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Meox Homeodomain Proteins Are Required for *Bapx1* Expression in the Sclerotome and Activate Its Transcription by Direct Binding to Its Promoter

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The axial skeleton of vertebrates derives from the sclerotomal compartment of the somites. Genetic analysis has demonstrated that the transcription factors Pax1, Pax9, Meox1, Meox2, and Bapx1 are all required for sclerotomal differentiation. Their hierarchical relationship is, however, poorly understood. Because *Bapx1* expression in the somites starts slightly later than that of the *Meox* genes, we asked whether *Bapx1* is one of their downstream targets. Our analysis of *Meox1*; *Meox2* mutant mice supports this hypothesis, as *Bapx1* expression in the sclerotome is lost in the absence of both Meox proteins. Using transient-transfection assays, we show that Meox1 activates the *Bapx1* promoter in a dose-dependent manner and that this activity is enhanced in the presence of Pax1 and/or Pax9. Furthermore, by electrophoretic mobility shift and chromatin immunoprecipitation experiments, we demonstrate that Meox1 can bind the *Bapx1* promoter. The palindromic sequence TAATTA, present in the *Bapx1* promoter, binds the Meox1 protein in vitro and is necessary for Meox1-induced transactivation of the *Bapx1* promoter. Our data demonstrate that the *Meox* genes are required for *Bapx1* expression in the sclerotome and suggest that the mechanism by which the Meox proteins exert this function is through direct activation of the *Bapx1* gene.

In vertebrates, the axial skeleton originates from the sclerotome of somites, which lie on either side of the neural tube and the notochord. Soon after their formation, the epithelial somites start to differentiate into a ventromedial mesenchymal compartment, the sclerotome, and a dorsolateral epithelial compartment, the dermomyotome. The sclerotome gives rise to the vertebral bodies and intervertebral disks of the vertebral column, the ribs, and the neural arches. This entire process requires the coordinated action of various regulatory molecules, including morphogenetic regulators of the homeodomain type (8) such as *Meox1* and *Meox2* (also known as *Mox1* and *Mox2*, respectively). These two genes constitute a subfamily of antennapedia-like homeobox-containing transcription factors which are expressed in a wide range of mesodermal structures (3, 4).

Meox1 is expressed in the presomitic mesoderm that will form the somites, in the epithelial somites, and in the sclerotome and dermomyotome of developing somites, whereas *Meox2* is expressed in the epithelial somites and in the sclerotome (3, 4). Mice lacking the *Meox1* gene show vertebral abnormalities, including hemivertebrae, occipitovertebral fusions, and kinks in the tail (B. S. Mankoo, unpublished observations), indicating that *Meox1* is required for the proper formation of the sclerotome derivatives. In contrast, *Meox2* null mice exhibit defects in limb musculature but no vertebral abnormalities (13). Nevertheless, the recent analysis of mutant mice doubly deficient in *Meox1* and *Meox2* shows that *Meox2* is also involved in the formation of the axial skeleton, as there is a dramatic increase in the severity of the sclerotomal defects compared with the single *Meox1* null mice. In animals lacking both Meox proteins, the sclerotome is specified but does not differentiate properly (14). While these studies have clearly established the function of the *Meox* genes in sclerotome differentiation, their mechanisms of action and their downstream target genes still need to be determined.

Bapx1, the mouse homologue of *Drosophila bagpipe*, is another homeobox-containing gene expressed in the sclerotome of somites (25), with a domain of expression that overlaps that of the *Meox* genes. Functional and genetic analyses have shown that *Bapx1*, like *Meox1*, is essential for chondrogenic differentiation and for the proper formation of the axial skeleton (1, 9, 11, 16, 26). *Bapx1* expression in the sclerotome is dependent on the activity of two paired box transcription factors of the Pax family, encoded by *Pax1* and *Pax9* (21), whose expression in the sclerotome is also required for proper formation of the vertebral column (19, 27). It has recently been shown that Pax1 and Pax9 bind and activate the *Bapx1* promoter (21). Furthermore, Pax1 interacts with Meox1 in a yeast two-hybrid system and in pulldown assays (22). Together, these data clearly indicate that Pax1, Pax9, Bapx1, Meox1, and Meox2 are all key players in the genetic cascade that leads to the formation of the axial skeleton. The precise functional relationships among them, however, have only been established for Pax1/Pax9 and Bapx1 (21).

