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# Binding of SP1 to the Immediate-Early Protein-Responsive Element of the Human Cytomegalovirus DNA Polymerase Promoter

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**Human cytomegalovirus (HCMV), a member of the herpesvirus family of DNA viruses, encodes two major immediate-early (IE) transcription factors, IE72 and IE86, that are important for regulated expression of the viral genome. The purpose of this study was to identify the host cellular components required for regulation of the HCMV DNA polymerase promoter (UL54) by HCMV IE proteins. Extensive mutagenesis defined a DNA element located between −54 and −43 relative to the transcription start site that was required for both basal transcriptional activity and transactivation by viral IE proteins. A single copy of the UL54 −54/−43 sequence enhanced the responsiveness of a heterologous minimal promoter to HCMV IE proteins. Fractionation of extracts prepared from uninfected cells led to the isolation of two cellular proteins with apparent molecular masses of 95 and 105 kDa that bound specifically to the UL54 −54/−43 element. Biochemical and immunochemical analyses identified this protein as the transcription factor SP1. Although initial inspection of the UL54 −54/−43 sequence did not predict an SP1 binding site, subsequent analyses indicated that it is indeed a nonconsensus GC box. We propose that SP1 is required to direct basal levels of promoter activity and that SP1-regulated transcription complexes allow the entry of HCMV IE proteins into the transcription cycle.**

Human cytomegalovirus (HCMV) is a ubiquitous opportunistic human pathogen of the herpesvirus family. HCMV is of medical significance because of its association with diseases that render the patient immunocompromised (1). Typical of herpesviruses, HCMV gene expression is regulated temporally after infection and can be divided into immediate-early (IE), early, and late phases (8, 34, 35, 54). Viral IE genes are the first to be expressed and encode transcriptional regulators that are required for the subsequent expression of early and late genes (21, 35, 38, 46, 55).

The most abundant and perhaps most important transcriptional regulators encoded by HCMV are the IE72 and IE86 proteins (for reviews, see references 35 and 46). Although a large body of work has been devoted to the study of these factors, their precise mechanism of action is unknown. IE72 and IE86 arise from alternatively spliced RNAs transcribed from the major IE enhancer-promoter of HCMV (47, 49). IE72 and IE86 have a common amino-terminal domain but diverge at their carboxyl termini (49). Structure-function studies have shown that the carboxyl-terminal region of IE86 is required for transcriptional activation, DNA binding, homodimerization, and protein-protein interactions (5, 6, 22, 39, 48, 58). The IE86 protein is a potent transcriptional activator of both viral and cellular promoters (9, 11–13, 17, 33, 36, 40, 44, 45, 48, 53). Although IE72 has been characterized as a strong transactivator on its own in heterologous systems (14, 16, 53), its action on homologous promoters is less direct, most often augmenting IE86-dependent transactivation (9, 11, 33).

Many mechanisms for IE86 activation have been proposed, and from these studies, it is apparent that IE86 can exert its influence at various stages of the transcriptional cycle. The IE86 protein binds DNA specifically (2, 29, 30, 42), and DNA binding sites for IE86 that reside upstream of the TATA box in the 2.2-kb HCMV promoter are important for maximal IE86 transcriptional activation (2, 28, 42, 43). The relevance of sequence-specific DNA binding by IE86 in transcriptional activation is not clear, as the distribution of IE86 binding sites in the viral genome is difficult to assess due to the heterogeneous nature of the IE86 binding site. Recruitment of IE86 to promoters that lack IE86 DNA binding sites could occur by association of IE86 with a cellular factor that binds directly to either an enhancer or a promoter element. Two examples of promoter recruitment via enhancer elements include the association of herpesvirus protein VP16 with the cellular enhancer-binding proteins OCT1 and HCF (56) and the binding of adenovirus E1A protein to cellular zipper proteins (31). Alternatively, direct interactions between IE86 and components of the basal transcription complex (5, 13, 22, 32) at the gene's promoter may be sufficient for recruitment of IE86 and fulfillment of its regulatory function.

In addition to the virally encoded IE factors, the expression of early and late HCMV genes requires cellular transcription factors. Several cellular factors including AP1, USF, and CREB have been implicated by the presence of their DNA binding sites in individual HCMV early promoters (9, 26, 28, 41, 52). We sought to identify the cellular factors required for IE protein transcriptional activation of the HCMV DNA polymerase gene (UL54), an essential prototypical early gene that has been characterized extensively by Stenberg and coworkers (23, 24, 46). Targeted mutagenesis within the UL54 promoter suggested that a novel 8-bp inverted repeat element (IR1) is required for activation by IE proteins (24). A 4-bp substitution within IR1 diminishes transactivation by IE proteins and abrogates binding of cellular proteins (24). Further analyses indicated that neighboring sequences are also important for maximal responsiveness to IE proteins (23). Introduction of the identical 4-bp mutation into a recombinant virus revealed

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that the IR1 element is critical at early, but not late, times after infection (23).

Because the specific aim of our work was to identify cellular proteins required for transcriptional regulation of the UL54 promoter by IE proteins, we chose to study DNA-protein complexes that bind specifically to the same UL54 sequences required for transactivation by IE proteins. In order to define precisely the DNA sequences required for transactivation by IE proteins, we performed a systematic, unbiased mutagenesis of the UL54 promoter. Consistent with previously published work (23, 24), our experiments identified a DNA element that maps between −54 and −43 relative to the transcription start site. Electrophoretic mobility shift assay (EMSA) experiments with nuclear extracts from HCMV permissive or nonpermissive cells revealed the presence of cellular proteins that bind to the −54/−43 promoter element. Biochemical and immunochemical analyses identify this cellular protein as transcription factor SP1.

