Analysis of proteins binding to the proximal promoter region of the human cytomegalovirus IE-1/2 enhancer/promoter reveals both consensus and aberrant recognition sequences for transcription factors Sp1 and CREB

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ABSTRACT

Expression of the immediate early 1 and 2 gene (IE-1/2) of human cytomegalovirus, an important pathogen in immunosuppressed patients, is controlled by a strong enhancer/promoter. To define the promoter domain within this large *cis*-active region of about 550 nucleotides, DNA-protein interactions were studied. DNase I footprinting experiments using procaryotically expressed transcription factor Sp1 revealed an extensive interaction of this transcription factor with both consensus and aberrant recognition elements within the IE-1/2 promoter region. Protection of these Sp1 binding sites could also be observed when nuclear extracts prepared from HeLa cells and permissive human fibroblast cells were used. After *in vitro* mutagenesis of Sp1 targets and transient expression of mutagenized CAT-expression plasmids, however, no significant reduction in CAT activities was found. By analyzing a series of 5' deletion mutants of the IE-1/2 promoter region, a strong *cis*-acting element was localized between nucleotides –94 and –78, upstream of sites that interact with Sp1. Gel retardation experiments demonstrated binding of recombinant transcription factor CREB to this motif which reveals it as an aberrant CREB recognition sequence. Thus, this study identifies several previously unknown binding sites for transcription factors Sp1 and CREB within the proximal promoter region of the IE-1/2 gene, which differ markedly in their relevance for constitutive promoter function.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the β -subgroup of herpesviruses, is of major clinical importance in immunosuppressed patients such as transplant recipients or patients suffering from AIDS (1). The gene expression of HCMV is regulated in a cascade that can be subdivided into three phases,

commonly referred to as immediate early (IE), early (E) and late (L). Some of the proteins expressed at IE times of infection, for example the 82 kd IE-2 protein of HCMV, are required for activation of promoters of the subsequent temporal class (2). Thus, these proteins seem to exert a crucial role to initiate the viral replicative cycle. Herpesviruses have evolved divergent strategies to express these regulatory genes efficiently. Herpes simplex virus, for example, belonging to the subclass of α -herpesviruses, carries a protein within the viral particle that *trans*-activates IE promoters after the virus has entered the cell (3). In contrast, all β -herpesviruses investigated in detail so far utilize strong transcriptional enhancer elements to initiate IE gene expression (4–6). In the genome of human cytomegalovirus which is the prototype of the subclass of β -herpesviruses, this enhancer/promoter element is located upstream of the immediate early 1 and 2 (IE-1/2) gene region (UL123–122) (7) that shows a complex regulation during the viral replicative cycle involving both transcriptional and post-transcriptional mechanisms (8–13). The high degree of conservation of the enhancer/promoter sequence between different wild-type strains suggests that this element plays an essential role for initiation of viral gene expression (14). The complex modular structure is one feature of the HCMV enhancer/promoter which approximately extends to 550 nucleotides (nt) upstream of the IE-1/2 transcription start. Four types of repetitive sequence elements with 17, 18, 19, and 21 bp, respectively, that are overlapping in part, contribute to its complexity (15). DNase I footprinting experiments revealed the formation of a large nucleoprotein complex on the enhancer region, thus suggesting a high density of protein binding sites (16). By gel retardation assays and functional studies transcription factors could be identified that bind to some of those repetitive motifs. The 19 bp motif, for example, acts as a binding site for ATF/CREB factors and confers c-AMP responsiveness to the enhancer (17). The 18 bp repeat is involved in stimulation of enhancer activities by the phorbol ester TPA and seems to associate with factors of the NF- κ B family (18). Although it could be demonstrated that these factors bind to isolated motifs,

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the presence of overlapping target sites may influence the binding pattern as has been demonstrated recently (19). Therefore, the knowledge about the interaction of defined transcription factors in the context of the HCMV enhancer/promoter sequence is limited at present.

This study was aimed to define protein binding sites within the promoter domain of the IE-1/2 enhancer/promoter. Although it is not possible to separate the enhancer from the promoter domain precisely, previous expression studies using two 5' deletion constructs of the IE-1/2 enhancer/promoter suggested that enhancer functions are located upstream of -65, whereas the promoter extends between +7 and -65 (20). Several potential protein binding sites are contained within the IE-1/2 promoter region including two GC-box motifs as consensus binding sites for transcription factor Sp1 (21), a half site of the 19 bp palindrome as contained within the enhancer domain and a sequence resembling a CCAAT-box. Previous studies, however, using DNase I protection analysis in conjunction with fractionated HeLa cell extracts, detected only weak interaction of nuclear proteins with this region (20). Here we show that transcription factor Sp1 interacts extensively with several consensus and aberrant recognition sequences of the IE-1/2 promoter region. In contrast to a number of other promoter systems, functional studies with both mutagenized sequences and 5' deletion constructs could not detect a significant contribution of Sp1 binding sites to the constitutive activity of the IE-1/2 promoter region. However, a strong *cis*-acting element between -94 and -78 was identified, that bound prokaryotically expressed transcription factor CREB in gel retardation assays. Thus, the high density of protein binding sites as reported for the enhancer domain seems to be present also in the IE-1/2 promoter region with strong functional differences between individual elements.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized by the phosphoramidite procedure using a Cyclone DNA synthesizer (Biosearch, San Rafael, Ca., U.S.A.). The following oligonucleotides (5' to 3' sequences, numbers refer to nucleotide positions relative to the IE-1/2 cap site, double stranded oligonucleotides are indicated by //) were used for cloning, PCR, site directed mutagenesis, or for competition in DNase I footprinting experiments: -77/-15 EP: ACTCGAGAAGCTTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA-TAAGCAGAGCT//CTGCTTATATAGACCTCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGGCGGAA-GCTTCTCGAGTAGCT; -92/-63 EP: CGAGAAATGTCGTAACCACTCCGCCCCATTGACG//TCGACGTCAATGGGGCGGAGTTGTTACGACATTTCTCGAGCT; -62/-44 EP: AGCTTAGATCTCAAAATGGGCGTAGGCGTG//TCGACACGCCTACCGCCCATTTGAGATCTA; -94/-78 EP: C-GAGCAAAATGTCGTAACAACG//TCGACGTTGTTACGACATTTTGTCTCGAGCT; SV40-Sp1: AGCTTAGATCTGA-ACTGGGCGGAGTTAGGG//TCGACCCTAACTCCGCCCA-GTTCAGATCTA; Oct: AGCTTAGATCTGGTAATTTGCA-TTTCTAAG//TCGACTTAGAAATGCAAAATTACCAGATCTA; 19bp: CGAGCCCATTGACGTCAATGGGG//TCGACCCCATTGACGTCAATGGGCTCGAGCT; IVM-74: TAACAACTCCCCCCCATTGA; IVM-54: CAAATGGGGGGTA-

