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The IE2 Regulatory Protein of Human Cytomegalovirus Induces Expression of the Human Transforming Growth Factor β1 Gene through an Egr-1 Binding Site

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Increases in transforming growth factor β1 (TGF-β1) mRNA and biological activity in the early phase of human cytomegalovirus (CMV) infection in fibroblasts are paralleled by increased TGF-β1–chloramphenicol acetyltransferase (CAT) reporter gene activity. To determine how CMV infection transactivates the TGF-β1 promoter, we examined the effects of the cotransfected IE2 regulatory protein of human CMV on 5′-deleted TGF-β1 promoter-CAT reporter genes in transient DNA transfection assays. Two upstream TGF-β1 promoter regions each containing an Egr-1 consensus site were shown to be important for IE2-induced transactivation in a cell type that displayed greatly reduced nonspecific activity. Furthermore, transfer of an Egr-1 site from between positions −125 and −98, but not point mutant versions of this site, to a heterologous promoter also conveyed IE2 responsiveness. Addition of an IE2 expression vector or use of the U373 A45 astrocytoma cell line expressing IE2 also produced synergistic stimulation of GAL4–Egr-1-mediated activation of a target promoter containing GAL4 binding sites. The 80-kDa IE2 protein present in A45 cells proved to selectively bind to glutathione *S*-transferase (GST)–Egr-1 beads. The results of in vitro protein binding assays also revealed that an intact in vitro-translated IE2 protein bound directly to the GST–Egr-1 fusion protein through the zinc finger domain of the Egr-1 protein and that this binding activity was abolished by deletion of parts of the zinc finger DNA-binding domain. Similarly, the Egr-1 protein was found to associate preferentially with a small region within the C-terminal half of the IE2 protein adjacent to the DNA-binding and dimerization domains that are important for both transactivation and downregulation. We conclude from these observations that IE2 may regulate transcription of the TGF-β1 gene as well as other potential cellular targets by virtue of its ability to interact with the Egr-1 DNA-binding protein.

When cytomegalovirus (CMV) infects a permissive cell, three broad categories of viral genes, termed immediate early (IE), early, and late genes, are transcribed progressively (55, 57). Two major nuclear proteins, IE1 (72 kDa) and IE2 (80 kDa), as well as a minor 55-kDa form of IE2 are produced by alternative splicing from the major IE (MIE) gene during permissive infection in the presence of cycloheximide and in some nonpermissive cell types. The IE1 mRNA consists of exons 1 through 4 of the MIE gene, and the IE2 mRNA consists of exons 1, 2, 3, and 5. Therefore, the IE1 protein and both IE2 proteins have 85 N-terminal amino acids in common, which are encoded by exons 2 and 3 of the IE1 transcription unit (48, 57, 59, 60).

The role of the IE1 protein is not understood, but the 80-kDa IE2 protein is a powerful transactivator in transient-cotransfection assays and induces expression of many heterologous (1, 2, 13, 26, 49, 51, 63) and homologous (7, 35) promoters. Previous mapping studies have identified two distinct activation domains of the IE2 protein, an NH_2-terminal 51-amino-acid domain (codons 25 to 85) and the 33-amino-acid COOH-terminal domain (codons 544 to 579) (48). Some of the relatively nonspecific transactivation obtained with the IE2 protein is thought to be mediated by TFIID acting through direct interaction of IE2 with the C terminus of the TATA-binding protein (TBP) component of TFIID (25). Unlike IE2, the highly acidic IE1 protein does not interact with TBP (25) and displays only weak transactivator characteristics but associates with metaphase chromosomes (33).

Not only does IE2 act as a positive regulator of CMV (8, 9, 35, 36, 47, 49, 57, 58), but it also represses the CMV MIE promoter through binding to a specific palindromic 14-bp target sequence, which is located between nucleotides −12 and +5 (9, 10, 35, 36, 47, 49). The IE2 protein alone transactivates several human CMV (HCMV) early and late promoters (14, 26, 32, 37, 40, 55, 58), but the IE1 protein, which usually has no effect on the early promoters by itself, often produces a synergistic effect together with the IE2 protein. Other HCMV minor IE and virion proteins, including TRS-1, US3, UL36-37, pp71, and UL69 (11, 33, 52, 56, 65), have also been reported to have regulatory or transactivator properties or to synergize with IE2, although, as for IE1, their exact contributions to the infection process are unknown.

Transforming growth factor β (TGF-β) has been shown to play a predominantly negative role in the proliferation of many cell types (41, 50, 54, 61). For example, it acts to inhibit growth of myeloid, lymphoid, epithelial, and endothelial cells at the level of G_1 arrest and is also a potent deactivator of cytokine-activated macrophage functions. Therefore, upregulation of active secreted TGF-β could produce both immunosuppression and either paracrine or autocrine effects that could be

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important for regulating or mediating HCMV latency and reactivation, especially in monocyte-macrophage cell types (16, 17, 43, 46, 64). The human TGF-β1 promoter has been cloned and contains functional AP-1 (27, 30) and Egr-1 (28, 29) binding sites. Many factors, including tumor suppressor proteins (15, 31), growth factors (28), and viral transactivators (30, 66), regulate production of TGF-β1 through these and other sites. TGF-β1 also autoregulates its own expression indirectly by a process mediated through the AP-1 binding sites responding to induced Jun-Fos complexes (27, 45).

In an earlier study, it was reported that HCMV infection induces endogenous TGF-β1 production in human fibroblasts (42). TGF-β1 mRNA and biological activity were increased two- and fivefold, respectively, during the early phase of CMV infection. In addition, expression from two different TGF-β promoter-driven luciferase reporter genes was enhanced between 50- and 700-fold by subsequent HCMV infection in transient DNA cotransfection assays in human fibroblasts, U937 cells, or U373MG astrocytoma cells. Similarly, a U373MG-derived cell line (A45) capable of expressing both IE1 and IE2 also stimulated TGF-β–luciferase expression 36-fold compared with that of the control U373 Neo^r cell line (AN). In the present study, we demonstrate that the IE2 protein induces transcription of TGF-β1 in cotransfection assays through targeting to either of the Egr-1 binding sites in the TGF-β1 promoter. In addition, we show that a C-terminal segment of the IE2 protein interacts with the zinc finger domain of the Egr-1 protein by in vitro protein binding assays.

