Identification of Sequence Elements in the Human Cytomegalovirus DNA Polymerase Gene Promoter Required for Activation by Viral Gene Products

JULIE A. KERRY, MORRIS A. PRIDDY, AND RICHARD M. STENBERG*

Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501

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To determine the mechanisms involved in the regulation of human cytomegalovirus early gene expression, we have examined the gene that encodes the viral DNA polymerase (UL54, *pol*). Our previous studies demonstrated that sequences required for activation of the *pol* promoter by immediate-early proteins are contained within a region from –128 to +20 and that cellular proteins can bind to this activation domain. In this study, we demonstrate by competition analysis that binding of cellular proteins to *pol* is associated with an 18-bp region containing a single copy of a novel inverted repeat, IR1. Time course analysis indicated that viral infection increased the level of protein binding to IR1, concurrent with the activation of the *pol* promoter. Mutation of the IR1 element abrogated binding of cellular factors to the *pol* promoter and reduced by threefold the activation by immediate-early proteins. Similarly, mutation of IR1 rendered the promoter poorly responsive to activation by viral infection. Mutation of additional sequence elements in the *pol* promoter had little effect, indicating that IR1 plays the major role in *pol* promoter regulation. These studies demonstrate that the interaction between cellular factors and IR1 is important for the regulation of expression of the polymerase gene by viral proteins.

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that typically causes subclinical disease after primary infection. However, a dramatic increase in morbidity and mortality occurs with individuals who are immunosuppressed or immunocompromised and with newborns who have been congenitally infected (reviewed in reference 1). In addition, recent studies have demonstrated that HCMV and human immunodeficiency virus are capable of coinfecting cells *in vivo*, further implicating HCMV as a cofactor in the pathogenesis of AIDS (4, 41).

HCMV infection of permissive cells in culture leads to an ordered sequential expression of viral genes. Three characteristic phases of HCMV gene expression have been identified and are referred to as immediate early (IE), early, and late (12, 39, 66). The HCMV IE genes have been extensively studied, and their expression is thought to be essential for viral replication (25, 33, 54, 55, 58–60, 62, 63, 67, 68). In addition, IE proteins are regulators of both homologous (3, 6, 9, 10, 13, 26, 38, 49, 52, 56, 65) and heterologous (10, 18, 19, 22, 44, 51, 64) promoters. The major IE gene region encodes several transcripts as a result of differential usage of 3' ends and alternative splicing (54, 55, 58, 59). Although the mechanism of action of the IE proteins has yet to be fully determined, studies have demonstrated that IE72 and IE86 can act synergistically to activate HCMV early promoters (6, 13, 38, 56).

Early gene expression in HCMV-infected cells is less well characterized, although three subclasses of early genes have been defined (reviewed in references 50 and 54). The first subclass is transcribed early and repressed after the onset of virus DNA replication (53, 54). The second subclass is expressed at constant levels throughout the replicative cycle (6). The third subclass is transcribed at low levels early and

subsequently expressed at higher levels late (13, 24, 40, 42, 47). Clearly, multiple levels of regulation occur at early times after infection, and these three subclasses probably reflect an underestimate of the complexity of HCMV early gene expression.

To better understand the events that occur during the early phase of virus replication, we and others have examined the role of viral proteins in the regulation of HCMV early genes (6, 13, 28–30, 38, 52, 54, 65). These investigations have implicated specific sequences in conferring activation by IE proteins and revealed that cellular proteins likely play an important role in the regulation of some early genes. The *pol* gene, or UL54 (8), is a prototypical early gene in that expression is mandatory for virus DNA replication. Our previous studies demonstrated that the *pol* promoter is activated synergistically by IE72 and IE86 proteins (56). Deletion analysis of the *pol* promoter revealed that sequences from –128 to –52 are essential for the activation of this promoter by IE proteins (54). Also, cellular factors were found to bind to the activation domain of the *pol* promoter. In this study, we find that binding of cellular factors to the activation domain of the *pol* promoter is associated with a novel 8-bp inverted repeat sequence, IR1. Mutational analysis of putative regulatory elements in the activation domain indicates that binding of cellular factors to the IR1 sequence likely plays the major role in the regulation of the *pol* promoter by viral proteins.

MATERIALS AND METHODS

Cells and virus. The growth of human fibroblasts and the propagation of HCMV (Towne) were as described previously (58, 60).

