Cooperative Interactions between PBX, PREP, and HOX Proteins Modulate the Activity of the α2(V) Collagen (COL5A2) Promoter*

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Cell type-specific expression of the human α2(V) collagen (COL5A2) gene depends on a cis-acting element that consists of two contiguous protein binding sites (FPA and FPB) located between nucleotides −149 and −95, relative to the transcription start site. The present study focused on the characterization of the FPB-bound complex. DNA binding assays and cell transfection experiments revealed that the bipartite core sequence of FPB (5′-ATCAATCA-3′) binds the PBX1/PREP1, and HOXB1 proteins, and this in turn leads to promoter transactivation. In the presence of all three nuclear factors, cooperative interactions between recombinant PBX1 and PREP1 or PBX1 and HOXB1 result in binding of the heterodimers to FPB in vitro. Similarly, overexpression of different combinations of PBX1, PREP1, and HOXB1 transactivates FPB-driven transcription. In contrast to the composition of the FPB complex purified from COL5A2-positive cells, the FPB complex from COL5A2-negative cells contains PBX2 and PREP1 but lacks PBX1. However, PBX1 exogenously introduced into COL5A2-negative cells cannot stimulate FPB-driven transcription unless co-expressed with PREP1. Within the intrinsic limitations of the experimental model, our results indicate that combinatorial interactions among PBX and PREP or HOX proteins are involved in regulating tissue-specific production of collagen V.

Proper assembly of collagenous networks in the developing connective tissue is critically important for the formation and function of virtually every organ system (1). Collagens I and II are the major fibrillar structures that confer strength and resilience to noncartilaginous and cartilaginous tissues, respectively (1). Aside from spatiotemporal regulation of the respective genes, formation of collagens I and II fibers is under the control of a variety of cellular and structural elements (2). Among the latter are the so-called minor fibrillar collagens (types V and XI), which regulate fibrillogenesis by co-polymerizing with the major fibrillar collagens (types I and II) (3, 4).

Understanding the mechanisms that underlie the production of minor fibrillar collagens is therefore relevant to elucidating the genetic determinants responsible for stage- and tissue-specific diversification of the extracellular matrix.

Our previous work has shown that transcription of the human α2(V) collagen (COL5A2) gene is controlled by an evolutionarily conserved sequence located between nucleotides −149 and −95 (5). DNase I footprinting and the electrophoretic mobility shift assay (EMSA)† have divided this upstream promoter sequence into two contiguous cis-acting elements, termed FPB (−149 to −115) and FPA (−114 to −95). Deletion of the −149 to −95 region as well as nucleotide substitutions in FPA and FPB were shown to adversely affect COL5A2 promoter activity in cell transfection experiments. Moreover, the core sequence of FPB was subsequently noted to exhibit some homology with FP7, a cis-acting element of the α1(XI) collagen (COL11A1) promoter (6). Indeed, the EMSA documented the ability of FPB, but not its mutant version, to compete with FP7 for binding to nuclear protein(s) (6).

The three-amino acid loop extension (TALE) class of homeo-proteins constitutes a set of transcription factors that bind DNA in heterodimeric form. Members of this class include mammalian MEIS1 and PREP1, as well as Drosophila Exd and Caenorhabditis elegans Ceh-20 (7). These proteins are able to specifically interact with PBX, a homeodomain transcription factor that in turn is able to form high affinity DNA-binding complexes with several HOX gene products (reviewed in Refs. 8 and 9). Using different interaction surfaces PBX can form a trimeric complex with HOXB1 and PREP1 or MEIS1 (10–12). These ternary complexes have been shown to have important gene regulating properties in mouse and Drosophila embryos (10–12). Dimeric complexes of PBX with PREP1 interact with other homeodomain proteins like D PX1 on the somatostatin promoter, where they display cooperative functional effects on transcription and possibly on the human urokinase enhancer (13–16). Three different PBX genes (PBX1, 2, and 3) code for five proteins, two of which (PBX1b and PBX3b) arise by alternative splicing (17–19). So far no information is available on the function of the single PBX isoforms.

