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The Myc Intron-Binding Polypeptide Associates with RFX1 In Vivo and Binds to the Major Histocompatibility Complex Class II Promoter Region, to the Hepatitis B Virus Enhancer, and to Regulatory Regions of Several Distinct Viral Genes

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We demonstrated that MIF-1, identified initially as a binding activity that associated with the intron I element of the c-*myc* gene, consists of two polypeptides, the *myc* intron-binding peptide (MIBP1) and the major histocompatibility class II promoter-binding protein, RFX1. Using a polyclonal antiserum directed against either oligonucleotide affinity-purified MIBP1 or a peptide derived from RFX1, we showed that MIBP1 and RFX1 are distinct molecules that associate in vivo and are both present in DNA-protein complexes at the c-*myc* (MIF-1) and major histocompatibility complex class II (RFX1) binding sites. We have also found that MIBP1 and RFX1 bind to a regulatory site (termed EP) required for enhancer activity of hepatitis B virus. In addition, we have identified MIF-1-like sequences within regulatory regions of several other viral genes and have shown that MIBP1 binds to these sites in cytomegalovirus, Epstein-Barr virus, and polyomavirus. We have also demonstrated that the MIF-1 and EP elements can function as silencers in the hepatocarcinoma HepG2 and the cervical carcinoma HeLa cell lines. These findings indicate that MIBP1 and EP/RFX1 can associate in vivo and may regulate the expression of several distinct cellular and viral genes.

The product of the c-*myc* oncogene has been implicated in diverse cellular processes, including cell proliferation, differentiation, and tumorigenesis. The observation that c-Myc can heterodimerize with other cellular proteins to transactivate gene expression in vitro has suggested that c-Myc may function to modulate genes involved in cell growth and differentiation pathways (3, 4, 14). The regulation of the c-*myc* gene involves a complex interplay of *cis*- and *trans*-acting elements, and several nuclear proteins that interact with regulatory sequences found in the 5′ upstream and exon I untranslated regions of the c-*myc* gene have been identified (16, 31). In addition, a 20-bp region of intron I was defined as a binding site for a phosphoprotein, initially designated MIF-1, and it was demonstrated that binding to this site was abolished by a point mutation present in the corresponding region of a translocated Burkitt's lymphoma c-*myc* gene (34, 35). Adjacent binding sites in c-*myc* intron I were also identified and designated MIF-2 and MIF-3 (33), and somatic mutations found in Burkitt's lymphoma samples were shown to be frequently clustered within discrete domains that define these recognition sequences (33). These findings suggested that the mutations clustering in this region of the c-*myc* gene in Burkitt's lymphoma may be targeting specific regulatory elements for c-*myc* regulation, although direct evidence for this is lacking.

It was recently demonstrated that the RFX1 protein, which binds to the X box of the major histocompatibility complex (MHC) class II genes, the methylated DNA-binding protein (MDBP), and the EP protein, which binds to the enhancer of hepatitis B virus (HBV), all represent the same DNA binding activity (29, 36). The X-box sequence has been shown to play a role in regulating MHC class II gene expression (5, 27, 32), and the gene encoding the RFX1 protein has been isolated (19, 20) and shown not only to upregulate MHC class II gene expression but also to function as a transactivator of the HBV enhancer through interaction with the EP element (29). Since RFX1 was also shown to be identical to the MDBP molecule (36), and since the migration pattern of DNA-protein complexes at the MDBP site was similar to the migration of complexes formed at the MIF-1 site (15, 34, 35), it was suggested that MIF-1-binding protein could be identical to the RFX/EP/MDBP molecule (36, 37).

In this study, we demonstrated that the MIF-1 activity consists of at least two polypeptides, the *myc* intron-binding peptide (MIBP1) and the MHC class II promoter-binding protein, RFX1. We used a polyclonal antiserum raised against either oligonucleotide affinity-purified MIBP1 or the N-terminal peptide of the RFX1 molecule (20, 36) and demonstrated that MIBP1 and RFX1 are two distinct molecules that bind to the MIF-1 recognition sequence in intron I of the c-*myc* gene, the promoter region of MHC class II genes, and the regulatory regions of a several viral genes, including HBV, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and polyomavirus (Py) genes. Further, we showed that MIBP1 and RFX1 associate in vivo in HeLa cells. In this study, we also demonstrated that both the MIF-1 and EP recognition sites regulate the

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activity of a heterologous promoter in a position- and orientation-independent manner, and each can function as a silencer in hepatocarcinoma (HepG2) and cervical carcinoma (HeLa) cell lines. Taken together, our data suggest that in vivo association of MIBP1 and EP/RFX1 may modulate the recognition of specific DNA sequences and participate in the transcriptional regulation of several distinct cellular and viral genes.

MATERIALS AND METHODS

Plasmids and oligonucleotide probes. Double-stranded oligonucleotides were synthesized as follows: MIF-1, GATCTAGAGTAGTTATGGTAACTGGG; mutant MIF-1, GATCTAGAGTAGTTATGATTACTGG; EP, GATCCGTT GCTCGGCAACGGCCTA; mutant EP, GATCCCAACCTCGGCAACGGC CTA; CCAAT, GATCTTGACGTCCAATGAGCGCTTTG; CMV, GATCTG TATGTTCCCATAGTAACGCC; Py, GATCCAAAGCGGGTGCAGTAACA GGA; EBV-A, GATCCAAAGCGGGTGCAGTAACAGGA; EBV-B, GATCC GTTCGCGTTGCTAGGCCACCTTCA; RFX (HLA DR X-box sequence), GATCCCCTTCCCCTAGCAACAGATGA. The mutant nucleotide in each oligonucleotide is underlined. The MIF-1, mutant MIF-1, EP and mutant EP double-stranded oligonucleotides were multimerized, and fragments containing five tandem copies were gel purified and cloned into the *Bgl*II or *Bam*HI site of the pCAT-P vector (Promega). The locations of both restriction sites used for cloning, relative to the simian virus 40 (SV40) promoter, are shown in Fig. 7A. All reporter plasmids were confirmed by sequencing. The plasmids containing multimers with five copies of the oligonucleotides inserted in head-to-tail orientation in relation to the transcriptional unit of the SV40 promoter were defined as the sense constructs.