MATERIALS AND METHODS

Cell culture and DNA transfection. AN, A1b, and A45 cells, which originated from U373MG astrocytoma cells, were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin (GIBCO) per ml. For transfection experiments, U373MG cells were plated at a density of 10^6/100-mm-diameter dish and grown until 60 to 70% confluent. The following day, cells were transfected by the calcium phosphate coprecipitation technique. After the cells were incubated with the DNA solutions for 8 to 12 h, they were exposed to glycerol shock. Forty-eight hours later, the cells were scraped from the culture dishes and lysed by freeze-thawing. A 100-μg sample of this cell lysate was used for chloramphenicol acetyltransferase (CAT) assays (23). All transfections were repeated at least three times. For transfection into AN, A1b, and A45 cells, these cells were plated at a density of 2×10^5/60-mm-diameter dish 24 h before transfection. Four micrograms of G5E1b-luciferase or G5E1b-CAT reporter plasmid was transfected together with the indicated amount of control GAL4(1-147) or GAL4–Egr-1 construct by use of the Lipofectamine reagent (GIBCO-BRL) according to the manufacturer's instructions. For normalization of transfection efficiencies, a growth hormone expression plasmid (pSVGH) was included in cotransfection. Growth hormone expression was quantified by using a growth hormone detection kit (Nichols Institute).

Establishment of cell lines stably expressing IE1 and IE1 plus IE2 proteins. The IE1 expression vector consisted of the 4-kb *Cla*I fragment excised from pRL103 (33, 49) and inserted into the *Acc*I site of pUC19. The IE1 plus IE2 vector pRL45 was described previously (33, 49). U373MG astrocytoma cells were cotransfected by the calcium phosphate technique with the IE expression vector and the gene for neomycin analog (G418) resistance under the control of the simian virus 40 promoter at a ratio of 5:1. Forty-eight hours after transfection, the cells were trypsinized and placed in Dulbecco's modified Eagle's medium with 5% fetal calf serum containing 400 μg of G418 (GIBCO) per ml. Colonies, which appeared after 3 to 4 weeks, were screened by immunofluorescence using monoclonal antibody against HCMV early nuclear antigen (Chemicon 810) and were used without further subcloning. IE expression was analyzed by Western blotting (immunoblotting) with the same monoclonal antibody. The A1b and A45 cells were previously described briefly (12, 42).

Plasmid construction. Construction of plasmid pE4Δ-38-WT and the multiple base substitution mutants of the Egr-1 site (28), the human TGF-β1 promoter-CAT chimeric genes (28), and the in vitro transcription and in vitro translation vectors for IE2 (10) has been described previously. For expression of the Egr-1 gene in *Escherichia coli*, fragments of Egr-1, which were produced by PCR, were inserted in frame behind the glutathione *S*-transferase (GST) domain in plasmid pGEX-2T (Pharmacia) to make full-length GST–Egr-1 (amino acids 1 to 533), GST-E1 (amino acids 1 to 100), GST-E2 (amino acids 1 to 150), GST-E3 (amino acids 247 to 414), GST-E4 (amino acids 410 to 533), GST-E5 (amino acids 247 to 334), GST-E6 (amino acids 335 to 418), GST-E7 (amino acids 247 to 364), GST-E8 (amino acids 32 to 533), GST-E9 (amino acids 150 to 533), GST-E10 (amino acids 150 to 533), GST-E11 (amino acids 249 to 533), and GST-E12 (amino acids 349 to 533) plasmids. Plasmids pCJC190, pCJC229, and pCJC177, encoding polypeptides GST-IE2(290-579), GST-IE2(290-392), and GST-IE2(388-579), respectively, were constructed by inserting appropriate PCR-generated fragments of HCMV IE2 exon 5 at in-frame *Bgl*II restriction sites in derivatives of pGEX-3T (Pharmacia).

IE2 proteins generated by in vitro transcription and translation. The black beetle virus (BBV) leader region vector that was used for synthesis of [^{35}S]methionine labeled HCMV MIE gene-based polypeptides by in vitro transcription and translation has been described previously (8, 10). Construction of plasmids containing the intact BBV/IE2(1-579) and BBV/IE1(1-495) genes in pCJC186 and pCJC181 and the IE2 subfragments BBV/IE2(1-143) in pCJC185, BBV/IE2(86-290) in pCJC182, BBV/IE2(290-579) in pGH313, and BBV/IE2(346-579) in pCJC176 has also been described by Chiou et al. (10). A human herpesvirus 6 (HHV-6) equivalent of HCMV BBV/IE2(346-579), referred to as pCJC201, was prepared in the same pGH255 background vector by PCR synthesis of a 630-bp DNA fragment using the parent plasmid pE89 and primers based on DNA sequence information obtained from John Nicholas (unpublished data) (44). These plasmids were used as templates for RNA synthesis by T7 RNA polymerase followed by translation in rabbit reticulocyte extracts (Promega).

GST–Egr-1 and GST-IE2 fusion proteins. GST–Egr-1 and three HCMV GST-IE2 fusion proteins expressed in *E. coli* were partially purified by adsorption to glutathione-Sepharose beads in the presence of the detergent *N*-laurylsarcosine (Sarkosyl) and Triton X-100 as has been described previously (18).

GST affinity chromatography. For the in vitro binding assays, various GST–Egr-1 or GST-IE2 fusion proteins were adsorbed onto glutathione-Sepharose 4B beads (Pharmacia) in 400 μl of NETN buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40; pH 8.0). Samples of each protein (0.5 to 1.0 μg) bound to Sepharose were preincubated with and without ethidium bromide (10 to 400 μg/ml) for 30 min. Then the samples were shaken for 1 h at room temperature with 5 to 10 μl of [^{35}S]methionine-labeled in vitro-translated protein. The beads were washed four times in NETN buffer and boiled for 3 min in 2× sodium dodecyl sulfate (SDS) electrophoresis loading buffer before fractionation on 4 to 20% Tris-glycine gels (Novex). The gels were rinsed in 10% acetic acid, dried, and exposed to X-ray film for autoradiography.

