Nucleic Acids Research, Vol. 19, No. 13 3715–3721

Formation of several specific nucleoprotein complexes on the human cytomegalovirus immediate early enhancer

Hans Helmut Niller and Lothar Hennighausen*
Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bldg 10, Rm 9N113, Bethesda, MD 20892, USA

Received January 14, 1991; Revised and Accepted June 5, 1991

ABSTRACT

The major immediate early enhancer of the human cytomegalovirus (HCMV) is composed of unique and repeated sequence motifs, which interact with different nuclear proteins, thus forming a large nucleoprotein complex. Using DNAase I protection analysis, we determined at the nucleotide level the interactions of B cell and HeLa cell nuclear proteins with transcription factor binding sites in the enhancer/promoter. In agreement with in vivo activity, protein binding to the 18 bp repeats (κB element) was found predominantly with B cell extract. Competition for proteins with individual transcription factor binding sites allowed us to define boundaries of closely spaced and overlapping binding sites, and to group binding proteins into several classes. Using gel mobility shift assays, we could show that proteins, which bind to the 17 bp repeat, also bind to a classical NF1 site. In addition, several novel binding sites were identified. The presence of overlapping binding sites, together with differences in the occupation of the 18 bp repeats in the two cell types, suggest that the HCMV major IE enhancer has several possibilities of forming nucleoprotein complexes.

INTRODUCTION

cHuman cytomegalovirus (HCMV) is a member of the herpesvirus family and can cause a wide spectrum of diseases. The major immediate early enhancer of the HCMV is among the strongest transcriptional elements in vivo and in vitro (1) and possibly contributes to the expression of the viral immediate early (IE) genes in numerous different cell types (2, 3). Features of this enhancer are its large size and complexity. Four types of different sequence repeats, with 17, 18, 19, and 21 base pairs (bp), respectively, are interspersed three to five times within the enhancer region between −524 and −65 upstream of the transcription initiation site (2, 4, 5). The 17 bp repeat contains the sequence motif 'TGGCA' which resembles a recognition half site for the transcription factor NF1 (6, 7). The 18 bp repeat contains a binding site (GGGACTTTCC) for NFκB (8, 9), a transcription factor (9, 10, 11, 12) which contributes to the specificity of immunoglobulin expression in B cells (9) but is also present in non-B cells (10, 11, 12, 13). The 19 bp repeat contains a cyclic AMP response element (CRE: TGACGTCA) (14, 15, 16, 17, 18), and the 21 bp repeat resembles an SP1 binding site (19). The interaction of nuclear proteins from HeLa cells with these repeated sequence motifs and with unique sequences has been demonstrated in DNAase I protection experiments, and the existence of a large nucleoprotein complex on the enhancer was proposed (1). In vitro transcription competition assays with oligonucleotides comprising protein binding sites of the enhancer established that the individual binding sites within the enhancer interact with transcription factors (20).

Since the HCMV enhancer contains multiple closely spaced and possibly overlapping transcription factor binding sites it is conceivable that the availability of factors in conjunction with steric interferences in the nucleoprotein complex could result in the formation of distinctly different complexes on the enhancer. To test this hypothesis we compared nucleoprotein complexes formed with either HeLa or B cell nuclear proteins. Furthermore, using oligonucleotides containing transcription factor binding sites as competitors we analyzed the formation of different nucleoprotein complexes on the enhancer/promoter in the absence of defined transcription factors. This approach allowed us to determine cell specific differences in in vitro binding to the different enhancer elements, to define the borders of overlapping transcription factor binding sites, and to group the protein binding sites.

MATERIALS AND METHODS

Reagents

Enzymes were from Biolabs, Boehringer Mannheim, BRL, or Stratagene. Radiochemicals were obtained from NEN. Cell culture media were from Biofluids. Oligonucleotides were from The Midland Certified Reagent Company, Midland, Texas. All other chemicals were from Biorad, BRL, Mallinckrodt, or Sigma.

Tissue culture

IM9 cells (an immunoglobulin secreting B myeloma cell line) were obtained from ATCC, and HeLa S3 cells from B. Moss (NIH). HeLa cells were maintained in suspension cultures of

* To whom correspondence should be addressed

DMEM, and lymphoid cells in RPMI 1640, each containing 10% fetal calf serum, 2 mM glutamine, 50 units penicillin per ml and 50 μg streptomycin per ml under 5% CO_2 and 37°C.