GGCGTGT; CREB-A: GCATCGTCGACGATGACCATGG-ACTCTGGAGC; CREB-B: ATTCTAAGCTTAATCTGACT-TGTGGCAGTAAAGG.

Plasmid constructions

Construction of plasmid pRR55 has been described previously (22). Plasmids pRR56/3, pRR56/4 and pRR56/5 (17) containing IE-1/2 enhancer/promoter sequences from -187 to +52, -94 to +52 and -65 to +52 in front of the CAT reporter gene, respectively, were obtained from R. Rüger (Erlangen). Cloning reactions were performed according to standard procedures (23). To obtain a series of 5' deletions of the IE-1/2 promoter region, the inserts of plasmids pRR56/4 and pRR56/5 were cloned as *Hind*III - *Bam*HI fragments into plasmid pHM72 which contains restriction sites for *Sac* I, *Hind* III, *Cla* I and *Bam* HI in front of the CAT gene in the BlueScribe vector (Stratagene, Heidelberg, Germany). The resulting constructs were designated p-94/+52EPCAT and p-65/+52EPCAT, respectively. To create p-76/+52EPCAT, plasmid p-65/+52EPCAT was digested with the restriction enzyme *Sac* I, which cleaves in the multiple cloning site of the vector and at -14 within the IE-1/2 enhancer/promoter. The vector fragment was ligated to the oligonucleotide -76/-15 EP. Plasmid p-14/+52EPCAT was generated by self-ligation of the vector fragment of p-66/+52EPCAT. The construction of deletion plasmids p-57/+52EPCAT and p-38/+52EPCAT has been achieved by cloning *Hind* III - *Hpa* II fragments of plasmids p388152 and p388301, respectively, into the CAT expression vector pHM72 which had been digested with *Hind* III and *Cla* I. Plasmids p388152 and p388301 had been created by *Bal* 31 shortening of IE-1/2 enhancer/promoter sequences (24). The insertion of oligonucleotide -94/-78EP into the CAT expression vector puPCAT (17) followed by dimerization of the cloned sequence was performed as described previously (17). The coding sequences of transcription factor CREB were amplified from HeLa cell RNA by RT-PCR using primers CREB-A and CREB-B as described (12) and subsequently cloned into the prokaryotic expression vector pROS (25). All plasmid constructs were confirmed by DNA sequence analysis using a commercially available T7 sequencing kit (Pharmacia, Freiburg, Germany). Analysis of the amplified CREB c-DNA revealed that it corresponds in sequence to CREB-A as described by Berkowitz and Gilman (26).

Mutagenesis

In vitro mutagenesis of GC-box elements within the IE-1/2 promoter region was performed according to Kunkel (27) using the Muta-gene M13 *in vitro* mutagenesis kit (Bio-Rad Laboratories, München, Germany). DNA of plasmid p-94/+52EPCAT was transformed into *E. coli* CJ236 and single stranded, uracil containing DNA was harvested after superinfection with the helper phage M13 KO7. After phosphorylation, oligonucleotides IVM-74 and IVM-54 were either used singly or in combination for synthesis of the mutagenic DNA strand. Annealing conditions, reactions for synthesis of the complementary DNA strand and transformation of *E. coli* MV1190 were performed as described by the manufacturer.

Nuclear extract preparation and preparation of procaryotic expressed Sp1 and CREB

Nuclear extracts of 10 g of HeLa cells and 1 g of primary human fibroblast cells were prepared as described previously (22).

Transcription factor Sp1 was expressed in *E. coli* JK50 using the Sp1 expression vector Sp1-778c (kindly provided by A. Courey and R. Tjian, University of California, Berkeley) and extracted as described by Kadonaga *et al.* (28,29). Transcription factor CREB was also expressed in *E. coli* JK50 as a lacZ fusion protein and partially purified as described by Urban *et al.* (30) except that after solubilization of the protein the urea was removed by dialysis against buffer Z (20 mM HEPES, pH 8.0, 1 mM MgCl₂, 20 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1 mM EDTA, 20% glycerol). As negative control in DNase I

footprinting and gel retardation experiments extracts of *E. coli* JK50 containing only the vectors used for expression of Sp1 (BlueScript) and CREB (pROS) were prepared in parallel.