## MATERIALS AND METHODS

**Plasmid construction.** The pLA12 parental plasmid containing the −270/+200 region of the UL54 promoter was constructed by amplification of the promoter region from HCMV AD169 genomic DNA (ABI) with the forward LO-35 and the reverse LO-34 primers: LO-35, 5′-GCGCGAGCTCGCGGTGGCCTC CGTG-3′, and LO-34, 5′-GAAGATCTGTAGCCGTTTTTGCGATG-3′. These primers were designed on the basis of the DNA polymerase promoter sequence published by Kouzarides et al. (27). The resulting DNA fragment was cloned into the pGL-2 basic vector (Promega). Deletion constructs were generated by PCR with the reverse primer LO-34 as a 3′ anchor. Oligonucleotide scan mutagenesis was performed by a two-step PCR method. The −54/−43 E1B and the E1B-luciferase (luc) reporter constructs were generated by inserting oligonucleotides containing the consensus core E1B TATA element with and without a single copy of the UL54 SP1 binding site into the pGL-2 basic vector.

**Cells and transfection.** Transfections were performed by the calcium phosphate precipitation method. Cells were plated in six-well plates on the evening before transfection at the following densities: U373 and HeLa, $5 \times 10^5$/ml; HepG2, $5 \times 10^4$/well. Beta-galactosidase activity produced by a control plasmid was measured and used to normalize transfection efficiency. The plasmid RL45 (a kind gift from Gary Hayward) was used to direct the expression of HCMV IE proteins and has been described previously (39). Cells were exposed to the calcium phosphate precipitate for 6 to 7 h, washed with phosphate-buffered saline, and refed with fresh medium. Cells were harvested in 200 μl of cell lysis buffer (Promega) 36 to 40 h posttransfection. All DNAs used for transfection were purified by CsCl gradient ultracentrifugation.

**Protein purification.** Nuclear extracts from human foreskin fibroblasts and U373 and HepG2 cells were prepared from ~$10^7$ cells. Cells were grown to confluency in a T-175 culture flask and were harvested by scraping. After centrifugation at 1,500 × *g* at 4°C, cells were resuspended in an equal volume (usually 75 to 100 μl) of ice-cold buffer C (20 mM HEPES [pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 μM $ZnCl_2$). Cell pellets were then sonicated for 1 min in a Brinkmann cup sonication unit. Samples were incubated for 1 h on ice and then centrifuged at 14,000 rpm in an Eppendorf 5451C centrifuge for 15 min. Protein concentration was determined by a standard Bradford miniassay (Bio-Rad).

HeLa cell nuclei were purchased from Cellex Biosciences, and nuclear extracts were prepared by the procedure of Dignam et al. (10). Nuclear extract prepared from $2.5 \times 10^{10}$ cells was applied to an SP Sepharose column equilibrated with buffer D (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 20% glycerol, 50 μM $ZnCl_2$) plus 200 mM KCl. The bound material was step eluted with buffer D containing 350 mM KCl. The protein peak was pooled and dialyzed to 200 mM KCl and bound to single-stranded (ss) DNA-Sepharose (Pharmacia) equilibrated in buffer D plus 200 mM KCl. The ssDNA column was eluted with a linear gradient of 0.2 to 0.6 M KCl. The EMSA activity eluted at 350 mM KCl. Active fractions were pooled, dialyzed, and fractionated further on DNA affinity columns. DNA affinity column resins contained 1.5 to 1.7 mg (per ml) of double-stranded (ds) oligonucleotides containing the wild-type −65/−35 region of the UL54 promoter bound to streptavidin-agarose beads (Sigma). The 5′ end of the top (sense) strand was biotinylated. Active fractions from the ssDNA column were preincubated with 3 mg of the 3.3 mutant oligonucleotide (see Fig. 2A) per ml and 50 ng of carrier DNA [poly(dI-dC)–poly(dI-dC) (Pharmacia)] per ml. Column washes containing 3 mg of the 3.3 mutant oligonucleotide per ml were performed to compete out nonspecific DNA binding. Bound material was eluted with 550 mM KCl and assayed directly in EMSAs.

Wheat germ agglutinin (WGA) affinity chromatography was performed as described previously (20). WGA resin was purchased from Vector Laboratories. Primary antibodies to SP1, SP2, SP3, and SP4 were purchased from Santa Cruz Biotechnology. Polyclonal antisera and monoclonal antibodies to IFI16 were a kind gift from Joseph Trapani (7). Immunoblots were developed with the Amersham ECL horseradish peroxidase detection system.

**DNA binding assays.** $^{32}$P-end-labeled ds oligonucleotide probe corresponding to the −65/−35 region of the UL54 promoter was generated by incubating 5 pmol of annealed oligonucleotides with polynucleotide kinase (Promega) in the presence of [γ-$^{32}$P]ATP (NEN; 6,000 Ci/mmol). Labeling efficiencies typically resulted in a specific activity of 5 cpm/fmol. DNA binding reactions were performed in a final volume of 25 μl and in 1× reaction buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mg of bovine serum albumin per ml, 0.05% Nonidet P-40, 5% glycerol, 50 μM $ZnCl_2$, 1 μg of poly(dI-dC)–poly(dI-dC)]. Binding was allowed to proceed for 30 min at room temperature. For oligonucleotide competition experiments, a 100-fold molar excess of ds mutant oligonucleotide was added to the reaction mixture and allowed to bind proteins for 5 min prior to addition of $^{32}$P-labeled wild-type probe. For antibody supershift experiments, 1 to 100 ng of purified polyclonal antibodies was added to the reaction mixture after 15 min of incubation. Reactions were then allowed to proceed. Electrophoresis through 5% polyacrylamide gels containing 0.5× Tris-borate buffer and 2% glycerol was performed at 150 V for 3 h.

**Renaturation of DNA binding activity after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).** The procedure used was a modification of that described by Ossipow et al. (37). Purified extracts (50 to 70 μg) were subjected to electrophoresis on SDS–8% polyacrylamide minigels at 150 V for 2 h. Gel slices were excised and homogenized in 3 volumes of elution-renaturation buffer (20 mM HEPES [pH 7.6], 5 mg of bovine serum albumin per ml, 100 mM KCl, 0.1 mM EDTA, 0.1 mM $ZnCl_2$) with the use of a Teflon pestle. Samples were incubated for 4 h at 37°C, after which the supernatant was removed and supplemented with glycerol to a final concentration of 20%. The eluted samples (2 to 5 μl) were assayed directly for DNA binding activity by EMSA.