Cell culture and DNA transfections. HepG2 and HeLa cells were obtained from the American Type Culture Collection. 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, 2.0×10^6 HepG2 or 1×10^6 HeLa cells were plated in 100-mm-diameter dishes and incubated overnight at 37°C. The cells were transfected by calcium phosphate precipitation (6) for 5 h, using either 5 μg of pCAT-P or pCAT-P containing five copies of the indicated DNA binding site. One microgram of pGL_2-Luc (Promega) was cotransfected to control for transfection efficiency. At 42 h after glycerol shock, the dishes were washed with cold phosphate-buffered saline and scraped, and the cell pellets were resuspended in 100 μl of 100 mM KH_2PO_4 (pH 7.8) with 1 mM dithiothreitol (DTT) and stored at −20°C. Chloramphenicol acetyltransferase (CAT) and luciferase assays were performed as described previously (13).

Preparation of nuclear and whole-cell extracts. Nuclear extracts were prepared according to the modified method of Schreiber et al. (26). 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 DTT, 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 sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Both buffers contained freshly prepared aprotinin and leupeptin (each at 0.02 μg/μl). Supernatants were cleared by centrifugation after 15 min at 4°C and were frozen in aliquots at −70°C. Nuclear extracts used for immunoblots and immunoprecipitation were prepared according to the method of Dignam (8). The whole-cell extract was prepared by resuspending 1 g of cell pellet in 3 ml of buffer A (10 mM HEPES [pH 7.9], 10 mM $MgCl_2$, 1 mM DTT) and then incubating the mixture for 30 min at 4°C. The cell suspension was then adjusted to 0.35 M KCl, and the mixture was agitated gently for 20 min at 4°C and centrifuged for 15 min at 4°C. The supernatant was collected, glycerol was added to 20%, and aliquots were stored at −70°C.

Production of antisera specific for MIBP1 and RFX1 protein. Rabbit antiserum was raised against oligonucleotide affinity-purified MIBP1 polypeptide from HeLa cells. A nuclear protein preparation enriched for MIBP1 (originally designated MIF-1) (35) was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the 138/142-kDa band representing MIBP1 polypeptide was visualized with Coomassie blue and excised as a single band before injection into the popliteal lymph node of a New Zealand White rabbit (Hazleton Washington, Inc.). To prepare the antiserum for the RFX1 polypeptide, we synthesized a peptide corresponding to the N-terminal domain of the RFX1 molecule (residues 3 to 21) (20). The peptide was then injected subcutaneously into the New Zealand White rabbits (Hazleton Washington, Inc.).

Electrophoretic mobility shift assay. Mobility shift assays were performed as described previously (33, 34). One-tenth of a nanogram of ^{32}P-labeled duplex oligonucleotide probe (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 DTT, 3% Ficoll, and 1 μg of poly(dI-dC) or 2 μl of whole-cell extract (25 mg of protein per ml) in 20 μl of the reaction mixture containing 10 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 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 labeled oligonucleotide probe. The reaction mixture was then incubated for 15 min at room temperature and analyzed by PAGE (4% polyacrylamide gel).

Immunoblot and immunoprecipitation analysis. HeLa nuclear extracts (200 μg of protein per lane) were immunoprecipitated by using Sepharose A beads as previously described (7). The immunoprecipitates were washed with modified radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS), subjected to SDS-PAGE (7.5% polyacrylamide gel), transferred to a nitrocellulose filter, immunoblotted with the MIBP1 antiserum (1:250 dilution) or RFX1 antiserum (1:1,000 dilution), and then detected with ^{125}I-protein A as described previously (6). Blocking of antiserum was performed by incubating 10 μg of RFX1 peptide for 1 h at 4°C with the MIBP1 or RFX1 antiserum before immunoblotting.

RESULTS

MIBP1 and RFX1 are distinct polypeptides which associate in vivo. It has recently been reported that a protein which binds to the X box in the promoter region of MHC class II genes, RFX1, was the same as the HBV enhancer-binding protein, EP, and MDBP (29, 36). Since the migration of DNA-protein complexes at the MDBP site was similar to the migration of complexes formed at the MIF-1 site in gel mobility shift assays (15, 34, 35), it was suggested that the MIF-1-binding protein may be identical to the cloned RFX1/EP/MDBP molecule (36, 37). To determine whether the MIF-1-binding protein (designated MIBP1) and RFX1 comigrate on SDS-PAGE, we performed immunoblot analysis using both MIBP1 and RFX1 antisera (Fig. 1A). HeLa nuclear extracts were resolved on an SDS–7.5% polyacrylamide gel, and proteins transferred to a nitrocellulose membrane were incubated either with preimmune serum (lanes 1 and 4), RFX1 antiserum (lane 2), or MIBP1 antiserum (lane 5). In addition, MIBP1 and RFX1 antisera were preincubated with a synthetic peptide derived from the N-terminal region of the RFX1 polypeptide as controls (lanes 3 and 6). We detected a 160-kDa polypeptide with the MIBP1 antiserum, while a smaller polypeptide of 130 kDa was recognized by the RFX1 antiserum (lanes 2 and 5). In addition, the synthetic peptide used in developing the RFX1 antiserum blocked recognition of the 130-kDa band but did not block detection of the 160-kDa MIBP1 polypeptide (lanes 3 and 6). Since we had originally demonstrated by Southwestern (DNA-protein) blot analysis (35) that a 138-kDa polypeptide binds to the MIF-1 recognition sequence, we reexamined our original HeLa nuclear extracts as well as the oligonucleotide affinity-purified protein preparation by using the MIBP1 antiserum. We detected a 160-kDa polypeptide in nuclear extract used as the starting material but a 138-kDa polypeptide in the oligonucleotide affinity-purified protein fraction, suggesting either degradation or cleavage of the 160-kDa MIBP1 polypeptide.

