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Interactions of the Transcription Factors MIBP1 and RFX1 with the EP Element of the Hepatitis B Virus Enhancer

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We previously demonstrated that MIBP1 and RFX1 polypeptides associate in vivo to form a complex that binds to the MIF-1 element in the c-*myc* gene and the major histocompatibility complex class II X-box recognition sequence. We now show that the EP element, a key regulatory sequence within hepatitis B virus enhancer I, also associates with MIBP1 and RFX1. Using polyclonal antisera directed against either oligonucleotide-purified MIBP1 or a peptide derived from the major histocompatibility complex class II promoter-binding protein RFX1, we showed that MIBP1 and RFX1 are both present in the DNA-protein complexes at the EP site. In addition, while the EP element can act cooperatively with several adjacent elements to transactivate hepatitis B virus expression, we demonstrated that the EP site alone can repress transcription of simian virus 40 promoter in a position- and orientation-independent manner, suggesting a silencer function in hepatocarcinoma cells.

The human hepatitis B virus (HBV) infects hepatocytes, induces acute and chronic liver disease in humans, and has been linked to hepatocellular carcinoma (3, 22, 37). The genome of HBV contains four major open reading frames encoding the core (C), surface (S), DNA polymerase, and X proteins (11). Expression of the S, C, and X genes (1, 14, 36), as well as the 3.5-kb pregenomic mRNA used during viral replication (19), has been shown to be under the control of HBV enhancer I, implicating its role in the regulation of both transcription and replication of HBV. Enhancer I contains functional binding sites for several cellular factors (4, 8, 28) that act synergistically in liver cells (12, 15, 21, 38). Functional analysis of the enhancer I complex demonstrated that a 20-bp sequence, referred to as the EP element, is required for efficient enhancer I function (8, 14, 27, 38). For example, mutations within this element, including the substitution of four nucleotides (nt) within the 5′ inverted repeat as well as deletion of the EP sequence from the enhancer region, were shown to result in loss of enhancer function (8, 12, 14, 16, 38). An adjacent sequence which binds the hepatocyte-specific nuclear factor-4 (HNF-4) and the retinoid X receptor (RXRα), function together with the EP site to confer liver-specific enhancer activity (12, 15, 20, 21). Although the EP element showed no intrinsic enhancer activity in the regulation of the globin gene promoter (8), it suppressed simian virus 40 (SV40) promoter activity in a position- and orientation-independent manner in hepatocarcinoma HepG2 cells (30).

The EP element was also found in the enhancer or promoter regions of several other viruses, including the polyomavirus (27, 30). A nuclear protein, termed EF-C, that has been previously described as a polyomavirus enhancer binding activity was shown to be the same as the HBV enhancer binding protein, EP (26, 27). Moreover, it was reported that the EP or EF-C activity corresponds to a protein designated RFX1 (34), which binds to an essential regulatory X-box sequence in the promoter region of major histocompatibility complex (MHC) class II genes (31, 32). RFX1 was shown to bind to the EP and EF-C recognition sites and to play a role as a transactivator of HBV enhancer I (6, 34).

We have recently shown that the RFX1 protein also associates in vivo with myc intron binding polypeptide (MIBP1) and that both can bind to the X-box sequence of the MHC class II promoter and the MIF-1 site in the intron I region of the c-*myc* gene. In addition, we have also shown that MIBP1 binds to the EP-like sequences present in the cytomegalovirus, Epstein-Barr virus, and polyomavirus regulatory regions (30). Since RFX1 binds to the EP site (34) and since RFX1 binds MIBP1 in vivo (30), we examined whether both polypeptides can associate with the HBV EP enhancer sequence. Using specific antisera raised against oligonucleotide-affinity-purified MIBP1 and the N-terminal peptide of RFX1, we demonstrated that both MIBP1 and RFX1 are components of an HBV(EP) binding complex. In addition, we showed that five tandem repeats of the HBV EP element can repress transcriptional activity of the SV40 promoter in an orientation- and position-independent manner in all hepatocarcinoma cell lines tested.

MATERIALS AND METHODS

Plasmids and oligonucleotide probes. Double-stranded wild-type and mutant oligonucleotides were synthesized as described previously (30, 41), and their sequences are shown in Fig. 2C. The MIF-1, mutant MIF-1, EP, and mutant EP double-stranded oligonucleotides were multimerized, and fragments containing five tandem copies were cloned into the *Bgl*II or *Bam*HI site of the pCAT-P vector (Promega) as described previously (30).

