The Cyclin E Promoter Is Activated by Human Cytomegalovirus 86-kDa Immediate Early Protein

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Human cytomegalovirus (HCMV) activates cyclin E/Cdk2, which regulates cell cycle progression in G₁ and S phase of the cell cycle. HCMV activation of cyclin E/Cdk2 can be demonstrated in cells that are refractory to normal mitotic stimuli. This observation suggests that the virus has some means to overcome the stringent control on expression of cell cycle progression factors that is characteristic of cells in the G₀ state. One of the mechanisms involved in activation of cyclin E/Cdk2 is the induction of cyclin E expression. We report here that HCMV induces cyclin E expression through a transcriptional mechanism. The cyclin E gene is activated by the HCMV 86-kDa immediate early gene product (IE86), which directly binds to nucleotide sequences within the cyclin E promoter. An IE86 DNA-binding mutant neither binds nor activates the cyclin E promoter. IE86-binding sites within the cyclin E promoter are required for IE86-mediated activation, and deletion of the IE86-binding site inhibits IE86 activation of the cyclin E promoter. We also demonstrate that mutation of the known E2F-binding sites in the cyclin E promoter does not block activation by HCMV or IE86. These data provide a molecular mechanism for HCMV induction of cyclin E and represent the first report of IE86 directly binding to a cellular promoter.

Activation of cyclin-dependent kinase 2 (Cdk2) is essential for progression through the G₁ phase of the cell cycle (1–4). Activation of Cdk2 is a complex process that may be controlled in part by subcellular localization of the catalytic subunit (5), the abundance of cyclin kinase inhibitors such as p27⁰Kip (6, 7), and the expression of the gene that encodes a regulatory subunit, cyclin E (1, 2, 4, 8, 9). Under certain circumstances, induction of cyclin E is the rate-limiting phenomenon in Cdk2 activation (10–13). Cyclin E is induced in mid-G₁, and there is evidence that this G₁ induction is associated with and perhaps due to activation of the cyclin E promoter (14–16). Beyond that, there is not a great deal known about regulation of the cyclin E promoter.

We have become interested in regulation of cyclin E expression by human cytomegalovirus (HCMV). A pathogenic herpesvirus, HCMV infects the majority of the population and is highly morbid in perinatal infection and in patients with suppressed immune functions. HCMV has the ability to promote exit from a G₀ state, and some aspects of cell cycle progression can be demonstrated in HCMV-infected cells, which arrest in a state that resembles late G₁ or early S phase (17–19). During this process, the virus affects a selective activation of cyclin E/Cdk2 (17, 20). Activation of cyclin E/Cdk2 kinase activity is essential for virus replication (21). This activation results from nuclear uptake of Cdk2 (22), inhibition of the cyclin kinase inhibitor p21⁰Cip (17), and induction of cyclin E (17, 20). In many respects, HCMV activation of Cdk2 mimics the effects of serum growth factors. However, there is an important and interesting difference, the virus can activate cyclin E/Cdk2 in cells that are severely contact-inhibited and therefore completely resistant to growth factor stimulation (17).

We have begun to investigate the molecular mechanisms that account for the ability of HCMV to activate cyclin E/Cdk2 complexes. We were motivated by the assumption that the virus might use cellular signaling pathways to this end, and elucidation of such pathways might provide new information into processes whereby mitotically quiescent cells can be recruited into proliferative populations. At the same time, we were intrigued by the possibility that HCMV might have some unique means to overcome the tight control on cell cycle progression factors that is characteristic of cells in G₀. The kinetics of induction of cyclin E during HCMV infection suggest that the products of one or more of the immediate early (IE) genes might play a role in cyclin E expression. Induction of cyclin E occurs about 8 h after infection, when immediate early genes are abundantly expressed, and other classes of HCMV genes are only minimally expressed (23). UV inactivation studies indicate that one or more HCMV genes is required for induction of cyclin E (17). The rate of inactivation by UV indicates that the mutagenic target may be an immediate early gene.

