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The Homeodomain Pbx2-Prep1 Complex Modulates Hepatocyte Nuclear Factor 1α-Mediated Activation of the *UDP-Glucuronosyltransferase 2B17* Gene

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ABSTRACT

UDP glucuronosyltransferases (UGT) are expressed in a wide range of tissues in which their levels of expression and distribution are dependent on cell-type specific regulatory mechanisms. The presence of a hepatocyte nuclear factor (HNF) 1 binding site in the proximal promoters of several *UGT2B* genes has been shown to contribute to their expression in liver cells and possibly other HNF1-containing cell types. In some of these *UGT2B* genes, a putative pre-B cell homeobox (Pbx) transcription factor binding site is found directly adjacent to the functional HNF1 site. To determine whether this putative Pbx site contributes to the regulation of *UGT2B* expression, we chose the *UGT2B17* gene and investigated the capacity of its Pbx site to bind specific transcription factors and alter promoter activity. The *UGT2B17* Pbx site matches a consensus Pbx site known to bind members of the Pbx, Hox, Meis, and Prep1 families of homeodomain-containing proteins and has previously been shown to bind nuclear proteins in DNaseI footprint assays. In this study, we used gel shift and functional assays to show that a Pbx2-Prep1 heterodimer can bind to the *UGT2B17* Pbx site and interfere with the binding of HNF1α to its site adjacent to the Pbx site. This interaction of Pbx2-Prep1 and HNF1α results in down-regulation of HNF1α-mediated activation of the *UGT2B17* promoter. Modulation of transcription by restricting the binding of transcriptional effectors to their target site is a novel role for Pbx2-Prep1 complexes.

The UDP glucuronosyltransferases (UGT) are a family of membrane-bound enzymes that catalyze the transfer of glucuronic acid to hydrophobic compounds. This process of glucuronidation alters the biological activity of these compounds and facilitates their excretion as water-soluble conjugates (Mackenzie, 1995). The presence of multiple forms of UGT with differing but often overlapping substrate specificities allows the glucuronidation of a wide range of endogenous and xenobiotic compounds, including bilirubin, steroids, drugs, and environmental pollutants. UGTs are classified into two families, UGT1 and UGT2, based on their nucleotide sequence (Mackenzie et al., 1997). The human UGT1 protein family consists of nine members encoded by a single gene locus on chromosome 2. Each member arises from the splicing of a distinct first exon to a set of common exons 2 to 5 (Gong et al., 2001). In contrast, the human UGT2 protein family is encoded by separate genes and is further subdivided into two subfamilies: the UGT2A family contains one member (UGT2A1), expressed in olfactory epithelium, and the UGT2B family contains seven members (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28), which have a wide tissue distribution (Turgeon et al., 2001). The UGT2B enzymes catalyze the glucuronidation of bile acids, fatty acids, and steroid hormones, as well as xenobiotics such as opioids, phenols, and carcinogens (Tukey and Strassburg, 2000). In particular, UGT2B enzymes glucuronidate a wide range of C18 and C19 steroids, including estrogens, androsterone, testosterone, and dihydrotestosterone (Hum et al., 1999). There is much accumulated evidence suggesting that steroid metabolic enzymes can regulate the level and physiological effect of steroids in their target tissues (Labrie, 1991). Because both the level and activity of steroids within steroid responsive tissues are likely to be altered by glucuronidation, UGT enzymes have been suggested to play an important role in the steroid metabolic pathway.

UGT2B enzymes have been shown to be distributed throughout extrahepatic tissues, including the kidney, ovary,

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ABBREVIATIONS: UGT, UDP glucuronosyltransferase, HNF, hepatocyte nuclear factor; bp, base pair(s); Pbx, pre-B cell homeobox; Prep1, pre-B cell homeobox regulating protein 1; Oct-1, octamer transcription factor-1; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; wt, wild-type.

lung, small intestine, mammary gland, testis, and prostate (Turgeon et al., 2001). Within these tissues, the levels and distribution of UGT2B forms vary considerably and are probably determinants of the extent and potential for glucuronidation. The variation in UGT expression suggests that tissue specific transcription factors may be important in the regulation of UGTs. Previous studies have shown that the liver enriched transcription factor HNF1α is a potent activator of several *UGT* genes, including the rat *UGT2B1* (Hansen et al., 1997), the human and mouse *UGT1A1* (Bernard et al., 1999), the human *UGT2B7* (Ishii et al., 2000), and the human *UGT2B17* (Gregory et al., 2000) genes. The recent partial characterization of the *UGT2B4*, *UGT2B10*, *UGT2B11*, and *UGT2B15* genes has shown that the HNF1 site is conserved in sequence and location (Turgeon et al., 2000). HNF1α is a homeodomain-containing protein which forms homodimers, or heterodimers with HNF1β, and regulates the transcription of liver-specific and extrahepatic genes through a palindromic consensus binding site (5′-GTTAATNATTAAC-3′) (Mendel and Crabtree, 1991). Although HNF1α was originally described as liver-specific, it is also expressed in several extrahepatic tissues (kidney, small intestine, colon, pancreas, and stomach) and may contribute to gene expression in these tissues (Blumenfeld et al., 1991). The presence of the HNF1 site seems to be essential for expression of UGT2B7 and UGT2B17 in the liver, as mutation of this site abolishes promoter activity in the liver cell line HepG2 (Gregory et al., 2000; Ishii et al., 2000). Ishii et al. (2000) has also shown that HNF1α-mediated activation of the *UGT2B7* promoter is enhanced by the ubiquitous transcription factor Oct-1. These data suggest that HNF1α is likely to be an important regulator of *UGT2B* genes in HNF1α-containing tissues.

