc., Vineland, N.J.). They were grown in equal volumes of Dulbecco modified Eagle medium and Ham F-12 medium containing 10% fetal calf serum and were >98% pure as determined by staining with antiglial fibrillary acidic protein antibody. Virus stock of HSV-2 (strain G) and HSV-1 (strain KOS) were prepared and titered as described previously (56).

Plasmids. All chimeric CAT constructions were prepared in pCATB′ (40) containing the CAT structural gene without eucaryotic promoter sequences. The target construction pICP10-cat contains a 649-base-pair (bp) (−544 to +105 relative to the mRNA cap site) fragment of HSV-2 (strain

TABLE 1. Summary of mutant synthetic oligonucleotides of the IE110 consensus virion response element

Sequence[a]	Binding site type[b]	Mutation[c]
CTGCATGCTAATGAGATTCTTG	O^+/T^+	None
CTGCATGCTAATGA*CCG*TCTTG	O^+/T^-	11,12,13
CTGCATGCT*CC*TGAGATTCTTG	O^-/T^-	6,7
CTGCA*GC*CTAATGAGATTCTTG	O^-/T^+	2,3

[a] Represents right-hand side 22 bp from the sense strand of the 44-bp double-stranded dimer.

[b] Abbreviations: O^+/T^+, wild-type IE110 element; O^+/T^-, a mutant oligomer lacking the TAATGARAT motif; O^-/T^-, an oligomer lacking both the OTF-1 and TAATGARAT motifs; O^-/T^+, an oligomer mutated to affect the OTF-1 motif.

[c] Mutation position (italicized and underlined codons in sequence column) relative to the start of the consensus element (boldface codons in sequence column).

333) DNA (0.554 to 0.558 m.u.) inserted 5′ to the CAT structural gene. pICP10-cat-initiated transcription will direct mRNA production and translation of the bacterial CAT enzyme. pICP10**R**-cat contains the promoter region of pICP10-cat in the reverse orientation. The construction of parental and deleted target constructs pIE175-cat and pIE175d108-cat was previously described (43). The structure and location of the virus sequences in plasmids pXhoIC, pIGA15, pGH62, pRL45, and pGH17a have been described previously. pXhoIC contains the gene for the IE175 protein of HSV-1 (40). pIGA15 for the IE110 protein and pGH62 is derived from pGR212b which contains the HSV-1 gene for Vmw65 (41, 42). pRL45 contains the human cytomegalovirus (HCMV) IE1 and IE2 transcription units (45). pGH17a contains the entire HSV-2 gene (coding and promoter) for the ICP10 protein (0.533 to 0.584 m.u.) (23) (Fig. 1).

DNA transfection. Cells were plated 24 h prior to transfection into six-well cluster dishes (6 mm by 35 mm; Costar, Cambridge, Mass.) at 10^5 cells per well. DNA transfection was performed by the calcium phosphate precipitation method and employed a glycerol boost (41). Routinely, transfection mixtures contained 1.0 μg of target supercoiled plasmid and equimolar *trans*-activator DNA. Parallel cotransfections employed pBR322 as a nonspecific DNA to equalize nucleic acid concentration effects. CAT-transfected cells were harvested 40 to 44 h posttransfection in CAT harvest buffer (40 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA) and stored at −20°C until assayed. Cells transfected for immunofluorescence were transferred into eight-well slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) 24 h posttransfection and allowed to grow at 37°C until fixation.

Antibodies. Monoclonal antibody (MAb) 58S specific for IE175 was obtained from M. Zweig (National Cancer Institute, Frederick, Md.). Its preparation and specificity were described (55). MAb 30 specific for ICP10 (L. Aurelian, P. Terzano, C. C. Smith, T. Chung, A. Shamsuddin, S. Costa, and C. Orlandi, Cancer cells, vol. 7. Molecular diagnostics of human cancer, in press), was prepared in our laboratory from an 8 h-HSV-2-infected cell extract.

Immunofluorescence. Slides were fixed in acetone at 40 h posttransfection. They were stained with primary MAb (1 h, 37°C) followed by fluorescein-conjugated goat anti-mouse immunoglobulin G (1 h, 37°C) as described (56).

CAT assays. All harvesting and CAT assays were performed as described previously (40). Routine assay conditions employed 0.2 μCi of [^{14}C]chloramphenicol substrate and an incubation time of 60 min. For quantitative estimates of CAT activity and fold activation, the appropriate sections were cut from the thin-layer chromatography plate and placed in toluene 2,5-diphenyloxazole-12,4-bis(5-phenyl-oxazole) benzene scintillation fluid and the radioactivity was counted in an LKB 216 liquid scintillation counter. Quantitative comparisons were made by measuring the amounts of chloramphenicol-acetate product with enzyme levels on the linear part of the curve for product formation versus extract concentration and time. pSV_2CAT was used as a positive

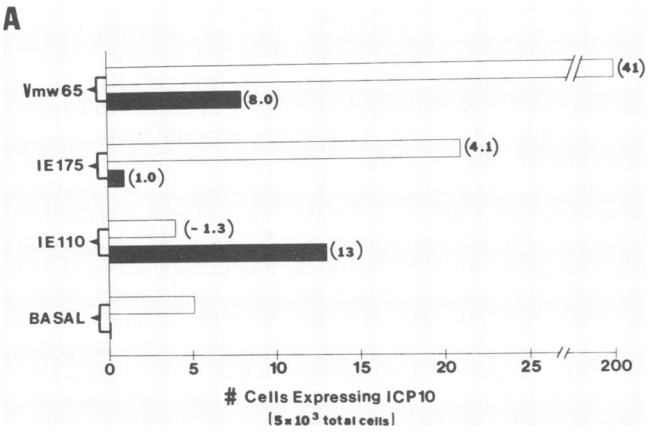
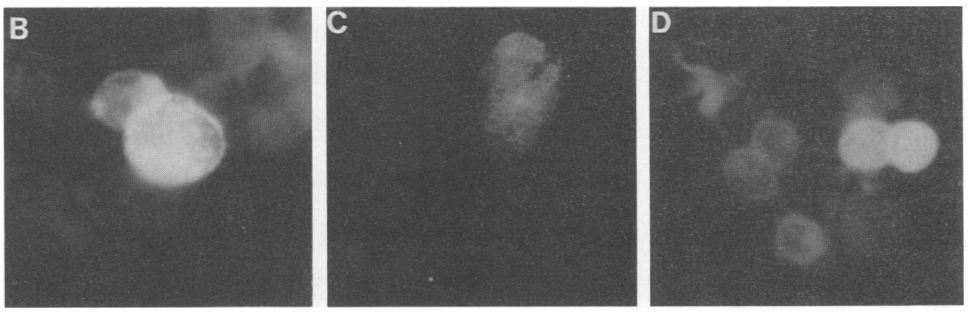


FIG. 2. ICP10 expression from the intact gene is activated by cotransfection with Vmw65-encoding DNA. (A) The 293 (□) and Vero (■) cells were cotransfected with pGH17a and intact regulatory genes, pGH62, pXhoIC, pIGA15, that respectively encode Vmw65, IE175, and IE110 proteins, or pBR322 (to assess basal expression) and stained with MAb 30. Typically, 5×10^3 cells were scored in each well, and results are shown as the average number of staining cells from three separate experiments. Parentheses represent fold activation (+) or inhibition (−) of basal expression. (B) The 293 cells cotransfected with pGH17a and pGH62 and stained with MAb 30. (C) Vero cells cotransfected with pGH17a and pGH62 and stained with MAb 30. (D) HSV-2-infected Vero cells (8 h postinfection) stained with MAb 30.



control, and the parent (pCATB') was used as a negative control (40).