The immediate early genes encode transcription factors that control both viral and cellular promoters (reviewed in Refs. 24 and 25). Although the mechanism by which immediate early gene products regulate transcription is not completely understood, there is increasing evidence that such factors act through direct DNA interaction and through protein-protein interactions with viral and cellular proteins. Among these cellular proteins are members of the E2F family of transcription factors, which interact with HCMV immediate early gene products (26, 27). The cyclin E promoter can be regulated by E2F family members, under certain circumstances (14–16). The kinetics of cyclin E induction, the ability of immediate early gene products to activate cellular promoters, and the ability of

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IE72 to interact with E2F suggests that one or more immediate early gene product may be involved in activation of cyclin E during HCMV infection.

Our objectives were to identify HCMV gene product(s) required for induction of cyclin E and to characterize the molecular mechanism that accounts for this activation. We report here that HCMV induction of cyclin E involves activation of the cyclin E promoter. Somewhat surprisingly, HCMV activation of the cyclin E promoter is independent of the E2F sites within the promoter. Cyclin E transcription is activated by the 86-kDa HCMV immediate early gene product (IE86), which interacts with a DNA sequence within the cyclin E promoter. Although IE86 is known to bind and regulate several viral promoters, it has previously been assumed that protein-protein interactions account, entirely, for IE86 effects on cellular genes. This is the first report of activation of a cellular promoter by direct binding of IE86.

MATERIALS AND METHODS

Plasmids and Reporter Constructs—Two sets of cyclin E promoter constructs were used in these studies. The first set (p10–4Luc, pMut

| pMut 1+II+III Luc) (provided by Dr. Robert Weinberg) has been described previously (14) and contains the wild-type cyclin E promoter sequence from –363 to +1007 (p10–4Luc) or the cyclin E promoter sequence (–363 to +1007) with three mutated E2F sites at –354, –292, and –282 (pMut 1+II+III). The second set of constructs (pE(–207)Luc and pMe(–207)Luc) was constructed by subcloning the promoter from p CycECAT or p CycECAT/2F2x2– ) (provided by Dr. Joseph Nevins) which contains either the wild type cyclin E promoter sequence from –207 to +77 or the cyclin E promoter sequence with two mutated E2F sites at –16 and +7 (15). A HindIII/XhoI fragment (–207 to +77) of the cyclin E promoter was cloned into the HindIII and XhoI sites of Bluescript KS+ (Stratagene). These plasmids were digested with SacI and KpnI, and the promoter fragment was ligated into the SacI and KpnI sites of pGL3Basic (Promega) luciferase reporter plasmid. The luciferase reporter was designated pE(–207)Luc or pMe(–207)Luc. HCMV immediate early expression plasmids (pRSV55, pRSV72, pRSV86, pRep9Amcs86, pRep5Amcs86/Mn) were a generous gift from Dr. Jay Nelson and have been described previously (26, 28). pRSLV40-Luc was purchased from Promega. p8DE DEL-Luc was constructed by using oligonucleotides (5‘-GATCCCTGTGACTTGCCGCCCGCCG and 5’-GA- TCTCGGCGGGCCGCCGTCCT) as PCR primers from –207 to +35 from pE(–207)Luc. The PCR product was gel-purified and ligated into pCR2.1 (Invitrogen). The deleted promoter (–207 to +77) was used as probe in all DNase I protection experiments. This construct is designated pE(–207) to +77. A mutant promoter (–207 to +77) containing a mutation in the E2F binding site was constructed by annealing the two primers and cloned into the SacI and KpnI sites of pGL3Basic (Promega) luciferase reporter plasmid. The mutant luciferase reporter was designated pME(–207)Luc or pME(–207)Luc. HCMV immediate early expression plasmids (pRSV55, pRSV72, pRSV86, pRep9Amcs86, pRep5Amcs86/Mn) were a generous gift from Dr. Jay Nelson and have been described previously (26, 28). pRSLV40-Luc was purchased from Promega. p8DE DEL-Luc was constructed by using oligonucleotides (5′-GATCCCTGTGACTTGCCGCCCGCCG and 5′-GA- TCTCGGCGGGCCGCCGTCCT) as PCR primers from –207 to +35 from pE(–207)Luc. The PCR product was gel-purified and ligated into pCR2.1 (Invitrogen). The deleted promoter (–207 to +35) was excised from pCR2.1 by KpnI/SacI digestion, and the promoter fragment was cloned into pGL3Basic that had been digested with KpnI and SacI. This construct is designated pE(–207) to +35/Luc. To reconstruct the wild type promoter, a double-stranded oligonucleotide (5′-GCCCGCCGCCCACCAGGTGCTC/ 5′-ACTGGGCGGCGGCGGGCCGTCCT) was subcloned into pGL3Basic (Promega). The reconstructed wild type promoter is designated pE(–207) to +35/Luc. To reconstruct the wild type promoter, a double-stranded oligonucleotide (5′-GCCCGCCGCCCACCAGGTGCTC/ 5′-ACTGGGCGGCGGCGGGCCGTCCT) was subcloned into pGL3Basic (Promega). The reconstructed wild type promoter is designated pE(–207) to +35/Luc.