Cell culture and DNA transfections. HepG2 cells were obtained from the American Type Culture Collection. Huh-7 and Huh-4 cells were derived from a patient with hepatocarcinoma and were kindly supplied by C. Harris (Laboratory of Carcinogenesis, National Cancer Institute). The BV173 cells, kindly provided by J. Rowley (Department of Genetics, University of Chicago) were derived from a patient with chronic myelogenous leukemia and contain two copies of the Philadelphia chromosome (29). All cell lines were grown in Dulbecco's modified Eagle's medium (Biofluids) with 10% fetal calf serum and 50 mg (each) of streptomycin and penicillin per ml. For each transfection, 1.5×10^6 Huh-7 cells and 2.5×10^6 Huh-4 cells were plated in 100-mm-diameter dishes and incubated overnight at 37°C. The cells were transfected by calcium phosphate precipitation (7) for 5 h with 5 μg of the indicated reporter plasmid and 4 μg of salmon sperm DNA. One microgram of pGL_2-Luc (Promega) was cotransfected to control for transfection efficiency. Cells were subjected to glycerol shock and harvested, and chloramphenicol acetyltransferase (CAT) and luciferase assays were performed as described previously (13, 40).

Preparation of nuclear protein extracts. Nuclear extracts were prepared ac-

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cording to the modified method of Schreiber et al. (33). Pelleted cells were resuspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid [HEPES; pH 7.5], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), and Nonidet P-40 was added to a final concentration of 0.5%. Nuclei were pelleted quickly and resuspended in buffer C (20 mM HEPES [pH 7.5], 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Both buffers contained freshly prepared aprotinin and leupeptin (0.02 μg/μl each). Supernatants were cleared by centrifugation after 15 min at 4°C and were frozen in aliquots at −70°C.

Production of antiserum specific for MIBP1 and RFX1 protein. Rabbit antiserum was raised against oligonucleotide-affinity-purified MIBP1 polypeptide (30, 42) or a peptide corresponding to the N-terminal domain of the RFX1 molecule (residues 3 to 21) as previously described (30, 32).

Electrophoretic mobility shift assay. Mobility shift assays were performed as described previously (40, 41). ^{32}P-Klenow end-labeled duplex oligonucleotide probe (0.1 ng [approximately 10^4 cpm per lane]) was incubated with 2 μl of nuclear extract (1 mg of protein per ml) in 10 μl of the reaction mixture containing 10 mM HEPES (pH 7.8), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 3% Ficoll, and 1 μg of poly(dI-dC). In supershift antibody experiments, the reaction mixture containing the nuclear extract was preincubated for 5 min at room temperature with 1 μl of either preimmune serum, serum raised against MIBP1 protein, or serum raised against a peptide derived from the N-terminal domain of RFX1, before the addition of the indicated oligonucleotide probe. The reaction mixture was then incubated for 15 min at room temperature and analyzed by 4% polyacrylamide gel electrophoresis and autoradiography.

RESULTS

MIF-1 and EP elements form similar DNA-protein complexes. To compare the DNA binding properties of the MIF-1 and EP elements, we synthesized oligonucleotide probes representing MIF-1 and EP recognition sites (Fig. 1A) and compared their migration patterns in gel mobility shift assays. The EP and MIF-1 oligonucleotide probes used in this study were previously identified by DNAse footprinting and exonuclease protection assays as the sequences required for protein binding (4, 41). Since the EP element is located in the HBV enhancer region, which is activated in hepatic cells, we compared the migrations of DNA-protein complexes of EP and MIF-1 sites with nuclear extracts derived from the HepG2 hepatocarcinoma cell line. We did not detect any qualitative difference in the migration of these complexes (Fig. 1B); however, protein binding appeared to be of a higher affinity at the EP site than at the MIF-1 site (Fig. 1B, lanes 1 and 2 and 5 and 6, and 1C, lanes 2 and 6). We have tested the specificity of the MIF-1 and EP complexes and demonstrated that each could be blocked by competition with unlabeled MIF-1 or EP oligonucleotides (Fig. 1B and C, lanes 3 and 7), while binding could not be blocked by unrelated sequences (Fig. 1B and C, lanes 4 and 8). We performed a dose-dependent cross-competition ranging from 0.1 to 10 ng of unlabeled competitor oligonucleotides (Fig. 2) representing molar ratios of unlabeled to labeled oligonucleotides from 1- to 100-fold. Using MIF-1 as a probe and 2 μg of HepG2 nuclear protein extract, we observed that 0.1 ng of unlabeled EP oligonucleotide partially inhibited protein binding, while 1 ng was sufficient to completely abrogate the DNA-protein complex (Fig. 2A, lanes 10 and 11). In contrast, residual protein binding was still detected with 1 ng of the unlabeled MIF-1 oligonucleotide (Fig. 2A, lane 3). Similar results were observed when the EP oligonucleotide was used as a probe and competition was performed with unlabeled MIF-1 and EP oligonucleotides (Fig. 2B). Mutant MIF-1 (2 of 21 nt changed) and mutant EP (4 of 20 nt changed) oligonucleotides (Fig. 2C) did not show any activity as competitors (Fig. 2A, lanes 5 to 8 and 13 to 16). These mutant oligonucleotide probes were established previously by substitution of the critical contact points required for protein binding (9, 41).