Gel retardation assay. Assays were performed as described (46), with extracts of HEp-2 or Vero cells mock infected or infected with HSV-2 (strain G) or HSV-1 (strain KOS), respectively. HSV-2 infection was done at a multiplicity of infection of 20 PFU per cell for 4 h at 37°C. HSV-1 infection was done at the same multiplicity of infection but for 8 h. Cells were washed twice with phosphate-buffered saline (pH 7.2) and extracted by high-salt lysis of isolated nuclei according to Dignam et al. (10). The HSV-1-infected cell extract was prepared by ammonium sulfate selection as described (50), and the 33%-ammonium-sulfate fraction was used in these studies. Virion extract was prepared by Nonidet P-40 extraction of purified virions according to the method of Marsden et al. (32).

Typical gel retardation reaction mixtures contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 0.6 mM dithiothreitol, 2.3 mM $MgCl_2$, 80 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg of bovine serum albumin per ml, 4% glycerol, labeled DNA (5,000 cpm; 5 fmol), 1.25 μg poly(dI-dC)-poly(dI-dC), and 2 μg of cell protein extract in a total volume of 25 μl. When used, virion preparation (1.4 μg) and MAb 58S (1:1,250 dilution in reaction buffer) were added at the start of incubation. The concentrations of poly(dI-dC)-poly(dI-dC) (0.05 to 10 μg), cell protein extract (1 to 10 μg), virion preparation (0.7 to 3.5 μg), and MAb 58S (1:5,000 to 1:625) were optimal, as determined by previous titration with each DNA fragment. Reaction mixtures were incubated at 25°C for 10 min and electrophoresed on a 3.5% polyacrylamide gel (30:1, bis) containing 45 mM Tris-borate and 1.25 mM EDTA for 2 to 3 h with a constant current of 20 mA. Gels were dried and exposed for autoradiography.

DNA restriction fragments for gel retardation analysis were radioactively labeled with [^{32}P]dCTP by fill-in reaction with the Klenow form of *E. coli* DNA polymerase. Labeled DNA fragments were purified by 8% polyacrylamide gel electrophoresis in 90 mM Tris-borate–2.5 mM EDTA. DNA was purified from gel by overnight elution (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate) and subsequent centrifugation to separate DNA from gel fragments. Supernatant was phenol and chloroform extracted and then was precipitated in 2 volumes of ethanol. Synthetic oligonucleotides used as DNA competitors (Table 1) were prepared from 26-base-pair single-stranded oligonucleotides. Each sequence contained an 8-base-pair palindrome at the 3' end which was used to produce self-annealed forms of the oligomers and extended by T4 DNA polymerase into 44-base-pair double-stranded head-to-head dimers. Oligonucleotides were heated (65°C, 10 min), slowly cooled, and passed through an Elutip-d column tip (Schleicher & Schuell, Inc.) to remove single-stranded oligonucleotides (C. M. J. apRhys, D. M. Ciufo, E. A. O'Neill, T. J. Kelly, and G. S. Hayward, submitted for publication).

DNA sequencing methods and analysis. DNA sequence was identified by the Sequenase (USA Biochemicals, Cleveland, Ohio) dideoxy-chain termination method (51). Single-stranded synthetic oligonucleotide-priming fragments complementary to sequenced regions were prepared (Operon Technologies, San Paolo, Calif.), and sequencing was performed on M13 recombinant bacteriophage containing the ICP10 promoter region. Sequence data was compiled and analyzed with the Cyborg database manager (International Biotechnologies Inc.).

RESULTS

Vmw65 stimulates ICP10 protein expression from the intact target gene. To define the effects of *trans*-activator genes on the expression of ICP10, a fragment of the HSV-2 genome encoding ICP10 (pGH17a) was cotransfected into Vero and 293 cells with equimolar concentrations of pXhoIC, pIGA15, or pGH62, respectively, encoding IE175, IE110, and Vmw65. ICP10 expression was determined by immunofluorescent staining with MAb 30. In both cell lines, ICP10 expression, defined as the number of expressing (staining) cells, was significantly enhanced by cotransfection with DNA encoding Vmw65. DNA encoding IE175 did not increase the number of cells expressing ICP10, while such an increase was observed with IE110-encoding DNA but only in Vero cells (Fig. 2A). The staining pattern was similar to that observed in HSV-2-infected cells and included both cytoplasmic and intranuclear reactivity (Fig. 2B through D).

Virion *trans*-activating factor (Vmw65) stimulates transcription from chimeric ICP10 promoter-CAT constructions. The transcriptional regulation pattern of ICP10 expression was studied by preparing a recombinant plasmid (pICP10-cat) containing the CAT structural gene under the regulation of the ICP10 promoter (−544 to +105; Fig. 1), and the results are summarized in Fig. 3. They represent the average of six independent experiments. *trans*-Activation of the pICP10-CAT target by pIGA15 (IE110) and pRL45 (HCMV IE2) was maximal at low input doses of the activating DNA (0.1 μg, 10- to 15-fold activation) and decreased at higher doses (3.0 μg, fivefold activation). Vmw65-induced CAT expression was observed at low doses of the activator DNA (pGH62; 0.1 μg, threefold activation), and it increased with increasing doses of pGH62 DNA (3.0 μg, 12-fold activation), peaking beyond the concentration range shown in Fig. 3. This may reflect the weak basal expression of the Vmw65 promoter (C. apRhys, unpublished results), such that high DNA concentrations are required in order to produce the levels of *trans*-activator that can induce a response similar to those observed with IE110 and HCMV IE2. In contrast to the other *trans*-activator genes studied in these series, IE175 DNA did not enhance CAT expression from pICP10-cat at low doses and at high doses IE175 DNA appeared to have a small negative effect on CAT enzyme accumulation.

