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Cellular Factors Controlling the Activity of Woodchuck Hepatitis Virus Enhancer II

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Woodchuck hepatitis virus (WHV) efficiently induces hepatocellular carcinoma in chronically infected hosts. A key step in hepatocarcinogenesis by WHV is insertional activation of the cellular N-*myc* gene by integrated viral DNA. WHV enhancer II (En II) is the major *cis*-acting element involved in this activation. Here we characterize this viral enhancer element and define the cellular factors involved in its activity. WHV En II activity is strongly liver specific and maps to an 88-nucleotide DNA segment (nucleotides 1772 to 1859) located 5′ to the pregenomic RNA start site. Genetic analyses and electrophoretic mobility shift assays indicate that the enhancer contains three subregions important to its activity. The core elements of the enhancer are recognition sites for the liver-enriched factors HNF1 and HNF4; together, these signals account for the bulk of En II activity as well as its strong liver specificity. Multimerization of either recognition site produced strong activity even in the absence of other En II sequences. 5′ to these elements is a binding site for the ubiquitous Oct-1 transcription factor, which further augments enhancer activity ca. twofold.

Hepatitis B viruses (hepadnaviruses) are hepatotropic DNA viruses that cause acute and chronic hepatitis and are strongly associated with the development of liver cancer (14). A central event in the viral life cycle is the transcription of the viral DNA by host RNA polymerase II. This step not only produces the mRNAs that engender the viral proteins but also produces the RNA template for reverse transcription. Production of the latter is strongly liver specific and is an important determinant of the hepatotropism that is the hallmark of this viral family (14, 22, 45).

The regulation of hepadnavirus transcription has been most extensively studied in human hepatitis B virus (HBV). This work has revealed the existence of four viral promoters (68), which separately drive the production of transcripts encoding the pre-S1, pre-S2/S, pre-C/C, and X proteins (the C mRNAs also encode the P [polymerase] protein and serve as the template for reverse transcription). In addition, two major enhancer elements in HBV have been defined by their ability to up-regulate homologous or heterologous promoters in transient-cotransfection assays (53, 68). The first of these, enhancer I (En I), maps upstream of the X mRNA start sites (47) and is preferentially active in liver cells (2, 19, 47), although it retains substantial activity in nonhepatic cells in some contexts (9, 57, 62). Deletions of En I lower the levels of genomic and (to a limited extent) subgenomic (S) RNAs (18), suggesting that it is a contributor to the function of several viral promoters; however, the fact that En I sequences are within the transcribed regions of these RNAs complicates the interpretation of these studies. Major roles for cellular transcription factors RF-X, NF1, HNF3, HNF4, and C/EBP at En I have been defined both genetically and biochemically (15, 35–37, 48, 58). More recently (5, 67, 68, 70–72), a second enhancer (En II) has been identified in HBV, located 5′ to the genomic RNA start sites and at least partially coextensive with upstream activating elements of the core promoter (69). This enhancer is strongly liver specific (72) and is known to influence genomic RNA transcription; whether it plays a wider role in regulation of other viral promoters in vivo is unknown. Several factors present predominantly in the liver, including HNF3, HNF4, and C/EBP, are implicated in its function (20, 30, 71). Only a single enhancer element has been described in the avian hepadnaviruses to date (7, 29).

Woodchuck hepatitis virus (WHV) is a mammalian hepadnavirus closely related to HBV. Like its human counterpart, it produces acute and chronic hepatitis, but it is even more potently oncogenic than HBV: virtually 100% of animals chronically infected from birth with WHV will develop hepatocellular carcinoma (40, 46). Buendia and colleagues (12, 64) have shown that a key step in this oncogenic process is the insertional activation of the cellular proto-oncogene N-*myc2* by integrated viral DNA sequences; such activation can occur even over long distances (ca. 200 kb) in vivo (10). Although N-*myc* insertional activation is not a common feature of carcinogenesis in other hepadnaviruses (17, 43), the mechanism by which it occurs in WHV infection remains of great interest. We (60) and others (9a) have examined the role of viral DNA sequences in N-*myc* activation. These studies indicate that WHV sequences corresponding to HBV En II are the major activators of the N-*myc2* promoter (11). In fact, they suggested that the organization of enhancer elements in WHV is rather different from that in HBV. For example, sequences corresponding to HBV En I are nearly inactive in transient-cotransfection assays on several promoters and are not further up-regulated by expression of WHV X protein (57a, 60); in HBV, the basal En I activity is significant, and further up-regulation by X is readily demonstrable (68). These and other findings suggest that in addition to its role in N-*myc2* activation, WHV En II might play an even more central role in the viral replicative program. Therefore, we have undertaken a detailed genetic and biochemical analysis of WHV En II, the results of which are presented below.

MATERIALS AND METHODS

Plasmids, oligonucleotides, and oligonucleotide probes. The E1B TATA-CAT plasmid is based on pSP72 (Promega). The chloramphenicol acetyltransferase (CAT) gene was excised as a *Hin*dIII-*Bam*HI fragment from pSV2cat and re-

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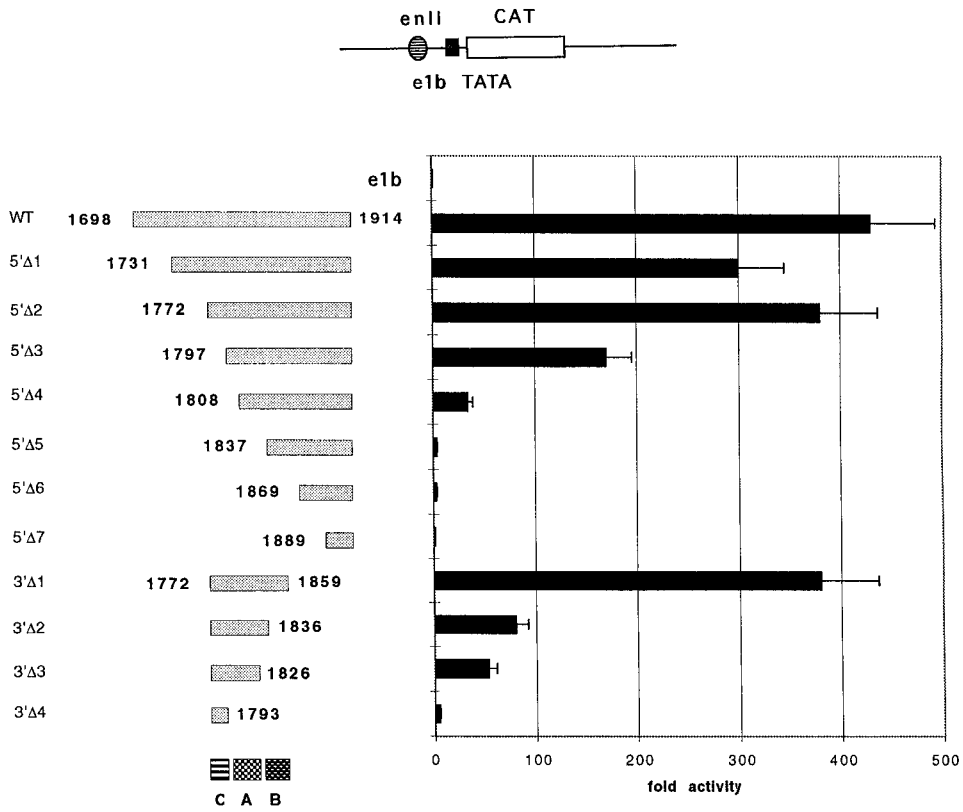

FIG. 1. Mapping of WHV En II. En II region DNA segments were cloned into the E1B TATA-CAT vector diagrammed at top. Schematic depictions of the extent of the 5′ (top) and 3′ (bottom) En II deletion mutants are shown on the left; stippled boxes denote WHV DNA sequences, and nucleotide numbers corresponding to the termini of the fragments are indicated. Boxes at the bottom depict the extents of the IIC, IIA, and IIB regions. The histogram displays the results of CAT assays of each construct, normalized to the level of CAT produced by the E1B TATA-CAT vector (arbitrarily set at 1.0), as described in Materials and Methods. WT, wild type.

cloned in the *Cla*I-*Bgl*II region of pSP72. A synthetic minimum TATA box derived from adenovirus E1B was inserted into the *Xba*I-*Bam*HI site of the resulting recombinant to generate pE1B TATA-CAT.