Though the *Meox1*, *Meox2*, and *Bapx1* expression domains largely overlap, the initiation of *Bapx1* expression in the somites starts slightly later than that of *Meox1* and *Meox2* (3, 25). In addition, *Meox1* is still expressed in the sclerotome of

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Bapx1-deficient embryos (12), indicating that Bapx1 is not upstream of Meox1. These data suggest the possibility that *Bapx1* activation may also depend on the *Meox* activity. To test this hypothesis, we analyzed *Bapx1* expression in mouse embryos deficient for all *Meox* gene function and observed that *Bapx1* is lost in the sclerotome of *Meox1*; *Meox2* double mutant mice, indicating that it is genetically downstream of the *Meox* genes. In addition, we investigated whether Meox1 protein can directly regulate *Bapx1* expression by activating its promoter. In transient-transfection assays, we show here that Meox1 activates the *Bapx1* promoter in a dose-dependent manner and that this activation is enhanced by the presence of Pax1 and/or Pax9. We demonstrate that Meox1 binds in vitro to a TAATTA core motif present in the 5′ region of the *Bapx1* gene and that this region is necessary for Meox1-induced transactivation of the *Bapx1* promoter. Furthermore, using chromatin immunoprecipitation (ChIP), we confirm that Meox1 binds to both the mouse and human *Bapx1* promoters. These data demonstrate that the *Meox* genes are upstream of *Bapx1* in the genetic pathways that lead to chondrogenic differentiation and axial skeleton formation and suggest that the Meox proteins control *Bapx1* expression through direct activation of its promoter.

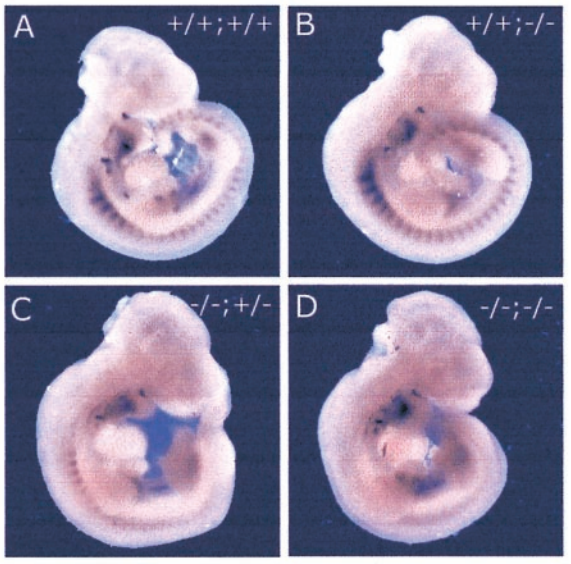

FIG. 1. Meox proteins are required for *Bapx1* expression in the sclerotome. Whole-mount in situ hybridization for *Bapx1* on embryonic day 9.5 mouse embryos with the indicated genotypes. (A) Wild-type control, $Meox1^{+/+}$; $Meox2^{+/+}$; (B) mutant $Meox1^{+/+}$; $Meox2^{-/-}$. Note that the expression of *Bapx1* in the sclerotome is weaker in the $Meox1^{-/-}$; $Meox2^{+/-}$ embryo (C), whereas in the $Meox1^{-/-}$; $Meox2^{-/-}$ mutant, expression is totally lost (D).

MATERIALS AND METHODS

In situ hybridization. *Meox1*; *Meox2* mutant embryos were generated and genotyped as previously described (13, 14). Whole-mount in situ hybridization was performed with a mouse *Bapx1* probe (12) as described previously (17).

