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RFX1 Is Identical to Enhancer Factor C and Functions as a Transactivator of the Hepatitis B Virus Enhancer

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Hepatitis B virus gene expression is to a large extent under the control of enhancer I (EnhI). The activity of EnhI is strictly dependent on the enhancer factor C (EF-C) site, an inverted repeat that is bound by a ubiquitous nuclear protein known as EF-C. Here we report the unexpected finding that EF-C is in fact identical to RFX1, a novel transcription factor previously cloned by virtue of its affinity for the HLA class II X-box promoter element. This finding has allowed us to provide direct evidence that RFX1 (EF-C) is crucial for EnhI function in HepG2 hepatoma cells; RFX1-specific antisense oligonucleotides appear to inhibit EnhI-driven expression of the hepatitis B virus major surface antigen gene, and in transfection assays, RFX1 behaves as a potent transactivator of EnhI. Interestingly, transactivation of EnhI by RFX1 (EF-C) is not observed in cell lines that are not of liver origin, suggesting that the ubiquitous RFX1 protein cooperates with liver-specific factors.

The control of hepatitis B virus (HBV) gene expression involves complex protein-DNA and protein-protein interactions that are not clearly understood. Yet this control is crucial for the pathogenesis of HBV-induced hepatitis and hepatocarcinogenesis, and it represents an interesting model for liver-specific gene regulation. Expression of the HBV genes encoding the surface, core, polymerase, and X proteins is under the control of two enhancers, enhancer I (EnhI) and EnhII, acting cooperatively (18, 32, 34, 37, 42). EnhI plays an essential role in the control of all HBV promoters (18). It can be divided into two regions, the basal enhancer module and accessory modules (14, 38), each containing several functionally important binding sites for ubiquitous and/or liver-specific transcription factors (1, 4, 14, 38). Mutagenesis experiments have demonstrated that EnhI function is strongly dependent on an inverted repeat known as the enhancer factor C (EF-C; also called EP) site situated within the basal enhancer module (4, 14, 25, 38). Because of this crucial role, considerable effort has been devoted to the study of the nuclear factor (EF-C [EP]) that binds to the HBV EF-C site and might be functionally involved in the control of HBV gene expression. However, in the absence of the cloned gene, a direct demonstration of the functional role of EF-C has until now not been possible.

RFX1 is a novel DNA-binding factor, initially cloned by virtue of its affinity for the X-box motif of HLA class II promoters (26, 27). An examination of known *cis*-acting sequences revealed that the RFX1 target site in the X-box motif shows imperfect homology with EF-C site (Fig. 1A), and we therefore investigated a possible relationship between RFX1 and EF-C. Surprisingly, we find that RFX1 is in all respects indistinguishable from EF-C. RFX1 and EF-C have identical DNA-binding requirements and target site specificity. Moreover, affinity-purified EF-C is recognized efficiently by an RFX1-specific antiserum.

Availability of the cloned RFX1 (EF-C) gene has now made possible a direct exploration of its functional role in the liver-specific activity of the HBV enhancer. RFX1-specific antisense oligonucleotides known to inhibit the synthesis of RFX1 compromise EnhI-driven expression of the HBV major surface antigen (HBsAg) gene in HepG2 hepatoma cells. Furthermore, in transient cotransfection experiments, RFX1 is a potent transactivator of EnhI in HepG2 cells. Together with the biochemical evidence, these results firmly establish that RFX1 is identical to EF-C and provide the first direct demonstration of its role as a transactivator for EnhI. Interestingly, although RFX1 is a ubiquitously expressed factor, transactivation of EnhI by RFX1 is not observed in nonliver cells, suggesting that RFX1 must cooperate with liver-specific factors.

MATERIALS AND METHODS

Plasmids and oligonucleotides. pHBV1004 contains the transcription units for the major surface mRNA and the X mRNA of HBV (genome subtype adyw) under the control of their own promoters and EnhI (29). Mutant pHBV1004-C containing a disrupted RFX1 (EF-C)-binding site was constructed by polymerase chain reaction amplification of two fragments overlapping the RFX1 (EF-C) site at positions 1149 to 1163. The primers for amplification of the first DNA fragment (bp 742 to 1175) were 1004-C1 (5′-GATGATGTG GTATTGGGGGCCAAGTCTGTAC-3′ and 1004-C2 (5′-AC GTAGGCCATATAGGCCGGGGTAAAGGTTC-3′. Primers for amplification of the second DNA fragment (bp 1137 to 1672) were 1004-C3 (5′-ATTATGGCCTATATGGCCGGTC AGGTCTCTG-3′ and 1004-C4 (5′-GAGTCCAAGAGTCCT CTTATGTAAGACCTT-G3′). Primers 1004-C2 and 1004-C3, containing the base substitutions to be inserted in the RFX1 (EF-C)-binding site, were designed to create a unique *Sfi*I site (underlined). Amplified DNA fragments were digested with *Sfi*I and *Nsi*I (C1-C2 fragment) or *Sfi*I and *Sph*I (C3-C4 fragment) and ligated simultaneously into *Nsi*I-*Sph*I-digested pHBV1004. pRSVneo contains the neomycin resistance gene under the control of the Rous sarcoma virus promoter. For transactivation assays, a full-length RFX1 cDNA was subcloned into the pSG5 expression vector

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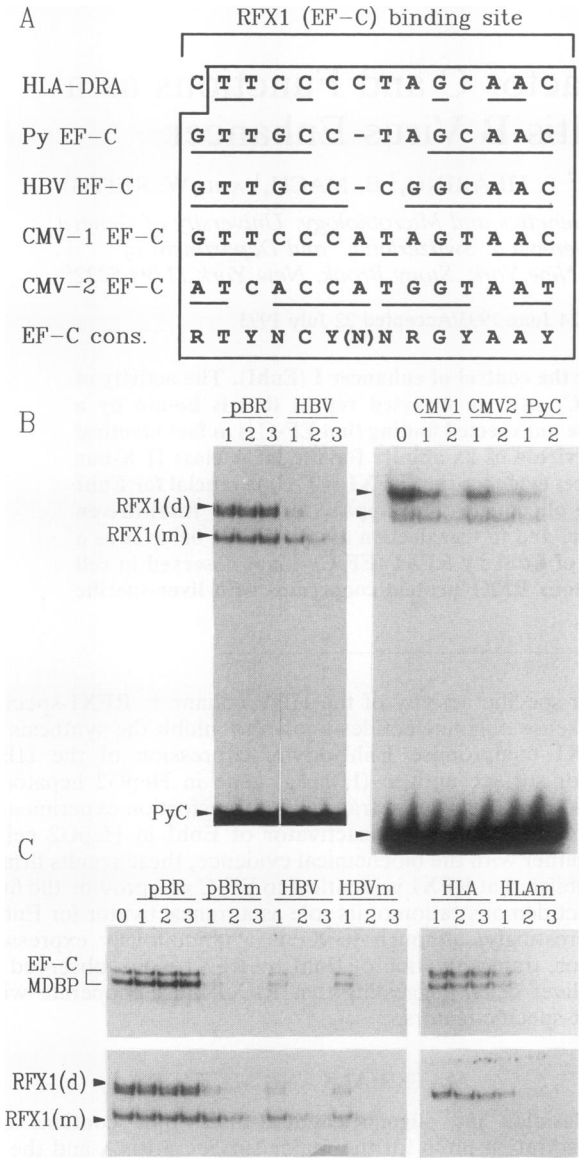