DNase I protection and gel retardation analysis

DNase I protection analysis was performed using a modification of the protocol reported by Bohmann *et al.* (31). The *Hind* III–*Sac* I fragment of plasmid pRR56/3 (containing IE-1/2 enhancer/promoter sequences between –187 and –15) or the *Hind* III–*Xba* I fragment of plasmid pRR56/4 (containing IE-1/2 enhancer/promoter sequences between –94 and +52) were used as probes. Fragments were labeled by filling in with Klenow fragment at the *Hind* III (non-coding strand) or *Xba* I sites (coding strand) or by dephosphorylation at the *Hind* III site (coding strand) followed by treatment with polynucleotide kinase in the presence of γ -³²P-dATP. Protein extracts (5 μ l, 5–10 μ g/ μ l) were preincubated with 1 μ g of poly(dI)-poly(dC) (Pharmacia, Freiburg, Germany) on ice for 10 min. In control reactions, extract was substituted by 5 μ l of protein dialysis buffer (buffer Z, see above). For competition experiments 50–200 ng of specific, double stranded competitor oligonucleotide were included. Reactions (10 μ l) contained 10 mM HEPES, pH 7.9, 20 mM KCl, 4 mM MgCl₂, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM DTT and 10% glycerol. End-labeled DNA (20,000 cpm) was added within 2 μ l of H₂O and incubation continued for 10 min. 2 μ l of DNase I (Boehringer), freshly diluted in cold H₂O to concentrations between 5 ng/ μ l and 200 ng/ μ l, were then added and the reactions were incubated for 90 s at 20°C. DNase I digestion was stopped by the addition of 100 μ l of stop buffer containing 0.2 M NaCl, 1% SDS, 20 mM EDTA, 50 μ g/ml tRNA and 2.5 μ g/ml proteinase K. After a 15 min period at 37°C, 200 μ l of phenol/chloroform/isoamylalcohol (25:24:1) were added. The aqueous phases were re-extracted with chloroform/isoamylalcohol (24:1), ethanol precipitated and loaded onto 6% acrylamide-urea sequencing gels. For localization of protected regions a Sanger sequencing reaction was run in parallel. Gel retardation assays were performed as described (17).

Transfection and CAT-assays

Transfection of HeLa cells and primary human foreskin fibroblasts was performed by the calcium phosphate co-precipitation procedure as described previously (22). Cells were harvested 48 h post-transfection and CAT activity was assayed as described (22). After autoradiography, acetylated and non-acetylated forms of ¹⁴C-chloramphenicol were quantified by liquid scintillation counting. Each transfection was repeated at least three times and representative results are shown.

RESULTS

Sp1 binds to consensus and aberrant recognition sequences at the IE-1/2 promoter of human cytomegalovirus

The IE-1/2 promoter region of human cytomegalovirus contains two GC-box elements (GGGCGG) that are known as consensus binding sites for transcription factor Sp1. To test whether these sequence elements act as target sites for Sp1 in the context of the IE-1/2 promoter, DNase I footprinting experiments were performed. Procaryotically expressed Sp1 was used as a source of protein to be able to study protein-DNA interactions of an isolated factor in the absence of any other eukaryotic DNA binding protein that might influence the observed protection pattern. For this experiment, the *Hind* III–*Sac* I DNA fragment

Fig. 1. DNase I protection analysis of the IE-1/2 promoter region with procaryotically expressed transcription factor Sp1. A) and B) The *Hind* III–*Sac* I fragment of plasmid pRR56/3 (enhancer/promoter sequences from –187 to –15) labeled at the upper strand and lower strand, respectively, was used as probe. Lanes 1, no added extract; lanes 2 and 3, 5 μ l of *E. coli* extract containing Sp1; lanes 4, 5 μ l of *E. coli* extract without Sp1. C) The *Hind* III–*Xba* I fragment of plasmid pRR56/4 (enhancer/promoter sequences from –94 to +52) labeled at the upper strand was used as probe. Lane 1, no added extract, lane 2, 5 μ l of *E. coli* extract containing Sp1; lane 3, 5 μ l of a 1 : 10 dilution of *E. coli* extract containing Sp1; lane 4, 5 μ l of a 1 : 100 dilution of *E. coli* extract containing Sp1; lane 5, 5 μ l of a 1 : 1000 dilution of *E. coli* extract containing Sp1; lane 6, 5 μ l of a 1 : 10000 dilution of *E. coli* extract containing Sp1; lane 7, 5 μ l of *E. coli* extract without Sp1. D) DNA sequence of the IE-1/2 promoter region between –159 and –20. Sequences protected from DNase I cleavage by Sp1 are marked by white letters on black background. Detected footprints (filled bars) and descriptions of the end-labeled DNA fragments (open rectangles indicate repeat motifs of the enhancer/promoter, brackets indicate the location of GC-boxes or G-rich elements) are shown diagrammatically on the right of the autoradiographs: TATA, TATA-box; GC, GC-box; GT, G-rich element; 18, 18 bp repeat element; 19, 19 bp repeat element; 19*, truncated 19 bp element. Numbers refer to nucleotide positions of the presented sequence relative to the IE-1/2 transcription start.