Plasmids for transactivation and ChIP assays. (i) Expression plasmids. The Meox1 expression plasmid pMeox1 contains a Myc-tagged mouse *Meox1* cDNA amplified by PCR and cloned in pcDNA1 (Invitrogen) downstream of the cytomegalovirus promoter. The Meox2 expression plasmid pMeox2 contains the entire coding region of the mouse Meox2 cDNA cloned into pCDNA3 (Invitrogen), downstream of the cytomegalovirus promoter. Similarly, the Pax1 and Pax9 expression plasmids contain mouse *Pax1* and *Pax9* coding sequences, respectively, cloned in pcDNA3 (Invitrogen).

(ii) Reporter plasmids. p5.3Bp-luc, p2.8Bp-luc, p1.9Nk-luc, p0.9Bp-luc, and p0.7Bp-luc, containing genomic sequences of the mouse *Bapx1* gene at 5′ end positions −5285, −2762, −1947, −880, and −748 and 3′ end position +109, respectively (+1 is the first nucleotide in the published cDNA sequence, with GenBank accession number U87957), cloned into the pGL3-Basic vector (Promega) with the firefly luciferase gene as the reporter, have been described elsewhere (21). The pGL3-Promoter vector (Promega) directs firefly luciferase expression by the simian virus 40 (SV40) promoter. The pRL-SV40 vector (Promega) contains the reporter *Renilla* luciferase gene upstream of the SV40 early enhancer-promoter and was used to normalize the transfection efficiency among different experiments. pVNC3EGFP codes for the green fluorescence protein (GFP) (20) and was used to monitor the transfection efficiency in the ChIP assays.

Transient-transfection assays. NIH 3T3 mouse embryonic fibroblasts were plated in six-well plates in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum (Gibco-BRL). When the cells reached about 35% confluence, the DNA was transfected with Lipofectamine Plus reagent (Invitrogen). In each experiment, 0.5 μg of firefly reporter plasmid was cotransfected with different amounts of pMeox1, pPax1, pPax9, or pcDNA3 control vector. The total amount of transfected DNA was made equal in each experiment by using pcDNA3. In addition, 20 ng of pRL-SV40 vector was always cotransfected for normalization. Forty-two hours after transfection, when the cells were at about 100% confluence, cell extracts were collected and firefly and *Renilla* luciferase activities were measured (dual luciferase reporter assay; Promega). The firefly luciferase activity in each sample was normalized to that of *Renilla* luciferase to correct for variations in transfection efficiency. For each assay, two to nine experiments were performed in duplicate.

EMSA. The electrophoretic mobility shift assay (EMSA) was performed as previously described (21). Meox1, Meox2, and luciferase (control) proteins were synthesized with the TNT-coupled wheat germ extract system (Promega). For most of the reactions, 1 μl (0.5- to 2-μl volumes were also tested and gave similar results) of protein translation extracts was incubated at room temperature for 15 min with each corresponding ^{32}P-labeled double-stranded oligonucleotide in 15 μl of binding reaction mixture. In competition assays, a 250- or 500-fold excess of unlabeled double-stranded oligonucleotide was incubated at room temperature for 15 min before addition of the labeled oligonucleotide. In reactions including antibodies, 1 μl of rabbit polyclonal antibody against Meox1 (4), 1 μl of rabbit polyclonal antibody against Meox2 (4), or 1 μl of anti-Pax1 goat polyclonal antibody (M-19; Santa Cruz Biotechnology) as a control was added and incubated for 20 min at room temperature before addition of the probe. DNA fragments A, B, and C (shown in Fig. 4A) were obtained by digestion of the p0.9Bp-luc plasmid: HindIII/BbvCI 370-bp fragment A, BbvCI/Eco47III 375-bp fragment B, and Eco47III/NotI 426-bp fragment C. The sequences of oligonucleotides B4, B5, B6, B7, and B8 from the *Bapx1* promoter region are indicated in Fig. 6. The oligonucleotides m1, m2, m3, m4, m2/3, and m2/3/4 are like oligonucleotide B7 but with the mutations indicated in Fig. 7A. The sequence of the control oligonucleotide SP1 is ATTCGATCGGGGCGGGGCGAGC.