Orientation effects were studied with a chimeric CAT construction containing the ICP10 promoter in the reverse orientation (pICP10**R**-cat). In this case, basal expression (defined as the percent conversion by the CAT enzyme in cells transfected with pICP10**R**-cat alone) was 0.5%. IE175 cotransfection had no effect on CAT expression (0.4% conversion). IE110 (pIGA15) and Vmw65 (pGH62) had a minimal effect (1.8% and 1.1% conversion, respectively). However, we do not consider the latter to be significant, since basal expression from the construction containing the ICP10 promoter in the correct orientation (pICP10-cat) was 1.3% conversion. The low levels of expression from pICP10**R**-cat presumably reflected the presence of *cis*-elements that are operative in both orientations, albeit at significantly reduced levels when in reverse orientation.

To determine if the Vmw65-mediated enhancement of CAT expression from pICP10-cat was analogous to that seen for the IE175 promoter, we compared the effect of pGH62 on pICP10-CAT, pIE175-CAT, and a deletion mutant of the IE175 promoter region lacking Vmw65 responsiveness (pIE175d108-cat [43]). Essentially identical levels of CAT activity were induced from pICP10-cat and pIE175-cat, while there was no activation from pIE175d108-cat at any of

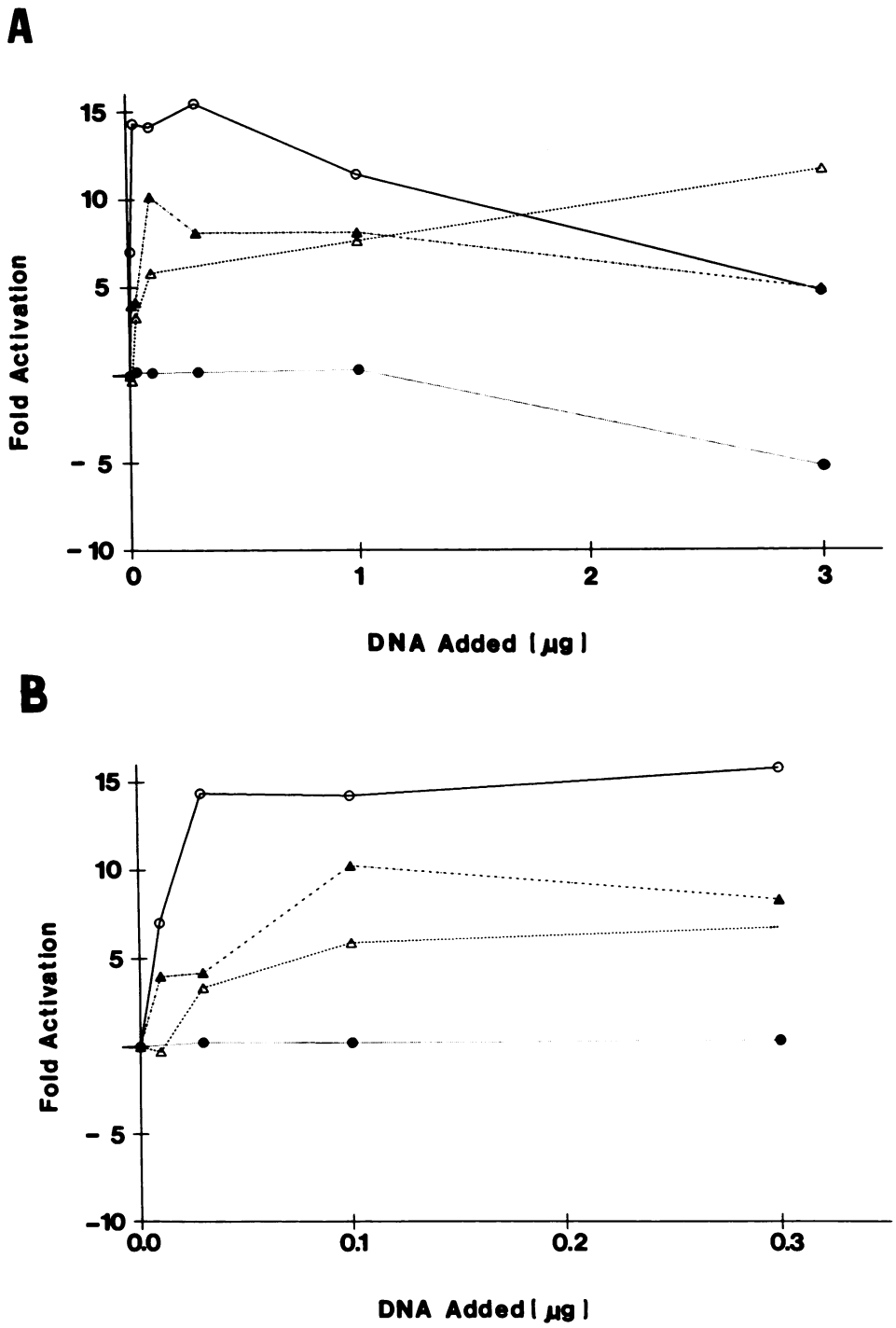


FIG. 3. Dose-response analysis of pICP10-cat activation. (A) Vero cells were cotransfected with pICP10-CAT (1.0 μg) and increasing amounts (0.01 to 3.0 μg) of DNA encoding IE110 (○), IE175 (●), Vmw65 (△), or HCMV IE2 (▲). Results are shown as fold activation above basal expression. (B) Detailed representation of the lower (0.01 to 0.3 μg)-concentration range of activator DNA. Symbols are explained in Fig. 3A.

the doses studied in this series (Fig. 4). We interpret these findings to suggest that response elements necessary for Vmw65 activation are similar in the IE175 and ICP10 promoters. The high basal expression of pIE175d108-cat is due to higher levels (2.0 versus 1.0 μg) of transfected pIE175d108-cat target DNA.

Effect of host cell type on pICP10-cat activation. The influence of cellular factors on the activation of pICP10-cat was examined by determining the activation potential of DNA encoding IE110, IE175, Vmw65, or HCMV IE2 in three additional cell types, i.e., an adenovirus-transformed human line (293), a primary, relatively specialized neuronal cell (rat astrocytes), and a human laryngeal carcinoma line, HEp-2. The results shown in Fig. 5 and 6 for astrocytes and