Plasmid DNAs. The IE expression vector pSVH, which expresses proteins from the major IE gene region, and the target *pol* promoter-chloramphenicol acetyltransferase (CAT) construct have been previously described (13, 56). Deletion constructs used in this analysis were generated by our previ-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, 700 W. Olney Rd., Norfolk, VA 23501. Phone: (804) 446-5684. Fax: (804) 624-2255.

TABLE 1. Oligonucleotides used to introduce specific base changes in the *pol* promoter

Name	Wild type element ^a	Sequence of the mutagenic oligonucleotide ^b
DRM	-99 CGGGACCACCGT	5'-GTCCGCCGAC <u>CGGGTACCCGT</u> GACGTCGGGC
ATFM	-87 GACGTC	5'-GGACCACCGT <u>GAATTC</u> GGGCAGCACC
CAATM	-73 CCAA	5'-GTCCGGCAGCAC <u>CCCGG</u> GACACGTCGTTAC
IRM	-55 AGGCTCCG	5'-CGTCGTTAC <u>AGATATCG</u> CCTTCTCTACG

^a Wild-type sequence element contained within the *pol* promoter, with the location relative to the cap site indicated.

^b Locations of the sequence elements are indicated in boldface, and the nucleotide changes introduced into the *pol* promoter are underlined.

ously published approach (56) and have been described elsewhere (54).

Generation of site-directed mutants. Mutations in the sequence elements within the *pol* promoter were generated by using the Altered Sites in vitro mutagenesis system (Promega Corporation). The promoter region of pPolCAT was cloned into pALTER-1 by digesting pPolCAT with *Hind*III, isolating the 450-bp promoter fragment, and ligating it into *Hind*III-digested pALTER-1. Mutagenesis was subsequently carried out on the single-stranded DNA as suggested by the supplier. Oligonucleotide primers used in the mutagenesis reactions were synthesized on a Cruachem PS250 DNA synthesizer and are shown in Table 1.

Transient expression assays. Transfection of human fibroblasts, subsequent superinfection with HCMV, and CAT assays were performed as previously described (13, 56).

Preparation of nuclear extracts. Human fibroblasts were infected with HCMV at 20 to 25 PFU per cell at confluence. After a 1-h adsorption, virus was removed and overlaid with fresh growth medium (Eagle's minimal essential medium containing 10% newborn calf serum). Extracts were prepared by a modification (61) of the Dignam procedure (14). Briefly, cells were washed in Tris-buffered saline (57) and harvested at a density of 5×10^6 cells per ml by scraping into buffer A containing 0.5% Nonidet P-40 and protease inhibitors (aprotinin [2 µg/ml], leupeptin [0.5 µg/ml], pepstatin [1 µg/ml], and phenylmethylsulfonyl fluoride [1 mM]). Cells were lysed on ice for 10 min by gentle pipetting, and the nuclei were removed by centrifugation at $1,500 \times g$. Nuclei were resuspended at $\sim 1.5 \times 10^7$ nuclei per ml in buffer C containing protease inhibitors and incubated on ice for 30 min with occasional gentle pipetting. The extract was centrifuged at $50,000 \times g$; the supernatant was removed, dialyzed against buffer D, and stored at -80°C .

Gel mobility shift analysis. Nuclear extracts (5 or 10 µg) were incubated with 5 µg of poly(dI-dC) · poly(dI-dC) and competitor DNAs for 15 min at room temperature in 50% buffer D (14). Radiolabeled probe was added (10,000 to 20,000 cpm, 0.5 to 1 ng of DNA), and the incubation continued for 30 min. Samples were subjected to polyacrylamide gel electrophoresis in standard $1 \times$ Tris-borate-EDTA (58). Gels were dried and subjected to autoradiography.

The activator probe was isolated after restriction digestion of pPolCAT with *Hind*III and *Pst*I and polyacrylamide gel electrophoresis (58). Labeling of 5' ends with [γ - ^{32}P]ATP was done as described previously (58). Subfragments of the activator fragment were isolated in a like manner after digestion with the indicated restriction enzyme. The IR1 probe was generated by heating two 18-mers (5'-GTTACAGGCTCCGCCTTC and 5'-GGAAGGCGGAGCCTGTAA) to 70°C for 15 min in 20 mM Tris-HCl (pH 7.5)–50 mM NaCl–10 mM MgCl_2 and subsequently allowing the reaction mixture to cool to room temperature. The annealed product was repaired with [α - ^{32}P]dCTP and Klenow polymerase and then 5' radiolabeled

with [γ - ^{32}P]ATP as described above. The 48-bp LIR probe was generated by annealing two 48-mers (5'-GGGAAAGACA CGTCGTTACAGGCTCCGCCTTCCTACGAGGAAAGTG TT and 5'-TTCCCAACACTTTCCTCGTAGGAAGGCCGA GCCTGTAACGACGTGTCT) as described above, and the DNA was subsequently 5' radiolabeled with [γ - ^{32}P]ATP. All radiolabeled oligonucleotides used in these analyses were subsequently purified by nondenaturing polyacrylamide gel electrophoresis.