All WHV En II deletion mutants depicted in Fig. 1 were subcloned into either the *Hin*dIII site or the *Hin*dIII-*Xba*I region of pE1B TATA-CAT. Wild-type WHV En II and most of its deletion mutants (Fig. 1) were prepared by PCR, in which the 5′-end primer was 5′-CCAAGCTTTCCGGTCCGTGTTGCTTGG TCTTC-3′ (primer EnII 5′) and the 3′-end primer was 5′-TTTCTAGATGC ATTTATGCCTACAGCCTCCTAATA-3′ (primer EnII 3′) for the wild type. Deletions 5′Δ1 and 5′Δ2 were also prepared by PCR, in which the 5′-end primers were 5′-CCAAGCTTGACTTGCGAACCATGGATTCCAC-3′ (primer 5′D1) and 5′-CCAAGCTTGCATGCAAATCGTCAACTTGGC-3′ (primer 5′D2), respectively, along with the primer EnII 3′. Other 5′ deletion mutants (5′Δ3 to 5′Δ7) were generated by the exonuclease III and mung bean nuclease methods (34) based on a WHV *Sph*I fragment from nucleotides nt. 1797 to 1914. Some of the 3′ deletion mutants in Fig. 1 were also prepared by PCR with the 5′ primer D2 as the 5′-end primer and 5′-TTTCTAGAGCCCTCCTCCCATTTAGTT AATAATTG-3′ (primer 3′D1) for 3′ deletion mutant 1 (3′Δ1), 5′-TTTCTA GATTTAGTTAATAATTGATCTTTTA-3′ (primer 3′D2) for 3′ deletion mutant 2 (3′Δ2), and 5′-CCAAGCTTTATATAAGGAGTCCAAAGGTCCTTAC TTGGA-3′ (primer 3′D3) for 3′ deletion mutant 3 (3′Δ3) as the antisense primers. 3′ deletion mutant 4 (3′Δ4) was prepared by hybridization between primer 5′D2 and 5′-GGAAGCTTGCCAAGTTGACGATTTGCATGCA-3′ (primer 3′D4). Plasmids bearing concatemers of the IIC, IIA, or IIB regions were prepared by annealing primers 5′D2 and 5′-GGAAGCTTGCCAAGTT GACGATTTGCATGCA-3′ for IIC, 5′-CCAAGCTTCCAAGTAAGGACCTT TGGACTCCTTATATA-3′ and 5′-CCAAGCTTTATATAAGGAGTCCAAA GGTCCTTACTTGGA-3′ for IIA, and 5′-TTTCTAGACAATTATTAACTAA ATGGGAGGAGGGC-3′ and 5′-TTTCTAGAGCCCTCCTCCCATTTAGTT AATAATTG-3′ for IIB, followed by a fill-in reaction with Klenow fragment. The products were then digested with *Hin*dIII (for the IIC and IIA constructs) or *Xba*I (for the IIB construct). The cleavage products were then ligated into the *Hin*dIII site (for the IIC and IIA fragments) or the *Xba*I site (for the IIB fragment) of pE1B TATA-CAT. The structures of all deletion mutants and concatameric clones were confirmed by DNA sequencing.

The canonical HNF1-binding site was created from synthetic oligonucleotides by annealing 5′-CTAGAATTAAATATTAACT-3′ with 5′-CTAGAGTTAATA TTTAATT-3′, which represent the human fibrinogen beta HNF1-binding site (31). The C/EBP consensus binding site was constructed by annealing 5′-GATCC AATTGGGCAATCAGG-3′ and 5′-GATCCCTGATTGCCCAATTG-3′ (27).

The DNA corresponding to the WT HBV HNF4/EF-C site was derived from the HBV En I sequence and was generously provided by Patrick Hearing, who referred to it as the GB/EF-C element in an earlier publication (15).

Oligonucleotides used in the regional competition analyses for IIA region (see Fig. 4) were 5′-AGCTTCCAAGTAAGGACCTTTGGA-3′ (sense) and 5′-AGCTTCCAAAGGTCCTTACTTGGA-3′ (antisense) (nt 1797 to 1814) for IIA5′, 5′-GATCCAAGGACCTTTGGACTCG-3′ (sense) and 5′-GATCCG AGTCCAAAGGTCCTTG-3′ (antisense) (nt 1803 to 1818) for IIAm, and 5′-GATCCTTGGACTCCTTATATAG-3′ (sense) and 5′-GATCCTATATAAGG AGTCCAAG-3′ (antisense) (nt 1811 to 1826) for IIA3′.

The mutated OCT-1/OTF site in fragment IIC (ATGCAAAT to GTT ACTTG) was introduced into the primer denoted 5′D2 and its antisense oligonucleotide as described previously (39).

Oligonucleotide probes for gel shift analyses were prepared either by annealing of complementary synthetic oligonucleotides or by gel purification following

TABLE 1. Ability of cell lines to support En II function

Cell line	CAT activity	
	pE1B TATA-CAT	pEn II TATA-CAT
Hepatic		
HepG2	1	430
Huh7	0.69	34
aML	2.3	67
Nonhepatic		
HeLa	1.6	1.4
NIH 3T3	2.3	2.6

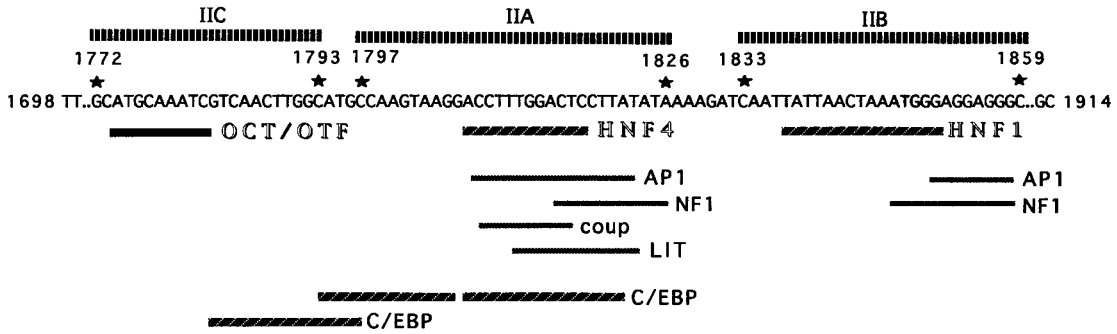

FIG. 2. Potential transcription factor-binding sites within En II. The WHV En II sequence was scanned for potential transcription factor-binding sites by the Pearson and Fasta method. Stars mark the boundaries of the IIC, IIA, and IIB regions, whose extents are also depicted in bars displayed above the DNA sequence. Immediately below the sequence are shown the positions of the Oct-1, HNF4, and HNF1 sites documented to be recognized by their cognate factors in this work. Below them are shown the predicted positions of other potential factor-binding sites proposed by computer-assisted sequence inspection; many of these nominees, however, shared only incomplete homology with their canonical binding sites.

excision from plasmid recombinants; termini were end labeled by fill-in reactions with the Klenow fragment of DNA polymerase I (44).

Transfection and CAT assay. HepG2 human hepatoma cells (10^6) were seeded, 1 day before transfection, on 6-cm dishes (Corning) containing 3 ml of Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, 100 μg of streptomycin per ml, and 100 U of penicillin G per ml. The next day, the medium was changed 3 to 4 h before transfection and 1 μg of each plasmid construct was transfected into the cells by the calcium-phosphate coprecipitation method. The cells were incubated for 16 h and then washed twice with serum-free Dulbecco's modified Eagle's medium. The medium was replaced with 4 ml of serum-containing Dulbecco's modified Eagle's medium and incubated at 37°C in a 5% CO_2 atmosphere for another 48 h. Then, the cells were washed twice with phosphate-buffered saline (PBS) without calcium and magnesium [PBS(−)] and harvested in 1.5-ml Eppendorf tubes. The cells were pelleted at 5,000 × *g* at 4°C for 5 min and suspended in 0.25 M Tris-HCl (pH 7.8). Cytoplasmic protein was extracted by three cycles of freezing and thawing (in an ethanol–dry-ice bath

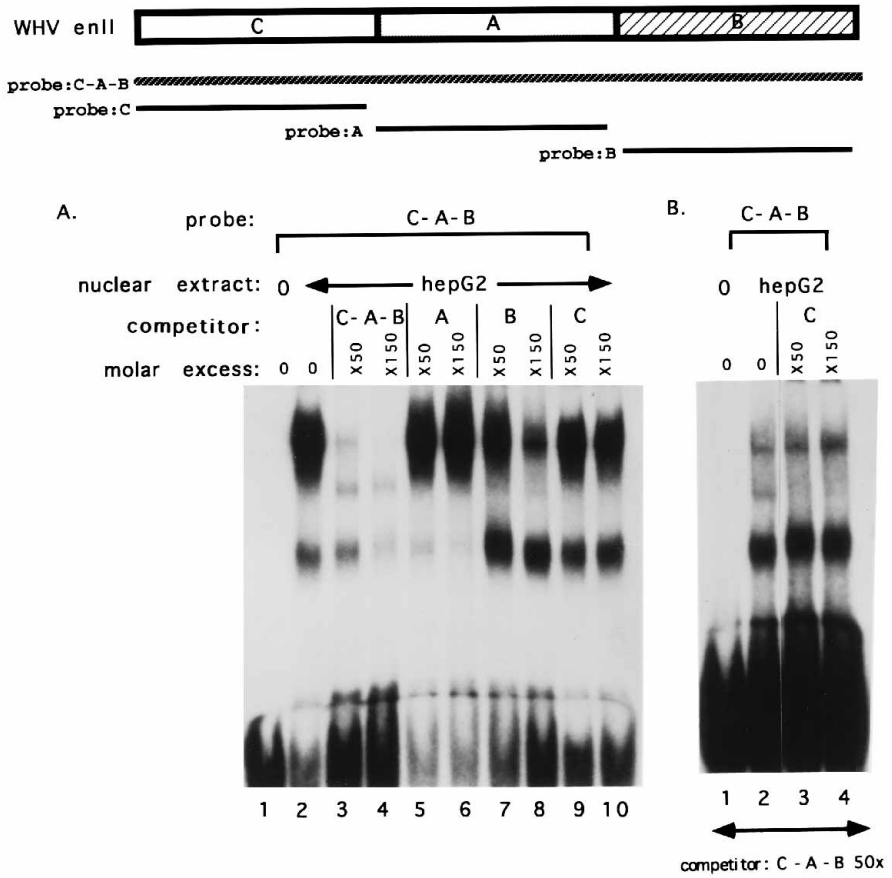

FIG. 3. Factors binding the entire En II region. A schematic depiction of the labeled probe (denoted C-A-B), which spans the full En II region, and its competitors (the individual IIC, IIA and IIB fragments) is shown above the gels. (A) Gel retardation assay with labeled C-A-B fragment and HepG2 nuclear extract. Above each lane, the unlabeled competitor fragment and its relative molar ratio to the probe are indicated. (B) Gel retardation assay with labeled C-A-B fragment and competition by the indicated amounts of the IIC competitor fragment. To display the middle DNA-protein complex optimally, a 50-fold molar excess of unlabeled C-A-B fragment was added to each sample.

