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Binding of Nuclear Factor EF-C to a Functional Domain of the Hepatitis B Virus Enhancer Region

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Nuclear factor EF-C is present in extracts prepared from human HepG2 liver cells and from other, nonliver cell lines and binds to the hepatitis B virus and polyomavirus transcriptional enhancer regions in vitro. An inverted repeat (5′-GTTGCNNNGCAAC-3′) is located within both binding regions. Diethyl pyrocarbonate interference binding assays and competition binding experiments using altered binding sites demonstrated that EF-C contacts symmetrical nucleotides within the inverted repeat. Mutations that changed the length of the spacer region between the arms of the inverted repeat were introduced in the hepatitis enhancer region. Introduction of 1 or 2 base pairs between the repeats did not affect EF-C binding, but deletion of 1 base pair or introduction of 3 to 9 base pairs reduced binding dramatically. Introduction of 10 base pairs restored partial EF-C binding ability. These and other results suggest that EF-C binding is stabilized by dimerization. In vivo assays for enhancer function using these mutants demonstrated that the EF-C binding site is a functional and important component of the hepatitis B virus enhancer region.

There is considerable interest in the molecular biology of hepatitis B virus (HBV) because of its association with human disease, as a causative agent of acute and chronic hepatitis, and with the development of primary hepatocellular carcinoma (2, 19, 36, 47). However, little is known about regulation of viral gene expression. Direct genetic analyses of HBV have been hampered by the lack of a cell culture system for propagation of the hepadnaviruses; however, the molecular cloning and DNA sequencing of the human HBV genomes (16, 17, 31, 54) as well as those of the ground squirrel hepatitis virus (GSHV) (40), duck hepatitis virus (DHV) (29), and woodchuck hepatitis virus (WCHV) (18) have allowed for alternative approaches to the study of the hepadnavirus life cycle (1, 39, 46, 58).

Although HBV DNA is partially single stranded in the viral particle, superhelical DNA molecules are believed to be the template for RNA transcription by the host cell RNA polymerase II, since the appearance of this molecular form of the genome precedes the appearance of viral RNAs (48, 53). Two major families of transcripts have been identified in infected livers and liver cell lines transfected with cloned viral genomes (6, 7, 13, 58). These overlapping transcripts include the four major open reading frames of the virus (see reference 20 for a review).

Both in vitro transcriptional analyses (34) and the results of transfection experiments using subgenomic fragments of HBV spanning these transcriptional start sites and cloned adjacent to reporter genes (38, 43, 44, 50) have demonstrated the presence of multiple promoter elements in this virus. Further genetic analyses are needed to define the promoters more precisely. A glucocorticoid responsive element has been identified in the viral genome (52) and, independent of these DNA sequences, a transcriptional enhancer region (Fig. 1) has been defined (42, 49). The enhancer has the unique feature of being located in the coding region of the putative polymerase of the virus, spanning approximately nucleotides (nt) 1081 to 1321 (numbering according to reference 18). This region has been implicated in the transcriptional regulation of HBV transcripts for the core protein and surface antigens (4, 8, 25, 42) as well as for transcripts initiating 5′ to the X-protein-coding region (50), as assayed by transient-expression analyses. What role the enhancer might play in the regulation of transcription of these various promoters in viral infection is not known. HBV is a liver-specific virus, although viral DNA has also been detected in bone marrow and mononuclear blood cells (12, 30, 37), suggesting a role of these cells in viral production. Data from several groups (11, 49, 55) demonstrated that the enhancer is active in cell lines other than those derived from liver, suggesting that the enhancer may not be solely responsible for the hepatotropic phenotype of the virus.

We previously described a protein, termed EF-C, that is present in nuclear extracts prepared from murine and human cell lines and binds to the C element of the polyomavirus enhancer region (33). The polyoma EF-C binding site contains a 6-base-pair (bp) inverted repeat, with 3 nucleotides positioned between the arms of the repeat (see Fig. 5). Binding of EF-C to this region results in a region of nuclease protection that symmetrically overlaps the inverted repeat and spans approximately 20 bp. A similar inverted repeat is located in the enhancer region of HBV subtype ayw (see Fig. 5). In this report, we demonstrate that EF-C binds to the HBV inverted repeat in vitro and that the EF-C site is an important functional component of the HBV enhancer region in vivo. Analysis of EF-C binding by using diethyl pyrocarbonate (DEPC) interference assays as well as competition binding experiments using similar but divergent sites present in other hepatitis virus genomes demonstrate that EF-C makes contacts with symmetrical nucleotides in the inverted repeat. Results of binding experiments using mutants that contain an altered number of nucleotides in the spacer region suggest that binding of EF-C is stabilized by dimerization.

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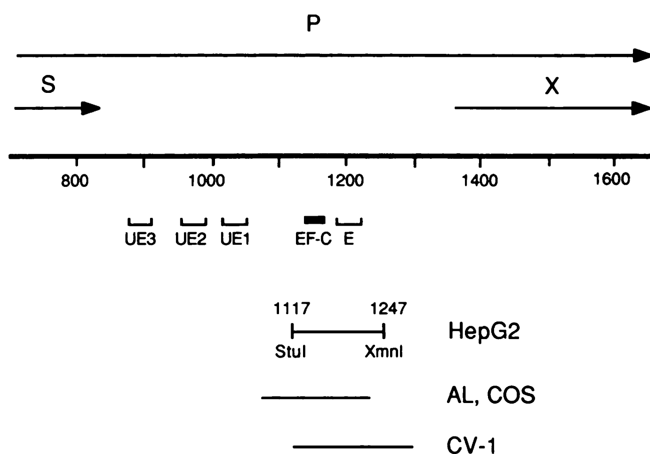

FIG. 1. HBV enhancer region. The HBV enhancer regions defined by Tognoni et al. (49) in monkey CV-1 cells and by Shaul and Ben-Levy (41) in human Alexander liver cells (AL) and monkey COS cells are indicated. The HBV enhancer region is located between the S-antigen (S) and X-protein (X) open reading frames and within the polymerase (P)-coding sequences. The *Stu*I-*Xmn*I enhancer region fragment (nt 1117 to 1247) used in this study in HepG2 liver cells is shown. The EF-C binding site and those of several other nuclear factors (E, UE1, UE2, and UE3) that bind to this region (41) are indicated. Nucleotide sequences are numbered according to Galibert et al. (18).

MATERIALS AND METHODS

Cells, nuclear extracts, and plasmid transfections. HepG2 cells were grown in minimal essential medium containing 10% fetal bovine serum. HeLa cells were grown in Dulbecco modified essential medium containing 10% calf serum. F9 stem cells were grown in Dulbecco modified essential medium containing 10% fetal bovine serum; F9 cells were grown on plates precoated with gelatin.

Nuclear extracts were prepared as described previously (33). Briefly, cells were harvested, washed, and lysed in LB (0.3 M sucrose, 10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.5 mM dithiothreitol) containing 0.4% Nonidet P-40. Isolated nuclei were washed in LB minus Nonidet P-40, and nuclear proteins were extracted with 0.3 M NaCl as described previously (3) except that the 0.1 M NaCl wash was omitted. Eluted proteins were precipitated by using ammonium sulfate at 45% saturation. Protease inhibitors (5 μg of aprotinin per ml, 5 μg of leupeptin per ml, and 0.5 mM phenylmethylsulfonyl fluoride) were included in all buffers during lysis and extraction. Extracts were dialyzed for 4 h against 20 mM Tris (pH 7.5)–50 mM NaCl–25% glycerol–0.1 mM EDTA–0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride.