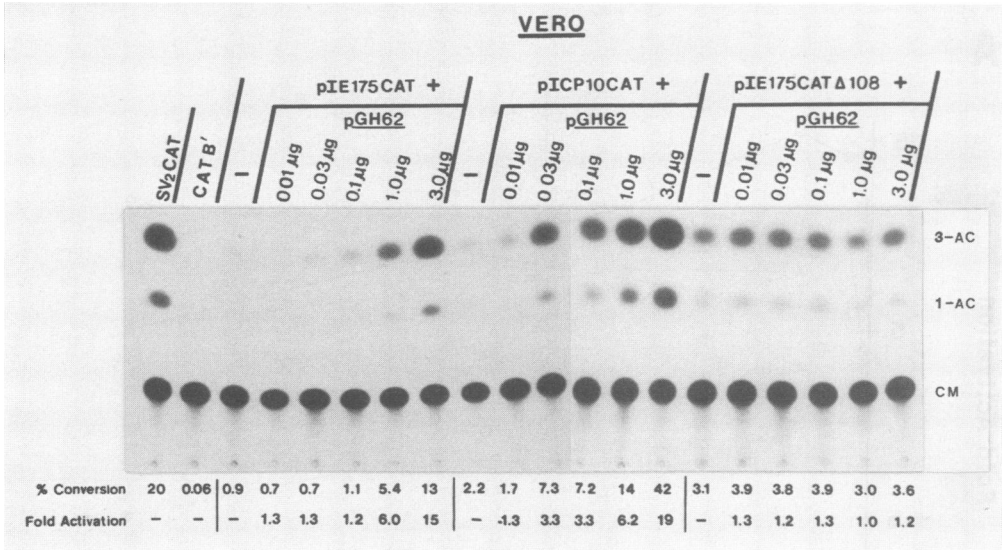


FIG. 4. Stimulation of pICP10-cat expression by Vmw65 is similar to that of pIE175-cat. Vero cells were cotransfected with target genes pIE175-cat (1.0 μg), pICP10-cat (1.0 μg), and pIE175d108-cat (2.0 μg) and increasing quantities (0.01 to 3.0 μg) of Vmw65-encoding DNA. Quantitative estimates of percent conversion and fold activation are shown below each sample.

293 cells respectively, indicate activation patterns for pGH62 cotransfection similar to those observed in Vero cells (Fig. 3). Virtually identical results were also observed in HEp-2 cells (basal; 0.9% conversion, activated 3.4 and 7.6% conversion at 0.1 and 1.0 μg pGH62, respectively). Identical dose responses for pGH62-induced activation of pICP10-cat, compared with those for pIE175-cat and pIE175d108-cat, were obtained in 293 and Vero cells (data not shown), supporting the interpretation that the ICP10 promoter, like that for IE175, contains Vmw65 response elements operative in all cell types studied. However, pXhoIC had a negligible (twofold) effect on pICP10-cat activity in primary astrocytes and 293 cells at all doses tested in these series. This differs from the reduced levels of pICP10-cat activity observed in Vero cells at higher pXhoIC doses, suggesting that the inhibition may be cell type restricted. Different results from those seen in the Vero cells were also obtained in rat astrocytes and 293 cells cotransfected with pIGA15, in that CAT activity was minimally (twofold) enhanced at low doses and minimally reduced (twofold) at high doses of pIGA15 (Fig. 5 and 6). We interpret these data to indicate that IE110-mediated activation of pICP10-cat activity is affected by host-cell type. In both Vero cells and astrocytes, cotransfection with pRL45 (HCMV IE2) activated expression from

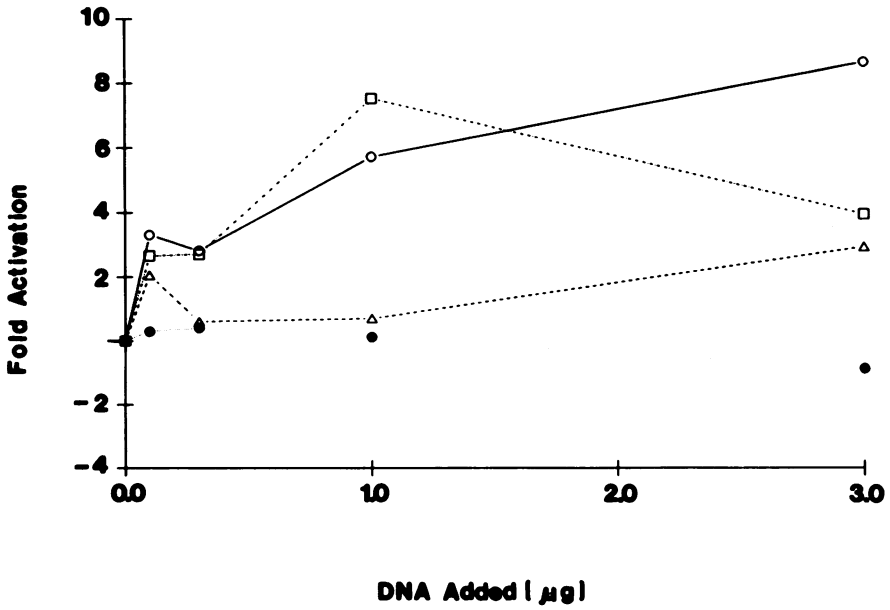


FIG. 5. Vmw65 but not IE110 activates pICP10-cat expression in primary astrocytes. Cells were cotransfected with pICP10-cat (1.0 μg) and increasing (0.1 to 3.0 μg) amounts of DNA encoding Vmw65 (○), IE175 (△), IE110 (●), or HCMV IE2 (□). Results are shown as fold activation above basal expression.

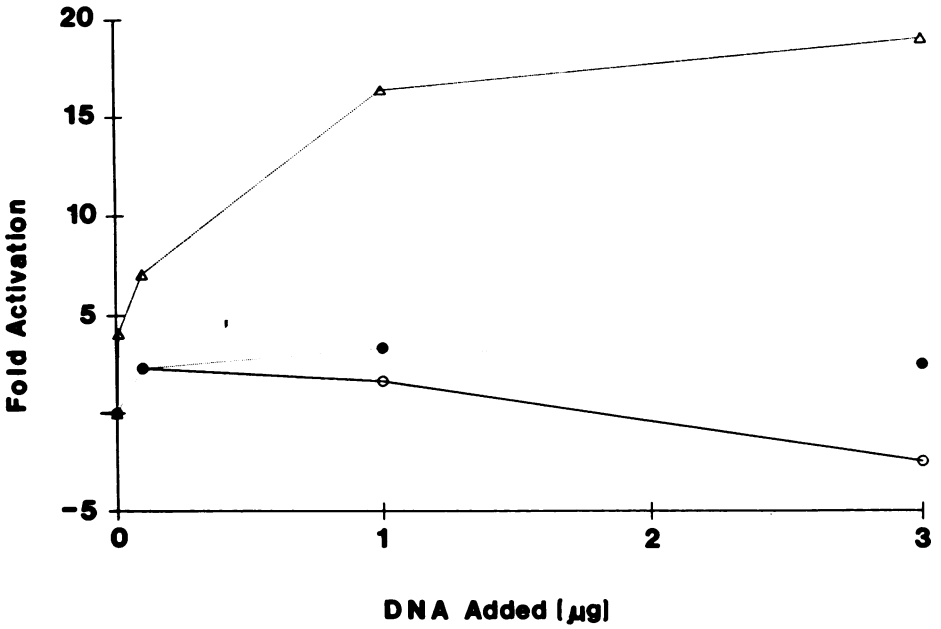


FIG. 6. Vmw65 but not IE110 activates pICP10-cat expression in 293 cells. Cells were cotransfected with pICP10-cat (1.0 μg) and increasing (0.01 to 3.0 μg) amounts of DNA encoding Vmw65 (△), IE175 (●), or IE110 (○). Results are shown as fold activation above basal expression.

pICP10-cat. However, the response was maximal at a lower concentration (0.1 μg) in Vero cells (Fig. 3), compared with that in astrocytes (1.0 μg [Fig. 5]).