Plasmids

pΔ0CAT contained sequences from nucleotide (nt) −1142 to nt +54 of the IE 1 gene of HCMV (AD169) in front of the structural CAT gene from pA10CAT2 (21). pΔ1CAT (pRR56/1) containing sequences from nt −526 to nt +54 of the HCMV IE 1 gene in front of the CAT gene was obtained from R. Rüger (Erlangen). pFSCAT containing sequences from nt −524 to nt +7 of the HCMV IE1 gene in front of the CAT gene was obtained from J. Nelson (San Diego). The core promoter (nt −55 to nt +7) plasmid was described as promoter (18). Plasmids p17CMVCAT, $p18_3$CMVCAT, $p19_6$CMVCAT, and pNF1CMVCAT contained the respective transcription units in pUC13 or pUC18 context. The respective oligonucleotides were cloned in either a BamH1 or Sma1 site immediately upstream of the core promoter in front of the structural CAT gene (21), or directly used for in vitro footprinting analyses. The doublestranded oligonucleotides were GATCCAGTACTTGGCAGTACATCAA // GATCTTGATGTACTGCCAAGTACTG containing the 17 bp repeat of HCMV from nt −373 to nt −356, CCAATAGGGACTTTCCAT // ATGGAAAGTCCCTATTGG containing the 18 bp repeat of HCMV from nt −428 to nt −411 in $p18_3$CMVCAT, CAGATCTGGCCCATTGACGTCAATAATCAGATCTG // CAGATCTGATTATTGACGTCAATGGGCCAGATCTG containing the 19 bp repeat of HCMV from nt −469 to nt −451 in p19CMVCAT, GCCCATTGACGTCAATAAT // ATTATTGACGTCAATGGGC containing the 19 bp repeat as a tandem hexamer in $p19_6$CMVCAT (This oligo was also used for the competition in DNAase I protection analyses), TATGTTCCCATAGTAACGCCAATAG // GATCCTATTGGCGTTACTATGGGAACA containing the unique region of HCMV from nt −446 to nt −421, AGATCTTGACTCAAGGCCT // AGGCCTTGAGTCAAGATCT containing an AP1 consensus site (22), and GATCTGGCACTGTGCCAAG // GATCCTTGGCACAGTGCCA containing an NF1 consensus site (6).

Plasmids purified over two CsCl gradients were essentially supercoiled and their integrity and purity were verified by sequence analysis.

Transfections

5 μg of each plasmid were incubated with 5×10^6 cells for 15 min in two ml of serum free Dulbecco's Modified Eagle's Medium containing 250 μg per ml DEAE Dextran, 0.1 mM chloroquindiphosphate and 50 mM Tris-HCl at pH 7.3 at 37°C in a shaking waterbath (100 rpm). Cells were then washed two times with 10 ml RPMI 1640 medium containing 10% fetal calf serum, followed by incubation for 36 hours under standard conditions.

CAT assays

Cytoplasmic proteins were extracted as described (21) and CAT activity was measured within the linear range of conversion using ^{14}C-chloramphenicol. Acetylated and non acetylated forms were separated using thin layer chromatography followed by scintillation counting. Each experiment was performed in duplicate.

Nuclear extracts

Nuclei were prepared using a combination and modification of two methods (23, 24). After harvesting and washing the cells they were resuspended in 5 pellet volumes of 0.3 M sucrose and 2% Tween 40 in buffer A (without antipain, leupeptin, and pepstatin A) and frozen in liquid nitrogen. After thawing and gently homogenizing the suspension, it was layered onto 0.5 M sucrose in the same buffer and centrifuged at 3000 g in a swinging bucket rotor. The nuclear extracts were prepared as described (24).

Gel mobility shifts

DNA fragments of 43 bp and 36 bp length were excised from plasmids p17CMVCAT and pNF1CMVCAT respectively with appropriate restriction enzymes and purified via polyacrylamide gel electrophoresis. Gel retardation assays were performed as described (24) with the following modifications: 7 μg of nuclear proteins, 1 μg poly dIdC, 0.1 ng of Klenow labeled fragment, and in the indicated cases a 40 fold molar excess of unlabeled competitor DNA fragment, were incubated in 25 μl of the same buffer as used for DNAase I protection analysis.

Constructing the enhancer chromatography column

5'-biotinylated enhancer DNA from nt −524 to nt +54 of the HCMV IE I gene was generated using polymerase chain reaction (PCR) on a Perkin Elmer Cetus PCR machine. As primers were used one 24-mer oligonucleotide containing a 5'-biotin group (The Midland Certified Reagent Company) starting at nt −524 and one 21-mer oligonucleotide ending at nt +54. PCR conditions were as described by the manufacturer with the following modifications: 100 μl reaction volume contained 10 units of Taq polymerase, 1.2 μg of the 5'-biotin primer, 1.05 μg of the 3'-primer with a concentration of 2.15 mM $MgCl_2$, and 700 μM each of the four desoxynucleotides. Biotinylated enhancer DNA was separated from remaining primers and concentrated through filtration with a centricon 100 filter (Amicon), and linked to avidin agarose at a ratio of 1 μg DNA per μl resin by mixing it on a rotating wheel overnight in 2 M KCl. Effectivity of coupling was controlled by inclusion of a small amount of radioactively labeled DNA.

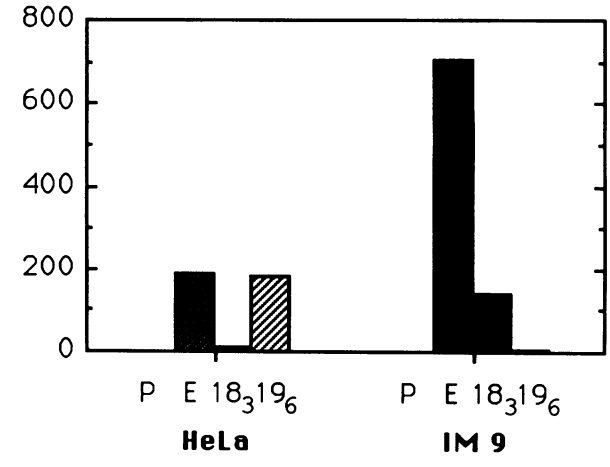

Fig. 1. Activities of HCMV transcriptional elements in HeLa S3 and IM9 B cells. The cell types and constructs are depicted on the abscissa and relative CAT activities are shown on the ordinate. The activity of the core promoter is set at 1 in both cell types. The activities of the plasmids $p18_3$CMVCAT, $p19_6$CMVCAT, and pFSCAT (enhancer) are related thereto.