DNA transfections were performed by the calcium phosphate precipitation method (57). HepG2 cells were split the day before transfection. The cells were transfected with 1 μg of plasmid DNA and 19 μg of salmon sperm carrier DNA per 100-mm-diameter dish. After incubation overnight with the calcium phosphate precipitate, the cells were washed with Tris-buffered saline solution, Tris-buffered saline containing 3 mM EGTA, and Tris-buffered saline. Fresh medium was added, and cell extracts were prepared 42 h later. Chloramphenicol acetyltransferase (CAT) enzyme levels were assayed as described previously (23). Transfections were performed 8 to 10 times each, using two different plasmid DNA stocks. The results were quantitated by excision of the acetylated and nonacetylated [^{14}C]chloramphenicol forms from the chromatogram and direct scintillation counting.

In vitro DNA-protein binding reactions and DEPC interference assays. Filter-binding assays were performed as described previously (33). Binding reactions (50 μl) were performed in 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5)–50 mM KCl, 5 mM $MgCl_2$–2 mM dithiothreitol–50 μg of bovine serum albumin per ml, and reaction mixtures contained 1.6 fmol of ^{32}P-labeled probe DNA (HBV probe, 15,000 cpm; EF-C probe, 11,000 cpm), 10 μg of HepG2 cell nuclear extract, 1.3 μg of homopolymer mix (10) as a nonspecific competitor for DNA-binding proteins, and increasing concentrations of cold competitor DNA, as indicated. The HBV probe DNA was the *Eco*RI-*Sau*96 fragment from pUC18-HBV (see below) containing the HBV *Stu*I-*Sau*96 fragment (subtype ayw nt 1117 to 1223; 124 bp); the EF-C probe DNA was the *Eco*RI-*Hin*dIII fragment from pUC9-EFC (33) containing a 29-bp oligonucleotide of the polyomavirus EF-C site in the pUC9 polylinker region (polyomavirus A2 nt 5150 to 5178; 65 bp). Probe DNAs were end labeled at the *Eco*RI site of the vector with [^{32}P]dATP by using Klenow polymerase. The nuclear extract and homopolymer DNA were preincubated at room temperature for 15 min; the probe DNA and specific competitors were then simultaneously added, and the reaction mixture was allowed to incubate for 30 min at room temperature. Specific binding was determined by a nitrocellulose filter-binding assay (33) and is expressed as percent DNA bound when no cold competitor was present in the binding reaction. The competitor DNAs were pBR (*Eco*RI-*Eco*RV fragment of pBR322; 185 bp), HBV (same as the probe DNA), and EF-C (same as the probe DNA). Plasmid pUC18-HBV contains the HBV subtype ayw *Stu*I-*Bam*HI fragment (nt 1117 to 1402) in pUC18 between the *Sma*I and *Bam*HI sites. A clone containing the HBV ayw genome was kindly provided by Chuck Tackney (Imclone Systems, New York, N.Y.).

Conditions for saturation binding of EF-C to the HBV enhancer region were determined by the filter-binding assay, and DNase I footprint analysis was performed as described elsewhere (33). The HBV ^{32}P-labeled probe was the same as described above. Binding reaction mixtures (250 μl) contained 3 fmol of probe DNA (30,000 cpm), 50 μg of HepG2 cell nuclear extract, and 6.5 μg of homopolymer DNA. Conditions for binding were as described above. After the binding reaction, DNase I (Worthington Diagnostics) in 25 mM HEPES (pH 7.5)–100 mM $MgCl_2$–100 mM $CaCl_2$ was added at the indicated concentrations (final concentrations were 2.3 mM $CaCl_2$ and 9.4 mM $MgCl_2$), and the reaction mixture was incubated for 5 min at room temperature. The reactions were terminated and analyzed in a denaturing polyacrylamide gel.

Binding reactions for mobility shift assays (20 μl) were performed in 25 mM HEPES (pH 7.5)–50 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–50 μg of bovine serum albumin per ml, and reaction mixtures contained 1.5 fmol of ^{32}P-labeled probe DNA (10,000 cpm; EF-C probe as described above), 2 μg of HepG2, HeLa, or F9 stem cell nuclear extract, and 2 μg of poly(dI-dC) (Sigma Chemical Co.). Reactions were performed as described for the filter-binding assay. Specific competitor DNAs were added simultaneously with the probe DNA at the indicated concentrations. DNA-protein complexes were resolved in a 4% polyacrylamide gel (acrylamide-bis ratio, 30:1) run in 25 mM Tris (pH 8.3)–25 mM boric acid–0.5 mM EDTA at 4°C essentially as described previously (5). The competitors were as follows:

EF-C (same as described above); HBV (same as described above); WCHV, an *Rsa*I-*Pvu*I fragment from pSP65-WCHV (WCHV nt 1211 to 1391; plasmid pSP65-WCHV contains one copy of the WCHV genome in plasmid pSP65 and was a gift of Bill Mason and Jesse Summers, Fox Chase Cancer Center, Philadelphia, Pa.); GSHV, a *Hin*cII fragment from pBA27-3 (GSHV nt 2506 to 2736; plasmid pBA27-3 contains a trimer insertion of the GSHV genome in pBR322 and was a gift from Don Ganem, University of California, San Francisco); DHV1, an *Eco*RV-*Tth*111I fragment from p2.3 (nt 718 to 904; plasmid p2.3 contains a dimer insertion of the DHV genome in pBR322 and was a gift of Don Ganem); DHV2, an *Rsa*I-*Nco*I fragment from p2.3 (nt 2141 to 2351); SV, an *Eco*RI-*Hin*dIII fragment from pSV-dlOP-CAT (9; see below; contains one 72-bp repeat, the 21-bp repeats and early promoter region, and pUC polylinker sequences; a gift of Peter Tegtmeyer and Molly Cox, State University of New York, Stony Brook); *myc*, an *Acc*I-*Sma*I fragment from pSV2CAT(BgSm3′) (35; *myc* sequences from nt −616 to −428; a gift of Ken Marcu and Gary Weisinger, State University of New York, Stony Brook); and HBV-DL-C, -DL-1, and -IS-1 through -IS-10, the *Eco*RI-*Bam*HI fragments from the respective CAT plasmids containing these mutant enhancer regions (see below; contains HBV *Stu*I-*Xmn*I fragment, ayw nt 1112 to 1247).

DEPC interference assays were performed as described previously (45).