It has been previously reported that c-Abl binds to the EP element and that nuclear extracts from BV173 cells, which lack the c-Abl protein, did not form DNA-protein complexes when incubated with the EP oligonucleotide probe (9). Therefore, we investigated whether DNA-protein complexes would form by using BV173 protein extracts with the MIF-1 and the EP oligonucleotide probes. In contrast to previously published results (9), DNA-protein complexes were detected in the BV173 nuclear extracts, which were indistinguishable from those of HepG2 cells, by both the MIF-1 and EP probes (Fig. 1C). Similarly, it was recently shown that BV173 cells contain the EP binding activity indistinguishable from that of Molt-4 cells (2). In addition, supershift analyses with four independent c-Abl antibodies (clone 8E9 from PharMingen and clones Ab-1, -2, and -3 from Oncogene Science) did not demonstrate evidence of c-Abl in either EP or MIF-1 complexes (data not shown). These results suggest that c-Abl is not a component of the MIF-1 or EP complexes and that c-Abl activity is not required for the DNA-protein interaction at these sites.

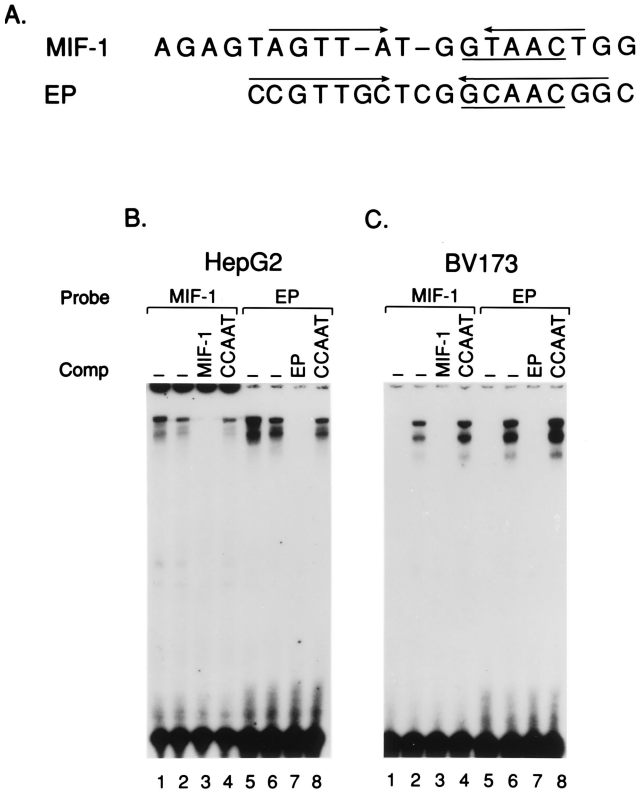

FIG. 1. Comigration of MIF-1 and EP DNA-protein complexes in a mobility shift assay. (A) Comparison of MIF-1 and EP binding sequences. Inverted repeats are indicated by overline arrows. Sequences with the strongest homology between the EP and MIF-1 sites are underlined. (B) Analysis of MIF-1–EP binding complexes. MIF-1 and EP oligonucleotide probes were used with 10 μg (lanes 1 and 5) or 2 μg (lanes 2 and 6) of HepG2 nuclear extract, respectively. Competition (Comp) was performed with 10 ng of either MIF-1 (lane 3), EP (lane 7), or CCAAT oligonucleotides (lanes 4 and 8). (C) BV173 nuclear protein extracts were used as indicated for panel B.

MIBP1 and RFX1 are both present in the EP DNA-protein complexes. We previously showed that MIF-1 binding activity consists of at least two polypeptides, MIBP1 and RFX1, which interact in vivo and bind to the c-*myc* (MIF-1) and MHC class II (RFX) recognition site (30). To determine whether both polypeptides are also present in the EP complex, we examined whether antisera raised against either oligonucleotide-affinity-purified MIBP1 or a peptide derived from RFX1 would cross-

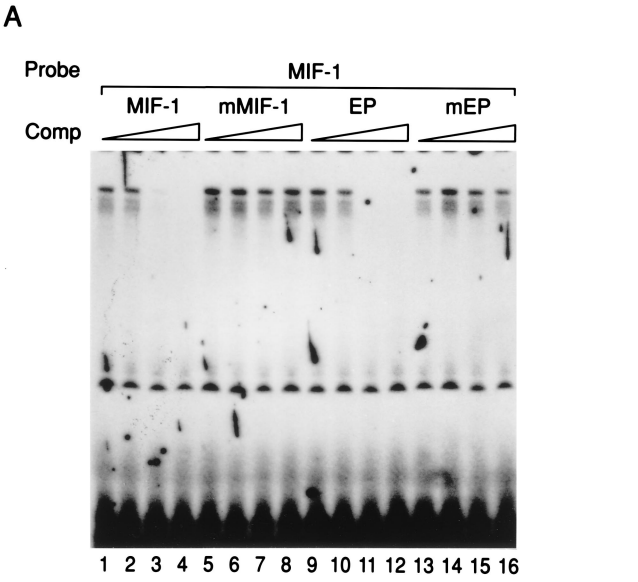

FIG. 2. MIF-1 and EP sequences cross-compete for binding in the mobility shift assays. (A and B) Cross-competition analysis of MIF-1 binding activity (A) and EP binding activity (B). HepG2 nuclear extracts were preincubated with either 0, 0.1, 1, or 10 ng of unlabeled competitor as indicated by the triangle above each set of competitors. (C) Nucleotide sequences of the wild-type MIF-1, wild-type EP, mutant MIF-1 (mMIF-1), and mutant EP (mEP) probes. The mutant nucleotide sequences of MIF-1 and EP are underlined.