## RESULTS

**Mutagenesis of the UL54 promoter.** Initial analyses of UL54 promoter deletion mutants indicated that sequences located between −90 and −30 relative to the transcription start site were essential for promoter function and transactivation by IE proteins and that sequences within the −270 to −90 region were dispensable for both functions (data not shown). In order to analyze further the role of the −90/−30 region of the UL54 promoter, we carried out three successive rounds of oligonucleotide scanning mutagenesis of a promoter fragment that contained sequences from −270 to +200 relative to the start site of transcription. In these studies, each promoter derivative was fused to the luc reporter gene and its level of expression was measured in transient transfection experiments. Activated transcription was measured by cotransfecting the various UL54 promoter-luc fusion constructs with a vector that expresses IE proteins from the major IE promoter (IE72/IE86).

In our initial experiments, transfection assays were performed in parallel in the glioblastoma cell line U373, in the hepatocyte-derived cell line HepG2, and in HeLa cells. In the first round of scanning mutagenesis, DNA segments of various lengths (average of 10 bases per mutant) were mutated by purine-pyrimidine exchanges within the −91 to −20 promoter region. Six mutants were generated, and their activity was measured in the transfection assays described above. In all three cell lines, the mutation of sequences from −91 to −56 had no significant effect on either basal promoter activity or transcriptional activation by IE proteins. In contrast, mutants with substitutions between −56 and −47, −46 and −32, and −31 and −22 were defective in both basal promoter function and transactivation by IE proteins (Fig. 1A). The −31/−22 mutant disrupted a TATA box sequence, suggesting that the UL54 TATA element is functional. In the next round of scanning mutagenesis, blocks of 5 bp were mutated within the −56 to −32 promoter region. Analysis of these promoter derivatives in transient transfection assays defined a 15-bp region from −56 to −41 that is required for transactivation by IE proteins and optimal basal promoter activity (Fig. 1B). Again, the results obtained were qualitatively identical in all three cell lines (data not shown), indicating that the cellular factors that function through this promoter region are expressed ubiqui-

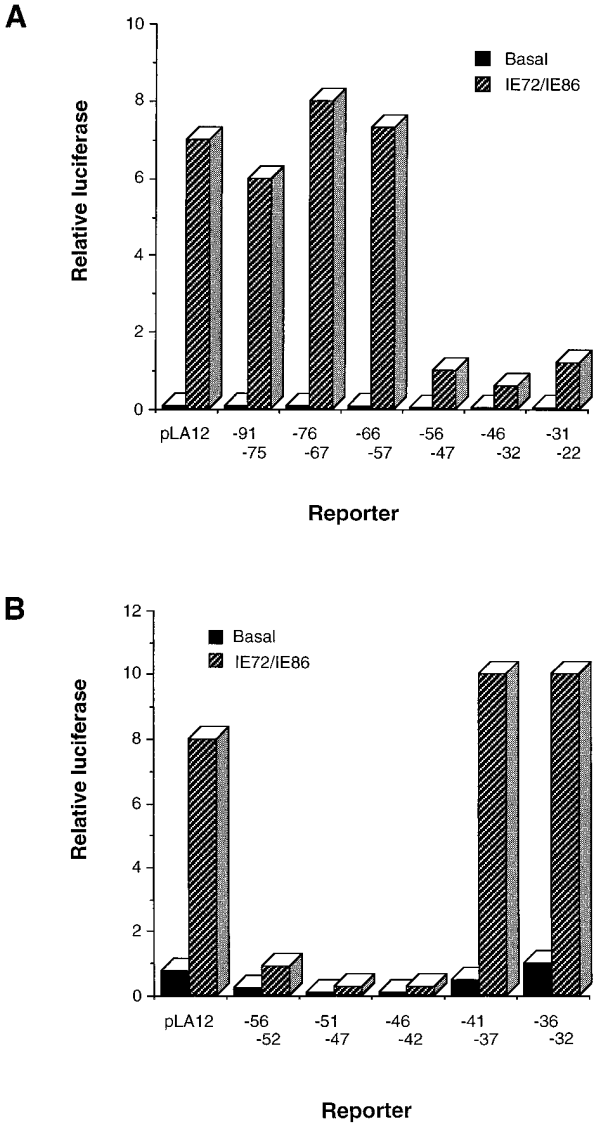


FIG. 1. (A) Mutagenesis scan of the −91 to −22 region of the UL54 promoter. Transient transfection assays were used to measure the activity of various promoter mutants fused to the luc reporter gene. The name of each reporter construct specifies the region of the promoter with the purine-to-pyrimidine substitutions. pLA12 is the parental construct, which contains UL54 sequences from −270 to +200. Each reporter was assayed either by itself (basal transcription) or together with an expression vector for IE proteins (IE72/IE86; activated transcription). Transfections were performed as described in Materials and Methods. (B) Mutagenesis scan of the −56 to −32 region of the UL54 promoter. The names of the reporter constructs specify the region of the promoter with purine-pyrimidine substitutions. The activity of each reporter was measured in transient transfection experiments that were normalized with a beta-galactosidase internal control. Data obtained with U373 cells are shown in both panels.

tously. In order to more precisely define the *cis*-acting DNA elements required for IE transactivation, we subjected the −56/−41 promoter region to a third round of scanning mutagenesis in which 2-bp segments were mutated by purine-pyrimidine exchange. Eight mutants were generated (labeled 3.1 to 3.8 in Fig. 2A) and tested in transfection assays as described above. Mutations 3.2 to 3.7 severely reduced IE activation, whereas mutations 3.1 and 3.8 had a minimal effect (Fig. 2B). Thus, our analysis defined the −54/−43 region as the

