JOURNAL OF VIROLOGY, Aug. 1993, p. 4981–4991
0022-538X/93/084981-11$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 67, No. 8

Human Cytomegalovirus Immediate-Early Gene 2 Protein Interacts with Itself and with Several Novel Cellular Proteins

BETH A. FURNARI,[1,2] ERIC POMA,[1,2] TIMOTHY F. KOWALIK,[2] SHU-MEI HUONG,[2] AND ENG-SHANG HUANG[1,2,3,4]*

Departments of Microbiology and Immunology[1] and Medicine,[3] Curriculum of Genetics,[4] and UNC Lineberger Comprehensive Cancer Center,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received 8 February 1993/Accepted 18 May 1993

The human cytomegalovirus immediate-early gene product 2 (IE2) is able to transactivate homologous and heterologous promoters alone or augmented by immediate-early gene product 1 (IE1). IE2 has also been shown to autoregulate the major immediate-early promoter by directly binding to a *cis* repression signal located between the TATA box and the cap site. However, IE2 has not been shown to act directly through a specific DNA sequence in transactivating various promoters. To understand whether IE2 can be indirectly involved in DNA sequence-specific transactivation through interactions with other transcriptional factors, we performed a study of the interactions of IE2 with cellular proteins. In order to study these interactions, IE cDNAs were subcloned into a bacterial expression vector, pGEX2T, by polymerase chain reaction amplification to produce fusion proteins which were full-length as well as proteins which contained various functional domains. We were able to demonstrate IE2's ability to interact directly or indirectly with several cellular proteins ranging from >200 to 14 kDa through glutathione S-transferase-fusion protein precipitation and far-Western analysis. These interactions have been mapped to domains within IE2 which are known to be necessary for either transactivation or both transactivation and autoregulation. All of the IE2-associated proteins are nuclear proteins, and a subset are phosphorylated. In vitro-synthesized ^{35}S-IE2 protein and bacterially expressed IE2 fusion proteins were used to study IE2-IE2 interaction by binding assay and far-Western analysis. IE2-IE2 interactions were mapped to a domain containing a putative helix-turn-helix motif located near the C terminus of IE2, between amino acids 456 and 539. However, IE2 was unable to directly interact with either IE1, an alternatively spliced variant of IE2 (55 kDa), or IE2 deletion mutants that did not contain the multimerization domain.

Like the genomes of all members of the herpesvirus family, the viral genome of human cytomegalovirus (HCMV) is expressed in a sequential fashion. HCMV lytic gene expression is divided into three categories: immediate-early (IE), early, and late (10, 42, 68, 69). IE gene expression which occurs in the absence of de novo protein synthesis includes the major IE (1, 24, 56, 58), UL36-38 (28, 62, 63, 71), and US3 (70) genes. The major IE region consists of two transcription units, IE1 and IE2, located downstream of the major IE promoter (MIEP) (56–58). The IE1 transcription unit consists of the first four exons and codes for a 1.95-kb mRNA that translates into a 72-kDa protein (56, 58). The second transcriptional unit, designated IE2, includes exons 1 through 3 and exon 5. Transcripts produced from this region encode several mRNAs of 2.25, 1.70, and 1.40 kb and, upon translation, result in proteins of 84, 55, and 40 kDa, respectively (54, 56–58). All of the major IE proteins share a N terminus of 85 amino acids derived from exons 2 and 3, with the exception of the 40-kDa protein that originates solely from exon 5 (57). Interestingly, the functional characteristics of the different IE proteins differ dramatically even though they share many domains.

IE1 has been shown to function as a transactivator of the MIEP via NF-κB (7, 51). Additionally, IE1 transactivates several heterologous promoters and augments the transactivation activity of the 84-kDa IE2 protein on various cellular and viral promoters (4, 11, 15, 17, 19, 41, 49, 53, 64, 67). The domains in IE2 which are required for transactivation are located between amino acids 1 and 97 and between amino acids 195 and 579 (41, 48, 49, 55). Autoregulation of the MIEP through the C-terminal half of IE2 (amino acids 290 to 579) occurs via a *cis* repression signal (CRS) located between the TATA box and the transcription start site (18, 39, 47–49, 55). Recently, IE2 has been demonstrated to bind directly to the CRS element (35).

An alternatively spliced version of IE2, the 55-kDa protein (54), appears to function as a transcriptional activator of the MIEP, probably by a mechanism other than directly through the CRS element (2), and seems to be incapable of activating expression of many early genes (41, 48, 55). The primary difference between the 84- and 55-kDa proteins is an internal deletion of amino acids 365 to 519. Thus, the abilities of the 84-kDa IE2 protein to transactivate heterologous and homologous promoters as well as downregulate the MIEP correlate with the deleted region in the 55-kDa protein. These conclusions have been further documented through the use of insertional mutations of IE2 in transient transfection assays (2, 55). An N-terminal truncation of IE2, the 40-kDa protein (amino acids 341 to 579), which is expressed at late times of infection (54), retains only the downregulation function (18, 47, 49).

Identification of protein-protein interactions between cellular and/or viral proteins with IE2 is the first step in understanding the mechanisms utilized by the various forms of IE2 to transactivate and autoregulate promoters. In the present study, we set out to (i) determine whether IE2

* Corresponding author.

specifically interacts with cellular and/or viral proteins, (ii) identify the domains of IE2 responsible for these interactions, and (iii) demonstrate which cellular proteins directly interact with IE2. To study protein-protein interactions between IE2 and cellular proteins, a fusion protein binding assay consisting of a glutathione S-transferase (GST)-IE2 chimera protein incubated with precleared, labeled lysates was employed. This procedure, which has been successfully utilized by others (5, 26), would allow for the detection of both weak and strong protein-protein interactions. Through this assay, we were able to identify several protein-protein interactions between IE2 and cellular proteins ranging from >200 to 14 kDa. Chimeric IE2 deletion constructs that were based on the known domains required for transactivation, autoregulation, or both allowed for the localization of regions in IE2 required for these protein-protein interactions. Through far-Western analysis, a subset of cellular proteins directly interacting with IE2 was detected. Serendipitously, IE2 was found to interact with another IE2 molecule through a C-terminal domain that contains two putative amphiphilic α-helices separated by a short β-turn. Oligomerization between IE2 and IE1 or the 55-kDa IE2 protein, which does not encode the required C-terminal domain, was not detected.

MATERIALS AND METHODS

Cell culture. HCMV Towne strain, passages 36 to 39, was propagated as described previously (21). Human embryonic lung fibroblasts (HEL) were grown in Dulbecco's modified minimal essential medium with high glucose (DMEM-H) supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Radioisotopic labeling. HEL cultures grown to 80% confluency were fed fresh medium 24 h prior to labeling with either [^{35}S]methionine (1,200 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) or ^{32}Pi (P_i, 285 Ci/mg; ICN, Costa Mesa, Calif.). For ^{35}S labeling, HEL cells were washed twice with phosphate-buffered saline (PBS [pH 7.5]) (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl) and incubated in methionine-free medium supplemented with 2% fetal bovine serum and [^{35}S]methionine (100 μCi/ml of medium) for 24 h. For ^{32}P labeling, HEL cells were washed twice with Tris-buffered saline (TBS) (25 mM Tris [pH 8.0], 150 mM NaCl) and preincubated in phosphate-free DMEM-H supplemented with 2% dialyzed fetal bovine serum for 2 h. Fresh phosphate-free medium supplemented with 2% dialyzed fetal bovine serum and $^{32}P_i$ (830 μCi/ml of medium) was added to cells, and the mixture was incubated for 3 to 4 h. Labeled cells were then washed twice with either ice-cold PBS or TBS and scraped from the surface of the roller bottles in 20 ml of buffer. The cells were then pelleted and washed once more. The cell pellet was resuspended in 1 ml of ice-cold EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate supplemented with aprotonin [Sigma, St. Louis, Mo.] and leupeptin [Boehringer Mannheim, Indianapolis, Ind.] [10 μg/ml each], 1 mM phenylmethylsulfonyl fluoride [PMSF] [Sigma]) and incubated at 4°C for 1 h. The debris was removed by microcentrifugation for 20 min at 4°C. The supernatant was aliquoted and stored at −70°C. Labeling efficiency was determined by trichloroacetic acid precipitation.