In the present study, we identify the nuclear factors binding to the FPB element of COL5A2 and demonstrated that they differ from those that interact with the FP7 element of COL11A1. The results implicate members of the homeotic complex (HOX) and the TALE class of homeoproteins in COL5A2 regulation, specifically PBX1/2, PREP1, and HOXB1. We dem-

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‡ This abbreviations used are: EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; COL5A2, α2(V) collagen gene; COL11A1, α1(XI) collagen gene; bp, base pair; TK, thymidine kinase.
onstrate that cooperative interactions between PBX1 and PREP1 or between PBX1 and HOXB1 lead to binding to FPB of the resulting heterodimers and to transactivation of the COL5A2 promoter. Having in mind the intrinsic limitations of the experimental design employed in this study, we suggest that different combinations of PBX, PREP, and HOX proteins may correlate with tissue-specific expression of the COL5A2 gene.

MATERIALS AND METHODS

Cell Cultures and Transfection Experiments—The human cells used in this study were the COL5A2-positive rhabdomyosarcoma line A-204 and the COL5A2-negative fibrosarcoma line HT-1080 (5). A-204 were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and HT-1080 in minimum essential medium supplemented with 10% fetal calf serum. Purification and transfection of plasmid DNA into cells using FuGene 6 (Roche Molecular Biochemicals) and assessment of chloramphenicol acetyltransferase (CAT) reporter gene activity were performed according to standard protocols (20). Chimeric constructs included the plasmid containing the −150-bp promoter of COL5A2 (−150COL5A2/CAT) and three copies of FPB subcloned 5′ to the basal promoter of the thymidine kinase (TK) gene (FPB,TK/CAT) (5, 21). Mutations were introduced either in the COL5A2 (m1COL5A2/CAT and m2COL5A2/CAT) or TK (m3FB3TK/CAT) promoters. Wild type and mutant constructs were engineered using standard methodologies of DNA manipulation (20). CAT activities were normalized against the activity of a co-transfected vector that expresses β-galactosidase under the control of the cytomegalovirus promoter. Expression vectors for PBX1, PREP, PREP1-HD, and HOX proteins have been described before (22, 23). Transfections were performed multiple times in duplicate, and the statistical value of the resulting data was evaluated by the Student’s t test.

In Vitro Binding Assays—Crude nuclear extracts were purified according to our published protocol (6). Production of recombinant proteins was carried out using 4 μg of plasmid DNA and a commercial in vitro transcription/translation kit according to the manufacturer's recommendations (Promega, Madison, WI). Approximately 5 μg of crude nuclear extracts and between 2 and 8 μl of the reticulocyte lysate were used in the EMSA together with ~20,000 dpm of labeled oligonucleotides. Unlabeled competitors were added in 100-fold molar excess. Synthetic peptides QPQIYPWMRKLH and QPQIYPFMRKLH were added at the concentrations detailed in the figure legends. When appropriate, nuclear extracts were preincubated with antibodies before addition of the labeled probe. Antibodies against PBX proteins and PREP1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and those against HOXB1 from Babco (Berkeley, CA). Anti-MEIS1 antibodies were kindly provided by Dr. M. Cleary (University of California, San Francisco). The sequences of the oligonucleotide probes used in this study are listed in Fig. 1.

RESULTS

The PBX-PREP1 Complex Binds to FPB but Not to FP7—A combination of cell transfections and DNase I footprinting experiments originally identified FPB (−149 to −115) as a major contributor to cell type-specific transcription of the minimal COL5A2 promoter (5). Methylation interference analysis narrowed the region of contact with nuclear proteins to around the bipartite sequence 5′-ATCAATCA-3′ located within the −130 to −115 region (Fig. 1). Inspection of the complementary sequence of the FPB motif revealed that it corresponds to the recognition sequence 5′-TGATTGATT-3′ of the PBX homeodomain (24–26). Based on this evidence, we assessed the possible involvement of PBX proteins in COL5A2 regulation.

