Blood coagulation Factor X and its activated form Factor Xa play an essential role in the midphase of the clotting cascade. To delineate the mechanisms governing the liver-specific expression of Factor X, we have previously characterized the complete 2.8 kilobase pairs of the 5'-flanking region of Factor X and demonstrated that the first 209 base pairs is sufficient to confer maximal promoter activity in HepG2 cells, a hepatoma cell line that expresses Factor X. We have also shown that mutations at ACTTTG and CCAAT elements located at −56 to −51 and −120 to −116, respectively, significantly reduce the promoter activity. In this report, we demonstrate that Factor X mRNA is primarily but not exclusively expressed in the liver. Using DNase I footprinting analysis, we determine four protein binding sites within the 209-base pair fragment, designated site 1 (−73 to −44), site 2 (−128 to −94), site 3 (−165 to −132), and site 4 (−233 to −169). Using mobility shift assays in combination with competition and supershift experiments, we demonstrate that hepatocyte nuclear factor 4 and Sp1 bind at site 1, the site which contains the ACTTTG element. Methylation interference assays reveal that HNF-4 and Sp1 contact adjacent sites with minor overlap. HNF-4 and Sp1 appear to bind site 1 in a mutually exclusive fashion. We also demonstrate that HNF-4 can transactivate the Factor X promoter in HeLa cells; mutation at the adjacent Sp1 site further increases the transactivation. Heteromeric transcription factor NF-Y was identified as the protein that binds the CCAAT box at site 2. We conclude that HNF-4 and NF-Y play crucial roles in modulating the activity of the proximal promoter of Factor X.

The vitamin K-dependent proteins F.VII, F.IX, F.X and prothrombin are the precursors of the major enzymes of the coagulation cascade. In order to have activity in coagulation, these proteins must undergo γ-carboxylation of glutamic acid residues at the N terminus, a reaction catalyzed by the enzyme γ-glutamyl carboxylase, which requires vitamin K as a cofactor (1). The vitamin K-dependent clotting factors are synthesized predominantly or exclusively in the liver, but the mechanisms controlling liver-specific expression are not understood in detail. We have chosen F.X as a paradigm for study, because of its central role in coagulation (when activated, it converts prothrombin to thrombin) and because, in contrast to the case of F.IX, a well characterized cell line exists, HepG2 cells, which expresses F.X (2). In previous work (3), we had defined the start sites of transcription of the F.X gene and had also carried out a functional characterization of the F.X promoter, which demonstrated that the proximal 209 bp of the promoter were adequate to confer maximal activity in HepG2 cells. Using site-directed mutagenesis and reporter gene assays, we defined two areas within the F.X promoter that bound proteins from HepG2 nuclear extracts and that were required for promoter activity. In this report, we have used DNase I footprinting to define 4 protein-binding sites in the proximal promoter. Using gel shift assays with nuclear extracts or purified proteins, and supershift assays with well characterized antibodies, we have determined the identity of the transcription factors that bind at the two proximal sites. In the case of the most proximal site, site 1, we show that two transcription factors, one ubiquitous (Sp1), and the other found in only a few tissues (HNF-4) bind at the site in a competitive fashion; we present evidence that HNF-4 binds at a similar site in the promoters of F.VII and F.IX. For site 2, which contains a CCAAT box, we have shown that the ubiquitous transcription factor NF-Y binds at this site; this is distinct from the transcription factor that binds at the CCAAT box in the F.IX promoter, which occupies a similar position with respect to the translation start site.

**MATERIALS AND METHODS**

Northern Blot Analysis—Human multiple tissue Northern blots were purchased from Clontech (Palo Alto, CA). Blots were hybridized with a random primer-labeled F.X DNA (1.3-kb EcoRI-EcoRI fragment) in ExpressHyb solution (Clontech) at 65 °C for 1 h, washed, and exposed together on a single piece of film overnight. Blots were then stripped and reprobed with labeled human β-actin cDNA and exposed to film for 20 min. Densitometric scanning of the signals was performed on a Molecular Dynamics Densitometer (Sunnyvale, CA) using the ImageQuant program.

