An Abd-B Class HOX-PBX Recognition Sequence Is Required for Expression from the Mouse Ren-1c Gene*

Expression from the mouse Ren-1c gene in As4.1 cells is dependent on a proximal promoter element (PPE) located at approximately −60 and a 241-base pair enhancer region located at −2625 relative to the transcription start site. The PPE (TAATAAATCAA) is identical to a consensus HOX-PBX binding sequence. Further, PBX1b has been shown to be a component of a PPE-specific binding complex present in nuclear extracts from As4.1 cells. The binding affinities of different paralog HOX members to the PPE were examined in the absence or presence of PBX1b. HOXB6, -B7, and -C8 failed to bind the PPE alone but showed weak affinity in the presence of PBX1b. In contrast, HOXD10 and to a lesser degree HOX9 bound the PPE with high affinities regardless of whether PBX1b was present. Abd-B HOX members, including HOXD10, -A10, -A9, -B9, and -C9, are expressed in As4.1 cells. The ability of HOX and PBX1b to form a ternary complex with PREP1 on the PPE is also demonstrated both in vivo and in vitro. Point mutations in the HOX or PBX half-site of the PPE disrupted the formation of the HOX-PBX complex and dramatically decreased transcriptional activity of the Ren-1c gene demonstrating that both the HOX and PBX half-sites are critical for mouse renin gene expression. These results strongly implicate Abd-B class Hox genes and their cofactors as major determinants of the sites of renin expression.

Renin is an aspartyl protease, which, as part of the renin-angiotensin system, plays a critical role in the maintenance of blood pressure and electrolyte balance by converting angiotensinogen to angiotensin I (1). Additionally, the renin-angiotensin system, plays a critical role in the maintenance of blood pressure and electrolyte balance by converting angiotensinogen to angiotensin I (1). Furthermore, it is thought to be involved in the regulation of blood pressure and electrolyte balance by converting angiotensinogen to angiotensin I (1). Renin expression is subject to complex developmental and tissue-specific regulation (for review, see Ref. 5). In murine fetuses, kidney renin transcripts can be detected as early as 14.5 days post coitum in the newly developing arteries (6). On development of the renal arterial tree, expression shifts to cells located in nascent portions of the growing arteries until expression is restricted to a small population of modified smooth muscle cells of the afferent arteriole proximal to the glomerulus called juxtaglomerular cells. In adult mice, renin is also expressed in adrenal gland, submandibular gland, gonads, and coagulating gland.

Some mouse strains have only a single renin gene (Ren-1c), whereas other strains have two copies, a Ren-1 locus with allele Ren-1d and a duplicated locus with Ren-2 (7, 8). Although these mouse renin genes are approximately equivalently expressed in the adult kidney, their expression patterns are different in some extra-renal tissues (9).

Identification of cis-acting sequences in the mouse renin 5′-flanking region has been accelerated by the isolation of a kidney tumor-derived As4.1 cell line from transgenic mice containing the mouse Ren-2 5′-flanking sequence fused to SV40 T antigen (10). As4.1 cells are capable of expressing high levels of renin mRNA (10) and secreting active renin protein (11). By transiently transfecting these cells with wild-type or mutant Ren-1d-chloramphenicol acetyltransferase (Ren-CAT) constructs, two regions in the Ren-1c 5′-flanking sequence were found to be necessary for high level expression of mouse renin gene in addition to a TATA box, the PPE located at −60 bp and a 241-bp enhancer located 2.6 kb upstream of the transcription start site (12). The PPE was shown to bind As4.1 cell nuclear proteins in electrophoretic mobility shift assays (EMSA). Further competition assays indicated that the minimal sequence required for protein binding included N1(3)TAATAAAT. Mutation of the PPE in the Ren-CAT construct containing a 4.1-kb mouse renin sequence dramatically reduced the chloramphenicol acetyltransferase activity in transfection assays suggesting a critical role of this element in the regulation of mouse renin gene expression.