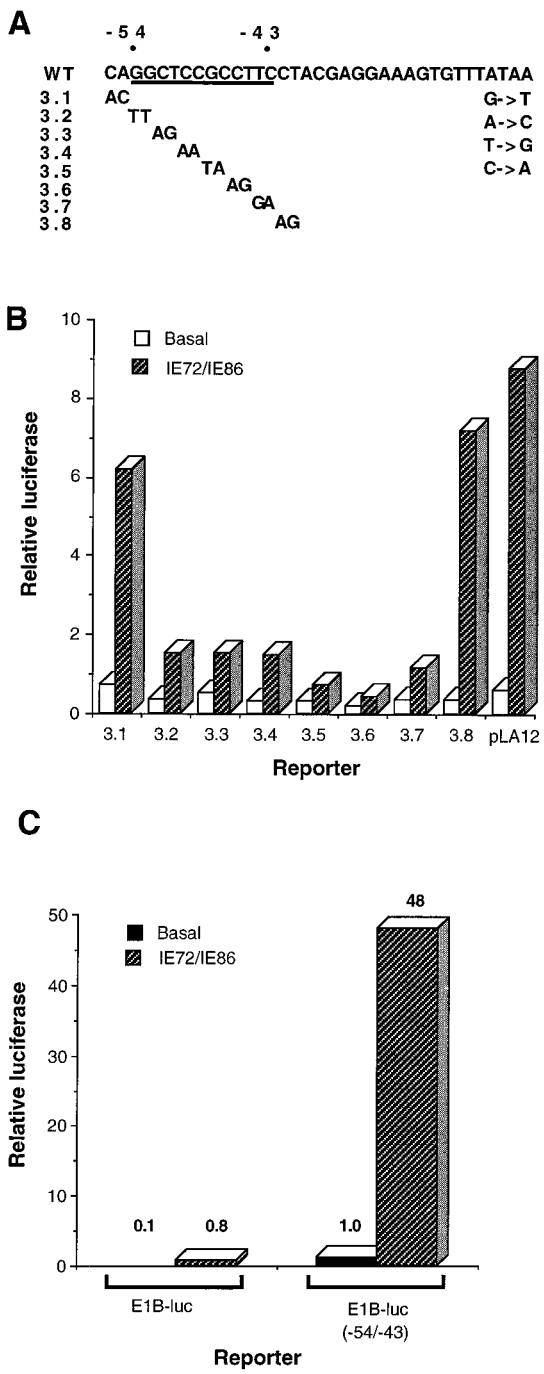


FIG. 2. Mutagenesis scan analysis of the −60/−41 region of UL54 defines the minimal UL54 promoter region essential for IE transactivation. (A) Eight mutants were generated through purine-pyrimidine exchanges in 2-bp stretches (named 3.1 to 3.8, shown at left). Corresponding base substitutions are shown underneath the parental sequence. The minimal region required for IE86 transactivation is underlined with a solid line, and the positions of the 5′ and 3′ boundaries are indicated at the top. WT, wild type. (B) Transient transfection analysis of mutants 3.1 to 3.8. U373 cells were transfected with each reporter construct either in the absence (basal transcription) or in the presence (IE72/IE86; activated transcription) of an expression vector for IE proteins. luc values were normalized to a beta-galactosidase internal control. (C) The −54/−43 DNA element confers IE86 inducibility on a heterologous promoter. One copy of the −54/−43 element was introduced upstream of the adenovirus E1B core promoter region (−30/+1) linked to the luc reporter gene (E1B-luc). Transactivation by IE proteins of the minimal E1B-luc and the chimeric reporter that contains the −54/−43 element was examined by transient transfection as described in Materials and Methods. Fold activation is shown above bars.

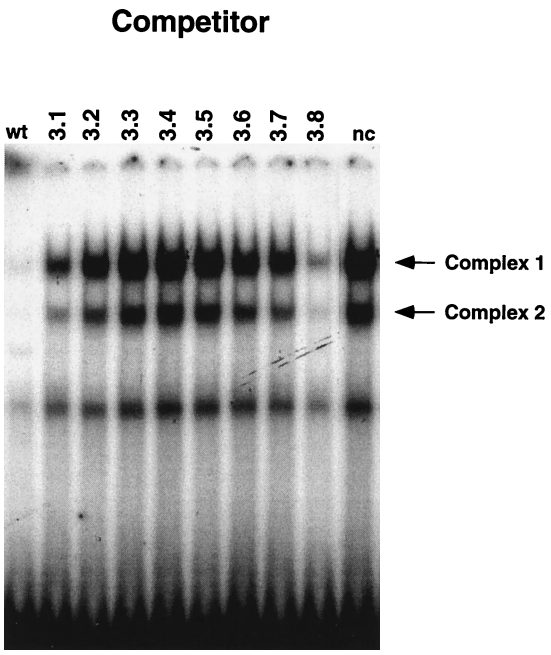


FIG. 3. Cellular factors bind specifically to the −54/−43 UL54 element. Oligonucleotides containing the mutations described for Fig. 2B were used to compete with DNA-protein complexes formed with 1 μg of nuclear extract and a $^{32}$P-labeled DNA probe containing the −65/−35 region of UL54. A 100-fold molar excess of the competitor oligonucleotide shown at the top of the figure was used in each case. wt, wild-type oligonucleotide; nc, no competitor. Complexes 1 and 2 are indicated by arrows.

minimal *cis* DNA element required for optimal transactivation of the UL54 promoter by IE proteins.

We wished to determine if this *cis* DNA element could confer IE86 responsiveness on a minimal heterologous promoter. To address this possibility, we placed one copy of the −54/−43 DNA element upstream from the E1B core promoter fused to the luc reporter gene and examined its inducibility by IE proteins. Results from a representative experiment are shown in Fig. 2C. The minimal E1B promoter-luc reporter construct showed low basal activity and was poorly stimulated by IE proteins (Fig. 2C). Interestingly, in the presence of the −54/−43 element IE proteins transactivated the E1B-luc reporter construct more than 48-fold (Fig. 2C). Insertion of the −54/−43 UL54 DNA element also increased basal promoter activity about 10-fold. Mutants within the −54/−43 element that disrupted transactivation by IE proteins always reduced basal promoter activity. These observations suggest that the −54/−43 element recruits a transcription factor or factors that stimulate both basal transcription and IE-activated transcription and that the functional interaction between these transcriptional regulatory proteins can occur in the context of a heterologous core promoter.