Cell Culture, Virus Infection, and Transfections—Human diploid embryonic lung fibroblasts (LU), passage 12–20, or U-373 astrocytoma cells from the American Tissue Type Collection (ATCC HTB-17) were cultured in Eagle’s minimum essential medium with Earle’s salts, 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and used for immunoblotting, as described previously (5, 17, 21). Recombinant IE86 and DNase I Protection Analysis—Recombinant wild type and mutant zinc finger IE86 was kindly provided by Dr. Jay Nelson. Recombinant IE86 was purified via nickel chromatography as described previously (28, 30). A fragment of the human cyclin E promoter (–207 to +77) was used as probe in all DNase I protection experiments. This construct is designated pE(–207) to +77. A mutant oligonucleotide (5′-CTGTCATTGGCCCGCCGGCCTTGC and 5′-ATC- CGCCGCTGCCCCCTACACC) was double-stranded and end- labeled with T4 polynucleotide kinase and used in the PCR reaction to generate full-length double-stranded probes from the plasmid pE(–207)Luc. The labeled probe was gel-purified and used for footprinting using Promega’s Core Footprinting kit as described by the manufacturer. Briefly, binding reactions (50 µl) contained 25 µl of binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl; 12.5 mM MgCl2; 1 mM EDTA; 20% glycerol; 1 mM dithiothreitol), 1 or 3 µl of recombinant IE86 (500 ng/µl), 1 µl of probe (–25,000 cpm/µl), and double-distilled H2O adjusted to 50 µl. Binding reactions were incubated for 10 min at 4°C prior to the addition of probe and for 10 min at 4°C after the addition of probe. Ca2+ and Mg2+ (25 µM each) were added to the binding reaction and incubated at room temperature for 1 min. DNase I (0.1 unit) was added to the reaction and incubated at room temperature for 1.5 min. The reaction was stopped by addition of stop solution (200 mM NaCl; 30 mM EDTA; 1% SDS; 100 µg/ml yeast RNA). DNA-protein complexes were extracted with phenol/chloroform and precipitated with 100% ethanol at 37°C. DNA was collected by sedimentation, redissolved in 10 µl of water, and electrophoresed on a 5% polyacrylamide gel. After electrophoresis, the gels were dried and exposed to x-ray film.