DNase I Footprint Analysis—Nuclear extracts from HepG2 and HeLa cells were prepared according to a modified Dignam method (4, 5). FXpGH-279 (3) was digested with XbaI, dephosphorylated, and labeled with [γ-32P]ATP by T4 polynucleotide kinase. The F.X promoter fragment was released by KpnI digestion, resolved by polyacrylamide gel electrophoresis, eluted onto a DEAE membrane, and purified using the Wizard DNA clean up kit (Promega, Madison, WI). Approximately 6 × 10^6 cpm (10 ng) of probe was incubated with 50 μg of nuclear extracts. DNase I footprint analysis was performed as described by Lichtsteiner et al. (6). Maxam and Gilbert (7) sequencing ladders were run alongside to serve as markers.

Electrophoretic Mobility Shift Assay and Methylation Interference Assay—Nuclear extracts from human and rat tissues were prepared as described by Gorski et al. (8). Double-stranded oligonucleotide probes were prepared by end-labeling with [γ-32P]ATP and T4 polynucleotide kinase. Indicated amounts of nuclear extracts were preincubated on ice for 10 min with 2.5 μg of poly(dI-dC), 1 μg of salmon sperm DNA, and 3 μg of bovine serum albumin in 20 μl of binding buffer containing 25 mm HEPES (pH 7.6), 5 mm MgCl2, 0.1 mm EDTA, 0.75 mm dithiothre-
Factor X mRNA Is Expressed Mainly, But Not Exclusively, in the Liver—Bahnak et al. (15) had demonstrated previously by Northern blot analysis that human liver and HepG2 cells express F.X mRNA, but kidney, ME-180 (human cervical cancer line), and BT-2 (human breast cancer cell line) do not. In order to evaluate the longstanding assumption that the expression of F.X is liver-specific, we carried out a comprehensive analysis of the tissue distribution of the F.X mRNA. Among 16 tissues evaluated (Fig. 1A), liver contains the highest amounts of F.X mRNA; however, F.X message is also present in RNA from heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, and colon. The only three tissues which do not contain F.X mRNA in this analysis are brain, placenta, and lymphocyte.

Four Protein-protected Sites—In previous work (3) we have used reporter gene assays in HepG2 cells (a hepatoma cell line (2)) to establish that maximal promoter activity is conferred by a 209-bp fragment immediately upstream from the first translated ATG. In order to map the cis elements within this sequence required for promoter activity, DNase I footprinting was carried out using nuclear extracts from HepG2 cells, which express F.X, and from HeLa cells, which do not. The results are shown in Fig. 2. Using the HepG2 nuclear extracts, four protected sites were delineated: site 1, −65 to −44; site 2, −128 to −94; site 3, −165 to −132; and site 4, −195 to −169. Sites 2–4 are also protected by HeLa nuclear extracts. The HeLa nuclear extract protects a site in the same region as site 1, but it is clearly different. The HeLa nuclear extract-protected site extends from −73 to −47; differences between this footprint and that obtained with HepG2 nuclear extracts are most easily appreciated at the boundaries of the footprint. The nucleotide sequence of the protected regions is shown in Fig. 2B. Since HepG2 and hepatocyte nuclear extracts are not identical in...
site 1, gel mobility shift assays were performed using nuclear extracts from a number of different tissues. An oligonucleotide probe spanning site 1 was radiolabeled and incubated with 10 µg of nuclear extract (Fig. 3B). Two major bands are seen (labeled A and B), suggesting the formation of two DNA-protein complexes. The identity of the most intense signal varies as a function of cell type, with complex A being more abundant in HeLa (a cervical carcinoma cell line), H4B (a brain tumor cell line), and spleen extracts (lanes 2, 3, and 5) and complex B more abundant in HepG2, liver, and kidney extracts (lanes 1, 4, and 6).