**Identification of cellular proteins in uninfected extracts that bind specifically to the −54/−43 DNA element.** In order to identify cellular proteins that bind selectively to the UL54 promoter, we performed EMSAs with HeLa nuclear extracts by using the −65/−35 region of UL54 as a probe. Two specific DNA-protein complexes could be resolved. The slower-migrating complex (Fig. 3, complex 1) was more abundant than the faster-migrating complex (complex 2). Similar results were obtained with nuclear extracts from all of the cell lines used previously in transfection assays. This EMSA pattern is similar to that observed by Stenberg and coworkers using human foreskin fibroblast nuclear extracts and a similar probe (22, 23).

To address the specificity of these DNA-protein complexes, competition analyses with the characterized UL54 promoter mutants were performed. A 50-fold excess of unlabeled −65/−35 oligonucleotides bearing the same set of mutations described in linker scan 3 (Fig. 2A) was assayed for the ability to compete with the formation of DNA-protein complexes obtained with the wild-type UL54 probe. The results of a representative competition experiment are shown in Fig. 3. Mutants 3.1 and 3.8 competed effectively with the formation of both complexes 1 and 2. In contrast, mutants 3.2, 3.3, 3.4, 3.5, 3.6, and 3.7 had no effect on the EMSA pattern. We also performed the reciprocal experiment in which the mutant oligonucleotides were labeled and used to test HeLa nuclear extracts for DNA binding activity. The results of these experiments were consistent (data not shown) with those described for Fig. 3. These analyses indicate that the DNA sequences required for the formation of complexes 1 and 2 map to the −54/−43 region of UL54. Thus, we conclude that the DNA sequences required for IE transactivation of the UL54 promoter are identical to those required for the formation of complexes 1 and 2.

**Purification of cellular factors that bind to the −54/−43 UL54 promoter element.** Because the cellular factors that bind to the UL54 promoter element seemed to be widely expressed, we decided to use HeLa cell nuclear extracts as the source of cellular factors. Nuclear extracts were prepared from 2.5 × $10^{10}$ cells and fractionated sequentially over SP Sepharose, ssDNA-agarose, and two successive DNA affinity steps (see Materials and Methods for details). The SP Sepharose and ssDNA-agarose steps were used to remove abundant nonspecific DNA-binding proteins that interfere with DNA affinity chromatography. The pool of DNA binding activity recovered

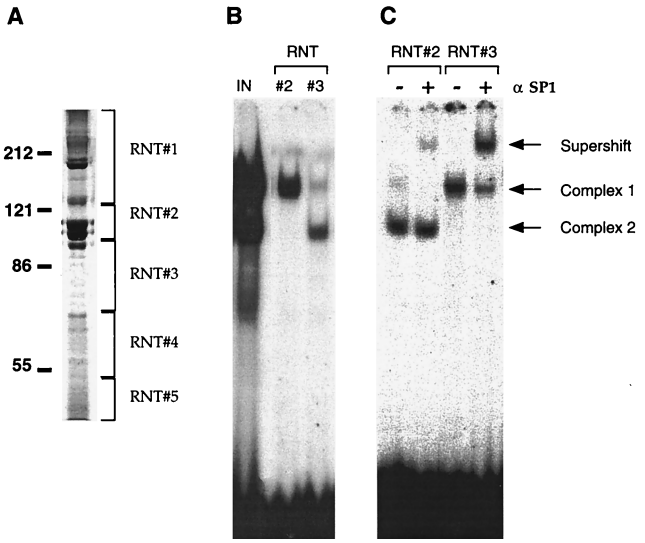


FIG. 4. SP1 binds to the −54/−43 UL54 element. (A) Reconstitution of DNA binding activity from SDS-polyacrylamide gel slices. The most highly purified fraction containing specific DNA binding activity was fractionated by SDS-PAGE and analyzed by silver staining. The polypeptides localized to gel regions (indicated by brackets RNT1 to -5) were excised and subjected to elution and renaturation as described in Materials and Methods. (B) EMSAs of renatured samples RNT2 and -3 contain specific DNA binding activity. IN, input. (C) Change in mobility of DNA-protein complexes formed with RNT2 and -3 by SP1 polyclonal antibodies. Lanes with a plus sign received 100 ng of SP1 antibodies. Lanes with a minus sign received equivalent amounts of control antibodies. The SP1 antibody-dependent supershift and complexes 1 and 2 are indicated by arrows.