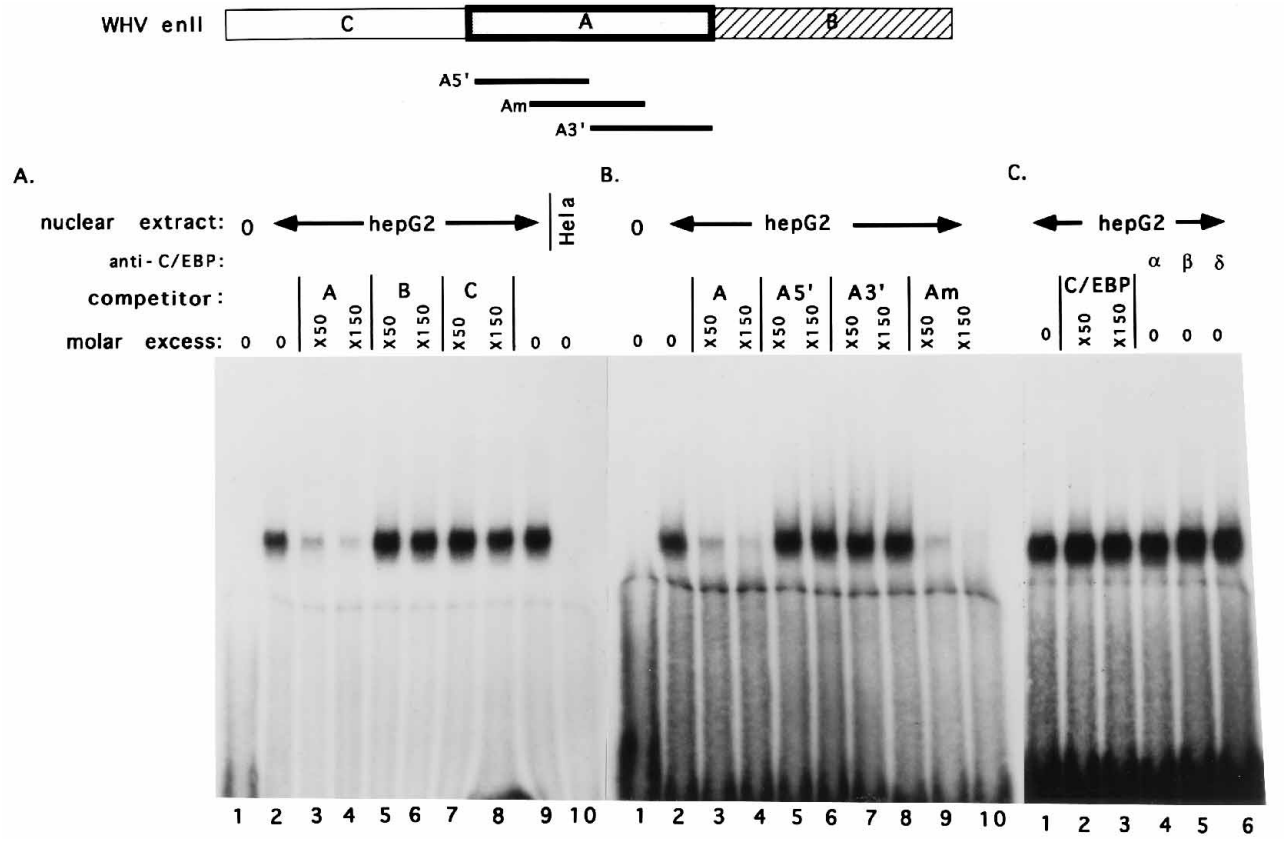

FIG. 4. EMSA analysis of factors binding the IIA region. A schematic presentation of the IIA region and its subregions IIA5′, IIAm, and IIA3′ is shown above the gels. (A) IIA probe is inhibited by IIA but not by IIB or IIC DNA. Lanes: 1, no extract. 2 to 9, the IIA probe was incubated with HepG2 nuclear extract in the presence of the indicated unlabeled competitor fragments; 10, HeLa nuclear extract. (B) EMSA with IIA subregion competitors. The IIA probe was incubated with HepG2 nuclear extract in the presence of the indicated unlabeled competitor fragments. (C) EMSA supershift analyses. Labeled IIA probe and HepG2 extract was incubated without (lanes 1 and 4 to 6) or with (lanes 2 and 3) C/EBP competitor; in lanes 4 to 6, antibodies to C/EBP alpha, beta, or delta were added.

and a 37°C water bath). Insoluble material was removed by centrifugation at 12,000 × *g* and 4°C for 5 min, and the supernatant was transferred to another tube. Deacetylase was eliminated by incubation at 65°C for 10 minutes; the resulting white cloudy precipitate was centrifuged at 12,000 × *g* and 4°C, and the supernatant was transferred to a fresh tube. The protein concentration was measured by a Bradford assay (Bio-Rad), calibrated with immunoglobulin G standards of known concentration. Protein (35 mg) from each extract was incubated for 3 h at 37°C in 100 μl of reaction mixture containing 0.5mM *n*-butyryl coenzyme A (Sigma) and 75 nCi of [^{14}C]chloramphenicol (New England Nuclear). The acetylated form of [^{14}C]chloramphenicol was extracted in the organic phase of hexane-xylene (2:1) and counted in a scintillation counter (model LS 7000; Beckman). All assays were performed in duplicate, and each duplicate assay was replicated at least twice. The data are shown as fold activation compared with the CAT activity driven by pE1B TATA-CAT. The basal activity of pE1B TATA-CAT in HepG2 cells is extremely low: ca. 0.5 to 0.75% of that of pSV2 CAT in the same cell line under the same conditions. This very low basal activity contributes strongly to the large fold activation mediated by En II.

Nuclear extract preparation. Nuclear extract was prepared from HepG2 (~10^9), HeLa, and Rat1 cells. After being washed with PBS(−), the cells were harvested by centrifugation at 250 × *g* and 4°C for 10 min. The pelleted cells were resuspended in 5 volumes of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride) and kept on ice for 10 min. The cells were centrifuged again at 250 × *g* and 4°C for 10 min and resuspended in 3 volumes of buffer A. Nonidet P-40 was added to 0.05%, and the cells were homogenized with 20 strokes of a tight-fitting Dounce homogenizer to release the nuclei. The nuclei were pelleted at 250 × *g* and 4°C for 10 min and resuspended in 1 ml of buffer C (5 mM HEPES [pH 7.9], 26% [vol/vol] glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). NaCl was added to 300 mM, and the pellet was mixed well by inversion. The mixture was kept on ice for 30 min and centrifuged at 24,000 × *g* and 4°C for 20 min. The supernatant, following determination of the protein concentration by the Bradford assay, was aliquoted, snap-frozen in dry-ice–ethanol, and stored at −70°C.

EMSA. Nuclear extract (10 to 12 μg) was used for each electrophoretic mobility shift assay (EMSA). Binding mixture (20 μl) was made up in 20 mM HEPES (pH 7.9)–1 mM $MgCl_2$–4% Ficoll (Ficoll-Paque; density, 1.077 g/ml [Pharmacia])–0.5 mM dithiothreitol–50 mM NaCl–2 μg of poly(dI-dC) (Pharmacia)–1 μg of bovine serum albumin (Sigma)–5 to 10 fmol of filled-in labeled oligonucleotide probe. The reaction mixture was incubated at room temperature for 30 min. For competition analyses, either specific or nonspecific competitor was added to the reaction mixture without the probe and preincubated for 30 min. Then the probe was added, and the mixture was incubated for another 30 min. In antibody-mediated supershift or binding-inhibition experiments, the antibody was incubated in the reaction mixture without probe for 30 min and then the probe was added and incubated for another 30 min. Samples were run on 4% polyacrylamide gels (in 0.25× conventional Tris-Borate-EDTA buffer) which were prerun at 10 V/cm for 2 h. Following electrophoresis, the gels were dried on filter paper (Whatman 3MM) and subjected to autoradiography.