Hox genes are members of the homeobox family of transcription factors and control many aspects of morphogenesis and cell differentiation in animals (13). In vertebrates, there are 39 Hox genes organized in four clusters (A, B, C, and D) on separate chromosomes with members of each cluster classified into as many as 13 paralog groups based on sequence similarity (14). Hox gene products can bind DNA as monomers or heterodimers with three-amino acid loop extension (TALE) class homeod melanins. This work was supported by National Institutes of Health Grants HL48459 (to K. W. G.), CA16056, and HD36416 (to S. C. P.).

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1 The abbreviations used are: PPE, proximal promoter element; EMSA, electrophoretic mobility shift assay; bp, base pair(s); kb, kilobase pair(s); RT-PCR, reverse transcription-polymerase chain reaction; CREB, cAMP-response element-binding protein.
main proteins including PBX and MEIS on HOX-PBX or HOX-MEIS recognition sequences (15–22). Interactions with PBX or MEIS proteins increase both DNA binding affinity and specificity for HOX proteins. Although the HOX-MEIS recognition sequences have not yet been identified in any natural genes, the HOX-PBX binding sequences have been found in genes such as Hox (23, 24), α2(V) collagen (25), Eph receptor EphA2 (26), and fork head (fkh) (27). Moreover, MEIS or its homolog PREP1 interacts with PBX through its amino-terminally located HM domain (28–30). This interaction is essential for PBX nuclear translocation. Several studies have also demonstrated that MEIS or PREP1 can form ternary complexes with HOX and PBX (31–36), and this trimeric complex has been shown to play an important role in regulating several HOX-responsive genes (31, 34–36).

In this report we show that the renin gene contains a functional HOX-PBX binding site in its promoter region. Homeodomain proteins PBX1b and HOX family members (preferentially HOX9 and -10) can bind this element in vitro and in As4.1 cells. Both PBX and HOX binding sites are necessary for the expression of the Ren-1 gene. Moreover, we have demonstrated that PREP1 can form a ternary complex with HOX and PBX1b on this site.

**EXPERIMENTAL PROCEDURES**

**Analysis of Hox Gene Expression Using RT-PCR—Total RNA from As4.1 cells was isolated by TRIzol reagent (Life Technologies, Inc.), and first strand cDNA was then synthesized using SUPERSCRIPT™ Preamplification System (Life Technologies, Inc.). The homeodomain regions of Hox genes were amplified from the first strand reaction product by PCR. The degenerate PCR primers used were designed against the first (5′-agctaagacgtaagacgagcgtcgtg/ctg/agcacgatgagcctcct-3′) and third (5′-agctgctggagtggagagggatgctgta-3′)′-GTGTT-3′) α-helices. The 150-bp fragment resulting from RT-PCR was isolated and subcloned. Sequences of individual clones were then determined.

**Plasmid Constructions—** Plasmids −4.1R1, −4.1M, −4.1mb, −4.1mp, and −4.1mp2 were constructed by inserting the wild-type or mutant Ren-1′ sequences from −4100 to +6 bp into pGLO2-basic (Promega). The reporter construct 3XR1-TA was made by inserting three copies of Ren-1′ into pGLO2-basic-derived plasmid containing the adenovirus E1b TATA box (37).

Full-length cDNA for Ren-2 was isolated from As4.1 cells and cloned into pcDNA3.1myc-His(+)/vector (Invitrogen), which contains a carboxyl-terminal Myc epitope. Full-length cDNA for PREP1 was also cloned from As4.1 cells and inserted in pcDNA3.1V5-His(+)/vector (Invitrogen), which has a carboxyl-terminal V5 epitope. Full-length cDNA for PBX1b was cloned in the same vector without incorporating any epitope. These plasmids were used in in vitro transcription/translation and EMSAs. For expression in mammalian cells, a stop codon was added immediately before the Myc or V5 epitope sequence in the HOXD10 or PBX1 expression vector, so that the carboxyl-terminal Myc or V5 epitope was not translated. VP16-HOXD10 or VP16-PBX1b was constructed by inserting HOXD10 or PBX1b full-length cDNA, respectively, in pVP16 (CLONTECH) so that a nuclear localization signal and a VP16 activation domain were fused amino-terminally to HOXD10 or PBX1b. VP16-PREP1 and VP16-HM were similarly constructed. VP16-PREP1 contained a truncated PREP1 with residues 1–75 deleted, whereas VP16-HM contains the HM region of PREP1 from residues 75 to 557.