Construction of HBV enhancer region insertion and deletion mutants. Mutants with insertions and deletions between the arms of the inverted repeat were constructed by using an *Hpa*II site that overlaps the spacer region between the inverted repeat. Plasmid pUC18-HBV (*Stu*I-*Bam*HI; nt 1117 to 1402) was digested partially with *Hpa*II, followed by a repair reaction using Klenow polymerase and subsequent ligation. This introduced 2 nt in the spacer region (see Fig. 7, IS-2) and generated a unique *Sst*II site. HBV-IS-8 was constructed by digestion of HBV-IS-2 with *Sst*II, followed by Klenow repair and subsequent insertion of a *Cla*I linker. HBV-IS-10 was constructed by digestion of HBV-IS-8 with *Cla*I, followed by Klenow repair and ligation. HBV mutants DL-C, DL-1, IS-1, and IS-3 through IS-9 were generated by treating HBV-IS-8 DNA, linearized with *Cla*I and repaired by using Klenow polymerase, with S1 nuclease under conditions such that 1 to 10 bp was removed from each end of the linear DNA. The DNA was subsequently repaired by using Klenow polymerase and ligated. The sequence of each mutant was determined by nucleotide sequence analysis.

The *Stu*I-*Xmn*I fragment of each mutant was inserted in the vector pSV-dlOP-CAT (9) between the unique *Kpn*I and *Bam*HI sites. Plasmid pSV-dlOP-CAT contains one complete simian virus 40 (SV40) 72-bp repeat, flanked by unique *Kpn*I and *Bam*HI sites at the upstream and downstream ends, respectively, fused to the 21-bp repeats and early promoter sequences of SV40 joined to the CAT-coding sequences (23). pSV-dlOP-CAT contains a deletion of T-antigen site I (*Bgl*I-*Hin*dIII). The HBV fragments were inserted such that the *Stu*I site (nt 1117; Fig. 1) was adjacent to the SV40 21-bp repeats.

RESULTS

EF-C binds to the HBV enhancer region. To determine whether EF-C bound to the HBV inverted repeat, a DNase I protection analysis was performed by using a ^{32}P-labeled DNA fragment that contained the HBV enhancer region (*Stu*I-*Sau*96I; nt 1117 to 1223; Fig. 1) and a nuclear extract prepared from the human liver cell line HepG2. The binding activities observed in these cells may be relevant to the expression of the virus in a productive infection, although HBV enhancer activity is not restricted to liver cells (11, 49, 52). Binding of a factor(s) in the HepG2 nuclear extract to the HBV enhancer region resulted in a region of nuclease protection of approximately 20 nt that included the HBV inverted repeat (Fig. 2A). No other regions of protection on this fragment were observed with use of this nuclear extract. DNase I protection of a fragment that contained the polyomavirus EF-C binding site also was observed by using nuclear extracts prepared from HepG2 cells (data not shown); the footprint obtained was indistinguishable from EF-C footprints obtained with the polyomavirus EF-C site and other cell extracts (33).

To test whether the same protein was binding to the HBV and polyomavirus enhancer regions, DNA competition experiments were performed, using a nitrocellulose filter-binding assay to detect DNA-protein complexes (33). Figure 2B shows the results of competition experiments using a ^{32}P-labeled HBV enhancer fragment (*Stu*I-*Sau*96I; nt 1117 to 1223). The homologous HBV fragment and a 29-bp oligonucleotide of the polyomavirus EF-C site (cloned in the pUC9 polylinker region) competed with equal efficiency for binding to the labeled HBV enhancer region. The specificity of this competition was demonstrated by the lack of competition for binding in assays with a nonspecific fragment derived from pBR322. The residual binding obtained in the competition assays (20 to 30% of the percent DNA bound) represented nonspecific binding to the probe DNA; similar levels of binding were observed in the assay with a nonspecific probe (data not shown). The results of competition experiments using a ^{32}P-labeled polyomavirus EF-C binding site are shown in Fig. 2C. Both the HBV and polyomavirus fragments competed equally for binding to the polyomavirus enhancer region. These results, in conjunction with the nuclease protection analyses, demonstrated that the same factor in HepG2 extracts, EF-C, bound to the HBV and polyomavirus enhancer regions. Furthermore, these results support the hypothesis that the inverted repeat is an important element of the EF-C binding site.

EF-C makes symmetrical contacts within the inverted repeat. Identical patterns of DNase I protection of the polyomavirus EF-C binding site were observed in assays with nuclear extracts prepared from human HepG2 and HeLa cells and from murine F9 embryonal carcinoma stem cells (33; Fig. 2 and data not shown), suggesting that the same factor was binding in these extracts. In a mobility shift assay to detect DNA-protein complexes (5, 14, 21), binding to the polyomavirus and HBV EF-C sites was examined. The results of experiments using nuclear extracts prepared from these three cell lines in binding reactions with a ^{32}P-labeled polyomavirus EF-C site are shown in Fig. 3.

Two prominent complexes were detected by using the HepG2 cell nuclear extract (dots in Fig. 3), but at least five separate, neighboring species were observed on longer exposure of the gel (data not shown). All of these complexes were specifically competed against by the homologous polyomavirus site and the HBV enhancer region but not by a control pBR322 fragment. One prominent complex that comigrated with the slowest-migrating HepG2 species was observed in assays with the HeLa cell extract. Several other complexes that also comigrated with the HepG2 species were observed with the HeLa cell extract on longer exposure of the gel (data not shown). These complexes were also specifically competed against by the polyomavirus and HBV