For ^{35}S-labeled, infected HEL lysate, HEL cultures grown to 80% confluency were fed 24 h prior to HCMV infection and labeling with [^{35}S]methionine. Before being labeled, HEL cultures were infected with a multiplicity of infection of 2 for 2 h. The medium was removed and replaced with medium supplemented with [^{35}S]methionine (100 μCi/ml of medium) for 24 h and processed as described above.

Cytoplasmic and nuclear fractions from ^{35}S-labeled cells were prepared essentially as described previously (12). Following ^{35}S labeling, HEL cells were scraped and suspended in ice-cold PBS. The cells were pelleted and washed twice with PBS. The cell pellet was resuspended in PCV buffer {10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] 1 mM dithiothreitol [DTT], 10 mM KCl, 100 mM NaF, 200 μM sodium orthovanadate, 10 μg of aprotonin per ml, 10 μg of leupeptin per ml, 1 mM PMSF}, incubated on ice for 10 min, and pelleted. The cells were resuspended in PCV buffer and Dounce homogenized briefly. The suspension was centrifuged at 16,000 rpm in a Sorvall HB4 rotor, and the cytoplasmic fraction was removed and stored at −70°C. The pellet was resuspended in 1 ml of nuclear resuspension buffer (20 mM HEPES [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 20% glycerol, 10 μg of aprotonin per ml, 10 μg of leupeptin per ml, and 1 mM PMSF) for every 10^9 cells and incubated on ice for 20 min with agitation. NaCl was added to a final concentration of 0.4 M to solubilize the nuclei, and the extract was centrifuged at 150,000 × *g* for 90 min. The nuclear extract was dialyzed twice against 20 mM dialysis buffer (20% glycerol, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1 mM PMSF), and aliquots were stored at −70°C.

GST fusion constructions. pGEX.IE1, pGEX.IE2-55K, pGEX.IE2-84K, pGEX.IE2.1-539, pGEX.IE2.1-291, pGEX.IE2.1-100, pGEX.IE2.97-579, pGEX.IE2.97-539, pGEX.IE2.97-456, pGEX.IE2.285-539, and pGEX.IE2.532-579 (the amino acid coding region of each construct is indicated by the number after pGEX.IE2) were cloned by polymerase chain reaction (PCR) amplification of the appropriate coding regions from IE cDNA clones pGEM4-1T (IE1), pGEM4-2T (IE2; 84 kDa), and pRSVIE2-55 (IE2; 55 kDa; gift from Jay Nelson) with combinations of the following primers: 5′IE1&2, 5′ ACGAATTCGGATCCATGGAGTCCTCTGCCAAGAG 3′; 5′IE2.97, 5′ ACGAATTCGGATCCATGGGTATCGATTCCAGTAGC 3′; 5′IE2.285, 5′ ACGAATTCGGATCCATGGCATCCGTGACCTCGAGC 3′; 5′IE2.532, 5′ ACGAATTCGGATCCATGTTCATGCTGCCTATCTACGAG 3′; 3′IE1, 5′ ACGAATTCGGATCCTGATTAGTGGGATCCATAACAGTAAC 3′; 3′IE2, 5′ ACGAATTCGGATCCTGATTACTGAGACTTGTTCCTCAG 3′; 3′IE2.100, 5′ ACGAATTCGGATCCTGATTAGGAATCGATACCGGCATGATTG 3′; 3′IE2.291, 5′ ACGAATTCGGATCCTGATTAGGCTCGAGGTCACGGATG 3′; 3′IE2.456, 5′ ACGAATTCGGATCCTGATTACACTTTGGGTGGATGTGTC 3′; and 3′IE2.539, 5′ ACGAATTCGGATCCTGATTACGTCTCGTAGATAGGCAGCATG 3′.

The 5′ amplimers contain a *Bam*HI site adjacent to a initiation codon (underlined) such that the resulting product would be cloned in frame with the GST gene. The 3′ amplimers contain a *Bam*HI site adjacent to a stop codon (TGA, underlined) for easy detection of cloned inserts oriented in the wrong direction. PCR reactions were composed of 10 ng of DNA template, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM Mg_2SO_4, 0.1% Triton X-100, 100 μg of bovine serum albumin per ml, 200

μM deoxynucleoside triphosphate (Pharmacia, Piscataway, N.J.), 10 pmol of each amplimer, and 1 U of Vent DNA polymerase (New England BioLabs, Beverly, Mass.) overlaid with mineral oil. Amplification was performed with a program consisting of 2 initial cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min followed by 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and 2 final cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min to create the various constructs mentioned above. A sample of each PCR reaction was analyzed by gel electrophoresis, and the remainder was purified with Geneclean (Bio 101 Inc., La Jolla, Calif.) according to the manufacturer's specifications. The isolated PCR-amplified DNA was digested with *Bam*HI for at least 2 h and subjected to gel electrophoresis through a 1% agarose gel. The PCR fragments were isolated from agarose by Geneclean as specified by the manufacturer and ligated into the *Bam*HI site of the pGEX-2T (Pharmacia) polylinker, which had been treated with calf intestinal phosphatase (Promega, Madison, Wis.). Double-stranded sequencing was performed on positively expressed constructs with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and Sequentide (Amersham) as directed by the manufacturer.

Expression and purification of GST fusion proteins. Expression of GST fusion proteins was as described by Smith and Johnson (52). Fresh overnight cultures of *Escherichia coli* [BL21(DE3)pLysS; Novagen] transformed with either pGEX-2T or one of the recombinants were diluted 1:20 in Luria-Bertani medium with both chloramphenicol (34 μg/ml) and ampicillin (50 μg/ml). After 2 h of growth in a 37°C shaker, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 0.1 mM, and incubation was continued for an additional 2 h. Cultures were pelleted by centrifugation at 2,500 × *g* for 10 min at 4°C and resuspended in 1 ml of ice-cold 1× PBS for every 10 ml of original culture volume; aliquots of 1 ml were stored at −70°C. Prior to each experiment, an aliquot was lysed by rapid thawing at 37°C and mild sonication. After centrifugation at 14,000 × *g* for 2 min at 4°C, fusion proteins were recovered from the supernatant by rocking with 25 μl of glutathione-Sepharose 4B beads (Pharmacia) for 15 min at 4°C. The glutathione-Sepharose beads complexed with fusion proteins were washed three times with ELB^+ (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 5 mM EDTA, 0.5 mM DTT, 1 mM PMSF). For analysis of bound fusion proteins, the beads were boiled in Laemmli sample buffer (31) and loaded onto a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. After electrophoresis, fusion proteins were visualized by Coomassie blue staining.

IE2 binding assay. Following expression and recovery of GST fusion proteins on glutathione-Sepharose beads, the bead-fusion protein complexes were equilibrated with three washes of ELB^+ buffer. The beads were incubated at 4°C for 2 h with either [^{35}S]methionine- or $^{32}P_i$- labeled whole-cell, nuclear, cytoplasmic, or programmed rabbit reticulocyte lysates (see below) precleared with GST-coated beads. The bead-associated complexes were then washed five times in 1 ml of ELB^+ buffer, pelleted, and boiled for 2 min in Laemmli sample buffer. Interacting proteins were resolved by electrophoresis through an SDS–10% polyacrylamide gel and visualized by either fluorography or autoradiography.

In vitro transcription and translation. PCR was used to amplify the coding regions of IE1 and IE2 from the IE cDNA clones pGEM4-1T (IE1) and pGEM4-2T (IE2; 84 kDa) with the following primers: 5′IE1&2Hind, 5′ CCCAAGCTTC CACCATGGAGTCCTTCTGCCAAGAG 3′; 3′IE1Sal, 5′ CTCGTCGACGTGGGATCCATAACAGTAAC 3′; and 3′IE2Sal, 5′ CTCGTCGACCTATGTACAAGAGTCCATG TC 3′.