To determine whether MIBP1 and RFX1 can interact in vivo, we immunoprecipitated HeLa nuclear extracts with the MIBP1 antiserum and analyzed the immunoprecipitates by gel electrophoresis and immunoblotting with the RFX1 antiserum (Fig. 1B). We found, after stringent washing conditions (0.1% SDS and 1% NP-40 in the wash buffer), that RFX1 coimmunoprecipitated with MIBP1 from human cells (lane 1). Proteins immunoprecipitated with the MIBP1 antiserum and then immunoblotted with the MIBP1 antiserum were used as positive controls (lane 2). These results suggest that the MIBP1 and RFX1 antisera recognize two distinct polypeptides and that MIBP1 and RFX1 associate in vivo in HeLa cells.

MIF-1, EP, and RFX sites bind MIBP1 and RFX1 polypeptides and form similar DNA-protein complexes. To compare

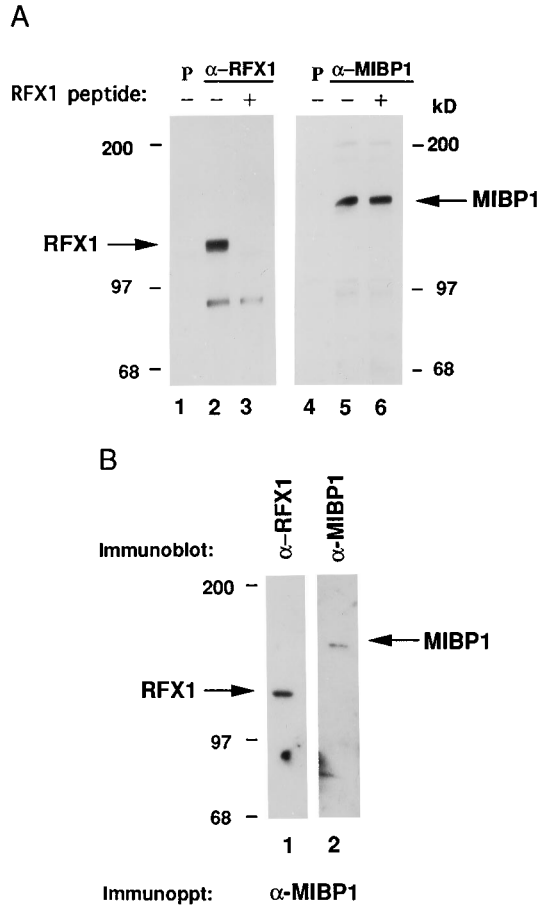

FIG. 1. MIBP1 and RFX1 are distinct proteins that associate in vivo. (A) Immunoblot analysis of MIBP1 and RFX1 from HeLa nuclear extracts. The 130-kDa RFX1 and 160-kDa MIBP1 polypeptides are indicated by the arrows. Preimmune serum (P) is shown in lanes 1 and 4. (B) Immunoprecipitation of HeLa nuclear extracts with MIBP1 antiserum (α-MIBP1; lanes 1 and 2) followed by immunoblotting with RFX1 antiserum (α-RFX1; lane 1) or MIBP1 antiserum (lane 2).

the DNA-protein complexes between the c-*myc* (MIF-1), HBV (EP), and MHC class II (RFX) sites, we synthesized oligonucleotide probes representing all three recognition sequences and compared the migration patterns of DNA-protein complexes in gel mobility shift assays (Fig. 2). We observed that the migration patterns for MIF-1, EP, and RFX were similar for two of the three DNA-protein complexes (Fig. 2A). Three bands (A, B, and C) were resolved at the MIF-1 recognition site (lane 1), while only bands A and B were detected with the EP and RFX binding sequences (lanes 2 and 3). Equal amounts of HeLa whole-cell protein extracts were incubated with the three oligonucleotide probes; however, stronger binding was observed at the EP site than at the MIF-1 and RFX sites, suggesting a higher binding affinity to the EP sequences. Since the EP element is located in an HBV enhancer region which is activated in hepatic cells, we compared the DNA-protein complexes of all three sites in the HepG2 hepatocarcinoma cell line and observed similar results (data not shown). High-resolution mobility shift gels, however, demonstrated differences in the migration patterns of the A complexes, with complex A formed with the RFX oligonucleotide probe migrating slightly faster than the complexes formed with the EP and MIF-1 sites (Fig. 2B). To test the specificity of these