react with the EP DNA binding complex. We confirmed the specificity of the anti-MIBP1 serum and have shown that anti-MIBP1 serum does not cross-react with RFX1 and that anti-RFX1 antibody does not cross-react with MIBP1 (30). EP and MIF-1 oligonucleotides were incubated with HepG2 nuclear extract or with oligonucleotide-purified MIBP1 and with antiserum specific for MIBP1 and RFX1 or the corresponding preimmune serum. We found that the MIBP1 and RFX1 antiserum specifically retarded the migration of the upper band of the complexes (Fig. 3 and 4, respectively) while the control preimmune serum showed no effect (Fig. 3 and 4, lanes 2 and 5). The supershifted complexes were also not generated after incubation of preimmune or immune serum with the MIF-1 and EP probes in the absence of protein extract (data not shown). A supershift pattern was obtained with both HepG2 nuclear extracts (Fig. 3A and 4A, lanes 3 and 6) and with the oligonucleotide-affinity-purified MIBP1 (Fig. 3B and 4B, lanes 3 and 6) (42). The observation that anti-RFX1 antibody supershifts the purified MIBP1 complexes suggested that the RFX1 copurified with MIBP1 on cation exchange and MIF-1 oligonucleotide-affinity chromatography (42). To test this possibility, we performed Western blot (immunoblot) analysis with anti-RFX1 serum and showed that we could detect RFX1 in the purified MIBP1 fraction (data not shown). This result was not surprising, since we have shown previously that RFX1 interacts with MIBP1 in vivo and both bind to the MIF-1 and RFX sites (30). Neither the MIBP1 antiserum nor the RFX1 antiserum supershifted the control CCAAT DNA-protein complex (Fig. 3C and 4C). Together, these results suggest that the EP regulatory site of the HBV enhancer I consists of at least two polypeptides, MIBP1 and RFX1.

Intrinsic silencer activity of the EP element in the hepatocarcinoma cells. We have previously shown that MIF-1 and EP recognition sites regulate the activity of a SV40 promoter in a position- and orientation-independent manner, and each can function as a silencer in a hepatocarcinoma (HepG2) cell line (30). Since the EP element is required for the transactivation of the HBV enhancer I activity, we asked whether the intrinsic silencer activity of the EP and the MIF-1 recognition sites observed in HepG2 cells is a common phenomenon in other hepatocarcinoma cell lines. Huh-7 hepatocarcinoma cells were transfected with constructs containing five repeats of the wild-type MIF-1 or EP elements cloned in either the sense or antisense orientation (in relation to the transcriptional unit of the SV40 promoter), with the mutant MIF-1 and EP elements in the sense orientation, or with the parental control plasmid (Fig. 5A). CAT activity was assayed 42 h after transfection. We observed that constructs containing the sense and antisense EP elements displayed about 3-fold reduction of CAT expression in Huh-7 cells compared with that of the parent vector (calculated as the mean of six transfections) (Fig. 5A, lanes 1, 5, and 7); however, a 10-fold reduction in CAT activity was observed compared with that of the construct with the mutant EP sequence (Fig. 5A, lanes 5 to 7). We observed the same pattern of CAT suppression in Huh-7 cells with reporter constructs that had the EP and MIF-1 multimers subcloned 2 kb upstream from the SV40 promoter region (Fig. 5B). In contrast, the MIF-1 element, which acts as a silencer in HepG2 cells (reference 30 and data not shown), did not show a significant effect in Huh-7 cells (Fig. 5A, lanes 1, 2, and 4).

We have also evaluated EP activity in two additional hepatoma cell lines which were infected with HBV (18). We transfected, into Hep3B and Huh-4 cell lines, plasmids with the EP and MIF-1 sites inserted either adjacent to or 2 kb upstream from the SV40 promoter and observed that the EP and MIF-1 elements exhibited 3.5- and 1.5-fold reductions of CAT activity, respectively, in Hep3B cells (data not shown), and 10.5- and 4.5-fold reductions of CAT activity, respectively, in Huh-4