In our recent research, endogenous HNF1α has been shown to regulate the *UGT2B17* gene in the liver cell line HepG2, but not in the prostate cell line LNCaP, in which HNF1α is not expressed (Gregory et al., 2000). UGT2B17 seems to have a primary role in the glucuronidation of androgens in the prostate, where its expression is regulated by epidermal growth factor, dihydrotestosterone, and cytokines (Guillemette et al., 1997; Levesque et al., 1998). However, the mechanisms through which these effectors act have not yet been elucidated. UGT2B17 is expressed at similar levels in the liver and prostate (Beaulieu et al., 1996), suggesting that an HNF1α-independent mechanism regulates UGT2B17 expression in prostate cells. DNaseI footprinting of the *UGT2B17* promoter with LNCaP cell nuclear extracts revealed the presence of a protein binding site that is directly adjacent to the HNF1 site (−31 to −39 bp). Protein binding to this site using HepG2 nuclear extracts is masked by binding of HNF1α complexes to the adjacent HNF1 site (Gregory et al., 2000). This binding site matched the consensus sequence for a Pbx binding site (TTGATTGAT) (Knoepfler and Kamps, 1997) and is also found conserved in the proximal promoters of *UGT2B10*, *UGT2B11*, and *UGT2B15* but not *UGT2B4* and *UGT2B7* (Turgeon et al., 2000). Pbx belongs to a homeodomain gene family containing a three-amino acid loop extension. The closely related Pbx1, Pbx2, and Pbx3 proteins are able to bind cooperatively to DNA with members of the Hox and Meis families and alter their DNA binding specificities (Knoepfler et al., 1997). Pbx factors also bind to the Meis1-like protein, Prep1, independently of DNA to enhance the transcriptional activity of Pbx-Hox and Pbx-Meis1 complexes (Berthelsen et al., 1998b).

In this work, the *UGT2B17* Pbx site was found to bind both Pbx2 and Prep1 using LNCaP and HepG2 nuclear extracts in EMSA assays. Although the Pbx2-Prep1 complex was not able to activate the *UGT2B17* promoter directly, this complex was able to attenuate HNF1α-mediated activation of the promoter by binding to the Pbx site and restricting the binding of HNF1α to its adjacent HNF1 site. This restriction of the binding of transcriptional effectors to their target sites is a novel role for Pbx-Prep complexes.

Experimental Procedures

Materials. Restriction enzymes and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). Poly(dI-dC) was purchased from Sigma Chemical Co. (St. Louis, MO), G-25 columns from Amersham Biosciences (Piscataway, NJ), and [^{32}P]ATP from Geneworks (Adelaide, Australia). Cell culture reagents and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). pGL3-basic, pRL-null, and the dual-luciferase detection kit were obtained from Promega (Madison, WI). Polyclonal antibodies to HNF1α, HNF1β, Pbx1/2/3, Pbx1, Pbx2, and Pbx3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antiserum raised to full-length Prep1 protein was kindly provided by Dr. Norio Kagawa (Vanderbilt University, Nashville, TN). The Prep1 antibody does not recognize in vitro translated Meis1 on Western blots and is considered to be specific for Prep1 (Norio Kagawa, personal communication). The HNF1α, Pbx2 and Pbx1b, and Prep1 expression plasmids were kindly provided by Drs. Gerald Crabtree (Stanford University, Stanford, CA), Chris Murre (University of California, San Diego, CA), and Bernard Peers (University de Liege, Liege, Belgium) respectively.

Methods

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from HepG2 and LNCaP cells essentially as described by Schreiber et al., (1989). Complementary oligonucleotides were annealed and end-labeled with [^{32}P]ATP using T4 polynucleotide kinase and purified by elution through G-25 columns (Amersham Biosciences, Piscataway, NJ). Electrophoretic mobility shift assays (EMSA) were carried out using 5 μg of nuclear extract, 1 μg of poly(dI-dC), and unlabeled competitor oligonucleotides (if needed) in a 15-μl reaction mixture containing 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10% glycerol for 10 min on ice. Labeled probe (50,000 cpm, 0.5–1 ng) and 2 μg of specific antibody (if needed) were added to the reaction mixture, which was incubated for a further 30 min at room temperature. The resulting DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels at 4°C in 0.5× 45 mM Tris borate, pH 8.3, and 4 mM EDTA.

Construction of Plasmids. A 692-bp promoter fragment (−650/+42) of the *UGT2B17* promoter was generated by PCR from a previously isolated cosmid clone (Gregory et al., 2000) and directionally cloned into the *Kpn*I and *Mlu*I sites of the pGL3 basic vector (Promega, Madison, WI). The primers used for this construction were −692 forward 5′- TTTTGGTACCTAAAATTCACAATGCAAGCC- 3′ and reverse 5′-AGAGACGCGTTGGTCTTATGCAATGCTTC-3′ (restriction sites shown in underlined type). Promoter constructs extending only to the 5′ border of the HNF1 site (−52/+42) containing both intact and mutated HNF1 or Pbx sites were generated as above using the forward primers HNF1 wild-type, 5′-AATTGGTACCGTTATATTTTAACTTGATTGAT-3′; HNF1 mutant, 5′-AATTGGTACCG**CCC**TATTTTAACTTGATTGAT-3′; Pbx mutant, 5′-AATTGGTACCGTTATATTTTAACTT**TT**TTGAT-3′; and the original reverse

primer (*Kpn*I sites are underlined, mutations are underlined and bold).

Cell Culture and Transfection. HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, a 0.1-mM mixture of nonessential amino acids (all from Invitrogen), and 80 μg/ml gentamicin at 37°C in 5% CO_2. LNCaP cells were cultured in RPMI medium supplemented with the same additives as above and under the same conditions. HepG2 and LNCaP cells were subcultured to a confluence of 60% into 24-well plates, 24 h before transfection. Transfections were carried out with 0.5 μg of promoter construct, 0.2 μg of HNF1, Pbx2, Prep1 expression vector and 0.05 μg of pRLnull (internal control for transfection efficiency) using LipofectAMINE 2000 according to the manufacturers protocol. After 16 h incubation, the DNA-LipofectAMINE 2000 mixture was removed and fresh media was added to the cells. Transfections were harvested after a further 24-h incubation and assayed for promoter activity using the dual luciferase assay system according to the manufacturer's protocol (Promega). Luciferase activity was measured using 20 μl of lysate in a 96-well plate on a Packard TopCount luminescence and scintillation counter (Mt. Waverley, Victoria, Australia).