Polyclonal anti-HNF4 antibody was originally generated by Sladek et al. (49) and was provided with their permission by P. Hearing (15). Polyclonal anti-C/EBP alpha, beta, and delta antibodies were provided by Steve McKnight (1, 27), and monoclonal anti-Oct-1 antibody and the purified Oct-1 protein expressed in *Escherichia coli* were provided by Greg Peterson, Tularik, Inc., South San Francisco, Calif. Anti-HNF1 antibodies were the gifts of T. Chouard and M. Yaniv (3, 50). Polyclonal anti-HNF1-alpha antibody rHCt-284, although raised against rat HNF1, cross-reacts with human HNF1-alpha; antibody rH-183 recognizes only the rat HNF1-alpha. Antibody rHNt-283 cross-reacts with both human and rat HNF1-alpha and HNF1-beta.

RESULTS AND DISCUSSION

Genetic definition and characterization of WHV En II. To define the functional boundaries of WHV En II, we first carried out a deletion analysis. A large (217-bp) WHV DNA segment spanning the region corresponding to human HBV En II (WHV nt 1698 to 1914 in the numbering system of

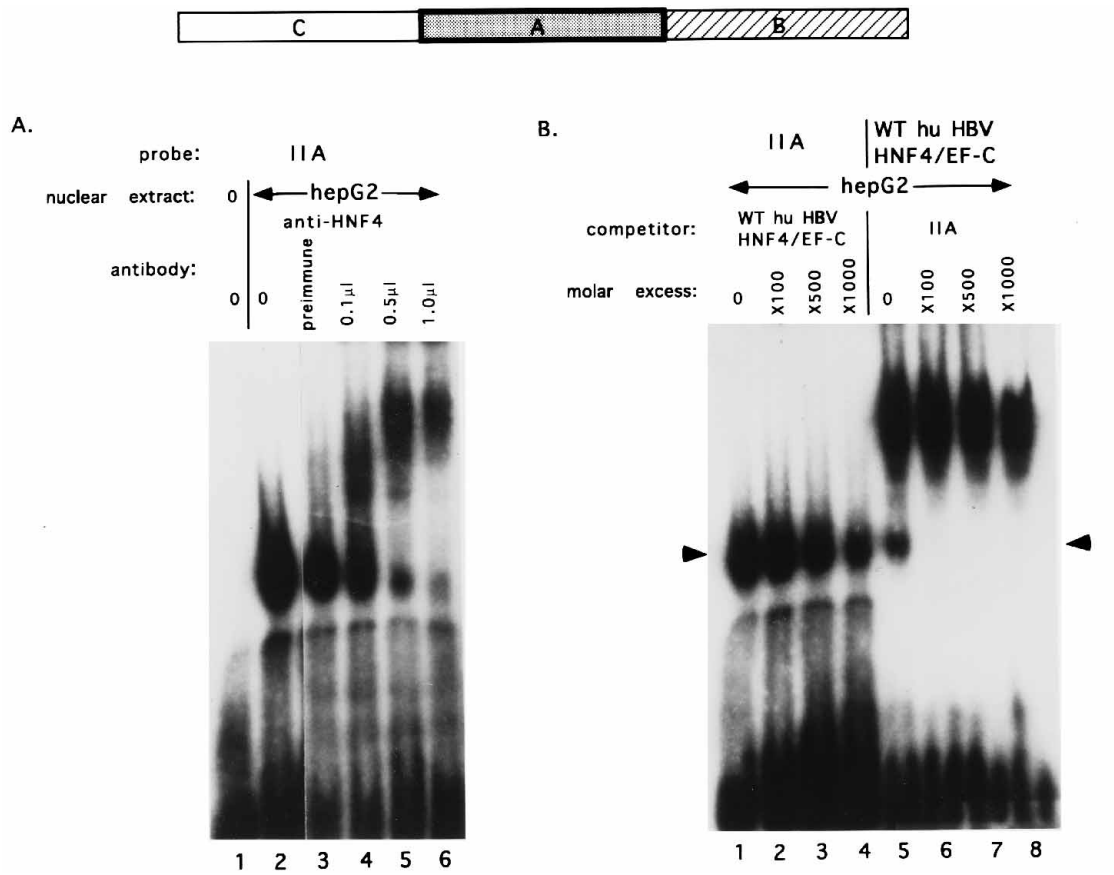

FIG. 5. HNF4 interacts with fragment IIA. The three cellular transcription factor-binding sites in WHV En II are diagrammed at the top. (A) EMSA supershift anayses with anti-HNF4 antibody. Labeled IIA fragment was incubated with the indicated nuclear extract without (lanes 1 and 2) or with (lanes 3 to 6) the indicated antibody. (B) Labeled IIA probe (lanes 1 to 4) or labeled HBV HNF4/EF-C probe (lanes 5 to 8) were incubated with the indicated cold competitor fragments in HepG2 extracts. Molar ratios of competitor to probe are displayed above each lane.

Kodama et al. [25]) was cloned into pE1B TATA-CAT, a plasmid in which CAT expression is driven by a minimal promoter (TATA box) from adenovirus E1B. The resulting plasmid was transfected into HepG2 human hepatoblastoma cells, a cell line known to be permissive for WHV replication (45) (a fact which we independently verified for our subline of HepG2 (data not shown)). At 48 h later, the cells were assayed for CAT activity; cells transfected by pE1B TATA-CAT were assayed in parallel. As shown in Fig. 1, a 400-fold increase in CAT activity was observed in the presence of this WHV fragment. In other experiments, enhancement of a cellular N-*myc* promoter was independent of the position and orientation of similar WHV sequences (reference 60 and data not shown). Serial deletions from the 5′ end of the fragment were then examined. No consistent loss of enhancer activity was encountered up to nt 1772, but further deletion to nt 1797 resulted in a ca. twofold loss of activity. Deletion to nt 1808 resulted in a major loss of enhancer function, and further deletion to nt 1837 completely inactivated En II activity. Deletions from the 3′ end showed no effect of removal of sequences from nt 1914 to 1859. However, deletion to nt 1836 decreased activity by ca. 75%; deletion of an additional 10 nt had little effect, but deletion to nt 1793 completely inactivated the enhancer. Taken together, these results suggest that the enhancer can be divided into three functional subregions, spanning nt 1772 to 1793, 1793 to 1836, and 1836 to 1859. We refer to these as regions IIC, IIA, and IIB, respectively. Deletion of region IIC reduces activity ca. twofold, indicating that regions IIA and IIB account for the bulk of the enhancer activity. Deletion of region IIB reduces activity fourfold, and all remaining activity is abolished by additional lesions in region IIA. Region IIC appears to be inactive in the absence of regions IIA and IIB (cf. Fig. 1). The region of WHV defined here as En II spans from −164 to −75 relative to the cap site of WHV pregenomic RNA. Although the minimal pregenomic (core) promoter has not been rigorously defined for WHV, this enhancer region, as for HBV En II, probably overlaps upstream elements of the core promoter (69).

WHV En II activity is strongly liver specific. Using construct 3′Δ1, which contains the smallest wild-type En II region (nt 1772 to 1859), we examined the ability of several hepatic and nonhepatic cell lines to support En II function. As shown in Table 1, En II was highly active in the human hepatocellular lines HepG2 and Huh7 and in the mouse hepatocyte line aML; all three of these lines support WHV replication. By contrast, only basal levels of CAT activity were directed by En II-based constructs in WHV-nonpermissive human or rodent lines of nonhepatic origin, including HeLa and NIH 3T3.

The DNA sequence of the 88-nt WHV En II element was then scanned by computer analysis for potential transcription factor-binding sites, using the Pearson-Fasta method (38). As expected, numerous candidate binding sites were suggested, including potential sites for liver-enriched factors (e.g., C/EBP [1, 8, 27, 56, 66], HNF-1 [3, 13, 26], and HNF-4 [33, 49]), as well