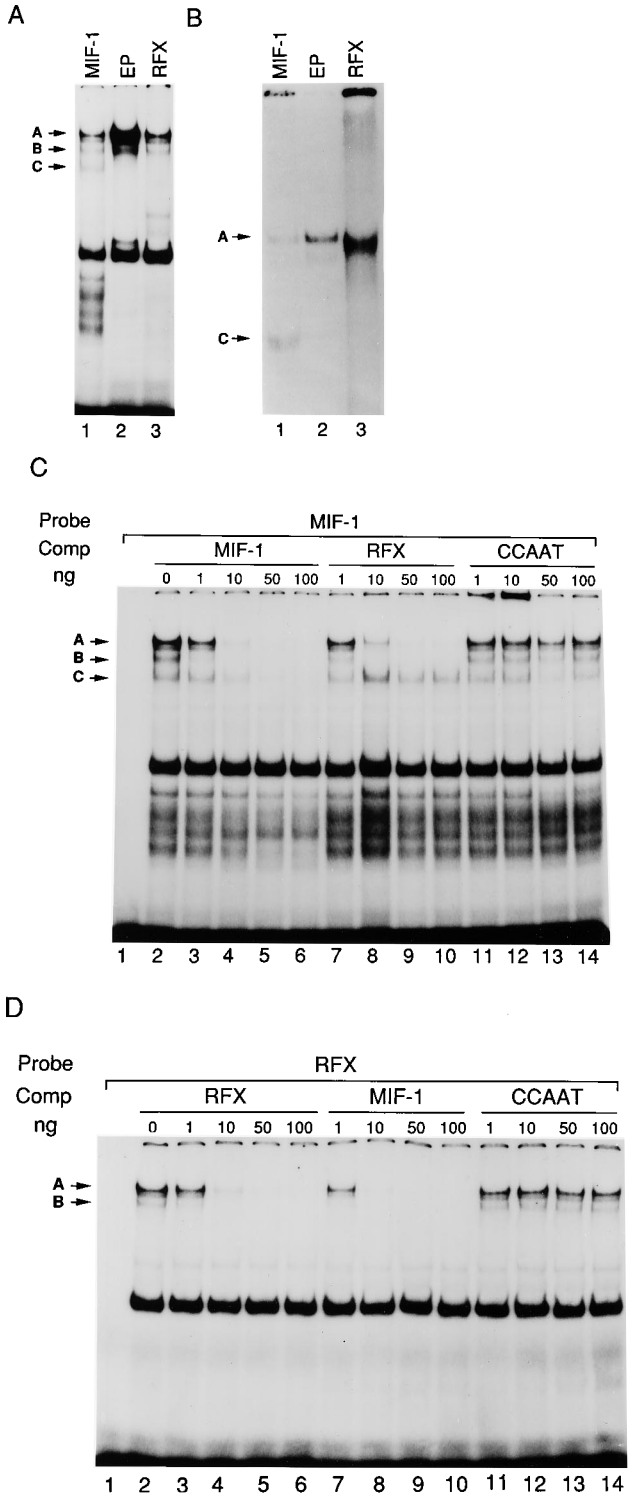

FIG. 2. Comparison of DNA-protein complexes between the MIF-1, EP, and RFX recognition sites. (A) Analysis of DNA-protein complexes with MIF-1, EP, and RFX binding sequences, using HeLa whole-cell extract. (B) High-resolution mobility shift assay of the MIF-1, EP, and RFX complexes. (C) Cross-competition analysis of MIF-1 with RFX sequences. (D) Cross-competition analysis of RFX with MIF-1 sequences. Specific DNA-protein complexes are indicated by arrows as A, B, and C. Comp, competitor.

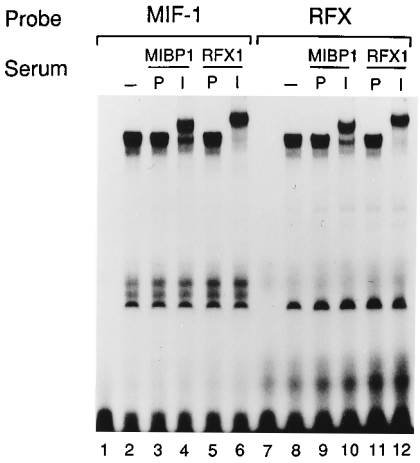

FIG. 3. Supershift analysis of MIF-1 and RFX DNA-protein complexes. MIBP1 and RFX antisera recognize DNA-protein complexes formed with the MIF-1 oligonucleotide (lanes 4 and 6) and with the RFX oligonucleotide (lanes 10 and 12). HeLa nuclear extracts were preincubated in the absence of serum (−) or in the presence of either preimmune serum (P) or MIBP1-specific or RFX1-specific immune serum (I). Lanes 1 and 7, MIF-1 and RFX probes in the absence of nuclear extracts.

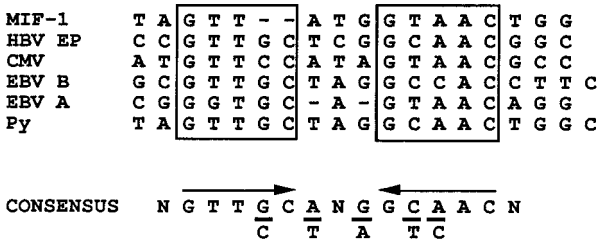

FIG. 4. Similarity in MIF-1 binding sequences in viral and c-*myc* regulatory regions.

complexes, we used MIF-1, EP, and RFX as probes and competitors in the mobility shift assay. We found that MIF-1 and RFX cross-compete for protein binding in the A and B complexes (Fig. 2C and D, lanes 2 to 10), while protein binding in complex C was blocked only by competition with MIF-1 (Fig. 2C, lanes 2 to 6). An unrelated competitor oligonucleotide showed no effect (Fig. 2C and D, lanes 11 to 14). Similar cross-competition was observed with the EP probe (data not shown).