The 5′IE1&2Hind amplimer contains a *Hin*dIII restriction site adjacent to a Kozak sequence before the start codon (underlined) to allow for efficient translation, and the 3′ amplimers contain a *Sal*I restriction site (underlined). PCR amplification and isolation of products were conducted as described above. Following *Hin*dIII-*Sal*I double digestion, the PCR products were cloned directionally into the *Hin*dIII and *Sal*I site of pBS+ (Stratagene, La Jolla, Calif.). The resulting phagemids, pBS+.IE1 and pBS+.IE2, linearized with *Eco*RI, were in vitro transcribed with the T3 RNA polymerase in vitro transcription kit (Promega) in the presence of 0.5 mg of bovine serum albumin per ml. Approximately 2 μg of RNA was added to a reaction containing 35 μl of micronuclease-treated rabbit reticulocyte lysate (Promega), 1 μl of 1 mM amino acids minus methionine, 1 μl of RNasin (Promega), 6 μl of [^{35}S]methionine (1,200 Ci/mmol, 10 mCi/ml; Dupont, NEN Research Products), and water to a 50-ml total volume. The reaction was incubated at 30°C for 2 h, and an aliquot examined by SDS-polyacrylamide gel electrophoresis (PAGE) was visualized by autoradiography.

Western blot (immunoblot) analysis. Cellular proteins interacting with IE2 were enriched by running cellular extracts from approximately 10^8 cells over a GST-Sepharose column three times to remove GST binding proteins and then through a GST-IE2-Sepharose column three times. The loaded GST-IE2 column was washed with several column volumes of ELB^+, and the IE2 interacting proteins were eluted with increasing concentrations of salt (350, 450, 550, 650, and 800 mM NaCl). A fraction of each elutent was boiled in Laemmli sample buffer and loaded onto SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose as described by Towbin et al. (65). The blots were blocked for 1 h in 5% (wt/vol) nonfat dry milk dissolved in TBS–0.1% Tween 20 and washed four times with TBS–0.1% Tween 20. The blots were then reacted with a 1:1,000 dilution of an antiTATA-binding protein (TBP) antibody (gift from Ray Jupp and Jay Nelson of the Oregan State University, Portland) for 1 h at room temperature. After extensive washing with TBS–0.1% Tween 20, the blots were probed with a 1:5,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham). Following extensive washing, the blots were developed by enhanced chemiluminescence (Amersham) according to manufacturer's specifications.

Far-Western analysis. pGEX-2TK.IE2 was cloned by PCR amplification with the 5′IE1&2 and 3′IE2 amplimers and pGEM4-2T (IE2; 84 kDa) and was then inserted into the *Bam*HI site of the pGEX-2TK (5, 25) polylinker in frame with the GST gene containing a heart muscle kinase site (GSTK). Expression of GSTK-IE2 proteins was conducted as described above. Following isolation on glutathione-Sepharose 4B (Pharmacia) beads, GSTK and GSTK-IE2 were labeled with [γ-^{32}P]ATP (150 μCi; 6,000 Ci/mmol; Amersham) and heart muscle kinase (Sigma) as previously described (5, 25). Proteins from cellular lysates were separated by SDS–10% PAGE and electroblotted onto nitrocellulose in 192 mM glycine–25 mM Tris–and 0.01% SDS. After transfer and blocking, proteins were renatured in situ (5) and then reacted with labeled GSTK or GSTK-IE2 as described previously (25). The blot was then washed extensively with hybridization buffer (25), dried, and autoradiographed.

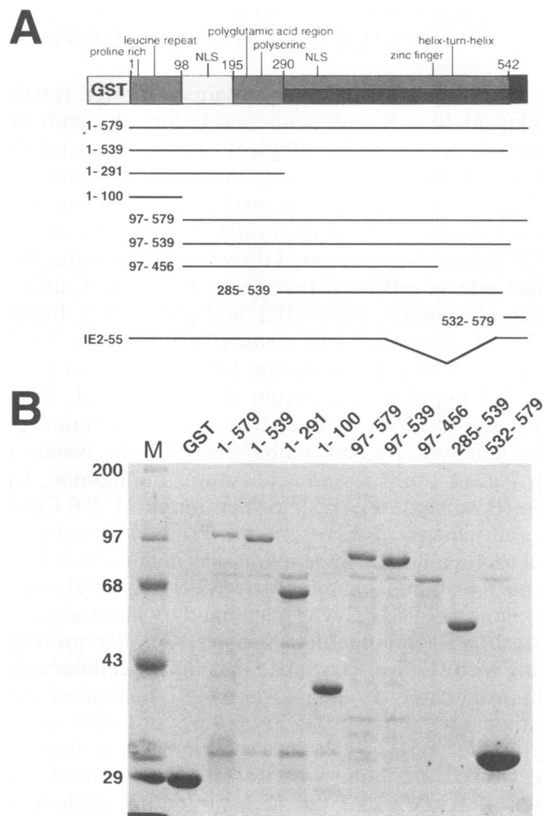

FIG. 1. Construction, expression, and purification of GST fusion proteins. (A) Diagrammatic representation of the GST-IE2 fusion protein and deletion mutants used in this study. Transactivation, repression, and C-terminal acidic domains are represented by the light-grey, dark-grey, and black regions, respectively. pGEX.IE2, pGEX.IE2.1-539, pGEX.IE2.1-291, pGEX.IE2.1-100, pGEX.IE2.97-579, pGEX.IE2.97-539, pGEX.IE2.97-456, pGEX.IE2.285-539, pGEX.IE2.532-579, and pGEX.IE2-55K were cloned by PCR amplification of the appropriate coding regions which correspond to known functional domains (39, 41, 47–49, 55) of IE2. (B) Bacterial clones containing the pGEX-2T or the recombinant pGEX-2T clones were grown in the presence of IPTG, sonicated, and incubated with glutathione-Sepharose beads. After purification and elution, the recombinant proteins were electrophoresed on an SDS–10% polyacrylamide gel and stained with Coomassie blue.

RESULTS

Expression of IE2 (84 kDa) and IE2 deletions as GST fusion proteins. To test the ability of the 84-kDa IE2 protein to interact with cellular proteins, a series of GST-IE2 fusion proteins that corresponded to either the 84-kDa IE2 or previously described functional domains of IE2 was generated (Fig. 1A). The complete coding region and domains of IE2 were PCR amplified from an IE2 cDNA and cloned into a procaryotic expression vector, pGEX2T. After IPTG induction, the fusion protein was affinity purified on glutathione-Sepharose beads and analyzed for expression on a SDS-polyacrylamide gel stained with Coomassie blue (Fig. 1B). Expression of the pGEX2T.IE2-84K clone results in a protein of approximately 110 kDa, and the fusion deletions corresponded to their predicted molecular weights. The fidelity of the recombinant GST-IE2 fusion proteins was confirmed by Western blot analyses using either a Dupont monoclonal antibody against early nuclear protein (recognizes both IE1 and IE2), an IE2-specific monoclonal antibody (12IE2), or a rabbit polyclonal peptide antibody against the C terminus of IE2 (57). The IE antibodies correctly recognized the bacterially expressed fusion proteins but not GST alone (data not shown).

The 84-kDa IE2 protein interacts with several cellular proteins. To identify cellular proteins which interact with the 84-kDa IE2 protein, the GST-IE2 fusion protein (Fig. 1B, 1-579) bound to glutathione-Sepharose beads was used as an affinity matrix to pull down protein complexes that may interact with IE2. As illustrated in Fig. 2A, minimal background was detected when precleared [^{35}S]methionine- (lane 1) or $^{32}P_i$- (lane 2) labeled HEL lysates were incubated with GST coated beads. However, several proteins ranging from >200 to 19 kDa affinity purified with GST-IE2-coated beads from the [^{35}S]methionine-labeled HEL lysate (lane 3). IE2-associated phosphoproteins of >200, 150, 60, 37, 34, and 19 kDa could be detected in the $^{32}P_i$-labeled HEL lysate incubated with GST-IE2-coated beads (lane 4). Unproportional phosphorylation of proteins was observed with proteins of sizes around 37 and 34 kDa. The interaction between IE2 and pp37 or pp34 may depend on their phosphorylation states. After a longer exposure, a few background bands of 58, 45, and 25 kDa were detected when labeled lysates were incubated with GST-coated beads. These particular protein interactions appear to have been enhanced in the presence of GST-IE2 but were considered to be nonspecific and were not included in this study.

