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Binding of transcription factors and creation of a large nucleoprotein complex on the human cytomegalovirus enhancer

(*in vitro* transcription/protein–DNA interaction/nuclear trans-acting factors)

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ABSTRACT **The effect of the human cytomegalovirus immediate early region 1 enhancer on transcription was studied *in vitro* with HeLa cell nuclear extract. Stimulation of *in vitro* transcription mediated by the enhancer element involves its recognition by specific trans-acting factors present in the nuclear extract. DNase I protection analysis was used to determine at the nucleotide level those enhancer sequences that interact with nuclear factors. At least nine sites of protein–DNA interaction were detected over ≈400 base pairs of enhancer sequence. The regions of nuclease protection are associated with 21-, 19-, 18-, and 17-base-pair repeat elements as well as with a unique sequence, creating a large nucleoprotein complex. The relationship between the protein binding and the activity of the immediate early region 1 enhancer is discussed.**

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is the etiologic agent of a wide spectrum of human diseases (1, 2). Upon infection, the HCMV genes are expressed in three sequential phases (reviewed in ref. 3). The immediate early (*IE*) genes are transcribed immediately after infection and their expression is not dependent on prior viral protein synthesis (4–7). The most abundant IE RNA is transcribed from a single gene, the *IE1* gene, that encodes a nuclear phosphoprotein with an apparent molecular mass of 72 kDa (8–10). The regulatory sequences governing the expression of the *IE1* gene have been shown to contain an enhancer located between nucleotides −524 and −117, which is significantly stronger than the simian virus 40 enhancer in a variety of different cell lines (11, 12). There is evidence suggesting the activity of enhancer elements is mediated by the interaction with multiple nuclear factors (13–19). We therefore asked whether the HCMV IE1 enhancer could activate transcription *in vitro* and whether such activation would require binding of trans-acting factors. Furthermore, we asked whether specific sequence motifs of the HCMV enhancer are recognized *in vitro* by nuclear proteins from HeLa cells. Here, we have attempted to answer these questions and to relate our findings to other viral and cellular enhancer elements.

MATERIALS AND METHODS

Preparation of DNA. The recombinant plasmids pPSCAT, pSSCAT and pCMV(−524)CAT, pCMV(−65)CAT were obtained from J. Nelson and R. Rüger, respectively. These constructs contain various 5′-flanking sequences of the HCMV (strain AD169) *IE1* gene linked to the bacterial chloramphenicol acetyltransferase gene (see Fig. 1*A*). Plasmids pE1, pE2, and pE3 contain BAL-31 deletion fragments (prepared by R. Rüger) overlapping the enhancer and promoter region of the *IE1* gene that were cloned into the *Hin*dIII and *Bam*HI sites of pTZl8R (see Fig. 1*A*). The competitor fragments (E1, E2, and E3) were gel-purified from their respective plasmids after cleavage with *Eco*RI and *Hin*dIII restriction enzymes.

Nuclear Extract Preparation and Fractionation. Nuclear extract from HeLa cells was prepared as described by Dignam *et al.* (20). The fractionation of the nuclear extract on columns of heparin-agarose (Bio-Rad) and DEAE-Sepharose CL-6B (Pharmacia) was performed according to the procedure of Dynan and Tjian (21). The heparin-agarose and DEAE-Sepharose columns were step-washed with 0.4 M and 0.225 M KCl, respectively. The flow-through fractions (HAO.1, DEO.1) and the step-washed fractions (HAO.4, DEO.225) from the column chromatography were used in the DNase I protection analysis.

***In Vitro* Transcription Assays.** The incubation mixture for the transcription reaction (25 μl) contained 12 mM Hepes (pH 7.9); 10% (vol/vol) glycerol; 1.6 mM dithiothreitol; 0.12 mM EDTA; 60 mM KCl; 2 mM $MgCl_2$; 5 units of placental ribonuclease inhibitor (Amersham); 100 μM ATP, CTP, and GTP; 30 μM UTP; [^{32}P]UTP (100,000 cpm/pmol); and 0.5 μg of template DNA. After incubation for 45 min at 30°C, the reaction was stopped and processed as described by Dignam *et al.* (20). The ^{32}P-labeled RNA was subjected to electrophoresis in a 4% sequencing gel.