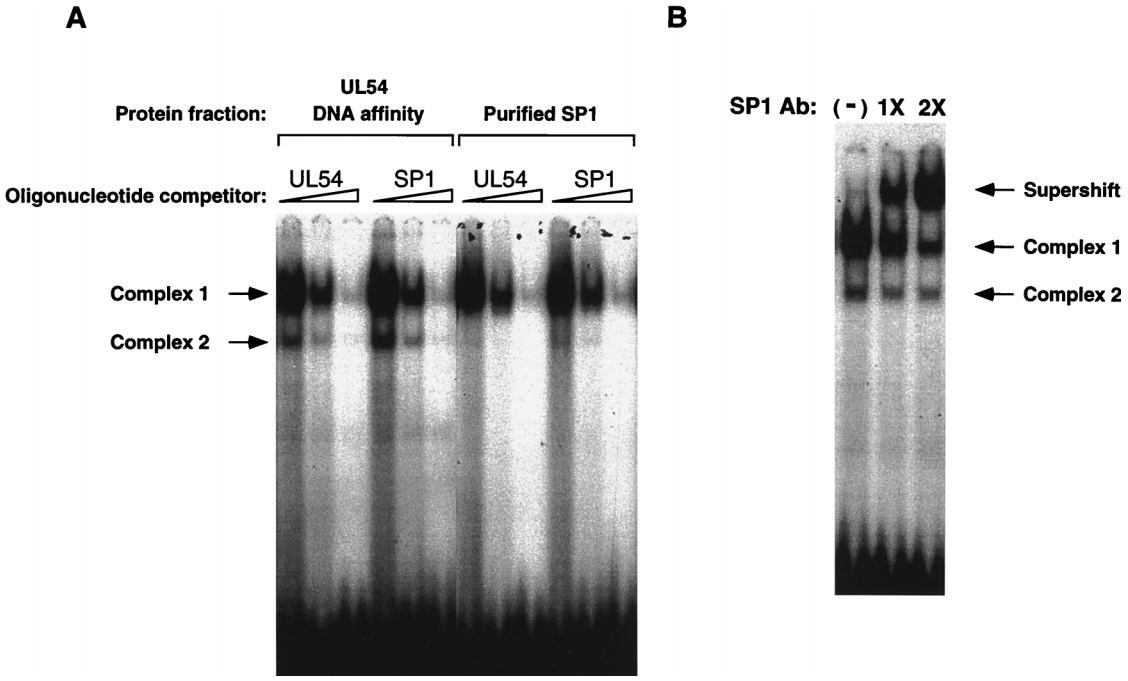


FIG. 5. (A) An oligonucleotide containing a consensus SP1 site effectively competes with the binding of cellular proteins to the UL54 promoter. Triangles show lanes that received increasing amounts (10-, 50-, and 100-fold molar excess) of the competitor oligonucleotide indicated at the top of the figure. The brackets group reactions according to the protein fraction used to form the DNA-protein complexes. Arrows indicate the mobility of complexes 1 and 2. EMSA conditions were as described in Materials and Methods. (B) Effect of SP1 antibody (SP1 Ab) on DNA-protein complexes formed with a partially purified protein fraction (supershift). EMSA conditions were as described in Materials and Methods. DNA binding reaction mixtures that received no SP1 antibody, 50 ng of SP1 antibody, or 100 ng of SP1 antibody are labeled (−), 1×, and 2×, respectively.

from the ssDNA-agarose step was loaded onto a DNA affinity column prepared as described in Materials and Methods. A wash step with mutant oligonucleotide was included to displace proteins that bound nonspecifically to the DNA affinity matrix. Several proteins were present in active fractions after the second affinity step, as visualized by silver staining of SDS-polyacrylamide gels (Fig. 4A). The proteins most enriched by the purification protocol migrated between the 86- and 121-kDa standards and exhibited apparent molecular masses of 95, 105, and 110 kDa. An abundant protein with an apparent molecular mass of about 180 kDa was retained nonspecifically, as indicated by the fact that it was strongly competed by mutant oligonucleotide washes and its elution did not correlate with specific DNA binding activity (Fig. 4A and data not shown).

To identify the polypeptides in this population responsible for DNA binding activity, we subjected DNA affinity-purified material to a denaturation-renaturation protocol after SDS-PAGE. This method has been used successfully to characterize many DNA-binding proteins (37). The protein bands present in the SDS gel shown in Fig. 4A were subdivided into several gel slices (RNT1 to -5 [Fig. 4A]) such that every protein in the mixture was analyzed. Proteins were then eluted, renatured, and assayed for DNA binding activity. Individual fractions and all combinations were tested to account for the possibility that multiple proteins were required for the formation of DNA-protein complexes 1 and 2. The EMSAs indicated that gel slices RNT2 and -3 contained the polypeptides that form complexes 1 and 2, respectively (Fig. 4B). Proteins eluted from other gel slices had no detectable DNA binding activity (data not shown). The formation of complex 1 with proteins eluted from RNT3 is likely a result of contamination with proteins present in RNT2 during excision of the gel slices.

The denaturation-renaturation experiments demonstrate that polypeptides present in RNT2 and RNT3 are responsible for specific DNA binding to the −54/−43 DNA element. Polypeptides with apparent molecular masses of 105 and 110 kDa were present in RNT2, and a polypeptide of about 95 kDa was present in RNT3. This protein pattern is similar to that of HeLa cell-purified transcription factor SP1, which migrates as a doublet of 95 and 105 kDa in SDS-PAGE (4). This observation, together with the fact that the −54/−43 DNA element resembles the GC boxes bound by members of the SP1 family of transcription factors, prompted us to investigate whether these polypeptides were in fact SP1. To address this possibility, proteins from RNT2 and -3 were first allowed to bind to the wild-type probe and subsequently incubated with either polyclonal antibodies to SP1 or a control antibody (Fig. 4C). The SP1 antibody specifically altered the mobility of, and therefore recognized, complex 1, which was formed with either RNT2 proteins or a crude protein fraction (Fig. 4C and Fig. 5B). Polyclonal antibodies directed against SP1-related proteins SP2, SP3, and SP4 did not recognize either DNA-protein complex, even at a 10-fold higher immunoglobulin G concentration (data not shown). Furthermore, the use of an SP1 monoclonal antibody generated results identical to those obtained with polyclonal anti-SP1 (data not shown). SP1 antibodies did not recognize complex 2, suggesting that this complex may be formed by the unglycosylated 95-kDa form of SP1 present in RNT3.