The Protein Giving Rise to Complex B Is HNF-4—To demonstrate that the formation of both complexes A and B is specific, a self-competition experiment was carried out (Fig. 3C, lanes 1–5). When labeled site 1 oligonucleotide was incubated with liver nuclear extract, addition of unlabeled site 1 effectively competes away both complexes A and B. Inspection of the oligonucleotide sequence protected at site 1 on the DNase I footprint reveals similarity to a previously described strong binding site for the hepatocyte-specific transcription factor HNF-4. When an oligonucleotide sequence known to bind HNF-4 (the APF-1 sequence derived from the 5'-flanking region of the apolipoprotein CII gene, used as an affinity ligand in the initial purification of HNF-4 from hepatocyte nuclear extracts (9)) is used as cold competitor in a gel mobility shift assay, the B complex is markedly attenuated while the A complex is unaffected (Fig. 3C, lanes 6–10). To develop further evidence for the presence of HNF-4 in the B complex, in vitro translated HNF-4 was tested in a gel mobility shift assay. HNF-4 cDNA subcloned into Bluescript SK (--) was transcribed using T3 RNA polymerase and the transcribed RNA translated in a rabbit reticulocyte lysate system. Results of a gel shift assay using the programmed lysate are shown in Fig. 3C, lanes 13 and 14. Lane 11 is a control showing the results of the site 1 oligonucleotide incubated with nuclear extracts from human liver. Complex B is seen as before. In lane 13, the same oligonucleotide is incubated with the HNF-4 programmed lysate, giving rise to a single band with mobility identical to the B complex. The unprogrammed lysate, on the other hand, shows no complex formation. When antisera to the carboxyl terminus of HNF-4 was incubated with HepG2 nuclear extract and the site 1 oligonucleotide, the B complex disappears and two slower moving complexes are formed (lane 12). Since the carboxyl terminus of HNF-4 has no homology to any known transcription factor, it is unlikely that the “supershift” arises from interaction of the antisera with some other transcription factor. Taken together, the competition experiment with the APF-1 oligonucleotide, the gel shift with the programmed lysate, and the supershift data suggest that HNF-4 interacts with the site 1 oligonucleotide to give rise to the B complex.

The Common ACTTTG Motif in Promoters of Factors VII, IX, and X Recognizes the Same Transcription Factor, HNF-4—Site 1 contains an ACTTTG motif which is present in the promoters of F.VII, F.IX, and F.X at a similar location relative to the translation start sites. We have previously demonstrated that mutation of this motif severely affects F.X promoter activity in reporter gene assays (3). To demonstrate that this conserved element binds the same protein, a gel mobility experiment was carried out with labeled oligonucleotides from all three promoters (Fig. 3D). The data show that probes from the three promoters and the APF-1 probe all give rise to a complex with mobility identical to complex B (lanes 1–4). The intensity of the B band varies significantly among probes; the complex is much more abundant with the F.X probe than with the probes for F.IX or F.VII. Addition of HNF-4 antiserum supershifts com-
FIG. 3. Site 1 binds two distinct proteins and forms two DNA-protein complexes. A, sequences of the oligonucleotides used in Fig. 3 are shown. Only one strand is shown for each oligonucleotide. The numbering system uses translation start site as +1. B, an oligonucleotide spanning -68 to -39 of the Factor X gene (FXSite1) was labeled and incubated with 10 μg of nuclear extract (N.E.) from cell lines and rat tissues as indicated. Two DNA-protein complexes are designated complex A and complex B. The position of the free probe is marked as F. C, a 26-oligonucleotidespanningsite 1was incubated with 12 μg of nuclear extract (N.E.) from human liver (lanes 1–12), 2 μl of in vitro translated HNF-4 in rabbit reticulocyte lysate (lane 13), or 2 μl of unprogrammed rabbit reticulocyte lysate (lane 14). Lanes 2–5 contain unlabeled site 1 oligonucleotide as cold competitors in 20 ×, 100 ×, 500 ×, and 1000 × molar excess. Lanes 7–10 contain unlabeled APF-1 (a strong HNF-4 binding site derived from -287 to -266 of apolipoprotein C III gene) as cold competitors in 20 ×, 100 ×, 500 ×, and 1000 × molar excess. Lane 12 contains 1 μl of HNF-4 antiserum. The positions of the antibody-HNF-4-DNA complexes are indicated by SS. D, probes containing the ACTTTG common motif from Factors VII, IX, X, and APF-1 were incubated with 12 μg of human liver nuclear extracts (N.E.) (lanes 1–8). One μl of HNF-4 antiserum was added in lanes 5–8. Positions of the immune complexes (supershift) are marked as SS. D, probes containing the ACTTTG common motif from Factors VII, IX, X, and APF-1 were incubated with 12 μg of human liver nuclear extracts (N.E.) (lanes 1–8). One μl of HNF-4 antiserum was added in lanes 5–8. Positions of the immune complexes (supershift) are marked as SS. Two DNA-protein complexes specific to the APF-1 probe are marked by asterisks. E, the Factor X site 1 probe was incubated with 15 μg of nuclear extract (N.E.) from either HepG2 (lanes 1–3) or HeLa cells (lanes 4–6). In lanes 2 and 5, 200 × molar excess of unlabeled Sp1 consensus oligonucleotide was added. In lanes 3 and 6, 1 μl of Sp1 antibody was added. The position of a minor complex is denoted by an asterisk.
plex B in all four probes (lanes 5–8). It should also be noted that the APF-1 probe forms two residual bands (marked **) after the HNF-4 supershift. These may represent nuclear receptors COUP-TF and ARP-1, which have previously been shown to recognize the APF-1 oligonucleotide (27).