Results

A Specific Protein Complex Forms on the Putative Pbx Region of the UGT2B17 Promoter. Protein binding to the *UGT2B17* promoter has been previously examined by DNaseI footprinting using HepG2 and LNCaP nuclear extracts (Gregory et al., 2000). Two protein binding regions were identified, the first corresponding to a functional HNF1α site (−40 to −52 bp) footprinted with HepG2 nuclear extracts, and the second to a region directly adjacent to the HNF1 site footprinted with LNCaP nuclear extracts (−31 to −39 bp). On closer examination of this sequence, it was revealed to contain a motif (TTGATTGAT) with strong homology to the consensus binding site identified for Pbx proteins (Knoepfler and Kamps, 1997). To determine whether proteins were able to specifically bind to the putative Pbx binding region, an EMSA was performed using a labeled Pbx probe (Fig. 1) with LNCaP and HepG2 nuclear extracts. An intense DNA-protein complex was observed with both LNCaP and HepG2 nuclear extracts as shown in Fig. 1, lanes 1 and 7. The formation of the radiolabeled DNA-protein complex was reduced by the addition of increasing amounts of unlabeled Pbx probe (Fig. 1, lanes 2–4 and 8–10), with a 50-fold molar excess of probe completely abolishing complex formation (lanes 3 and 9). The addition of a 50- or 200-fold molar excess of an unlabeled mutant Pbx probe, containing a GA-to-TT mutation known to prevent Pbx binding (Chang et al., 1996) (Fig. 1), was unable to compete with the DNA-protein complex formation (Fig. 1, lanes 5, 6 and 11, 12). Taken together, these results indicate that protein binding to the Pbx site was specific to the intact Pbx site and occurs in both LNCaP and HepG2 cell lines.

The Complex Binding to the UGT2B17 Pbx Region Consists of Heterodimers of Pbx2-Prep1 and Pbx1b-Prep1. To identify the proteins in the Pbx DNA-protein complex, an EMSA was performed using specific antibodies to members of the Pbx family. Five different isoforms of Pbx (Pbx1a, Pbx1b, Pbx2, Pbx3a, Pbx3b) have been identified so far; they are derived from the alternate splicing of three different genes (Monica et al., 1991). In this assay, antibodies to the amino-terminal regions of Pbx1 and Pbx3 were used. These antibodies would identify both the Pbx1a and -1b forms and the Pbx3a and -3b forms, respectively. The Pbx1b and -3b forms (about 40 kDa) are truncated forms of Pbx1a and -3a and are formed from the splicing of the carboxyl-terminal ends of the Pbx1a and -3a forms (about 50 kDa) (Monica et al., 1991). A Pbx2-specific antibody and a Pbx1/2/3 antibody recognizing the Pbx1a, -2, and -3a isoforms were also used. Addition of the Pbx1 antibody decreased the mobility of part of the LNCaP Pbx complex (Fig. 2, lane 8) and to a lesser degree the HepG2 Pbx complex (Fig. 2, lane 2), suggesting that Pbx1 composes part of the Pbx DNA-protein complex. Interestingly, the addition of antibody revealed that the Pbx complex consists of two complexes (A and B), the lower complex B being supershifted by the Pbx1 antibody. In contrast, the Pbx2 antibody supershifted the upper complex A, formed with both HepG2 and LNCaP nuclear extracts (Fig. 2, lanes 3 and 9). These results suggest that complex B contains the smaller Pbx1b isoform (40 kDa) and not the larger Pbx1a form (50 kDa). The Pbx1/2/3 antibody supershifted only complex A (Fig. 2, lanes 5 and 11), confirming the presence of the Pbx2 form in this complex and the absence of the Pbx1a and -3a forms in complex B. The Pbx3-specific antibody was unable to supershift either complex A or B, indicating that this protein is not present in the complex (Fig. 2, lanes 4 and 10). An antibody raised to the known Pbx binding partner, Prep1, was also added to the Pbx complex and was found to supershift both complexes A and B. This suggests that the *UGT2B17* Pbx site is able to bind both Pbx2-Prep1 (complex A) and Pbx1b-Prep1 (complex B) heterodimers.

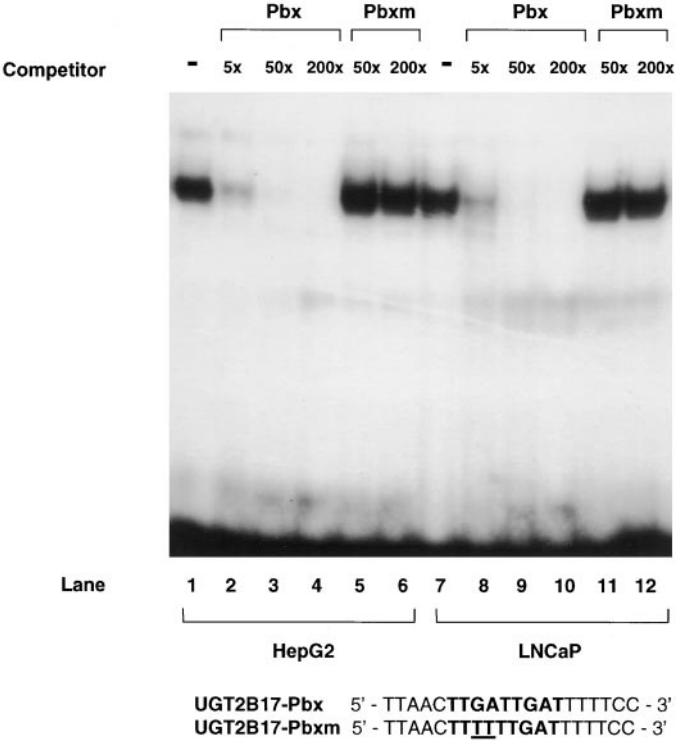

Fig. 1. A specific protein complex binds to the Pbx site in the *UGT2B17* promoter. EMSAs were performed using ^{32}P-labeled Pbx oligonucleotides (50,000 cpm per lane; sequence shown) incubated with 5 μg of HepG2 or LNCaP nuclear extracts followed by resolution on a 4% nondenaturing polyacrylamide gel. Unlabeled competitors were added at either a 5-, 50- or 200-fold molar excess before the addition of the labeled Pbx probe. The sequence of the oligonucleotides are shown with the Pbx binding site (bold) and its corresponding mutant site (underlined) indicated.