Nuclear extracts purified from COL5A2-positive cells were incubated with antibodies against members of the PBX family and then employed in the EMSA using the FPB oligonucleotide as a probe. FPB has been previously shown to yield a pattern that consists of two closely migrating complexes (Fig. 2A) (5). The faster migrating complex (F in Fig. 2A) was supershifted by the anti-PBX1 antibody, whereas the intensity of the slower migrating complex (S in Fig. 2A) decreased after preincubation with the anti-PBX2 antibody. By contrast, there was no effect

Fig. 1. Nucleotide sequence of the COL5A2 element examined in this study. At the top is a schematic representation of the proximal promoter with the sequence of FPA and FPB, and the core element of the former indicated by bold letters. The nucleotide substitutions of the mutant oligonucleotides m1, m2, and m3 are italicized in the sequences. The FP7 element of the COL11A1 promoter is shown at the bottom for comparison purpose.

Fig. 2. Characterization of the FPB complex by the EMSA. A and B, incubation of the radiolabeled FPB oligonucleotide with nuclear proteins from A-204 cells after addition of antibodies (a) against PBX1, PBX2, PBX3, PREP1, and MEIS1 or in the presence of 100-fold molar excess of the unlabeled FPB (B). The retarded bands of the FPB complex are indicated by the letters S and F. They correspond to FPB2-PREP1 and PBX1a-PREP1 or PBX1b-PREP1 heterodimers, respectively. The arrow points to the position of the supershifts. C, competition with the indicated molar excess of mutant oligonucleotides m1 and m2 (see Fig. 1).
PBX-containing complexes (Fig. 2A). PBX proteins heterodimerize with MEIS1 or PREP1, and these cooperative interactions stabilize PBX binding to target DNA sites (13, 14, 23, 27–30). Specific antibodies were therefore employed to characterize the PBX1 partner(s) in the FPB complex. Preincubation of the A-204 nuclear extracts with anti-PREP1 antibody, but not with anti-MEIS1 antibody eliminated formation of both F and S complexes (Fig. 2B). Binding specificity was documented by the lack of competition by the oligonucleotides with single (5′-ATCAAgCA-3′) or double (5′-cgCAATCA-3′) substitutions at FPB sites known to interact with PBX-containing complexes (Fig. 2C) (8).

It has been recently reported that different combinations of PBX, PREP1, and MEIS1 proteins synergize on a particular promotor element by binding to closely adjacent sequences (10, 12). A similar situation may exist in the COL5A2 promoter given that FPA and FPB are in close proximity to each other and are both required for optimal promoter activity (Fig. 1) (5). Accordingly, the antibody interference test was repeated using the longer 73-bp probe that includes FPA and FPB (Fig. 1). The 73-bp probe has been shown to yield an additional and slower migrating doublet (complex SS in Fig. 3A), in addition to the FPB doublet (Fig. 2). Unlike the FPB bands, however, the SS doublet was not affected by preincubation with any of the antibodies (Fig. 3A). Altogether, these in vitro results implicated binding of PBX-containing dimers to FPB and excluded binding of MEIS1 to the 73-bp long cis-acting element of COL5A2.

Co-expression of the COL5A2 and COL11A1 genes in selected tissues is responsible for the formation of collagens VXI heterotypic fibrils, whose contribution to matrix assembly and function is yet to be determined (1). We have previously reported that the FPB sequence effectively competes binding of nuclear proteins to FP7, a cis-acting element of COL11A1 (6). To test whether the two genes share regulatory elements that are bound by the same trans-acting factors, we analyzed the composition of the FP7 complex using the same set of antibodies employed in the characterization of the FPB complex. Neither the anti-PBX nor anti-PREP1 antibodies supershifted the FP7 complex, thus demonstrating that distinct nuclear proteins recognize closely related DNA motifs in two coordinately expressed collagen genes (Fig. 3B).

PBX1-PREP1 and PBX1-HOXB1 Heterodimers Bind to FPB—In addition to interacting with PREP1, PBX1 dimerizes with HOX proteins via the pentapeptide motif YPWMK (31–35). Moreover, PREP1 and PBX1 can also form ternary complexes with HOX proteins (10, 12, 23). In light of these possibilities, we tested whether HOX proteins participate in the formation of the FPB complex and, if so, whether they give rise to an alternative heterodimer of PBX1 or a ternary complex that includes both PBX1 and PREP1. To simplify the analysis, only the longer PBX1a isoform (herein referred to as PBX1) was used in the assays (19, 30).