Since MIBP1 and RFX1 associate in vivo, we examined whether MIBP1 and RFX1 polypeptides are the components of the DNA-protein complexes at MIF-1, EP, and RFX sites by testing whether the MIBP1 and RFX1 antisera altered the migration of these complexes. The MIF-1 or RFX oligonucleotide was incubated with HeLa nuclear extracts supplemented with either the immune (anti-MIBP1 or anti-RFX1) or preimmune serum before gel electrophoresis. The supershift analysis was performed under optimal conditions for antibody protein interaction (described in Materials and Methods) but not for resolution of the A, B, and C complexes as shown in Fig. 2. We observed that the MIBP1 antiserum specifically retarded the migration of the upper band (complex A) at both the MIF-1 and RFX sites (Fig. 3, lanes 4 and 10). The control preimmune serum showed no effect (lanes 3 and 9). In addition, antiserum raised against a synthetic peptide derived from the N-terminal portion of the RFX1 molecule also supershifted the complexes formed at the MIF-1 and RFX sites (lanes 6 and 12), while the preimmune serum showed no effect (lanes 5 and 11). The RFX1 but not MIBP1 antiserum supershifted the B complex (lanes 4, 6, 10, and 12); however, at higher dilutions, the RFX1 antiserum continued to supershift the A complex but could no longer supershift the B complex (data not shown). The antisera to MIBP1 and RFX1 did not alter migration of the control CCAAT DNA-protein complex (data not shown). In addition, both MIBP1 and RFX1 antisera supershifted the DNA-protein complexes at the EP site (data not shown and reference 29). Thus, MIBP1 immunoreactivity was present in MIF-1, RFX1, and EP complexes; conversely, the RFX1 antiserum recognized DNA-protein complexes formed by the MIF-1 EP and RFX sites. These results and the immunoprecipitation analysis suggest that MIBP1 and RFX1 associate in vivo and bind to the MIF-1, EP, and RFX recognition sequences.

Consensus MIF-1-like sequences are present in viral regulatory regions. The EP site in the HBV enhancer sequence exhibits homology to a set of inverted repeats found in the regulatory regions of CMV and Py (18, 29, 36). In addition, the DNA-protein complexes formed at the EP site are similar to the complexes formed with the Py enhancer element C, designated EF-C (1, 17). It was proposed, therefore, that EP and EF-C represent the same protein which binds to the enhancers of CMV, HBV, and Py (18, 29). Since MIF-1 and EP form similar DNA-protein complexes (Fig. 2A), we compared the MIF-1 binding sequences with the binding sites present in the regulatory regions of HBV, CMV, and Py (Fig. 4). In addition, a computer search using the Blast program identified two similar sites in the EBV genome (termed EBV-A and EBV-B) that fit the consensus sequence that we have established for the MIF-1 and viral sites (Fig. 4). Two identical EBV-A sites are located at positions 13971 to 13991 and 17042 to 17062, and the two identical EBV-B sites are located at positions 14259 to 14279 and 17332 to 17350. All sites are located upstream of the two EBNA promoters (positions 14352 and 17424) (25, 30). To determine whether MIBP1 also binds to sites found in CMV, Py, and EBV, we constructed synthetic oligonucleotides containing each of these sequences and compared their retardation patterns in gel mobility shift assays (Fig. 5A). We found that the migrations of these complexes were indistinguishable between the MIF-1, EP, CMV, Py, and EBV-B synthetic oligonucleotide probes, while the complexes were not detected with the EBV-A sequences (Fig. 5A, lanes 3 to 14). The quality of the protein extracts was confirmed by a control CCAAT-binding oligonucleotide probe (lane 2). In addition, proteins bound to the CMV, Py, and EBV-B sequences were cross-competed against by the MIF-1 and the EP probes but not by the CCAAT-specific sequences (Fig. 5B). Similarly, proteins bound to MIF-1 and EP sequences were cross-competed against by the CMV, Py, and EBV-B probes but not by EBV-A and the CCAAT probes (Fig. 5C). To determine whether MIBP1 was also present in the CMV, Py, and EBV-B complexes, we tested whether antiserum raised against oligonucleotide affinity-purified MIBP1 would alter the migration of these DNA-protein complexes. The CMV, Py, and EBV-B oligonucleotides were incubated with HeLa nuclear extracts supplemented with either the immune or the preimmune serum before gel electrophoresis. The MIBP1 antiserum, but not preimmune serum, retarded the migration of the upper band of the CMV, Py, and EBV-B complexes (Fig. 6), generating a supershift pattern identical to that observed with the MIF-1 and RFX complexes (Fig. 3). Migration of the DNA-protein complexes formed at the CCAAT oligonucleotide was unaltered by either the preimmune serum or the antiserum specific