Pbx2-Prep1 Does not Activate the UGT2B17 Promoter. Having shown that Pbx2-Prep1 and Pbx1b-Prep1 can bind to the *UGT2B17* Pbx site, we tested the functional significance of these interactions in transient transfection assays. A 692-bp *UGT2B17* promoter fragment (−650/+42) was coupled to the luciferase reporter gene and transfected into HepG2 and LNCaP cells. The promoter activity of the −650/+42 construct was approximately 3-fold higher than the promoterless pGL3 basic in both cell lines (Fig. 3, A and B). Because of the higher amount of the Pbx2-containing complex compared with the Pbx1b-containing complex, cotransfection experiments were initially performed using Pbx2 and Prep1 expression vectors. When cotransfected with the Pbx2 or Prep1 vectors separately, no significant change in the activity of the −650/+42 promoter was observed in either cell line. Cotransfection with both the Pbx2 and Prep1 vectors together resulted in a slight decrease in −650/+42 activity in the LNCaP cell line, with no comparable effect observed in the HepG2 cell line (Fig. 3, A and B). The same result was achieved when a Pbx1b expression vector was used in place of the Pbx2 vector (data not shown). These results suggest that Pbx2-Prep1 and Pbx1b-Prep1 complexes do not activate the *UGT2B17* promoter and may slightly inhibit promoter activity. We proceeded, therefore, to investigate other mechanisms by which Pbx/Prep1 might influence the *UGT2B17* promoter. In the glucagon gene, a Pbx1-Prep1 heterodimer has been shown to interact with the glucagon enhancer element G3 and attenuate Pax6-mediated activation of the glucagon promoter (Herzig et al., 2000). The G3 enhancer element is composed of two adjacent but distinct binding domains, A and B, which bind the Pax6 (domain A) and the Pbx1-Prep1 (domain B) independently of each other. The proximity of the Pbx and HNF1 sites in the *UGT2B17* promoter prompted us to investigate whether Pbx2-Prep1 could modulate HNF1α-mediated activation of the promoter.

Pbx2-Prep1 Competes with HNF1α for Binding to the HNF1-Pbx Region. One possible mechanism by which Pbx2-Prep1 may be modulating promoter activity is via competitive interaction with HNF1α at the HNF1-Pbx site. Having established that Pbx2-Prep1 can bind to the Pbx site (see

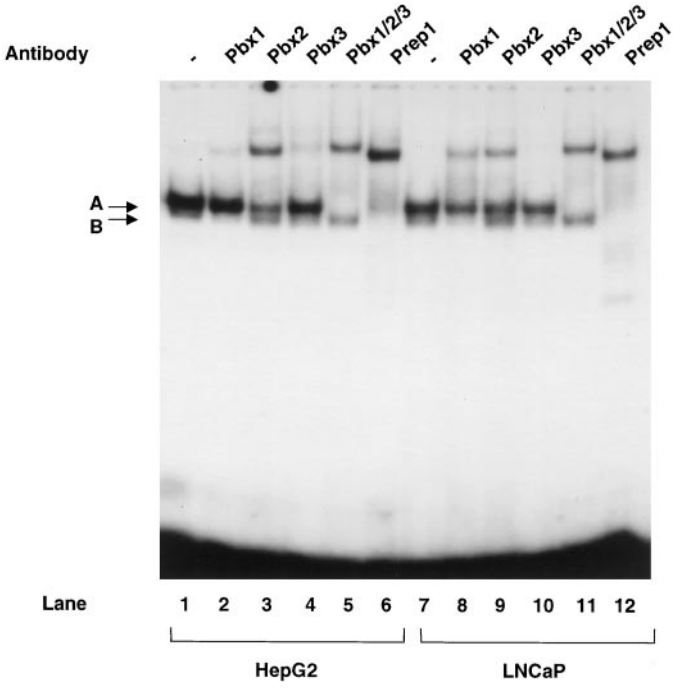

Fig. 2. Pbx2/Prep1 and Pbx1b/Prep1 heterodimers bind to the Pbx site in the *UGT2B17* promoter. EMSAs were performed using ^{32}P-labeled Pbx oligonucleotides (50,000 cpm per lane; sequence shown in Fig. 1) incubated with 5 μg of HepG2 or LNCaP nuclear extracts and 2 μg of either a Pbx1 (recognizing the Pbx1a and -b isoforms), Pbx2, Pbx3, Pbx1/2/3 (recognizing the Pbx1a, -2, and -3a isoforms), or Prep1 specific antibody followed by resolution on a 4% nondenaturing polyacrylamide gel. Antibodies were added after the addition of labeled probe and incubated for 45 min before loading. The doublet band consists of two protein complexes labeled A and B (as indicated with arrows).