The cellular proteins complexed to GST-IE2 were washed with increasing salt concentrations in order to better define the stability of binding in the complexes. At NaCl concentrations of 250 mM (the salt concentration of the ELB$^+$ solution), the GST-IE2 and cellular protein complexes remained intact. When salt concentrations reached between 350 and 550 mM, proteins with molecular masses of 150, 105, 95, 85, 68, and 60 kDa and small portions of proteins of 52, 45, 42, 40, 37, 34, and 32 kDa disassociated. The remaining peptides with sizes of 74, 72, 70, and 62 kDa and the majority of proteins of 52, 45, 42, 40, 37, 34, 32, and 27 kDa remained bound to the GST-IE2 Sepharose beads even when salt concentrations in the ELB$^+$ buffer reached as high as 0.8 M (data not shown). The 45- and 42-kDa proteins existing within the complexes could be detected in GST-Sepharose controls and were considered to be nonspecific.

The function and nuclear localization of IE2 suggest that these cellular proteins would also be located in the nucleus. To determine whether these proteins were nuclear or cytoplasmic, ^{35}S-labeled HEL cells were harvested to yield nuclear and cytoplasmic fractions. IE2 binding assays were performed on both fractions and whole cell lysate for comparison. As seen in Fig. 2B, the detection of cellular proteins that interact with IE2 was enhanced in the nuclear fraction (lane 4) and not visible in the cytoplasmic fraction (lane 3). Some faint bands were detected in the cytoplasmic fraction that comigrate with similar bands from the nuclear fraction. There are two possible explanations for this observation: (i) there was some leakiness of proteins from the nuclei during preparation of the cytoplasmic fraction or (ii) IE2 is interacting with proteins that were recently translated and had not been translocated to the nucleus. Interestingly, the infected lysate (24 h postinfection) used in the IE2 binding assay (Fig. 2B, lane 5) generated a banding pattern similar to that seen in uninfected lysate (lane 2); however, the intensity of some bands was lower, suggesting possible competition between the endogenous IE2 in the infected lysate with GST-IE2 or decreasing of these proteins synthesized upon

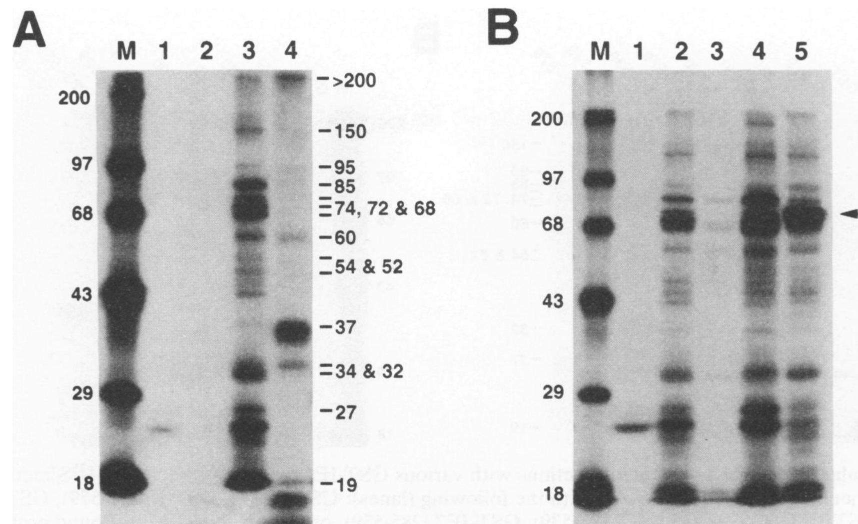

FIG. 2. Detection of cellular protein-IE2 protein interactions by IE2 binding assays. (A) Autoradiograph showing the results of a IE2 binding assay with either [^{35}S]methionine- and ^{32}P$_i$-labeled HEL extracts precleared with GST-coated beads. Precleared [^{35}S]methionine-labeled lysate was incubated with either GST- (lane 1) or GST-IE2- (lane 3) coated beads, and precleared ^{32}P$_i$-labeled lysate was incubated with either GST- (lane 2) or GST-IE2- (lane 4) coated beads. Interacting proteins were resolved by SDS-10% PAGE and visualized by fluorography. (B) IE2 binding assay conducted with either precleared ^{35}S-labeled whole-cell lysate (lane 2), cytoplasmic fraction (lane 3), nuclear fraction (lane 4), or 24-h-infected, whole-cell lysate (lane 5) in the presence of either GST- (lane 1; ^{35}S-labeled whole-cell lysate is shown) or GST-IE2-coated beads.

viral infection. The 80-kDa protein that is present only in the infected lysate may be either a cellular protein induced by infection or a viral protein which can interact with IE2. The latter seems to be favored since further data in this report demonstrate IE2-IE2 protein interactions. These experiments demonstrate the ability of IE2 to interact specifically, either directly or indirectly, with approximately 16 cellular proteins that are nuclear in origin, a subset of which are phosphorylated. Additionally, the infected lysate exhibits a new IE2 protein interaction with either an induced cellular protein or a virus-specific protein.

Mapping cellular protein-IE2 protein interactions to domains of IE2. Regions of IE2 responsible for transactivation of early promoters and autoregulation of the MIEP have been mapped through transient transfection assays by several groups (48, 49, 55). We hypothesize that these domains within IE2 would be involved in protein-protein interactions that elicit the observed regulation of various promoters. To characterize proteins that interact with these domains of IE2, several GST-IE2 deletion contructs were designed (Fig. 1A). The expressed GST-IE2 deletions (Fig. 1B) were incubated with either [^{35}S]methionine- (Fig. 3A) or ^{32}P$_i$- (Fig. 3B) labeled HEL lysates as previously described. Two major groups of cellular proteins required different functional domains of IE2 for protein-protein interactions. The assemblage that appeared to interact with a region (amino acids 97 to 290) encompassing a transactivation domain included 150-, 72-, and 54-kDa proteins. Another assemblage which appeared to interact with a region (amino acids 285 to 539) required for transactivation and autoregulation by IE2 included proteins of >200, 52, 37, 34, and 32 kDa. A 60-kDa protein interacted with the N-terminal transactivation domain (amino acids 1 to 100). The first 85 amino acids of IE2 are shared with IE1; however, preliminary data suggest the interaction between the 60-kDa protein and IE2 requires an additional 15 amino acids (86 to 100) since this cellular protein failed to interact with GST-IE1 (data not shown). Amino acids 1 to 100 appeared to be dispensible for the interaction between IE2 and the >200-, 68-, 52-, 37-, 34-, 32-, and possibly the 150-, 72-, and 54-kDa proteins. However, both the 95- and 85-kDa proteins require the full-length IE2 for their interaction. The 74- and 27-kDa proteins interacted with all GST-IE2 deletions and are considered nonspecific. In addition to the interactions described above, new bands appeared with various IE2 deletions. Since these bands were not represented in the pool of proteins which interacted with the full-length IE2, the authenticity of their interaction with IE2 is questionable and assumed to be nonspecific. The results from these experiments are summarized in Table 1.

These experiments reveal the ability of IE2 to interact with what appears to be two complexes and several additional, individual proteins. These proteins associate with functional domains required for transactivation or both transactivation and autoregulation.