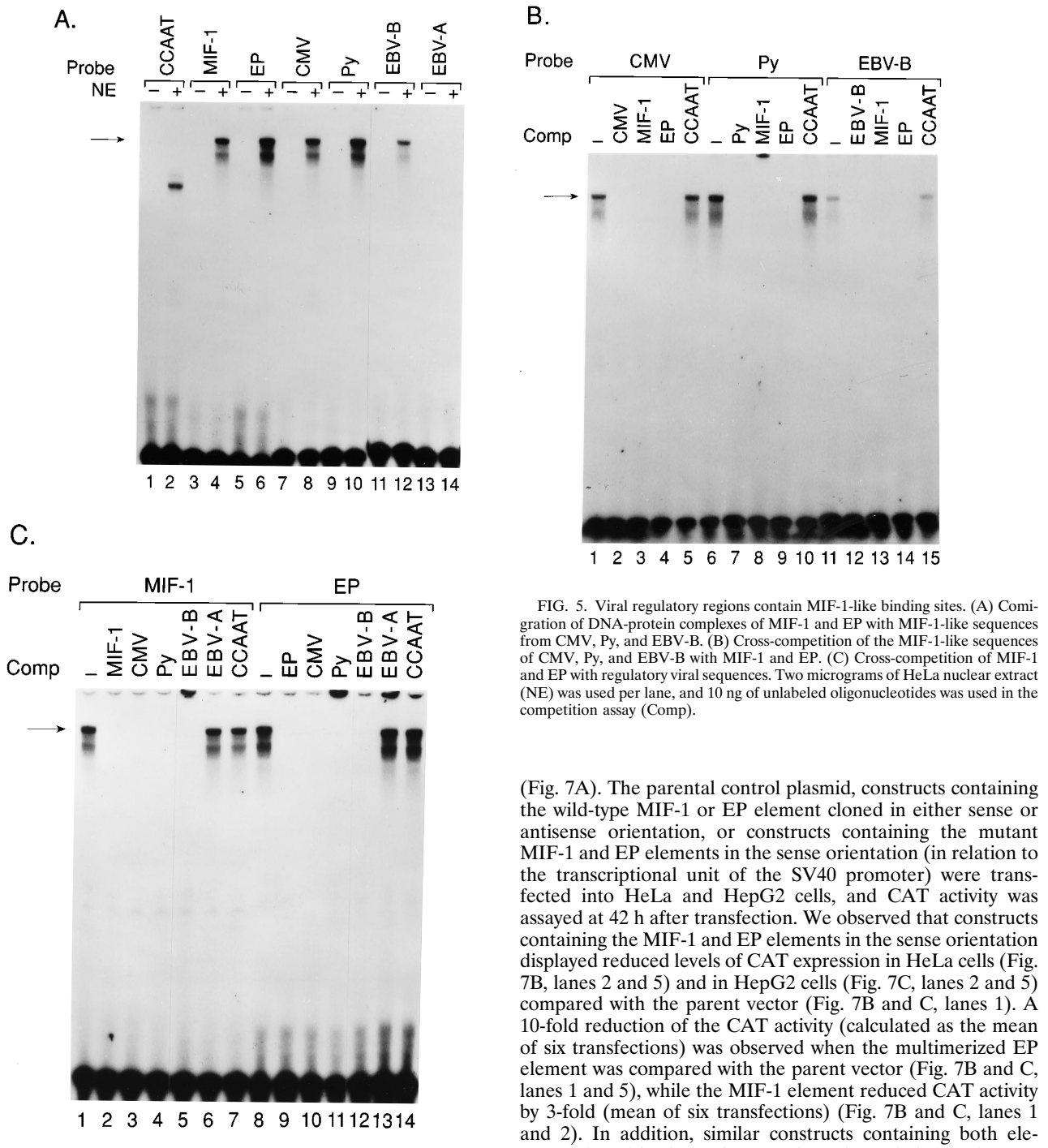

FIG. 5. Viral regulatory regions contain MIF-1-like binding sites. (A) Comigration of DNA-protein complexes of MIF-1 and EP with MIF-1-like sequences from CMV, Py, and EBV-B. (B) Cross-competition of the MIF-1-like sequences of CMV, Py, and EBV-B with MIF-1 and EP. (C) Cross-competition of MIF-1 and EP with regulatory viral sequences. Two micrograms of HeLa nuclear extract (NE) was used per lane, and 10 ng of unlabeled oligonucleotides was used in the competition assay (Comp).

for MIBP1. These data suggest that MIBP1 binds to the regulatory elements of these different viral genes.

MIF-1 and EP sites exhibit silencer activity in HepG2 and HeLa cells. To test whether the MIF-1 recognition site could mediate transcriptional regulation and to compare its potential transcriptional activity with that of the EP element, we prepared synthetic multimers of either MIF-1 or EP, as well as the mutant MIF-1 and EP, which are inactive in the binding assay (references 34 and 35 and data not shown). The oligonucleotide multimers were cloned in the *Bgl*II site adjacent to the SV40 promoter region in the CAT expression vector pCAT-P (Fig. 7A). The parental control plasmid, constructs containing the wild-type MIF-1 or EP element cloned in either sense or antisense orientation, or constructs containing the mutant MIF-1 and EP elements in the sense orientation (in relation to the transcriptional unit of the SV40 promoter) were transfected into HeLa and HepG2 cells, and CAT activity was assayed at 42 h after transfection. We observed that constructs containing the MIF-1 and EP elements in the sense orientation displayed reduced levels of CAT expression in HeLa cells (Fig. 7B, lanes 2 and 5) and in HepG2 cells (Fig. 7C, lanes 2 and 5) compared with the parent vector (Fig. 7B and C, lanes 1). A 10-fold reduction of the CAT activity (calculated as the mean of six transfections) was observed when the multimerized EP element was compared with the parent vector (Fig. 7B and C, lanes 1 and 5), while the MIF-1 element reduced CAT activity by 3-fold (mean of six transfections) (Fig. 7B and C, lanes 1 and 2). In addition, similar constructs containing both elements cloned in the antisense orientation relative to the promoter region revealed a similar pattern of CAT repression (Fig. 7B and C, lanes 1, 4, and 7). In contrast, the construct containing multimers of the mutant EP site displayed activity indistinguishable from that of the parent vector (Fig. 7B and C, lanes 1 and 6), while the MIF-1 mutant exhibited partial activity compared with the parent vector (Fig. 7B and C, lanes 1 and 3). This partial activity may be explained by the previously reported observation that the two point mutations present in the MIF-1 mutant oligonucleotide allow trace protein binding in the gel mobility shift assay compared with the wild-type probe (34, 35). To test whether the multimerized MIF-1 and EP elements could regulate transcription at a distance, we

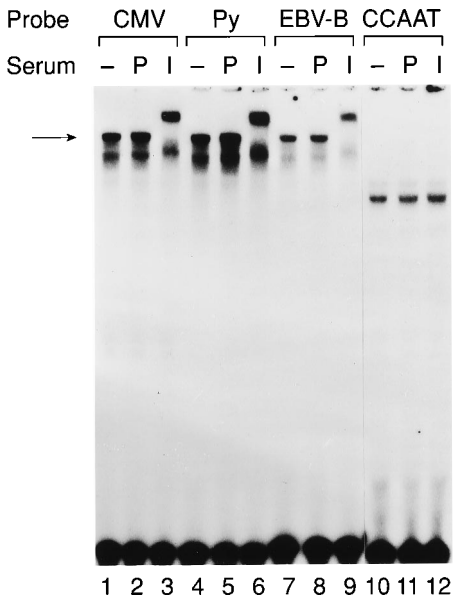

FIG. 6. MIBP1 antiserum recognizes the CMV, Py, and EBV DNA-protein complexes. HeLa nuclear extracts were preincubated in the absence of serum (−) or in the presence of either preimmune serum (P) or MIBP1-specific immune serum (I). The arrow shows the major DNA-protein complex that is supershifted by the MIBP1 antiserum.