Fractionation of crude nuclear extracts

Crude nuclear proteins were passed over the enhancer DNA resin at a ratio of 100 μg protein per μg DNA, and, after extensive washing of the resin with DNAase I protection buffer, proteins were eluted with 2 M KCl. The eluted proteins were dialyzed for four hours as described (24, p. 723), and flowthrough, wash, and eluate fractions were stored at −70°C.

DNAase I protection analysis

Plasmids pΔ0CAT and pΔ1CAT were cut with appropriate restriction enzymes, endlabeled with Klenow enzyme, recut and labeled fragments containing the regulatory sequences of HCMV purified from a native 5% polyacrylamidegel. pΔ0CAT was labeled at XbaI of the polylinker sequence at the 3′-end of the HCMV sequences, and pΔ1CAT was labeled at HindIII at the

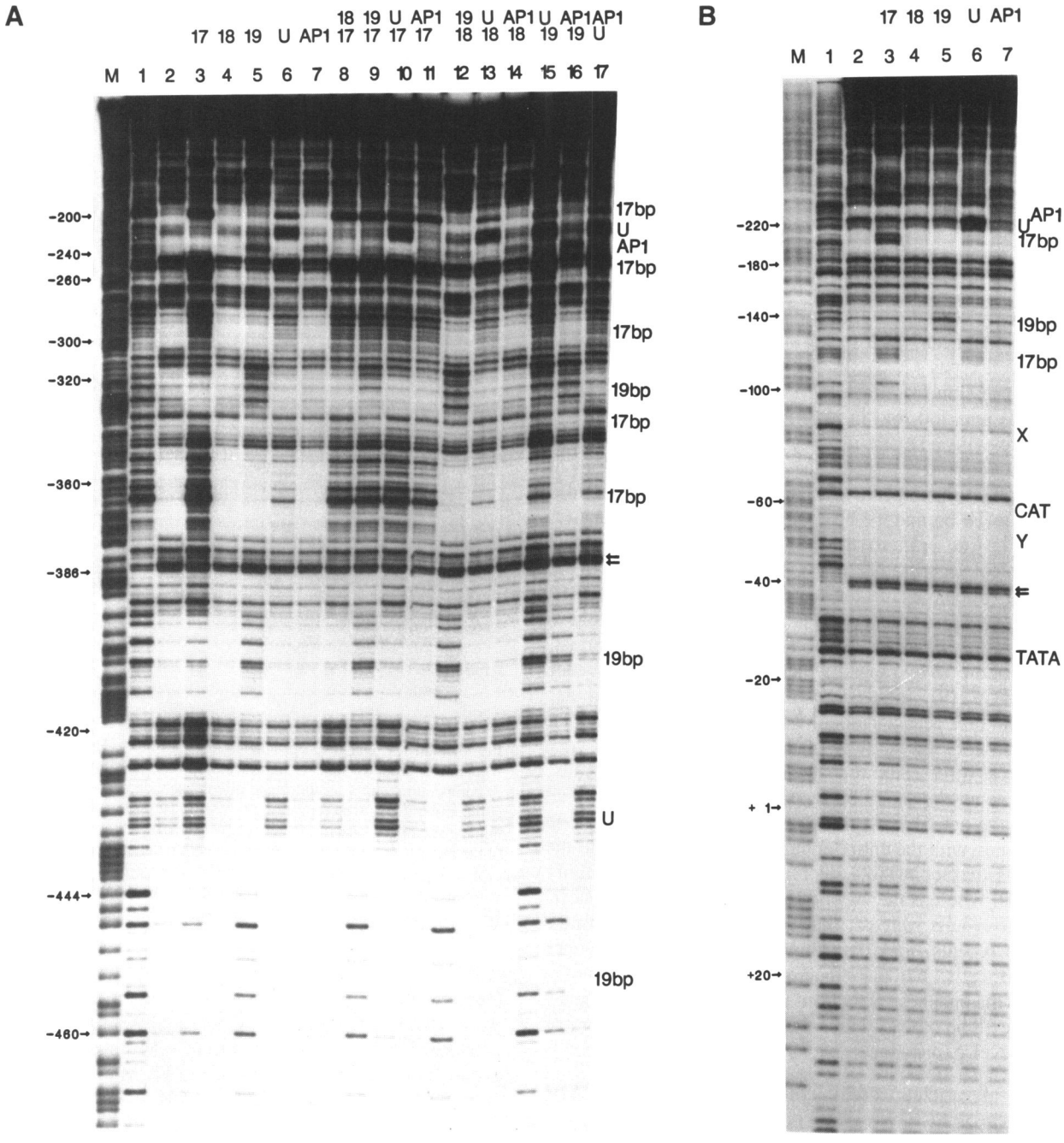

Fig. 2. Panel **A**: DNAase I protection analyses of the HCMV upstream region with HeLa cell proteins. lane 1: unprotected probe, lane 2: protected probe, lane 3: competition with 1 μg unlabeled oligonucleotide of the 17 bp repeat, lane 4: competition with 18 bp repeat oligo, lane 5: 19 bp repeat oligo, lane 6: unique binding site oligo, lane 7: AP1 site oligo, lane 8: competition with 0.5 μg each of 17 bp and 18 bp repeat oligos, lane 9: 17 bp and 19 bp repeat oligos, lane 10: 17 bp repeat and unique binding site oligos, lane 11: 17 bp repeat and AP1 site oligos, lane 12: 18 bp and 19 bp repeat oligos, lane 13: 18 bp repeat and unique binding site oligos, lane 14: 18 bp repeat and AP1 site oligos, lane 15: 19 bp repeat and unique binding site oligos, lane 16: 19 bp repeat and AP1 site oligos, lane 17: unique binding and AP1 site oligos. Panel **B**: DNAase I protection analyses of the HCMV promoter proximal region with HeLa cell proteins. lane 1: unprotected probe, lane 2: protected probe, lane 3: competition with 1 μg unlabeled oligonucleotide of the 17 bp repeat, lane 4: competition with 18 bp repeat oligo, lane 5: 19 bp repeat oligo, lane 6: unique binding site oligo, lane 7: AP1 site oligo.

5'-end of the HCMV sequences. For DNAase I footprinting 7 μg proteins from the eluate fraction were incubated with 3 μg poly dIdC at room temperature for 15 min, then with the indicated amounts of unlabeled doublestranded synthetic oligonucleotides for 5 min, then for 20 more min in the presence of 8 ng of endlabeled fragment (50000 cpm) in 12 mM Hepes-KOH, pH 7.9, 10% glycerol, 1.6 mM dithiothreitol (DTT), 0.12 mM EDTA, 60 mM KCl, 6 mM $MgCl_2$, the reaction volume was 50 μl. Then the DNA was digested with 1 unit of DNAase I (Boehringer, Mannheim) at RT for 1 min. The reaction was stopped by addition of 100 μl of stop solution (100 mM Tris HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS, 10 μg proteinase K, 1 μg E. coli DNA), incubation for 15 min at 37°C, and denaturation at 90°C for 2 min. The digestion products were phenol extracted, ethanol precipitated, and separated on a 6% sequencing gel. Maxam and Gilbert A+G sequencing markers (25) of the corresponding probes were used to locate the footprint regions.