Specific response elements in the ICP10 promoter region. The reported sequence analysis of the ICP10 gene (57) extends only 179 bp upstream of the cap site. In order to better interpret our data, we extended the sequence analysis to include upstream sequences not previously described. The findings are shown in Fig. 7. Based on data showing that the start of transcription of the HSV-1 5.0-kilobase mRNA is 23 nucleotides downstream of the TATA box (35) and on the homology between HSV-1 and HSV-2 (38), we conclude that the promoter fragment inserted into pICP10-cat begins approximately 544 bp upstream of the RNA initiation site and ends 105 bp downstream of this site. Our sequence data differ from that of Swain and Galloway (57) only at nucleotide +77 by including an additional cytosine residue. The canonical TATA homology (GATAAAA) is located 23 bp upstream of the cap site. Potential basal expression elements include one consensus octamer transcription factor (OTF-1) binding site (ATGCAAAT; beginning at nucleotide −129) (54), two consensus AP-1 binding elements (TGACTCAG; nucleotides −62 and −94) (31), and five possible SP-1 binding elements (GGCGGG; nucleotides −242, −303, −337, −344, and −393) (4). Vmw65 activation has been suggested to occur via various types of response elements, at least three of which (5, 26) are represented here. At nucleotide −122, (ATGCAAATGGGATTC) the first motif contains a consensus OTF-1 element that overlaps with a near consensus version of the Vmw65 activation element TAAT GARATTC. The second is immediately upstream (nucleotide −142) and consists of the sequence GCGGAAAC (59). The third element (ACGTGG, at nucleotide −168) is a possible alpha-H2–alpha-H3 (GCCACGTGG [26]) binding element in an inverted orientation. Finally, at nucleotide −321 we find the core element ATCGTC previously shown to be involved in IE175 binding to DNA (12, 50).

The ICP10 promoter forms complexes that include the Vmw65 protein. To begin addressing the question of how ICP10 is regulated as an IE gene and identify proteins that bind to the ICP10 promoter, we used gel retardation analysis with extracts of HSV-2 (G)-infected and mock-infected HEp-2 cells. In this assay, labeled DNA fragments are incubated with the protein fractions and subjected to gel electrophoresis. Specific protein-DNA complexes are de-

-535
GTCGACAGGCTGTACCGCTGGCAGCCGGATCTGCCGGGGCGCCCTACCGCACGCACCTGC
S

-475
ACATACGCCGCCTTCGCAGAGCTGGGTGTCATGCCAGACAACAGCCCCCGCTGTCTGCAC

-415 SP-1
CGCACCGAGCGGTTTGGGGCGGTCGGCGTTCCGGTTGTCATCCTGGAGGGCGTGGTGTGG

-355 SP-1 SP-1 IE 175 SP-1
CGCCCGGCGGGTGGCGGGCCTGCGCGTGATCGTCTATTGACGACGGCCGCCCAACCCGAG
E

-295 SP-1
CGACCTTCCCCTCCCACTTTCCCCCCCCCCCCTCCTACACACCAACTCCGCCCTCGCCGT

-235 ALPHA H2/H3
CTTGCCGTGCGCGGCCCCGTGCGTCCGTCTCAATAAAGCCAGGTTAAATCCGTGACGTGG

-175 'GCGGAAAC' OTF-1 \TAATGARAT
TGTGTTTGGCGTGTGTCTCTGAAATGGCGGAAACCGACATGCAAATGGGATTCATGGACA

-115 AP-1 AP-1
CGTTACACCCCCCTGACTCAGGAGATAGGCATATCCTCCTTAGATTGACTCAGCACACGA

-55
TCGCACCCCACCCCTGTGTGCCGGGGATAAAAGCCAACGCGGGCGGTCTGGGTTACCACA
P

+5
ACAGGTGGGTGCTTCGGGGACTTGACGGTCGCCACTCTCCTGCGAGCCCTCACGTCTTCG

+65
CCCACCGACTTCCTGTTGCGTTCCTGTCGGCCGGTGCTGTCCTGTCGAC

FIG. 7. Sequence analysis of ICP10 promoter fragment in pICP10-cat. Distance from the cap site is shown above first nucleotides in each row. Potential regulatory elements are shown in boldface with binding factor named above each relevant DNA sequence. *Eag*I, *Sal*I, and *Pvu*I sites, pertaining to gel retardation analysis (shown in Fig. 9 through 11), are listed below their respective sites as E, S, and P, respectively.

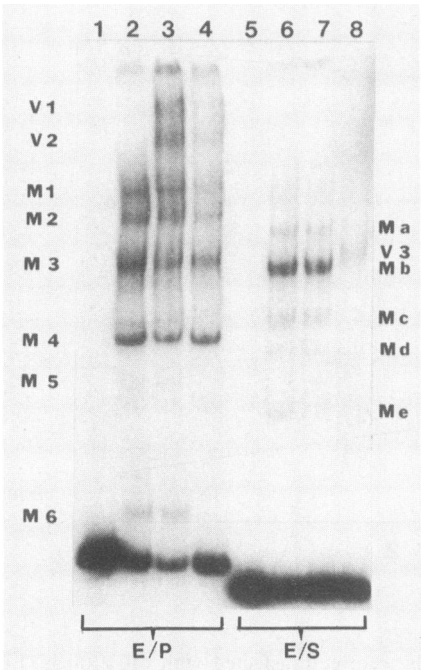


FIG. 8. TAATGARAT containing ICP10 promoter DNA forms novel complexes with uninfected cell extract and virion lysates. TAATGARAT containing (E/P, lanes 1 through 4) or lacking (E/S, lanes 5 through 8) DNA fragments (lanes 1 and 5) were incubated either with cell extracts from uninfected (lanes 2 and 6) or HSV-2-infected (lanes 4 and 8) cells or with a mixture of uninfected cell extract and virion lysate (lanes 3 and 7). Bands M1 through M6 and Ma through Me represent complexes formed by the E/P and E/S fragments, respectively, with uninfected cell extracts. V1, V2, and V3 represent complexes believed to contain HSV regulatory proteins.