As a means of addressing further the contribution of SP1 to the formation of complexes 1 and 2, we performed competition experiments with a wild-type −54/−43 oligonucleotide and an SP1 consensus GC box oligonucleotide on DNA-protein complexes formed with either the DNA affinity-purified fraction or purified SP1 protein (Fig. 5A). The ability of the −54/−43 and SP1 consensus oligonucleotides to compete with formation of

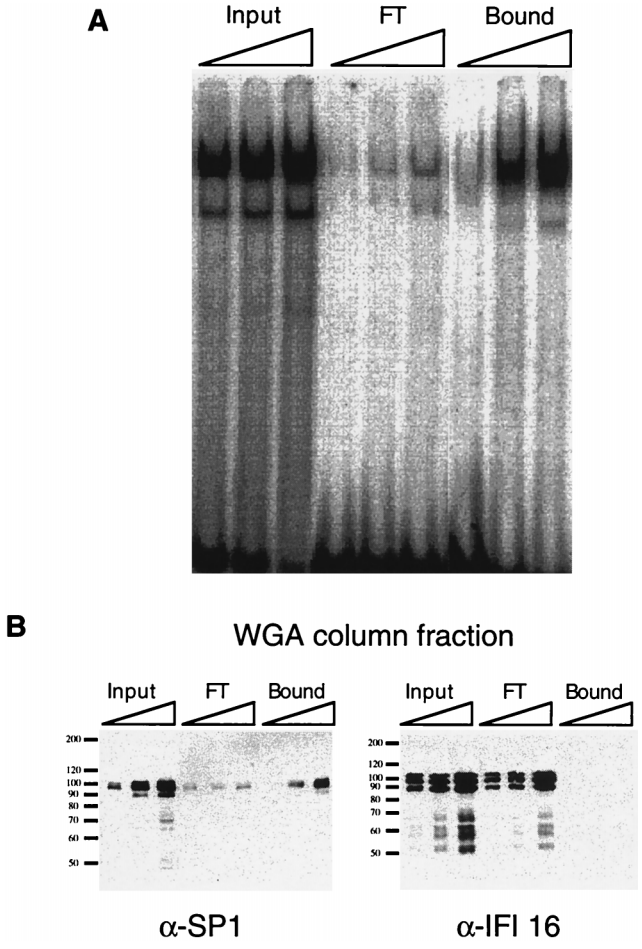


FIG. 6. WGA affinity chromatography depletes extracts of UL54 −54/−43-specific DNA binding activity. (A) EMSA of WGA affinity column fractions. Triangles indicate the addition of increasing amounts (1, 2, and 4 μl) of column input, flowthrough (FT), and bound material to DNA binding reaction mixtures as indicated. EMSA conditions were as described in Materials and Methods. (B) SP1 and IFI 16 immunoblot of WGA affinity column. Increasing amounts (0.5, 1, and 2 μl) of each sample were loaded for Western blot analysis as indicated by the triangle above the lane. α-SP1, SP1 antibodies; α-IFI 16, IFI 16 antibodies. Molecular size markers are indicated at the left of each panel in kilodaltons.

DNA-protein complexes with either affinity-purified UL54 protein or purified SP1 was indistinguishable. It is noteworthy that purified SP1 protein produced a single DNA-protein complex with a mobility that is identical to that of complex 1 (Fig. 5A).

Microsequence analyses of the 110-kDa polypeptide indicated that it is the product of the IFI 16 gene (7). This gene is expressed constitutively in most tissues, and its expression appears to be induced by gamma interferon in myeloid cells (7, 51). Immunoblot experiments with IFI 16 antibodies indicated that HeLa-derived IFI 16 comigrated with the 110- and 105-kDa polypeptides retained in our affinity columns. However, IFI 16 protein bound to DNA affinity columns prepared with mutant oligonucleotides and did not bind to the WGA column (data not shown and Fig. 6). The opposite was true for SP1, which binds to a WGA column and does not recognize UL54 mutant oligonucleotides and whose elution correlated exactly with specific DNA binding activity. Furthermore, IFI 16 antibodies did not alter the mobility of either complex 1 or 2, and to date, we have not obtained any evidence that either HeLa cell-derived or recombinant IFI 16 protein can bind DNA in a sequence-specific manner. We conclude that IFI 16 is an abundant protein that binds nonspecifically to GC-rich DNA and is not involved in the formation of either complex 1 or complex 2.

**Depletion of SP1 and its corresponding DNA binding activity from cellular extracts.** SP1 is one of a few nuclear proteins known to be glycosylated (19). We took advantage of this property of SP1 to strengthen the evidence that it binds to the −54/−43 DNA element. Affinity chromatography on WGA columns has been used extensively for the purification of SP1 from nuclear extracts (20). We fractionated nuclear extracts over a WGA affinity column. As shown in Fig. 6A, most of the DNA binding activity was retained in the WGA column and eluted selectively with *N*-acetylglucosamine. Immunoblot analysis indicated that the DNA binding activity of the input, flowthrough, and bound WGA fractions correlated perfectly with the concentration of SP1 protein present in the various fractions (Fig. 6B). In contrast, IFI 16 was not retained by the WGA column (Fig. 6B). In agreement with published results, silver staining of the WGA-bound fraction revealed that the 105- and 95-kDa SP1 polypeptides are retained selectively in this column (data not shown). These experiments strongly suggest that SP1 is responsible for the formation of complexes 1 and 2. WGA-purified SP1 produced primarily complex 1, in agreement with the notion that complex 2 is formed by the less abundant unglycosylated SP1 protein.

A comparison of the consensus SP1 GC and GT boxes with the antisense strand of the −54/−43 element is shown in Fig. 7. This alignment indicates that the similarity between the GC box and the −54/−43 element is quite high in the core binding region. Mismatched bases show a purine preservation (adenine in UL54). Moreover, the mutagenesis scan (Fig. 1 and 2) and the EMSA analyses (Fig. 3) of the UL54 −54/−43 sequence correlate with the integrity of the SP1 binding site. Although the UL54 GC box has a different primary sequence from that of the consensus GC box, our data show that purified SP1 protein binds with similar affinity to both elements (Fig. 5A). Taken together, our experiments indicate that SP1 is the cellular factor responsible for abetting the action of IE proteins at the UL54 promoter.

## DISCUSSION

HCMV IE proteins are believed to be essential for the expression of early and late viral genes. In this study, we examined the cellular factor requirement for the function of IE proteins on a model early promoter. Our data show that transcription factor SP1 plays a critical role in UL54 promoter activity and in transactivation by IE proteins. Several lines of evidence support this conclusion. First, consistent with previously published results (23, 24), we found that the DNA sequences required for basal promoter activity and transactivation by IE proteins map to a single GC box located between

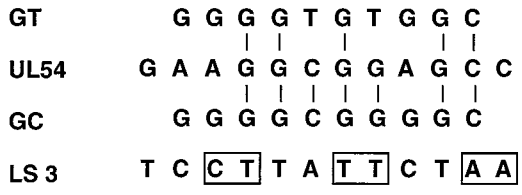


FIG. 7. Alignment of the UL54 SP1 element with consensus GC and GT boxes. The consensus GT and GC sequences shown are as described in reference 25.