inserted the multimers 2 kb upstream from the SV40 promoter region in the *Bam*HI site (Fig. 7A) and transfected these constructs into HepG2 cells. We observed a pattern of reduction in CAT expression (Fig. 7D) similar to that for multimers inserted adjacent to SV40 promoter region (Fig. 7B and C). These data, therefore, demonstrate for the first time that the MIF-1 element in intron I of the c-*myc* gene is capable of mediating transcriptional activity and also suggest that both the MIF-1 and EP elements can function as transcriptional silencers in HeLa and HepG2 cells, downregulating transcription in an orientation- and position-independent manner.

DISCUSSION

MIF-1 was initially identified as a binding activity that associated with a 20-bp sequence in the intron I region of the c-*myc* gene. Using specific antisera, we have now demonstrated that MIF-1 polypeptides MIBP1 and RFX1 are part of the MIF-1 complex. In addition, we have shown that MIBP1 and RFX1 associate in vivo and form DNA-protein complexes at c-*myc* (MIF-1) and MHC class II (X-box) recognition sequences. RFX1, which binds to the promoter region of MHC class II genes, was recently cloned and shown to be identical to the EP factor required for the enhancer activity of HBV (29). It was also demonstrated that MDBP shared biochemical characteristics as well as immunoreactivity with RFX1 (36). Therefore, it was proposed that a single gene product represents the binding and functional activities of RFX1, MDBP, and EP (29, 36). Since MDBP and MIF-1 (15, 34, 35) DNA-protein complexes were shown to migrate similarly in mobility shift assays, it was further suggested that MIF-1 might represent the same gene product as well (37). Using a polyclonal antiserum derived against either oligonucleotide affinity-purified MIBP1 or a peptide derived from RFX1, we have demonstrated that the 130-kDa RFX1 and the 160-kDa MIBP1 are distinct molecules which associate in vivo and may exist as heterodimers to bind to the MIF-1 or RFX recognition sequence. In addition, we showed that MIF-1-like sequences are also present in the regulatory regions required for promoter and enhancer activities of several distinct DNA viral genes, including the HBV, CMV, EBV, and Py genes (Fig. 4).

Examination of the DNA-protein complexes formed with the MIF-1, EP, or RFX oligonucleotide probe demonstrated both similarities as well as differences in binding patterns in the mobility shift assay. We found that three DNA-protein complexes A, B, and C, formed with the MIF-1 oligonucleotide, while only two complexes (A and B) were detected with the RFX and the EP oligonucleotide probes. We also observed minor but reproducible differences between the migration patterns of the A complex formed at the RFX, EP, and MIF-1 sites; however, both MIBP1 and RFX1 antisera reacted with components present in the A complex at all three sites. RFX1 contains a protein dimerization domain, and it was shown that the protein can homodimerize or heterodimerize with other members of the RFX family, including RFX2 and RFX3, in an in vitro system (20, 24). Therefore, the subunit compositions of potential homo- or heterodimers of RFX1 and its family members and/or MIBP1 may account for differences in the migration of the A complex. The polypeptides present in the B complex seem to be distinct from those in the A complex, since the RFX1 but not the MIBP1 antiserum could supershift the B complex. Further, at higher dilutions, the RFX1 antiserum continued to supershift the A complex but could no longer supershift the B complex (data not shown). These observations suggest that complex B may contain polypeptides with structural similarity to RFX1 (such as the other members of the RFX family [12, 26]); however, the exact composition of complex B remains to be established. Analysis of the C complex, which is present only with the MIF-1 binding site, indicated that it consists of two polypeptides, p105 and p115, which also interact with the MIF-2 recognition sequence, located adjacent to the MIF-1 binding site (33, 33a). The nature of these two polypeptides and their possible role in the interaction with MIBP1 and/or RFX1 remain to be established.

Analysis of the structural and functional properties of RFX1 has predicted that its DNA-binding and dimerization domains are located in different regions of the protein and are functionally independent of each other (20). Thus, it has been proposed that the RFX1 homodimer, which has two independent DNA-binding domains, could interact simultaneously with two different binding (X-box) sequences located far apart on the MHC class II DR gene. Such interactions would cross-link the sites leading to formation of a loop, thus bringing regulatory sequences situated far upstream and downstream into the vicinity of the transcription initiation site (20). A similar model has been described for the PRDII-BF1 protein containing two widely separated zinc finger motifs that could bind simultaneously to two copies of the same recognition sequence in the beta interferon gene (12). Similarly, an EP-like sequence was previously found in the upstream region of the murine c-*myc* gene (18), and we have identified a MIF-1-like sequence located about 600 bp upstream from the promoter region of the human c-*myc* gene which interacts with both the MIBP1 and RFX1 molecules (33a). Isolation of the gene that encodes MIBP1 would facilitate further characterization of the RFX1-MIBP1 interaction and would allow a more direct examination of their role in DNA binding and in the regulation of c-*myc*, MHC class II, and viral gene expression.