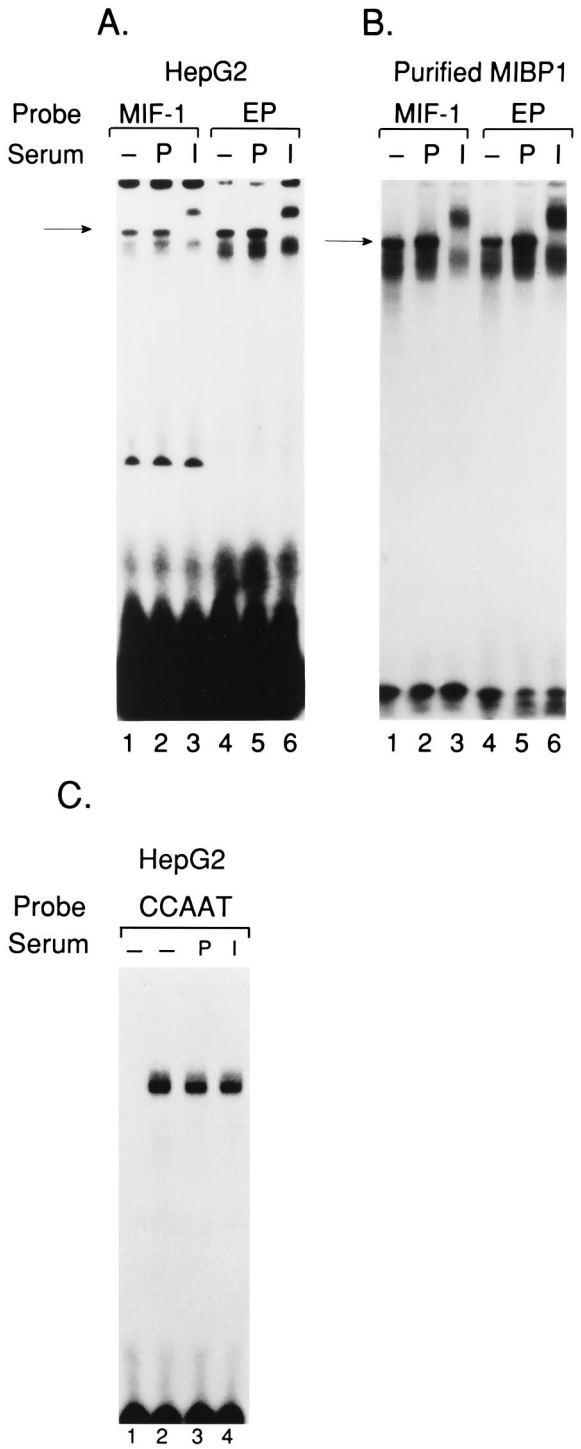

FIG. 3. MIBP1 antiserum induces supershift of the MIF-1 and EP complexes. HepG2 nuclear extracts (A) and purified MIBP1 protein (B) were preincubated in the absence of serum (−) or in the presence of either preimmune serum (P) or MIBP1-specific immune serum (I). (C) Migration of the DNA-protein complex formed at the CCAAT oligonucleotide was not affected by either preimmune serum or MIBP1-specific immune serum. The arrows show the major DNA-protein complex that is supershifted by the MIBP1 antiserum.