Far-Western blotting. Samples (10 μg) of the GST–Egr-1 fusion proteins were resolved by 4 to 20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose. The blots were probed with 100 μl of reticulocyte lysate containing in vitro translated [^{35}S]methionine-labeled IE2.

Western blots. AN, A1b, and A45 cells grown in 150-mm-diameter dishes were lysed at 4°C in lysis buffer (25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μg of aprotinin per ml, and 50 μM leupeptin). The lysates were centrifuged at 12,000 × *g* for 15 min, and equal amounts (1 mg) of protein lysates were incubated with GST–Egr-1 fusion proteins bound to Sepharose beads. The bound proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CMV antibody (0.5 μg/ml). Immunity-specific bands were visualized by enhanced chemiluminescence (Amersham).

RESULTS

Transactivation of the TGF-β1 gene by IE2 can be mediated through the Egr-1 binding site. To identify the sequences responsible for regulation by IE2, we tested a series of deletion variants of the human TGF-β1 promoter linked to CAT. The structures of the TGF-β1–CAT chimeric plasmids used in this study have been described elsewhere (28). The chimeric plasmids were cotransfected into U373MG cells with an expression vector expressing CMV IE2 (CMV-IE2). Addition of IE2 protein induced the expression of the TGF-β1–CAT reporter genes containing 5′ sequences out to positions −160 and −119 by 10- to 12-fold compared with addition of control CMV vector sequences alone (Fig. 1A, lanes 3 to 6). The induction was totally abolished when the deletion reached nucleotide position −71 (Fig. 1A, lanes 7 and 8), suggesting that sequences responsible for the IE2-mediated transcriptional regulation in the TGF-β1 promoter reside in an 89-bp fragment between nucleotides −160 and −71. Similar results were obtained in a CCL-64 mink lung epithelial cell line, with lower nonspecific background (data not shown). The IE2 mutant construct pCJC120, in which amino acids between positions 290 and 345 were deleted, failed to activate the TGF-β1 promoter (Fig. 1B). It has previously been demonstrated that the region between positions −160 and −71 in the TGF-β promoter contains two independent Egr-1 binding sites as well as

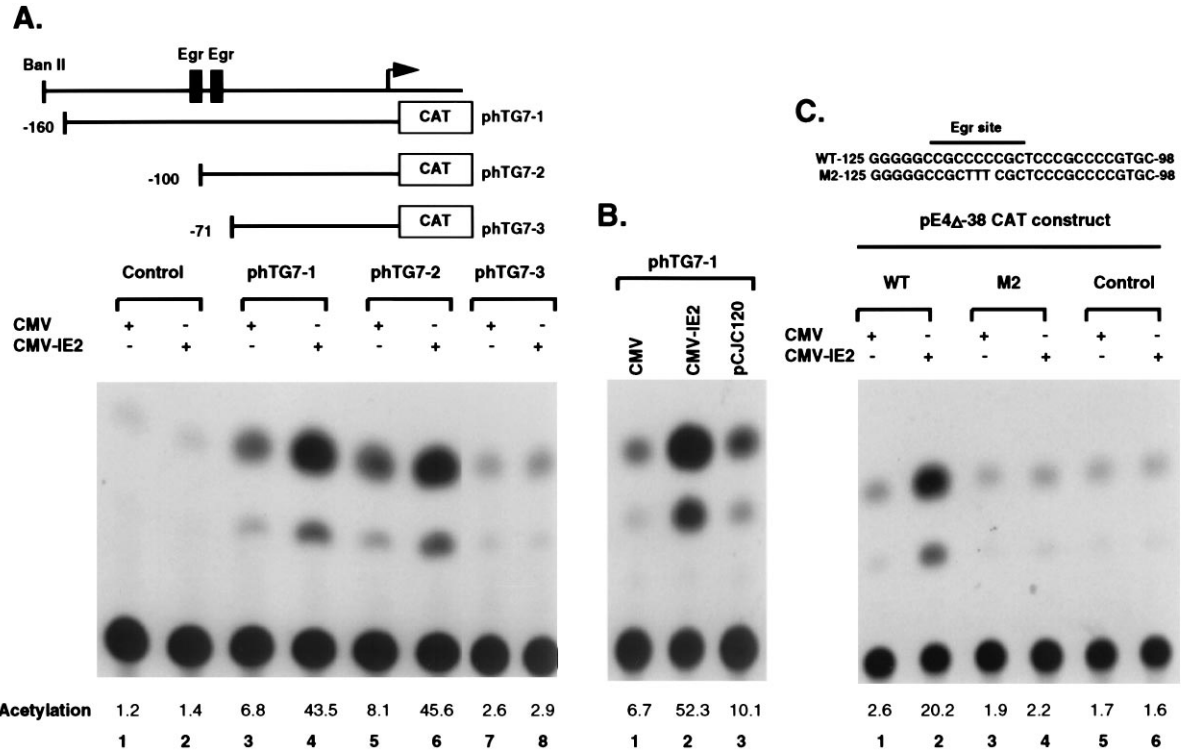

FIG. 1. Identification of an IE2-responsive element in the human TGF-β1 promoter. (A) Target reporter plasmids (10 μg) containing a series of 5′-deleted variants (phTG7-1, phTG7-2, and phTG7-3) of the human TGF-β1 promoter or a promoterless control plasmid (pSVoCAT) were cotransfected into U373MG cells with 10 μg of a control CMV MIE enhancer containing plasmid (lanes 1, 3, 5, and 7) or 10 μg of the same vector (plasmid pEQ326) expressing the IE2 protein (lanes 2, 4, 6, and 8) and assayed for CAT activity. (B) TGF-β1 promoter construct phTG7-1 was cotransfected into U373MG cells with 10 μg of either IE2 mutant construct pCJC120 (lane 3) or wild-type IE2 expression plasmid (lane 2). (C) Target reporter plasmids (10 μg) containing the TGF-β promoter element from positions −125 to −98 inserted into a minimal adenovirus E4 promoter-CAT gene (pE4Δ-38-WT-CAT) and base substitution variants (M1 and M2) that have been described previously (28) were cotransfected into U373MG cells together with 10 μg of either the CMV MIE enhancer containing only vector sequences (CMV) or the CMV IE2 expression plasmid (CMV-IE2). WT, wild type.