tected by changes in electrophoretic mobility of the DNA fragments. The ICP10 promoter was cut with *Eag*I and *Pvu*I, to generate the E-P 257-bp fragment containing the TAATGARAT element involved in Vmw65 binding, and with *Sal*I and *Eag*I to generate the E-S 224-bp fragment that contains the ATCGTC core sequence involved in IE175 binding. The fragments were end labeled with ^{32}P and assayed in gel retardation. Both DNA fragments formed several complexes with proteins from mock-infected cells (Fig. 8). Reproducibly identifiable complexes formed with the E-P fragment are designated M1 through M6 in order of increasing electrophoretic mobility (Fig. 8, lane 2). These experiments were performed in the presence of competitor poly(dI-dC)-poly(dI-dC) to reduce nonspecific binding, suggesting that the complexes are formed by proteins with a greater affinity for the ICP10 promoter than for poly(dI-dC)-poly(dI-dC). Two bands of lower mobility, designated V1 and V2 (which presumably reflect complex formation with Vmw65), were observed when the E-P fragment was incubated with mock-infected cell extract mixed with the lysed virion preparation containing Vmw65 (Fig. 8, lane 3) or infected cell extract (Fig. 8, lane 4).

Five complexes (Ma through Me) in order of increasing electrophoretic mobility were also formed by interaction of the mock-infected cell extract with the E-S fragment (Fig. 8, lane 6). Incubation of the E-S fragment with the infected cell extract revealed a band of somewhat lower mobility than Mb, designated V3 (Fig. 8, lane 8). This band was not observed when E-S was incubated with mock-infected cell extract mixed with the lysed virion preparation (Fig. 8, lane 7), suggesting that the V3 complex is due to the interaction of the E-S fragment with an infected cell protein that is not a structural component of the virion.

An OTF-1-nuclear factor III binding element is necessary for Vmw65 complex formation. TAATGARAT sites in the IE110 promoter overlap with ATGCTAAT motifs that are similar to the immunoglobulin and histone H2b octamer response elements (OTF-1) and to nuclear factor III binding sites in the adenovirus 2 origin of replication (39; apRhys et al., submitted). Since a similar overlap between the TAATGARAT and OTF-1 response elements is present in the ICP10 promoter (E-P fragment), these experiments sought to determine the relative contribution of the TAATGARAT versus that of the OTF-1 response elements towards formation of the V1 and V2 complexes. E-P DNA was mixed with mock-infected cell extracts and virion lysates in the presence (or absence) of various concentrations (5 to 500 fmol = 1- to 100-fold excess) of the oligonucleotide competitors shown in Table 1. Formation of both the V1 and V2 bands was effectively competed by incubation with the OTF-1-TAATGARAT consensus element of the IE110 promoter even at equimolar (5 fmol) concentrations. Concomitantly, there was an apparent increase in the levels of detectable complexes M4, M5, and M6 (Fig. 9, lanes 4 through 7). Mutations in the 3′ end of the competitor oligonucleotide that affect the TAATGARAT but not the OTF-1 motif had no effect on competing ability (Fig. 9, lanes 8 through 11) at any concentration, while mutations affecting the 5′ end OTF-1 but not the TAATGARAT element competed with the formation of both complexes (Fig. 9, lanes 16 through 19) but only at higher concentrations (50 to 500 fmol). An oligonucleotide mutated to affect both the OTF-1 and TAATGARAT elements had no competing activity at 5- and 50-fmol concentrations and was only partially competitive at the highest concentrations (200 to 500 fmol) (Fig. 9, lanes 12 through 15).

The ICP10 promoter forms complexes that include IE175. In order to further define the interaction of the E-S fragment of the ICP10 promoter with infected cell proteins, it was assayed in gel retardation with extracts of mock-infected or HSV-1-infected Vero cells enriched for IE175 by infection in the presence, reversal, or both, of cycloheximide and selective ammonium sulfate precipitation. The E-P DNA fragments and a fragment generated by *Sph*I-*Bam*HI cleavage of the IE110 promoter that contains a defined IE175 binding site (ATCGTC) but no Vmw65 response element (50) were used as negative and positive controls, respectively. The results are shown in Fig. 10. Three bands (V4 through V6) were seen when the IE110 fragment was mixed with the HSV-1-infected (Fig. 10, lane 7) but not mock-infected (Fig. 10, lane 6) cell extract. Addition of MAb 58S, specific for IE175 (55), to the infected cell extract retarded migration (Fig. 10, lane 8), consistent with the interpretation that the DNA-protein complex contains IE175. Although significantly weaker, one band of approximately equal mobility to V4 was observed in mixtures containing the E-S fragment and HSV-1-infected cell extracts (Fig. 10, lane 3). Addition of the 58S antibody retarded migration (Fig. 10, lane 4) in a pattern essentially identical to that observed for the IE110 DNA fragment (Fig. 10, lane 8). Complex formation was not observed when E-S DNA was incubated with an extract of mock-infected cells (Fig. 10, lane 2). Furthermore, a complex similar to V4 was not formed by incubation of the E-P fragment with the infected cell extract, as suggested by the observation that there was no further retardation in the