**Cell Culture and Transient Transfections—** As4.1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transfected using FuGENE 6 (Roche Molecular Biochemicals). For each transfection in a 35-mm culture dish, 2.2 μg of DNA including 0.5 μg of reporter plasmid, 0.5 μg of each expression plasmid, nonspecific plasmid if necessary, and 0.2 μg of plasmid containing theRen-2 virus promoter driving β-galactosidase were mixed with 4.4 μl of FuGENE reagent. Forty-eight hours after transfection cells were harvested and measured for luciferase and β-galactosidase activities using the Luciferase Assay System (Promega) and Galacto-Light Plus™ chemiluminescent reporter assay (Tropix), respectively. The luciferase activity is normalized with the average ± S.D. of at least three separate experiments.

**In Vitro Transcription and Translation—** HOX, PBX1b, and PREP1 proteins were in vitro transcribed/translated by the T7N Coupled Wheat Germ System (Promega). Parallel reactions containing [35S]methionine were performed to correct differences in translation efficiency between experiments. All translation results represent the average ± S.D. of at least three separate experiments.

**RESULTS**

**The Ren-1′ PPE Is a HOX-PBX Protein Binding Site—** The consensus sequence for HOX-PBX binding is NTGATTTNAT. Using in vitro site selection assays, the preferred binding site for PBX and HOX6–10 paralogs was shown to be NTGATTTTATN (or inverting N′N′ATAAATCAn) (18, 19). The sequence is a precise match to the highly conserved PPE in mouse Ren-1 and Ren-2 and rat and human renin promoters (Fig. 1). To test whether the PPE is a HOX-PBX binding site,
supershift EMSA was performed with antibodies against PBX1/2/3, which recognizes isoforms of PBX proteins (PBX1a, PBX2, and PBX3a), PBX1, PBX2, and PBX3 (Fig. 2). Only PBX1 antiserum, which recognized both PBX1a and PBX1b, was able to supershift the R1-As4.1 nuclear protein complex. The results demonstrated that the PPE contains the PBX binding site and suggest that PBX1b is the major PBX species binding at the PPE.

To determine which HOX protein might bind to the PPE, the Hox gene complement in As4.1 cells was surveyed by amplifying the highly conserved homeodomain region using RT-PCR. A total of 45 clones was assessed, and results from the analysis are shown overlaid on an organizational map of the mouse Hox gene cluster (Fig. 3). The results showed that predominantly (33 of 45) Abd-B classes 9 and 10 are represented, and specifically HOXD10, -A10, -A9, -B9, and -C9 are expressed in As4.1 cells.

To test whether HOX and PBX proteins can bind to the PPE, EMSA was performed with in vitro synthesized HOXB6, -B7, -B8, -B9, and -D10 proteins plus and minus in vitro synthesized PBX1b (Fig. 4). Each HOX protein contained a Myc carboxy-terminal epitope so that the complex formed by the R1 probe and the HOX protein could be supershifted by antibody against the Myc epitope. PBX1b alone was not capable of binding the PPE (Fig. 4, lane 1). HOXB6 (Fig. 4, lane 2), -B7 (Fig. 4, lane 6), and -C8 (Fig. 4, lane 10) did not bind to R1 detectably, however, in the presence of PBX1b they were able to bind weakly (more apparent with longer exposure, Fig. 4, lanes 3, 7, and 11, and data not shown). However, HOXB9 and especially -D10 gave rise to prominent shifts with or without PBX1b (Fig. 4, lanes 14, 15, 18, and 19). The Hox and HOX-PBX1b complexes were able to be supershifted by Myc antiserum (Fig. 4, lanes 16 and 20), whereas the HOX-PBX1b complex was disrupted by the addition of antibody against PBX1 (Fig. 4, lanes 17 and 21).