Identification of direct protein interactions between cellular proteins and IE2 via far-Western blotting. The binding assay utilized in the previous experiments was unable to distinguish between direct and indirect IE2 protein interactions. To identify cellular proteins directly interacting with IE2, a far-Western blotting technique was employed with IE2 as a probe to detect direct interactions. The coding region of IE2 was subcloned into pGEX-2TK (5, 25), which contains a heart muscle kinase domain downstream of the thrombin cleavage site. Initially, a renatured Western blot of a GST-IE2 binding assay was probed with ^{32}P-labeled GSTK or GSTK-IE2 fusion protein. This resulted in a heavily labeled band of approximately 110 kDa plus lower bands on a short exposure which probably corresponded to bacterially expressed GST-IE2; however, GSTK-IE2 did not cross-react with GST alone. This result indicated that IE2 may be capable of interacting with itself; this will be discussed later. To avoid potential background interference introduced by GST-IE2, whole-cell HEL lysates were Western blotted along with an IE2 binding assay from ^{35}S-labeled HEL

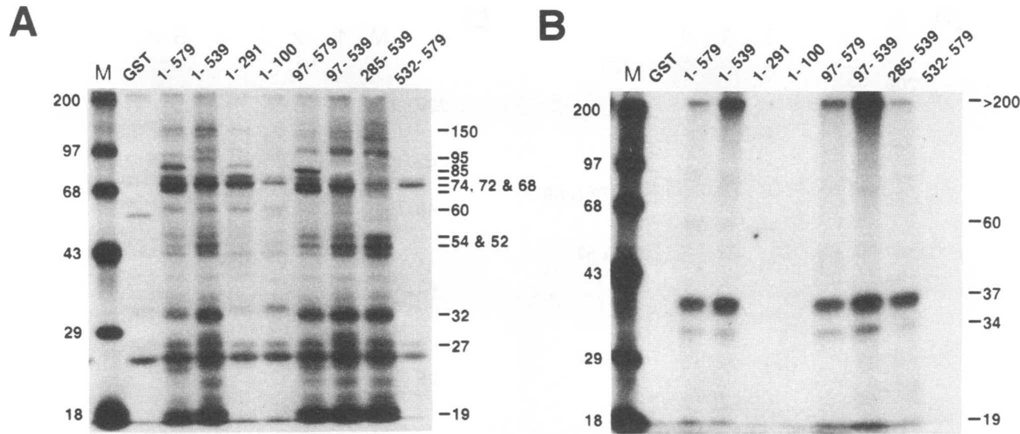

FIG. 3. Detection of cellular protein-IE2 protein interactions with various GST-IE2 deletions. Precleared [^{35}S]methionine- (A) or $^{32}P_i$- (B) labeled HEL lysates were incubated with beads coated with the following (lanes): GST (GST), GST-IE2 (1-579), GST-IE2 (1-539), GST-IE2 (1-291), GST-IE2 (1-100), GST-IE2 (97-579), GST-IE2 (97-539), GST-IE2 (285-539), or GST-IE2 (532-579). Bound proteins were separated by SDS–10% PAGE and visualized by fluorography (A) or autoradiography (B).

lysates. The whole-cell HEL proteins resolved by SDS-PAGE were transferred to nitrocellulose, renatured, and probed with ^{32}P-labeled GSTK-IE2. The results were compared with those of an IE2 binding assay resolved on the same SDS-polyacrylamide gel and transferred along with the whole-cell lysates. As illustrated in Fig. 4A, IE2 directly interacts with a subset of cellular proteins (150-, 95-, 74- or 72-, 60-, 54-, or 52-, 34-, or 32-, 27-, and 19-kDa proteins) similar in size to those identified in the binding assays. In addition, a new cellular protein of approximately 120 kDa was found to interact with IE2 directly and not with the control probe, GSTK, by this method. The nature of this

TABLE 1. Summary of cellular protein interactions with IE2 deletions

Protein (size [kDa])	Interaction with IE2 with the following amino acids deleted[a]							
	1–579	1–539	1–291	1–100	97–579	97–539	285–539	537–579
>200[b]	+	+	−	−	+	+	+	−
150[c]	+	+	+	−	+	+	−	−
95[d]	+	−	−	−	−	−	−	−
85[d]	+	−	−	−	−	−	−	−
74[e]	+	+	+	+	+	+	+	+
72[c]	+	+	+	−	+	+	−	−
68[f]	+	−	−	−	+	−	−	−
60[g]	+	+	+	+	−	−	−	−
54[c]	+	+	+	−	+	+	−	−
52[b]	+	+	−	−	+	+	+	−
37[b]	+	+	−	−	+	+	+	−
34[b]	+	+	−	−	+	+	+	−
32[b]	+	+	−	−	+	+	+	−
27[e]	+	+	+	+	+	+	+	+
19[b]	+	+	−	−	+	+	+	−

[a] +, interaction; −, no interaction.
[b] A group of cellular proteins found to interact with IE2 between amino acids 285 and 539.
[c] Another group of proteins found to interact with IE2 between amino acids 97 and 285.
[d] Proteins requiring full-length IE2 for their interaction with IE2.
[e] Cellular proteins found to interact with all deletions and assumed to be nonspecific.
[f] Cellular protein requiring amino acids 97 to 579 for its interaction with IE2.
[g] A protein found to interact with the N terminus between amino acids 1 and 100.

protein interaction is unknown, although this result was consistent between experiments. However, these results indicate that several cellular proteins interact directly with IE2.

It was previously reported that IE2 can interact directly with in vitro-translated TBP (17). To demonstrate that GST-IE2 is able to interact with TBP made in vivo, whole-cell HEL lysates were passed over a GST affinity column prior to being loaded on a GST-IE2 affinity column. Elutions of increasing salt concentrations were collected and subjected to Western blot analysis using an αTBP antibody (gift of Ray Jupp and Jay Nelson). As shown in Fig. 4B, αTBP antibody reacted with a protein of approximately 38 to 40 kDa. This protein eluted from the GST-IE2 column with salt concentrations of up to 450 mM suggesting a stable protein-protein interaction between IE2 and TBP. The molecular mass of this protein corresponds to recombinant human TBP expressed in rabbit reticulocyte lysates (27, 46). This experiment demonstrates that IE2 can associate with human TATA-binding protein made in vivo in agreement with a previous report of results with in vitro-made protein (17).

The C-terminal region of the 84-kDa IE2 protein conveys IE2-IE2 protein interactions. To investigate the possibility of IE2-IE2 interactions, IE2 binding assays were performed with rabbit reticulocyte lysates programmed with either IE1 or IE2 (84 kDa) in vitro-transcribed RNA. As shown in Fig. 5A, the ^{35}S-labeled proteins did not interact with either beads alone (lanes 3 and 4) or GST-coated beads (lanes 5 and 6). However, IE2-programmed lysate did interact with GST-IE2- (lane 10) but not with GST-IE1- (lane 8) or GST-IE2-55K-coated beads (lane 11). It was also noted that IE1-programmed lysates did not interact with either GST-IE1 (lane 7) or GST-IE2 (lane 9). To confirm these results, far-Western analyses were performed on immobilized GST, GST-IE1, and GST-IE2 proteins with either ^{32}P-labeled GSTK or GSTK-IE2 proteins. As seen in Fig. 5B, GSTK-IE2 interacted directly with GST-IE2 but not with GST or GST-IE1.

The IE2-IE2 interacting domain was located by using the previously described GST-IE2 deletions plus an additional deletion (GST-IE2.97-456) that removed a potential coiled-coil motif. As seen in Fig. 6, IE2-IE2 interaction occurred

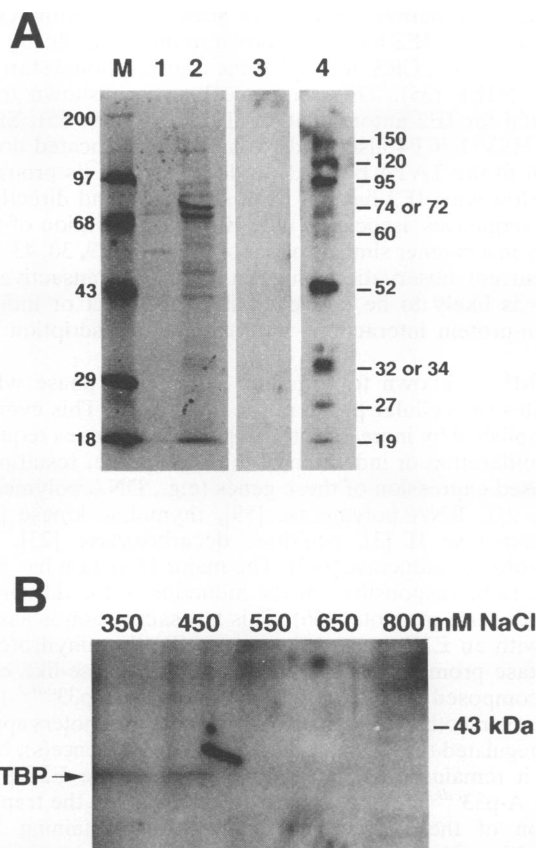

FIG. 4. Direct interactions between several cellular proteins and IE2. (A) Western blot of HEL cell lysate which was renatured and probed with ^{32}P-labeled GSTK (lane 3) or GSTK-IE2 (lane 4). Far-Western blots were compared to an IE2 binding assay of precleared, [^{35}S]methionine-labeled lysates incubated with either GST- (lane 1) or GST-IE2- (lane 2) coated beads and resolved by electrophoresis on the same SDS–10% polyacrylamide gel and electroblotted onto nitrocellulose. The IE2 binding assay and far-Western blots were visualized by autoradiography. (B) Western blot analysis with an αTBP polyclonal antibody was performed on fractions of IE2-associated proteins eluted from a GST-IE2 column by increasing salt concentrations. A protein with a molecular mass similar to that of TBP was found associated with IE2 in the 350 mM and 450 mM salt elutions.