RESULTS

Cell specific activity of the 18 bp and 19 bp enhancer repeats

The HCMV enhancer has a strong transcriptional activity in a number of cell types (2, 3). In our experiments the enhancer increased expression from the HCMV promoter about 190-fold in HeLa cells and 700-fold in IM9 B cells as compared to the core promoter (Fig. 1). To identify enhancer elements which confer activity in both cell types, the transcriptional activities of the 18 bp and 19 bp repeats were analyzed. Oligonucleotides containing the 18 bp and 19 bp repeats were ligated to an IE1 core promoter (nt −55 to nt +7) in front of the bacterial chloramphenicol acetyltransferase (CAT) gene. Six copies of the 19 bp repeat, which contains the cAMP response element (CRE) TGACGTCA (14), stimulated the IE1 promoter about 6-fold in B cells, and about 190-fold in HeLa cells (Fig. 1). In contrast, three copies of the 18 bp repeat, which contains the κB sequence GGGACTTTCC (9), induced expression from the promoter about 140-fold in B cells and about 9-fold in HeLa cells (Fig. 1). Thus in HeLa cells the activity of six 19 bp repeats was very strong, whereas three 18 bp repeats had little activity. In B cells, on the other hand, the six 19 bp repeats had little transcriptional activity and the activity of three 18 bp repeats was about one fifth of that seen with the entire enhancer. This suggests that the 19 bp repeats may contribute strongly to the enhancer activity in HeLa cells, whereas the 18 bp repeats may have little effect. Further these results suggest, that the 18 bp repeats, contrary to the 19 bp repeats, may contribute strongly to the enhancer activity in B cells, but also that other repeat elements may contribute to the enhancer activity in B cells, or that the sequence context of the 18 bp repeats within the enhancer is more favorable for a high level expression.

Formation of nucleoprotein complexes on the HCMV enhancer

The differential utilization of the 18 bp and 19 bp repeats in HeLa and in B cells, together with previous work from this laboratory (1, 26, 20), led us to investigate differences of the nucleoprotein complexes formed with proteins from the two cell types.

Methodical approach. DNAase I protection analyses using crude nuclear extracts revealed that the HCMV regulatory region between nt −524 and nt +54 was already extensively protected