potential Sp1 sites (28). To examine whether one of the Egr-1 binding sites in the TGF-β1 promoter might be directly involved in IE2-mediated transcriptional regulation, we cotransfected IE2 with chimeric target reporter genes containing Egr-1 sequences between positions −125 and −98 ligated to adenovirus E4Δ-38 promoter-CAT vector (22). A base substitution mutant of the Egr-1 binding site is described in the top panel of Fig. 1C. When pE4Δ-38-WT, which contains the wild-type sequences, was cotransfected with CMV-IE2, a 13-fold increase in CAT activity was observed (Fig. 1C). Mutation that affected the central core of the Egr-1 site (positions −119 to −114) greatly abolished (M2) activation by CMV-IE2. These findings confirm that the presence of an Egr-1 binding site is important for the IE2-mediated transactivational activation and imply that this effect may be additive for the two Egr-1 sites within the context of the whole Egr-1 promoter (Fig. 1A).

IE2 stimulates GAL4-mediated Egr-1 transactivation. To determine whether the Egr-1 protein was directly involved in IE2-mediated transcriptional activation of TGF-β1, we performed cotransfection experiments with a GAL4–Egr-1(1-533) chimeric protein containing the whole Egr-1 coding region, together with a series of control GAL4 fusion proteins. Plasmids expressing the fusion proteins were cotransfected with a standard luciferase reporter target gene (G5E1b-Luc) containing five GAL4 binding sites placed upstream from the adenovirus E1b TATA box. Either the IE2 expression plasmid (CMV-IE2) or the control expression vector containing MIE enhancer sequences only (CMV-Vect) was then added to these transfection mixtures. G5E1b-Luc is one of the few heterologous target promoters that fails to respond significantly to cotransfected IE2, even in Vero cells (49a). As expected, no increase in luciferase activity was observed when a control plasmid encoding the minimal GAL4 DNA-binding domain [GAL4(1-147)] was cotransfected with CMV-IE2 (Fig. 2). Cotransfection with GAL4–Egr-1 alone, which contains its own activation domain, increased luciferase levels threefold in U373MG cells (Fig. 2). However, the luciferase activity was further stimulated up to nearly 20-fold by inclusion of IE2 as well (Fig. 2). Transactivation by GAL4-CREB was also slightly stimulated in the presence of IE2 (Fig. 2), but no stimulation was observed with either GAL4-ATF1 or GAL4-VP16 in the presence of IE2 (Fig. 2).

A similar experiment was carried out using stable DNA-transfected Neo^r cell lines expressing HCMV IE1 alone (A1b) or IR1 plus IE2 (A45) and a control Neo^r cell line (AN), all of which originated from U373MG human astrocytoma cells (42). When the GAL4–Egr-1 chimeric construct and G5E1b-CAT were cotransfected into these cell lines, the IE1-plus-IE2-containing cell line (A45) induced GAL4–Egr-1-dependent transcription up to 7.3-fold (Fig. 3, lane 12), whereas neither the IE1 line (A1b) nor the control (AN) cells gave an increase in G5E1b-CAT activity (Fig. 3, lanes 3, 4, 7, and 8). Control experiments with GAL4(1-147) in place of GAL4–EGR-1(1-533) failed to yield any transactivation effects even in A45 cells (Fig. 3, lanes 7 and 8). These results suggest that Egr-1 can specifically mediate IE2-induced transcriptional activation.

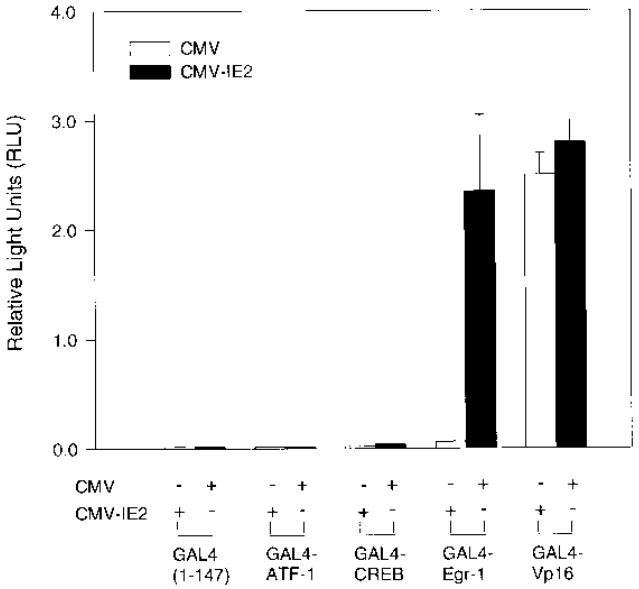

FIG. 2. Stimulation of GAL4–Egr-1 transactivation by IE2. An E1b-Luc reporter plasmid containing five GAL4 binding sites (G5E1b-Luc) was cotransfected into U373MG cells with GAL4–Egr-1(1-533) or other GAL4 fusion proteins in the presence of either control vector DNA containing the CMV MIE enhancer alone (CMV) or the CMV IE2 expression vector (pEQ326; CMV-IE2). The results are expressed as relative light units.

Egr-1 can interact directly with an 80-kDa IE2 protein from A45 cells. To demonstrate that the A45 cells do indeed express the appropriate IE2 protein, we prepared whole-cell extracts from AN, A1b, and A45 cells and performed Western blot experiments using an anti-CMV IE monoclonal antibody (Chemicon 810) that recognizes an epitope present in exons 2 and 3, which are common to IE1 and IE2. The antibody proved to recognize both a single 68-kDa IE1 protein band present in both A1b and A45 extracts and a triplet 80- to 100-kDa set of IE2 bands present only in the A45 extracts (Fig. 4, lane 4). The presence of a triplet 80- to 100-kDa set of IE2 bands in A45 cellular extract was also probed by Western blotting with monoclonal antibody 12E2, specific to IE2 (data not shown).