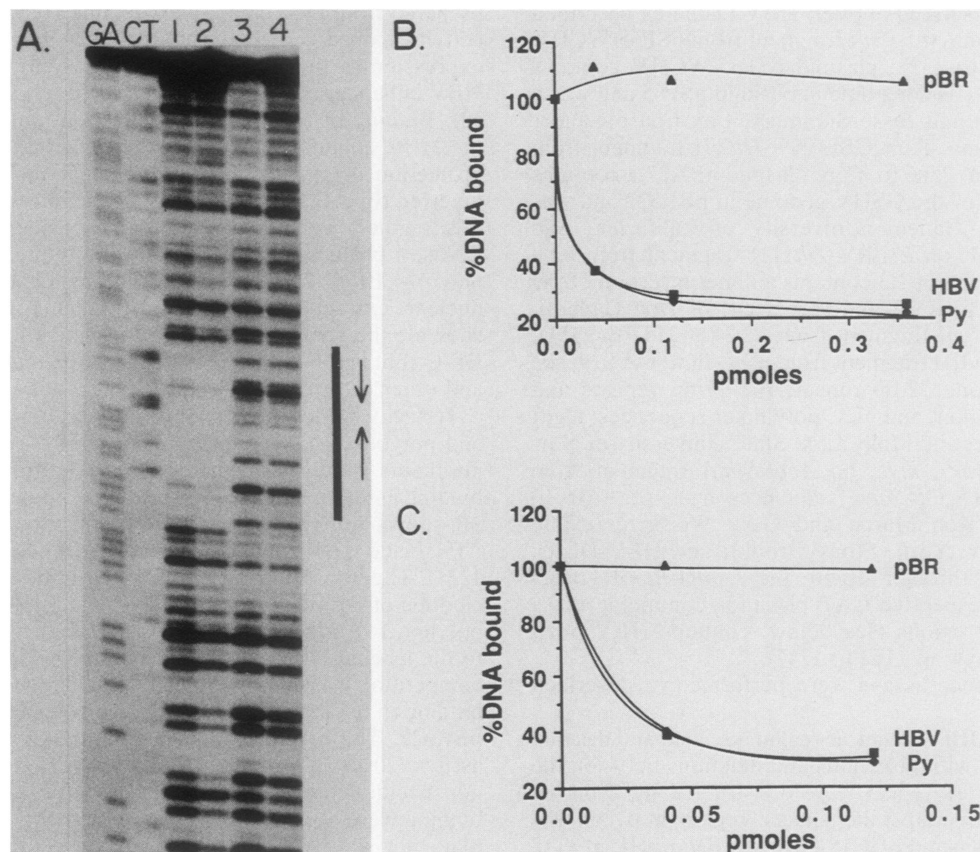

FIG. 2. Binding of EF-C to the HBV enhancer region. (A) DNase I footprint analysis of the HBV enhancer region. A DNA fragment containing the HBV enhancer region (*Stu*I-*Sau*96; nt 1117 to 1223) was end labeled and used in a binding reaction containing nuclear extract prepared from HepG2 cells and homopolymer DNA as a nonspecific competitor. Conditions for saturation binding of EF-C to the HBV DNA were optimized by using a filter-binding assay as described in Materials and Methods. After the binding reaction, the samples were digested with DNase I, and the products were analyzed in a denaturing polyacrylamide gel. Lanes: 1 and 2, DNase I ladders obtained after incubation of the HBV probe DNA with HepG2 nuclear extract; 3 and 4, DNase I ladders obtained when the HBV probe DNA was treated with DNase I in the absence of nuclear extract. Concentrations of DNase I were 0.48 (lane 1), 1.2 (lane 2), 0.024 (lane 3), and 0.048 (lane 4) U/ml. GA and CT are homologous sequencing ladders. Symbols: ▮, region of protection; ↓, ↑, inverted repeat within the EF-C binding site. (B and C) Competition binding experiments using the HBV and polyomavirus EF-C sites. (B) Results obtained by using binding reaction mixtures containing a fixed concentration of ^{32}P-labeled HBV enhancer region probe (1.6 fmol; 15,000 cpm; nt 1117 to 1223; 124 bp), HepG2 cell nuclear extract and homopolymer DNA, and increasing concentrations of competitor DNA fragments (shown as picomoles). Specific binding was determined by a nitrocellulose filter-binding assay and is expressed as percent DNA bound when no competitor DNA fragments were present in the binding reaction; 100% bound was 600 cpm (4% of input). The competitor DNAs were pBR (the *Eco*RI-*Eco*RV fragment of pBR322; 185 bp); HBV (same as the labeled probe; 130 bp); and polyomavirus (Py) (a 29-bp oligonucleotide containing the polyomavirus EF-C site [Py-EFC probe; sequences in Fig. 5 plus 6 polyomavirus flanking nt and additional pUC polylinker sequences; 65 bp]). (C) Competition for binding to a ^{32}P-labeled polyomavirus fragment containing the EF-C binding site (1.6 fmol; 11,000 cpm; 65 bp). Binding reactions and competitor fragments were as described for panel B; 100% bound was 330 cpm (3% of input).

fragments. A single complex was detected with the F9 stem cell extract. This complex was also specifically competed against by the polyomavirus EF-C site and HBV enhancer region but migrated with distinct mobility in comparison with the complexes observed with the HepG2 and HeLa cell extracts. The altered mobility of the complex formed by using the F9 cell extract did not appear to reflect a species-specific difference of EF-C, since binding patterns identical to those obtained with the HeLa and HepG2 cell extracts were obtained by using extracts prepared from murine L cells and 3T3 cells (data not shown). These results showed that the binding activity observed in human HeLa cells and murine F9 cells, like that found in HepG2 nuclear extracts, bound to both the polyomavirus and HBV EF-C binding sites.

DEPC interference assays (45) were used to identify EF-C contact sites in these complexes. DEPC was used to modify G and A residues in the ^{32}P-labeled polyomavirus EF-C probe, and the modified probe was used in binding reactions with the extracts described above. After electrophoresis, free (unbound) and complexed (bound) DNAs were eluted from the gel, cleaved at the modified bases with piperidine, and analyzed on a denaturing polyacrylamide gel. Contact sites on both strands of the probe were analyzed for the prominent complexes shown in Fig. 3.

Identical patterns of interference were observed with all of the complexes examined: the single major species observed with the HeLa and F9 cell extracts and the upper two species detected with the HepG2 cell extract (Fig. 4). A summary of these results is shown in Fig. 5. EF-C contacted symmetrical G and A residues with respect to the inverted repeat. Six of eight contact sites observed were within the repeats, while two contact points were the outer 2 nt of the 3-bp spacer region. Identical symmetrical contact sites were observed

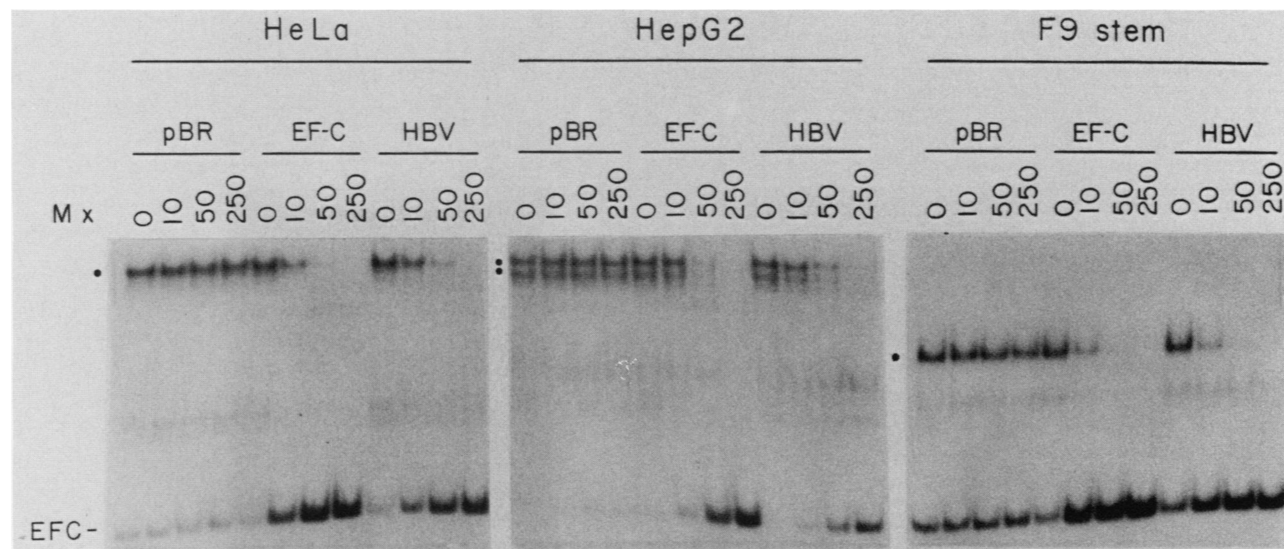

FIG. 3. EF-C binding results obtained by using different cell extracts. A ^{32}P-labeled Py-EFC probe (see legend to Fig. 2C) was used in binding reactions with nuclear extracts prepared from HeLa, HepG2, and the F9 embryonal carcinoma stem cell lines. Binding reactions contained the ^{32}P-labeled Py-EFC probe, nuclear extract, poly(dI-dC) as a nonspecific competitor, and increasing molar concentrations of competitor DNA fragments (10-, 50-, and 250-fold molar excess). The products of the binding reaction were electrophoresed in a low-percentage polyacrylamide gel. The competitor were the same as for Fig. 2B. ●, Specific complexes described in the text.

when a DEPC-modified HBV enhancer region probe was used in the analysis (data not shown). With the HBV EF-C site, one of the contact points in the spacer region was a G residue rather than an A (Fig. 5). Identical results also were obtained with respect to EF-C contact with G residues by using dimethyl sulfate-modified polyomavirus and HBV probes (data not shown).