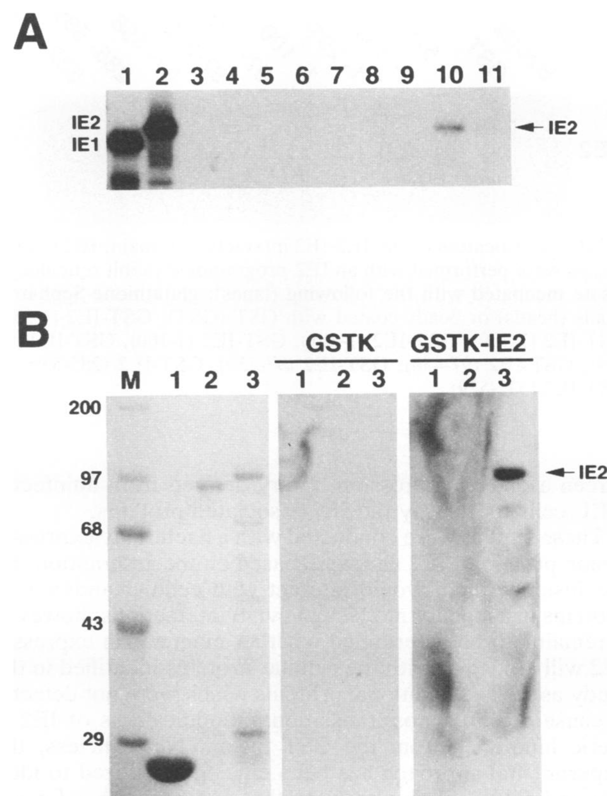

FIG. 5. Detection of IE2-IE2 interactions. (A) [^{35}S]methionine-labeled IE1- (lane 1) or IE2-programmed (lane 2) rabbit reticulocyte lysates were used in IE2 binding assays. IE1- and IE2-programmed rabbit reticulocyte lysates were incubated with glutathione-Sepharose beads alone (lanes 3 and 4, respectively) or with beads coated with GST (lanes 5 and 6, respectively), GST-IE1 (lanes 7 and 8, respectively), GST-IE2 (lanes 9 and 10, respectively), or GST-IE2.55K (IE2-programmed lysate only, lane 11). (B) Purified and eluted GST (lane 1), GST-IE1 (lane 2), and GST-IE2 (lane 3) were resolved by SDS–10% PAGE in triplicate for far-Western analysis. The first panel is an SDS-10% polyacrylamide gel stained with Coomassie blue; the second and third panels are duplicate Western blots reacted with ^{32}P-labeled GSTK and GSTK-IE2, respectively.

with the following deletions: 1 to 539, 97 to 579, 97 to 539, and 285 to 539. This interaction was not detected with the 97 to 456 deletion, which suggested that amino acids 456 to 539 were required. This region contains two amphipathic α-helices separated by a short β-turn as predicted by the Chou-Fasman algorithm (9).

DISCUSSION

The mechanism(s) by which IE2, alone or in cooperation with IE1, transactivates viral and cellular promoters and autoregulates the MIEP is unknown at this time. It is known that IE2 binds directly to the CRS element at the cap site in the MIEP, but the mechanism of downregulation is speculative. IE2 has not been shown to bind to specific sequences in other promoters, and the current thought is that IE2 may direct its influence through protein-protein interactions. Thus, the first step in deciphering the mechanism(s) utilized by IE2 is to identify the cellular and/or viral proteins that interact directly or indirectly with this transactivator-repressor.

IE2 interaction with cellular proteins. In this report, we demonstrate several cellular proteins ranging from >200 to 19 kDa that interact with the 84-kDa IE2 protein. Of these, a subset were identified as phosphoproteins and all appeared to be nuclear proteins. Additionally, a protein of approximately 80 kDa appears to be specific to infected lysates. The cellular protein-IE2 interactions, including two distinct protein complexes and several individual proteins, were mapped to specific functional domains of IE2 known to be required for transactivation or both transactivation and autoregulation. By far-Western analysis, a subset of proteins (150, 95, 74 or 72, 60, 54 or 52, 34 or 32, 27 and 19 kDa) was demonstrated to interact directly with IE2. As a control for the binding assays, GST-IE2 was shown to interact with TBP made in vivo by affinity purification. Thus, it is quite possible that the 37-kDa protein detected only in the $^{32}P_i$ HEL lysate might be TBP, based on its size and phosphorylation. The far-Western technique is currently being used to

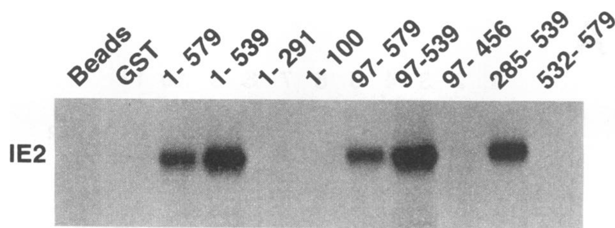

FIG. 6. Location of the IE2-IE2 interaction domain. IE2 binding assays were performed with an IE2-programmed rabbit reticulocyte lysate incubated with the following (lanes): glutathione-Sepharose beads (beads) or beads coated with GST (GST), GST-IE2 (1-579), GST-IE2 (1-539), GST-IE2 (1-291), GST-IE2 (1-100), GST-IE2 (97-579), GST-IE2 (97-456), GST-IE2 (97-539), GST-IE2 (285-539), or GST-IE2 (532-579).

screen a cDNA expression library derived from uninfected HEL cells to identify the IE2-associated proteins.

These studies were conducted with a bacterially expressed fusion protein of IE2 and were based on the assumption that the fusion protein would interact with cellular and/or viral proteins in a manner modeling in vivo interactions; however, it remains to be determined whether mammalian expressed IE2 will associate with the cellular proteins identified in this study as well as additional proteins which were not detected because of either posttranslational modifications of IE2 or steric hindrance from the GST fusion. Nevertheless, this experimental approach has been efficiently utilized to identify and clone cellular proteins that interact with c-*fos* and the retinoblastoma protein, p105-Rb (5, 25, 26). In addition, it is possible that these results of fusion precipitations are not free protein-protein interactions but rather DNA-mediated protein-protein interactions in which small pieces of contaminating DNA nonspecifically bridge proteins. To exclude this possibility, we have performed fusion precipitation in the presence of 100 μg of ethidium bromide per ml as described by Lai and Herr (32). No major variation of the protein binding pattern was found between fusion precipitations with ethidium bromide and controls. However, higher background noise was observed (data not shown). Therefore, the phenomenon of DNA-mediated protein-protein interaction should be very limited in this study. The nature of the far-Western experiments raises the possibility that certain proteins that directly interact with IE2 may go undetected because of their inability to properly renature. This shortcoming was demonstrated by our inability to detect mammalian-expressed TBP by far-Western analysis.