FIG. 1. RFX1 and EF-C have identical binding site specificity. (A) Sequence alignment between the X-box region of the HLA-DRA gene, the polyomavirus (Py), HBV, CMV1, and CMV2 EF-C sites, and the EF-C consensus sequence. R, purine; Y, pyrimidine; N, any nucleotide; (N), a nucleotide that may be present or absent. Nucleotides matching the EF-C consensus sequence are enclosed by a box. Nucleotides forming inverted repeats are underlined. The RFX1-binding site in the DRA gene (bracket at the top) coincides precisely with the region showing homology to EF-C sites. (B) Binding of in vitro-synthesized human RFX1 to the polyomavirus EF-C oligonucleotide was analyzed by EMSA. Positions of free DNA (PyC) and DNA bound by RFX1 dimers (d) and monomers (m) are indicated. Unlabeled competitor oligonucleotides containing a sequence from pBR322 (pBR) or the EF-C site of HBV were added during the binding reactions at a 10-fold (lanes 1), 50-fold (lanes 2), or 250-fold (lanes 3) molar excess. Competitor oligonucleotides containing the CMV1, CMV2, and polyomavirus (PyC) EF-C sites were added at a 10-fold (lanes 1) or 50-fold (lanes 2) molar excess. (C) Binding of EF-C in a B-cell nuclear extract (upper panels) and in vitro-translated human RFX1 (lower panels) was analyzed by EMSA using the polyomavirus EF-C oligonucleotide as a probe. Binding reaction mixtures contained no competitor (lanes 0), a 10-fold (lanes 1), 50-fold (lanes 2), or 250-fold (lanes 3) molar excess of the methylated or nonmethylated pBR and HBV oligonucleotides (left panels), or a 50-fold (lanes 1), 250-fold (lanes 2), or 500-fold (lanes 3) molar excess of the methylated or nonmethylated HLA oligonucleotide (right panels).

(Stratagene). The alkaline phosphatase reporter plasmid pSV2AP was used to monitor transfection efficiency as described previously (16).

pHBV-WT contains the HBV enhancer (subtype ayw) fused to the 21-bp repeats and early promoter sequences of simian virus 40 joined to the chloramphenicol acetyltransferase (CAT)-coding sequences (25). Mutant pHBV-IS9 is a derivative of pHBV-WT and contains an insertion of nine nucleotides between the two inverted repeats of the EF-C-binding site (25).

To construct plasmids pHRFX1 and pMRFX1 used for in vitro transcription-translation, full-length human (26, 27) and mouse (25a) cDNAs were inserted into plasmid pT7-7 (36), and the ATG translation initiation codon of the vector was eliminated to allow initiation at the ATG codon of the cDNA insert.

Preparation of double-stranded oligonucleotides used for electrophoretic mobility shift assays (EMSAs) has been described previously (28). The sequence of the PyC oligonucleotide containing the polyomavirus EF-C site (underlined) is GGCCAGTTGCCTAGCAACTAATT. The HBV, cytomegalovirus 1 (CMV1), CMV2, HLA, and pBR oligonucleotides have been described elsewhere (9, 21, 25, 41, 43, 44). Sequences of the nonmethylated HBV, HLA, and pBR322 sites are as follows: HBV, cGTTGCCCGGCAACg; HLA, gGCCGTCATGGCGCCc; and pBR322, gATCGTCA CGGCGATc. In the methylated versions of these sites, the underlined CpG dinucleotides contain 5-methylcytosine on both strands. The Y-box oligonucleotide contains nucleotides −89 to −49 of the DRA promoter.

Unmodified deoxyoligonucleotides (14 to 20 bp) used for antisense experiments were synthesized by means of an automatic DNA synthesizer (Applied Biosystems) and purified on Sep-pak columns (Millipore). The antisense oligonucleotides A1, A2, A3, A5, and A6 contain, respectively, nucleotides 87 to 100, 94 to 109, 310 to 323, 573 to 586, and 928 to 942 of the noncoding strand of the published RFX1 sequence (27). The sense oligonucleotide S5 contains nucleotides 586 to 573 of the coding strand. Neither sense nor antisense sequences were identical to any other known nucleotide sequence available in the EMBL data base.

Nuclear extract preparation, in vitro synthesis of RFX1, EMSAs, and methylation interference assays. Preparation of nuclear extract from Raji B cells has been described elsewhere (28). Plasmids pHRFX1 and pMRFX1 were linearized and transcribed with T7 RNA polymerase, and the resulting RNA was translated in vitro as described previously (27). To test the effect of sense and antisense oligonucleotides on translation, the oligonucleotides were added to in vitro translation reactions at a concentration of 25 μM, and translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). EMSAs and methylation interference assays were done as described previously (26–28). For analysis of proteinase K-resistant cores, binding reactions were allowed to proceed for 30 min and then digested for 15 min at 0°C with 25 μg of proteinase K per ml prior to gel electrophoresis. For testing reactivity of the RFX1 antiserum, 30-min binding reaction mixtures were supplemented with either antiserum or preimmune serum at a final dilution of 1/200 and then incubated

for a further 15 min at 0°C prior to gel electrophoresis. Production and characterization of the RFX1 antiserum is described elsewhere (17).