DNase I Protection Analysis. Fragments E1 and E2 were labeled at their 3′ ends using the large fragment of DNA polymerase I and [α-^{32}P]dGTP and at their 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP. Protection analyses were performed in the same buffer component as in the *in vitro* transcription reaction, with the nucleoside triphosphates and ribonuclease inhibitor omitted and the reaction volumes increased to 50 μl. DNase I protection analyses were performed as described by Ohlsson and Edlund (22).

RESULTS

The HCMV *IE1* Gene Promoter Is Transcriptionally Active *in Vitro*. Run-off assays were performed to determine whether the crude nuclear extract prepared from the nuclei of uninfected HeLa cells contained the necessary factors for initiating and sustaining transcription from the HCMV IE1 promoter. The DNA templates pPSCAT and pSSCAT, containing the IE1 promoter and 5′-flanking region, were cleaved with the restriction endonucleases *Pvu* II or *Eco*RI, which truncate the template 165 or 260 base pairs downstream of the IE1 cap site, respectively, generating run-off transcripts that could be visualized by electrophoresis. For all the templates tested, the size of the run-off transcripts obtained in the cell-free assay corresponded to the expected length (Fig. 1*B*). As shown by the sensitivity to α-amanitin (0.5 μg/ml), the transcription observed is dependent on polymerase II (Fig.

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Abbreviations: HCMV, human cytomegalovirus; IE1, immediate early region 1.