PBX1, PREP1, and HOXB1 proteins were translated in vitro, and the binding patterns of different combinations of them were examined by the EMSA. As expected, no binding was observed with PREP1 alone, and very little was observed with PBX1 alone (Fig. 4A, lanes 3 and 4). By contrast, addition of PREP1 and PBX1 together yielded an intense band migrating comparably to the S complex in crude nuclear extracts (Fig. 4A, lanes 1 and 6). A faster migrating complex was observed with HOXB1 alone, and a slower complex was observed with the HOXB1-PBX1 combination (Fig. 4A, lanes 4 and 5). We also noted that the migration of the PBX1-HOXB1 complex is slightly different from that of the PBX1-PREP1 combination (Fig. 4A and B, lanes 6 and 7). Addition of increasing amounts

![Fig. 3. Characterization of the FPA/FPB and FP7 complexes by the EMSA. A, incubation of the radiolabeled 73-bp oligonucleotide with nuclear proteins from A-204 cells after addition of antibodies (α), or in the presence of 100-fold molar excess of unlabeled 73-bp sequence (lane AB). The position of the supershifts is signified by the arrow. The doublet band of the FPA complex is identified by the letters SS. B, incubation of radiolabeled FP7 oligonucleotide with nuclear proteins from A-204 cells after addition of the same set of antibodies (α) as in A or in the presence of 100-fold molar excess of unlabeled FP7 (lane 7) or FPB (lane B) oligonucleotides. Control samples are indicated by (−).](http://www.jbc.org/)

when the nuclear extracts were preincubated with anti-PBX3 antibody (Fig. 2A).
of HOXB1 to the PBX1-PREP1 combination had no noticeable effect on the migration or the intensity of the heterodimer, thus apparently excluding the formation of a ternary complex (Fig. 4B). Binding specificity was supported by the lack of binding of the recombinant proteins to mutant FPB oligonucleotides (data not shown). Preincubation of each of the samples with the various antibodies further corroborated binding specificity as they supershifted or inhibited formation of the retarded complexes (Fig. 4C). The sole exception was the much weaker effect observed with the combination of the three nuclear proteins when preincubated with the anti-HOX antiserum (Fig. 4C, lane I). This last result was at variance with the strong anti-HOX antibody effect observed in the PBX1-HOXB1 combination (Fig. 4C, lane 5). Because PBX1-HOXB1 and PBX1-PREP1 co-migrate, the effect of the anti-HOX antibodies may conceivably be minor if PBX1-HOXB1 dimers represent only a minor fraction of the retarded band. Indeed, a faint supershift at the same location as in the sample with the PBX1-HOXB1 combination could be seen after overexposure of the EMSA sample in which the three nuclear proteins had been preincubated with the anti-HOX antibody (data not shown).

The above results excluded the formation on the FPB probe of a ternary complex inclusive of HOXB1. This conclusion was further corroborated by two additional lines of evidence. First, formation of retarded complexes by recombinant proteins was challenged with increasing amounts of the peptide QPQIYPW-MRKLH, which has been previously shown to mediate the PBX-HOX interaction (31). As a control, the same samples were preincubated with a mutant version of the HOX peptide that contains F in place of the obligatory W residue (31). The EMSA documented the ability of the wild type, but not the mutant peptide, to disrupt the recombinant PBX1-HOXB1 complex (Fig. 5A). Surprisingly, we found that the wild type peptide specifically eliminates the recombinant PBX1-PREP1 heterodimer as well (Fig. 5A). Consistent with the above findings, increasing amounts of the peptide also interfered with the formation of the S and F complexes in crude nuclear extracts from A-204 cells (Fig. 5B). Along these lines, the anti-HOXB1 antibodies had no effect on the binding of nuclear proteins to FPB (data not shown).