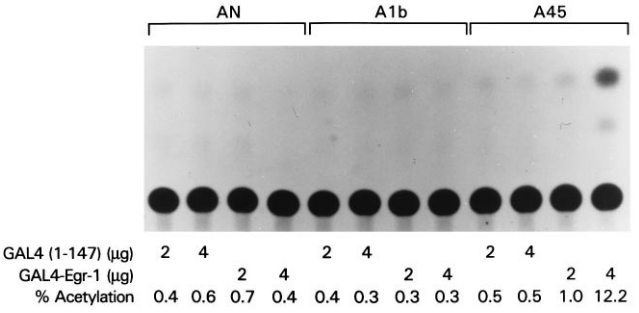

FIG. 3. Stimulation of GAL4-mediated Egr-1 transactivation in an IE2-expressing cell line. Four micrograms of a target G5E1b-CAT plasmid and 2 to 4 μg of either GAL4(1-147) or GAL4–Egr-1(1-533) were cotransfected into three stably transfected Neo^r U373MG astrocytoma cell lines containing vector sequences only (AN; lane 4), expressing HCMV IE1 (A1b; lanes 5 to 8), or expressing HCMV IE1 and IE2 (A45; lanes 9 to 12). Percent acetylation of [^{14}C]chloramphenicol in the extracts is given below each lane. Construction of GAL4–Egr-1(1-533) and the U373 cell lines is described in Materials and Methods.

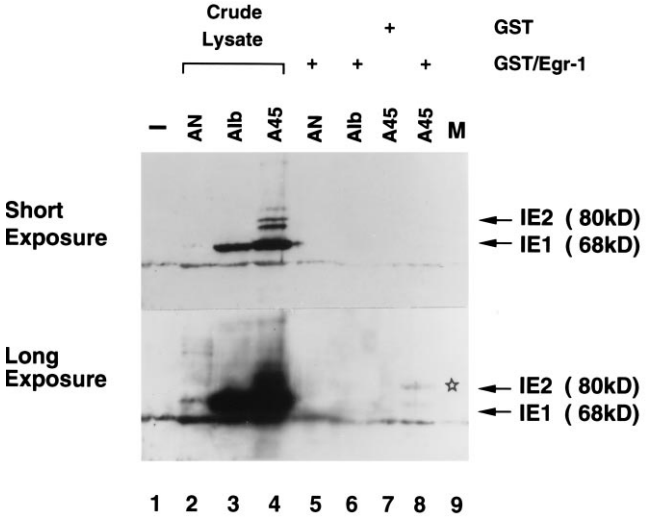

FIG. 4. Evidence that both the IE1 and the IE2 proteins are expressed in the A45 cell lines. Western blotting analysis of AN, A1b, and A45 cell extracts was done with Chemicon 810 monoclonal antibody, which recognizes both IE1 and IE2, after electrophoresis through an SDS–9% polyacrylamide gel. Unfractionated total cell lysates were prepared from AN, A1b, and A45 cell lines. Ten micrograms each of the AN, A1b, and A45 cell lysates was loaded directly onto the gel as positive input control samples (lanes 2 to 4, respectively). One milligram of each cell lysate was then mixed with 10 μg of GST–Egr-1 fusion protein bound to Sepharose beads. After extensive washing, the bound proteins were subjected to 4 to 10% gradient SDS-PAGE and Western blotting analysis with Chemicon 810 monoclonal antibody (lanes 5, 6, and 8). Similarly, 1 mg of the A45 cell lysate was mixed with 10 μg of GST-2T Sepharose beads, which contain GST protein only, and then subjected to Western blotting analysis in parallel with the other sample (lane 7). The position of positive signal in A45 cells (star) is indicated. Lane 9, molecular mass markers.

None of these protein bands were present in the control AN cell extracts (Fig. 4, lane 2).

Subsequently, a GST–Egr-1(1-533) fusion protein bound to glutathione-Sepharose beads was mixed with the lysates from AN, A1b, and A45 cells, and coprecipitated proteins were analyzed by Western blot analysis with the anti-CMV IE antibody. The 80-kDa IE2 protein from A45 cells was preferentially bound and recovered from the GST–Egr-1 beads (Fig. 4, lane 8). The IE1 protein in A1b and A45 cells bound only weakly to the GST–Egr-1 protein beads (Fig. 4, lanes 7 and 8).

Furthermore, no cellular proteins reacting with the antibody were recovered from the A45 cells with GST-only beads or from the AN cells with GST–Egr-1 beads (Fig. 4, lanes 5 and 7). These results show that the Egr-1 protein can bind either directly or indirectly to the intact 80-kDa IE2 protein present in the A45 cell line.

Egr-1 interacts directly with the C terminus of HCMV and HHV-6 IE2 proteins in in vitro binding assays. An intact 80-kDa [^{35}S]methionine-labeled HCMV IE2 protein prepared by in vitro translation (Fig. 5A, lane 1) was found to bind to GST–Egr-1(1-533) protein by GST-affinity chromatography (Fig. 5A, lane 6). IE2 binding was not detected with the agarose beads containing GST alone (data not shown). When polypeptides representing three segments of IE2 (amino acids 1 to 143, 86 to 290, and 290 to 579) were used in the experiment, only the 35-kDa C-terminal domain of IE2(290-579) interacted with the GST–Egr-1 fusion protein (Fig. 5A, lane 4). Neither the 25-kDa IE2(1-143) nor the 35-kDa IE2(86-290) N-terminal polypeptide was precipitated (Fig. 5A, lanes 7 and 8). A polypeptide containing a related 133-amino-acid segment from the homologous IE2 protein of HHV-6 (Fig. 5A, lane 5)