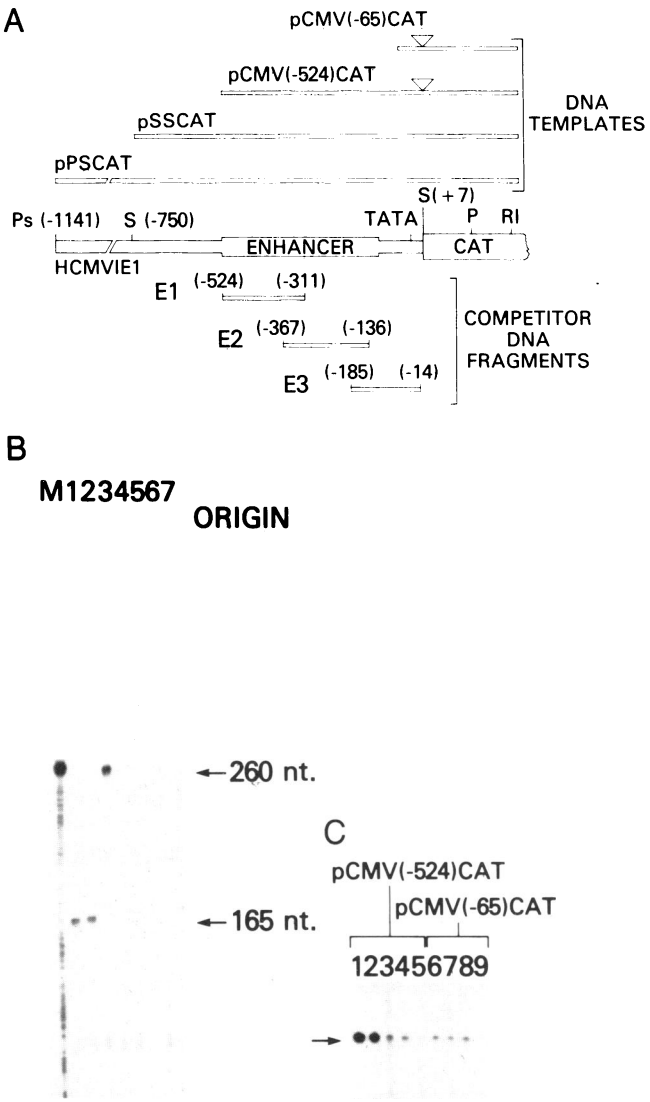

FIG. 1. (*A*) Schematic representation of the recombinant templates and competitor DNA. The central figure shows the upstream region of the HCMV *IE1* gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. The location of the enhancer region and the various restriction sites described in the text are marked. P, *Pvu* II; RI, *Eco*RI; Ps, *Pst* I; S, *Sau*3A. Numbers refer to the nucleotide position relative to the cap site of the *IE1* gene. ▽ indicates that the templates have an extra 55 base pairs of 5′ untranslated leader sequence from the *IE1* gene. (*B*) *In vitro* transcription from HCMV IE1 promoter. Run-off transcription assays contained 15 μl of crude nuclear extract and were performed as described. Electrophoretic analysis of *in vitro* transcription products of: lane 1, pSSCAT truncated with *Pvu* II; lane 2, pPSCAT truncated with *Pvu* II; lane 3, pPSCAT truncated at the *Eco*RI site; lane 4, no template; lane 5, transcription performed as in lane 2 in the presence of α-amanitin (0.5 μg/ml); lane 6, as in lane 2 but with α-amanitin (200 μg/ml); lane 7, as in lane 3 but with α-amanitin (0.5 μg/ml). Lane M, A+G reaction of a known sequence of 265-base-pair fragment (not CMV) as marker lane. Arrows indicate the run-off transcript bands and their sizes are correspondingly marked. nt, Nucleotides. (*C*) Activity of different competitor DNA on *in vitro* transcription from pCMV(−524)CAT and pCMV(−65)CAT templates. Nuclear extract (6 μl) was preincubated with ≈15 M excess of competitor DNA (over the template DNA) in 23 μl of transcription assay mix for 15 min on ice. Template DNA (0.3 μg) truncated at the *Hin*dIII and *Pvu* II sites and [^{32}P]UTP was added and transcription was allowed to proceed for 30 min at 30°C. The electrophoretic analysis of the competition assay products is shown. Lanes 1–5 and 6–9 contained the pCMV(−524)CAT and pCMV(−65)CAT templates, respectively, and the following lanes combined competitor DNAs. Lane 1, no competitor; lanes 2, pUC19 cut with *Hae* III; lane 3, E1; lane 4, E2; lane 5, E3; lane 6, pUC19 cut with *Hae* III; lane 7, E1; lane 8, E2; lane 9, E3. Arrow indicates the run-off transcript.

1*B*). To quantify the level of transcription, the amount of radioisotope in gel slices containing the transcript was determined by liquid scintillation counting. The calculated transcriptional efficiency of the system corresponds to 1–1.5 transcripts per template molecule per hr.

Stimulation of the HCMV IE1 Promoter by Its Enhancer Depends on Trans-Acting Factors. Resection of the *IE1* gene regulatory sequences from −524 to −65 results in a decrease by a factor of 25 in the *in vitro* transcriptional activity from the IE1 promoter (Fig. 1*C*, lanes 2 and 6). Activity was determined by densitometric scanning of the autoradiograph and by liquid scintillation counting of gel slices containing the transcript. Thus, the IE1 promoter is clearly dependent on the upstream enhancer sequences for efficient transcription *in vitro*. To determine whether the transcriptional stimulation involved a specific trans-acting factor(s) we applied a competition assay. *In vitro* transcription of pCMV(−524)CAT and pCMV(−65)CAT was carried out in the presence of a number of DNA fragments to monitor the competition for transcription factors (Fig. 1 *A* and *C*). Transcription from the HCMV promoter in pCMV(−524)CAT could be selectively competed for by DNA fragments containing enhancer sequences—i.e., E1 and E2 (Fig. 1*C*, lanes 2–4). The fragment E3, containing part of the enhancer and promoter sequences, competed more efficiently than the enhancer fragments (lanes 3–5). To ensure that the competition effects observed were specific, several competitors were tested using the same extract and DNA template preparations. Fig. 1*C* (lanes 1 and 2) shows that pUC19 fragments did not compete for factors necessary for transcription from pCMV(−524)CAT. Competition with fragments E1 and E2 that did contain enhancer sequences resulted in a decrease of transcriptional activity from a promoter with an enhancer, but had no effect on the transcription from an enhancer-less template (Fig. 1*C*, lanes 2–4 and 6–8). These data strongly suggest the interaction of specific nuclear factors with IE1 enhancer sequences resulting in transcriptional stimulation. The reduction of transcriptional activity by enhancer fragments E1 or E2 as competitor resulted in the transcriptional rate normally seen with an enhancer-less template (Fig. 1*C*, lanes 3, 4, and 6). This observation suggests that the *in vitro* stimulatory effect of the enhancer involves, in a major part, the binding of trans-acting factors.