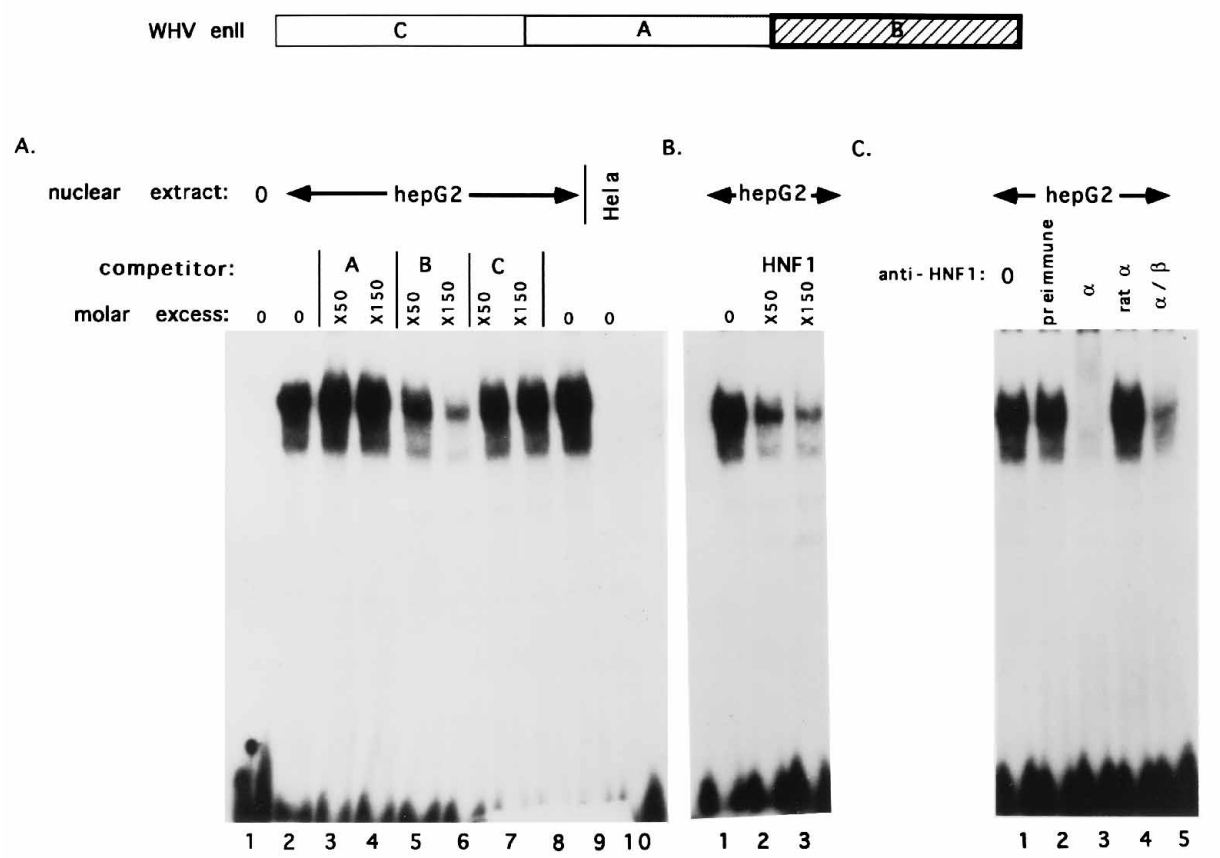

FIG. 6. EMSA analysis of IIB-binding factors. The three cellular transcription factor-binding sites in WHV En II are diagrammed at the top. (A) IIB-binding factors are not inhibited by IIA and IIC DNA. Labeled IIB probe was incubated with the indicated nuclear extract. Above each lane, the unlabeled competitor fragment and its relative molar ratio to the probe are indicated. (B) Competition analyses with a canonical HNF1-binding site. Labeled IIB probe was incubated with HepG2 extract and the indicated molar ratio of an unlabeled oligonucleotide corresponding to the human beta-fibrinogen HNF1-binding site (see Materials and Methods). (C) Anti-HNF1 antibodies inhibit IIB EMSA. Labeled IIB probe was incubated with HepG2 nuclear extract in the presence of the indicated antibody. Antibody in lane 3, raised to a rat HNF-1, cross-reacts with human HNF1-alpha; that in lane 4 is specific for rat HNF1-alpha, while that in lane 5 recognizes rat and human HNF1-alpha and -beta.

for as more ubiquitous factors (e.g., Oct-1 [23, 24, 52], NF-1 [21], AP1 [28], COUP-TF [59, 63], and LIT [4]) (Fig. 2). Many of these candidate sites, however, had only partial homology to known recognition elements or involved transcription factor families whose recognition sites are fairly degenerate. Nonetheless, the analysis was useful as a guide to the experimental evaluation of candidate binding factors; in fact, all three of the factors that we experimentally demonstrated as active in binding to En II were among the set predicted by computer-assisted sequence inspection (Fig. 2).

Binding factors for the whole WHV En II sequence. As a first step to assessing the complexity of the cohort of factors binding WHV En II, we examined the full 88-bp element for binding factors by EMSA. For this, we used nuclear extracts prepared from the human hepatoblastoma line HepG2, which is known to support correct WHV transcription and replication (45). The intact En II fragment encompassing regions IIC, IIA, and IIB was end labeled with ^{32}P and incubated with HepG2 nuclear extract; complexes were then fractionated by nondenaturing acrylamide gel electrophoresis. Figure 3A shows the results of this analysis. As shown in lane 2, three complexes are formed under these conditions (the central complex is obscured by the heterogeneous slowly migrating complex but is more evident when the latter is partially inhibited, as in Fig. 3A (lane 3) and Fig. 3B). All three bands are inhibited by excess unlabeled En II fragment (lanes 3 and 4) but not by nonspecific competitors (not shown). Further competition analysis with probes from the IIC, IIA, and IIB subregions (lanes 5 to 10) allowed tentative assignment of the complexes to different regions of En II. For example, the most slowly migrating complex was effectively inhibited by fragment IIB (lanes 7 and 8) but not by the other fragments, whereas the most rapidly migrating complex was inhibited only by fragment IIA (lanes 5 and 6). The middle band was specifically inhibited only by fragment IIC (Fig. 3B, lanes 2 to 4). These assignments were confirmed in all cases by direct binding experiments to individually labeled subfragments (see below).

HNF-4 binds the IIA sequence. To search for a factor(s) recognizing the IIA region, we used a IIA-specific probe in similar EMSA experiments. A single complex was detected on this probe in gel retardation assays with HepG2 nuclear extracts (Fig. 4A, lane 2). This complex was absent in similar experiments with HeLa cell nuclear extract (lane 10) and Rat1 cell nuclear extract (data not shown), consistent with its arising from the binding of a liver-specific factor. To determine which subregion of fragment IIA was involved in binding, we prepared smaller fragments termed IIA5′, IIAm, and IIA3′, whose extents are schematically depicted in the diagram in Fig. 4. Each of these unlabeled subfragments was used to compete with ^{32}P-labeled fragment IIA for binding to the factor (Fig.

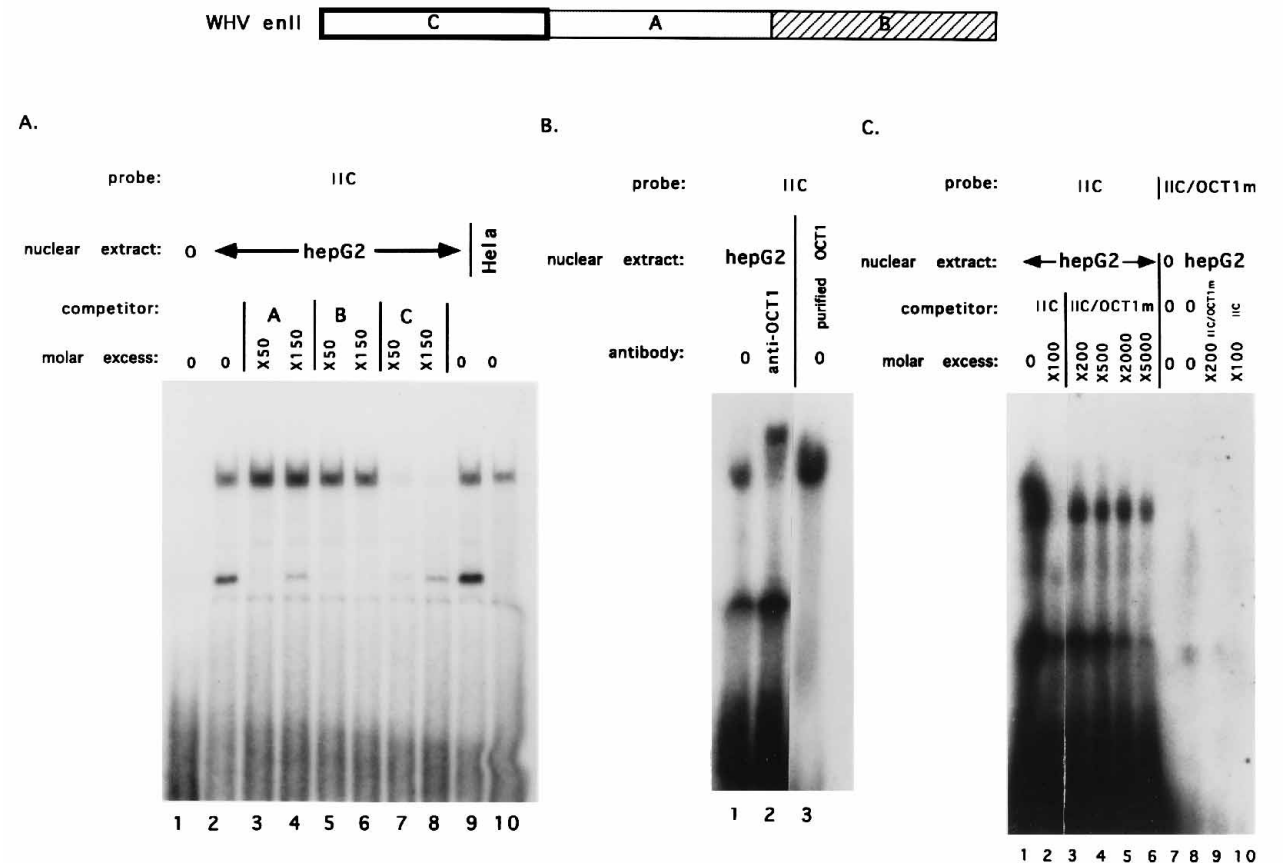

FIG. 7. EMSA of IIC-binding factors. The three cellular transcription factor-binding sites in WHV En II are diagrammed at the top. (A) IIC-binding factor is not inhibited by IIA or IIB DNA. Labeled IIC probe was incubated with the indicated extract in the presence of the indicated competitor; the molar excess of the competitor with respect to the probe is shown above each lane. (B) Oct-1 interacts with the IIC region. Labeled IIC probe was incubated with HepG2 nuclear extract (lanes 1 and 2) or purified Oct-1 (lane 3). In lane 2, anti-Oct-1 antibody was also added. (C) IIC DNA bearing mutations in the Oct-1-binding site does not bind to the IIC-binding factor. Lanes: 1 to 6, labeled IIC probe was incubated with the indicated ratio of either wild-type (lane 2) or mutant (lanes 3 to 6) IIC competitor DNA; 7 to 10, labeled mutant IIC probe was incubated with the indicated extract and competitors.