IR1 and IRM competitors were produced by annealing the appropriate oligonucleotides (for IRM, 5'-GTTACAGATA TCGCCTTC and 5'-GGAAGGCGATATCTGTAA). The DR1ATF competitor fragment was generated by annealing a 42-mer (5'-CCGAAGCTTCGGGACCACCGTGACGTCGG GCAGAAGCTTGCC) with a 15-mer (5'-GGCAAGCTTCT GCCC) and repairing the single-stranded region with deoxynucleoside triphosphates and Klenow polymerase as described previously (58).

Binding site analysis. Transcription factor-binding sites within the *pol* promoter were analyzed by using the motif search program within the MacDNASIS 3.0 sequence analysis package (Hitachi).

RESULTS

Identification of binding domains in the activation domain of the *pol* promoter. Our previous analysis of the *pol* promoter (54) revealed that the sequences required for activation by IE proteins were contained within a region from -128 to $+20$ which we termed the activation domain (Act; Fig. 1C). Gel mobility shift analysis demonstrated that cellular factors bind to the activation domain and that the binding profiles of infected and uninfected cells were similar (54). The activation domain of the *pol* promoter contains several sequence elements which could potentially bind cellular proteins and play a role in the regulation of *pol* expression (Fig. 1A). To determine which of these elements might be involved in binding putative regulatory proteins, we used specific subfragments of the activation domain as competitors in our gel shift assay (Fig. 1B and C).

The results shown in Fig. 1 confirm our previous finding, indicating that three cellular protein-DNA complexes, A, B, and C, are formed when the activation domain is used as a probe in the gel shift assay (Fig. 1B, lane 2). As expected, the intact activation domain fragment competed very efficiently for binding to the radiolabeled probe (Fig. 1B, lanes 3 and 8). Similar results were observed with use of a shorter fragment (competitor 70; Fig. 1C) derived from the activation domain, which contains the putative CAAT and TATA elements as well as a copy of an inverted repeat, IR1 (Fig. 1B, lanes 4 and 9). In contrast, a fragment containing only the TATA element (Nla-1; Fig. 1B, lane 15) or the CAAT box (Nla-3; Fig. 1B, lane 16) was unable to compete for binding.

These data suggest that the IR1 element is involved in

FIG. 1. (A) Sequence of the activation domain of the *pol* promoter. Direct and inverted repeats (DR1 and IR1), as well as the ATF, CAAT box, and TATA homologies, are underlined. Relevant restriction endonuclease sites are indicated. +1 indicates the cap site. (B) Identification of a protein-binding domain(s) within the 148-bp *pol* activation domain. A radiolabeled activation domain fragment was incubated with infected (I) or mock-infected (M) nuclear extracts isolated at 4 h after infection. Reactions were incubated in the presence or absence of a 50-fold molar excess unlabeled competitor DNA as indicated. Competitor 70 is a 70-bp fragment isolated by using an *AatII* and *AvaI* restriction enzyme digest of the activator fragment (Act; panel C). Similarly, Nla-1, -2, and -3 were isolated by using an *NlaIV* digest of Act. IR1, IR1^{Act} oligonucleotide competitor; DR1ATF, DR1ATF oligonucleotide competitor; NS, 147-bp nonspecific competitor fragment; A, B, and C, specific protein-DNA complexes; P, probe. (C) Summary of the data shown in panel B, indicating the sequence elements contained within the competitor DNAs. A schematic of the activation domain (Act) and its associated elements is shown at the top. Competitor fragments and their associated elements are as indicated. Ability to compete is indicated as + or -.

binding of cellular proteins to the activator region. To confirm this, an 18-bp oligonucleotide (-61 to -43) containing the IR1 sequence (IR1^{Act} oligonucleotide) was used as a competitor in the gel shift assay. The IR1^{Act} oligonucleotide efficiently competed for all three of the protein-DNA complexes formed with the full-length activator fragment (Fig. 1B, lane 21). A control 42-bp oligonucleotide containing the DR1ATF element was unable to compete for binding (Fig. 1B, lane 22).