Nuclear Proteins Bind to Multiple Sequence Motifs in the HCMV IE1 Enhancer. Since trans-acting factors mediate the enhancer activity by binding to specific sequences within the enhancer region, DNase I protection experiments were performed to investigate whether any specific sequence motifs are recognized by factors present in the active extract. Initially, "footprint" analysis of fragments E1 and E2 using crude nuclear extract resulted in an extensive protection pattern (data not shown). To clearly distinguish regions of protection, the crude nuclear extract was fractionated with columns of heparin-agarose and DEAE-Sepharose and footprint analyses were performed on the eluted fractions. Similar to other investigators (22), we have titrated the amount of protein and nonspecific competitor DNA [poly(dI·dC)] to the labeled DNA fragment. An example of the titration analysis is shown for the first column fraction, the heparin-agarose flow-through fraction (HAO.1) for both strands of fragment E1. Using this approach, we detected a protected region between nucleotides −447 and −424 (site III) on the E1 fragment (Fig. 2). However, with the same approach, the E2 fragment did not yield a clear region of DNase I protection (data not shown). Clear protection of site III was observed on only one strand. Similar observations for some of the binding sites detected on the immunoglobulin heavy-chain enhancer have been reported (18).

The same analysis was applied to the other column fractions, a summary of which is shown in Fig. 3. Multiple regions

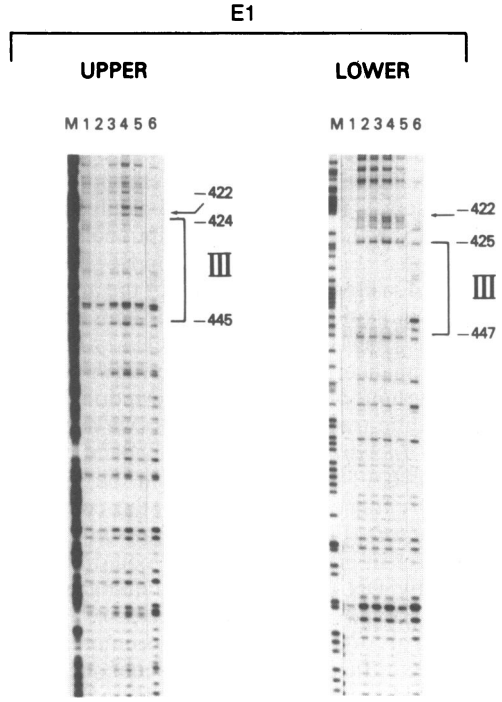

FIG. 2. Footprint titration analyses of fragment E1 using HAO.1 nuclear protein fraction. Binding reactions were carried out as described, and Maxam and Gilbert (A+G) sequencing markers (lanes M) of the fragment were used to locate the footprint region. The fragment was labeled at the 5′ end (upper) or 3′ end (lower) at approximately nucleotide position −524. The following amounts of HAO.1 fraction were used: lane 1, 1 μl; lane 2, 5 μl; lanes 3–5, 10 μl. The following amounts of poly(dI·dC) were used: lanes 1–3, 250 ng; lane 4, 500 ng; lane 5, 1000 ng. Lanes 6, control lanes; DNase I digestion of the fragment in the presence of 20 μg of bovine serum albumin and 500 ng of poly(dI·dC). Numbers represent the distance in base pairs located upstream from the IE1 cap site. Brackets and roman numerals designate the protected region, which is shown in Fig. 4. Arrows indicate sites of enhanced DNase I cleavage.

of protection are clearly seen with the HAO.4 and DEO.1 column fractions, while only weak binding activity is observed with the DEO.225 fraction (Fig. 3). In addition to the protected regions, hypersensitive sites of DNase I cleavage are observed in proximity to the protected areas (Figs. 3 and 4). Such DNase I hypersensitive sites are characteristic of nearby protein–DNA interactions (14, 17, 18). A total of nine sites of protein–DNA interaction have been mapped over ≈400 base pairs of the enhancer region. A comparison of the protected regions suggests that five different sequence motifs are involved in the binding of nuclear proteins. These are three 12-base-pair palindromic sequences, ATTGACGTCAAT, located within the 19-base-pair repeats; three 13-base-pair sequences, TTGGCAGTACATC, located within the 17-base-pair repeats; a sequence, TGGCN_5GCCCA, associated with part of the 21-base-pair repeats; two 6-base-pair sequences, CTTTCC, located within the 18-base-pair repeats; and a unique sequence, TTCCCATAGTAACGCC, at site III (Fig. 4).