Purification of HeLa cell EF-C. Nuclear extracts from suspension cultures of HeLa cells were prepared by the method of Dignam et al. (3). Nuclear extract was dialyzed against DB-100 (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 100 mM KCl, 20% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The dialysate was clarified by centrifugation at 25,000 × *g* for 20 min and applied to a DEAE-cellulose column (~500 mg of total protein; 10-mg/ml bed volume) equilibrated in DB-100. EF-C activity was in the flowthrough fraction (~200 mg of total protein), which was dialyzed against 20 mM potassium phosphate (pH 6.8)–50 mM KCl–5 mM $MgCl_2$–20% glycerol–5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–0.1% 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate. The cleared dialysate was loaded on a hydroxylapatite column (bed volume, 6 mg of protein per ml) equilibrated in phosphate dialysis buffer. Bound proteins were eluted with a 20 to 400 mM potassium phosphate gradient. EF-C-containing fractions were pooled (~25 mg of total protein) and dialyzed against DB-100 containing 5 mM dithiothreitol. The cleared dialysate was loaded on a double-stranded calf thymus DNA column (bed volume, 5 mg of protein per ml) equilibrated with DB-100. Bound proteins were eluted with a 100 mM to 1 M KCl gradient in dialysis buffer. EF-C-containing fractions were pooled (~5 mg of protein), dialyzed against dialysis buffer containing 150 mM KCl (DB-150), and applied to an EF-C site-specific affinity column containing a multimerized oligonucleotide of the polyomavirus EF-C site in the presence of 100 μg of single-stranded calf thymus DNA per ml. The column was washed in DB-150, and bound proteins were eluted with dialysis buffer containing 1 M KCl (designated the B1 fraction; ~50 μg of total protein). EF-C was enriched ~10,000-fold during purification, with ~10% recovery of activity.

Cell culture and transfections. The human hepatoma cell line HepG2 and the fibroblastic cell line 143B were grown in Dulbecco modified essential medium supplemented with 2 mM L-glutamine, antibiotics, and 10% fetal calf serum at 37°C in 5% CO_2. DNA transfections were performed by calcium phosphate precipitation followed 4 h later by a glycerol shock. To obtain stable transfectants, HepG2 cells cotransfected with pRSVneo and pHBV1004 or pHBV1004-C were split after 42 h and resuspended in complete medium supplemented every 48 h with 1 mg of Geneticin per ml. After 4 weeks of antibiotic selection, surviving clones were expanded and assayed for secretion of HBsAg into the cell culture supernatant. The B-lymphoma cell line Raji was grown in RPMI 1640 medium supplemented as specified above and transfected by electroporation with a 250-V/960-mF pulse (Gene Pulser; Bio-Rad).

Detection of HBsAg and human albumin secretion. HBsAg and human albumin were detected in the supernatant of transfectants by enzyme-linked immunosorbent assay (ELISA) (Abbot Laboratories and Immundiagnostic, respectively) according to the manufacturers' instructions. Albumin contained in fetal calf serum is not recognized by the monoclonal antibody supplied with the ELISA kit. Cell supernatants were diluted to fall within the linear range of the human albumin detection assay.

Antisense inhibition in cell culture. HepG2 clones transfected with the wild-type (pHBV1004) or mutated (pHBV1004-C) construct were plated in 48-well plates at a density allowing growth to subconfluence after 3 days. Cells were always plated in triplicate. Twelve hours after plating, culture medium was replaced with Dulbecco modified essential medium containing 2.5% fetal calf serum, and oligonucleotides were added at a concentration of 25 μM. Cell viability was monitored and remained above 98% at all times. Culture supernatants were recovered after 1, 2, 3, or 4 days, and evaporation losses were compensated for where required prior to measurement of HBsAg and human albumin concentrations.

Transactivation of EnhI by RFX1 (EF-C). Cells were cotransfected as described above with the wild-type (pHBV-WT) or mutated (pHBV-IS9) plasmid and increasing doses of pSG5RFX1 or the control plasmid pSG5. The total amount of plasmid DNA was kept constant in all transfections. Cell extracts were prepared 42 h after transfection, measured for protein concentration, and assayed for CAT activity as described previously (13). The results were quantitated by excision of the acetylated and nonacetylated [^{14}C]chloramphenicol forms from the chromatograms and direct scintillation counting. All transfections were performed several times in duplicate or triplicate, using several different plasmid preparations.

RESULTS

RFX1 and EF-C have identical target site specificity and binding requirements. The RFX1-binding site in the HLA-DRA class II promoter has been positioned precisely on the basis of methylation interference experiments and the effect of promoter mutations that inhibit binding (15, 26, 27). This binding site exhibits homology with a set of inverted repeats called EF-C (also referred to as EP) sites (Fig. 1A). EF-C sites are *cis*-acting inverted repeats present in several viral enhancers, including the enhancers of polyomavirus, HBV, and CMV, and they derive their name from the nuclear protein, EF-C (or EP), that binds to them. EF-C (EP) was first identified on the basis of its binding to the EF-C site situated in enhancer element C of polyomavirus (24) and was subsequently shown to bind also to the EF-C sites of HBV and CMV (4, 9, 14, 25).

In view of the homology between EF-C sites and the RFX1-binding site in the DRA promoter, we explored the relationship between RFX1 and EF-C. We first analyzed the ability of RFX1 to bind to EF-C sites. EMSAs were performed with in vitro-synthesized RFX1 and an oligonucleotide probe containing the EF-C site of polyomavirus. As observed previously with use of the DRA site (27), complexes corresponding to the binding of both homodimers and monomers are detected (Fig. 1B). These complexes are abolished efficiently by oligonucleotides containing the HBV, polyomavirus, CMV1, and CMV2 EF-C sites (Fig. 1B). Direct binding to the HBV, CMV1, and CMV2 sites can also be demonstrated by using the corresponding oligonucleotides as probes (data not shown). All of the viral EF-C sites are thus indeed high-affinity binding sites for RFX1. In fact, the results of direct binding studies and comparative competition experiments have shown that affinity of RFX1 for sites such as the HBV and polyomavirus EF-C sites is greater than that for the major histocompatibility complex class II X-box sites (data not shown).

EF-C is indistinguishable from another nuclear complex called MDBP (methylated DNA-binding protein) (9, 19, 21, 41, 43, 44). Both EF-C and MDBP exhibit a unique and characteristic site-specific methylation-dependent DNA-binding activity at certain sites, a feature that has not been

described for any other nuclear or cloned DNA-binding proteins. EF-C (MDBP) binds to certain sites (e.g., a site in pBR322 and a site in the several HLA class I genes) only when the sites contain methylated C residues at CpG dinucleotides; binding to other sites (e.g., the HBV EF-C site) is independent of whether the CpG dinucleotides are methylated (9, 21, 41, 43, 44). To extend further the comparison of binding site specificity between RFX1 and EF-C (MDBP), we determined whether recombinant RFX1 also exhibits this site-specific methylation-dependent DNA-binding activity. Recombinant RFX1 or a B-cell nuclear extract (as source of EF-C [MDBP]) was incubated with the polyomavirus EF-C probe in the presence of either the methylated or nonmethylated versions of the pBR, HLA, and HBV competitor oligonucleotides (Fig. 1C). As observed in other cells (9, 25, 43), EF-C complexes in crude B-cell extracts are detected as a doublet. The results obtained for RFX1 and EF-C (MDBP) are identical. Competition by the HBV oligonucleotide is efficient and unaffected by CpG methylation, while the pBR and HLA oligonucleotides compete only when they are methylated. RFX1 thus has the same site-specific and methylation-dependent/independent binding activity as does EF-C (MDBP). Affinity of RFX1 for the methylated pBR site is comparable to that for the nonmethylated HBV and polyomavirus EF-C sites. As observed for MDBP (43), affinity for the methylated HLA site is approximately fivefold lower, as judged from the excess of unlabeled oligonucleotide required to obtain complete competition. Binding to the methylated pBR and HLA sites can also be demonstrated directly for both RFX1 and EF-C (MDBP) by using the methylated pBR (see Fig. 3B) or HLA (data not shown) oligonucleotides as probes.