Electrophoretic Gel Mobility Shift Assays—Double-stranded oligonucleotides were generated for IE86 response elements. The binding site within the cyclin E promoter, designated CcnE, consisted of nucleotides from +35 to +77 with the sequence (5′-AGCAAGCCGGCCGGCCGCGC- GGCGGGCCGGCCGGCGGTCCT) as PCR primer from –207 to +35 from pE(–207)Luc. The PCR product was gel-purified and ligated into pCR2.1 (Invitrogen). The deleted promoter (–207 to +35) was excised from pCR2.1 by KpnI/SacI digestion, and the promoter fragment was cloned into pGL3Basic that had been digested with KpnI and SacI. This construct is designated pE(–207) to +35/Luc. To reconstruct the wild type promoter, a double-stranded oligonucleotide (5′-GCCCGCCGCCCACCAGGTGCTC/5′-ACTGGGCGGCGGCGGGCCGTCCT) was constructed. The cis-repression signal within the HCMV major immediate early promoter (designated CRS) consisted of a pair of synthetic oligonucleotides with the sequence (5′-GGTTATAGAACCTGGCATG/5′-CTAGACTGTACGGGTTCATCTCAA- AGGAC) and a mutant cis-repression signal (designated mCRS) was constructed with the sequence (5′-GGCGCGGTAGCCGTGATG/5′-CTAGATCTGACGGGTTCATCGAC). Oligonucleotides were end-labeled with [γ-32P]ATP plus T4 polynucleotide kinase and purified by acrylamide gel electrophoresis. Binding reactions (12 µl) contained 6 µl of binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl; 12.5 mM MgCl2; 1 mM EDTA; 20% glycerol; 1 mM dithiothreitol), 2 µl of recombinant IE86 (500 ng/µl), 1 µl of labeled probe (–1.25 fmol), and H2O to a final volume of 12 µl. Reactions were incubated for 25 min at room temperature and resolved on a 6% polyacrylamide sequencing gel. The gel was dried and exposed to x-ray film.

RESULTS

HCMV Infection Induces Cyclin E mRNA Expression—Serum-starved, quiescent LU cultures were infected with HCMV.
Cells were harvested at various times after infection and assayed for cyclin E mRNA abundance. Cyclin E RNA expression was undetectable in uninfected cells (0 h), as shown in Fig. 1. However, cyclin E mRNA was induced by 8 h and reached maximal levels 12–16 h after infection. No increase in glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was observed, indicating that the virus does not induce all cellular transcripts. Expression of Cdk2 was unaffected, as previously reported (17). The kinetics of cyclin E mRNA induction closely paralleled those observed for the induction of cyclin E protein, as shown in the lower panel of Fig. 1. The data suggest that induction of cyclin E by HCMV may involve increased transcription of the cyclin E gene.

**HCMV Activates the Cyclin E Promoter**—Two previously characterized cyclin E promoter constructs were used to determine if HCMV activates the human cyclin E promoter when transfected in human LU fibroblasts. The first reporter, designated p10–4Luc, includes the nucleotides from –363 to –207 and contains two mutant cyclin E promoters and the conventional luciferase gene in p10–4Luc.

Transfection experiments were done using another human cell line (U-373) that is also permissive for HCMV infection (21). U-373 cells have a much higher efficiency of transfection than LU cells and are therefore more useful for promoter analysis. U-373 cells transfected with either p10–4Luc or pE(–207)Luc exhibited a dramatic increase in cyclin E promoter activity upon HCMV infection, as shown in Fig. 3, A and B. All subsequent experiments were therefore carried out with the more easily transfected U-373 cell line. The cyclin E promoter can be regulated by E2F (14–16), and HCMV can regulate at least one cellular promoter by activation of E2F (26). We were therefore interested in determining if activation of the cyclin E promoter by HCMV was dependent on the E2F sites within the promoter. There are five reported E2F sites within the human cyclin E promoter from –363 to +1007 (14–15), as illustrated in Fig. 2A. Two mutant cyclin E promoter constructs were used to determine what effect these E2F sites had on HCMV activation of the cyclin E promoter. pMutI++III Luc, is a derivative of p10–4Luc with mutated E2F sites at –354, –292, and –282, whereas pME(–207)Luc is deleted of E2F sites upstream of –207 and contains two mutated E2F sites at –16 and +7 (see Fig. 2A). These constructs were transfected into U-373 cells, which were then infected with HCMV. As shown in Fig. 3, A and B, mutation of the E2F sites did not inhibit HCMV activation of the cyclin E promoter. In fact, a slight increase in HCMV activation was consistently observed with E2F mutant constructs. These results demonstrate that HCMV infection can activate the cyclin E promoter, independent of those E2F sites that have been shown to affect cell cycle regulation of this gene. Furthermore, these data indicate that elements from –363 to –207 and from +1007 to +77 are not required for HCMV activation, since deletion of these sequences did not diminish HCMV activation of the promoter.