DISCUSSION

The HCMV IE1 enhancer element exhibits the strongest known enhancing activity *in vivo* (11, 12). However, in a few cases (for example, undifferentiated teratocarcinoma cells), the IE1 promoter/enhancer upstream region is not active (23). The requirement for the IE1 enhancer sequences to stimulate transcription *in vivo* has been reproduced *in vitro* by using a HeLa cell nuclear extract (Fig. 1). The transcriptional efficiency of the IE1 enhancer containing templates in our assay system corresponds to ≈1 transcript per template molecule per hr. This efficiency is ≈15-fold higher than that for the adenovirus major late promoter (24, 25), one of the most efficient templates assayed *in vitro*. The ability of enhancer elements to stimulate transcription *in vitro* has been demonstrated in only a few cases (14, 16–18). For example, the stimulatory effect mediated by the intensively studied simian virus 40 and immunoglobulin heavy-chain enhancers is between 3- and 10-fold (16–18). In our *in vitro* transcription system, the HCMV IE1 enhancer-dependent stimulation is at least 25-fold. Therefore, the IE1 enhancer element exhibits not only the strongest enhancing activity *in vivo*, but also *in vitro*.

There is experimental evidence suggesting the action of enhancers is mediated by binding of trans-acting factors (13–19). The results of the *in vitro* transcription-competition experiments show that binding of transcription factors is a major requirement for the IE1 enhancer activity (Fig. 1*C*). We have taken the approach, by DNase I protection analysis, of determining sequence elements within the IE1 enhancer that interact with nuclear proteins from the active extracts. We identified at least nine sites of protein–DNA interaction on the IE1 enhancer sequence (Figs. 2–4).

Two lines of evidence support the conclusion that the observed DNase I protected regions on the HCMV IE1 enhancer are due to the binding of several distinctly different nuclear factors: (*i*) the DNA target sequences as delineated by the protected regions fall into at least five different classes of sequence motifs; and (*ii*) the various binding factors exhibit different biochemical fractionation properties—for example, the selective fractionation of the factor binding to site III in the HAO.1 fraction. As illustrated in Fig. 4, it is apparent that a large nucleoprotein complex is created by the binding of multiple factors to the IE1 enhancer. Such an arrangement suggests that the close juxtaposition of enhancer proteins may allow protein–protein interactions or be part of a mechanism for modulating enhancer protein activity. It is interesting to note that *in vitro* transcription reconstitution experiments with the various column fractions suggest the requirement for the interaction of multiple transcription factors (data not shown, available on request).

It has been suggested that the 19- and 18-base-pair repeats are required for enhancer activity while the 17-base-pair repeats may be associated with an inhibitory element (26). In this connection, fragment E1 and E2 contain both the 19- and 18-base-pair repeat elements, which may explain the competition experiment in which both fragments independently reduced the transcriptional activity of an enhancer containing template to the same extent.

An important issue to be addressed is whether the protected regions have any sequence similarities to other enhancer sequences or known sites of protein–DNA interactions. The protected 6-base-pair motif (CTTTCC) is part of an 11-base-pair sequence (GGGACTTTCCA), which also occurs in the mouse CMV, simian virus 40, lymphotropic papovavirus, and immunoglobulin κ gene enhancers as well as in the human immunodeficiency virus long-terminal repeat. It is noteworthy that relatively weak protection of the entire 11-base-pair motif, juxtaposed to sites IV and VIII, is observed in the HAO.4 fraction (Fig. 3, lanes 1). In the simian virus 40 enhancer, the 6-base-pair motif, termed GT-I, which corresponds to the "enhancer core" sequence (27), binds a factor and is essential for *in vivo* activity in HeLa cells (16, 28). The immunoglobulin κ gene enhancer also contains the 11-base-pair element, which binds a factor specific to κ-producing B cells (29). The 13-base-pair motif and sites I and VII resemble the nuclear factor 1 binding consensus sequence (30). Nuclear factor 1 is known to bind to several other enhancer elements, such as in the papovaviruses BK