of plasmid pRR56/3 spanning nucleotides -187 to -15 of the IE-1/2 enhancer/promoter was labeled at the 5' end of the upper strand and incubated with *E. coli* extracts with or without Sp1. Proteins were allowed to bind to the promoter and DNase I was used to digest the unprotected phosphodiester backbone of DNA. After size fractionation of the resulting fragments in denaturing polyacrylamide gels and autoradiography several protected regions were visible in reactions that contained Sp1 (Fig. 1, A, lanes 2 and 3). No protection was detectable when bacterial extract lacking Sp1 was used (Fig. 1, A, lane 4). Protection was observed from -63 to -28 and from -85 to -66 relative to the IE-1/2 transcription start. These areas clearly cover the two GC-box elements at -54 and -74 of the IE-1/2 promoter. An additional strong protection was located between -158 and -139 overlapping a 19 bp repeat element of the IE-1/2 enhancer. Sequence inspection revealed a G-rich element in the center of this protection that, however, differed from the Sp1 consensus by substitution of the central C of the GC-box by T (Fig. 1, D). To confirm the observed protection pattern, footprinting reactions were performed with the same DNA fragment labeled at the lower strand by filling in the *Hind* III site with Klenow fragment. As shown in Fig. 1, B, identical regions were found to interact with Sp1. Whereas approximately 20 nt of DNA were protected from DNase I cleavage by binding of Sp1 to the G-rich element at -148 and the -74 GC-box, the protection at the -54 GC-box covered more than 35 nucleotides. To clarify whether additional Sp-1 target sites are located in this region, DNase I footprinting was done by using the *Hind* III-*Xba* I fragment of plasmid pRR56/4 that was labeled at the *Xba* I site (upper strand). This fragment spanned nucleotides -94 to $+52$ of the IE-1/2 promoter and allowed therefore a more precise analysis of the protein binding pattern in the vicinity of the IE-1/2 transcription start. Protection by Sp1 at the -54 GC-box was found to extend to the IE-1/2 TATA-box where a G-rich element (GGTGGGAGG) was located immediately upstream of the TATA-box (Fig. 1, C, D). In this experiment several dilutions of the Sp-1 protein were used in order to analyse the binding site occupancy at different protein concentrations. However, this did not reveal major differences in the affinity of Sp1 to their respective target sequences within the IE-1/2 promoter region (Fig. 1, C). Therefore, four binding sites for transcription factor Sp1 could be identified within the IE-1/2 enhancer/promoter region between -180 and $+50$ with about equal binding affinities. Whereas two of these sites conform to the consensus GGGCGG, the other two targets differ in having a central T instead of a C.

DNase I protection analysis with HeLa cell nuclear extracts reveals a complex protection pattern with overlapping binding sites for Sp1 and ATF/CREB factors

The human cytomegalovirus IE-1/2 enhancer has a high density of potential protein binding sites. As competition of transcription factors for some of these sites has been shown to occur (19), the presence of a complex mixture of DNA binding proteins may influence binding site occupancy. Therefore, we asked the question whether we can observe protection of Sp1 binding sites within the IE-1/2 promoter region when using crude nuclear extracts. First, we decided to prepare nuclear extracts from HeLa cells because Sp1 is known to be abundantly present in HeLa cells (21). The *Hind* III-*Sac* I fragment of plasmid pRR56/3 labeled at its 5' end was used as probe in DNase I protection experiments. Procarotically expressed Sp1 was analysed in parallel to be able to correlate binding sites of crude nuclear

extracts with binding sites of isolated Sp1 (Fig. 2, A, lane 2). Competition experiments were performed to further demonstrate the specificity of Sp1 binding. When an oligonucleotide corresponding to a known Sp1 binding site of the SV40 promoter (SV40-Sp1) was preincubated with *E. coli* extract containing Sp1, protection was not observed in DNase I footprinting reactions (Fig. 2, A, lane 3). Nuclear extracts of HeLa cells gave a rather complex profile of protection and DNase I hypersensitive sites

Fig. 2. DNase I protection analysis of the IE-1/2 promoter region with HeLa cell nuclear extracts. The *Hind* III-*Sac* I fragment of plasmid pRR56/3 (enhancer/promoter sequences from -187 to -15) was used as probe. A) Lanes 1, 5 and 9, no added extract; lanes 2 and 3, $5 \mu\text{l}$ of *E. coli* extract containing Sp1; lane 4, $5 \mu\text{l}$ of *E. coli* extract without Sp1; lanes 6 to 8, $5 \mu\text{l}$ of HeLa cell nuclear extract; lanes 3 and 8, competition with 100 ng of the SV40-Sp1 oligonucleotide. B) Lanes 1, 4 and 15, no added extract; lanes 2 and 3, $5 \mu\text{l}$ of *E. coli* extract containing Sp1; lanes 5 to 14, $5 \mu\text{l}$ of HeLa cell nuclear extract; lane 3, competition with 100 ng of the SV40-Sp1 oligonucleotide; lanes 6 to 8, competition with 50, 100, and 200 ng, respectively, of the oct oligonucleotide; lanes 9 to 11, competition with 50, 100 and 200 ng, respectively, of the SV40-Sp1 oligonucleotide; lanes 12 to 14, competition with 50, 100, and 200 ng, respectively, of the 19 bp repeat oligonucleotide. Diagrams of the end-labeled DNA fragment are shown on the right of the autoradiographs (for a detailed description see figure 1).