Hagemeier et al. (17) demonstrated the ability of IE2 to interact with the C terminus of in vitro-made TFIID, and our studies confirm that in vivo-expressed TBP interacts with IE2. It is possible that IE2 may also interact with additional members of the preinitiation complex since proteins similar in size to others in the preinitiation complex were found to interact with IE2 in this study. The interaction between TFIID and IE2 is similar to that of the IE protein of adenovirus, E1A (36), and other herpesvirus IE proteins, including Z of EBV (38).

Other IE proteins (E1A, ICP4, and Z) have been shown to have transactivation mechanisms in addition to those involving direct interactions with TFIID. These include binding to a specific DNA sequence in the case of ICP4 and Z (6, 14, 16, 22, 37, 38, 43, 45, 60, 61) and interacting directly or indirectly with another DNA-bound transactivator(s), thereby functioning as a bridging molecule between the transactivator and the preinitiation complex, as seen in E1A with ATF-2 (40). Recently, IE2 has been shown to bind specifically to a DNA sequence, CRS, located at the transcriptional start site of the MIEP (35). This sequence has been shown to be essential for IE2 autoregulation (18, 39, 47, 49, 55). Similiarly, HSV-1 ICP4 also binds to a sequence located downstream of the TATA box (43) to downregulate its promoter (50). However, IE2 has not been shown to bind directly to DNA sequences associated with the transactivation of promoters in a manner similar to that of ICP4 (14, 29, 30, 43, 44). Our current observations suggest that IE2 transactivating ability is likely to be conveyed through direct or indirect protein-protein interactions with cellular transcription factors.

HCMV is known to stimulate a G_1/S-like phase which activates the cellular proliferation machinery. This event is accomplished by increasing the expression of genes required for proliferation or induction of the G_1/S phase, resulting in increased expression of these genes (e.g., DNA polymerase α [20, 21], RNA polymerase [59], thymidine kinase [13], topoisomerase II [3], ornithine decarboxylase [23], and dihydrofolate reductase [66]). The major IE region has been shown to be responsible for the induction of the dihydrofolate reductase promoter (66). This transactivation is associated with an E2F recognition site within the dihydrofolate reductase promoter that is bound by an S-phase-like complex composed of E2F, p107, cyclin A, and $p33^{cdk2}$ (66). Also, several other cellular and HCMV early promoters appear to be regulated through an E2F recognition sequence(s); however, it remains to be determined whether the E2F-p107-cyclin A-$p33^{cdk2}$ complex is involved directly in the transactivation of these promoters. Promoters containing E2F recognition sites such as the adenovirus E2 promoter can be transactivated by IE2 alone, although this can be augmented by IE1 (19, 49, 64). Thus, IE2 is a likely candidate for stimulating E2F-mediated transactivation. Our observations from binding assays found proteins (>200, 150, 120, 60, and 34 kDa) with molecular masses similar to those of the E1A- and E2F-associated proteins which interact with IE2. It is likely that the 32- to 34-kDa IE2-binding proteins may be CDK2- or CDC2-related proteins. We are currently investigating the ability of IE2 to interact directly with cellular proteins known to be involved in regulating the transactivating ability of E2F.

Location of the domain responsible for IE2-IE2 interactions. By IE2-binding assays and far-Western analysis, we demonstrated an IE2-IE2 interaction in a domain required for both transactivation and autoregulation. This domain is located between amino acids 456 and 539 and is predicted to contain two amphipathic α-helices separated by a short β-turn. This motif is located adjacent to a putative TFIIIA-type zinc finger (41) (Fig. 7). It remains to be determined whether the zinc finger motif is responsible for recognition of the CRS element by IE2; however, the close proximity of these putative elements may play a significant role in autoregulation and transactivation. Site-directed mutagenesis is being performed on these α-helical regions to determine their role in the IE2-IE2 interaction. Preliminary results from UV-cross-linking studies using in vitro-translated ^{35}S-IE2 protein in the presence of ditho-bis-succinimidylpropionate indicate IE2 interactions may be in the form of a dimer. The interaction after cross-linking can be disrupted in the presence of DTT (our unpublished data). Further studies using expressed proteins to confirm IE2 multimerization and to elucidate its functional role in vivo are needed.

The experiments presented in this report also demonstrate

```
351  PNVQTRRGRV KIDEVSRMFR NTNRSLEYKN LPFTIPSMHQ VLDEAIKACK
401  TMQVNNKGIQ IIYTRNHEVK SEVDAVRCRL GTMCNLALST PFLMEHTMPV
451  THPPEVAQRT ADACNEGVKA AWSLKELHTH QLCPRSSDYR NMIIHAATPV
501  DLLGALNLCL PLMQKFPKQV MVRIFSTNQG GFMLPIYETA TKAYAVGQFE
551  QPTETPPEDL DTLSLAIEAA IQDLRNKSQ*
```

FIG. 7. Location of a putative zinc finger and helix-turn-helix motif in the C terminus of the 84-kDa IE2 protein. IE2-IE2 interactions were mapped to a region between amino acids 456 and 539. This region contains an amphipathic α-helix (amino acids 463 to 482), a short β-turn (amino acids 483 to 488), and then another α-helix (amino acids 489 to 513). A putative zinc finger (amino acids 428 to 452) is located upstream of the helix-turn-helix motif.

the inability of IE2 to form heterodimers with IE1. However, these results do not discount the ability of IE2 to interact with IE1 indirectly by other means. The 55-kDa IE2 protein fails to transactivate HCMV early promoters as well as downregulate the MIEP (18, 41, 48, 55). This protein contains neither the zinc finger nor the helix-turn-helix motif and does not dimerize with IE2 (Fig. 5A, lane 11). The 40-kDa IE2 protein contains both motifs and would be expected to dimerize with the 84-kDa IE2 protein. This late IE2 variant is capable of downregulating the MIEP but fails to transactivate cellular and HCMV early promoters (18, 47, 49) and thus most likely functions as a trans-dominant repressor. The bovine papillomavirus expresses a similar protein, E2-TR, which contains the dimerization and DNA binding domains but not the transactivation region (8, 33, 34). Preliminary results from DNA electrophoretic mobility shift assays using the IE2 CRS element of MIEP indicate that IE2 multimerization is not essential for IE2 binding to the CRS sequence (our unpublished data).

Further characterization of the domain within IE2 that is required for IE2-IE2 interaction will be necessary to determine whether IE2 oligomerization is required for transactivation, autoregulation, or both. Additionally, identification of the cellular proteins which interact with IE2 will be of utmost importance in elucidating the mechanism(s) involved in transactivation of homologous and heterologous promoters by IE2.

ACKNOWLEDGMENTS

We thank Ray Jupp and Jay Nelson for the plasmid pRSVIE2-55 and the TBP monoclonal antibody and Andy Yurochko for helpful discussion.

This investigation was supported by grants A12717 and CA21773 from the National Institutes of Health. T.F.K. is a recipient of a Damon Runyon-Walter Winchell cancer research fellowship (DRG-1097).