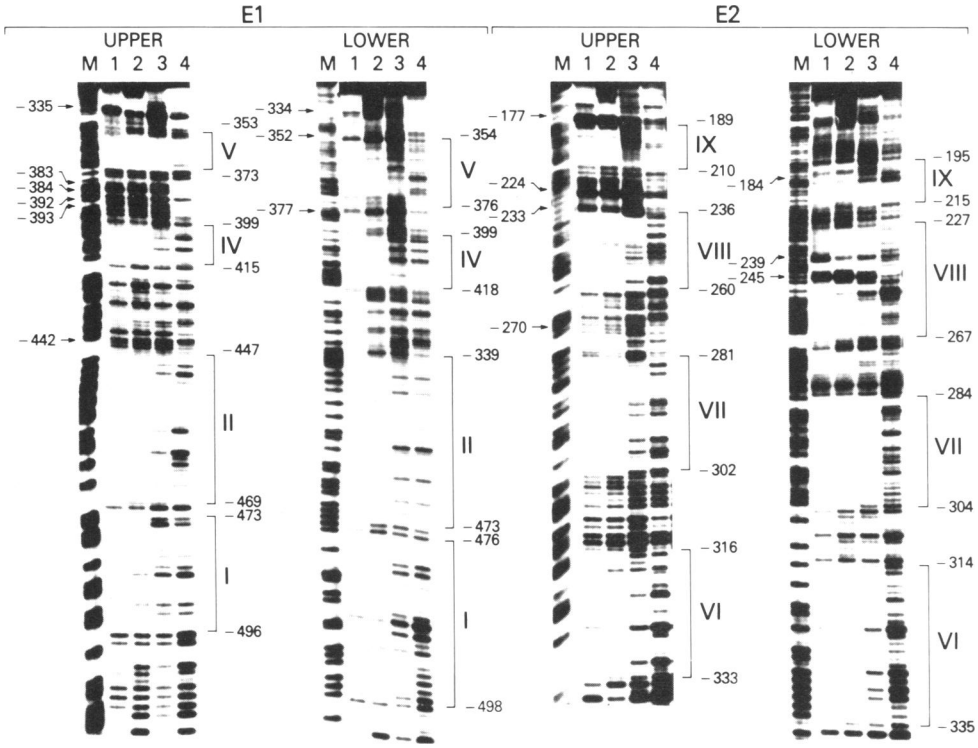

FIG. 3. DNase I protection analyses of the E1 and E2 enhancer fragments using different column fractions from the fractionation of HeLa cell crude nuclear extract. Binding reactions were carried out as described, and Maxam and Gilbert sequencing markers (lanes M) of the corresponding probes were used to locate the footprint regions. The fragments were labeled at their 5′ end (upper) or 3′ end (lower) at approximately nucleotide position −524 for E1 and −367 for E2. The following amounts of HAO.4, DEO.1, and DEO.225 column fractions were used: lane 1, 35 μg; lane 2, 13 μg; lane 3, 19 μg; lane 4, control lane; 750 ng of nonspecific competitor [poly(dI·dC)] was used for all the reactions.

and JC (31, 32) as well as 5′ to the HCMV IE1 enhancer (33). In addition, sites I and VII also contain sequence similarities to the recognition sequence of the transcription factor Sp1 (34). However, the biochemical fractionation properties of the nuclear protein binding to sites I and VII suggest that it is not Sp1 (unpublished data). Binding site III and the