**HCMV IE86 Activates the Cyclin E Promoter**—UV-irradiated HCMV cannot induce cyclin E (17), indicating that expression of a viral gene is required. The kinetics of UV inactivation indicated that the viral gene is relatively insensitive to UV and might therefore be an immediate early gene (31). The kinetics of cyclin E induction in virus-infected cells are consistent with this proposition, as shown in Fig. 1. Cyclin E/luciferase reporter constructs were co-transfected into U-373 cells along with plasmids that express HCMV immediate early gene products IE86, IE72, and IE55. Although IE72 and IE55 were abundantly expressed, neither protein activated cyclin E/luciferase more than 2-fold (data not shown). However, IE86 was a robust activator of cyclin E promoter activity, as shown in Fig. 4 (circles). Cyclin E/luciferase activity increased about 30-fold when transiently co-expressed with wild type IE86. IE86 also activated the cyclin E promoter that had been deleted of E2F sites (squares). This observation is consistent with the data shown in Fig. 3 and indicates that HCMV activation of cyclin E transcription is not mediated by E2F-dependent processes.

Nelson and co-workers (28, 30) have described a zinc finger mutant of IE86 that cannot bind to the cis-repression signal in the HCMV major immediate early promoter. Although this
mutant cannot bind DNA, it retains the ability to activate HCMV promoters by protein-protein interactions (28). The zinc finger mutant of IE86 failed to activate the cyclin E promoter (Fig. 4, triangles) at concentrations that were optimal for activation of the cyclin E promoter by wild type IE86. The zinc finger mutant IE86, like wild type IE86, activated the SV40 promoter/enhancer 2–4-fold, on average. However, the amount of either wild type or mutant IE86 required to activate the SV40 promoter/enhancer was 10 times greater than that required to activate cyclin E (data not shown). The observation that the zinc finger mutant IE86 can activate the SV40 promoter/enhancer is consistent with the report that the zinc finger mutant can still participate in protein-protein interaction with core transcription factors (28, 30).

IE86 Directly Interacts with the Cyclin E Promoter—DNase I footprinting was done to investigate the interaction of purified, recombinant IE86 with the cyclin E promoter from nucleotides −207 to +77. The DNase I footprint revealed a strong IE86-binding site located from approximately +35 to +66, indicated by the vertical bar in Fig. 5A. A second potential binding site was observed around and somewhat upstream of +35. The major and minor IE86-binding sites could be displaced by addition of a 5-fold molar excess of unlabeled probe (Fig. 5A, lane 5). Recombinant IE86 mutated in the zinc finger was also used for DNA footprinting. As shown in Fig. 5B, the addition of mutant zinc finger IE86 protein did not protect the cyclin E promoter (lane 2), whereas the wild type IE86 bound to the region from +35 to +66 (lane 2).