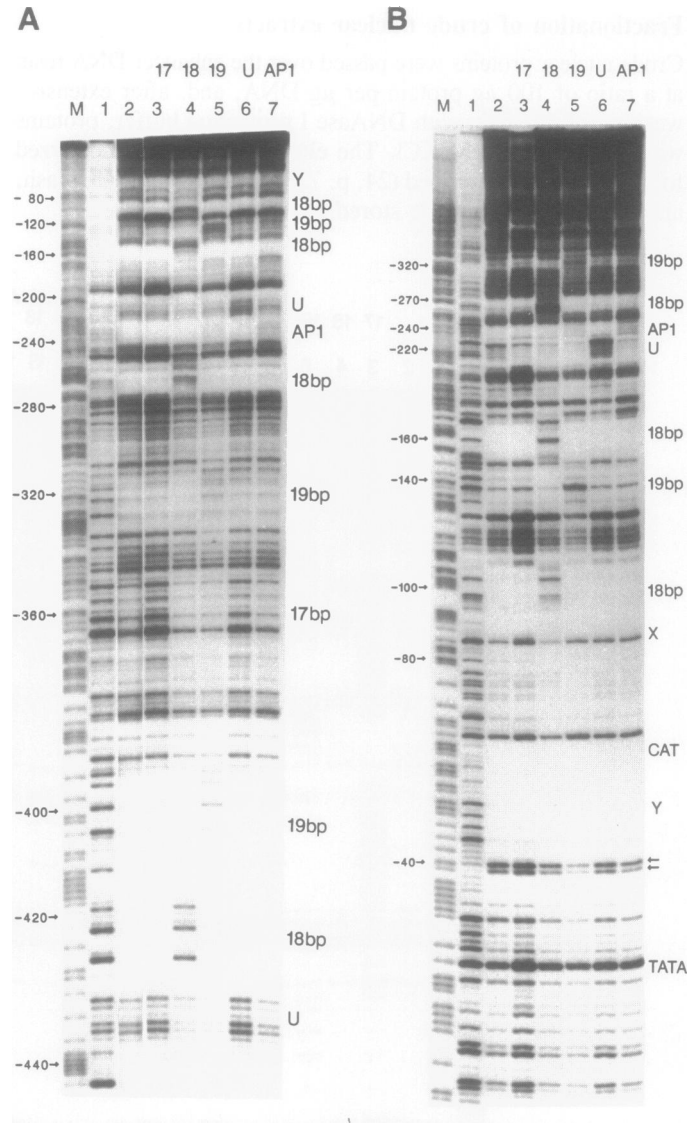

Fig. 3. Panel A: DNAase I protection analyses of the HCMV upstream region with B cell proteins. lane 1: unprotected probe, lane 2: protected probe, lane 3: competition with 1 μg unlabeled oligonucleotide of the 17 bp repeat, lane 4: competition with 18 bp repeat oligo, lane 5: 19 bp repeat oligo, lane 6: unique binding site oligo, lane 7: AP1 site oligo. Panel B: DNAase I protection analyses of the HCMV promoter proximal region with B cell proteins. lane 1: unprotected probe, lane 2: protected probe, lane 3: competition with 1 μg unlabeled oligonucleotide of the 17 bp repeat, lane 4: competition with 18 bp repeat oligo, lane 5: 19 bp repeat oligo, lane 6: unique binding site oligo, lane 7: AP1 site oligo.

with low amounts of nuclear protein (1, data not shown). To clearly define individual protected sites crude nuclear proteins were fractionated on an avidin agarose resin with HCMV enhancer DNA (from nt −524 to nt +54) linked to it via a biotin group. Footprinting analyses of the HCMV regulatory regions were performed with the HeLa and B cell proteins eluted from this resin. In accordance with earlier observations (1, 26) the analyses revealed a pattern of densely packed and possibly overlapping binding sites. In order to define borders and types of different binding sites in both extracts, we investigated protection patterns on the enhancer/promoter in the presence of oligonucleotides containing specific binding sites. Incubation of nuclear proteins with specific oligonucleotides prior to adding the template resulted in the formation of nucleoprotein complexes devoid of the corresponding transcription factors.

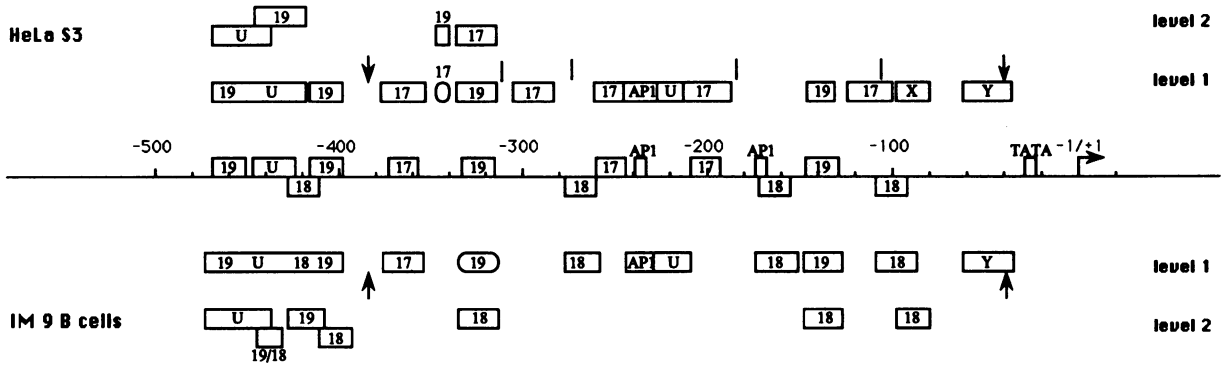

Fig. 4. Schematic summary of the DNAase I protection analyses. The line in the middle of the figure represents HCMV regulatory sequences from nt −524 to nt +54. Numbers and stipples above the line indicate sequence locations. Boxes attached to the line indicate the location of the 17 bp, 18 bp, and 19 bp repeats, two consensus AP1 sites, and a TATA box, each as deduced from sequence homologies (2), and a unique protein binding site (U) as described (1). The arrow at −1/+1 indicates the start of transcription (5). The upper half of the figure shows the results for HeLa S3 proteins, the lower half for IM9 B cell proteins. The boxes at level 1 in both halves of the figure show the protection pattern on the DNA with the eluate fractions from both cell types. Rounded boxes indicate weaker footprints. The numbers in the boxes indicate the oligonucleotide which abolishes the respective footprint when used as a competitor in the DNAase I protection analysis. The boxes X and Y are not disappearing in any of the competition assays. The arrows and bars at level 1 indicate strong and weak DNAase I hypersensitive sites respectively. The boxes at level 2 of each half of the figure show footprints which are getting stronger upon competition with the indicated oligonucleotide.