ChIP assay. For the ChIP experiments, extracts were prepared from Meox1-overexpressing human Hek293 cells. Cells were plated on P60 dishes and transfected with ExGen 500 transfection reagent (Fermentas) with either 3 μg of the Meox1 expression vector pMeox1 or 3 μg of pcDNA3 as a control. When indicated, 1.5 μg of the p0.9Bp-luc plasmid, containing sequences of the mouse *Bapx1* promoter, was cotransfected. In all cases, 0.3 μg of the pVNC3EGFP plasmid, encoding GFP, was included to monitor the transfection efficiency. Forty-two hours after transfection, when the cells were at about 100% confluence, the cells were cross-linked with 1% formaldehyde for 10 min.

Chromatin extraction and immunoprecipitations were performed according to the Upstate Biotechnology protocol for ChIP. Briefly, chromatin was fragmented by sonication and precleared with a salmon sperm DNA-protein A-agarose 50% slurry (Upstate Biotechnology) for 2 h at 4°C. Immunoprecipitation was performed by incubation with rabbit polyclonal anti-Meox1 antiserum or with preimmune serum as a control at 4°C overnight. Salmon sperm DNA-protein A-agarose was then added, and rotation was continued for another 2 h. After the immune complexes were washed, the cross-link was reverted by addition of NaCl to 200 mM and heating at 65°C overnight. DNA was treated with proteinase K, extracted with phenol-chloroform, ethanol precipitated, washed, and resuspended in 50 μl. As positive control, the input DNA, representing 25% of each immunoprecipitation reaction, was removed after sonication to omit the immunoprecipitation step. One microliter of each DNA sample was used in the PCRs that were performed at 58°C for annealing for 40 cycles, except that 25 cycles were used to amplify the *mBapx1* promoter. Primers are as follows: *mBapx1*