A search of the GenBank sequence library revealed sequences similar to those of the EF-C inverted repeats in the WCHV and GSHV genomes (Fig. 5). The DHV genome has diverged through evolution in comparison with the HBV, WCHV, and GSHV genomes (28). The DHV genome contained two weaker sequence similarities to the inverted repeat: one sequence maintained the correct spacing between the arms of the repeat but had several mismatches in comparison with the polyomavirus and HBV sequences (DHV1; Fig. 5), while the second sequence similarity contained an additional 9 bp in the spacer region (DHV2; Fig. 5). Two partial homologies to the EF-C inverted repeat were found in the murine c-*myc* 5′ regulatory region and in the SV40 enhancer region (Fig. 5). These sequences both contained one arm of the inverted repeat that was identical to the polyomavirus EF-C site, but the second arm of the repeat contained two or more mismatches in comparison with the polyomavirus site. Competition binding experiments were performed by using a ^{32}P-labeled polyomavirus EF-C site, HepG2 cell nuclear extract, and different DNA fragments containing the sequences shown in Fig. 5 (Fig. 6).

A DNA fragment containing the GSHV sequence competed nearly as well as the homologous EF-C site and HBV enhancer region for EF-C binding. Of the sequences shown in Fig. 5, the GSHV sequence was the most similar to the EF-C binding site, as determined by DEPC interference assays (one purine transition in comparison with the HBV EF-C site; Fig. 5). The GSHV sequence was reduced three- to fivefold in EF-C binding in comparison with the polyomavirus or HBV EF-C site. The WCHV fragment, DHV1 and -2, SV40 early promoter-enhancer fragment, and c-*myc* fragment competed either very poorly or not at all for EF-C binding even at the highest concentration of competitors used. These fragments were reduced at least 100-fold in EF-C binding in comparison with the polyomavirus and HBV sites. These results are consistent with the EF-C binding site consensus sequence 5′-GTTGC(T/C)NG(G/A)CAAC-3′. The GSHV sequence had a nearly perfect match to the HBV EF-C site and bound EF-C efficiently. Each of the other competitors tested that bound EF-C poorly or not at all contained at least two mismatches at EF-C contact sites. The results obtained by using the c-*myc* and SV40 competitor fragments also demonstrated that EF-C did not bind efficiently to only one arm of the inverted repeat, since these sites each contained one perfect half-site (Fig. 5). This finding suggested that EF-C binding may be stabilized by dimerization.

Mutants that alter the spacing between the arms of the inverted repeat. To examine EF-C binding in vitro in greater detail and to assess the functional role of the EF-C site in the HBV enhancer region in vivo, we constructed mutants containing alterations in the spacing between the arms of the inverted repeat in the context of the wild-type HBV enhancer region (*Stu*I-*Xmn*I fragment; Fig. 1). Mutants containing a deletion of 1 bp and an insertion of 1 to 10 bp in the spacer region were generated (DL-1 and IS-1 through IS-10; Fig. 7). Each of the mutant sequences maintained all of the contact sites for EF-C binding, as determined by DEPC interference assays. A mutant that contained a 16-bp deletion of the EF-C binding site was also generated (DL-C; Fig. 7). Mutant fragments were used in competition binding experiments in vitro and were introduced in an SV40-based CAT expression vector for analysis of enhancer function in vivo.

The results of in vitro competition binding experiments using these mutants are shown in Fig. 8. The binding reactions contained a ^{32}P-labeled polyomavirus EF-C site, HepG2 cell nuclear extract, and competitor DNAs at the indicated concentrations. The wild-type HBV enhancer fragment competed efficiently for EF-C binding at a 10-fold molar excess to the probe DNA, whereas the negative

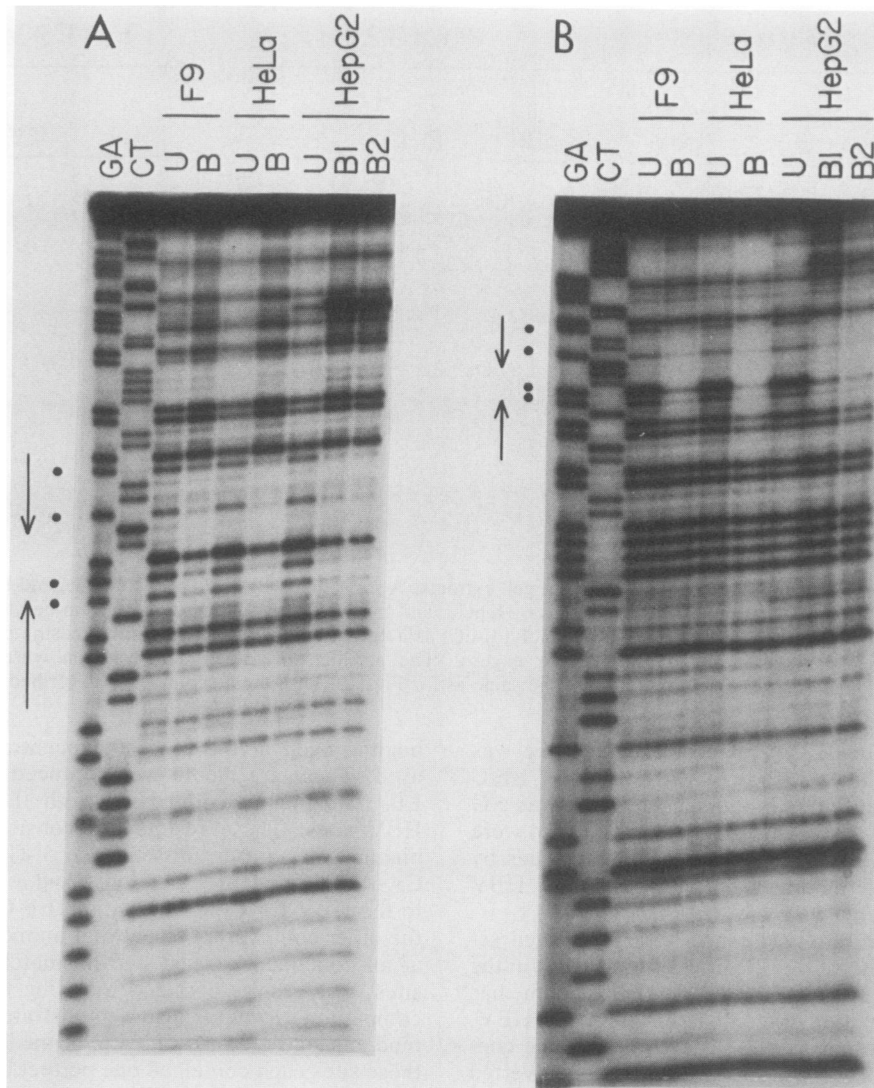

FIG. 4. Analysis of EF-C binding, using a DEPC interference assay. ^{32}P-labeled Py-EFC probes (see legend to Fig. 2C), labeled on either strand (A and B), were partially modified at G and A residues with DEPC and used in binding reactions with nuclear extracts from F9, HeLa, and HepG2 cells. The products of the reactions were electrophoresed in a low-percentage polyacrylamide gel and visualized by autoradiography. The unbound (U; free) and bound (B; complexed) DNAs were eluted, cleaved at the modified bases with piperidine, and analyzed in a denaturing polyacrylamide gel. Symbols: ●, specific contact nucleotides homologous to the Maxam and Gilbert sequencing ladders; ↓, ↑, EF-C inverted repeat.