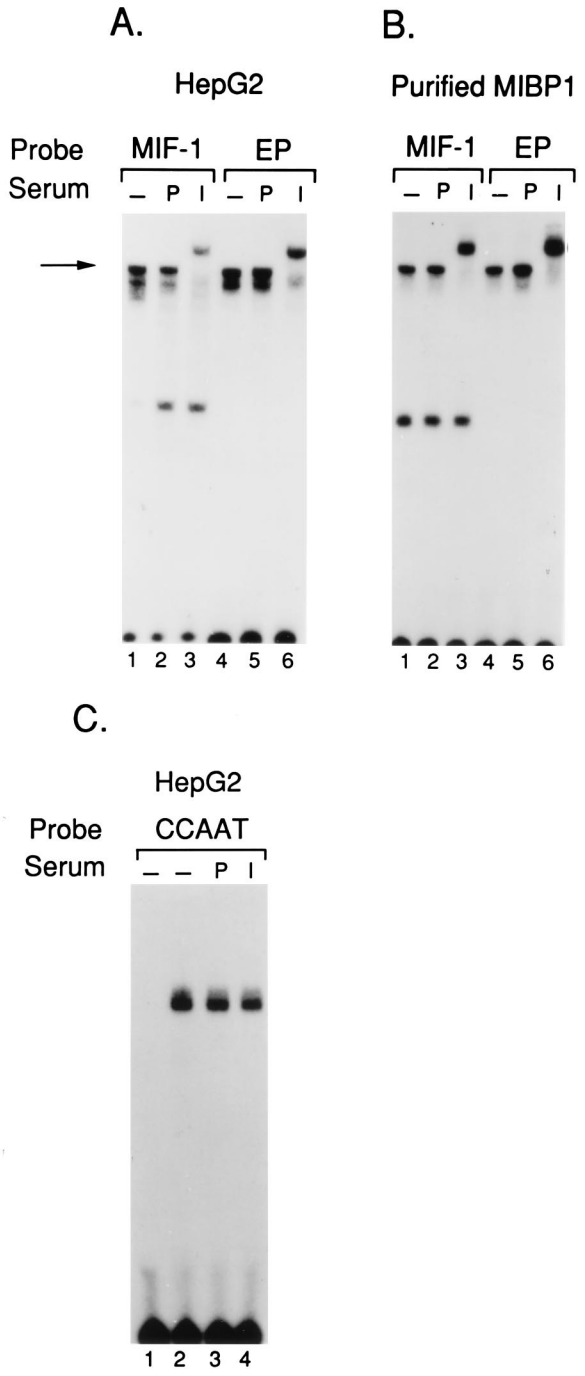

FIG. 4. RFX1 antiserum induces supershift of the MIF-1 and EP complexes. HepG2 nuclear extracts (A) and purified MIBP1 protein (B) were preincubated in the absence of serum (−) or in the presence of either preimmune serum (P) or RFX1-specific immune serum (I). (C) Migration of the DNA-protein complex formed at the CCAAT oligonucleotide was not affected by either preimmune serum or RXF1-specific immune serum. The arrow shows the major DNA-protein complex that is supershifted by the RFX1 antiserum.

cells (Fig. 6A and B, respectively). Therefore, cell lines such as HepG2 and Huh-4, which support high levels of EP suppressor activity (10-fold repression) also exhibit MIF-1 suppressor activity, although to a lesser extent. Conversely, cell lines such as Huh-7 and Hep3B, which support lower levels of EP activity (threefold repression), also show low levels of MIF-1 activity. Therefore, the EP element exhibited silencer activity in all four hepatoma cells tested, and the degree of promoter repression was independent of HBV status.