Our previous studies had demonstrated that a second copy of the IR1 sequence is found upstream of the activation domain of the *pol* promoter at -225 (54). Deletion of this region of the *pol* promoter has no detrimental effect on promoter activity in transient assays (54). In addition, this

region does not efficiently compete for binding in our gel shift assay (see Fig. 3). Examination of the sequences surrounding the two copies of the 8-bp IR1 element reveals that they are divergent. Therefore, the IR1 sequence found within the activator region of the *pol* promoter will subsequently be referred to as IR1^{Act}, and that upstream will be referred to as IR1^{US}.

The finding that an oligonucleotide containing the IR1^{Act} sequence could compete for the major protein-binding activity of the activation domain implicated this sequence in binding cellular factors. To extend this finding, we created a mutation in IR1^{Act} by site-directed mutagenesis (Table 1). The effect of this mutation was assessed in the context of the entire activa-

FIG. 2. Mutational analysis of the protein-binding domain(s) within the 148-bp *pol* activation domain. A radiolabeled 148-bp activation domain fragment was incubated with mock-infected (M) nuclear extracts as described in the legend to Fig. 1. Competitor DNAs: Act, unlabeled activator region; ActIRM, unlabeled activator region containing a 4-nt mutation in the IR1 sequence; ActATFM, unlabeled activator region containing a 2-nt mutation in the ATF consensus sequence; NS, 147-bp nonspecific competitor fragment; IR, 18-bp oligonucleotide containing the IR1^{Act} sequence; IRM, 18-bp oligonucleotide containing the IR1^{Act} sequence with a 4-nt mutation; DR1ATF, 42-bp oligonucleotide containing the DR1 and ATF sequence elements. Mutations are as indicated in Table 1. A, B, and C, specific protein-DNA complexes; P, probe.

tion domain (ActIRM) for its ability to compete with the wild-type fragment in a gel shift assay (Fig. 2). A 4-nucleotide (nt) substitution within the central region of the IR1^{Act} sequence (AGGCTCCG to AGATATCG) abrogated the ability of the ActIRM fragment to compete (Fig. 2, lane 4). In addition, an 18-bp oligonucleotide of this region containing the same mutation (IRM) was unable to compete for binding (Fig. 2, lane 8). In contrast, mutation of 2 nt within the ATF sequence of the activator fragment (TGACGTCG to TGAATTCG) had no effect on the ability of the ActATFM fragment to compete for binding (Fig. 2, lane 5). This mutation has been previously shown to eliminate binding to ATF sites (23). These data strongly suggest that the IR1 element in the activation domain of the *pol* promoter can bind cellular factors.

Direct binding of cellular proteins to the IR1 sequence. We have demonstrated by competition analysis that the IR1^{Act} element is involved in the binding of cellular factors to the activation domain of the *pol* promoter. To demonstrate direct binding of cellular factors to the IR1^{Act} element, we used the IR1^{Act} oligonucleotide as a probe in a gel shift analysis. Nuclear extracts prepared from cells infected for 4 h formed two specific protein-DNA complexes, A and B, with the IR1^{Act} oligonucleotide probe (Fig. 3A, lane 2), indicating that at least two different proteins or multiprotein complexes bind to this element. A third, minor band migrating just above band A was observed. The activation domain fragment competed for binding to the IR1^{Act} oligonucleotide probe (Fig. 3A, lane 5), but another DNA fragment of identical size did not (Fig. 3A, lane 6). Presence of an excess of the upstream region of the *pol* promoter (−425 to −128) which includes IR1^{US} resulted in a slight reduction in the overall level of binding (Fig. 3A, lane 7). However, an unrelated fragment of similar size resulted in a similar reduction in binding, indicating that this was a nonspecific effect (Fig. 3A, lane 8). In addition, no binding was detected to an IR1^{Act} oligonucleotide probe which contained a 4-nt substitution in the IR1 sequence (data not shown).