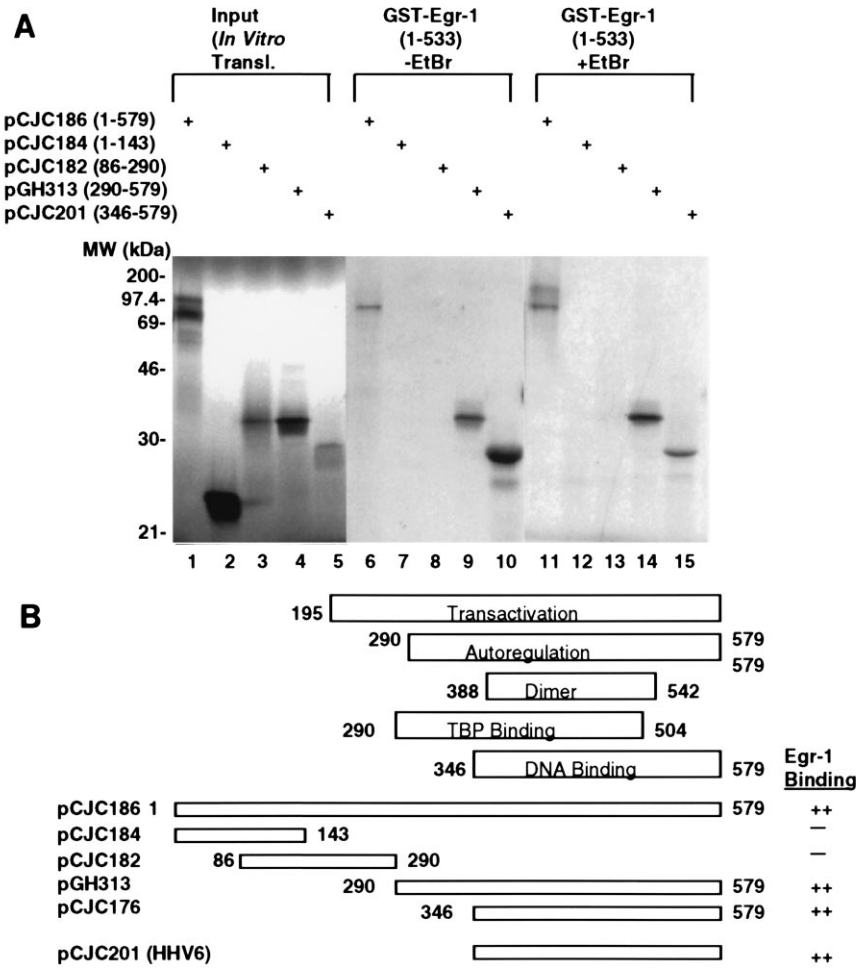

FIG. 5. The Egr-1 protein interacts with the in vitro-translated C-terminal domain of the IE2 protein by GST affinity chromatography. (A) Intact IE2 and three IE2 deletion mutants plus an HHV-6 IE2 C-terminal fragment were transcribed in vitro with T7 polymerase and translated in the rabbit reticulocyte system in the presence of [^{35}S]methionine (lanes 1 to 5). The bacterially expressed GST–Egr-1(1-533) was incubated with the amount of radiolabeled input protein shown in lanes 1 to 5 in either the presence (lanes 11 to 15) or the absence (lanes 6 to 10) of 400 μg of ethidium bromide (EtBr) per ml to eliminate DNA-mediated interactions (34). After extensive washing, the coprecipitated radiolabeled proteins were resolved on a 9% polyacrylamide gel. (B) Diagram illustrating the structure of the labeled IE2 protein probes used in panel A.

also bound to GST–Egr-1 beads with efficiency equal to that of the HCMV C-terminal polypeptide (Fig. 5A, lane 10). Note that the exactly equivalent 133-amino-acid C-terminal segment of HCMV IE2(346-579) was also positive (data not shown).

The C-terminal region of IE2 between amino acids 346 and 579 contains the DNA-binding and dimerization domain (10). Some in vitro protein interactions detectable in these types of assays have proved to be mediated by nonspecific DNA-binding interactions of both partners with DNA present in the extracts (25). Therefore, to examine whether the observed Egr-1–IE2 binding involves a DNA-mediated effect or was an authentic physical interaction of the two proteins, we carried out the experiment in the presence of ethidium bromide to disrupt potential DNA-protein interactions (34). However, even in the presence of a high level of ethidium bromide (400 μg/ml), the binding of either intact HCMV IE2(1-579) or HCMV IE2(290-579) to GST–Egr-1 was essentially unaffected (Fig. 5A, lanes 11 and 14), whereas the binding of HHV-6 IE2(346-579) was somewhat diminished (Fig. 5A, lane 15). In contrast, binding to a parallel control sample of the in vitro-translated Epstein-Barr virus Zta protein (8), which bound to GST–Egr-1 with an efficiency similar to that of IE2(290-579) in the absence of ethidium bromide, was nearly abolished after the addition of ethidium bromide (data not shown).

IE2 binds to a zinc finger domain of Egr-1 in in vitro binding assays. To identify which domain of Egr-1 binds to the IE2 protein, five GST–Egr-1 deletion chimeras, GST–Egr-1(1-533), GST–Egr-1(1-100), GST–Egr-1(1-150), GST–Egr-1(247-414), and GST–Egr-1(410-533), were constructed, using internal PCR primers (Fig. 6A). All proteins from these chimeras were produced from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *E. coli* bacterial hosts and analyzed by SDS-PAGE (data not shown). After the proteins were blotted onto nitrocellulose, a far-Western blot analysis using the [^{35}S]methionine-labeled intact 80-kDa IE2 protein was performed. Figure 6B shows that the IE2 protein can bind to GST–Egr-1(1-533) and GST–Egr-1(247-414) but not to the other chimeras. To further map the GST–Egr-1(247-414) domain to which IE2 binds, a GST affinity assay which showed that IE2 can bind to the GST–Egr-1(335-418) construct, which contains three zinc finger domains, was performed (Fig. 6C). Neither GST–Egr-1(247-334) nor GST–Egr-1(247-364) displayed any IE2 binding affinity.