Protection with HeLa cell proteins. Using the competition assay with HeLa cell proteins we could define one weakly and five strongly protected regions as 17 bp repeat homologs (Fig. 2A, B, lanes 2, 3; fig. 4). No changes in protection were observed upon competition with the 18 bp repeat oligonucleotide (Fig. 2A, B, lanes 2, 4; fig. 4). Competition with the 19 bp repeat oligonucleotide defined the proteins binding to the four complete 19 bp repeats as belonging to one group (Fig. 2A, B, lanes 2, 5; fig. 4). Competition with the unique binding site oligonucleotide revealed the already known binding site at nt −440 and an additional one at nt −220 (Fig. 2A, B, lanes 2, 6; fig. 4). The finding that the unique oligonucleotide could also compete for 17 bp repeat binding protein (Fig. 2A, B, lanes 6) may be due to the 'TGGC' sequence at nt −426, which seems sufficient to bind a 17 bp related protein. Competition with the AP1 oligonucleotide revealed one AP1 binding site at nt −233. The AP1 consensus site at nt −174 was not protected (Fig. 2A, B, lanes 2, 7; fig. 4). The already described footprint at nt −90 (26) and the footprint from nt −62 to nt −35 were not competed for by the different oligonucleotides and are designated X and Y respectively (Figs. 2B, 4). Footprint Y, though, probably extends an already described footprint from nt −65 to nt −50 (26).

Using the DNAase I protection competition assay we identified several overlapping binding sites, with the most pronounced ones located between nt 460 and nt −420. Competition with the unique binding site oligonucleotide extinguished the footprint at nt −440, but resulted in better protection of the neighboring 19 bp repeat at nt −460. Vice versa, competition with the 19 bp oligonucleotide extinguished the 19 bp footprint, but yielded in better protection over the unique region. Competition for binding to juxtaposed sites was most clearly seen in the presence of both oligonucleotides. The band at nt −444 was totally deprotected only upon competition with both the unique and 19 bp repeat oligonucleotides (Fig. 2A, lanes 1, 2, 5, 6, and 15).

Protection with IM 9 B cell proteins. Clear binding to only one 17 bp repeat (at nt −360) was detected also in B cells (Fig. 3A, lane 3). The 19 bp repeats, the two unique binding sites, the AP1

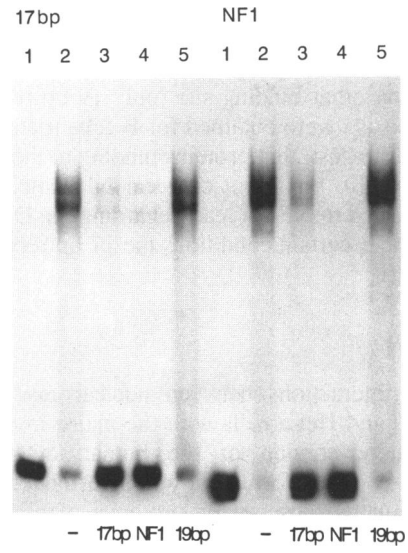

Fig. 5. Binding of nuclear proteins from HeLa cells to the 17 bp repeat and to an NF1 consensus site. Labeled restriction fragments containing the 17 bp repeat and an NF1 site were incubated with crude nuclear extracts from HeLa cells and a 40-fold molar excess of unlabeled restriction fragments containing the 17 bp repeat or the 19 bp repeat from HCMV or an NF1 consensus site as indicated. The resulting protein-DNA complexes were separated in a 4% polyacrylamidegel. Lanes 1: no protein added, lanes 2 to 5: with nuclear protein; lanes 3: competition with the 17 bp repeat, lanes 4: competition with the NF1 site, lanes 5: competition with the 19 bp repeat.

site, and the Y box were clearly protected also by B cell proteins (Figs. 3, 4). In contrast to the situation with HeLa cell proteins, however, we observed strong protection of all four 18 bp repeats with B cell proteins (Fig. 3A, B, lanes 2, 4). Specificity of binding was shown by competition with the respective oligonucleotides (Figs. 3, 4).

Similar to HeLa cell proteins, binding of B cell proteins to certain sites in the enhancer was more pronounced in the presence of oligonucleotides spanning juxtaposed binding sites. Although

these effects are weak, they deserve attention. The intensity of protection of the 19 bp element at nt −140 increased upon competition with the 18 bp repeat oligonucleotide (Fig. 3B, lane 4; fig. 4). A footprint around the area of the X box at nt −90 seen with HeLa extract, similarly appeared with B cell proteins after titrating κB proteins from their binding site around nt −100 with the 18 bp repeat oligonucleotide (Fig. 3B, lane 4; fig. 4). The protein(s) responsible for the footprint, however, may be different from the HeLa cell protein(s). The protection of the 19 bp repeat at nt −320 was not very strong, but became clear upon competition with the 18 bp repeat oligonucleotide (Fig. 3A, lanes 2, 4; fig. 4). This 19 bp repeat has no immediate 18 bp repeat neighbors, suggesting that there might be interactions of proteins which are not next to each other on the linear DNA.