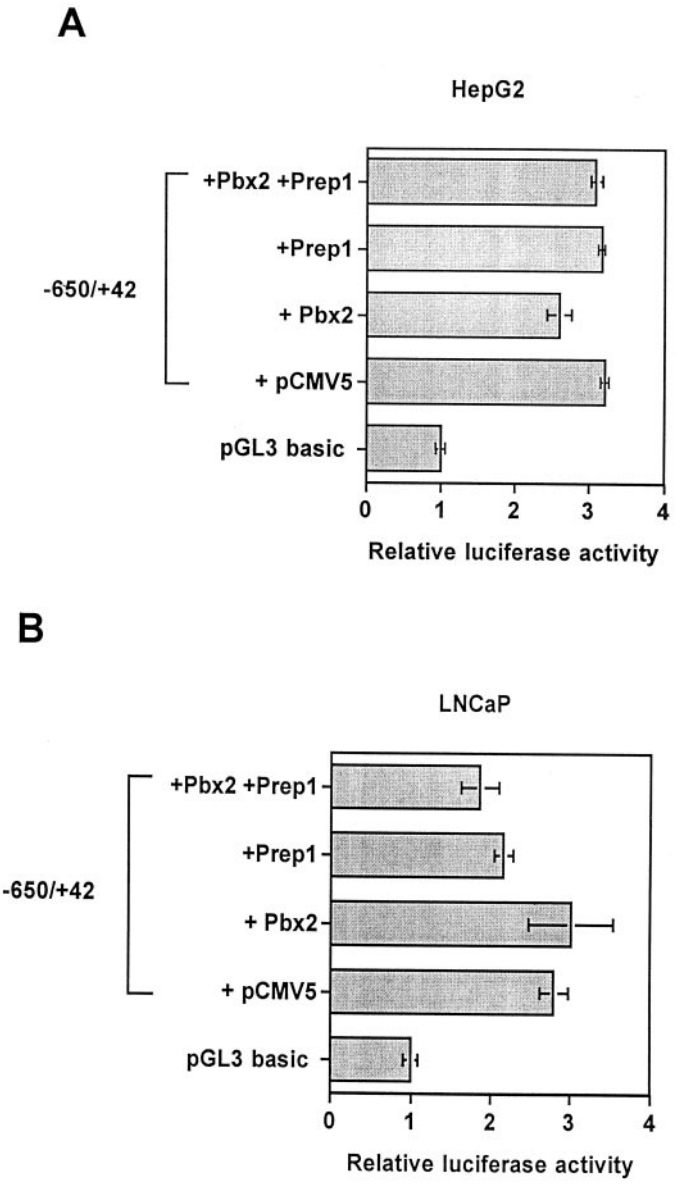

Fig. 3. Pbx2/Prep1 do not activate the UGT2B17 promoter. The proximal 650 bp of the *UGT2B17* promoter was cloned into the pGL3 basic firefly luciferase reporter vector and transiently transfected (0.5 μg) into HepG2 (A) and LNCaP (B) cells. Cotransfections were performed with 0.2 μg of pCMV5 (empty expression vector), Pbx2, Prep1, or a combination of Pbx2 and Prep1 with DNA amounts normalized to a total of 0.95 μg with pCMV5. Transfection efficiency was normalized using 0.05 μg of the pRLnull *Renilla reniformis* luciferase reporter vector. Cells were harvested 48 h after transfection and assayed for firefly and *R. reniformis* luciferase activities. Relative luciferase assays were graphed with comparison to pGL3 basic activity (set at a value of 1). Results are expressed as the mean of three transfection experiments with S.E.M. as shown.

Fig. 2), we tested whether this complex could also bind to its site when the adjacent HNF1 site is present, and whether it has any influence on HNF1α binding. An oligonucleotide containing both the HNF1 and Pbx binding sites was synthesized and used in EMSAs (Fig. 4B). When HepG2 nuclear extracts were added to the HNF1-Pbx probe, two separate complexes of differing mobility were observed (Fig. 4A, lane 1). Supershifting these complexes with HNF1α, Pbx2- and Prep1-specific antibodies showed that HNF1α is present in the upper complex (Fig. 4A, lane 2), and Pbx2 and Prep1 are present in the lower complex (Fig. 4A, lanes 4 and 6). The Pbx complex formed with the HNF1-Pbx probe has only one distinct band, which differs from the doublet band formed when the Pbx probe is used. The Pbx1 antibody is unable to supershift the Pbx complex formed with the HNF1-Pbx probe (Fig. 4A, lane 3), suggesting that this complex does not contain the smaller 40-kDa Pbx1b isoform, which is present in the complex formed on the Pbx probe (Fig. 2, lane 2). The Pbx3-specific antibody also did not supershift the lower complex, indicating that this protein is not present (Fig. 4A, lane 5).