4B); this showed that only the central subregion (fragment IIAm) was active in competition. Among the factors identified by sequence inspection in this region was the liver-enriched family of factors known as C/EBP. However, a canonical C/EBP-binding sequence (27) did not compete for IIA binding (Fig. 4C, lanes 1 to 3), nor did antibodies against C/EBP isoforms alpha, beta, or delta alter the formation or migration of the complex (Fig. 4C, lanes 4 to 6).

Again guided by the computer analysis of Fig. 2, we next tested the possibility that HNF4 was involved in binding to this region, since a predicted HNF4 site is contained within subfragment IIAm. Complexes formed on the IIA probe were efficiently supershifted by anti-HNF4 antibody in a dose-dependent fashion (Fig. 5A), strongly implying that HNF4 was an important participant in complex formation. In addition, IIA complexes were inhibited, albeit inefficiently, by a human HBV EnI fragment containing an HNF4 site (Fig. 5B, lanes 1 to 4). Since the HBV En I competitor used in this experiment also contained an RFX (EF/C) site (15), it was unclear if the competition observed was due to HNF4 or to RFX. To clarify this, we conducted the reciprocal competition experiment. Labeled HBV En I probe containing HNF4 and RFX (EF/C) sites was incubated with HepG2 nuclear extract in the presence of increasing amounts of unlabeled competitor IIA fragment. As shown in Fig. 5B, lanes 5 to 8, only the lower of the two complexes observed with this probe is inhibited by fragment IIA. This band is known from earlier work (15) to represent the HNF4-DNA complex (note that it comigrates with the similar complex formed on labeled IIA probe [lanes 1 to 4]). Together, these findings establish HNF4 (or a closely related factor) as the element involved in interaction with region IIA. We presume that the inefficiency of competition displayed by the HBV En I HNF4 site is due to a lower affinity of this particular site for HNF4 than that in the WHV IIA fragment.

HNF4 is a member of the superfamily of nuclear steroid receptors, a large group of ligand-activated transcription factors (33, 49). Although much is known about the binding specificity of HNF4 and its natural distribution, its presumed ligand is unknown. It is worth mentioning that some HNF4 recognition sites can also be bound by several other members of the superfamily; in HBV En I, for example, the HNF4 site can also interact with RXR alpha and COUP-TF (chicken ovalbumin upstream promoter transcription factor) (15, 16). In the WHV En II element, computer analysis of the IIA region identifies a potential COUP-TF site in this region (Fig. 2). However, the latter is unlikely to be important in En II binding, since HeLa cells, which contain the ubiquitous COUP-TF (65), lack a binding activity for fragment IIA.

HNF1 binds the IIB region. To identify the factor(s) bound to region IIB, we used IIB-specific probes in gel retardation assays. As shown in Fig. 6A, a heterogeneous band of complexes was formed on this probe, and all are efficiently inhib-

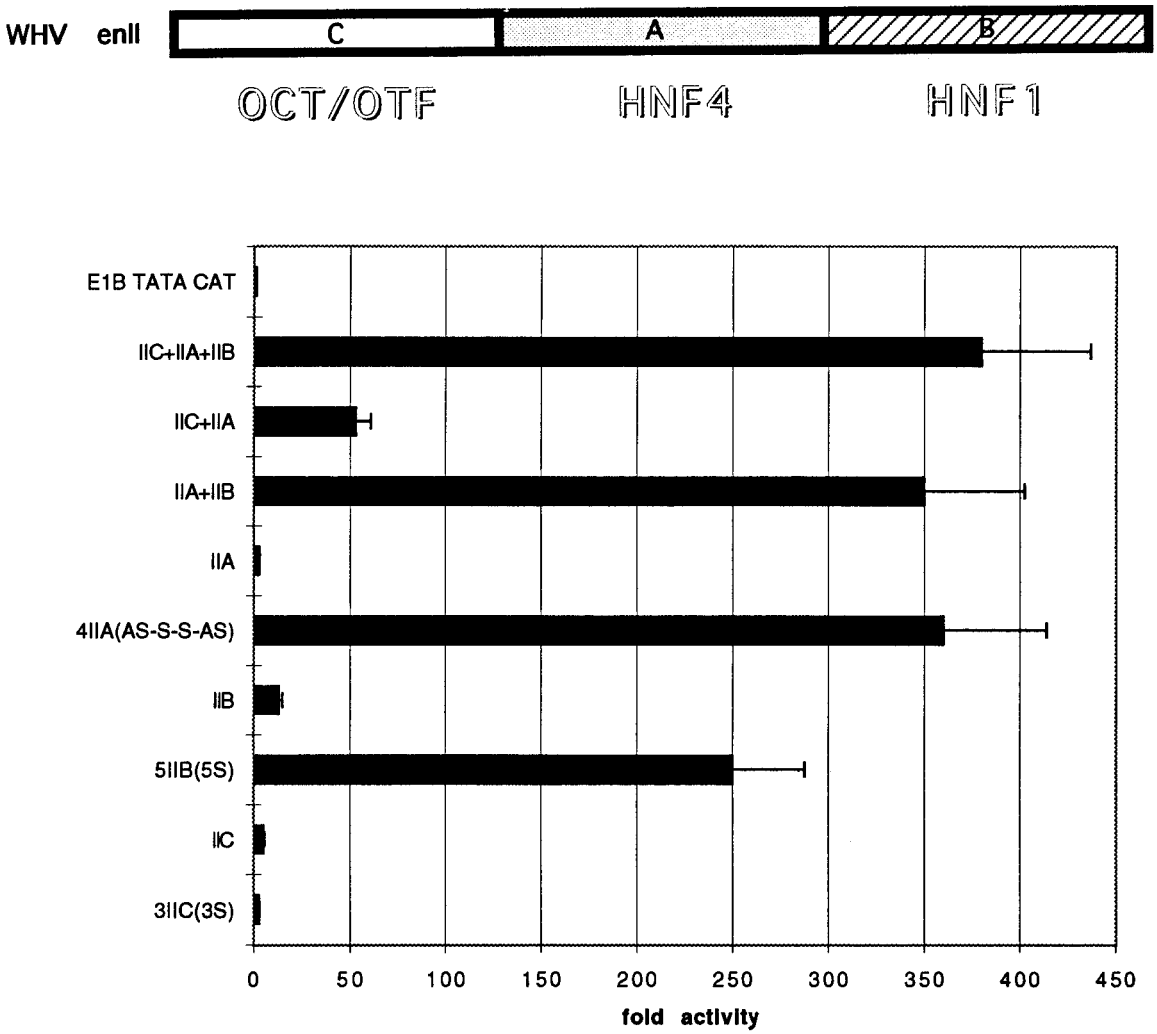

FIG. 8. Enhancer activity of individual subdomains of WHV En II. At left is indicated the structure of the En II fragments cloned 5′ to E1B TATA-CAT. 4IIA indicates four multimerized IIA-binding sites; 5IIB indicates five multimerized IIB sites; 3IIC denotes three multimerized IIC sites. In parentheses, the orientation of the individual sites in each multimer is indicated (IS [sense] and AS [antisense] denote the orientation of each fragment according to the direction of the CAT gene). The histogram displays the results of a CAT assay of each construct, normalized to the level of CAT produced by E1B TATA-CAT vector (arbitrarily set at 1.0), as described in Materials and Methods.

ited by homologous (IIB) but not heterologous (IIA or IIC) fragments. As in region IIA, the IIB recognition factor(s) appeared to be liver specific: no complexes were observed in HeLa extracts (Fig. 6A, lane 10) or Rat1 extracts (data not shown). Computer-assisted sequence inspection in this region identified a candidate HNF1 recognition site; this was an attractive candidate binding factor, because it is known to be relatively liver specific (3, 13, 26). Consistent with this identification, a canonical HNF1 site from the beta-fibrinogen gene efficiently inhibited the binding of the factor to labeled IIB probe (Fig. 6B).

Two isoforms of HNF1 are known. HNF1-alpha, the prototype factor of the family, is highly concentrated in the liver (although it is also found in selected extrahepatic sites such as the kidney) and is a potent activator of transcription (31, 32, 41, 42). Expression of HNF1-alpha in nonhepatic cells allows efficient activation of genes bearing HNF1 recognition sites (32, 41). HNF1-beta, also called v-HNF1, shares virtually identical DNA-binding properties with HNF1 alpha but differs at its C terminus, the locus of the major transcriptional activation domain; as a result, it is typically a less potent activator (32). Both polypeptides can dimerize, either with themselves or with each other, and both are expressed in HepG2 cells (32, 42, 50). The heterogeneity of the complexes formed on fragment IIB suggests that several HNF species may be involved in recognition of En II. To determine which HNF1 isoforms might be involved in WHV En II binding, we performed antibody supershift experiments. Addition of an antibody reactive with human HNF1-alpha strongly blocked complex formation on fragment IIB DNA; of the few remaining complexes observed, many were retarded in their mobility (Fig. 6C, lanes 1 to 3). An antibody reactive with both human HNF1-alpha and -beta isoforms also inhibited complex formation (lane 5), while preimmune serum (lane 2) or non-cross-reacting antibodies to rat HNF1-alpha had no effect (lane 4). We conclude that the majority of the complexes include at least one HNF1-alpha subunit.