with varying intensity on the IE-1/2 promoter region (Fig. 2, A, lanes 6, 7). Strong protection was observed for the -54 and -74 GC-box. The region upstream of these two GC-boxes was also protected. Two prominent DNase I hypersensitive sites were found at -160 and -126. Between those sites a strong protection which corresponded to the aberrant Sp1 binding element at -148 and a moderately protected region which corresponded to one half of the 19 bp repeat element between -147 and -129 were identified. When competition was performed with the SV40-Sp1 oligonucleotide a change of the protection pattern and of the DNase I hypersensitive sites occurred (Fig. 2, A, lane 8). Whereas part of the G-rich element protection disappeared, the 19 bp element between -147 and -129 was more completely protected. This was accompanied by a loss of DNase I hypersensitivity at -126. Additionally, no protection was now observed at the -54 and -74 GC-box whereas protection of the region between -125 and -90 became more intensive. To further analyze binding site occupancy at the -148 G-rich element we performed a footprint competition experiment with several specific competitor oligonucleotides. As shown in figure 2, B, lanes 6 to 8, there was no significant change in the footprinting profile obtained with HeLa extracts after competition with increasing amounts of an oligonucleotide that corresponded in sequence to an octamer factor binding site (oct oligonucleotide). The SV40-Sp1 oligonucleotide elicited the same alteration of the protection pattern as described above (Fig. 2, B, lanes 9-11). However, when the 19 bp oligonucleotide which has been previously shown to bind proteins of the ATF/CREB family of transcription factors (17) was used, the protection that covered the 19 bp repeat element was removed (Fig. 2, B, lanes 12-14). Thus, at least two distinct transcription factors, Sp1 and ATF/CREB, compete for binding in the region between -160 and -125.

Sp1 binding sites are also protected with nuclear extracts from permissive human fibroblasts

The HeLa cell system does not allow replication of human cytomegalovirus. Therefore, nuclear extracts from permissive human foreskin fibroblast were prepared and used in DNase I protection experiments with the *Hind* III-*Sac* I fragment of plasmid pRR56/3 as probe. Complete protection could be observed at the -54 and -74 GC-box (Fig. 3, A, lanes 2 and 3). Immediately upstream was a strong binding site from nucleotides -127 to -89 that covered the 18 bp repeat element between nt -109 to -92. In contrast to HeLa cell extracts, the 19 bp element between -147 and -129 and the G-rich element at -148 were not protected efficiently with HFF extracts, however, strong DNase I hypersensitive sites were located there. When competition was performed with the SV40-Sp1 oligonucleotide or oligonucleotides -92/-63EP and -62/-44EP (Fig. 3, B, lanes 6, 4, 3, respectively) which contained the GC-box elements of the IE-1 promoter, protection over the -74 and -54 GC-box decreased. No effect was observed on the binding of factors to the region between -127 and -89. As well, competition with the 19 bp and the oct oligonucleotide (Fig. 3, B, lanes 5 and 2) did not alter the footprint pattern of this DNA fragment. Thus, the protection profile obtained with HFF extracts differs from that with HeLa extracts mainly in the region upstream of nucleotide -89. Within HFF extracts, Sp1 seems to bind also to the GC-boxes of the IE-1/2 promoter, however not to the G-rich element at -148.

Sp1 binding sites can be mutated without altering the constitutive activity of the IE-1 promoter domain

Previous studies had shown that the IE-1/2 promoter domain between nucleotides -94 and +52 exerts a high constitutive activity when tested in transient expression assays in HeLa cells (20). To analyse the contribution of Sp1 binding sites to this effect, site-directed mutagenesis of the two GC-boxes within the IE-1/2 promoter was performed in order to abolish binding of Sp1 to each of the two sites. GC-box mutants were generated separately (M-54 and M-74) and in combination (M-54/-74) (Fig. 4, A). Base changes were introduced into the fourth position of the consensus binding site, thus changing the sequence from GGGCGG to GGGGGG. This mutation was previously shown to lower the Sp1 binding affinity 30-fold *in vitro* (32).

To demonstrate that this mutation had its intended effect on Sp1 binding, DNase I footprinting was performed using the wild-type sequence and the double mutant sequence (M-54/-74) as binding substrates. As expected, protection was severely diminished in case of the doubly mutated template M-54/-74 (Fig. 4, B, lane 4) when compared to the wild-type DNA (Fig. 4, B, lane 2).

Fig. 3. DNase I protection analysis of the IE-1/2 promoter region with nuclear extracts from primary human fibroblast cells. The *Hind* III-*Sac* I fragment of plasmid pRR56/3 (enhancer/promoter sequences from -187 to -15) was used as probe. A) Lane 1, no extract added; lanes 2 and 3, 5 μ l of HFF cell nuclear extract. B) Lane 1, no extract added; lanes 2 to 8, 5 μ l of HFF cell nuclear extract; lane 2, competition with 100 ng of the oct oligonucleotide; lane 3, competition with 100 ng of the -62/-44EP oligonucleotide; lane 4, competition with 100 ng of the -92/-63 oligonucleotide; lane 5, competition with 100 ng of the 19 bp repeat oligonucleotide; lane 6, competition with 100 ng of the SV40-Sp1 oligonucleotide. Diagrams of the end-labeled DNA fragment are shown on the right of the autoradiographs (for a detailed description see figure 1).