The 17 bp repeat binding proteins bind also to NF1 sites

17 bp repeat binding proteins seem to be major constituents of the nucleoprotein complex on the HCMV enhancer in HeLa cells. Since the 17 bp repeat is related to the binding site for NF1, we tested whether there was a cross competition for proteins between these binding sites. Complexes of HeLa cell proteins with the 17 bp repeat were diminished by competition with 40 fold excesses of unlabeled 17 bp repeat and NF1 site (Fig. 5), but not with any other of the binding sites used (only 19 bp repeat shown). Also complexes on the NF1 site were diminished by competition with the 17 bp repeat and extinguished with the NF1 site, but not any other binding site (only 19 bp repeat shown). Comparable results were obtained for B cells (data not shown). Therefore, we suggest, that proteins binding to the 17 bp repeat of HCMV can also bind to a classical NF1 site. Thus, these proteins are related to NF1 at least regarding the DNA sequence specificity. Under certain conditions the 17 bp repeat may even bind NF1.

DISCUSSION

We analysed interactions between nuclear proteins from B lymphoid cells and HeLa cells with the major immediate early enhancer/promoter region of the human cytomegalovirus. Nucleoprotein complex formation was investigated by DNAase I footprinting in the absence or presence of oligonucleotides spanning protein binding sites from the HCMV enhancer. In addition, the contribution of CRE (19 bp repeat) and κB (18 bp repeat) elements to transcriptional activity from the IE promoter was tested by transient expression assays. Taken together, it is clear that due to qualitative differences in protein binding between cell types alternative forms of nucleoprotein complexes can arise on the densely packed binding sites of the IE enhancer.

Cell specific differences

The κB element (18 bp repeat) was almost inactive in HeLa cells, but had strong activity in B cells. These in vivo results partly correlated with in vitro binding of nuclear proteins to the enhancer/promoter. The 18 bp repeat element formed sequence specific complexes only with proteins from B cells (for summary see Fig. 4). Previous work from this laboratory had shown partial protection of the 18 bp repeat (1, 26), and a strong contribution of the 18 bp element to in vitro transcriptional activity with HeLa cell protein (20). These apparent discrepancies may be due to differences in extract preparation and fractionation. They may also be explained by proteins, non identical to NF-κB, whose binding sites overlap with the 18 bp repeats, and are now recognized as overlapping sites. These sites are for example the 18 bp repeat around nt −100 with the X box around nt −90, or the 18 bp repeat around nt −420 with its neighboring sites. The weak activity of the 19 bp repeat in B cells cannot be explained by the lack of proteins binding to this sequence. It is possible, that the binding proteins are in an inactive state, whereas the corresponding proteins from HeLa cells are active. A protein like CREB, with its different phosphorylation states, could explain these results (27). This is supported by experiments, showing that CAT expression from plasmid $p19_6$CMVCAT increased only marginally in HeLa cells after cAMP stimulation, but approached the expression level from the enhancer plasmid in non stimulated B cells (data not shown). The differences in the protection of the 17 bp repeat in both cell types is probably due to lower amounts of 17 bp binding protein in B cells. Mobility shift experiments with B cell proteins corresponding to the HeLa experiment of figure 5 show, besides a similar cross competition, an overall weaker protein binding (data not shown). On the other hand, this difference may be due to structural restraints of the nucleoprotein complex on the enhancer with B cell proteins. At this point it is not clear that proteins from the two cell types binding to corresponding sites are the same or related. Further experiments are necessary to answer this question.

Crossrecognition of binding sites by proteins binding to 17 bp repeats and NF1 sites

The 17 bp repeat sequence is related to the NF1 binding site, and those two sequences showed cross competition for their respective binding proteins. This finding reopens the question whether the 17 bp repeats and several more protein binding sites in the enhancer do bind proteins related to the NF1 family of transcription factors. This is supported by work from Sippel's group (28, 7), which shows, that the sequence 'TGGCA' (related to the NF1 half site) can bind an NF1 related protein. The NF1 half site 'TGGCA' in the 17 bp repeats and related sequences in several more binding sites of the enhancer thus would seem sufficient to bind NF1 like proteins, although we do not exclude, that the complete 17 bp repeat in the context of the intact enhancer sequence might bind additional proteins. Earlier work (29) demonstrated binding of NF 1 proteins to the upstream region and one binding site in the downstream region, but not in the enhancer. This apparent contradiction may be explained by different affinities of the 17 bp repeats and the NF1 sites for NF1-like proteins and the higher sensitivity in our present experimental approach. Another study (30) investigating the NF1 binding to part of the HCMV IE1 upstream region does not exclude NF1 binding to the enhancer either. To proof or exclude, however, that the 17 bp binding protein is NF1 related, further experiments are required.

Competition for transcription factors reveals additional binding complexity to the enhancer

In vitro transcription in conjunction with competing oligonucleotides spanning enhancer binding sites had been used to identify transcription factor binding sites (20). We extended this approach and analyzed in vitro the formation of nucleoprotein complexes on the HCMV enhancer with HeLa and B cell proteins. Using this technique we could classify several protected regions as belonging to the 17 bp repeat, 18 bp repeat, 19 bp repeat, unique binding site, and AP1 families. The 19 bp repeats and AP1 site might share some binding protein (31), since the AP1 site binding was also competed for by the 19 bp

oligonucleotide. The protein complex on the AP1 site in the enhancer might be a heterodimer of one CREB- and one fos-related protein (32). The inability of the AP1 site at nt −174 to bind protein may be due to the sequence context of this site or in structural constraints of the protein complex on the enhancer, which may make it impossible for AP1 proteins to bind at this site.