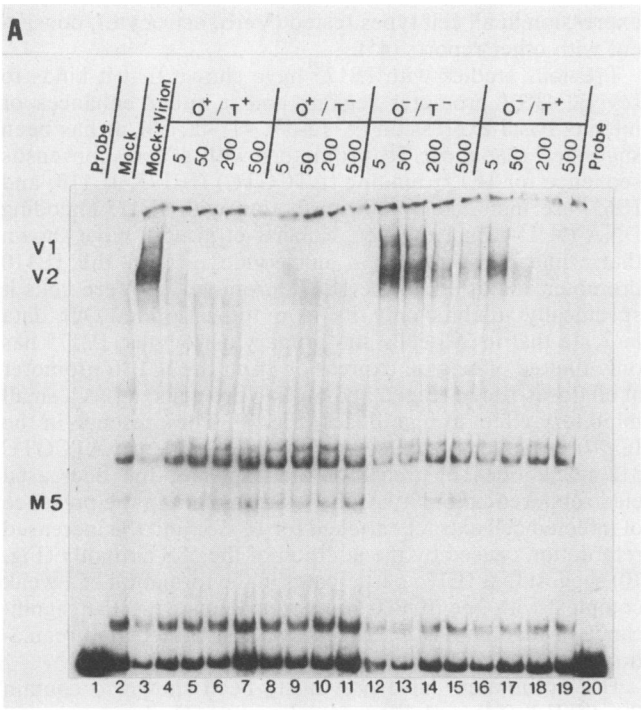


FIG. 9. OTF-1 motif is necessary for optimal binding of virion factor to ICP10 promoter DNA. (A) E-P DNA (5.0 fmol) was incubated with uninfected cell extract (lane 2), uninfected cell extract and virion lysate preparation (lane 3), or uninfected cell extract, virion lysate preparation, and competitor oligonucleotides (lanes 4 through 19). Oligonucleotides include the wild-type IE110 element (O^+/T^+, lanes 4 through 7), a mutant oligomer lacking the TAATGARAT motif (O^+/T^-, lanes 8 through 11), an oligomer lacking both the OTF-1 and TAATGARAT motifs (O^-/T^-, lanes 12 through 15), and an oligomer mutated to affect the OTF-1 motif (O^-/T^+, lanes 16 through 19). Concentrations of competitor (5 to 500 fmol) are shown above each lane. V1 and V2 represent complexes believed to contain HSV regulatory proteins. M5 represents a complex formed by the E-P fragment with uninfected cell extract. (B) Quantitative measurement of oligonucleotide competition with the formation of the V1 and V2 complexes. Radioactivity present in the V1 and V2 complexes was measured, and the results are expressed as the percent label in the complexes formed in the presence of the competitors, compared with the percent label in the V1 and V2 complexes formed in the absence of competitor. Symbols: ●, O^+/T^+; △, O^+/T^-; □, O^-/T^+; ▲, O^-/T^-; and ○, base line radioactivity measured from E-P DNA fragment mixed with uninfected cell extract at mobility of V1 and V2 complexes (lane 2).

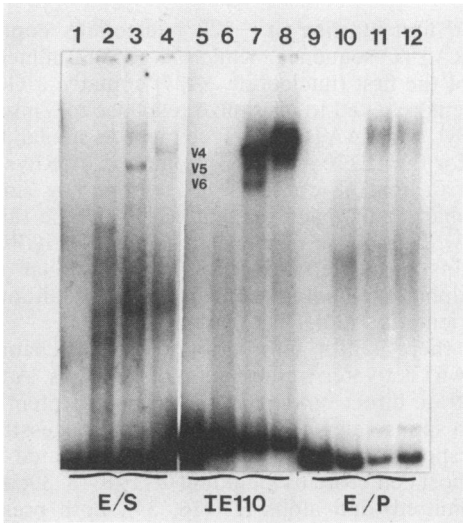


FIG. 10. Mobility shift of ICP10 promoter DNA complexes by IE175-specific antibody. DNA fragments E/S (lanes 1 through 4), E/P (lanes 9 through 12), and *Sph*I-*Bam*HI IE110 (lanes 5 through 8) were incubated with uninfected Vero cell extract (lanes 2, 6, and 10), IE175-enriched, HSV-1-infected cell extract (lanes 3, 7, and 11), or IE175-enriched HSV-1-infected cell extract and MAb 58S (lanes 4, 8, and 12). V4, V5, and V6 represent complexes believed to contain HSV regulatory proteins.

presence of MAb 58S (Fig. 10, lanes 11 and 12). Presumably, the complex formed by incubation of the E-P fragment with infected cell extract represents Vmw65 associated with OTF-1 and the octamer-TAATGARAT motif.

DISCUSSION

Synthesis of the large subunit of HSV ribonucleotide reductase (ICP6-ICP10) and accumulation of the enzymatic activity behave temporally as DE events, functions, or both (21, 37, 44). Paradoxically, however, previous studies from these (36, 56) and other (8, 22, 27) laboratories found that ICP6-ICP10 is synthesized under conditions (viz. cycloheximide reversal and nonpermissive temperature) that do not allow expression of other DE genes. The studies described in this report sought to address this apparent paradox by developing a better understanding of regulatory aspects of ICP10 regulation.

The salient feature of our data is the observation that expression from the ICP10 promoter is stimulated by cotransfection with pGH62 DNA that encodes a virion protein, Vmw65, previously shown to activate in trans the transcription of all five IE genes (5, 16, 17, 42, 43). Stimulation from the ICP10 promoter increased as a function of the pGH62 dose, and it was observed in all cell types studied in this series. This includes the 293 cells that contain endogenous *trans*-activating factors, thereby underscoring the dominance of the ICP10 response to Vmw65-mediated activation. pGH62-mediated enhancement of CAT expression from the ICP10 promoter was virtually identical to that observed from the IE175 promoter, but there was no detectable activation from pIE175d108-cat, a construction in which the *cis*-acting Vmw65-specific elements were deleted (Fig. 4).

Consistent with the interpretation that Vmw65-induced activation of the ICP10 promoter is mediated via *cis*-acting elements similar to those present in the IE175 promoter, sequence analysis revealed the presence of three elements showing homology to the consensus Vmw65 response ele-

ments. The first starting at −122 nucleotides contains a TAATGARATTC sequence, while the second immediately upstream of the first (nucleotide −137) contains a GCGGA AAC element believed to maximize response to Vmw65 (5). Significantly, our TAATGARAT element is similar to that described for the IE110 and IE63 promoters (apRhys et al., submitted) in that it contains an overlapping immunoglobulin-enhancer octamer element (OTF-1). The third potential Vmw65 response element present in the ICP10 promoter begins at nucleotide −168 and is similar to the alpha-H2-alpha-H3 binding element of IE63, although in inverted orientation (26).

To date, there is little information on the mechanism by which Vmw65 activates transcription of IE genes. Attempts to demonstrate direct binding of the Vmw65 protein to the *cis*-acting response elements have not been successful (32). Instead, responsiveness was shown to be mediated by the binding of host cell proteins including OTF-1 (18, 39; apRhys et al., submitted) and alpha-H1 (26, 34), both present in uninfected HeLa nuclear cell extracts. Elements containing the OTF-1-TAATGARAT overlapping motif present in the IE110 and IE63 promoters bind both factors (18, 26, 34, 39; apRhys et al., submitted). Consistent with recent publications (18; apRhys et al., submitted), we show that the involvement of Vmw65 in DNA-protein complex formation requires the OTF-1 consensus sequence for optimal binding. Thus, we found that the migration of the E-P DNA fragment (containing the OTF-1-TAATGARAT element) is retarded by the addition of virion preparations to mock-infected cell extracts (Fig. 8). Binding of the virion factor (presumably Vmw65) depends on the OTF-1 motif, as evidenced by competition experiments with oligonucleotides covering the entire binding element. The OFT-1 motif, however, may not be sufficient for Vmw65 complex formation, as V1 and V2 formation was partially competed with oligonucleotides containing a functional TAATGARAT but lacking the OTF-1 element. A similar conclusion was recently reached for the IE110 promoter by using purified OTF-1 and nuclear factor III preparations (18; apRhys et al., submitted).