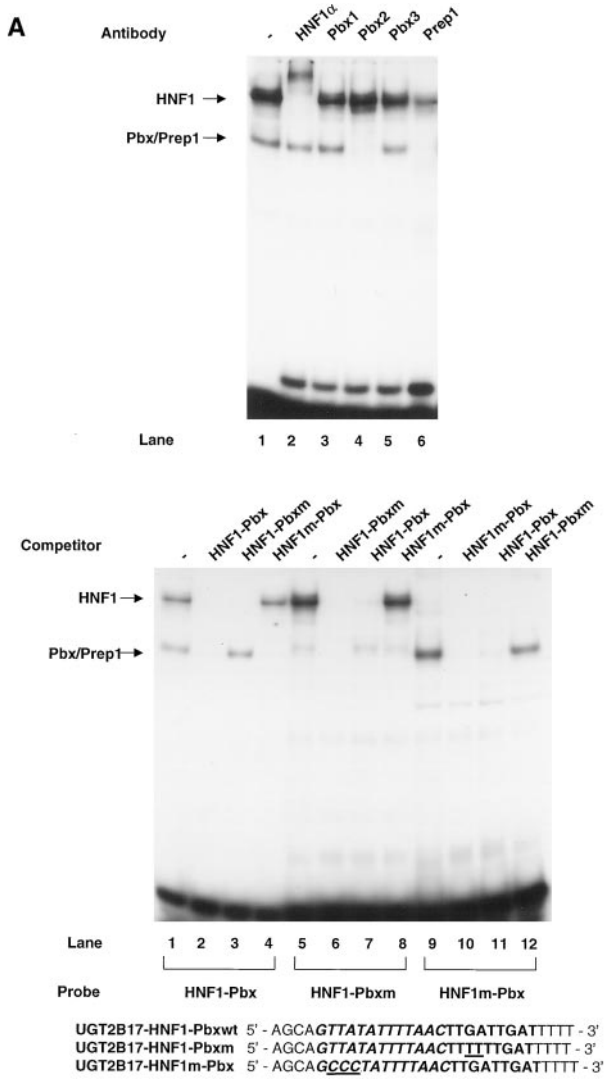

Fig. 4. Pbx2/Prep1 and HNF1α compete for binding to the HNF1-Pbx region in the *UGT2B17* promoter. EMSA's were performed using ^{32}P-labeled Pbx oligonucleotides (50,000 cpm per lane; sequence shown) incubated with 5 μg of HepG2 or LNCaP nuclear extracts followed by resolution on a 4% nondenaturing polyacrylamide gel. For supershift assays (A), 2 μg of HNF1α, Pbx1, Pbx2, Pbx3, or Prep1 specific antibodies were incubated with labeled probe and nuclear extract for 45 min before loading. For competition studies (B), unlabeled competitors were added at a 50-fold molar excess before the addition of the labeled Pbx probe. The sequence of the oligonucleotides are shown with the Pbx (bold) and HNF1 binding sites (bold and in italics) and the corresponding mutant sites (underlined) indicated. The HNF1 and Pbx/Prep1 complexes are indicated with arrows.

To determine whether Pbx2-Prep1 competes with HNF1α for binding to the HNF1-Pbx region, EMSAs were performed using HNF1-Pbx probes containing mutations in the HNF1 and Pbx sites that are known to affect the binding of these proteins to their respective sites (Fig. 4B). When the Pbx site was mutated (HNF1-Pbxm), there was a significant decrease in the intensity of the Pbx complex with a corresponding increase in the intensity of the HNF1 complex (Fig. 4B, lane 5), compared with the wild-type HNF1-Pbx probe (HNF1-Pbxwt; Fig. 4B, lane 1). Similarly, mutation of the HNF1 site (HNF1m-Pbx) resulted in a decrease in the intensity of the HNF1 complex and an increase in the intensity of the Pbx complex (Fig. 4B, lane 9). These results suggest that both HNF1 and Pbx2-Prep1 competitively inhibit the binding of each other to the HNF1-Pbx region. Competition studies were performed using these mutated oligonucleotides to determine whether HNF1α and Pbx2-Prep1 can bind independently to their respective sites. Figure 4B shows each of the complexes formed with the HNF1-Pbxwt, HNF1-Pbxm, and HNF1m-Pbx probes were able to be competed with by 50-fold molar excesses of identical probes (Fig. 4B, lanes 2, 6, and 10). In each case, the HNF1m-Pbx probe was unable to compete with the upper HNF1 complex (Fig. 4B, lane 4 and 8), and the HNF1-Pbxm probe was unable to compete with the lower Pbx complex (Fig. 4B, lanes 3 and 12). Taken together, these results confirm that HNF1α and Pbx2-Prep1 bind independently to their adjacent sites and interfere with each other for binding to these sites.

Pbx2-Prep1 Down-Regulates HNF1α-Mediated Activation of the *UGT2B17* Promoter through the Pbx Site. To attribute functional significance to the interference observed between HNF1α and Pbx2-Prep1, we focused on the HNF1-Pbx region using transfection assays. HNF1α has been previously shown to be an important regulator of the *UGT2B17* promoter in HepG2 cells but is not present in LNCaP cells (Gregory et al., 2000). Therefore, we used HepG2 cells in transfection studies to determine the role endogenous HNF1α and Pbx2-Prep1 have in *UGT2B17* promoter regulation. Promoter constructs were made with the HNF1-Pbx region defining the 5′ border of the promoter, containing either wild-type (−52/+42wt), mutant HNF1 (−52/+42HNF1m), or mutant Pbx (−52/+42Pbxm) sites. The mutations in the promoter constructs corresponded to those used in the EMSA studies (Fig. 4B). Most of the activity of the −52/+42 promoter region in HepG2 cells can be attributed to HNF1α activation through the HNF1 site; therefore, any measured changes in the activity of the −52/+42 construct are likely to be the result of changes in HNF1α activation efficiency (Gregory et al., 2000). Transfection of the −52/+42wt construct into HepG2 cells showed 3-fold activity over the pGL3 basic plasmid (Fig. 5). Mutation of the HNF1 site (52/+42HNF1m) did not significantly decrease promoter activity, but this may be caused by some residual binding of HNF1α to its site. Mutation of the Pbx site (52/+42Pbxm) increased promoter activity by 3- to 10-fold above the pGL3

basic plasmid. This increase in activity is likely to be caused by inability of endogenous Pbx2-Prep1 to bind to the mutated Pbx site, therefore allowing more endogenous HNF1α to bind and activate the promoter through the HNF1 site. To determine whether Pbx2-Prep1 can compete with exogenously added HNF1α, cotransfections of the −52/+42 constructs were performed with HNF1α, Pbx2, and Prep1 expression plasmids. Cotransfection with the HNF1α expression plasmid activated the −52/+42wt and −52/+42Pbxm constructs but had little effect on the 52/+42HNF1m construct (Fig. 5). Addition of Pbx2-Prep1 lowered HNF1α-mediated activation of the −52/+42wt construct by approximately 2-fold but did not lower activation of the −52/+42Pbxm construct, showing that the intact Pbx site is essential for Pbx2-Prep1 to down-regulate HNF1α-mediated activation of the *UGT2B17* promoter.