The second set of experiments examined binding of recombinant proteins to FPB in the presence of a PREP1 mutant. The mutant (PREP1-HD) still interacts with PBX1 but is unable to bind to DNA because of the deletion of the homeodomain (23). PREP1-HD has been previously shown to eliminate binding of the PBX1-PREP1 heterodimers to the autoregulatory element of the mouse HOXB1 gene while concomitantly favoring formation of the ternary PREP1-PBX-HOX complex (23). EMSAs were performed under conditions that discriminate between the PBX1-PREP1 and PBX1-HOXB1 heterodimers (complexes I and II in Fig. 5C, respectively) (23). They revealed that PREP1-HD did not form a DNA-binding complex with PBX1, and in the three-protein combination, this led to the selective loss of mostly band I that corresponds to the PBX1-PREP1 dimer (Fig. 5C). In conclusion, the DNA binding assays excluded formation of a ternary complex with crude nuclear extracts and demonstrated that, in the presence of all three recombinant proteins, FPB is only bound by PBX1-PREP1 and PBX1-HOXB1 heterodimers.

**Transactivation of the FPB Element by PBX1, PREP1, and HOXB1**—To provide a functional correlate to the DNA binding experiments, various combinations of the HOX and TALE proteins were expressed in A-204 cells co-transfected with different reporter plasmids. They included the wild type −150COL5A2 promoter and two mutant versions that harbor the nucleotide substitutions that eliminate protein binding. Furthermore, three copies of the wild type and mutant FPB sequence were linked to the basal TK promoter and tested as well.

Transcription from the −150COL5A2/CAT plasmid remained nearly the same when PBX1, PREP1, or HOXB1 were expressed singularly or using the PBX1-PREP1 combination (Fig. 5A). By contrast, a modest 2-fold increase was observed with the PBX1-HOXB1 combination, and a more robust 5-fold stimulation was observed with all three proteins (Fig. 6A). Transcriptional stimulation by the latter combination was slightly less when the PBX1b isoform was expressed in place of PBX1a (data not shown). Qualitatively similar results were obtained with the basal TK promoter harboring three copies of the FPB sequence (Fig. 6B). The specificity of the transactivation by the three-protein combination was documented in co-transfection experiments with mutant COL2A5 and TK promoter constructs (Fig. 6C). These last results are somewhat more consistent with cooperative binding of the three nuclear factors than with the additive contribution of the two PBX1-containing heterodimers.

The Composition of the FPB Complex Is Cell Type-specific—We have previously reported subtle differences in the migration pattern of the retarded bands that are formed between FPB and nuclear proteins from COL5A2-positive or COL5A2-
negative cells (5). We have argued that these differences may reflect the distinct composition of the FPB complexes in these two groups of cells. Antibodies against PBX1, PBX2, and PREP1 were therefore used to identify the binding factors in nuclear extracts purified from HT-1080 and Jurkat cells. This revealed that the nuclear factors that interact with FPB in these COL5A2-negative cells are different from those of the A-204 cell line. Both Jurkat and HT-1080 nuclear proteins yielded a single retarded band that contains PBX2 and PREP1 but no PBX1 or MEIS1 (Fig. 7). Taken at face value, the results therefore implicated PBX1 in COL5A2 gene activation.

To test the above hypothesis, we co-transfected the COL5A2 promoter together with the PBX1- and PREP1-expressing plasmids in HT-1080 cells. Although only the data of the PBX1a overexpression are shown here, the PBX1b plasmid yielded the same kind of results. Overexpression of PBX1 or PREP1 resulted only in a modest stimulation of the COL5A2 promoter (Fig. 8A). On the other hand, a robust ~4-fold stimulation of the −150COL5A2/CAT plasmid was observed with the combination of both PBX1 and PREP1 (Fig. 8A). Thus, COL5A2
promoter activity in HT-1080 cells may be limited by the amount of available PREP1 available to dimerize with PBX1. This conclusion was indirectly supported by the results of antibody interference experiments performed with nuclear extracts purified from HT-1080 cells transfected with PBX1 singularly or with PBX1 and PREP1. Overexpression of PBX1 in HT-1080 cells had no effect on the FPB binding pattern, as judged by the lack of supershift with the anti-PBX1 antibody (Fig. 8B). A PBX1-specific supershift was instead visible when PBX1 and PREP1 were co-expressed in HT-1080 cells (Fig. 8B). Furthermore, binding of PBX1-PREP1 heterodimers was not accompanied by the loss of the endogenous PBX2-PREP1 complex (Fig. 8B). We interpret these results to indicate that exogenously added PBX1 may not bind to FPB because the endogenous pool of PREP1 is already complexed with PBX2. Regardless of the interpretation, the DNA binding and co-transfection data correlate with variations in FPB composition and cell type-specific expression of COL5A2.