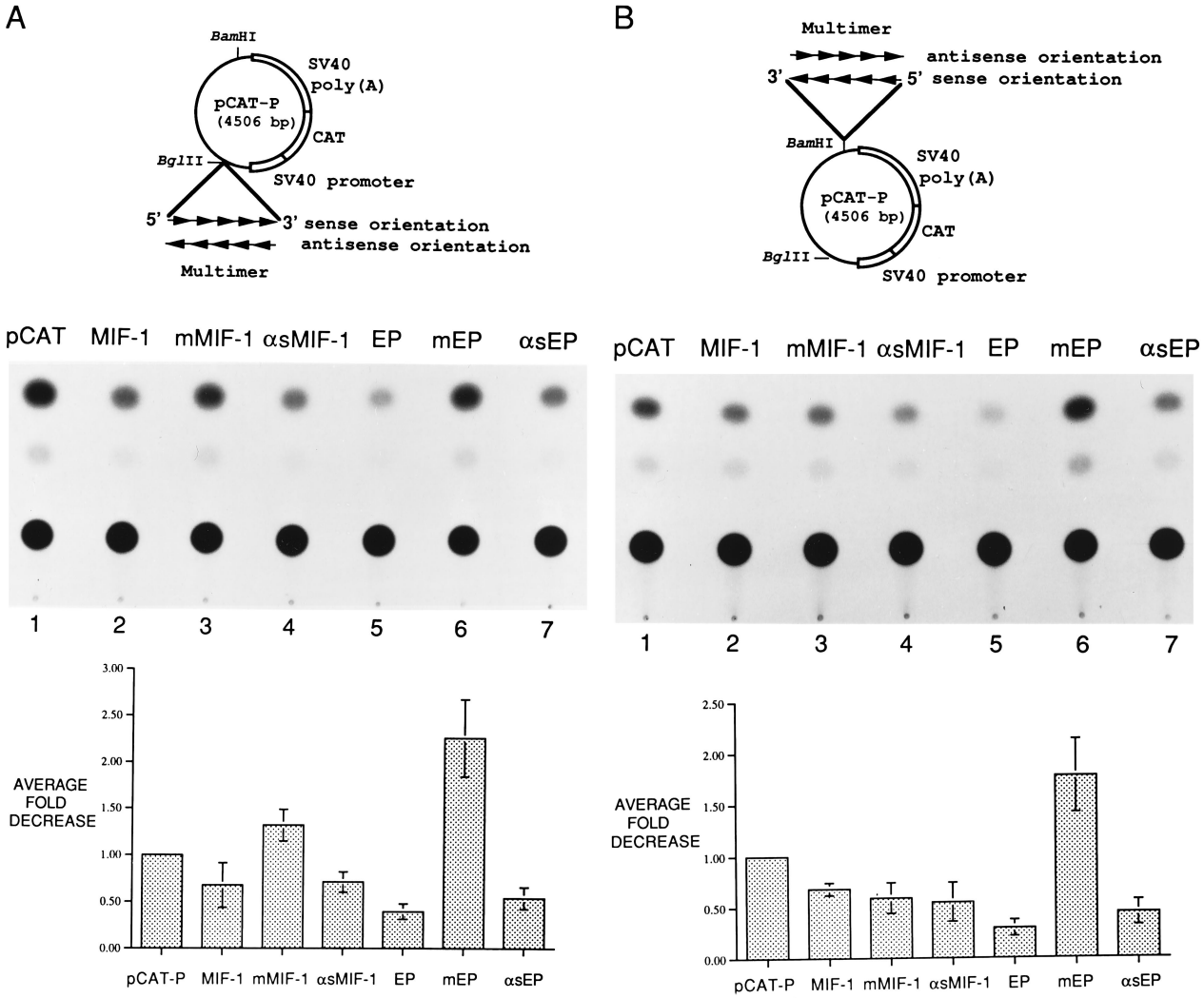

FIG. 5. Silencer activity of the EP binding site in Huh-7 cells. The MIF-1, mutant MIF-1 (mMIF-1), EP, and mutant EP (mEP) binding sequences were cloned into an SV40 CAT expression vector either in the proximity of or 2 kb upstream from the SV40 promoter. (A and B) CAT activity of cells transfected with the multimers cloned adjacent to (A) or 2 kb upstream from (B) the SV40 promoter. The bar graph reflects analysis of three independent experiments, each performed in duplicate and adjusted for transfection efficiency by cotransfection with the pGL_2-luciferase vector. αs, antisense orientation.

DISCUSSION

We have demonstrated that the nuclear proteins MIBP1 and RFX1 bind to the EP element, which is a key functional component of HBV enhancer I. MIBP1 was initially identified as the polypeptide that binds to the MIF-1 sequence in intron I of the c-*myc* gene (30, 41, 42). The RFX1 protein was independently isolated as the nuclear factor that binds and regulates the MHC class II promoter and the EP site of HBV enhancer I (32, 34). Similarities in the nucleotide sequences of the binding elements and in the migration patterns of the DNA-protein complexes of both RFX1 and MIBP1 suggested initially that the same protein activity may be responsible for binding to the MHC class II (X-box), HBV (EP), and c-*myc* (MIF-1) sites (34, 43, 44). We demonstrated, however, that the MIF-1 binding activity consisted of two polypeptides, MIBP1 and RFX1, that associated in vivo and that were both present in DNA-protein complexes at the c-*myc* (MIF-1) and MHC class II (RFX) binding sites (30). Using polyclonal antisera raised against either oligonucleotide-purified MIBP1 or a peptide derived from RFX1 (30, 32), we have now shown that both the

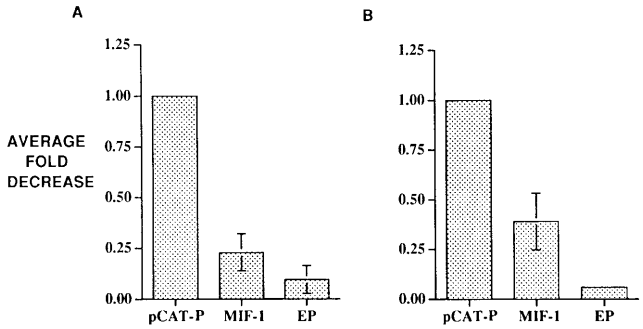

FIG. 6. Silencer activity of EP binding site in HBV-positive Huh-4 cells. The plasmids containing the MIF-1 and EP binding sequences were described in the legend to Fig. 5. (A and B) CAT activity of cells transfected with the multimers cloned adjacent to (A) or 2 kb upstream from (B) the SV40 promoter. The bar graph reflects analysis of three independent experiments each performed in duplicate and adjusted for transfection efficiency by cotransfection with the pGL_2-luciferase vector. sMIF-1, MIF-1 preincubated in the presence of preimmune serum.

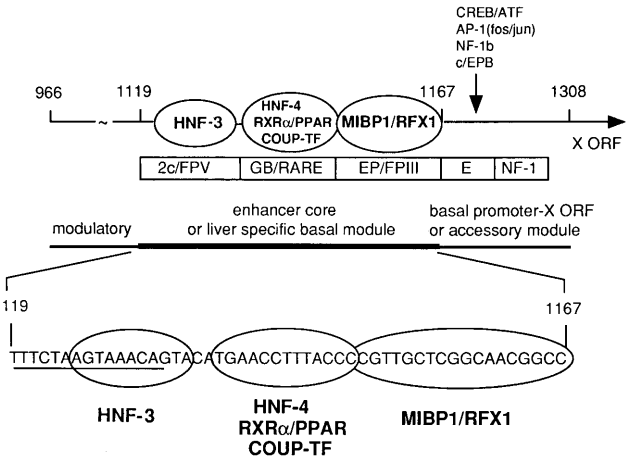

FIG. 7. Schematic representation of the functional domains, individual *cis* elements, and their binding factors in HBV enhancer I (14, 38). The nucleotide sequences for each of the binding sites located in the enhancer core and their binding proteins are shown. Underlined sequence represents a predicted 2c site (14), for which a binding protein has not yet been identified. ORF, open reading frame.

160-kDa MIBP1 and the 130-kDa RFX1 proteins bind to the EP element of HBV enhancer I.