FIG. 3. (A) Binding of proteins to the IR1 sequence. A radiolabeled 18-bp IR1^{Act} oligonucleotide probe was incubated with 4-h-infected (I) nuclear extracts as described in the legend to Fig. 1. Competitor DNAs were as described in the legend to Fig. 2 except for 147 (147-bp nonspecific competitor fragment), Rep (277-bp upstream *EcoRI-PstI pol* promoter fragment from −425 to −128 [54]), and 267 (267-bp nonspecific competitor fragment). Specific protein-DNA complexes A and B are indicated. P, probe. (B) Binding of proteins to the 48-bp LIR probe. Radiolabeled 48-bp LIR oligonucleotide was incubated with 72-h-infected nuclear extracts (I) as described in the legend to Fig. 1. Competitor DNAs were as described in the legends to Fig. 2 and 3A except for US, which is the 277-bp *EcoRI-PstI pol* promoter fragment (54). Specific protein-DNA complexes 1, 2, and 3 are indicated. P, probe.

Although we were able to detect binding of cellular factors to the 18-bp IR1^{Act} oligonucleotide, this binding appeared to be of a lesser affinity than that observed in gel mobility shift assays using a fragment corresponding to the entire activation domain (Fig. 2). In addition, only two protein-DNA complexes were consistently observed in gel shift analyses using the IR1^{Act} oligonucleotide. However, when the IR1^{Act} oligonucleotide was used as a competitor for binding to the full-length activation domain fragment, we observe strong competition of the three DNA-protein complexes. It is possible that binding affinity is associated with the size of the probe. To examine this possibility, we used as a probe in a gel shift assay a longer oligonucleotide of 48 bp (LIR) that included the IR1^{Act} sequence (Fig. 3B). This probe extends from immediately 3' of the CAAT box (-68) to 5' of the TATA element (-25) and thus includes no other known DNA-binding protein domain other than the IR1^{Act} sequence (Fig. 1A). These studies demonstrate that nuclear extracts from both infected and uninfected cells were able to form three specific protein-DNA complexes with the LIR probe (Fig. 3B, lane 2). A qualitative assessment of the level of binding observed for two of the three complexes indicates a higher level of affinity than that observed with the 18-bp IR1^{Act} oligonucleotide probe. Binding of cell proteins to the LIR probe was competed for efficiently by the intact activation domain fragment as well as the IR1^{Act} oligonucleotide (Fig. 3B, lanes 3 and 5) but not by sequences in which the IR1 motif had been mutated (Fig. 3B, lanes 4 and 6). As was observed with the IR1^{Act} oligonucleotide probe, presence of an excess of the upstream region of the *pol* promoter resulted in a slight reduction in the overall level of binding (Fig. 3B, lane 7), as did an unrelated fragment of similar size (Fig. 3B, lane 8). Taken together, these data conclusively demonstrate that the IR1^{Act} element is associated with the binding of cellular proteins to the *pol* promoter.

Time course analysis. Our data indicate that the IR1^{Act} element within the *pol* promoter is involved in binding cellular factors. To correlate binding activity with *pol* promoter activation, cells were transfected with the pPolCAT reporter plasmid, superinfected with HCMV, and harvested at various times after infection for CAT (Fig. 4A). Activation of the *pol* promoter was observed at 24 h postinfection, with maximal activation at 72 h (Fig. 4A). In the presence of phosphonoacetic acid (PAA), an inhibitor of viral DNA replication, reduced levels of *pol* promoter activity were detected. This result indicates that viral DNA replication is required for full activation of the *pol* promoter in transient assays. This pattern of expression is similar to that observed when *pol* mRNA levels were examined in cells infected with the AD169 strain of HCMV (21, 34, 35).

At similar times after infection, nuclear extracts were isolated from human fibroblasts and examined for binding activity (Fig. 4B). At 12 and 24 h after infection, a decrease in protein binding to the *pol* promoter, to approximately 20% of that observed at 4 h, was observed. However, at later times in infection (48 and 72 h), a significant increase in the overall level of cellular proteins binding to the *pol* promoter was observed. In particular, the level of protein-DNA complex B was enhanced almost threefold compared with the level found in mock-infected cells. A comparison of protein-binding activity with promoter activation indicates that the major increase in binding activity parallels the increase in *pol* promoter activity. In addition, the presence of PAA was found to inhibit both the increase in binding activity and the level of *pol* promoter activation.