Discussion

Although the importance of Pbx in regulating cell fate and segmental patterning during embryonic development is well known (Casares and Mann, 1998; Mercader et al., 1999; Ferretti et al., 2000), the physiological role of Pbx and its binding partners in adult tissues, such as the liver and liver-derived cells, is much more poorly understood. A recent study however, has indicated a role for Pbx/Meis1 complexes in modulating the thyroid hormone responsiveness of the malic enzyme [(*S*)-malate:NADP+ oxidoreductase] gene in chicken hepatocytes (Wang et al., 2001). In the current work, we show that the homeodomain proteins Pbx2 and Prep1 can bind to the *UGT2B17* Pbx site. Although these proteins do not activate the *UGT2B17* promoter, they are able to attenuate HNF1α-mediated activation of the promoter by binding to the Pbx site and restricting access of HNF1α. This modulation of HNF1α regulation of the UGT2B17 gene represents a novel target and function for Pbx2-Prep1 heterodimers in liver cells.

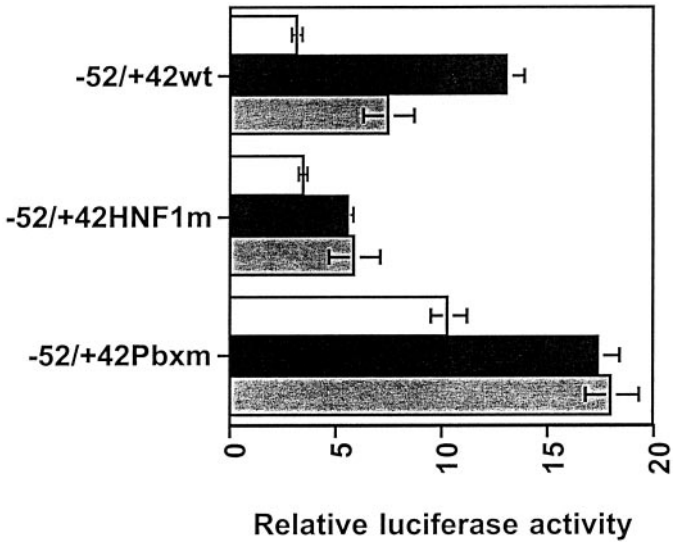

Fig. 5. Pbx2/Prep1 down-regulates HNF1α-mediated activation of the UGT2B17 promoter. Promoter fragments containing the proximal 52 bp of the *UGT2B17* promoter were generated by PCR containing no mutation (−52/+42wt), a HNF1 site mutation (−52/+42HNF1m), or Pbx site mutation (−52/+42Pbxm) and cloned into the pGL3 basic firefly luciferase reporter vector. These promoter constructs (0.5 μg) were transiently cotransfected into HepG2 cells with 0.2 μg of pCMV5 (empty expression vector, □), HNF1α (■), or a combination of HNF1α, Pbx2, and Prep1 (▨) with DNA amounts normalized to a total of 1.15 μg with pCMV5. Transfection efficiency was normalized using 0.05 μg of the pRLnull renilla luciferase reporter vector. Cells were harvested 48 h after transfection and assayed for firefly and *R. reniformis* luciferase activities. Relative luciferase assays were graphed with comparison to pGL3 basic activity (set at a value of 1). Results are expressed as the mean of three transfection experiments with S.E.M. as shown.

The demonstration of Pbx2-Prep1 as a negative regulator of HNF1α activation suggests that *UGT2B* expression may be lowered by the binding of Pbx2-Prep1 to the Pbx site. Of the six *UGT2B* genes isolated so far, four (*UGT2B10*, *UGT2B11*, *UGT2B15*, and *UGT2B17*) contain a Pbx site that is conserved in sequence and location with the *UGT2B17* Pbx site (Fig. 6). We postulate that the presence of this conserved Pbx site may in part be indicative of lower expression levels of these *UGT2B* genes in HNF1α-containing tissues, compared with *UGT2B* genes that do not contain the Pbx site (i.e., *UGT2B4* and *UGT2B7*). Examination of *UGT2B* expression in the liver by quantitative RT-PCR has shown that the expression of those UGT2B genes that do not contain a Pbx binding site (viz., *UGT2B4* and *UGT2B7*) is higher than those *UGT2B* genes with the Pbx-binding site (Congiu et al., 2002). This difference in expression may be important in maintaining circulating levels of steroids. Both UGT2B15 and UGT2B17, whose gene promoters have a Pbx-binding site, are closely related in sequence (95% identical in their coding regions) and have a similar substrate specificity (Turgeon et al., 2000). In particular, they glucuronidate androgens (C19 steroids) and androgen metabolites (e.g., testosterone, dihydrotestosterone) with high efficiency (Chen et al., 1993; Beaulieu et al., 1996). In contrast, UGT2B4 and UGT2B7 glucuronidate most androgens at a much lower efficiency than UGT2B15 and UGT2B17 (Turgeon et al., 2001). The other forms whose genes contain the Pbx-binding site (UGT2B10 and UGT2B11) are inactive toward steroids (Jin et al., 1993). Androgens are known to play major roles in cell growth and function in steroid responsive tissues. Lower levels of UGT2B15 and UGT2B17 in the liver, facilitated by the inhibitory action of Pbx2-Prep1 on HNF1α-mediated promoter activation, would ensure that hepatic clearance of androgens by glucuronidation is restricted, allowing unconjugated active androgens to reach their target sites in steroid responsive tissues. It is interesting to note that Pbx-binding sites are not found adjacent to the HNF1-binding sites of *UGT2B1* and *UGT2B2*; the two rat UGTs that are primarily responsible for the glucuronidation and clearance of androgens in the liver (Mackenzie and Rodbourn, 1990; Haque et al., 1991). This may, in part, account for the lower level of circulating androgens in rodents compared with humans (Guillemette et al., 1996).