IE86 binds to response elements in the HCMV major immediate early promoter and the 2.2-kilobase pair early HCMV promoter (32–34). No definitive IE86 consensus binding sequence has been identified, although all IE86-binding sites contain invariant CG residues at both ends of a 14-nucleotide sequence. IE86 binding to these elements protects 4–5 nucle-
hours after transfection, the cells were either mock-infected (calcium phosphate. Cultures were split 1:6 after transfection. Twenty four U-373 cells were co-transfected with 10 units. The data are the average of three dishes.

Promoter—Gel shift analysis was done to verify IE86 binding to the cyclin E promoter. U-373 cells were also transfected with either pE(-207)Luc or pMutII-IIIILuc, and assayed for cyclin E promoter activity (A). U-373 cells were also transfected with either pE(-207)Luc or pME(-207)Luc, as described above and assayed for cyclin E promoter activity (B). Data are expressed as light units of firefly luciferase per 10⁵ Rinella luciferase light units. The data are the average of three dishes ± S.D. Fig. 3. HCMV activates the cyclin E promoter independent of E2F sites. U-373 cells were co-transfected with 10 µg of p10-4Luc or pMutII-II-IIIILuc and 0.5 µg of pRLSV40-Rinella per dish using calcium phosphate. Cultures were split 1:6 after transfection. Twenty four hours after transfection, the cells were either mock-infected (solid bar) or HCMV-infected (striped bar). Cells were harvested 24 h after infection and assayed for cyclin E promoter activity (A). U-373 cells were also transfected with either pE(-207)Luc or pME(-207)Luc, as described above and assayed for cyclin E promoter activity (B). Data are expressed as light units of firefly luciferase per 10⁵ Rinella luciferase light units. The data are the average of three dishes ± S.D.

Deletion of the Cyclin E Promoter IE86 Response Element Inhibits Activation by IE86—The IE86-binding site was deleted from the cyclin E promoter, which was then assayed for IE86-dependent activation. A reporter construct, deleted of nucleotide sequences downstream of +35, was co-transfected with wild type IE86 expression vector, as shown in Fig. 8. The reporter that was deleted of the IE86-binding site (pE(-207)/ +35/Luc, cross-hatched bars) did not respond to concentrations of IE86 that were optimal for activation of the wild type promoter (pE(-207)/+77/Luc, filled bars). These data indicate that the +35/+66 IE86-binding element is responsible for the major portion of IE86-dependent activation of the cyclin E promoter. The zinc finger mutant IE86 failed to activate the deleted promoter (data not shown). This observation suggests that the residual activity that is observed when the deletion mutant is co-transfected with 5 µg of wild type IE86 involves IE86 DNA binding, possibly to weak IE86 DNA-binding sites upstream of +35. The deleted cyclin E promoter has not been inactivated. Basal activity of the deleted promoter is not significantly different from that of wild type, and both wild type and deleted promoters are activated by adenovirus to the same extent (data not shown).

The nucleotide sequences downstream of +35 are associated with the 5’ end of a CG-N₁₀-CG motif. The nucleotide sequences of the IE86-binding sites within the HCMV immediate early CRS, the HCMV 2.2-kilobase pair early promoter, and the cyclin E promoter are compared in Fig. 6B.

IE86 Binds an Oligonucleotide Derived from the Cyclin E Promoter—Gel shift analysis was done to verify IE86 binding to the cyclin E promoter +35/+66 element. An oligonucleotide corresponding to +32 to +75 was labeled and used as probe. This oligonucleotide is designated CcnE in Fig. 7. Incubation of the CcnE oligonucleotide with recombinant wild type IE86 resulted in a shifted nucleoprotein complex (lane 2). However, incubation of the oligonucleotide with recombinant mutant zinc finger IE86 did not result in a nucleoprotein shift (lane 6). To demonstrate specificity, various competition experiments were done with 50-fold molar excess unlabeled oligonucleotides. Unlabeled CcnE oligonucleotide effectively competed for IE86 binding (lane 3). An oligonucleotide corresponding to the well characterized IE86-binding cis-repression signal (CRS) of the HCMV major immediate early promoter (32, 34) also blocked IE86 binding to the CcnE oligonucleotide (lane 4). However, a CRS mutant oligonucleotide (mCRS) that is unable to bind IE86 (32, 34) was unable to compete for IE86 binding to the cyclin E promoter oligonucleotide (lane 5). These data indicate that binding of IE86 to the cyclin E promoter is nucleotide sequence-specific.