The Protein Giving Rise to Complex A Is Sp1—The oligonucleotide sequence protected at site 1 also contains a potential binding site for Sp1. We hypothesized that the A band represented a complex composed of Sp1 and the oligonucleotide. Several lines of evidence support this notion. First, addition of cold Sp1 competitor (Sp1 consensus oligonucleotide) to HepG2 or HeLa nuclear extracts results in disappearance of complex A (Fig. 3E, compare lane 1 with lane 2 and lane 4 with lane 5). Second, addition of an affinity-purified polyclonal antibody against Sp1 to labeled probe and HepG2 or HeLa nuclear extracts results in disappearance of complex A (Fig. 3E, lanes 3 and 6). The immune complex (supershift) is better appreciated in the HeLa nuclear extracts. A minor complex (marked *) persists in the HeLa nuclear extracts after the addition of Sp1 antibody (lane 6). This complex can be competed away by the Sp1 consensus oligonucleotide (lane 5) and likely contains an Sp1-like protein. Third, as discussed further below, addition of increasing amounts of purified Sp1 to liver nuclear extracts results in increasing intensity of complex A (Fig. 5, lanes 1–4).

HNF-4 and Sp1 Bind at Adjacent Sites on the Oligonucleotide—Methylation interference identifies guanines and adenines in the DNA binding site that, when methylated, interfere with binding of the protein; this method was used to determine the contact points within site 1 between the DNA and the bound transcription factor. An end-labeled probe (either sense or antisense strand) spanning F.X site 1 was methylated and incubated with either purified Sp1 or liver nuclear extracts as a source of HNF-4. For HNF-4, methylation at some sites results in complete exclusion from the bound fraction (closed circles, Fig. 4A) while modification at other sites results in partial exclusion from the bound fraction (open circles). Methylation interference for the sense strand with Sp1 demonstrates 4 critical guanine residues (marked with closed circles), while analysis with the antisense strand demonstrates an additional 2 residues. The sites identified as contact points are summarized in Fig. 4B. The binding sites for Sp1 and HNF-4 are clearly juxtaposed and may have minor overlap. Whether they bind in a mutually exclusive or cooperative fashion cannot be assessed from methylation interference studies.

HNF-4 and Sp1 Bind to Site 1 in a Competitive Manner—Two types of experimental evidence, gel mobility shift assays (Fig. 5) and transactivation experiments (Fig. 6), support the contention that HNF-4 and Sp1 bind to site 1 in a competitive manner. In the gel mobility shift assay, labeled site 1 oligonucleotide is incubated with human liver nuclear extract in the presence of increasing amounts of recombinant Sp1. As the amount of Sp1 is increased, the intensity of complex A is also increased, supporting the hypothesis (see above) that the protein giving rise to complex A is Sp1. It is also apparent that the intensity of complex B is gradually diminished by the addition of Sp1, suggesting that HNF-4 and Sp1 bind in a mutually exclusive manner.