Methylation interference experiments have shown that EF-C has symmetrically placed contact points situated in both halves of its palindromic target sites (9, 25). The same is observed for RFX1 when binding to a site such as the polyomavirus EF-C site is analyzed (Fig. 2A). The contact points obtained for RFX1 dimers are identical to those observed with EF-C, a finding that emphasizes further the identity in target site specificity between RFX1 and EF-C. RFX1 monomers bind preferentially to the right-hand EF-C half-site (only one minor inhibition and an enhancement are observed at the left half-site). A similar half-site preference at certain EF-C sites has been described for MDBP (35).

A final feature characteristic of EF-C (MDBP) is that two different proteinase K-resistant DNA-binding cores are generated at EF-C sites (35). The slower-migrating core reflects the fact that the protein binds as a dimer and corresponds in fact to a double core containing a proteinase K-resistant DNA-binding domain bound to each of the two EF-C half-sites (Fig. 2C) (35). The second, smaller core reflects the ability of EF-C (MDBP) to bind as a monomer and corresponds to a single proteinase K-resistant DNA-binding domain bound to only one of the two half-sites (Fig. 2C) (35). Identical results are obtained for RFX1 (Fig. 2B); treatment of RFX1-DNA complexes with proteinase K results in the formation of two cores (Fig. 2B and C, cores C2 and C1), which have been shown by methylation interference experiments to represent a double core (C2) having contact points in both EF-C half-sites and a single core (C1) having contact points in only one half-site. As expected from the methylation interference profile of intact RFX1 monomers, the C1 core has a clear preference for the right polyomavirus half-site.

RFX1 is present in affinity-purified nuclear EF-C. The binding data presented above suggested that RFX1 is identical to EF-C. To confirm this possibility, we performed an EMSA experiment to determine whether (i) RFX1 comigrates with EF-C and (ii) an antiserum specific for RFX1 cross-reacts with EF-C. The polyomavirus EF-C oligonucle-

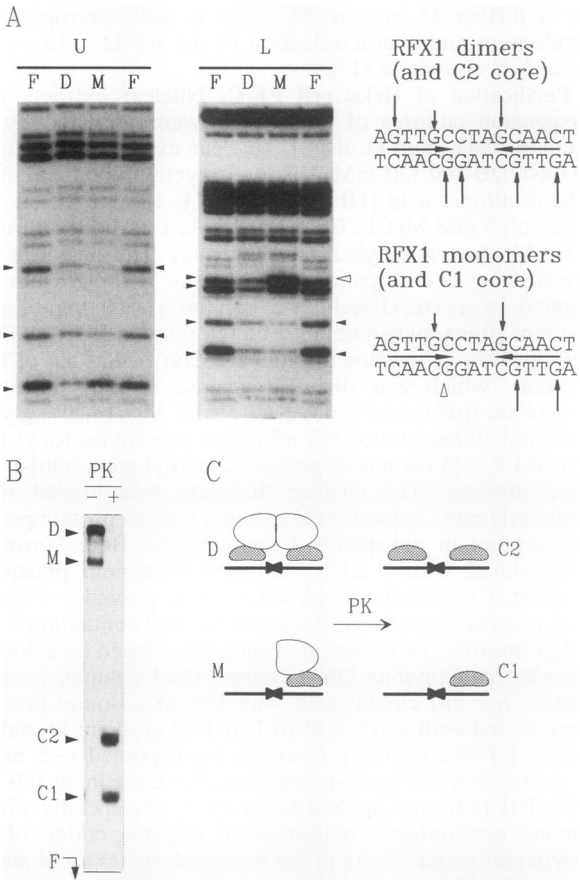

FIG. 2. Analysis of methylation interference contact points and proteinase K-resistant cores. (A) Contact points on the polyomavirus EF-C site were determined by methylation interference experiments for RFX1 dimers, RFX1 monomers, and the C1 and C2 proteinase K-resistant cores (see panel B). The gel for RFX1 dimers (D) and monomers (M) is shown at the left for the upper (U) and lower (L) DNA strands, and methylation interference profiles are summarized at the right. Arrows indicate G nucleotides at which methylation inhibits binding. The open arrowhead indicates a G residue at which methylation enhances binding of monomers. EF-C half-sites are underlined with arrows. (B) A preparation containing both RFX1 dimers (D) and monomers (M) was analyzed by EMSA using the polyomavirus EF-C oligonucleotide as a probe. Binding reactions were untreated (−) or treated (+) with proteinase K (PK) prior to gel electrophoresis. Positions of DNA bound by RFX1 monomers (M), dimers (D), and proteinase K-resistant cores (C1 and C2) are indicated. Free DNA (F) was allowed to run out of the gel in order to improve resolution. Methylation interference profiles of C1 and C2 are given in panel A. (C) Dimerization of RFX1 occurs via a C-terminal domain (unshaded region in the protein) that is sensitive to proteinase K and is structurally and functionally independent of the proteinase K-resistant DNA-binding domain (shaded region) (27). Binding of RFX1 dimers to the polyomavirus EF-C site involves strong interaction of each subunit with one of the two perfect half-sites. Monomers bind preferentially to the right half-site. Following treatment with proteinase K (PK), dimers give rise to a double core (C2) containing two independently bound proteinase K-resistant DNA-binding domains bound to both half-sites, and monomers give rise to a single core (C1) bound to the right half-site.