**DISCUSSION**

The fibrillar collagens represent an interesting example of genes that are constitutively expressed with widely disparate patterns in cell lineages of common embryonic origin (1). Not surprisingly, current evidence indicates that spatiotemporal regulation of the fibrillar collagen genes involves combinatorial interactions of ubiquitous and tissue-restricted transcription factors.
Collagen Regulation by Homeoproteins

Fig. 7. Characterization of FPB complexes in COL5A2-negative cells. EMSAs were performed using the FPB probe and nuclear proteins from HT-1080 (A) and Jurkat (B) cells preincubated with various antibodies (α) or in the presence of 100-fold molar excess of unlabeled FPB (β). Control sample is designated by (−). The positions of the FPB complex, the supershifts, and an unspecific band co-migrating with the latter are respectively indicated by the arrowhead, arrow, and asterisk.

Factors that bind to modularly organized cis-acting elements (2, 36). In most cases, these regulatory sequences are promoter-specific rather than being shared by coordinately expressed collagens. A case in point is the formation of distinct nuclear complexes by the closely related cis-acting elements of the COL5A2 and COL11A1 genes.

We have shown that FPB and FP7, although capable of competing with each other for protein binding, interact with different nuclear factors. This observation suggests that binding specificity is probably dependent on the context of the cis-acting element. Indeed, identical PREP1-PBX sites may or may not bind PREP1-PBX1a heterodimers depending on the nature of the surrounding sequences (11). We have provided in vitro evidence that PBX1/2, PREP1, and HOXB1 bind to FPB cooperatively by forming binary complexes and that selected combinations of them transactivate the COL5A2 promoter. Furthermore, we have shown that variations in the composition of the FPB-bound complex (specifically, the inclusion of different PBX proteins) correlate with cell type-specific expression of the COL5A2 gene. To the best of our knowledge and within the limitations of the experimental design employed, this is the first indication of distinct contributions to transcription by different members of the PBX family of proteins.

It is now well established that interactions between HOX and TALE proteins of the PBX/Exd subtype determine specificity and modulate affinity of DNA binding (8, 9, 29, 37–41). Indeed, there is ample genetic evidence suggesting that formation of these interacting complexes plays a key role in the cell lineage-specific interpretation of regulatory clues by the developing embryo (11, 12, 40–46). In addition to interacting with HOX proteins, PBX/Exd molecules heterodimerize with more distantly related members of the TALE family (MEIS/hth and Pknox1-PREP) to regulate expression of several genes (37). Cooperative interactions have also been reported to occur between HOX and MEIS-like proteins and among HOX, MEIS, and PBX and HOX, PBX, and PREP-1 (37). These various combinations of nuclear factors recognize slightly different binding sites with a spectrum of affinities and distinct transcriptional activities. Moreover, different PBX and MEIS protein isoforms with distinct spatiotemporal patterns are produced during mouse embryogenesis (43, 48). As a result, the range of DNA targets of this group of homeoproteins is very broad and has the potential of regulating a wide variety of genes.

Our transient transfection tests and in vitro DNA binding assays strongly indicate that cell type-specific activation of COL5A2 transcription depends on interactions among PBX1/2, PREP1, and HOXB1. However, the two experimental approaches we employed leave unresolved whether or not a ternary complex can also be formed on the FPB element. On the one hand, in vitro DNA binding tests exclude this possibility by documenting the mutually exclusive binding of different PBX-containing heterodimers; on the other hand, the co-transfection experiments are more in line with the idea of cooperativity among the three factors for maximal COL5A2 transactivation. Thus, although solely based on correlative evidence, the notion of a ternary complex has not been rigorously ruled out.