HNF-4 Transactivates the F.X Promoter in HeLa Cells, and Transactivation Is More Effective if Sp1 Binding Is Abolished—Compared with HepG2 cells, HeLa cells lack HNF-4, the liver-specific transcription factor that binds at site 1 (Fig. 3B). We sought to determine whether expression of HNF-4 in HeLa cells would result in increased expression of the F.X promoter in these cells. HeLa cells were cotransfected with a reporter gene plasmid containing 279 bp of F.X promoter coupled to a human growth hormone gene and an expression plasmid containing the cDNA for rat HNF-4 (pLEN45S). In control experiments, HeLa cells were cotransfected with the same reporter gene plasmid and an expression plasmid without the HNF-4 insert (pLEN(−)). Results are shown in Fig. 6 and demonstrate that the presence of HNF-4 results in a 2.0-fold increase in transactivation of the F.X promoter in HeLa cells. Mutation of the HNF-4 binding site within site 1 results in loss of the transactivation effect (1.2-fold induction in both FXpGH-mutΔACTTTG and FXpGH-mutACTTAG constructs), suggesting that the transactivation effect is indeed mediated by binding of the transcription factor to the HNF-4 binding site within site 1. Mutation of the adjacent Sp1 site (FXpGH-mutΔGGGGCG), which abolishes binding of Sp1 (data not shown), results in more effective transactivation by HNF-4 (3.5-fold versus 2.0 fold), again suggesting that the binding of Sp1 interferes with binding of HNF-4 at the adjacent site. When similar transactivation experiments were performed on reporter gene constructs containing the F.VII and F.IX promoters (FVIIpGH-309 and FIXpGH-420, see "Materials and Methods").
Methods), it was determined that HNF-4 transactivates the F.VII and F.IX constructs 4.5- and 3.8-fold, respectively (data not shown).

The Protein That Binds at Site 2 is NF-Y—DNase I footprinting had documented a protected region from −128 to −94. Previous work (3) had shown that mutation of the CCAAT sequence within this region resulted in a 10-fold reduction in expression from the F.X promoter to compete away the F.X complex. As demonstrated in lanes 12–16, the CCAAT box from the F.IX promoter fails to compete with the F.X CCAAT box. When the CCAAT sequence is mutated to AGCTA, the F.X cold competitor can no longer compete away the complex (lanes 7–11). Second, incubation of radiolabeled probes from either the F.X CCAAT box, or the Y box of the MHC class II gene Eα, with nuclear extracts from HepG2 or HeLa cells, results in complexes of the same mobility (lanes 1, 4, 5, and 8, Fig. 8C). Note that this complex is not of the same mobility as that formed when the F.IX CCAAT sequence (lanes 3 and 7), which occurs at approximately the same position in its promoter, is used as probe, suggesting that different proteins bind at the CCAAT boxes in F.IX and F.X. It had been demonstrated previously that this sequence in the F.IX promoter recognizes the unrelated transcription factor NF-1 (19).

Third, a monoclonal antibody against the first glutamine-rich domain of NF-YA (αNF-YA1a) and a polyclonal antisera against NF-YB (αNF-YB) (10) result in supershift of the complex formed by FXCCAAT wt (Fig. 8D). A faster migrating complex only present in the HepG2 nuclear extracts (Fig. 7) was not supershifted by αNF-YA1a, but was supershifted by αNF-YB. This complex likely contains the heterodimer of an alternatively spliced form of NF-YA (which lacks the first Q-rich domain (20)) with NF-YB. Taken together, these data sequences may determine which specific CCAAT-binding transcription factor binds at a particular site. The binding site for the ubiquitously expressed transcription factor NF-Y (CCAT-NF) closely matches a portion of the sequence found within the footprint at −128 to −94 (17). NF-Y binds as a heterodimer composed of two subunits, NF-YA and NF-YB (18). Three lines of evidence suggest that NF-Y is the transcription factor binding at site 2. First, on gel shift assays using FX CCAAT wt as the labeled probe, cold competition with an oligonucleotide based on the sequence of the CCAAT box (Y box) within the promoter of the MHC class II gene Eα, which was used as an affinity ligand in the initial purification of NF-Y (18), shows that this sequence competes effectively with the F.X sequence for binding of proteins in HepG2 nuclear extracts (Fig. 8B). When the Eα Y box sequence is used as cold competitor, the disappearance of the major band is similar to that seen when the F.X sequence is used as self-competitor (compare lanes 1–6 with lanes 17–21). We also tested the ability of the CCAAT box from the F.IX promoter to compete away the F.X complex. As demonstrated in lanes 12–16, the CCAAT box from the F.IX promoter fails to compete with the F.X CCAAT box. When the CCAAT sequence is mutated to AGCTA, the F.X cold competitor can no longer compete away the complex (lanes 7–11).