control fragment, DL-C, did not compete even at a 1,000-fold molar excess. Deletion of 1 bp in the spacer region (DL-1) eliminated EF-C binding. In contrast, mutant fragments containing 1- or 2-bp insertions (IS-1 and IS-2) bound EF-C as efficiently as did the wild-type enhancer region. Insertion of 3 bp (IS-3) reduced EF-C binding at least 100-fold, whereas insertion of 4 bp (IS-4) eliminated EF-C binding. EF-C binding to competitor fragments containing insertions of 5 through 8 bp (data not shown) and 9 bp (IS-9; Fig. 7) was at the level of the control fragment (DL-C). Interestingly, insertion of 10 bp in the spacer region (IS-10) restored EF-C binding to a level reduced about 20-fold compared with binding to the wild-type enhancer region.

The wild-type and mutant enhancer region fragments (*Stu*I-*Xmn*I; Fig. 1) were inserted in place of the SV40 enhancer region in a vector (pSV-dlOP-CAT; 9) that contains the SV40 early promoter-enhancer region fused to the coding sequences for the CAT gene (23). The function of the wild-type and mutant HBV enhancer regions was tested in HepG2 cells after transfection of plasmid DNAs by measuring the level of CAT enzyme activity in cellular extracts (Fig. 9).

The wild-type HBV enhancer region increased CAT expression 50-fold relative to the level obtained by using a plasmid lacking enhancer sequences. Deletion of the EF-C site (DL-C) reduced the activity of the HBV enhancer 10- to 15-fold. The results obtained with the deletion and insertion mutant enhancer regions in vivo were comparable to those obtained when these fragments were used as competitors for EF-C binding in vitro. Insertion of 1 or 2 bp in the spacer region between the arms of the inverted repeat (IS-1 and IS-2) did not reduce the activity of the enhancer region in comparison with that of the wild-type fragment. Insertion of 3 through 10 bp or deletion of 1 bp (IS-3, IS-4, IS-9, IS-10, and DL-1 [Fig. 9]; IS-5 through IS-8 [data not shown]) reduced enhancer activity about 10-fold. These results dem-

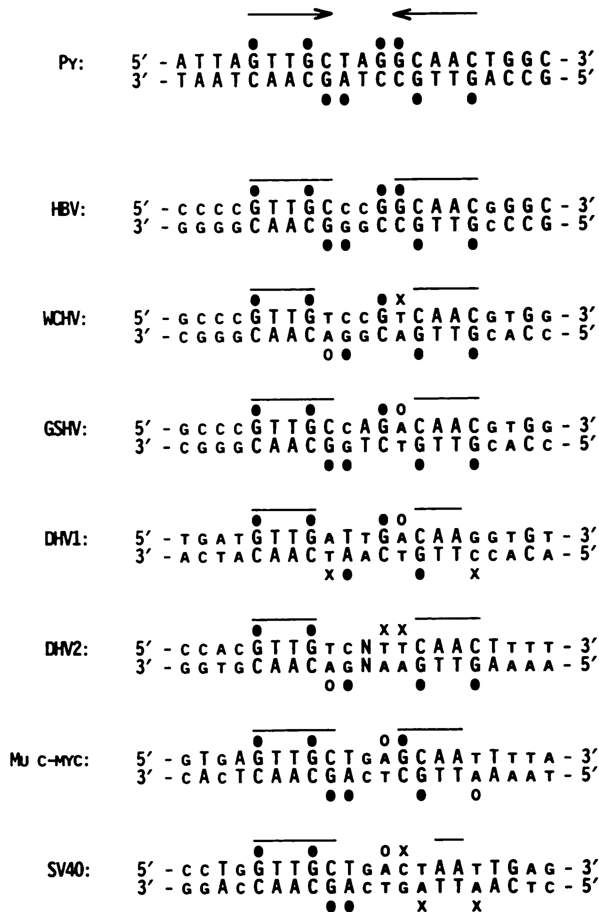

FIG. 5. EF-C binding site. Sequences of the polyomavirus (Py) and HBV EF-C binding sites are shown at the top. Arrows (Py) and lines (HBV) correspond to the inverted repeats described in the text; circles are the EF-C contact sites determined by dimethyl sulfate and DEPC interference assays. Listed below these sequences are similar sequences identified in a search of the GenBank sequence library; WCHV, nt 1275 to 1295 (18); GSHV, 2656 to 2676 (40); DHV1, nt 772 to 792 (29); DHV2, nt 2294 to 2323 (29) (N corresponds to 10 additional nt between the arms of the inverted repeat in this region of the DHV genome); Mu c-*myc*, −606 to −586 of the murine c-*myc* gene relative to the cap site of the P1 promoter at +1 (35); and SV40 enhancer region, nt 177 to 197. Large letters correspond to nucleotides that are identical to those in the polyomavirus sequences; small letters correspond to those that are different. Symbols: ●, nucleotides identical to those in the polyomavirus and HBV EF-C contact sites; ○, nucleotide transitions at the EF-C contact sites; ×, nucleotide transversions at these sites.

onstrate that the EF-C binding site is an important functional component of the wild-type HBV enhancer region. The observation that the activity of the IS-10 mutant enhancer region was not increased relative to the activities of the IS-3 through IS-9 mutant enhancer regions is not surprising, since the IS-10 mutation reduced EF-C binding in vitro 20-fold compared with results obtained for the wild-type HBV enhancer region.

DISCUSSION

The results of DNase I protection analysis and competition binding experiments (Fig. 2) demonstrate that nuclear factor EF-C binds to both the polyomavirus and HBV enhancer regions in vitro. The polyomavirus enhancer activates viral early- and late-region transcription as well as viral DNA replication, and the EF-C site is required for both aspects of enhancer function (references 27, 51, and 56 and references therein). We show in these analyses that the HBV EF-C site is a critical element in the HBV enhancer region: mutations that disrupt binding to the EF-C site in vitro reduce enhancer function in vivo at least 10-fold (Fig. 9). The polyomavirus enhancer region contains multiple, functionally redundant enhancer elements; the EF-C site in this region functions only in the context of other, adjacent binding motifs (56; P. Hearing, unpublished data). The same situation is true with the HBV enhancer region. While the EF-C site is an important component of this region, the integrity of other flanking elements is also required for enhancer region function (49; Hearing, unpublished data). Thus, it appears that EF-C functions in vivo in combination with other, distinct enhancer region motifs. The interaction of adjacent enhancer region motifs, which are independently nonfunctional but act together to generate a functional enhancer unit, has been described in the SV40 system (15, 32). By definition, the EF-C site would be an enhanson: an independent binding motif that cooperates with adjacent enhanson motifs to generate a functional enhancer element. We have no evidence that this cooperativity exists at the protein-DNA binding level, since we detect efficient EF-C binding in the absence of other binding proteins in vitro. Rather, cooperativity of adjacent elements may be reflected only at the functional level in vivo.