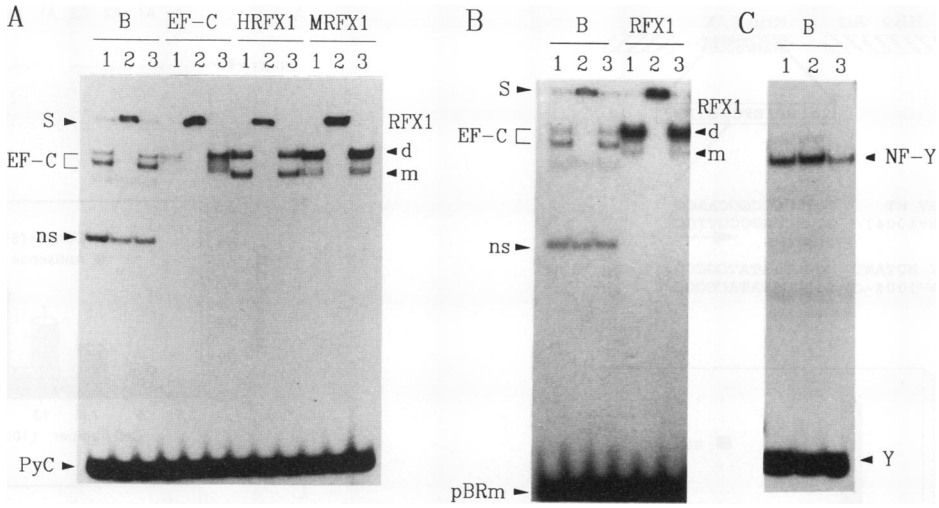

FIG. 3. RFX1 is present in the nuclear complex EF-C. (A) A B-cell nuclear extract (B), affinity-purified HeLa cell EF-C (EF-C), and in vitro-translated human (HRFX1) and mouse (MRFX1) RFX1 were analyzed by EMSA using the polyomavirus EF-C oligonucleotide as a probe. (B) As in panel A except that binding of in vitro-synthesized HRFX1 and EF-C in a B-cell nuclear extract was analyzed with the methylated pBR oligonucleotide as a probe. (C) As in panel A except that binding of NF-Y in a B-cell nuclear extract (B) was analyzed with an oligonucleotide (Y) containing the DRA Y box. Binding reactions were analyzed directly (lanes 1) or supplemented prior to gel electrophoresis with either RFX1 antiserum (lanes 2) or preimmune serum (lanes 3). Positions of free probe (PyC, pBRm, and Y) and probe bound by EF-C, RFX1 dimers (d), RFX1 monomers (m), NF-Y, and a nonspecific protein (ns) are indicated. Protein DNA-complexes supershifted by the RFX1 antiserum are indicated (complexes S, lanes 2).

otide or the methylated pBR oligonucleotide was incubated with in vitro-synthesized mouse and human RFX1, with a B-cell nuclear extract to detect crude EF-C, and with affinity-purified EF-C from HeLa cells, and protein-DNA complexes were resolved by gel electrophoresis (Fig. 3). Identical results are obtained with the HBV EF-C probe (data not shown). As observed in other cells (9, 25, 43), EF-C complexes in crude B-cell extracts are detected as a doublet of low electrophoretic mobility. With purified HeLa cell EF-C, only the upper band is visible, which is consistent with the observation that the upper band is the major EF-C complex detected in crude HeLa extracts (25). The complex corresponding to RFX1 homodimers comigrates with affinity-purified EF-C and with the upper band of the B-cell EF-C doublet (Fig. 3). RFX1 monomers migrate slightly faster than the lower band of the B-cell EF-C complex (Fig. 3).

To determine whether the RFX1-specific antiserum reacts with EF-C, binding reaction mixtures were supplemented with either the antiserum or preimmune serum prior to gel electrophoresis (Fig. 3). The RFX1 antiserum specifically retards migration of both dimeric and monomeric RFX1-DNA complexes. The same supershift is obtained for both the affinity-purified EF-C and the two EF-C complexes detected in the crude nuclear extract. The control preimmune serum reacts with neither RFX1 nor EF-C. The RFX1 antiserum is highly specific and does not react with the nuclear factor NF-Y (Fig. 3), with NF-S (17), or even with other highly homologous members of the RFX family (25a). We conclude that RFX1 is present in the EF-C complex and is responsible for its binding. Comigration suggests that the purified HeLa cell EF-C and the upper band of B-cell EF-C are RFX1 homodimers. The lower band detected in the B-cell extract contains RFX1 but migrates more slowly than RFX1 monomers. This lower complex is a heterodimer composed of RFX1 and another member (RFX3) of the RFX protein family (see Discussion).

The RFX1 (EF-C)-binding site of the HBV enhancer is required for expression of the HBV envelope protein. To study the effect of the HBV enhancer on the synthesis of the HBV envelope protein, HepG2 hepatoma cells were stably transfected with a plasmid containing the genes for the major surface antigen and X proteins of HBV, under the control of their own promoters and EnhI (pHBV1004). This transfection system has been shown to reproduce closely the natural HBV infection of hepatocytes (29). The role of the RFX1 (EF-C)-binding site was addressed by constructing a mutated plasmid (pHBV1004-C) in which the RFX1 (EF-C)-binding site is replaced with a random sequence (Fig. 4A). Following transfection, stable clones were selected and assessed by ELISA for the secretion of HBsAg into the culture medium. Figure 4B shows that HepG2 cells transfected with the plasmid containing the wild-type enhancer (pHBV1004) secrete substantial amounts of HBsAg. The amount of HBsAg secreted correlates well with the number of HBsAg-producing cells analyzed. In contrast, HBsAg was virtually undetectable in the supernatant of all clones transfected with the plasmid containing the mutated EF-C site (pHBV1004-C), even at the highest cell density. This drastic difference (up to 100-fold) in the level of HBsAg produced by the two types of transfectants indicates that synthesis of the major viral envelope protein is strictly dependent on the RFX1 (EF-C)-binding site of EnhI. These results confirm and extend those of transient transfection assays showing that disruption of the RFX1 (EF-C)-binding site leads to a 10-fold reduction in the activity of EnhI (25).

RFX1 antisense oligonucleotides inhibit expression of the HBV envelope protein. Unmodified oligodeoxynucleotides (14 to 20 bp), complementary to the sense or antisense (control) strand of the human RFX-1 gene, were first assayed for the ability to inhibit RFX1 synthesis in vitro. In vitro translation reactions containing a constant amount of full-length RFX1 mRNA were carried out in the presence of 25 μM various candidate antisense oligonucleotides, and the effect on translation was monitored by SDS-PAGE (Fig.