Oct-1 binds region IIC. A factor(s) interacting with the IIC region was similarly sought by EMSA experiments with IIC-specific probe (Fig. 7A). Once again, this probe detected a

single major complex (the minor, more rapidly migrating band in Fig. 7A, lanes 1, 4, 8, and 9, was not reproducibly observed, and its origin remains unclear). The major IIC complex was inhibited by homologous (IIC) but not heterologous (IIA or IIB) fragments (Fig. 7A, lanes 1 to 8). Formation of this complex did not seem to be liver specific, however, since similar complexes were detected with HeLa cell nuclear extract (lane 10). Although limited potential homologies to C/EBP recognition sequences were identified in this region (cf. Fig. 2), competition experiments with known C/EBP-binding sites and supershift experiments with anti-C/EBP antibodies all failed to implicate C/EBP alpha, beta, or delta in this interaction (data not shown). However, the computer also identified a sequence in this region with strong homology to an Oct-1 recognition site at the 5′-end region of IIC fragment (Fig. 2). Since Oct-1 is known to be a ubiquitous factor (23, 24, 52), we pursued it further as a candidate binding protein. Figure 7B (lane 3) shows that purified recombinant Oct-1 (a generous gift of Greg Peterson) can bind to the IIC fragment and that the resulting complex comigrates with those formed in HepG2 nuclear extracts (lane 1). Furthermore, the mobility of complexes formed in HepG2 extracts on IIC probe can be further retarded by the addition of a monoclonal antibody to Oct-1 (Fig. 7B, lane 2). Finally, we synthesized a mutant IIC fragment bearing multiple lesions in the core of the Oct-1-binding site (see Materials and Methods) and used this for binding and competition studies. Figure 7C shows that when this fragment was used as a probe, no labeled retarded complexes were formed (lanes 7 and 8); furthermore, when it was used as a competitor, the mutant sequence failed to compete for binding to labeled wild-type fragment IIC (lanes 1 to 6).

Enhancer activity of individual En II factor-binding sites. Finally, we examined the separated IIC, IIA, and IIB elements for their ability to activate a TATA box driving a CAT reporter following transient transfection into HepG2 cells. As shown in Fig. 8, single copies of IIA and IIC were nearly inactive whereas a single IIB (HNF1) site caused ca. 10-fold activation. Multimerization of the individual IIA (HNF4) or IIB (HNF1) sites, however, produced strong activation, virtually identical to that of the wild-type enhancer. Thus, these factors are independently able to activate transcription, a result in accord with the known properties of HNF1 and HNF4 (32, 34, 41). By contrast, multimerization of the IIC region did not up-regulate transcription; this is consistent with earlier reports in which multimerized Oct-1 failed to activate several polymerase II promoters, despite the presence of an N-terminal glutamine-rich activation domain active on other promoters (6, 51, 52, 54, 55). Thus, Oct-1 displays promoter-specific activation properties; whether any of the WHV promoters could be directly activated by Oct-1 bound at En II is a matter for future study. However, in the present context at least, Oct-1 must interact with other elements to mediate activation of a nearby TATA box. One such element is clearly HNF4, since linking a single copy of IIC to IIA produces a 20-fold enhancement of CAT expression over that driven by IIA alone (Fig. 8). The mechanistic details of this activation remain to be determined. One possibility is that the Oct-1 protein interacts with HNF4 bound to an adjacent site on the DNA or with another nuclear protein that augments HNF4 activity; many other models are possible, however.

Taken together, these and our previous studies (60) indicate that the transcriptional regulatory elements of WHV differ substantially from those of HBV (47, 61). The En I element in WHV appears much less active than its HBV counterpart. WHV En II, like HBV En II, is a potent enhancer that is strongly liver specific. However, the details of the deployment of individual transcription factors on the two enhancers differ considerably. HBV En II appears to be substantially more complex, with several recognition sites for C/EBP and HNF3, as well as individual sites for interaction with HNF4 and RFX-1 (17a, 30, 48, 71). The relative simplicity of WHV En II (Fig. 2) will facilitate the mutational analysis of the contributions of individual binding factors to viral replication in cultured cells and in whole-animal hosts. Such studies are currently in progress and should clarify the contributions of En II to transcription from the four classical viral promoters. In addition, given the importance of En II in the activation of N-*myc2* expression, lesions in En II may produce interesting phenotypes affecting oncogenesis in vivo.

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REFERENCES

1. **Agre, P., P. F. Johnson, and S. L. McKnight.** 1989. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. Science **246:**922–926.
2. **Antonucci, T., and W. Rutter.** 1989. Hepatitis B virus promoters are regulated by the HBV enhancer in a tissue-specific manner. J. Virol. **63:**579–585.
3. **Bach, I., M.-G. Mattei, S. Cereghini, and M. Yaniv.** 1991. Two members of an HNF1 homeoprotein family are expressed in human liver. Nucleic Acids Res. **19:**3553–3559.
4. **Carlsson, P., P. Eriksson, and G. Bjursell.** 1990. Two nuclear proteins bind to the major positive element of the apolipoprotein B gene promoter. Gene **94:**295–301.
5. **Chen, S.-T., P. La Porte, and J.-K. Yee.** 1993. Mutational analysis of hepatitis B virus enhancer. Virology **196:**652–659.
6. **Cleary, M. A., S. Stern, M. Tanaka, and W. Herr.** 1993. Differential positive control by Oct-1 and Oct-2: activation of a transcriptionally silent motif through Oct-1 and VP16 corecruitment. Genes Dev. **7:**72–83.
7. **Cresenzo-Chaigne, J., A. Pillot, A. Lillienbaum, M. Levrero, and E. Elfassi.** 1991. Identification of a strong enhancer element upstream of the pregenomic start site of the duck hepatitis B virus genome. J. Virol. **65:**3882–3885.
8. **Descombes, P., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell **67:**569–579.
9. **Elfassi, E.** 1987. Broad specificity of the hepatitis B virus enhancer function. Virology **160:**259–262.
9a. **Fourel, G., and M. Buendia.** Personal communication.
10. **Fourel, G., J. Couturier, Y. Wei, F. Apiou, P. Tiollais, and M.-A. Buendia.** 1994. Evidence for long-range oncogene activation by hepadnavirus insertion. EMBO J. **13:**2526–2534.
11. **Fourel, G., C. Transy, B. C. Tennant, and M.-A. Buendia.** 1992. Expression of the woodchuck N-myc2 retroposon in brain and liver tumors is driven by a cryptic N-myc promoter. Mol. Cell. Biol. **12:**5336–5344.
12. **Fourel, G., C. Trepo, L. Bougueleret, B. Henglein, A. Ponzetto, P. Tiollais, and M.-A. Buendia.** 1990. Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumours. Nature (London) **347:**294–298.
13. **Frain, M., G. Swart, P. Monaci, A. Nicosia, S. Stampfli, R. Frank, and R. Cortese.** 1989. The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell **59:**145–157.
14. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B virus. Annu. Rev. Biochem. **56:**651–667.
15. **Garcia, A. D., P. Ostapchuk, and P. Hearing.** 1993. Functional interaction of nuclear factors EF-C, HNF-4, and RXRa with hepatitis B virus enhancer I. J. Virol. **67:**3940–3950.
16. **Hall, R. K., F. M. Sladek, and D. K. Granner.** 1995. The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for inductin of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. Proc. Natl. Acad. Sci. USA **92:**412–416.
17. **Hansen, L. J., B. C. Tennant, C. Seeger, and D. Ganem.** 1993. Differential activation of *myc* gene family members in hepatic carcinogenesis by closely related hepatitis B viruses. Mol. Cell. Biol. **13:**659–667.
17a. **Hearing, P.** Personal communication.
18. **Hu, K., and A. Siddiqui.** 1991. Regulation of the hepatitis B virus gene expression by the enhancer element I. Virology **181:**721–726.
19. **Jameel, S., and A. Siddiqui.** 1986. The human hepatitis B virus enhancer requires *trans*-acting cellular factors for activity. Mol. Cell. Biol. **6:**710–715.