Data from EMSA and transfection studies showed that the Pbx2-Prep1 complex was able to bind to the *UGT2B17* Pbx site in prostate-derived LNCaP cells and cause a slight repression in the basal activity of the −650/+42 *UGT2B17* promoter (Figs. 2 and 3B). Although this repression was not caused by interference with HNF1α binding, Pbx2-Prep1 may be modulating the transcription activity of other unidentified factors in the LNCaP cell line, and possibly other HNF1α-negative cell lines. In support of this concept, Pbx-Prep1 complexes have been shown to repress the transcriptional activity of the glucagon gene in a cell-type–dependent manner (Herzig et al., 2000). Activation of this gene by multiple factors such as Pax6 and Ets-1 is repressed by Pbx-

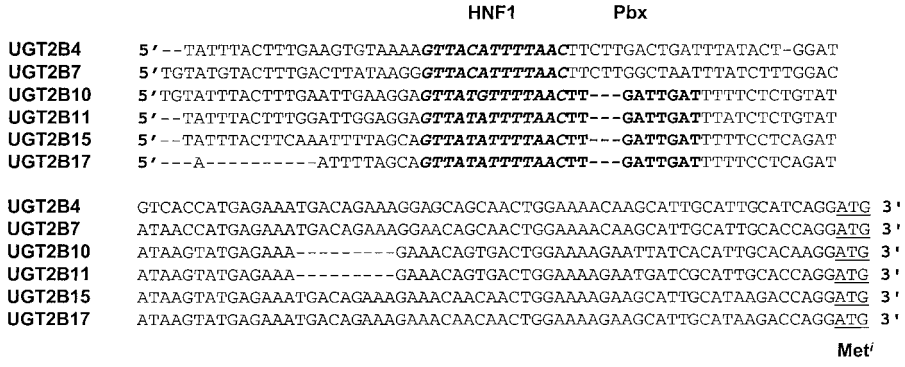

Fig. 6. Comparison of the proximal promoters of the *UGT2B* genes. The alignment of the proximal promoters of the *UGT2B* genes is shown. The HNF1 binding sites (bold and in italics), Pbx binding sites (bold), and the methionine initiation codons (underlined) are indicated.

Prep1 complexes in non–glucagon-producing cells (Herzig et al., 2000). However, this repression was not observed in glucagon-producing pancreatic cells when promoter activity was activated by a combination of Pax6, Ets-1, and HNF3β factors, suggesting that the ability of Pbx-Prep1 to inhibit transcription is dependent on the nature and combination of transcription factors that bind to the glucagon gene promoter (Herzig et al., 2000). The mechanism of inhibition of transcription of the glucagon gene by Pbx-Prep1 was not elucidated. In the HepG2 cell line, the mechanism of inhibition of HNF1α-mediated promoter activation was shown to be direct interference of HNF1α binding by Pbx2-Prep1 binding. Transfection studies showed that Pbx2-Prep1 interfered with but did not completely inhibit HNF1α activation of the promoter. This may reflect a difference in the relative binding affinities of these proteins for their respective sites and suggests that the relative levels of HNF1α and Pbx within a cell type are important factors determining the physiological relevance of this regulatory mechanism. It is well established that HNF1α is expressed at high levels in the liver and HepG2 cell lines (Mendel and Crabtree, 1991; Gregory et al., 2000), however, it is not known whether the liver expresses Pbx isoforms at similar levels to HepG2 cells.

In contrast to the repressive effect of Pbx-Prep1 complexes on the *UGT2B17* and glucagon genes, these complexes are generally observed to stimulate the activation of transcriptional effectors on other genes. In the case of the urokinase plasminogen activator gene (*uPA*), a Pbx-Prep1 heterodimer binds to an element termed COM that is essential for cooperative activation of the uPA enhancer by several Jun, Fos and ATF complexes (De Cesare et al., 1996; Berthelsen et al., 1998a). Deletion of the COM region resulted in a loss of phorbol ester inducibility mediated through the binding of these activator protein-1–like complexes to sites flanking the COM region (De Cesare et al., 1996). Pbx-Prep1 has also been shown to interact with the pancreatic homeodomain transcription factor PDX1 and modulate PDX1 activation of the somatostatin gene (Goudet et al., 1999). In a manner similar to that of the *UGT2B17* gene, Pbx-Prep1 proteins bind to a consensus Pbx binding site (termed UE-A) directly adjacent to the activator PDX1 binding site (Goudet et al., 1999). The Pbx-Prep1 complex enhanced PDX1 activation of the somatostatin minienhancer; however, the mechanism through which this synergism occurs was not determined. It is possible that Pbx-Prep1 may be cooperatively binding with PDX1, although gel-shift studies failed to demonstrate this interaction in the context of the in vitro system (Goudet et al., 1999). Cooperative binding of Pbx-Meis complexes to PDX1 has been shown occur on the elastase1 gene minienhancer (Liu et al., 2001) and this mechanism may also be responsible for the synergetic activation of the somatostatin promoter by PDX1 and the Pbx-Prep1 complex. In the *UGT2B17* gene, EMSA analyses with the HNF1-Pbx site showed that Pbx2-Prep1 and HNF1α bind independently to separate sites. They do not bind to each other to form trimeric protein complexes as observed with Pbx and other homeodomain proteins such as HOX (Shen et al., 1999; Penkov et al., 2000). Thus, the effect of Pbx-Prep1 complexes in modulating transcription factor activity may be dependent on the context in which they interact. Hence, it is feasible that in the presence of specific homeodomain transcription factors, such as members of the Hox or Meis family, stimulation rather than repression of the expression of *UGT2B* genes containing the conserved Pbx site may occur. Although the presence of Pbx2 and Prep1 were confirmed in HepG2 cells by EMSA studies, whether Hox or Meis is expressed in this cell line is not known. Further studies are needed to determine whether these factors are present in HepG2 cells and whether they interact with the UGT2B Pbx site.

In summary, the present study establishes a role for Pbx2-Prep1 complexes in repressing the activity of the *UGT2B17* gene in liver cells. The repression of HNF1α-mediated transcriptional activation by these complexes may be a general mechanism for the differential regulation of *UGT2B* genes in the liver and may help prevent the precipitous hepatic clearance of circulating steroids by glucuronidation.

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