Fig. 5. Increasing amounts of Sp1 diminish the binding of HNF-4 to site 1. Labeled Factor X site 1 oligonucleotide was incubated with 12 μg of human liver nuclear extracts (N.E.) in the presence of 0, 0.2, 1, and 2 footprint units (lanes 1–4) of recombinant Sp1.

Fig. 6. HNF-4 transactivates the Factor X promoter through the ACTTTG motif. Ten μg of FXpGH wild type and mutant constructs were cotransfected with 10 μg of either pLEN(−) (shaded bars) or pLEN4S (black bars) into HeLa cells. The expression level of FXpGH−279 in the presence of pLEN(−) was taken as 1. The expression level of FXpGH + pLEN4S was compared with that of FXpGH + pLEN(−), and the average -fold increase is shown at the top of each column. The results represent an average of three independent transfection experiments.
provide strong evidence that the protein binding at site 2 is NF-Y.

**DISCUSSION**

A longstanding but poorly documented assumption regarding procoagulant proteins has been that expression of these proteins is confined to the liver. Our data indicate that, although liver is the major site of F.X mRNA synthesis, F.X mRNA is also found in lung, heart, ovary, and small intestine. Similar findings have been reported for other vitamin K-dependent coagulation proteins: Jamison and Degen (21) have reported that prothrombin mRNA is found in uterus, placenta, kidney, spleen, and small intestine in addition to liver; and more recently, Stitt et al. (22) have reported the presence of protein S mRNA in uterus, heart, placenta, lung, smooth muscle, kidney, spleen, and ovary. Like thrombin and protein S, which have recently been demonstrated to activate intracellular signaling cascades through binding to specific cell surface receptors (a G protein-coupled receptor in the case of thrombin (23), the Tyro 3/Axl family of receptor tyrosine kinases in the case of protein S (22)), F.Xa has also been shown to bind to a specific cell surface receptor, EPR-1, found on monocytes and endothelial cells (24). Occupation of the EPR-1 receptor by F.Xa triggers a mitogenic response through a signal transduction process that is not yet well understood. Thus, in contrast to

**Fig. 8.** The protein which binds the CCAAT box in Factor X site 2 is NF-Y.

A, sequences of the oligonucleotides used are shown. B, FXCCAATwt oligonucleotide was incubated with 15 µg of nuclear extract (N.E.) from HepG2 cells (lanes 1–21). Unlabeled competitors added were FXCCAATwt (lanes 2–6), FXCCAATmut (lanes 7–11), FIXCCAATwt (lanes 12–16), and MHC class II Eα Y Box (lanes 17–21) at 10 ×, 50 ×, 100 ×, 500 ×, and 1000 × molar excess. C, labeled oligonucleotides as indicated were incubated with 15 µg of nuclear extract (N.E.) from HepG2 (lanes 1–4) or HeLa cells (lanes 5–8). D, labeled FXCCAATwt oligonucleotide was incubated with 15 µg of nuclear extract (N.E.) from HepG2 cells. Lanes 2–4 contain 0.5, 1, and 2 µg of monoclonal antibody αNF-YA1a, respectively. Lanes 5–7 contain 0.5, 1, and 2 µl of polyclonal antiserum αNF-YB, respectively. Positions of the immune complexes (supershift) are denoted SS1 and SS2.
F.VII and F.IX (25), vitamin K-dependent proteins for which expression is clearly confined to the liver, our data suggest that F.X belongs to another group of vitamin K-dependent proteins with a wider tissue distribution and more protean biological effects.