−54 and −43 relative to the transcription start site. This element is necessary and sufficient to support both basal promoter activity and transactivation by IE proteins in the context of promoter sequences from −270 to +200 relative to the start site (Fig. 1 and 2). In agreement with the ubiquitous distribution of SP1, identical results were observed with several different cell types. Second, competition analyses indicated that the UL54 sequences required for the formation of SP1-DNA complexes are identical to those required for transactivation by IE proteins and map to the −54/−43 element (Fig. 3). Third, renaturation of DNA affinity-purified material and SP1 antibody supershift experiments demonstrate that SP1 binds in a sequence-specific manner to the −54/−43 GC box (Fig. 4 and 5). Finally, fractionation of nuclear extracts on WGA affinity columns efficiently depletes specific DNA binding activity (Fig. 6).

Neither computer analyses nor visual inspection of UL54 promoter sequences predicted the presence of an SP1 binding site. This observation strengthens the notion that both genetic and biochemical approaches are required for identification of cellular factors that participate in the regulation of viral genes. Our findings are in agreement with and significantly extend previous studies of the UL54 promoter (23, 24, 46). Stenberg and coworkers proposed that a novel inverted repeat (IR1) serves as the critical element in UL54 regulation (23, 24). Our data indicate that the complete sequence corresponding to this putative inverted repeat element (−55/−48) lies within the SP1 binding site mapped in this study. The reported effect of a 4-bp mutation in IR1 (24) that destroys the 5′ half of the GC box is consistent with our data (Fig. 2). This IR1 mutation (23) is represented in our linker scan mutants 3.2, 3.3, and 3.4 (Fig. 2).

It has been suggested that SP1 activity is up-regulated upon HCMV infection (59). Up-regulation of SP1 activity occurs as an IE event and is thus independent of protein synthesis. Therefore, it is possible that regulation of SP1 activity might also contribute to the temporal regulation of the UL54 promoter. An additional mode of SP1 regulation might involve competition of DNA binding by IFI 16 or the SP3 protein, a transcriptionally inactive member of the SP family that binds to GC boxes and represses activation by SP1 (15). It is reasonable to speculate that, at early times after infection, the main role of SP1 is to facilitate association of the basal transcriptional apparatus and viral IE proteins with promoter sequences. This is consistent with the view that one of the functions of promoter-proximal DNA-binding proteins is to recruit chromatin remodeling factors and thereby activate transcription from promoters occluded by nucleosomes. This activity would not be required at late times after infection, as active replication of the viral genome might overcome transcriptional inhibition by chromatin. An alternative possibility is that newly synthesized copies of viral DNA that are still not assembled into chromatin serve as the template for transcription of late genes.

It is well established that SP1, like other promoter-proximal factors, does not elicit strong transactivation by itself. This is consistent with recent findings indicating that SP1 stimulates exclusively the rate of transcriptional initiation by RNA polymerase II and has no effect on transcriptional elongation (3). It is believed that strong transcriptional activation requires stimulation of both the rate of initiation and the efficiency of elongation (57). Typically, this is achieved with enhancer elements that can recruit multiple regulatory factors that can act on both phases of the transcription reaction. Moreover, it has been shown that activators that stimulate only elongation synergize best with those that stimulate only initiation and vice versa (3), in agreement with the notion that one way to achieve synergistic activation is by stimulation of different rate-limiting steps of the transcription reaction. The work presented here and that of Stenberg and coworkers indicate that, in addition to the TATA box, the SP1 binding site is the most important regulatory element of the UL54 promoter. This observation, together with the suggestion that SP1 can stimulate only the rate of transcription initiation, implies that IE proteins are involved either directly or indirectly in stimulation of the elongation efficiency of the UL54 gene. This view is consistent with the finding that transactivation by IE proteins of a minimal heterologous core promoter is augmented significantly in the presence of the UL54 SP1 binding site (Fig. 3C). We predict that similar synergism might be observed between IE proteins and other factors that stimulate only transcriptional initiation. A corollary exists with the E1A protein of adenovirus. It has been shown that the activation domain of E1A stimulates transcription elongation (3) and that the expression of HCMV IE proteins can functionally complement an E1A-deficient adenovirus (50), suggesting that these viral regulatory proteins have similar roles.

The mechanisms responsible for tethering IE86 to promoters that lack IE86 binding sites are still poorly understood. It has been shown that IE86 can establish protein-protein interactions in solution with both SP1 and the TATA-box binding protein (TBP) subunit of the basal transcription factor TFIID (5, 13, 22, 32). Furthermore, ternary complexes containing promoter DNA, TBP, and IE86 have been resolved in EMSAs, and the IE86 protein domains required for this association are also required for transcriptional activation (22). In contrast, we have not been able to observe an interaction between IE86 and DNA-bound SP1. It is possible that the physical interaction between IE86 and SP1 is weaker than the IE86-TBP interaction. However, it is likely that the IE86-TBP interaction is not sufficient for recognition and stable association of IE86 with viral promoters in vivo. It is conceivable that SP1-containing preinitiation complexes provide additional interaction interphases and the proper promoter topology required for the entry of IE86 into the transcription cycle. Consistent with this view is the observation that the interaction between SP1 and the TAF110 subunit within the TFIID complex occurs only in the context of DNA-bound SP1 (18). Thus, while it is clear that promoter-proximal factors are necessary for gene regulation by viral proteins, mechanistic details still remain unsolved. A better understanding of the mechanism of action of HCMV IE proteins will require the use of well-characterized natural target promoters and the analysis of the effects of these proteins during different stages of the transcription reaction. The work presented here provides a suitable framework for future mechanistic studies.

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