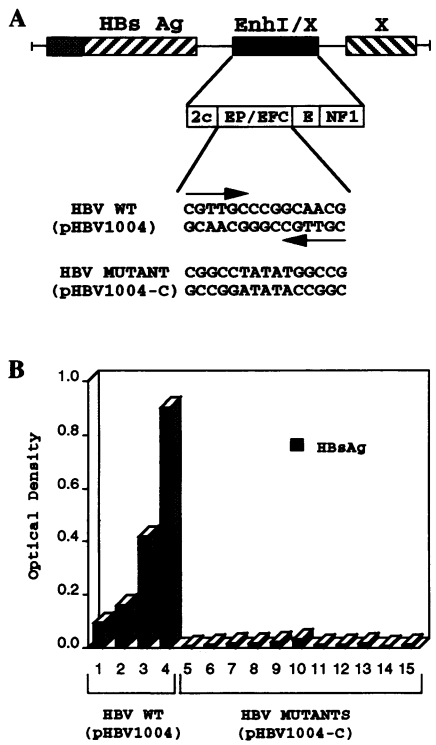

FIG. 4. The RFX1 (EF-C) site is essential for EnhI function. (A) Schematic representation of pHBV1004 showing the HBsAg gene and its promoter, the HBV X-protein gene and its promoter, and EnhI. The position of the EF-C (EP) site is shown with respect to other *cis*-acting sequences, the 2c (or HBLF), E, and NF1 sites present in EnhI. In the mutated pHBV1004-C construct, the palindromic EF-C site (arrows) has been replaced with an *Sfi*I site. WT, wild type. (B) Levels of HBsAg production by HepG2 cells stably transfected with the HBsAg gene under the control of the wild-type (WT; pHBV1004) or mutated (pHBV1004-C) enhancer. HBsAg was detected by ELISA of the cell culture supernatant 3 days after plating. Columns: 1 to 4, increasing numbers (5,000, 10,000, 20,000, and 40,000, respectively) of cells stably transfected with the wild-type plasmid (pHBV1004); 5 to 15, 40,000 cells of 11 independent clones transfected with the plasmid containing the mutated EF-C site (pHBV1004-C).

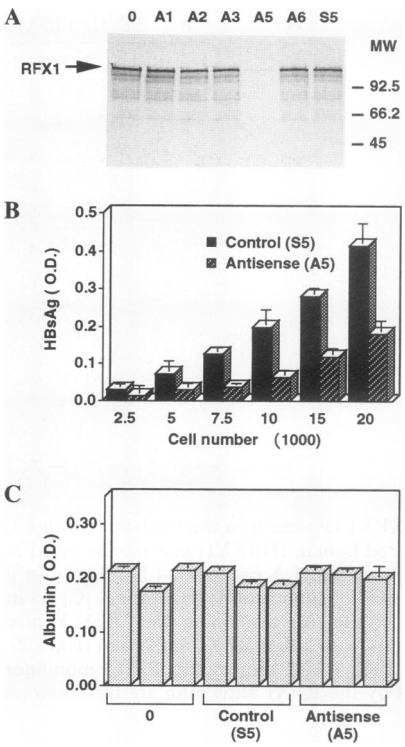

FIG. 5. Inhibition of HBsAg secretion by RFX1 antisense oligonucleotides. (A) Inhibition of in vitro translation of RFX1 by antisense oligonucleotides. Translation of RFX1 was performed in the absence (0) and presence of 25 μM sense (S5) and antisense (A1 to A6) RFX1 oligonucleotides. Translation products were analyzed by SDS-PAGE. Molecular weights (MW) are indicated in thousands. (B) Inhibition of HBsAg secretion by the RFX1-specific antisense oligonucleotide A5. HepG2 cells stably transfected with pHBV1004 were plated at the indicated optical densities (O.D.) and incubated with a single dose of the antisense oligonucleotide A5 (5′-GGTCAGCTGGAGGG-3′) or the control sense oligonucleotide S5 (5′-CCCTCCAGCTGACC-3′). HBsAg in the cell supernatant was measured by ELISA after 3 days. Results represent the mean and standard deviations of triplicate experiments. (C) Albumin secretion is not affected by RFX1-specific antisense oligonucleotides. Cells were treated as described above, and human albumin in the cell supernatant was measured by ELISA after 3 days. Results represent the mean and standard deviations of triplicate experiments.

5A). This approach allowed selection of an antisense oligonucleotide (A5) that proved to be efficient enough to result in an almost complete inhibition of full-length RFX1 synthesis (Fig. 5A). Specificity of this inhibition is demonstrated by the facts that no inhibition is obtained with the corresponding sense oligonucleotide control (S5) used at the same concentration (Fig. 5A) and that the antisense A5 oligonucleotide has no effect on the simultaneous translation of an unrelated control protein (data not shown).

The A5 antisense oligonucleotide was then tested for its capacity to inhibit HBsAg expression in HepG2 cells stably transfected with pHBV1004. Cells were plated at low density, and culture conditions were optimized to allow linear growth in a minimal volume. The control (S5) or antisense (A5) oligonucleotide was added as a single dose at a 25 μM concentration. Cells treated with the antisense RFX1 oligonucleotide A5 exhibit a strong reduction in HBsAg secretion compared with cells incubated in the absence of oligonucleotide or in the presence of the control sense oligonucleotide S5 (Fig. 5B). Inhibition of HBsAg secretion by a single dose of the A5 oligonucleotide attained 60 to 70%. Time course experiments showed that the inhibition by a single dose of antisense oligonucleotide was detectable only after 24 h (a period of time presumably required for intracellular depletion of RFX1 [EF-C]) and persisted for 3 days (data not shown).

The specificity of the inhibition of HBsAg secretion was studied by measuring the effect of the antisense or sense oligonucleotide on a control protein secreted by the same pathway. The synthesis of several proteins known to be secreted by HepG2 cells (22) was assessed by nephelometry (data not shown). Among them, human albumin was shown to be detectable in the supernatant of small numbers of cells at a concentration of 20 mg/liter. A highly sensitive ELISA was used to measure the secretion of albumin by oligonucleotide-treated or untreated HepG2 cells. Figure 5C demonstrates that neither the sense (S5) nor antisense (A5) oligonucleotide affects the secretion of albumin, indicating that the effect on HBsAg is specific and not due to a general

reduction in protein secretion. Together with the strong reduction in HBsAg synthesis obtained by disruption of the RFX1 (EF-C) site (Fig. 4), these experiments suggest that the RFX1-specific antisense oligonucleotide inhibits HBsAg synthesis by interfering with EnhI activity.