Cellular proteins have been shown to bind to alpha-H2-alpha-H3 (26) and GCGGAAAC (59) binding elements found in IE promoters. The latter is believed to potentiate Vmw65-induced activation (5). While their functions, if any, are unclear and the cellular proteins that they might bind are unknown, both the GCGGAAAC and the alpha-H2-alpha-H3 binding elements are present in the ICP10 promoter. Indeed, the E-P DNA fragment formed at least six identifiable complexes with extracts of uninfected cells. In competition experiments, several of these (M4 through M6) were inversely correlated with the formation of Vmw65-dependent complexes V1 and V2 (Fig. 9), possibly reflecting the presence in these bands of proteins that bind to one or both of these elements.

Transient expression assays with the HCMV IE2 and HSV IE110 genes have shown that these proteins function as nonspecific *trans*-activators in that they stimulate expression from both the major classes of HSV and heterologous promoters (41, 42, 45). The cotransfected IE110 gene stimulated expression from the ICP10 promoter in Vero cells but not in 293 cells or primary astrocytes. Additionally, the Vero cell stimulation showed a biphasic pattern in that it peaked at low rather than high doses of cotransfected activator DNA (Fig. 3). The mechanism responsible for these *trans*-activation patterns has not yet been defined and may involve posttranslational effects, mRNA stability, or transcriptional regulation. The HCMV IE2 protein was able to activate expression in all cell types tested (Vero, astrocyte), consistent with other reports (45).

Previous studies with IE175 have shown that it binds to several HSV promoter regions and it either enhances or inhibits basal expression (9, 15–17, 41, 42, 50). It has been suggested that those IE promoters with a core consensus sequence for IE175 binding (ATCGTC) (IE175, IE110, and IE63) are inhibited by cotransfection with IE175-encoding DNA (9, 15–17). However, Roberts et al. (50) have shown that while IE175 binds at nucleotide −60 in the IE110 promoter and at +1 in the IE175 promoter, in Vero cells it specifically inhibits only its own transcription. Our data indicate that in 293 cells and primary astrocytes, IE175 has only limited effects on expression from the ICP10 promoter at all doses tested (Fig. 5 and 6). In Vero cells, it has a small inhibitory effect at high doses (Fig. 3). The presence in the ICP10 promoter of the core consensus sequence ATCGTC 312 nucleotides upstream of the cap site, the decreased electrophoretic mobility of the E-S fragment in the presence of infected cell extract enriched for IE175, and the increased retardation caused by the addition of the 58S antibody (Fig. 10) suggest that IE175 participates in the formation of a weak complex with the ICP10 promoter. However, the significance of this binding with regard to IE175-mediated regulation from the ICP10 promoter remains unclear.

HSV promoters have previously been shown to contain the OTF-1 (54) and SP-1 (4) elements. However, to our knowledge ICP10 is the first HSV promoter recognized to contain AP-1 (31) elements. A consensus polyadenylation site (AATAAA) for the adjacent 50K virion protein gene (33) is located 198 nucleotides upstream of the cap site. The five potential SP-1 elements and the IE175 core-binding motif are located upstream of this polyadenylation site. This is not an uncommon finding in HSV genes. For instance, the entire 38K (RR2) promoter is located within the ICP10 coding region (57). In any case, the regulatory elements OTF-1-TAATGARAT, GCGGAAAC, alpha-H2-alpha-H3, and both AP-1 elements are distal to the polyadenylation site but upstream of the TATA element. Significantly, the OTF-1-TAATGARAT and one of the AP-1 elements are conserved in the ICP6 promoter (33). The AP-1 elements may play a role in ICP10 expression as an IE gene since AP-1 binding factors are constitutively expressed in most cell systems studied to date (14, 31, 48).

We are presently preparing deletion- and site-directed point mutations designed to specifically establish the role of the *cis*-response elements identified in this study. Until these data are obtained, final conclusions pertaining to the regulation of the ICP10 promoter are premature. However, consistent with previous suggestions (56), our findings indicate that under certain conditions ICP10 can be regulated as an IE gene, independent of IE175 (36). What, if any, is the significance of this observation with respect to HSV-2 infection? Expression of DE genes involved in viral DNA replication requires prior IE protein synthesis and coincides with irreversible changes in cell macromolecular metabolism that are likely to result in cell death (27, 28). ICP10 expression as a DE gene is therefore incompatible with long-term cell survival, such as would be the case in latently infected nerve cells. However, recent studies with an HSV-1 deletion mutant have shown that ICP6 expression is essential for virus growth and DNA synthesis in nondividing cells or in cells at 39.5°C (19), a conclusion that we have independently reached with a HSV-2 isolate that is defective in ICP10 expression at 39.5°C (Smith et al., submitted). Therefore, it might be predicted that if indeed ICP10 is required for the

establishment, maintenance, or both, of latency in cells of neuronal origin, it might be regulated as an IE gene. Our data in primary astrocytes are consistent with this interpretation.

Sequences that encode the N-terminal portion of ICP10 have been shown to have neoplastic potential (20, 23–25), possibly involving gene amplification or the rearrangement of oncogenes or of cell cycle regulatory proteins (49, 53) that are required for normal cell growth (Aurelian et al., in press). Indeed, HSV induces the replication of integrated simian virus 40 (SV40) genes in SV40-transformed cells (52) and the amplification of cotransfected SV40 DNA sequences in cells which are nonpermissive for SV40 DNA replication (7). Similarly, cells transformed by an HSV-2 isolate deficient in ICP10 synthesis at 39.5°C fail to express neoplastic potential at the nonpermissive temperature at early passages; at later passages, alternate (presumably cellular) functions required for anchorage-independent growth are induced and these compensate for the defect in ICP10 expression at 39.5°C (Smith et al., submitted). Because expression of DE genes is associated with the arrest of host-DNA replication, it is incompatible with induction of gene amplification and long-term cell survival; this predicts that if ICP10 is involved in neoplastic transformation, it should be regulated as an IE gene. Indeed, recent studies from our laboratory suggest that ICP10 contains a serine- and threonine-specific protein kinase activity apparently encoded within the amino-terminal domain unique to HSV (T. D. Chung, J. P. Wymer, C. Smith, M. Kulka, and L. Aurelian, submitted for publication). Regulation as an IE gene may be required for this function. In this context, it may be particularly significant that the ICP10 promoter contains two AP-1 consensus elements since the large T antigen of SV40 (14) and the oncogenic v-*fos* (48) and v-*jun* (3) proteins all interact either directly or indirectly with AP-1 binding factors.

ACKNOWLEDGMENTS

We thank Mabel Chiu, Dolores Cuifo, Andrew Lieberman, and Rita Fishelevich for excellent technical assistance, Richard Ambinder and Colette apRhys for the self-complementary oligolabeling procedure, Scott Roberts for the IE175-enriched, HSV-1-infected Vero cell extract, and Deniece Garnett for help with the figures.

This study was supported by Public Health Service grants CA-39691 to L.A. and CA-28473 to G.S.H. from the National Cancer Institute. J.P.W. is a Medical Scientist Scholar of the Life and Health Medical Research Fund.

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