The design of the GST bead affinity binding assay was re-

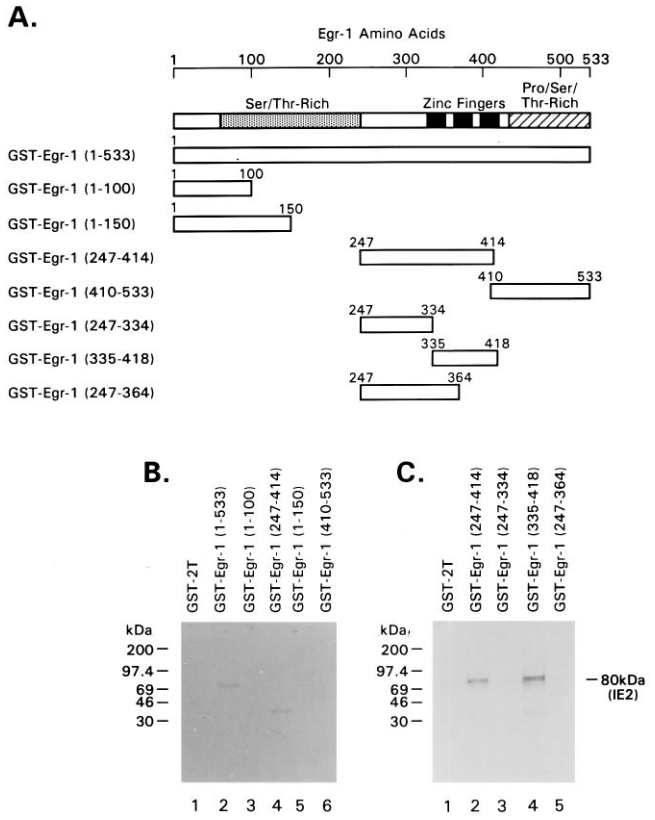

FIG. 6. Identification of an Egr-1 protein domain required for binding to the IE2 protein. (A) Diagram comparing the structures of the GST–Egr-1 fusion proteins used in panels B and C. All were expressed in *E. coli* and partially purified by using glutathione-Sepharose beads. (B) Far-Western blotting of the GST–Egr-1 deletion chimeras. Five different fragments of Egr-1, in the indicated GST fusion proteins, were separated by 4 to 20% gradient SDS-PAGE, blotted onto nitrocellulose, and then incubated with a [^{35}S]methionine-labeled in vitro-translated sample of intact IE2(1-579) protein. (C) For further definition of the IE2 binding domain by GST affinity chromatography, the [^{35}S]methionine-labeled IE2(1-579) protein synthesized in vitro was incubated with Sepharose beads containing the indicated GST–Egr-1 fusion proteins and bound protein was detected by SDS-PAGE and autoradiography.

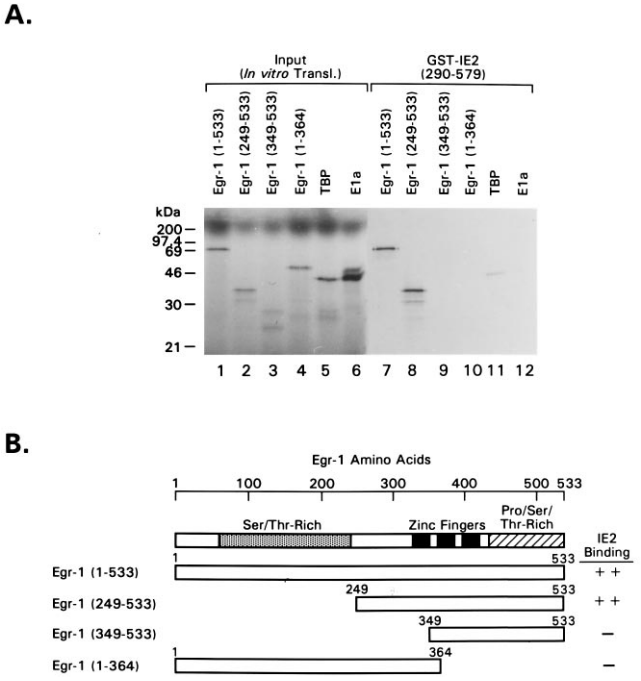

FIG. 7. Loss of the IE2 interaction by removal of zinc finger DNA-binding motifs from Egr-1. (A) The four Egr-1 deletion mutants diagrammed in panel B were synthesized in vitro and labeled with [^{35}S]methionine, then coprecipitated with the GST-IE2(290-579) fusion protein (lanes 7 to 10), and analyzed by SDS-PAGE (lane 11). [^{35}S]methionine-labeled TBP was used as a positive control in the GST affinity chromatography experiments, and [^{35}S]methionine-labeled E1a was used as a negative control (lane 12). The input in vitro-translated samples (10% of the amount used for GST binding) are shown in lanes 1 to 6. (B) Diagram illustrating the structures of the test Egr-1 in vitro-translated proteins used in panel A.

versed by using ^{35}S-labeled in vitro-translated forms of Egr-1 to identify which segment of Egr-1 was involved in binding to IE2. In the experiment whose results are shown in Fig. 7, a Sepharose bead-bound GST fusion protein containing the entire C-terminal half of IE2 was used. The results revealed that both the intact Egr-1(1-533) protein and the large C-terminal segment of Egr-1(249-533) bound to IE2. However, the smaller C-terminal segment of Egr-1(347-533) and the N-terminal segment of Egr-1(1-364) did not show any binding to IE2. In other versions of this experiment, the Egr-1(150-533) polypeptide also showed some binding activity (data not shown). ^{35}S-labeled in vitro-synthesized preparations of the intact human TBP and the adenovirus E1A protein served as positive and negative controls, respectively, for the binding specificity in this assay. The basic region of TBP has been shown to bind to the C terminus of IE2 previously in both GST bead precipitation and far-Western assays (5), whereas E1A does not (9a).

In a second set of experiments of this type (Fig. 8), the *E. coli* IE2 C-terminal domain was further subdivided into two segments, GST-IE2(290-390) and GST-IE2(388-579). When these reagents were used in the GST affinity bead binding assays, both the Egr-1(1-533) and the Egr-1(249-533) polypeptides bound to the GST-IE2(290-390) construct (Fig. 8, lanes 5 and 6), but they did not show any binding to the GST-IE2(388-579) construct (Fig. 8, lanes 9 and 10). The Egr-1(349-533) and Egr-1(1-364) constructs again failed to bind to either segment of IE2 (Fig. 8, lanes 7, 8, 11, and 12).