Fig. 4. Activation of the cyclin E promoter by HCMV immediate early gene products. Cells were transfected with 1 µg of wild type cyclin E reporter pE(-207)Luc or a derivative that contains mutations in two E2F-binding sites (pE(-207)Luc, as illustrated in Fig. 2A). Cyclin E reporters were co-transfected with various concentrations of expression vectors for wild type or zinc finger mutant (mZn) IE86. DNA concentrations were equalized by addition of vector DNA, as described under "Materials and Methods." Cells were harvested 24 h after transfection, and 5 µg of protein was assayed for luciferase activity. Data are expressed as light units of firefly luciferase per 10⁵ Rinella luciferase light units. The data are the average of three dishes ± S.D.
Cyclin E Promoter Activation by HCMV IE86

Fig. 5. IE86 binds to the cyclin E promoter. The antisense strand of the cyclin E promoter from −207 to +77 was end-labeled and used as probe for DNase I footprinting, as described under “Materials and Methods.” In the footprint shown in A, lanes 1 and 4 contain no IE86; lane 2 contains 50 ng of recombinant IE86, and lanes 3 and 5 contain 500 ng of IE86. Lane 5 also contains 5-fold molar excess of unlabeled −207 to +77 DNA. The major IE86-binding site from +35 to +66 is indicated by the solid line. B represents a footprint of the cyclin E promoter from −207 to +77 in which no protein (lanes 1 and 4), 500 ng of wild type IE86 (lane 2), or 500 ng of zinc finger mutant IE86 (lane 3) were used.

DISCUSSION

The most abundant HCMV immediate early proteins originate from two regions, IE1 (UL123) and IE2 (UL122), which are transcribed from the major immediate early promoter to yield several alternatively spliced RNAs. The most abundant of these mRNAs is derived from the IE1 locus and encodes a 72-kDa nuclear phosphoprotein (IE72). Two major immediate early transcripts arise from the IE2 region through differential splicing. These mRNAs encode 86- and 55-kDa proteins (IE86 and IE55). The IE1 and IE2 proteins (IE72, -55, and -86) have identical amino termini of 85 amino acids, which arise from exons 1 and 2 of the IE1 region. The 86- and 55-kDa proteins are identical in their amino acid sequence, except IE55 is deleted of 154 amino acids between residues 365 and 519 in IE86 (reviewed in Refs. 24 and 25). These 154 amino acids contain the zinc finger DNA-binding domain, so that IE55, unlike IE86, does not bind nucleotide sequence elements in HCMV promoters.

IE86 activates several cellular promoters including c-myc (36, 37), c-fos (36, 38), heat shock protein 70 (hsp70) (36, 39), and transforming growth factor-β1 (40). However, the mechanism whereby IE86 activates these genes is different from that demonstrated for HCMV promoters. IE86 activation of c-myc, c-fos, and hsp70 promoters requires no sequences upstream of the TATA box (36) and is thought to occur through IE86 interaction with basal transcription factors, such as TBP and TFIIB (30, 36, 41–43). The transforming growth factor-β1 promoter is activated by IE86 binding to Erg-1 (40). IE86 also interacts with transcription factors other than Erg-1, including p53, Rb, SP-1, Tef-1, c-jun, junB, ATF-2, NF-κB, p300, and UBF (24, 27, 44–46). Such observations have generated the notion that nucleotide-specific binding of IE86 does not occur with cellular promoters, despite the fact that this appears to be a predominant mode of regulation for HCMV promoters.