Involvement of the IR1 element in *pol* promoter regulation. Our data indicated that the protein binding to the *pol* pro-

FIG. 4. (A) Time course analysis of *pol* promoter activity. Human fibroblasts were transfected with the *pol* promoter-CAT construct (pPolCAT) and subsequently superinfected with 20 PFU of HCMV per cell as described previously (13). After adsorption, cells were incubated in the presence or absence of PAA (200 μ g/ml). At the indicated times, cells were harvested and subsequently assayed for CAT activity. Representative data from a single experiment are shown; values are indicated above the bars. (B) Time course analysis of protein-binding activity. A radiolabeled 148-bp activator fragment was incubated with 5 μ g of nuclear extracts prepared at the indicated times after infection. After adsorption of virus, cells were incubated in the presence or absence of PAA (200 μ g/ml). Protein-DNA complexes were then analyzed as described in Materials and Methods. Mock-infected nuclear extracts (lane M) were prepared at 72 h. A, B, and C, specific protein-DNA complexes; P, probe.

moter associated with the IR1 element roughly parallels the level of promoter activation observed in transient assays. As a more direct assessment of the role of IR1^{Act} in *pol* promoter activity, we examined the effect of a 4-nt substitution in the IR1^{Act} element on *pol* promoter activation by IE proteins. This mutation was previously shown to abrogate binding of a cellular factor(s) to the activation domain of *pol* (Fig. 2). The data in Fig. 5B demonstrate that mutation of the IR1^{Act} site (pIRMCAT) within the context of the entire *pol* promoter results in a decrease in the activity of the *pol* promoter by approximately threefold (3 ± 0.6 , seven replicate assays). This level of activity is approximately threefold greater than that of a basal *pol* promoter construct (pd177CAT) which contains only an additional 23 bp upstream of the *pol* TATA element (54).

The studies described above indicate that maximal levels of *pol* promoter activation in transient assays occurred at late

FIG. 5. (A) Schematic representation of *pol* promoter mutations (-425 to +1). Motifs identified within the promoter sequence are indicated. Motifs present within the mutated *pol* promoter, IRM, and the deleted minimal promoter, d177 (54), are also shown. (B) Activation of pPolCAT and mutants by IE proteins. The promoter-CAT plasmids were cotransfected in the presence (+) or absence (-) of IE proteins as described previously (56) and subsequently assayed for CAT activity. Values from a typical experiment of seven replicate experiments are shown, with data expressed as percentages of activity observed with the wild-type promoter (pPolCAT). (C) Time course of the activation of pPolCAT and mutants after superinfection with HCMV. The promoter-CAT plasmids were transfected into human fibroblasts and superinfected with virus as described previously (13). Cell extracts were prepared at the indicated times and subsequently examined for CAT activity. Data from a single representative experiment are shown; values are indicated above the bars. M, mock infection.

times after infection (Fig. 4). This finding indicates that there are factors in addition to the IE proteins which are required for full activation of the *pol* promoter. We therefore examined the effect of the IR1^{Act} mutation on activation of the *pol* promoter after viral infection. Under these conditions, the responsiveness of pIRM was similar to that observed with the basal promoter construct, pd177CAT (Fig. 5C). This result was consistently observed in three replicate experiments (pd177CAT, 20% of wild-type activity $\pm$ 8%; pIRM, 31% $\pm$ 7%). These data strongly indicate that binding of cellular

FIG. 6. Mutational analysis of putative responsive elements in the *pol* promoter. Promoter-CAT plasmids were transfected into human fibroblasts as described in the legend to Fig. 5 in the presence (+) or absence (-) of IE proteins. Forty-eight hours after transfection, extracts were prepared and subsequently analyzed for CAT activity. Mutations are as described in Table 1 and the legend to Fig. 5. Values from a single experiment of four replicate experiments are indicated, with data expressed as percentages of pPolCAT (POL) activity.

proteins by the IR1 element is necessary for full activation of the *pol* promoter.

Assessment of additional sequence elements in *pol* promoter regulation. Mutation of IR1^{Act} resulted in an abrogation of protein binding by this element and a concurrent decrease in the activity of the *pol* promoter. However, our previous analysis of *pol* promoter sequence elements indicated that the DR1ATF motif could confer responsiveness to the IE proteins when transferred to a basal promoter construct (54). In addition, under varied conditions, with different nonspecific competitors, direct binding of cellular proteins to the DR1ATF element could be detected (54) (data not shown). However, it should be noted that this sequence could not compete for the major activation domain protein-DNA complexes (Fig. 1). To determine if other sequence elements may also be involved in the regulation of the *pol* promoter, we undertook a systematic mutational analysis of the potential regulatory motifs in the activation domain of the *pol* promoter. The effects of these mutations were assessed within the context of the entire *pol* promoter (Fig. 6).