As already alluded to, the problem of solving the in vivo identity of the FPB complex was compounded by the unexpected finding that the QPIQYPWMRKLH peptide can also block formation of recombinant PBX1-PREP1 dimers. Taken at face value, this last result could be interpreted to indicate that the sites of PBX-PREP1 and PBX-HOX interaction may share some sequence homology. According to recent evidence, a possible candidate sequence is the extra long loop between the first and second α-helix of the TALE homeodomain (12). Should this assumption be correct, one could argue that the relative concentrations of PREP1 and HOX proteins may represent an additional level of regulation for the formation of specific combinations of PBX-containing heterodimers. Because PBX-PREP1 dimers interact via the amino-terminal regions, whereas PBX-HOX interactions require the homeodomain and nearby sequences, the postulated sequence homology is not readily apparent (22, 23). Conceivably, it may reside in the tertiary structure arrangement of PREP1 that brings together key amino acid side chains.

Although the precise nature of the in vivo complex remains to be determined, the binding specificity and its effect on promoter activity were corroborated by the concordant results of mutating the bipartite sequence of FPB (5′-ATCAATCA-3′) on DNA binding and promoter transactivation. The mutations (5′-cgCAATCA-3′ and 5′-ATCAATCA-3′) involve those nucleotides that are required for recognition by the PBX1 (TGAT) and HOX (TNA/TTC) proteins (34, 36). The FPB core most closely resembles the recognition sequence for PBX1 dimerized with HOX proteins of the paralog group 1 (29, 35). Consistent with this observation, we found that HOXA1 is the only other HOX member that binds to DNA after interacting with recombinant PBX1 (data not shown). However, the in vitro interaction is likely not to be physiologically relevant because co-expression...
nuclear extracts from HT-1080 cells transfected with combinations of genes was arbitrarily set at 1. Promoter activities were expressed as fold stimulation against the activity of the control sample, normalized against the co-transfected CMV-LacZ plasmid and expressed per mg of the indicated expression plasmids. Promoter activities were assessed by EMSA performed with the FPB probe and by gel shift analysis of HT-1080 cells with 2 mM 35S-labeled genomic DNA fragments containing the FPB regulatory region. The positions of the FPB complex, the supershifts with antibodies to the indicated proteins, and an unspecific band co-migrating with the latter are respectively indicated by the arrow, arrowhead, and asterisk.

A final interesting finding pertains to the relationship between the FPB complex and cell type-specific expression of COL5A2. Within the intrinsic limitation of the experimental model used in this study, exclusion of PBX1 from the FPB complex appears to be associated with lack of gene activity. Furthermore, overexpression of PBX1 alone cannot restore COL5A2 expression or lead to inclusion of PBX1 in the FPB-bound complex. Even though there is enough endogenous PREP1 to complex with PBX2, we found that inclusion of PBX1 in the FPB complex and promoter transactivation in COL5A2-negative cells require overexpression of both PBX1 and PREP1. Cell type-specific transcription of COL5A2 may therefore be modulated by changes in the relative amounts of the PBX proteins and/or their affinities for the pool of available PREP1 molecules. Additional levels of regulation may include the amounts and identities of the HOX partners and, consequently, the ability of the resulting PBX-HOX dimers to compete more or less effectively with PBX-PREP1 complexes for FPB binding.

The combinatorial alternatives of the FPB complex are compatible with the available data on HOX, TALE, and COL5A2 gene expression during mouse embryogenesis (47–50). COL5A2 transcripts are first detectable, widely and diffusely distributed in the intestinal and craniofacial mesenchyme of the 12.5-day embryo (50). HOXB1, PREP1, and PBX family members also display a widespread embryonic expression, which begins earlier in development (48, 49). Even though the mesenchymal tissues in which COL5A2 is expressed have not been analyzed, the available data are, however, consistent with a co-expression of COL5A2 and PREP1, PBX, and HOXB1 at later stages of development.

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PBX1 and PREP1 expressing plasmids were preincubated with the respective antibodies (a). The positions of the FPB complex, the supershifts and an unspecific band co-migrating with the latter are respectively indicated by the arrowhead, arrow, and asterisk.
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Cooperative Interactions between PBX, PREP, and HOX Proteins Modulate the Activity of the \( \alpha2(V) \) Collagen (COL5A2) Promoter

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