REFERENCES

1. **Akrigg, A., W. G. Wilkinson, and J. D. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. **2:**107–112.
2. **Baracchini, E., E. Glezer, K. Fish, R. M. Stenberg, J. A. Nelson, and P. Ghazal.** 1992. An isoform variant of the cytomegalovirus immediate-early auto repressor functions as a transcriptional activator. Virology **188:**518–529.
3. **Benson, J. D., and E. S. Huang.** 1990. Human cytomegalovirus induces expression of cellular topoisomerase II. J. Virol. **64:**9–15.
4. **Biegalke, B. J., and A. J. Geballe.** 1991. Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. Virology **183:**381–385.
5. **Blanar, M. A., and W. J. Rutter.** 1992. Interaction cloning: identification of a helix-loop-helix zipper protein that interacts with c-Fos. Science **256:**1014–1018.
6. **Chang, Y.-N., L.-Y. Dong, G. S. Hayward, and S. D. Hayward.** 1990. The Epstein-Barr virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif. J. Virol. **64:**3358–3369.
7. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus ie1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63:**1435–1440.
8. **Choe, J., P. Vaillancourt, A. Stenlund, and M. Botchan.** 1989. Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs. J. Virol. **63:**1743–1755.
9. **Chou, P. Y., and G. D. Fasman.** 1974. Prediction of protein conformation. Biochemistry **13:**222–245.
10. **DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan.** 1980. Pattern of transcription of human cytomegalovirus in permissively infected cells. J. Virol. **35:**277–286.
11. **Depto, A. S., and R. M. Stenberg.** 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. J. Virol. **63:**1232–1238.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
13. **Estes, J. E., and E. S. Huang.** 1977. Stimulation of cellular thymidine kinases by human cytomegalovirus. J. Virol. **24:**13–21.
14. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the HSV-1 regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **14:**6067–6082.
15. **Ghazal, P., J. Young, E. Giulietti, C. DeMattei, J. Garcia, R. Gaynor, R. M. Stenberg, and J. A. Nelson.** 1991. A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic transactivation by cytomegalovirus immediate-early proteins. J. Virol. **65:**6735–6742.
16. **Gruffat, H., N. Duran, and A. Sergeant.** 1990. The Epstein-Barr (EBV) ORIlyt enhancer is not B-cell specific and does not respond synergistically to the EBV transcription factors R and Z. J. Virol. **64:**2810–2818.
17. **Hagemeier, C., S. Walker, R. Caswell, T. Kouzarides, and J. Sinclair.** 1992. The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early protein transactivates heterologous promoters in a TATA box-dependent mechanism and interacts directly with TFIID. J. Virol. **66:**4452–4456.
18. **Hermiston, T. W., C. L. Malone, and M. F. Stinski.** 1990. Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. J. Virol. **64:**3532–3536.
19. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. **61:**3214–3221.
20. **Hirai, K., and Y. Watanabe.** 1976. Induction of alpha type DNA polymerases in human cytomegalovirus-infected WI-38 cells. Biochem. Biophys. Acta **477:**323–339.
21. **Huang, E. S.** 1975. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. **16:**298–310.
22. **Imbalzano, A. N., A. A. Shepard, and N. A. DeLuca.** 1990. Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. J. Virol. **64:**2620–2631.
23. **Isom, H. J.** 1979. Stimulation of ornithine decarboxylase by human cytomegalovirus. J. Gen. Virol. **42:**265–278.
24. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD169. J. Virol. **49:**363–370.
25. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham,**

M. A. Blanar, D. M. Livingston, and E. K. Flemington. 1992. Expression cloning of a cDNA encoding a retinoblastoma binding protein with E2F-like properties. Cell **70:**351–364.
26. **Kaelin, W. G. J., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston.** 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell **64:**521–532.
27. **Kao, C. C., P. M. Lieberman, M. C. Schmidt, Q. Zhou, R. Pei, and A. J. Berk.** 1990. Cloning of a transcriptionally active human TATA binding factor. Science **248:**1646–1650.
28. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, E. Preddy, and B. G. Barrell.** 1988. An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. Virology **165:**151–164.
29. **Kristie, T. M., and B. Roizman.** 1986. a-4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with the promoter-regulatory domains of a genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83:**3218–3222.
30. **Kristie, T. M., and B. Roizman.** 1986. DNA-binding site of major regulatory protein α4 specifically associated with promoter-regulatory domains of genes of the herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83:**4700–4704.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Lai, J.-S., and W. Herr.** 1992. Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. USA **89:**6958–6962.
33. **Lambert, P. F., N. L. Hubbert, P. M. Howley, and J. T. Schiller.** 1989. Genetic assignment of multiple E2 gene products in bovine papillomavirus-transformed cells. J. Virol. **63:**3151–3154.
34. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50:**69–78.
35. **Lang, D., and T. Stamminger.** 1993. The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE-1/2 enhancer-promoter. J. Virol. **67:**323–331.
36. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67:**365–376.
37. **Lieberman, P. M., and A. J. Berk.** 1990. In vitro transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr virus Zta protein. J. Virol. **64:**2560–2568.
38. **Lieberman, P. M., and A. J. Berk.** 1991. The Zta trans-activator protein stabilizes TFIID association with promoter DNA by direct protein-protein interactions. Genes Dev. **5:**2441–2454.
39. **Liu, B., T. W. Hermiston, and M. F. Stinski.** 1991. A *cis*-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. J. Virol. **65:**897–903.
40. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. Cell **61:**1217–1224.
41. **Malone, C. L., D. H. Vesole, and M. F. Stinski.** 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. **64:**1498–1506.
42. **McDonough, S., S. Staprans, and D. Spector.** 1985. Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. J. Virol. **53:**711–718.
43. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein a4 of herpes simplex virus. Science **239:**1531–1534.
44. **Muller, M. T.** 1987. Binding of the herpes simplex virus type 1 gene product ICP4 to its own transcription start site. J. Virol. **61:**858–865.
45. **Packham, G., A. Economou, C. M. Rooney, D. T. Rowe, and P. J. Farrell.** 1990. Structure and function of the Epstein-Barr virus BZLF1 protein. J. Virol. **64:**2110–2116.
46. **Peterson, M. G., N. Tanese, B. F. Pugh, and R. Tjian.** 1990. Functional domains and upstream activation properties of cloned human TATA binding protein. Science **248:**1625–1630.
47. **Pizzorno, M. C., and G. S. Hayward.** 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. J. Virol. **64:**6154–6165.
48. **Pizzorno, M. C., M. A. Mullen, Y. N. Chang, and G. S. Hayward.** 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. J. Virol. **65:**3839–3852.
49. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62:**1167–1179.
50. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α 4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62:**4307–4320.
51. **Sambucetti, L. C., J. M. Cherrington, G. W. Wilkinson, and E. S. Mocarski.** 1989. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8:**4251–4258.
52. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
53. **Staprans, S. I., D. K. Rabert, and D. H. Spector.** 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. J. Virol. **62:**3463–3473.
54. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63:**2699–2708.
55. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **64:**1556–1565.
56. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. **49:**190–199.
57. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56:**665–675.
58. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. **46:**1–14.
59. **Tanaka, S., S. Ihara, and Y. Watanabe.** 1978. Human cytomegalovirus induces DNA-dependent RNA polymerases in human diploid cells. Virology **89:**179–185.
60. **Taylor, N., E. Flemington, J. L. Kolman, S. Baumann, H. Speck, and G. Miller.** 1991. ZEBRA and a FOS-GCN4 chimeric protein differ in their DNA-binding specificities for sites in the Epstein-Barr virus BZLF1 promoter. J. Virol. **65:**4033–4041.
61. **Tedder, D. G., R. D. Everett, K. W. Wilcox, P. Beard, and L. I. Pizer.** 1989. ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. J. Virol. **63:**2510–2520.
62. **Tenney, D. J., and A. M. Colberg-Poley.** 1991. Expression of the human cytomegalovirus UL36-38 immediate early region during permissive infection. Virology **182:**199–210.
63. **Tenney, D. J., and A. M. Colberg-Poley.** 1991. RNA analysis and isolation of cDNAs derived from the human cytomegalovirus

immediate-early region at 0.24 map units. Intervirology **31**:203–214.

64. **Tevethia, M. J., D. J. Spector, K. M. Leisure, and M. F. Stinski.** 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology **161**:276–285.
65. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
66. **Wade, M., T. F. Kowalik, M. Mudryj, E. S. Huang, and J. C. Azizkhan.** 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol. Cell. Biol. **12**:4364–4374.
67. **Walker, S., C. Hagemeier, J. G. Sissons, and J. H. Sinclair.** 1992. A 10-base-pair element of the human immunodeficiency virus type 1 long terminal repeat (LTR) is an absolute requirement for transactivation by the human cytomegalovirus 72-kilodalton IE1 protein but can be compensated for by other LTR regions in transactivation by the 80-kilodalton IE2 protein. J. Virol. **66**:1543–1550.
68. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41**:462–477.
69. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate-early and early times after infection. J. Virol. **38**:446–459.
70. **Weston, K.** 1988. An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. Virology **162**:406–416.
71. **Wilkinson, G. W., G. A. Akriggs, and P. J. Greenway.** 1984. Transcription of the immediate early genes of human cytomegalovirus AD169. Virus Res. **1**:101–116.