Liver-specific transactivation of EnhI by RFX1 (EF-C). Since RFX1 (EF-C) is a ubiquitously expressed protein, direct complementation studies using cells deficient in RFX1 could not be carried out. We therefore assessed the effect of overexpression of RFX1/EF-C on the activity of EnhI. Plasmids containing a CAT reporter gene driven by the simian virus 40 early promoter fused to either the wild-type HBV enhancer (pHBV-WT) or an HBV enhancer mutated in the RFX1 (EF-C site) (pHBV-IS9) were cotransfected into HepG2 cells with an RFX1 expression vector (pSG5RFX1). When transfections conditions are optimized to allow a low basal level of expression of the wild-type enhancer construct (i.e., by using low amounts of pHBV-WT), cotransfection with pSG5RFX1 results in up to a 20-fold, dose-dependent increase in CAT activity (Fig. 6A and B). This transactivation is strictly dependent on the presence of an intact RFX1 (EF-C)-binding site, since no transactivation is obtained with the reporter plasmid containing the mutated enhancer (pHBV-IS9) (Fig. 6A and B).

Human B cells and fibroblasts have been shown to allow weak expression of the HBV enhancer (7). This finding was confirmed by transfection of pHBV-WT into Raji and 143B cell lines (Fig. 6C). Since RFX1 (EF-C) is a ubiquitously expressed factor, we explored whether transactivation of EnhI could also be observed in these nonliver cell lines. Contrary to the strong transactivation obtained with RFX1 (EF-C) in HepG2 hepatoma cells, no transactivation of EnhI could be obtained under any of the conditions tested in the Raji and 143B cell lines (Fig. 6C). This observation strongly suggests that transactivation of EnhI by the ubiquitous protein RFX1 (EF-C) is liver cell specific.

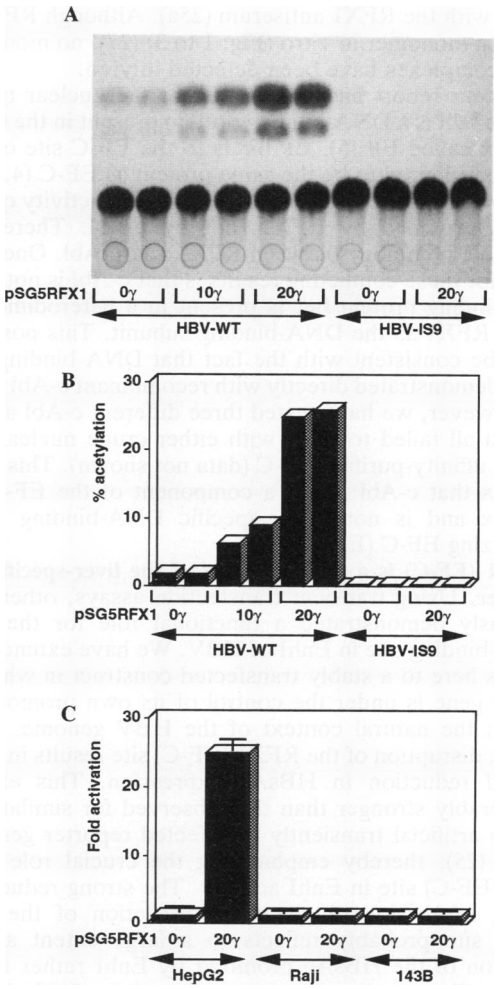

FIG. 6. Transactivation of EnhI by RFX1 (EF-C) is restricted to hepatoma cells. (A) Reporter plasmids (0.5 μg per plate) containing the CAT gene driven by the simian virus 40 early promoter and either the wild-type enhancer (pHBV-WT) or an enhancer mutated in the EF-C site (pHBV-IS9) were cotransfected with increasing amounts (0, 10, or 20 μg) of pSG5RFX1 into hepatoblastoma (HepG2) cells. CAT activity was measured after 2 days. Results a representative CAT assay are shown. (B) CAT activity of the experiment shown in panel A, expressed as percent acetylation of [^{14}C]chloramphenicol. (C) Transfections with 0.5 μg of pHBV-WT and 0 or 20 μg of pSG5RFX1 were performed in parallel with HepG2, Raji, and 143B cells. The means and standard deviations derived from three independent experiments are presented. Results are given as fold induction of CAT expression in the presence of 20 μg of pSG5RFX1 relative to that obtained without pSG5RFX1, i.e., with only 20 μg of the control plasmid pSG5.

DISCUSSION

RFX1 is identical to EF-C. EF-C sites are bound by nuclear complexes called EF-C, EP, and MDBP. EF-C, EP, and MDBP probably all represent the same binding activity (4, 5, 9, 14, 43). We have established here that EF-C (EP, MDBP) contains RFX1. An antiserum specific for recombinant RFX1 (17) reacts efficiently with affinity-purified EF-C as well as with EF-C in a crude nuclear extract (Fig. 3). Cross-reactivity with a homologous protein is unlikely because the RFX1-specific antiserum does not react with the other cloned members of the RFX protein family despite the fact that they share significant regions of homology (25a). The conclusion that RFX1 is present in EF-C (EP, MDBP) is further supported by the fact that they are indistinguishable by all criteria that have been examined. These include (i) expression in a wide variety of cell lines, including the cells (B lymphocytes) from which RFX1 was cloned (25, 27, 43), (ii) high-affinity binding to the polyomavirus, HBV, CMV1, and CMV2 EF-C sites (Fig. 1) (9, 25, 43, 44), (iii) binding as dimers (Fig. 2) (25, 27, 35), (iv) stronger interaction with one half-site of certain binding sites (Fig. 2) (35), (v) identical symmetrical contact points on the palindromic polyomavirus EF-C site (Fig. 2) (9, 25), (vi) presence of proteinase K-resistant DNA-binding cores (Fig. 2) (9, 35), (vii) CpG methylation-dependent binding to certain sites and CpG methylation-independent binding to EF-C sites (Fig. 1) (9, 21, 41, 43), (viii) molecular weight (purified EF-C has an estimated molecular weight of 140,000 [data not shown], and both nuclear and recombinant RFX1 migrate with an apparent molecular weight of 140,000) (17), (ix) inactivation of binding activity by alkylation with *N*-ethylmaleimide (reference 9 and data not shown), and (x) comigration in EMSA of affinity-purified EF-C and recombinant RFX1 homodimers (Fig. 3). The lower band of the EF-C complex detected in the B-cell nuclear extract represents a heterodimer consisting of RFX1 and another member (RFX3) of the RFX protein family which can heterodimerize with RFX1 (25a). This conclusion is based on the fact that the lower complex comigrates with in vitro-synthesized RFX1-RFX3 heterodimers and reacts with an antiserum specific for RFX3 as

well as with the RFX1 antiserum (25a). Although RFX1 can bind as a monomer in vitro (Fig. 1 to 3) (27), no monomeric RFX1 complexes have been detected in vivo.