**PREP1 Can Form a Ternary Complex with HOX-PBX on Ren-1′′ PPE—** TALE class homeodomain proteins PREP1 and MEIS have been shown to form ternary complexes with HOX-PBX (31–36). We examined whether PREP1 is a component of the As4.1 cell nuclear proteins that bind to the Ren-1′′ PPE. In supershift assays, the antibody against PREP1 supershifted the complex formed by Ren-1′′ PPE and As4.1 nuclear extracts (Fig. 5A). In vitro synthesized PREP1 was also tested for its ability to bind Ren-1′′ PPE in the presence of HOXD10 and PBX1b (Fig. 5B). The results indicate that, although PREP1 could not bind to PPE alone or in combination with PBX1b (data not shown), it forms a ternary complex with HOXD10 and PBX1b (Fig. 5B, lane 3). The ternary complex could be supershifted or disrupted by the addition of antibody against Myc, PBX1, or PREP1 (Fig. 5B, lanes 4–6). Moreover, the ternary complex formed by HOXD10-PBX1b-PREP1 has almost the same electrophoretic mobility as the complex formed by As4.1 cell nuclear proteins (Fig. 5B, lane 3 versus lane 7) further suggesting that the Ren-1′′ PPE binds a ternary complex including HOX, PBX1b, and PREP1.

To further assess whether HOX is a member of As4.1 cell nuclear proteins binding to PPE, we tested whether in vitro translated HOXD10 can complex with PBX and PREP1 in As4.1 cell nuclear extract on the PPE. One microliter of 10-fold diluted HOXD10 protein synthesized in vitro was mixed with As4.1 cell nuclear extracts in EMSA, and the presence of HOXD10 in the complex was confirmed by supershift assays with Myc antiserum (Fig. 5C). The results showed that the complex formed by this amount of HOXD10 alone was barely visible (Fig. 5C, lane 1). However, the addition of the same amount of HOXD10 to As4.1 cell nuclear extract resulted in the formation of a complex that paralleled in size the complex formed by As4.1 cell nuclear extract alone and was more intense (Fig. 5C, lane 3 versus lane 2). The sharp increase in complex intensity by adding HOXD10 suggests the possibility that As4.1 cell nuclear extract can provide factors that improve the ability of HOXD10 to bind the R1 PPE, and these factors include PBX1 and PREP1. Myc antiserum was capable of supershifting almost all the complex formed (Fig. 5C, lane 4) suggesting that the exogenous HOXD10 was in excess of endogenous HOX proteins binding to the PPE. Antibodies against PBX1 and PREP1 also supershifted the complex (Fig. 5C, lanes 5 and 6) indicating that the formation of HOXD10-PBX1b-PREP1 ternary complex.

Both HOX and PBX Half-sites Are Crucial for Ren-1′′ Gene Expression—Based on HOX-PBX crystal structure (38, 39), crucial contacts are made between PBX and the adenine at the nucleotide 4 position of the heterodimer recognition sequence and between HOX and the nucleotide 8 position of the recognition sequence (see Fig. 6A). We examined the effects of point mutations at these positions on the binding of As4.1 cell nuclear extracts in EMSA (Fig. 6, A and B). A single mutation of nucleotide 4 (Fig. 6A, R3) or double mutations at nucleotides 3 and 4 (Fig. 6A, R4) resulted in the formation of complexes with high mobility equivalent to binding of the HOX monomer alone (Fig. 6B, lanes 2 and 3). The same results were obtained using in vitro translated HOXD10-PBX1b (Fig. 6C, lanes 4 and 5). In contrast, the single nucleotide mutation at nucleotide 8 (Fig. 6A, R5) resulted in complete failure of complex formation with
either As4.1 cell nuclear extracts (Fig. 6B, lane 4) or in vitro translated HOXD10-PBX1b (Fig. 6C, lane 6). In competition assays (Fig. 6B), the R1 oligonucleotide competed with itself in the formation of the R1-As4.1 cell nuclear protein complex very well at a 100-fold excess (Fig. 6B, lanes 5 and 6). However, oligonucleotides with PBX half-site mutations exhibited only partial competition (Fig. 6B, lanes 7 and 8), whereas the oligonucleotide with the nucleotide 8 mutation failed to compete (Fig. 6B, lane 9). These results are consistent with previous
reports that HOX can bind its recognition site as monomer; however, PBX cannot bind to the HOX-PBX recognition sequence in isolation.