The results of dimethyl sulfate and DEPC interference binding assays (Fig. 4 and data not shown) demonstrate that EF-C contacts symmetrical nucleotides within the inverted repeats present in the polyomavirus and HBV binding sites. The symmetrical nature of these contact sites suggests that EF-C may bind to its cognate sequence as a dimer. The results of in vitro competition binding experiments (Fig. 2 and 6) are consistent with the EF-C binding site consensus sequence 5'-GTTGC(T/C)NG(G/A)CAAC-3'. The central base pairs of the spacer region and the nucleotides that flank the inverted repeat are variable in the three sites that bind EF-C efficiently (polyomavirus, HBV, and GSHV; Fig. 5 and 6) and therefore do not appear to be an essential part of the EF-C binding site. The fact that the SV40 and c-*myc* sequences bound EF-C poorly or not at all (Fig. 6) suggests that EF-C binding is stabilized by dimerization, since both of these sites have one perfect arm of the inverted repeat that shares a 6-of-6-bp match with the polyomavirus EF-C site (Fig. 5). We previously found that a perfect EF-C half-site present in the polyomavirus early promoter region also did not bind EF-C (33).

Further evidence to support the idea that EF-C binding is stabilized by dimerization was obtained by analyzing mutants with alterations in the spacing between the arms of the HBV inverted repeat (Fig. 7). Analysis of the EF-C binding properties of mutant fragments (Fig. 8) demonstrated that deletion of 1 nt or insertion of 3 through 9 nt in the spacer region reduced EF-C binding at least 100-fold. These mutant fragments maintained each of the contact sites that were defined in the modification-interference binding assays, yet were unable to bind EF-C. EF-C binding may have been blocked sterically with mutant DL-1. With mutants IS-3 through IS-9, we would predict that the proper EF-C alignment required for stable dimerization is lost, since the arms of the inverted repeat are rotated away from each other, thus preventing interaction of EF-C monomers between the half sites. By analogy to binding of the lambda *c*I repressor to the O_R operator region (26), EF-C may contain distinct domains

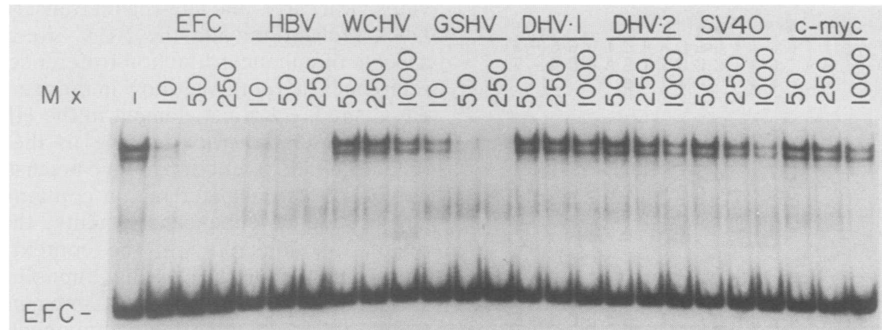

FIG. 6. Competition for EF-C binding, using altered sites. A mobility shift assay was used to measure competition for EF-C binding, using DNA fragments containing the sequences presented in Fig. 5. A ^{32}P-labeled Py-EFC probe (see legend to Fig. 2C) was used in binding reaction mixtures that contained HepG2 cell nuclear extract, poly(dI-dC), and increasing concentrations of competitor DNA fragments (10-, 50-, 250-, or 1,000-fold molar excess to the probe, as indicated). The products of the binding reactions were electrophoresed in a low-percentage polyacrylamide gel. The competitor DNA fragments used in the analysis are described in Materials and Methods.

that are involved in DNA binding and dimerization. This idea is supported by the binding studies with mutants IS-1, IS-2, and IS-10 (Fig. 7). Mutant fragments IS-1 and IS-2 bound EF-C as efficiently as did the wild-type fragment, yet the arms of the inverted repeat with these mutants were separated from each other by approximately 0.34 and 0.68 nm in distance and by approximately 35 and 70° in rotation. Flexibility in the binding of EF-C to DNA clearly exists with these mutants, which could be explained by flexibility in the protein itself between a DNA-binding domain and a dimerization domain. The fact that the IS-10 mutant DNA bound

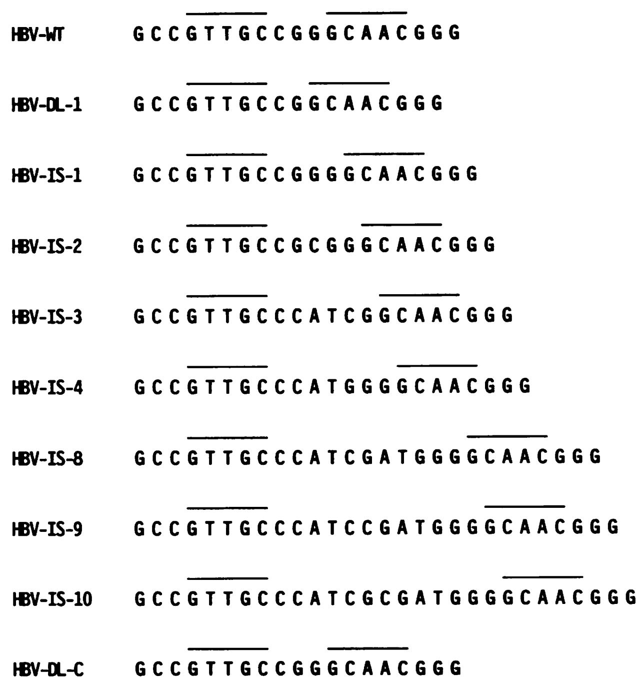

FIG. 7. Spacer region mutations in the HBV EF-C binding site. Deletion and insertion mutations were introduced within the spacer region of the HBV EF-C binding site as described in Materials and Methods. The arms of the HBV inverted repeat are indicated by lines above the sequences. The line under the sequence of mutant DL-C represents the nucleotides that were deleted. The mutant DNAs maintained each of the EF-C contact sites, as determined by DEPC interference assays.

EF-C significantly better than did the IS-9 mutant fragment (Fig. 8) also supports this idea. With the IS-10 mutant, the arms of the inverted repeat are separated by approximately one helical turn of the DNA with respect to their normal configuration in the wild-type fragment. The DNA-binding domains of two EF-C monomers at this site would be separated considerably on the DNA, yet binding is observed, albeit with reduced efficiency. This result is most easily explained by flexibility within the EF-C protein between binding and dimerization domains. Partial looping of the intervening DNA sequences with the IS-10 mutant may also be important for binding.