Fig. 4. Expression analysis of IE-1/2 promoter-CAT constructs after *in vitro* mutagenesis of GC-box elements. A) DNA sequence of GC-box mutants of the IE-1/2 promoter. WT, wild-type sequence; M-54, sequence of the -54 GC-box mutant; M-74, sequence of the -74 GC-box mutant; M-54/-74, sequence of the -54/-74 GC-box double mutant. Lanes 1, 5, 9, 13, A-reaction; lanes 2, 6, 10, 14, C-reaction; lanes 3, 7, 11, 15, G-reaction; lanes 4, 8, 12, 16, T-reaction. B) DNase I footprinting of the wild-type (WT) and the double mutant (M-54/-74) IE-1/2 promoter with *E. coli* extract containing Sp1. Lanes 1 and 3, no extract added; lanes 2 and 4, 5 μ l of *E. coli* extract containing Sp1 were added. C) CAT assay of wild-type and mutant IE-1/2 promoter-CAT constructs after transient expression in HeLa (lanes 1 to 4) and HFF cells (lanes 5 to 8). Lanes 1 and 5, wild-type plasmid p-94/+52EPCAT was used for transfection; lanes 2 and 6, GC-box mutant M-54 was used for transfection; lanes 3 and 7, GC-box mutant M-74 was used for transfection; lanes 4 and 8, GC-box double mutant M-54/-74 was used for transfection.

Wild-type CAT-expression plasmid p-94/+52EPCAT (which contains sequences between -94 and +54 relative to the IE-1/2 transcription start) together with the single mutants M-54 and M-74 and the double mutant M-54/-74 were then transfected into both non-permissive HeLa cells and permissive HFFs. After 48 h of transient expression CAT activities were determined. As shown in figure 4, C, no significant difference in activities could be observed between wild-type and mutant CAT expression vectors. This experiment was performed several times. As well, CAT reactions in which lower levels of CAT acetylation occurred did not reveal any difference (data not shown). Thus, the GC-box elements within the IE-1/2 promoter can be mutated without altering the constitutive activity of this domain in both HeLa and HFF cells.

5' deletion mutagenesis of the IE-1/2 promoter domain detects a strong *cis*-acting element located between -94 and -78

To further sub-localize *cis*-acting elements that mediate the strong constitutive activity of the IE-1/2 promoter domain, a series of 5'-promoter deletion plasmids containing the CAT gene as reporter was constructed. This was achieved by both subcloning *Bal* 31 deletion fragments of the IE-1/2 enhancer/promoter and by cloning of synthetic oligonucleotides into the CAT expression vector pHM72 (see Materials and Methods). The resulting constructs were designated according to the IE-1/2 promoter sequence inserted in front of the CAT gene (e.g. p-77/+52EPCAT contains IE-1/2 promoter sequences between -77 and +52 relative to the IE-1/2 transcription start) (Fig. 5, A). These promoter deletion plasmids were then tested in transient expression assays. HeLa cells were transfected with 20 μ g of each of the plasmids and CAT activities were determined 48 h later (Fig. 5, B and C). The removal of the GC-boxes at -74 and -54 as exerted by deletions from -77 to -65 and -57 to -38 lead to an only marginal decrease of CAT enzyme levels (Fig. 5, B, lanes 2 and 3, lanes 4 and 5). This confirms the results obtained after *in vitro* mutagenesis of the GC-boxes. The deletion, however, of sequences between -94 and -77 had a strong effect on promoter activity (Fig. 5, B, lanes 1 and 2). CAT enzyme levels were diminished by a factor of about 10. Identical profiles were obtained when CAT RNA levels were analyzed or when primary human fibroblasts were used for transient expression of these deletion constructs (data not shown). Thus, deletion analysis identifies a strong *cis*-acting domain located between -94 and -78, whereas GC-box elements could be removed without significant effects on IE-1/2 promoter function. In addition, the domain between -77 and -31 of plasmid p-94/+52EPCAT could be deleted entirely without reducing the activity of the IE-1/2 promoter markedly (data not shown).

To obtain further evidence for the *cis*-activity of the DNA element between -94 and -78, an oligonucleotide corresponding in sequence to this motif was synthesized (oligonucleotide -94/-78 EP). The oligonucleotide was inserted into the CAT expression vector puPCAT (43) upstream of IE-1/2 promoter sequences between -65 and +52 in front of the CAT gene. After dimerization of the inserted motif as described (43) both plasmid p-94/-78PCAT (containing a monomer of oligonucleotide -94/-78EP) and p2x-94/-78PCAT (containing a dimer of oligonucleotide -94/-78EP) were transfected together with plasmid puPCAT into HeLa cells. As shown in figure 6, a monomer and a dimer of oligonucleotide -94/-78 EP, stimulated the IE-1/2 promoter as contained within puPCAT, 5fold and 26fold, respectively. This confirmed that the domain between -94 and -78 can act *in cis* to induce promoter activities.

Recombinant CREB can interact with the -94/-78 motif

The central core of the -94/-78 motif is identical in 6 nucleotides with the octameric consensus sequence TGACGT-CA for transcription factor CREB as also contained within the 19 bp repeat element of the HCMV IE-1/2 enhancer. To test whether transcription factor CREB can interact with the -94/-78 element, a gel retardation experiment was performed (Fig. 7). Procarotically expressed CREB protein was incubated with 32 P-labeled oligonucleotides corresponding in sequence to the 19 bp element, to the -94/-78 motif and to a Sp1 binding site of the SV40 promoter (see Materials and Methods). Both

Fig. 5. Expression analysis of 5' promoter deletion plasmids. A) 5' deletion constructs of the IE-1/2 promoter region. The nucleotide sequence of the IE-1/2 promoter between -94 and -11 is shown on top; TATA- and CCAAT-box homologies and Sp-1 binding sites are boxed; the underlined sequence corresponds to the truncated 19 bp repeat element. IE-1/2 promoter sequences contained within each CAT-expression plasmid are indicated by filled bars. The 5' and 3' end points of promoter sequences are shown above the bars. The nomenclature of the 5' deletion plasmids is given on the right. B) Schematic diagram of CAT acetylation values observed after transient expression of 5' promoter deletion constructs in HeLa cells.