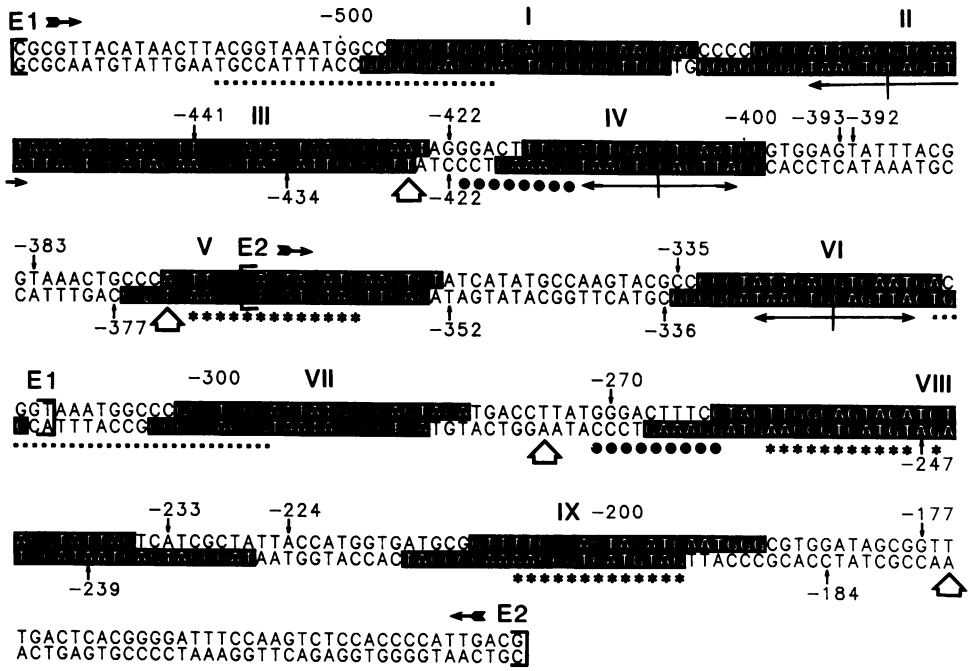

FIG. 4. Sites of protein–DNA interaction on the HCMV IE1 enhancer. DNA sequence of both strands of the enhancer region is shown. Numbers represent the distance in base pairs located upstream of the transcription start site (+1). Sequences protected from DNase I cleavage are marked by reverse printing. Roman numerals denote protected regions and the E1 and E2 fragment borders are indicated by brackets and horizontal arrows. Vertical arrows mark positions of enhanced protein-induced DNase I cleavage *in vitro*. Large open arrows indicate sites of *in vivo* DNase I hypersensitivity (23). Repeated sequence elements within the enhancer region are designated as follows: ••••, 21 base-pair repeat; <+>, 19-base-pair repeat; ●●●●●, 18-base-pair repeat; *****, 17-base-pair repeat. Underlining is only shown for those sequences that have 100% identity to each other.

19-base-pair repeat on the IE1 enhancer have no apparent homology to other enhancer elements or protein–DNA target sequences. However, the 19-base-pair repeat is conserved between mouse CMV and HCMV enhancer elements, strongly suggesting a functional significance with respect to the CMV viruses (35). Moreover, the 19-base-pair repeat and site III represent novel enhancer target sequences for nuclear factors. The occurrence of similar sequence motifs—for example, the 11-base-pair motif—and perhaps the binding of common enhancer factors suggests that there may be common functional components in all enhancers. More importantly, however, it is perhaps those sequence motifs—for example, the 19-base-pair repeat and binding site III—and corresponding nuclear factors, which are specific to a particular enhancer, that determine the differential activities of different enhancer elements.

This study has shown that the various motifs of the HCMV IE1 enhancer do indeed bind specific protein factors *in vitro* to create a large nucleoprotein complex. Binding of proteins to DNA sequences *in vivo* has been correlated with "active" chromatin, which is characterized by defined regions (≈30 base pairs) of hypersensitivity to nuclease attack (36–38). Regions of *in vivo* DNase I hypersensitivity located on the IE1 enhancer region of HCMV have been reported (23). Strikingly, these regions coincide with sites of enhanced DNase I cleavage that are associated with a number of the footprint regions observed *in vitro* (see Fig. 4). Since there is good agreement between the *in vivo* and *in vitro* DNase I hypersensitive sites this strongly suggests that the nucleoprotein complex detected *in vitro* also occurs *in vivo*.

In conclusion, we have shown that multiple nuclear proteins recognize specific sequence motifs on the HCMV IE1 enhancer generating a large nucleoprotein complex and that the binding of trans-acting factors to enhancer sequences is critical for its enhancing activity *in vitro*.

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