The effect of mutations that alter the spacing in the palindromic binding sites for nuclear factor I (NFI) and the thyroid hormone (T3) receptor have been analyzed. Insertion or deletion of 1 or 2 bp between the arms of the inverted repeat in the NFI binding site reduced NFI binding in vitro dramatically (24). In contrast, insertion of 1 through 6 bp between the arms of the inverted repeat of the estrogen response element T3 receptor binding site only slightly reduced T3 receptor binding in vitro; insertion of 9 bp reduced T3 receptor binding considerably (22). With the T3 receptor, selection for binding to sites with altered spacing has apparently occurred, since the T3 receptor binds efficiently to the growth hormone T3 response element and the vitellogenin A2 estrogen response element, which differ in sequence by the absence or presence, respectively, of a 3-bp gap at the center of the dyad symmetry (22). Flexibility in the spacer region of the EF-C binding site may also represent an adaptive feature of this enhancer motif.

The EF-C binding site in the human HBV genome is located within a region previously shown to have enhancer function in liver and nonliver cell lines (41, 49). EF-C sequence homologies located in other hepadnavirus genomes identified by a search of the GenBank sequence library (WCHV, GSHV, and DHV2; Fig. 5) are situated in comparable locations in the respective viral genomes. Of these sites, however, only the GSHV sequence efficiently competed for EF-C binding in vitro (Fig. 6). This result may reflect species-specific divergence of the EF-C binding site or protein. Although enhancer regions in the other hepadnaviruses have not yet been identified, the conservation of location and sequence of the EF-C binding sites in the HBV and GSHV genomes suggests conservation of a functional domain. We note that sequences in the vicinity of the EF-C homology in the GSHV and WCHV genomes are very

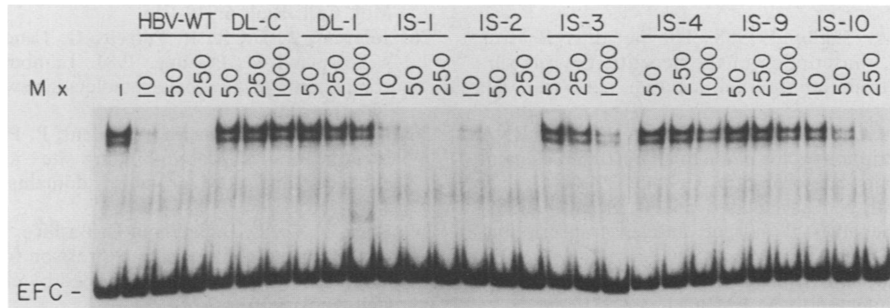

FIG. 8. Effect of spacer region mutations on EF-C binding in vitro. Competition binding experiments using a ^{32}P-labeled Py-EFC probe (see legend to Fig. 2C), HepG2 cell nuclear extract, and specific competitor DNAs were performed as described for Fig. 6. The competitor DNAs (HBV *Xmn*I-*Stu*I; nt 1117 to 1247), containing the mutations listed in Fig. 7, were included in the binding reactions at a 10-, 50-, 250-, or 1,000-fold molar excess, as indicated.

similar to sequences in the HBV enhancer region, which may reflect binding sites for additional enhancer factors (such as factor E; 41). This interpretation is complicated by the fact that the enhancer region is located within the coding sequences for the polymerase gene. Identification of enhancer regions in other hepadnavirus genomes will be required to address this point.

Shaul and Ben-Levy (41) identified a nuclear factor in hepatoma cells that binds within the HBV enhancer region fragment used in these analyses (factor E; Fig. 1). This binding site has some similarity to the polyomavirus EF-C binding site (41), yet EF-C did not bind to this region in our assays. Furthermore, the HBV enhancer region mutant DL-C, which contained a deletion of the entire EF-C binding site (Fig. 7) but maintains an intact E site, did not compete even weakly for EF-C binding (Fig. 8). Thus, we conclude that EF-C and E are distinct factors with distinct binding sites. Karpen et al. (26a) recently described a protein in nuclear extracts from HepG2 cells that binds to the HBV enhancer region. Methylation interference analysis of the binding site indicates that this protein is very likely EF-C (27).

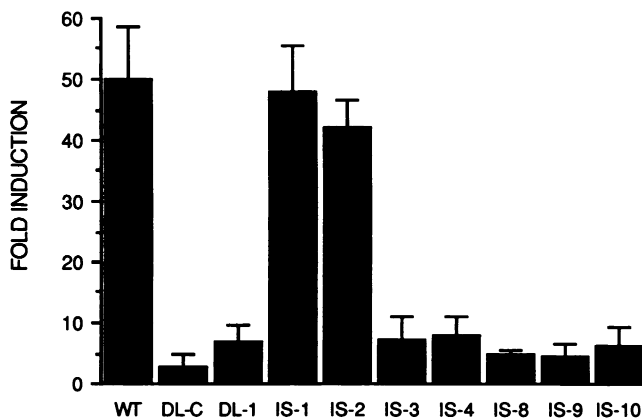

FIG. 9. Effect of spacer region mutations on HBV enhancer region function in vivo. Enhancer region fragments (*Xmn*I-*Stu*I; nt 1117 to 1247), containing the spacer region mutations listed in Fig. 7, were inserted upstream of the SV40 21-bp repeats in vector pSV-dlOP-CAT (9). This vector contains SV40 early promoter sequences fused to the CAT gene (23). CAT enzyme activity was measured in cell extracts after transfection of the expression plasmids into HepG2 liver cells. The results (mean values and standard deviations from multiple transfection experiments) are presented as fold induction of CAT expression obtained with each of the plasmids relative to that obtained with a plasmid lacking enhancer sequences.

In assays using the HepG2 and HeLa cell extracts, multiple complexes between the polyomavirus site and EF-C were observed in mobility shift assays (Fig. 3 and data not shown). A complex with completely distinct mobility was observed by using an F9 stem cell extract. DEPC interference assays demonstrated that identical EF-C contact sites were detected with all of the complexes (Fig. 4). The difference in mobilities of the complexes may reflect proteolysis of EF-C during extract preparation, protein modification, or the binding of additional factors. This difference does not appear to be species specific, since other murine cell extracts result in mobility shift patterns similar to those obtained by using the human cell extracts (Hearing, unpublished results). The pattern of the mobility shift obtained by using the F9 stem cell extract did not change on differentiation of F9 cells obtained by using retinoic acid and cyclic AMP (P. Ostapchuk and P. Hearing, unpublished results). The EF-C mobility shift pattern does not appear to correlate with EF-C enhancer activity in vivo. EF-C is functional in each of these cell lines, as judged by examination of EF-C enhancer activity in HepG2 cells (Fig. 9), HeLa cells (with use of the wild-type HBV enhancer region and the DL-C mutant region) (Ostapchuk and Hearing, unpublished results), and F9 stem cells (51). It will be of interest to analyze the nature of the heterogeneity observed with EF-C binding in the mobility shift assays and to determine whether modification of EF-C affects the activity of this protein.

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