Effects of these point mutations on expression from the Ren-1" promoter were also tested (Fig. 6D). The mutations were incorporated into plasmid −4.1R1. The nucleotide 8 mutation (−4.1mh) in the HOX protein binding site reduced the expression level of −4.1R1 by ~80%. The nucleotide 4 single mutation (−4.1mp) or nucleotides 3 and 4 double mutations (−4.1mp2) in the PBX half-site also resulted in an ~85% reduction in expression from the Ren-1" promoter. These results suggest that the binding of PBX and HOX to PPE is crucial for mouse renin gene expression.

HOXD10, PBX1b, and PREP1 Can Bind the Ren-1" PPE in As4.1 Cells—As4.1 cells were cotransfected with a reporter construct containing three copies of the Ren-1" PPE upstream of an E1b TATA box and expression vectors for HOXD10, PBX1b, and PREP1, and various combinations. Overexpression of these proteins had little effect on the transcription of the reporter gene (Fig. 7). However, fusion proteins VP16-HOXD10, VP16-PBX1b, and VP16-PREP1, which have a VP16 activation domain fused amino-terminally to HOXD10, PBX1b, and PREP1, respectively, were capable of activating reporter gene expression by 11-, 15-, and 30-fold. However, they did not activate a similar reporter gene with mutated Ren-1" PPE (data not shown). These results suggest that HOXD10, PBX1b, and PREP1 are capable of binding to the PPE in As4.1 cells. Moreover, the fusion protein VP16-HM containing only the VP16 activation domain and HM domain, which is the region in PREP1 interacting with PBX, was still capable of activating the reporter gene by 30-fold. The results agree with previous reports that the homeodomain of PREP1 is not necessary in the ternary complex formation on a HOX-PBX site (31).

**DISCUSSION**

In this report, we have demonstrated that the PPE of the renin gene is a HOX-PBX heterodimer binding site. First, the nucleotide sequences of PPE match perfectly to those of the HOX-PBX heterodimer recognition site. Second, Abd-B HOX9 and -10 members and PBX1b were shown to bind this element with high affinity in vitro. Finally, point mutation of a critical nucleotide either in the HOX or PBX half-site showed the expected effects on HOX-PBX binding in EMSA. In addition, these mutations dramatically reduced the transcriptional activity of Ren-1" gene suggesting that both PBX and HOX half-sites are necessary for renin gene expression.

The human renin PPE was previously identified as a Pit-1 site in GC cells (40). However, our results have shown that the PPE in the mouse renin promoter is not a Pit-1 binding site (12). The PPE in mouse is a poor consensus for Pit-1. Moreover, the As4.1 cell nuclear proteins were not capable of binding to a human Pit-1 consensus oligonucleotide. Furthermore, the major nuclear protein complex from As4.1 cells formed on mouse Ren-1" PPE could not be competed by the Pit-1 oligonucleotide, although it could be competed by the oligonucleotide containing the human PPE. These observations suggest that in As4.1 cells the primary proteins binding to the PPE are HOX and PBX, not Pit-1. Whether HOX and PBX proteins bind to the human PPE in GC cells has not been examined.