A recent report has suggested that the nuclear tyrosine kinase c-Abl is a DNA-binding protein present in the nuclear complex called EP (5). EP binds to the EF-C site of HBV and is considered to be the same protein as EF-C (4, 5, 14). We have shown here that the DNA-binding activity of EF-C (EP) is provided by RFX1, not by c-Abl. There is no detectable homology between RFX1 and c-Abl. One explanation for these conflicting results is that c-Abl is not itself a DNA-binding protein but is present in a heterodimer containing RFX1 as the DNA-binding subunit. This possibility would be consistent with the fact that DNA binding could not be demonstrated directly with recombinant c-Abl protein (5). However, we have tested three different c-Abl antibodies, and all failed to react with either crude nuclear EF-C (EP) or affinity-purified EF-C (data not shown). This finding suggests that c-Abl is not a component of the EF-C (EP) complex and is not a site-specific DNA-binding protein recognizing EF-C (EP) sites.

RFX1 (EF-C) is a transactivator of the liver-specific HBV enhancer. Using transient transfection assays, others have previously demonstrated a functional role for the RFX1 (EF-C)-binding site in EnhI of HBV. We have extended this analysis here to a stably transfected construct in which the HBsAg gene is under the control of its own promoter and EnhI in the natural context of the HBV genome. In this system, disruption of the RFX1 (EF-C) site results in up to a 100-fold reduction in HBsAg expression. This effect is considerably stronger than that observed for similar mutations in artificial transiently transfected reporter gene constructs (25), thereby emphasizing the crucial role of the RFX1 (EF-C) site in EnhI activity. The strong reduction in HBsAg expression obtained by disruption of the RFX1 (EF-C) site probably reflects to a large extent a direct activation of the HBsAg promoter by EnhI rather than an indirect effect mediated by activation of the HBV X gene. The X gene is present on the construct (pHBV1004) used to study the role of the RFX1 (EF-C) site. However, the RFX1 (EF-C) site is known to contribute only weakly to the activity of the X promoter (14), and its disruption should thus not strongly reduce X-protein expression. Moreover, complete elimination of X-protein synthesis by the introduction of a frameshift mutation into the X gene of pHBV1004 reduces HBsAg synthesis only by a factor of 2 to 5 (reference 29 and data not shown), an effect that is weak compared with the 100-fold reduction obtained by mutation of the RFX1 (EF-C) site. Finally, the inhibitory effect of the RFX1-specific antisense oligonucleotide is observed even in the absence of a functional X gene (data not shown).

Consistent with the importance of the RFX1 (EF-C) site and the biochemical evidence for identity between RFX1 and EF-C, we have demonstrated here that RFX1 (EF-C) is indeed a transactivator of EnhI. Overexpression of RFX1 (EF-C) in HepG2 hepatoma cells results in up to a 20-fold transactivation of EnhI, and this transactivation requires an intact RFX1 (EF-C) site. Perhaps even more significantly, interfering with the synthesis of endogenous RFX1 (EF-C) by an RFX1-specific antisense oligonucleotide appears to result in a specific inhibition of EnhI-driven expression of the HBsAg gene. The transactivation observed in transient transfections is thus unlikely to be an artifact due simply to overexpression of exogenous RFX1.

RFX1 belongs to a novel family of transcription factors that have essentially identical target site specificity and are capable of binding as heterodimers (25a). We are presently studying whether RFX1 functions at EnhI as a homodimer or as a heterodimer with other members of the RFX family.

Transactivation of EnhI by RFX1 (EF-C) is observed in HepG2 hepatoma cells but not in cells that are not of liver origin such as B cells and fibroblasts. This is surprising because RFX1 (EF-C) is a ubiquitously expressed factor and suggests that it may function in conjunction with liver-specific factors to activate EnhI. Cooperation between EF-C and liver-specific factors has also been postulated recently on the basis of the observation that the basal liver-specific enhancer module of EnhI requires both an intact EF-C site and adjacent sequences, the 2c, HBLF, and GB elements, which are bound by liver-enriched factors (10, 14, 38). Moreover, optimal EnhI activity also requires accessory regions containing target sites for the liver-specific factor C/EBP as well as for other factors such as NF1, AP1, and CREB (14, 38). Liver-specific EnhI activity may thus be determined to a large extent by the cooperative action of the ubiquitously expressed RFX1 and a set of liver-specific transcription factors.

EnhI is also transactivated by the non-DNA-binding HBV X-gene product (2, 12, 33, 39, 40). Transactivation by X requires the E region of EnhI. It is thought that X acts indirectly via proteins such as AP1, C/EBP, CREB, and ATF-2, which have potential binding sites within the E region (8, 20, 23, 31, 40). However, a requirement for the RFX1 (EF-C) site in transactivation by X has also been described (12), and we have confirmed this finding directly by cotransfection of an X-protein expression plasmid with wild-type and mutated EnhI-driven reporter plasmids (data not shown). In these experiments, no transactivation by X is obtained when the RFX1 (EF-C) site is disrupted, although the E site remains intact. This observation further confirms that RFX1 (EF-C) plays a key role in EnhI activity and suggests that it participates with proteins binding to the E site in mediating the effect of X.

RFX1 is likely to be a transcription factor for a variety of cell-specific and ubiquitously expressed viral and cellular genes. Functionally important RFX1 (EF-C)-binding sites are found in several viral enhancers (4, 9, 25, 43). The HBV site is conserved in hepadnaviruses from other species (4, 9, 25). The polyomavirus EF-C site is crucial for enhancer activity (15a). CMV enhancer activity can be inhibited in an in vitro transcription system by adding an excess of a competitor oligonucleotide containing the CMV1 RFX1 (EF-C) site (11). Finally, additional RFX1 (EF-C) sites are found in functionally important regions such as the *ori* core regions of polyomavirus and lymphotropic papovavirus, and the long terminal repeat of equine infectious anemia virus (4, 43). There are also RFX1 (EF-C) sites in several cellular genes (6, 27, 30, 43). A functional role for binding of RFX1 to the X-box motif of HLA class II genes has been documented (27). In particular, the RFX1-specific antisense oligonucleotide that compromises HBV EnhI function also inhibits induction of HLA class II genes by gamma interferon gamma (32a). Another gene in which RFX1 is implicated is the ribosomal protein L30 (rpL30) gene; RFX1 is present in the nuclear rpL30-α complexes that contribute to rpL30 promoter activity (30). Taken together, these observations suggest that RFX1 is likely to be a key transcription factor for a variety of viral and cellular genes, including both ubiquitously expressed (rpL30) and highly cell specific (HLA class II, HBV EnhI) genes. The finding that RFX1 is identical to the nuclear complexes (EF-C, EP, MDBP, rpL30α) detected in these systems thus opens the way for an

analysis of the function of RFX1 in the transcriptional control of a diverse set of genes.

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