We have determined that induction of cyclin E occurs through DNA binding of IE86 to the cyclin E promoter. The cyclin E promoter contains a prominent IE86-binding site between +35 and +66. The requirement for DNA binding activity for cyclin E activation is further supported by the observation that IE72 and IE55 fail to activate the cyclin E promoter to any significant extent. IE72 is not known to bind to DNA. IE55 is identical to IE86 except that it is deleted of the zinc finger DNA binding domain and therefore unable to bind DNA. Likewise, a DNA-binding mutant of IE86 neither binds nor activates the cyclin E promoter. This zinc finger mutant is capable of activating viral promoters through protein-protein interaction (30). In our hands, the zinc finger mutant activates the SV40 promoter/enhancer to about the same extent as does wild type IE86, although relatively high concentrations of either protein are required. The observations that neither the zinc finger mutant of IE86 nor the naturally occurring DNA-binding variant IE55 can activate the cyclin E promoter is consistent with a DNA-binding mechanism. Finally, deletion of the IE86-binding site within the cyclin E promoter inhibits IE86 activation of cyclin E promoter/reporter constructs.

The major IE86-binding site within the cyclin E promoter contains two overlapping CG-N10-CG motifs. It is known that IE86 binds weakly to half CG-N10-CG motifs within HCMV early promoters (33), and weak IE86 binding around +30 of the cyclin E promoter may involve part of a CG-N10-CG motif. The IE86-binding sites within the cyclin E promoter are somewhat different than those observed within HCMV early and immediate early promoters; the 10 internal nucleotides within the cyclin E promoter CG-N10-CG motifs are GC-rich, rather than AT-rich. The ability of IE86 to bind various DNA sequences has led to the idea that IE86 recognizes DNA structural features and is relatively sequence-tolerant (24, 33, 34), and our data are consistent with that proposition.

Deletion of the major DNA-binding site did not completely abolish activation of the cyclin E promoter. The activation that occurs upon co-expression of wild type IE86 and the deleted promoter may be due to weak interactions between IE86 and promoter sequences around and upstream of +30. The observation that the IE86 zinc finger mutant failed to activate the deleted cyclin E promoter suggests that DNA binding is required for the weak stimulation. This hypothesis has not been explored because the effect is relatively small. Deletion of the major IE86-binding site within the cyclin E promoter does not increase the basal promoter activity. This observation suggests that activation by IE86 does not occur through displacement of a cellular repressor bound to the cyclin E promoter.

The results presented in this report define a mechanism whereby HCMV is able to induce cyclin E expression. This is the first report demonstrating direct binding of IE86 to a cellular promoter, and our data challenge the prevailing view that activation of cellular promoters by HCMV immediate early proteins is invariably due to protein-protein interactions. The IE86-binding sites within the cyclin E promoter share many characteristics with IE86-binding sites within HCMV promoters. The most striking similarity is the presence of the CG-N10-CG motifs. IE86 does not bind with equal affinity to all CG-N10-CG motifs present within the cyclin E promoter, suggesting that sequence, context, or structure of these motifs
provides important, although poorly understood, structural constraints upon IE86 binding. IE86 binding to the immediate early CRS has been shown to occur through interaction with the minor groove and probably results in DNA bending (35). IE86 binding within the minor groove of the cyclin E promoter remains to be resolved but may explain some of the binding specificity for IE86.

In conclusion, we have shown that an HCMV immediate early protein, IE86, binds specifically to the cyclin E promoter. The activity of the promoter increases as a consequence, and cyclin E expression is induced. The potency of this induction is...
evidenced by the observation that HCMV can induce cyclin E in cells that are refractory to mitotic stimulation by serum growth factors (17). This ability to activate cell cycle progression genes in G₀ cells provides a model system for understanding how other hyperplastic stimuli may regulate gene expression in post-mitotic cell populations.

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