Fig. 6. Expression analysis of CAT fusion genes containing a monomer or dimer of the *cis*-acting element between -94 and -78. A) CAT-assay of the vector plasmid puPCAT and constructs containing a monomer (p-94/-78PCAT) or a dimer (p-2x-94/-78PCAT) of the -94/-78 element in front of the IE1/2 minimal promoter after transient expression in HeLa cells. Lane 1, plasmid puPCAT; lanes 2 and 3, plasmid p-94/-78PCAT; lanes 4 and 5, plasmid p2x-94/-78PCAT. B) Schematic diagram of CAT acetylation values observed after transient expression of CAT fusion genes.

the 19 bp motif and the -94/-78 motif gave rise to one major complex, migrating at an identical position for both probes (Fig. 7, lanes 3 and 6, respectively). No retarded band was visible when the SV40-Sp1 oligonucleotide was used (Fig. 6, lane 9), indicating the specificity of the observed interaction. In addition, protein extracts prepared from bacteria containing only the pROS vector used for expression of CREB did not result in any shifted bands (Fig. 7, lanes 2, 5 and 8). This showed that transcription factor CREB binds to the -94/-78 motif.

DISCUSSION

This study identified and characterized protein binding sites within the promoter and adjacent upstream region of the human cytomegalovirus immediate early 1 and 2 gene (IE-1/2). This

Fig. 7. Gel retardation analysis of the -94/-78 motif using procaryotically expressed transcription factor CREB. Lanes 1 to 3, an oligonucleotide corresponding in sequence to the 19 bp repeat motif of the HCMV IE-1/2 enhancer was used as probe; lanes 4 to 6, an oligonucleotide corresponding in sequence to the -94/-78 motif was used as probe; lanes 7 to 9, an oligonucleotide corresponding in sequence to a Sp1 binding site of the SV40 promoter was used as probe. Lanes 1, 4 and 7, no protein added; lanes 2, 5 and 8, *E. coli* extract without CREB was added; lanes 3, 6 and 9, *E. coli* extract with CREB was added.

promoter forms part of the complex IE-1/2 control region that contains several functional domains: the far most upstream domain between -1145 and -750 has been termed modulator as it has an influence on cell-type specific expression of the IE1/2 gene (33); between -750 and -550 there is a cluster of three NF-1 binding sites together with a unique protein binding site (34,35); the strong enhancer domain extends between -550 and -65 of the IE-1/2 cap site and extends into the RNA polymerase II promoter at approximately -65 (4,20). Two aspects make it important to identify the transcription factors interacting with the IE1/2 promoter. (i) The promoter with its basal transcription

machinery directs RNA polymerase II to start with transcription and therefore acts as the ultimate target for effects exerted by the modulator or enhancer domain. (ii) An element within the promoter has recently been identified as *cis*-reactive site for negative regulation by viral IE-2 proteins (8–10). As there is at present no evidence for a direct DNA/protein contact of IE-2 polypeptides, this effect may be due to an interaction with other transcription factors associated with this region. Therefore, the knowledge of the binding factors may help to elucidate these mechanisms.

Sequence inspection of the IE-1/2 promoter revealed several potential protein binding sites including two GC-box elements at –74 and –54 that are known as consensus recognition motifs for transcription factor Sp1 (21). To see whether Sp1 binds to these motifs we performed DNase I footprinting experiments with Sp1 protein expressed in *E. coli* and could identify four protections within the sequence under investigation. Two of these protections covered the GC-boxes at –74 and –54, whereas the two other sites differed from the Sp1 consensus sequence GGGCGG by having a central T instead of a C. An identical aberrant Sp1 binding site has also been described for the E2 promoter of bovine papilloma virus (36). As well, Sp1 binds to sites within the HIV-1 LTR that differ markedly from the consensus GGGCGG (37). Thus, the presence of aberrant Sp1 recognition sequences is not unique to the human cytomegalovirus IE-1/2 promoter. However, it shows that the search for consensus sequences does not suffice; studies on DNA-protein interactions are necessary to identify transcription factor binding sites.

The high density of potential protein target sites within the IE-1/2 regulatory region makes it possible that sterical factors exert a profound influence on the binding of transcription factors. Therefore, it was necessary to test whether the identified sites are accessible for Sp1 in the presence of other proteins interacting with DNA. When nuclear extracts of HeLa cells were used for DNase I footprinting, Sp1 sites were found to be protected. By competition with oligonucleotides having a consensus Sp1 recognition sequence it was possible to demonstrate that a factor with binding characteristics of Sp1 occupies these sites. Therefore, Sp1 interactions seem to dominate within the IE-1/2 promoter region and lead to an extensive protection pattern *in vitro*. This was also observed when nuclear extracts of permissive human fibroblasts were used. This differs from the results of previous DNase I footprinting studies where only weak protection was reported for the region between –80 and +1 (20). The fractionation of HeLa cell extracts used in these experiments, however, may have resulted in a loss of Sp1 binding activities (20). A recent study in which both HeLa- and B-cell extracts purified via an enhancer affinity column were used for DNase I footprinting detected protections in the region between –40 and –80 (19). As these protections could not be competed by several repeat motifs of the enhancer, they may correspond to the Sp1 binding sites identified in this study.