A direct test of whether HOXD10 protein is involved in the formation of the ternary complex on the mouse PPE in super-shift assays has not been possible because of the unavailability of specific antibody. However, other evidence suggests that HOXD10 is a major HOX protein binding to the PPE in As4.1 cells. HOXD10 is expressed in As4.1 cells. Moreover, in the presence of PBX1b, HOXD10 binds to the PPE in EMSA with higher affinity compared with other selected HOX paralog members, and the size of the complex mimics the endogenous complex formed in As4.1 cell nuclear extracts. Furthermore, a small amount of *in vitro* synthesized HOXD10 was capable of forming the HOXD10-PBX1b-PREP1 complex with endogenous PBX and PREP1. However, we cannot rule out a redundant role for other HOX family members such as HOX9 members, which are also expressed in As4.1 cells and recognize the same sequence motifs as HOXD10. Recent evidence suggests that although different HOX-PBX heterodimers have preferred sites *in vitro*, this binding specificity is not always utilized *in vivo* (41, 42). It has been suggested that the specificity of a HOX-PBX site might be affected by cofactor binding sites. Thus, the study of renin gene expression in mice lacking different HOX proteins will be helpful in identifying the specific HOX family members necessary for renin expression.

We showed that overexpression of HOXD10, PBX1b, PREP1, or various combinations was not able to activate a promoter containing three copies of Ren-1" PPE. These results are in agreement with previous results reported by Shen et al. (32) and Shanmugam et al. (33) that HOX-PBX-MEIS complex does not exhibit transcriptional activity on artificial promoters containing HOX-PBX binding sites. Thus, other cofactor binding sites may be necessary in the natural targets for the function of HOX-PBX-PREP1/MEIS. We also cotransfected −4.1R1 with HOXD10, PBX1b, PREP1, or various combinations into As4.1 cells. The results showed that the transcriptional activity of the reporter gene was not affected by co-expression of these proteins (data not shown). It is possible that a cofactor, which is necessary for HOX-PBX-PREP1-mediated expression from −4.1R1, is limiting. Therefore, overexpression of HOX-PBX-PREP1 would not further result in an increase in transcriptional activity. When the VP16 activation domain is attached to either HOXD10, PBX1b, or PREP1, it can substitute for the limiting cofactor to activate expression from construct 3XR1-1A.

How the PPE bound HOX-PBX1b-PREP1 complex interacts with other required transcriptional factors to regulate the mouse renin gene expression remains to be elucidated. It is possible that the PPE-binding proteins directly interact with a factor binding to an element located within or immediately outside the renal enhancer region located at −2.6 kb to bring the enhancer closer to the TATA box by looping out. Recent findings by Ryoo et al. (35), Ferretti et al. (36), and Jacob et al.
(34) have demonstrated that the vertebrate HoxB2 r4 or *Drosophila labial* (lab) gene enhancer contains a MEIS/PREP1 (or *Drosophila* homolog HTH) binding site located almost one competitive turn of the helix away from a HOX-PBX site. The MEIS/ PREP1/HTH site is required for the HOX-PBX-MEIS/PREP1 or HTH ternary complex formation. However, there is no PREP1/MEIS site found adjacent to the PPE in the *Ren-1* gene. Interestingly, surveys of the 4.1-kb *Ren-1* flanking sequences for PREP1/MEIS or PBX-PREP1/MEIS sites have revealed two such sites in proximity to the enhancer region. One of these MEIS/PREP1 sites may interact with the HOX-PBX site in the promoter region through the formation of the HOX-PBX-PREP1/MEIS ternary complex to bring the enhancer close to the transcriptional apparatus.

A recent report by Saleh et al. (43) suggests that the HOX-PBX complex recruits the coactivator CREB-binding protein to activate gene expression. A CREB/cAMP-response element modulator protein binding site within the enhancer located at 9.26 kb is critical for the enhancer function. Deletion or mutation of the CREB/cAMP-response element modulator protein binding site results in complete loss of enhancer activity. It is thus possible that the PPE bound HOX-PBX complex indirectly interacts with the CREB site within the enhancer through a common CREB-binding protein coactivator to activate *Ren-1* gene expression.

These findings strongly imply that renin and in larger scope the renin-angiotensin system are immediate downstream targets of the Class I *Hox* developmental control genes. As such, the production of angiotensin II, a classical pressor substance that also exhibits growth factor activities as mediated through the AT1 and AT2 receptor system (44), is regulated by an developmental control genes. As such, renin gene expression.

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