In previous work we had determined that the proximal 209 bp of the F.X promoter were adequate to confer maximal promoter activity in a reporter gene assay. In this work, we have used DNase I footprint analysis with nuclear extracts from HepG2 and HeLa cells to identify four protein-binding sites within this promoter element. A previously reported footprint analysis of this region carried out with HepG2 extracts identified only one of these sites, site 1 (26). The failure to detect other sites within the probe used by these investigators may reflect a difference in the quality and concentration of transcription factors in the nuclear extracts used, or it may be a function of the incubation conditions used, since failure to saturate a protein-binding site may render it difficult to detect on a footprint. For the same reasons, of course, we cannot state with certainty that the four sites we have mapped are the only protein-binding sites within this fragment.

We chose the two proximal protein-binding sites for further study. In previous studies, we had established the functional significance of both of these sites (3). Additionally, site 1 was of special interest, because it is the only one of the four for which the footprint differs between HepG2 and HeLa cells. We have now identified the cognate binding proteins for each site. Site 1 forms two DNA-protein complexes, designated A and B. Complex B is present only in nuclear extracts from liver, kidney, and HepG2 cells, whereas complex A is present (in varying amounts) with all extracts tested. Competition experiments, gel shift assays using in vitro translated HNF-4, and supershift experiments document that the protein giving rise to complex B is HNF-4. HNF-4 binds to the nuclear receptor superfamily of transcription factors; these are characterized by two highly conserved regions, one in the N terminus which binds DNA, and another in the C terminus that is required for ligand binding, dimerization, and activation. Because of the high degree of conservation in the DNA-binding domain, other members of the superfamily can demonstrate similar binding specificity. For example, COUP-TF and ARP-1 have been demonstrated to compete with HNF-4 for binding sites in the promoters of apolipoprotein CIII (27), ornithine transcarbamylase (28), and erythropoietin (29). In the supershift experiment presented in Fig. 3D, the two residual bands seen with the APF-1 probe (marked by asterisks next to lane 8) likely represent complexes containing these (or other) related proteins. The absence of any distinct residual bands in the supershift experiments with F.VII, F.IX and F.X (lanes 5–7) suggests that these promoters are probably not recognized by other members of the nuclear receptor superfamily.

Recently published data (30) demonstrate that HNF-4 forms stable homodimers and fails to heterodimerize with a number of nuclear receptors that were tested. In the supershift experiment presented here (Fig. 3C, lane 12), the presence of 2 supershifted complexes raises the question of heterodimerization, but the data presented by J. Iang et al. (30) make it more likely that the two complexes arise from the binding of either one (faster moving complex) or two (slower moving complex) antibody molecules to the homodimer in the supershift complex.

The promoters of F.VII, F.IX, and F.X all contain the ACTTTG motif and all bind HNF-4 (Fig. 3D). The molar amounts of labeled DNA were identical in these experiments.

\[ \text{H.-L. Hung and K. A. High, unpublished results.} \]

The absence of any distinct residual bands in the supershift experiments with F.VII, F.IX and F.X (lanes 5–7) suggests that these promoters are probably not recognized by other members of the nuclear receptor superfamily.

Thus HNF-4 has greater affinity for the F.X promoter than for the promoters of F.VII and F.IX. Comparison of the nucleotide sequences to a 13-bp consensus HNF-4 binding site proposed by Sladek (31) showed that the F.X promoter sequence gave the best match (12/13 nucleotides). The F.VII and F.IX promoters did not exhibit as close a match (11/13 and 10/13, respectively).

In addition to the binding experiments, we showed that HNF-4 can transactivate all three promoters in HeLa cells. The transactivation effect in our system ranged from 2-fold in the case of F.X to 4.5-fold in the case of F.VII. These results are different from those previously published by Reijnen et al. (32), who reported a >300-fold transactivation of the F.IX promoter by HNF-4 in cotransfection experiments using the same HNF-4 expression vector. Technical considerations likely account for the differences in results. To assess transactivation, we compared our results to a base-line control cotransfected with the expression plasmid without the HNF-4 insert. Use of mock-transfected cells as baseline, as reported by Reijnen et al. (32), results in a much higher fold transactivation effect.