Competition of factors for a target site could be observed at a G-rich element located at –148 that overlaps a 19 bp repeat motif of the enhancer domain. With HeLa cell extracts this G-rich element, that also binds procaryotically expressed Sp1, was strongly protected, whereas the adjacent 19 bp motif showed only weak interactions with nuclear factors. After competition with an Sp1 consensus site oligonucleotide, protection at the 19 bp motif increased whereas part of the G-rich element was no longer protected. Thus, transcription factors Sp1 and ATF/CREB compete for binding to this site. As the 19 bp element seems

to be responsible for the strong constitutive activity of the HCMV enhancer in some cell types (19,22), we will try to determine in further studies whether the overlapping Sp1 binding site can modulate the function of this motif. In contrast to HeLa cell extracts, HFF extracts did not protect this 19 bp motif and the G-rich element at –148. However, strong interactions were present in the region between –127 and –89 containing an 18 bp repeat motif. As has been shown for B-cell extracts (19), this observation demonstrates that alternative protein complexes can form on the HCMV enhancer depending on the cell type. Additional experiments will be required to determine whether these interactions of HFF extracts involve binding of nuclear factor NF-kappa-B and other factors.

In contrast to differences in the protection pattern of the region upstream of –80, the Sp1 binding sites of the IE1/2 promoter seemed to be protected equally well with HeLa and HFF extracts. Transcription factor Sp1 has been shown to enhance transcription by RNA polymerase II about 10–50 fold from a select group of promoters that contain at least one GC box (38). Responsive promoters of both cellular and viral origin include the promoter of the rat NF-1 gene (39), the human argininosuccinate synthetase (AS) promoter (40), the SV40 early promoter (41), and the long terminal repeat (LTR) of human immunodeficiency virus (HIV) (37). Whereas *in vitro* transcription assays were performed in most studies to demonstrate Sp1 responsiveness (37), it could also be shown that transcriptional activation occurs *in vivo* (40). To investigate the contribution of Sp1 to HCMV IE1/2 promoter function, Sp1 binding sites were mutagenized within a construct that contained sequences up to –94 of the IE-1/2 cap site. This sequence has been previously shown to exert a strong constitutive activity in HeLa cells (20). Surprisingly, mutagenized constructs did not show an altered activity after transient expression in both HeLa and HFF cells when compared to the wild-type CAT expression plasmid. Although the mutation of the GC-boxes of the IE-1/2 promoter caused a decrease of the affinity for Sp1 by a factor of 30 *in vitro* (32), the *in vivo* activity of Sp1 binding sites might not have been affected due to the high abundance of Sp1 within the cell. To exclude this, a series of 5' deletion constructs sequentially removing GC-box elements of the promoter was tested. After deletion of Sp1 binding sites the constitutive activity decreased only marginally thus confirming the results obtained after site directed mutagenesis. In addition, the domain containing the Sp1 binding sites could be deleted entirely without markedly reducing the activity of the IE-1/2 promoter. Therefore, Sp1 binding sites are not necessary for efficient constitutive promoter function at least within HeLa and HFF cells. At present, we cannot exclude that these binding sites are important in other cell types. A similar result was reported for the *in vivo* function of Sp1 sites within the HIV I LTR. After site directed mutagenesis of Sp1 targets and transient expression of the respective plasmids, only a minimal decrease of basal activities could be observed (42). However, in this system Sp1 sites seemed to be required for efficient induction of promoter functions by the viral *trans*-activator tat. Whether Sp1 exerts an analogous role for viral *trans*-activation or negative regulation in the HCMV IE-1/2 promoter remains to be determined.

Deletion analysis of the IE-1/2 promoter region could also identify a strong *cis*-acting element located between –94 and –78. Deletion of this motif resulted in an about 10fold reduction of CAT activities. As well, re-insertion of the element as synthetic oligonucleotide in one and two copies upstream of a minimal IE1/2 promoter confirmed the activating properties of this

sequence. Therefore, we were able to allocate the previously reported strong reduction in transcriptional activities of a deletion from -94 to -65 (20) to the domain from -94 to -78. This region was protected in DNase I footprinting experiments and interacts with nuclear proteins. As the central core of this motif (TGTCGTAA) has homology to the consensus sequence for ATF/CREB factors (TGACGTCA) we performed a gel retardation experiment using procaryotically expressed transcription factor CREB. This revealed that recombinant CREB binds to this motif although it does not conform to the consensus sequence. Therefore, similar to Sp1, an aberrant binding site for transcription factors of the ATF/CREB family which is located between -94 and -78, could be identified.

In summary, this study identifies several previously unknown binding sites for transcription factors Sp1 and ATF/CREB within the IE1/2 promoter region of human cytomegalovirus. Functional assays revealed that Sp1 does not have a strong influence on basal promoter activity whereas the aberrant CREB binding site upstream of the Sp1 domain exerts a strong transcriptional stimulation. Due to these results we would like to propose nucleotide -78 relative to the IE-1/2 cap site as a boundary of the HCMV enhancer domain whereas promoter functions are contained within the sequences downstream.

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