It is clear, that complete protection of some binding sites can only be obtained in the absence of proteins binding to juxtaposed and overlapping binding sites. The differences in the occupation of the 18 bp repeats in the two cell types together with the presence of overlapping binding sites suggest, that this enhancer has several possibilities of forming functional nucleoprotein complexes. These possibilities may depend on the presence and abundance of certain transcription factors under the respective conditions in the cell like cell type, stimulation and growth stage.

ACKNOWLEDGMENTS

H.H.N. was supported by the Deutsche Forschungsgemeinschaft and by the Fogarty International Center, NIH. This work was supported in part by funds from the Intramural AIDS Targeted Antiviral Program from the Office of the Director of the National Institutes of Health.

We thank Henryk Lubon for stimulating discussions, Jay Nelson for the HCMV enhancer plasmid pFSCAT, Rüdiger Rüger for the HCMV enhancer plasmid pRR56/1 (here named pΔ1CAT), Richard Case (The Midland Certified Reagent Company, Texas) for a gift of 5′-biotinylated oligonucleotides, and William Jakoby for most generous support.

REFERENCES

1. Ghazal, P., Lubon, H., Fleckenstein, B., and Hennighausen, L. (1987) Proc. Natl. Acad. Sci. USA, **84**, 3658−3662.
2. Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) Cell, **41**, 521−530.
3. Foecking, M. K., and Hofstetter, H. (1986) Gene, **45**, 101−105.
4. Thomsen, D. R., Stenberg, R. M., Goins, W. F., and Stinski, M. F. (1984) Proc. Natl. Acad. Sci. USA, **81**, 659−663.
5. Stenberg, R. M., Thomsen, D. R., and Stinski, M. F. (1984) J. Virol., **49**, 190−199.
6. Hennighausen, L., Siebenlist, U., Danner, D., Leder, P., Rawlins, D., Rosenfeld, P., and Kelly, T. jr. (1985) Nature (London), **314**, 289−292.
7. Rupp, R. A. W., Kruse, U., Multhaup, G., Göbel, U., Beyreuther, K., and Sippel, A. E. (1990) Nucleic Acids Res., **18**, 2607−2616.
8. Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W. G., and Mocarski, E. S. (1989) EMBO J., **8**, 4251−4258.
9. Sen, R., and Baltimore, D. (1986) Cell, **47**, 921−928.
10. Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Bauerle, P. A., and Israel, A. (1990) Cell, **62**, 1007−1018.
11. Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D. (1990) Cell, **62**,1019−1029.
12. Bours, V., Villalobos, J., Burd, P. R., Kelly, K., and Siebenlist, U. (1990) Nature (London), **348**, 76−80.
13. Hennighausen, L., Furth, P. A., and Pittius, C. W. (1989) Nucleic Acids Res., **17**, 8197−9206.
14. Montminy, M. R., Sevarino, K., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. USA, **83**, 6682−6686.
15. Hunninghake, G. W., Monick, M. M., Liu, B., and Stinski, M. F. (1989) J. Virol., **63**, 3026−3033.
16. Stamminger, T., Fickenscher, H., and Fleckenstein, B. (1990) J. Gen. Virol., **71**, 105−113.
17. Chang, Y.-N., Crawford, S., Stall, J., Rawlins, D. R., Jeang, K.-T., and Hayward, G. S. (1990) J. Virol., **64**, 264−277.
18. Niller, H. H., and Hennighausen, L. (1990) J. Virol., **64**, 2388−2391.
19. Dynan, W. S., and Tjian, R. (1985) Nature (London), **316**, 774−778.
20. Ghazal, P., Lubon, H., and Hennighausen, L. (1988) Mol. Cell. Biol., **8**, 1809−1811.
21. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol., **2**, 1044−1051.
22. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich P., and Karin, M. (1987) Cell, **49**, 729−739.
23. Bunce, C. M., Thick, J. A., Lord, J. M., Mills, D., and Brown, G. (1988) Anal. Biochem., **175**, 67−73.
24. Hennighausen, L., and Lubon, H. (1987) Methods Enzymol., **152**, 721−735.
25. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol., **65**, 499−560.
26. Ghazal, P., Lubon, H., and Hennighausen, L. (1988) J. Virol., **62**, 1076−1079.
27. Yamamoto, K. K., Gonzalez, G. A., Biggs W. H. III, and Montminy, M. R. (1988) Nature (London), **334**, 494−498.
28. Nowock, J., Borgmeyer, U., Puschel, A. W., Rupp, R. A. W., and Sippel, A. E. (1985) Nucleic Acids Res., **13**, 2045−2061.
29. Hennighausen, L., and Fleckenstein, B. (1986) EMBO J., **5**, 1367−1371.
30. Ghazal, P., Lubon, H., Reynolds-Kohler, C., Hennighausen, L., and Nelson, J. A. (1990) Virology, **174**, 18−25.
31. Hai, T., Liu, F., Allegretto, E. A., Karin, M., and Green, M. R. (1988) Genes Dev., **2**, 1216−1226.
32. Hoeffler,J. P., Deutsch, P. J., Lin, J., and Habener, J. F. (1989) Mol. Endocrinology, **3**, 868−880.