Based on competition with an Sp1 consensus oligonucleotide and on supershift by an Sp1 antibody, the protein in complex A that binds to site 1 is identified as Sp1. Sp1 is a ubiquitous transcription factor, but as is clearly evident in Fig. 3B, its concentration in different tissues varies considerably. These differences are supported by a study of the developmental expression of Sp1 in the mouse (33), which documents low levels of Sp1 in the liver, and 70-fold higher levels in the thymus, the highest expressing tissue. One possibility is that relative levels of HNF-4 and Sp1 may influence sites of expression of F.X, with the highest levels of expression being seen in the liver where HNF-4 levels are high and Sp1 levels are low.

Methylation interference analysis of the contact points of HNF-4 and Sp1 on site 1 indicates that the two transcription factors bind at adjacent sites with minor overlap. The contact region of HNF-4 includes at least 11 nucleotides centered around the ACTTTG element, whereas the contact region of Sp1 surrounds the GGGGC element. The overlap of the HNF-4 and Sp1 binding sites suggests that they may bind to site 1 in a mutually exclusive manner. This notion is further supported by the fact that addition of increasing amounts of Sp1 reduces the binding of HNF-4 in a gel mobility shift assay (Fig. 5). Based on this hypothesis, one would predict that Sp1 could repress expression of Factor X by excluding the binding of HNF-4 at the neighboring site and that loss of the Sp1 site might result in higher level expression. Experimental confirmation of the latter prediction can be seen in Fig. 6, where HNF-4 transactivation of the F.X promoter in HeLa cells is decreased by the presence of Sp1 (Fig. 6). In previous work (3), we had shown that mutation of the Sp1 site does not result in an increase in expression in HepG2 cells. This is most likely due to the fact that the concentration of Sp1 in HepG2 cells is low (Fig. 3B), so that loss of...
the Sp1 binding site has little effect. In contrast, in HeLa cells, where Sp1 is abundant, loss of the Sp1 binding site has a noticeable effect on transactivation. Whether repression by Sp1 plays any physiologic role in cells that express HNF-4 but express only low levels of F.X (e.g. kidney) is not yet clear.

Two lines of evidence support our contention that the ubiquitous transcription factor NF-Y binds at site 2. First, the specific complex formed between site 2 and HepG2 nuclear extracts can be competed away by a strong NF-Y binding site (the Y box from MHC class II E α gene), and second, the DNA-protein complexes can be supershifted against antibodies against NF-YA and NF-YB. It is of interest that the CCAAT box from the F.IX gene, which resides at a similar location in the F.IX promoter, cannot compete away the complex formed between site 2 and HepG2 nuclear extracts. Crossley and Brownlee (19) have demonstrated that the F.IX CCAAT box binds to NF-1, a transcription factor that is not structurally related to NF-Y (34). Thus in contrast to the situation with HNF-4, which binds to the conserved ACTTTG element in all three promoters (F.VII, F.IX, and F.X), the conserved CCAAT boxes of F.IX and F.X demonstrate binding to distinct and unrelated cognate proteins.

Our findings are summarized in Fig. 9. The proximal 209 bp of the F.X promoter, which are sufficient to confer maximal activity in HepG2 cells, contains four protein-protected sites as identified by DNase I footprinting. Only one of these, site 1, shows a unique footprint pattern when results with HepG2 nuclear extracts are compared with extracts from non-hepatic cell lines. We have demonstrated that site 1 binds two distinct transcription factors, HNF-4, a liver-specific factor, and Sp1, a ubiquitous factor. Methylation interference assays and transactivation experiments suggest that these two factors bind at overlapping sites in a competitive manner. In reporter gene assays in HepG2 cells, a mutation that abolishes HNF-4 binding (but does not affect Sp1 binding) reduces promoter activity to 17.2% of wild type, but mutation of the Sp1 site has virtually no effect (90.2%) (3). We have also demonstrated that the ubiquitous transcription factor NF-Y binds at site 2; a mutation at this site also markedly reduces promoter activity to 11.8% of wild type (3). These data confirm that sites 1 and 2 are critical for activity of the F.X promoter. Ongoing experiments are directed at determining the identity of the proteins that bind at sites 3 and 4 and their role in the regulation of F.X transcription.

Acknowledgments—We thank Frances Sladek, Weimin Zhong, and James Darnell, Jr. for providing the HNF-4 expression vector and antiserum. We also thank Diane Mathis for providing antibodies and antiserum to NF-Y.

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