As was observed previously, mutation of the IR1 sequence resulted in a significant decrease in the response of the *pol* promoter to IE proteins. Mutation of the ATF motif within the context of the *pol* promoter did result in a slight drop in the activity of the promoter (75.4% of wild-type activity $\pm$ 21%, nine replicate experiments). However, this decrease in activity is not of the same magnitude as that observed with the IR1^{Act} mutant (37.7% $\pm$ 8.7%, eight replicate experiments). Mutation of the DR1 element and the CAAT box resulted in a slight but consistent increase in *pol* promoter activity (DRM, 139% $\pm$ 65%; CAATM, 136% $\pm$ 24%; four replicate experiments), suggesting that these elements may act as repressors of *pol* promoter activity.

Similar results were observed when activation of the *pol* promoter was examined in the context of viral infection (Fig. 7). Mutation of the DR1 and CAAT elements in the *pol* promoter resulted in an increase in promoter activation by virus infection. In contrast, the ATF mutant displayed a level of activity similar to that of the wild-type promoter, indicating that this element may have little influence on *pol* promoter activity within the context of virus infection. Some variation in the overall level of activation of the *pol* promoter after superinfection was observed. However, only the d177 deletion mutant and the IR1 mutant consistently displayed a significant

FIG. 7. Time course of activation of pPolCAT and mutants after superinfection with HCMV. The promoter-CAT plasmids were transfected into human fibroblasts and superinfected with virus. Mutations are as described in Table 1 and the legend to Fig. 5. Cell extracts were prepared at the indicated times after infection and subsequently examined for CAT activity. Data from a single experiment of three replicate experiments are shown; values are indicated above the bars.

decrease in promoter activity following viral superinfection (Fig. 7). These data indicate that the IR1 element plays the major role in the regulation of the *pol* promoter by the viral proteins.

DISCUSSION

Early gene expression in HCMV-infected cells is a complicated process, and several subclasses of viral early genes can be distinguished (54). In this study, we have examined the regulation of expression of the early gene coding for the viral DNA polymerase (*pol*). A novel *cis*-acting element within the *pol* promoter has been shown to be responsible for the activation of the promoter by viral proteins. This element functions to bind protein complexes comprised of cellular proteins.

Previous investigations utilizing progressive deletion of the *pol* promoter revealed that sequences from -128 to +20 were required for maximal activation by IE proteins, with the region from -128 to -52 being essential for promoter activity (54). In this study, we have shown that cellular proteins bind to the IR1

element within this activation domain. Mutation of the IR1 repeat in the activation domain of the *pol* promoter results in abrogation of protein binding by this element and a concurrent decrease in the activity of the promoter. These results demonstrate conclusively that IR1^{Act} is involved in the regulation of the *pol* promoter.

Interestingly, binding of cellular proteins by the IR1^{Act} element could not be competed for by an excess of the upstream fragment (-425 to -128) which also contains a copy of IR1 (IR1^{US}). This finding indicates that sequences adjacent to the 8-bp IR1^{Act} repeat (AGGCTCCG) are also important for binding. Comparison of the IR1^{Act} and IR1^{US} element sequences revealed the presence of a palindrome, AGGCtc cGCCT, which overlaps the IR1^{Act} repeat but is not conserved in the upstream element. This palindromic sequence is present in the IR1 oligonucleotide that we have used in our binding studies but is disrupted in the IR1 mutants. We are currently characterizing the role of this palindromic sequence in IR1 binding activity.

A search of two transcription factor-binding site libraries (MacDNASIS 3.0) (17) did not reveal a previously identified sequence element with significant homology to either the IR1 repeat or the palindromic sequence. Thus, IR1 likely represents a novel transcription factor-binding site. The multiple bands seen in mobility shift assays using IR1^{Act} could be explained by oligomerization of a single binding protein or binding of multiprotein complexes to this element. Preliminary characterization of the protein(s) binding to the IR1 sequence is under way.

Previously, our data suggested a role for the DR1ATF motif in the regulation of the *pol* promoter (54). This hypothesis was based on experiments whereby the DR1ATF motif from the *pol* promoter was cloned into a basal promoter construct, immediately upstream of a TATA motif. This element was subsequently found to confer responsiveness to the viral IE proteins. Our current experiments show that the ATF element displays minimal responsiveness to the IE proteins in the context of the *pol* promoter. Similarly, the IR1 element displayed only low levels of activation by IE proteins when a single copy of this motif was cloned into the same basal promoter construct (27). This finding indicates that promoter context may play a significant role in the enhancer activity of some sequence elements. It is known that some transcriptional enhancer elements require multiple protein-protein interactions with diverse *trans*-acting factors in order to activate transcription (11, 15). Thus, altering the promoter context of particular sequence elements may have profound effects on their ability to regulate gene expression.