The active HBV enhancer I region (nt 966 to 1308) has been subdivided into three domains: the modulatory (nt 966 to 1119), core enhancer (nt 1119 to 1168), and basal promoter-X open reading frame regions (nt 1168 to 1308) (38). The EP site (also termed FPIII) (28) is located in the enhancer core subdomain (Fig. 7). Adjacent to the EP recognition sequence is the GB element (12), also termed RARE (21), which binds liver-enriched nuclear proteins HNF-4 (35) and RXRα (23), as well as a ubiquitous nuclear protein termed COUP-TF (39). The GB/RARE element and the EP site have been proposed to cooperate to confer liver specificity to the enhancer function (12). Adjacent to the GB element is a footprint site (FPV) (28) which also binds a liver-specific factor, initially termed HBLF and recently shown to be HNF-3 (5, 25, 38). In addition, overlapping the FPV site is another liver-specific site, termed 2c (14). Therefore, within a 50-nt region of the enhancer core there are four binding sites which bind at least seven nuclear factors (Fig. 7). The observation that three liver-specific sites, GB or RARE, FPV, and 2c can cooperate with the EP site to confer liver-specific enhancer function suggests a complex interaction between the liver-specific factors and the ubiquitously expressed MIBP1 and RFX1. Whether MIBP1 and/or RFX1 interacts with one or with several of these nuclear factors and whether such an interaction plays a role in the expression or replication of HBV, however, remains to be established.

We have previously shown that MIBP1 interacts with the regulatory regions of several viral genes, including the polyomavirus enhancer sequence (30), which is known to bind a protein termed EF-C (26). The EF-C protein has been shown to be identical to the EP binding protein, and thus, the EP binding protein has sometimes been referred to as the EF-C factor (27). In addition, it was recently reported that EP and EF-C represent the same activity as RFX1 (34). These observations suggest that MIBP1 and RFX1 may both bind to the polyomavirus enhancer region and that the EP or EF-C binding activities would consist, therefore, of at least two polypeptides, MIBP1 and RFX1.

Although the EP element was shown to be essential for efficient HBV enhancer function (8, 14, 27, 38), neither a single EP site nor a reporter construct with 7 to 10 tandem EP repeats, showed intrinsic enhancer activity in Alexander or HeLa cells when placed adjacent to the β-globin promoter (8). In contrast, we have shown that the presence of five tandem repeats of the EP element downregulates the SV40 promoter in a position- and orientation-independent manner in HepG2, Huh-7, Huh-4, and Hep3B cells. Whether this discrepancy is due to the difference between the promoters or cell lines used remains to be demonstrated. To exhibit transcriptional repression of the SV40 promoter, however, tandem EP repeats were required, since a single copy of the EP element was not sufficient for the silencer activity in HepG2 cells (data not shown). Similar results were reported in other studies of the SV40 enhancer, in which a single binding site which works in conjunction with the adjacent *cis*-acting sites did not exhibit transcriptional activity but could be active in a dimerized or multimerized form (10, 24). These studies suggested that the interaction between cooperating *cis*-acting elements and their binding factors could be compensated for by the duplication of a functional site which could then interact with itself to regulate transcription (17).

Although we observed that the EP element exhibited silencing activity on a heterologous promoter, other studies have reported that EP, in cooperation with adjacent *cis* elements, enhanced transcription in vivo (8, 14, 27, 38). Our results indicate that the silencing activity of the EP site may be restricted to hepatic cells, since activation of transcription was evident in cells of nonhepatic origin (data not shown). Thus, the activity of the EP element may depend on interactions with other cell-dependent factors.

Our results also showed that five tandem repeats of the EP site downregulated transcription in all hepatocarcinoma cells, although the levels of activity varied between different cell lines. In contrast, the MIF-1 element was active in some but not all cell lines tested. Cell lines which supported high levels of the EP silencer activity (10-fold downregulation) also demonstrated repression of MIF-1 activity; however, cell lines which supported lower levels of EP activity (3-fold downregulation) did not demonstrate activity with the MIF-1 site. A recent report (6) compared the affinities of protein binding between the EP or EF-C site and the MHC class II X-box sequence and concluded that proteins bound the viral EP and EF-C sites with a higher affinity than the MHC class II X-box sequence. This is in agreement with our data, since the EP viral site also appeared to interact with the protein complex with a higher affinity than the cellular MIF-1 site (Fig. 1 and 2). The 5′ half-site of the palindromic viral recognition site which is required for high-affinity protein binding (6), is different from the 5′ half-sites of both the X-box and the MIF-1 sequence (30). Therefore, the higher degree of silencing activity observed with the EP site compared with that of the MIF-1 sequence may result from preferential interaction of MIBP1 and RFX1 with both the 5′ and 3′ inverted repeats found in the EP site compared with the 3′ half-site interaction at the MIF-1 site.

The observation that the EP element was always active in all hepatic cells tested suggests that HBV may have evolved a higher-affinity binding site to compete efficiently with cellular *cis* elements for the low levels of RFX1 and MIBP1 found in eukaryotic cells. In addition, the observation that the EP site in cooperation with adjacent factors is required for liver-specific basal enhancer function, while, in contrast, the individual EP site acts as a silencer in the regulation of the promoter activity in liver cells, could lead to the differential activation or silenc-

ing of different viral genes and may be important for the replication of HBV.

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