DISCUSSION

The 80-kDa IE2 nuclear phosphoprotein encoded by the HCMV MIE gene behaves both as a nonspecific transactivator of heterologous reporter genes and as a specific repressor of its own promoter-enhancer region. It was recently demonstrated that HCMV infection induces TGF-β1 protein production and strongly transactivates TGF-β1 promoter-driven reporter genes in human fibroblasts (42). In this report, we demonstrate that the regulation of TGF-β1 promoter activity by IE2 can be mediated through an Egr-1-responsive element (4). These results suggest that TGF-β1 may be directly involved in HCMV pathogenesis.

Egr-1 is a Ser-Thr-Pro-rich nuclear phosphoprotein containing three zinc fingers of the Cys_2His_2 type, which bind to the target sequence CGCCCCGC (4, 38). It is also a cellular IE transcription factor that is responsive to cell division signals (62). The Egr-1 protein contains two distinct activation domains and a negative regulatory domain (20). The Ser-Thr-rich N-terminal domain of Egr-1 contains a strong transactivation function, while the Pro-Ser-Thr-rich C-terminal domain displays a weak transactivation function (20).

Our results also show that the Egr-1 and IE2 proteins physically interact both in DNA-transfected cell lines and in in vitro

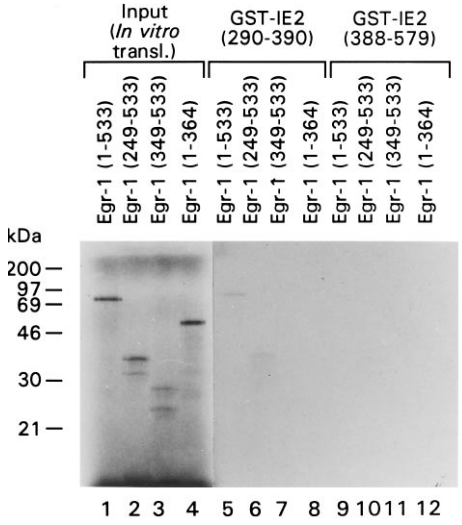

FIG. 8. Interaction of Egr-1 with GST-IE2 proteins does not require an intact IE2 DNA-binding or dimerization domain. The C-terminal binding region of IE2 was expressed as two separate GST fusion proteins and used for affinity chromatography with various segments of ^{35}S-labeled Egr-1 probes synthesized in vitro. The input samples (lanes 1 to 4) were compared with labeled protein recovered from GST-IE2(290-390) beads (lanes 5 to 8) or from GST-IE2(388-579) beads (lanes 9 to 12).

protein binding assays, but the data do not preclude the involvement of other factors in the transcriptional stimulation. The IE2 protein binds to the zinc finger domain of Egr-1 rather than to its activation domains, which may permit synergistic effects. Recently, it has been reported that p53 protein physically associates with the product of the *WT1* tumor suppressor gene, which contains four zinc fingers in its C terminus, and converts WT1 from a transcriptional activator to a transcriptional repressor (39). The zinc finger region of WT1 is a sequence-specific DNA-binding domain which recognizes the consensus site (5′-CGCCCCCGC-3′) of Egr-1. We have also found that IE2 interacts in vitro with p53 in both far-Western blotting and GST affinity binding assays through the same domain that was mapped in this study for Egr-1 (9a). Therefore, it will be interesting to see whether IE2 also interacts with WT1 and modulates the transcriptional activity.

Recent evidence suggests that the HCMV IE2 protein may bind to and interact functionally with a variety of cellular proteins and transcription factors (19, 53). Hagemeier et al. (24) and Sommer et al. (52) have also described in vitro binding between one or more domains of IE2 and the C-terminal pocket regions of the retinoblastoma gene product. Hagemeier et al. (25) suggested that IE2 can transactivate gene expression through a TATA motif and that the transactivation is mediated through the interaction of IE2 with the TBP component of TFIID in vitro. Caswell et al. (5) also showed that the C-terminal half of the IE2 protein interacts specifically with the C-terminal basic repeat domain of TBP.

The IE2 protein dimerizes through its C-terminal domain (10). Our results demonstrate that within the IE2 protein, the Egr-1 interaction domain maps to regions between amino acids 290 to 390 and 346 to 579. The overlap between them lies adjacent to the dimerization or oligomerization domain located at positions 388 to 506. This region also overlaps with the left-hand boundary of the DNA-binding domain between positions 346 and 388 (10). A sequence comparison of the C-terminal segment of six betaherpesvirus IE2 proteins reveals that this region contains one of the three most highly conserved amino acid motifs within these proteins (9), suggesting that this region is functionally very important.

The transcription of TGF-β1 is regulated by transcription factors, proto-oncogenes, and viral transactivators, including the Tax protein of human T-cell lymphotropic virus type 1 (30), the X protein of hepatitis B virus (66), and the Zta protein of Epstein-Barr virus (6). A variety of oncogene proteins, including Jun, Fos, Ras, and Src, selectively enhance the activity of the TGF-β1 promoter through AP-1 binding sites (3, 21, 27). AP-1 sites are also the targets for the activation of the TGF-β1 promoter by human T-cell lymphotropic virus type 1 Tax protein (30). In addition, it has been demonstrated elsewhere that the hepatitis B virus X protein also activates transcription of the TGF-β1 gene via the binding sites for Egr-1 (66). Since the promoters of many genes contain Egr-1 binding sites, disregulation of Egr-1 expression by viral transactivators such as HCMV IE2 or hepatitis B virus X protein may affect expression of multiple genes and lead to biologically significant changes of cell physiology. These data and evidence showing that TGF-β1 expression can also be induced by Epstein-Barr virus (6) and human T-cell lymphotropic virus type 1 (30) suggest that TGF-β1 may play an important role in the pathogenic processes seen in viral disease.

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Y. D. Yoo and C.-J. Chiou contributed equally to this work.

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