In addition, we found that mutation of the direct repeat element, DR1, or the putative CAAT box resulted in a slight but consistent increase in *pol* promoter activity. This effect was observed in both cotransfection and superinfection experiments. While these data are preliminary, they suggest that these sequence elements may act to repress *pol* promoter function. This notion is consistent with the recent studies of Huang et al., who demonstrate a functional negative regulatory element upstream of another HCMV early gene (22a). Further experiments will be required to confirm the role of these two elements in *pol* promoter regulation. Interestingly, there is some evidence suggesting that the CAAT box-binding factor, CTF-1, can interact directly with the 72-kDa IE1 protein (20). However, in these experiments, the CTF-1-IE72 interaction was associated with enhanced transcriptional activity. In addition, a CAAT box dyad symmetry has also been implicated in positive regulation of the HCMV early UL4 gene (22a). These results further implicate an important role for promoter context in enhancer activity.

Currently, we are testing the role of the *pol* promoter elements within a viral genomic context by constructing recombinant HCMV (31). These studies indicate that when inserted into the viral genome, the *pol* promoter-CAT construct is regulated similarly to the endogenous *pol* gene (31). The putative *pol* promoter regulatory sequence elements will be similarly assessed in this system. This analysis should help to determine the significance of these elements in *pol* promoter regulation in HCMV infection.

Similar studies to assess the role of IE proteins in early gene regulation have been performed on other HCMV early genes (6, 13, 28, 38, 52, 65). These investigations have implicated specific sequences in conferring activation by IE proteins and revealed that cellular proteins likely play an important role in the regulation of some early genes. The transcription factor USF is involved in activating the 2.7-kb early gene, whereas AP-1 is associated with activation of the 1.2-kb early gene (28, 30, 65). Similarly, the 2.2-kb class of early genes may be

regulated by the cellular factor E2F (52). Also, our recent studies on the human immunodeficiency virus long terminal repeats demonstrate that IE72 and IE86 synergistically activate this promoter and that this action occurs through the USF site (18). Our current studies are consistent with these aforementioned reports and further support the suggestion that during activation of early gene expression, the HCMV IE proteins do not bind directly to regulatory sequences but instead interact with cellular factors to indirectly regulate transcription (54).

Interestingly, both USF and E2F can also be influenced in a positive manner by adenovirus E1A protein (2, 7, 37, 43, 46, 48). It is possible that IE proteins, and in particular IE72 and IE86, work together to activate certain early promoters in an E1A-like manner. This view is supported by the studies of Tevethia and coworkers (51, 64), who demonstrated that DNAs capable of encoding IE1 and IE2 proteins complement adenovirus early region 1 deletion mutants. In addition, IE1 and IE2 proteins synergistically activate the adenovirus E2A promoter, further demonstrating that these two proteins are capable of substituting for E1A (64). E1A activation of transcription also involves a direct interaction of this protein with the TATA-binding protein TFIID (36). Recent evidence demonstrates that the IE proteins also interact directly with TFIID (19, 26), further supporting the concept that IE proteins and E1A act in similar manners.

A recent study by Klucher and colleagues has suggested that IE86 activates transcription *in vitro* by counteracting histone H1-mediated repression (29). However, this effect was not observed with the IE72 protein. Our data suggest that both IE72 and IE86 are required for the activation of some early promoters in permissive cells (32, 54). In addition, the IE proteins appear to function via specific sequence elements within the early promoters (this study and references 28, 30, 52, and 65). Indeed, the apparent release of histone repression as described (29) was shown to be promoter dependent, indicating that some degree of specificity of interaction was required. Therefore, it is likely that the IE proteins function together as transcriptional coactivators, providing a link between cellular transactivators and the basal transcriptional machinery (5, 16, 45). It is important to note that HCMV early promoters are not activated in uninfected cells or cells lacking IE proteins. Clearly, cellular transcription factors alone are insufficient for activating early promoter expression. This type of model is consistent with HCMV biology and could explain how HCMV IE proteins act through a diverse series of regulatory sequences to regulate HCMV early gene expression.

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