20. **Johnson, J. L., A. K. Raney, and A. McLachlan.** 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. Virology **208:**147–158.
21. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48:**79–89.
22. **Junker, M., P. Galle, and H. Schaller.** 1987. Expression and replication of the hepatitis B virus genome under foreign promoter control. Nucleic Acids Res. **15:**10117–10132.
23. **Kemler, I., E. Bucher, K. Seipel, M. M. Muler-Immergluck, and W. Schaffner.** 1991. Promoters with the octamer DNA motif (ATGCAAAAT) can be ubiquitous or cell type-specific depending on binding affinity of the octamer site and Oct-factor concentration. Nucleic Acids Res. **19:**237–242.
24. **Kemler, I., and W. Schaffner.** 1990. Octamer transcription factors and the cell type-specificity of immunoglobulin gene expression. FASEB J. **4:**1444–1449.
25. **Kodama, K., N. Ogasawara, H. Yoshikawa, and S. Murakami.** 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutional relationship between hepadnaviruses. J. Virol. **56:**978–986.
26. **Kuo, C. J., P. B. Conley, C.-L. Hsieh, U. Francke, and G. R. Crabtree.** 1990. Molecular cloning, functional expression and chromosomal localization of mouse hepatocyte nuclear factor 1. Proc. Natl. Acad. Sci. USA **87:**9838–9842.
27. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2:**786–800.
28. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA inducible enhancer element. Cell **49:**741–752.
29. **Liu, C., W. S. Mason, and J. B. E. Burch.** 1994. Identification of factor-binding sites in the duck hepatitis B virus enhancer and in vivo effects of enhancer mutations. J. Virol. **68:**2286–2296.
30. **Lopez-Cabrera, M., J. Letovsky, K.-Q. Hu, and A. Siddiqui.** 1991. Transcriptional factor C/EBP binds to and transactivates the enhancer II element of the hepatitis B virus. Virology **183:**825–829.
31. **Mendel, D. B., and G. R. Crabtree.** 1991. HNF-1, a member of a novel class of dimerizing homeodomain proteins. J. Biol. Chem. **266:**677–680.
32. **Mendel, D. B., L. P. Hansen, M. K. Graves, P. B. Conley, and G. R. Crabtree.** 1991. HNF1a and HNF1b (vHNF1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. Genes Dev. **5:**1042–1052.
33. **Mendelzon, D., F. Boissier, and M. M. Zakin.** 1990. The binding site for the liver-specific transcription factor Tf-LF1 and the TATA box of the human transferrin gene promoter are the only elements necessary to direct liver-specific transcription in vitro. Nucleic Acids Res. **18:**5717–5721.
34. **Miura, N., and K. Tanaka.** 1993. Analyses of the hepatocyte nuclear factor (HNF) 1 gene promoter: synergistic activation by HNF4 and HNF1 proteins. Nucleic Acids Res. **21:**3731–3736.
35. **Ori, A., D. Atzmony, I. Haviv, and Y. Shaul.** 1994. An NF1 motif plays a central role in hepatitis B virus enhancer. Virology **204:**600–608.
36. **Ori, A., and Y. Shaul.** 1995. Hepatitis B virus enhancer binds and is activated by the hepatocyte nuclear factor 3. Virology **207:**98–106.
37. **Ostapchuck, P., G. Schierle, and P. Hearing.** 1989. Binding of nuclear factor EF/C to a functional domain of the hepatitis B virus enhancer region. Mol. Cell. Biol. **9:**2787–2797.
38. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
39. **Perez-Mutul, J. M. M., and B. Wasylyk.** 1988. Mutational analysis of the contribution of sequence motifs within the IgH enhancer to tissue specific transcriptional activation. Nucleic Acids Res. **16:**6085–6096.
40. **Popper, H., L. Roth, R. Purcell, B. Tennant, and J. Gerin.** 1987. Hepatocarcinogenicity of the woodchuck hepatitis virus. Proc. Natl. Acad. Sci. USA **84:**866–870.
41. **Raney, A., A. Easton, D. Milich, and A. McLachlan.** 1991. Promoter-specific transactivation of hepatitis B virus transcription by a glutamine- and proline-rich domain of hepatocyte nuclear factor I. J. Virol. **65:**5774–5781.
42. **Rey-Coampos, J., T. Chouard, M. Yaniv, and S. Cereghini.** 1991. vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. EMBO J. **10:**1445–1457.
43. **Robinson, W. S.** 1994. Molecular events in the pathogenesis of hepadnavirus-associated hepatocellular carcinoma. Annu. Rev. Med. **45:**297–323.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Seeger, C., B. Baldwin, and B. C. Tennant.** 1989. Expression of infectious woodchuck hepatitis virus in murine and avian fibroblasts. J. Virol. **63:**4665–4669.
46. **Seeger, C., B. Baldwin, W. E. Hornbuckle, A. E. Yeager, B. C. Tennant, P. Cote, L. Ferrell, D. Ganem, and H. E. Varmus.** 1991. Woodchuck hepatitis virus is a more efficient oncogenic agent than ground squirrel hepatitis virus in common host. J. Virol. **65:**1673–1679.
47. **Shaul, J., W. Rutter, and O. Laub.** 1985. A human hepatitis B virus enhancer element. EMBO J. **4:**427–430.
48. **Siegrist, C. A., B. Durand, P. Emery, E. David, P. Hearing, B. Mach, and W. Reith.** 1993. RFX1 is identical to enhancer factor C and functions as transactivator of the hepatitis B virus enhancer. Mol. Cell. Biol. **13:**6375–6384.
49. **Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell, Jr.** 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. **4:**2353–2365.
50. **Sourdive, D. J. D., T. Chouard, and M. Yaniv.** 1993. The HNF1 C-terminal domain contributes to transcriptional activity and modulates nuclear localisation. C. R. Acad. Sci. Paris **316:**385–394.
51. **Stern, S., and W. Herr.** 1991. The herpes simplex virus trans-activator VP16 recognizes the Oct-1 homeo domain: evidence for a homeo domain recognition subdomain. Genes Dev. **5:**2555–2566.
52. **Strum, R. A., G. Das, and W. Herr.** 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. **2:**1582–1599.
53. **Su, H., and J.-K. Yee.** 1992. Regulation of hepatitis B virus gene expression by its two enhancers. Proc. Natl. Acad. Sci. USA **89:**2708–2712.
54. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell **60:**375–386.
55. **Tanaka, M., J.-J. Lai, and W. Herr.** 1992. Promoter-selective activation domains in Oct-1 and Oct-2 direct differential activation of an snRNA and mRNA promoter. Cell **68:**755–767.
56. **Theisen, M., R. R. Behringer, G. G. Cadd, R. L. Brinster, and G. S. McKnight.** 1993. A C/EBP-binding site in the transferrin promoter is essential for expression in the liver but not the brain of transgenic mice. Mol. Cell. Biol. **13:**7666–7676.
57. **Tognoni, A., R. Catteneo, E. Serfling, and W. Schaffner.** 1985. A novel selection approach allows precise mapping of the hepatitis B virus enhancer. Nucleic Acids Res. **13:**7457–7471.

57a. **Trujillo, A., J. Chang, and Y. Wei.** Unpublished data.

58. **Trujillo, M., J. Letovsky, H. Maguire, M. Lopez-Cabrera, and A. Siddiqui.** 1991. Functional analysis of a liver-specific enhancer of the hepatitis B virus. Proc. Natl. Acad. Sci. USA **88:**3797–3801.
59. **Tsai, S. Y., I. Sagami, H. Wang, M.-J. Tsai, and B. W. O'Malley.** 1987. Interactions between a DNA-binding transcription factor (COUP) and a non-DNA binding factor (S300-II). Cell **50:**701–709.
60. **Ueda, K., Y. Wei and D. Ganem.** 1996. Activation of N-myc2 gene expression by cis-acting elements of oncogenic hepadnaviral genomes: key role of enhancer II. Virology **217:**413–417.
61. **Valenzuela, P., L. Rall, M. Zaldivar, M. Quiroga, P. Gray, and W. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. ICN-UCLA Symp. Mol. Cell. Biol. **18:**57–70.
62. **Vannice, J., and A. Levinson.** 1988. Properties of the human hepatitis B virus enhancer: position effects and cell-type nonspecificity. J. Virol. **62:**1305–1313.
63. **Wang, L.-H., S. Y. Tsai, R. G. Cook, W. G. Beattie, M.-J. Tsai, and B. W. O'Malley.** 1989. COUP transcription factor is a member of the steroid receptor superfamily. Nature (London) **340:**163–166.
64. **Wei, Y., G. Fourel, A. Ponzetto, M. Silvestro, P. Tiollais, and M.-A. Buendia.** 1992. Hepadnavirus integration: mechanisms of activation of the N-*myc2* retrotransposon in woodchuck liver tumors. J. Virol. **66:**5265–5276.
65. **Wijnholds, J., J. Philipsen, and A. Geert.** 1988. Tissue-specific and steroid-dependent interaction of transcription factors with the oestrogen-inducible apoVLDL II promoter in vivo. EMBO J. **7:**2757–2763.
66. **Williams, S. C., C. A. Cantwell, and P. F. Johnson.** 1991. A family of C/EBP-related proteins capable of forming covalently linked lucine zipper dimers in vitro. Genes Dev. **5:**1553–1567.
67. **Yee, J.-K.** 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science **246:**658–661.
68. **Yen, T.-S. B.** 1993. Regulation of hepatitis B virus gene expression. Semin. Virol. **4:**33–42.
69. **Yuh, C.-H., Y.-L. Chang, and L.-P. Ting.** 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J. Virol. **66:**4073–4084.
70. **Yuh, C.-H., and L.-P. Ting.** 1990. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. **64:**4281–4287.
71. **Yuh, C.-H., and L.-P. Ting.** 1991. C/EBP-like proteins binding to the functional box-a and box-b of the second enhancer of hepatitis B virus. Mol. Cell. Biol. **11:**5044–5052.
72. **Yuh, C.-H., and L.-P. Ting.** 1993. Differentiated liver cell specificity of the second enhancer of the hepatitis B virus. J. Virol. **67:**142–149.