The identification of the MIF-1 recognition sequence as a protein binding site that is a target for mutations in Burkitt's lymphoma initially suggested that this region could function to negatively regulate c-*myc* expression (33, 34). The EP and the RFX sites, however, are positive elements required for the

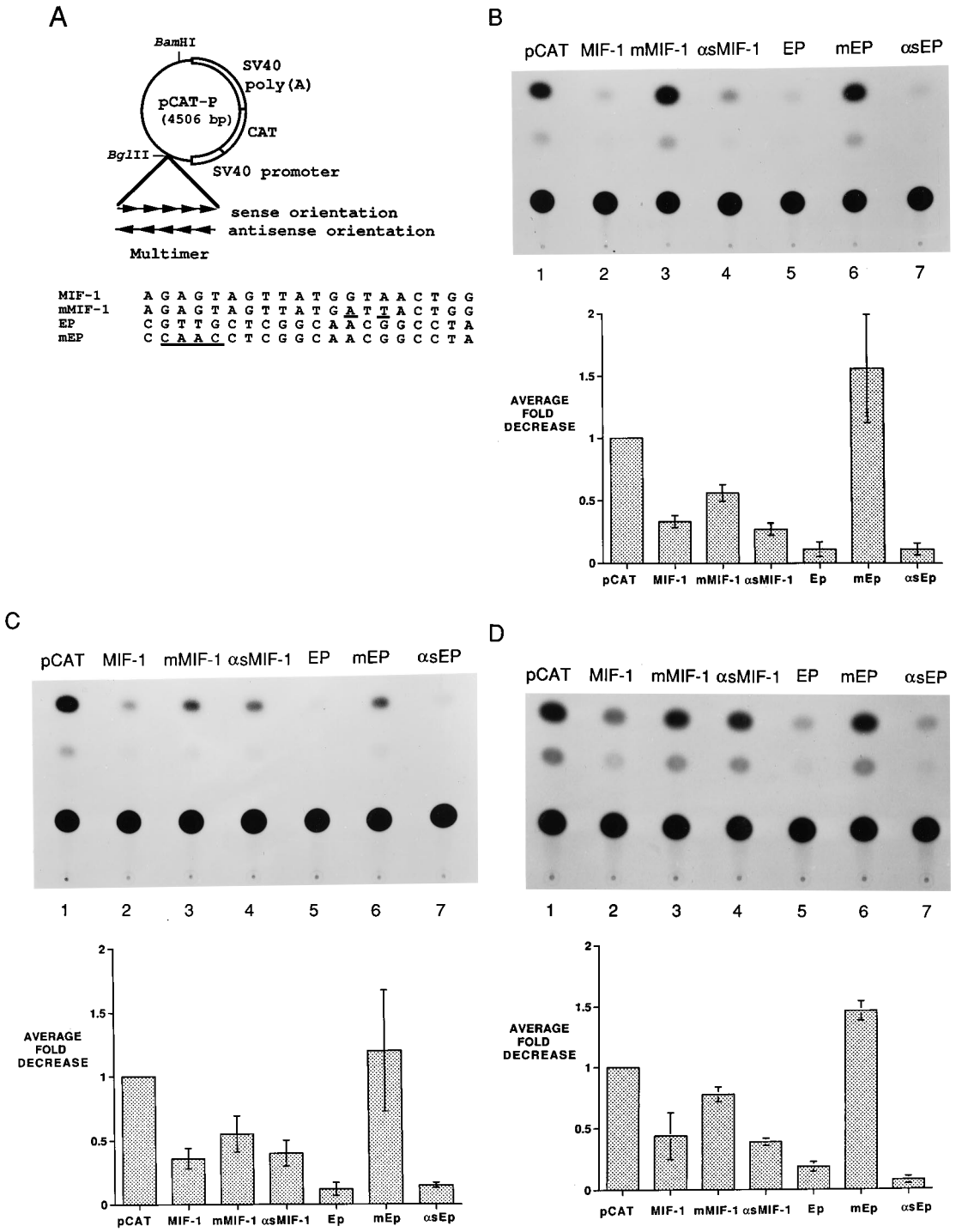

FIG. 7. Silencer activity of MIF-1 and EP binding sites. (A) The MIF-1, mutant MIF-1 (mMIF-1), EP, and mutant EP (mEP) binding sequences were cloned into an SV40 CAT expression vector either in the proximity of (*Bgl*II site) or 2 kb upstream from (*Bam*HI site) the SV40 promoter. The mutant nucleotide sequences in the binding sites of MIF-1 and EP are underlined. (B to D) CAT activity in transfected HeLa cells (B) or HepG2 cells (C) transfected with the multimers cloned into the *Bgl*II site or in HepG2 cells transfected with the multimers cloned at the *Bam*HI site (D). Bar graphs show analysis of three independent experiments each performed in duplicate in HeLa cells (B) or HepG2 cells (C and D), adjusted for transfection efficiency by cotransfection with the pGL_2-Luc vector.

promoter and enhancer activities of HBV and MHC class II genes, respectively (9, 27, 28, 32). In contrast, comparison of transcriptional activities between multimers of the MIF-1 and EP recognition sites demonstrated that each can act as a silencer when placed upstream from a heterologous SV40 promoter in HeLa and HepG2 cell lines. This discrepancy could be explained by the observation that the EP and RFX binding sites are both located adjacent to other functional *cis* elements (2, 9, 22) that act cooperatively to activate transcription (10, 21, 23). Similarly, the MIF-1 site is also located adjacent to three

additional protein-binding elements in the intron I of the c-*myc* gene, and it has been proposed that these sites work together to regulate promoter activity (33). To address the potential dual activator/repressor function of MIF-1/RFX EP elements, we transiently transfected multimerized MIF-1 and EP binding sites into different cell lines and found that the positive or negative effect on transcription is cell type dependent (33a). Therefore, our findings suggest that the activity of MIF-1 and EP/RFX1 in the regulation of c-*myc*, MHC class II, and HBV genes may depend on interaction with adjacent elements as well as with other factors that may work together in a cell-dependent manner.

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