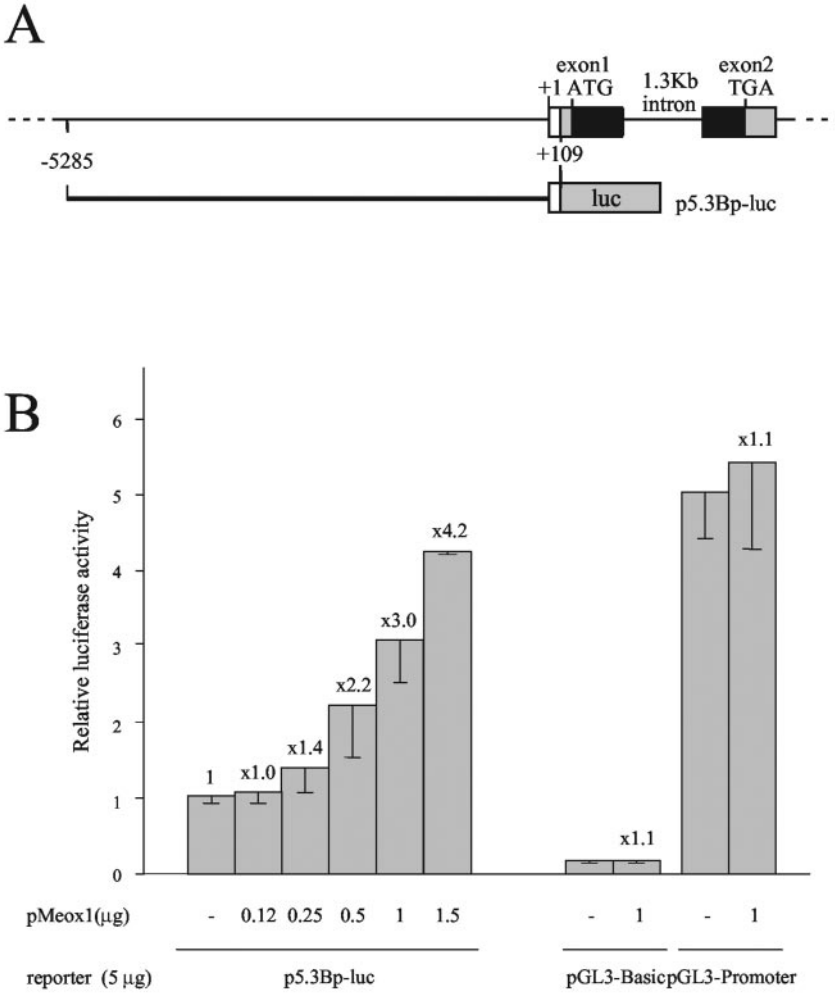

FIG. 2. Meox1 can transactivate the *Bapx1* promoter. (A) Scheme of the *Bapx1* genomic structure and of the p5.3Bp-luc reporter plasmid. The boxes indicate exons of *Bapx1* (top) and the luciferase (luc) reporter (bottom). The coding regions of *Bapx1* are shown by the closed boxes, together with the first ATG codon (position +277) and the stop codon (TGA) in exon 2. Plasmid p5.3Bp-luc contains a 5.4-kb (−5285 to +109) genomic fragment, including sequences upstream of the *Bapx1* promoter and part of the 5′ untranslated region, attached to the luciferase reporter. (B) Promoter activities of the p5.3Bp-luc, pGL3-Basic, and pGL3-Promoter constructs alone and cotransfected with the indicated amounts of expression plasmid for Meox1, pMeox1. Numbers above the bars indicate induction over basal activity. Error bars indicate the standard deviation.

forward, CCAATCCAATCAAACGTAAAATG, and *mBapx1* reverse, GGC GAAAACTCAACAGAAAGC (F1 and R1, respectively, in Fig. 6A), 214-bp fragment; *hBAPX1* forward, CAGAAATTCTCCCAAAGATGC, and *hBAPX1* reverse, TCTCCCTACAGTTTCGCCG, 198-bp fragment; and *hTRP1* forward, GACCTTTCATTCATTGGTTAATTC, and *hTRP1* reverse, TCCTTGTTT GCTGATGATAAGATC, 240-bp fragment.

RESULTS

***Bapx1* expression in the sclerotome is dependent on *Meox* gene function.** The expression domains of *Bapx1*, *Meox1*, and *Meox2* overlap in the sclerotome of the somites, but *Bapx1* activation starts slightly later than that of the *Meox* genes. For this reason, to test whether *Bapx1* is a downstream effector of the Meox proteins, we analyzed *Bapx1* expression in mouse embryos deficient in one or both Meox proteins (Fig. 1). In the absence of *Meox2* when *Meox1* was present (*Meox1*$^{+/+}$; *Meox2*$^{-/-}$), *Bapx1* expression was not observed to be different from that in the control embryos (Fig. 1B). However, when there was only one functional copy of *Meox2* in the absence of *Meox1* (*Meox1*$^{-/-}$; *Meox2*$^{+/-}$), *Bapx1* expression levels in the sclerotome were significantly reduced, although still present (Fig. 1C). Interestingly, in the absence of both Meox proteins (*Meox1*$^{-/-}$; *Meox2*$^{-/-}$, Fig. 1D), *Bapx1* expression in the sclerotome was totally lost, but it was maintained in the other expression domains (mandibular portion of the first branchial arch, splanchnic mesoderm, and mesenchyme of the limb buds) (compare Fig. 1D and A). These results indicate that *Bapx1* is genetically downstream of coordinated *Meox1* and *Meox2* gene function in the sclerotome.

***Meox1* activates the *Bapx1* promoter.** To determine whether the *Meox* genes maintain *Bapx1* expression by direct activation of its transcription or through the activity of intermediate regulators, we tested if Meox1 could activate the expression of the *Bapx1* gene in transient-transfection assays. For this purpose we employed plasmid p5.3Bp-luc, which contained a fragment

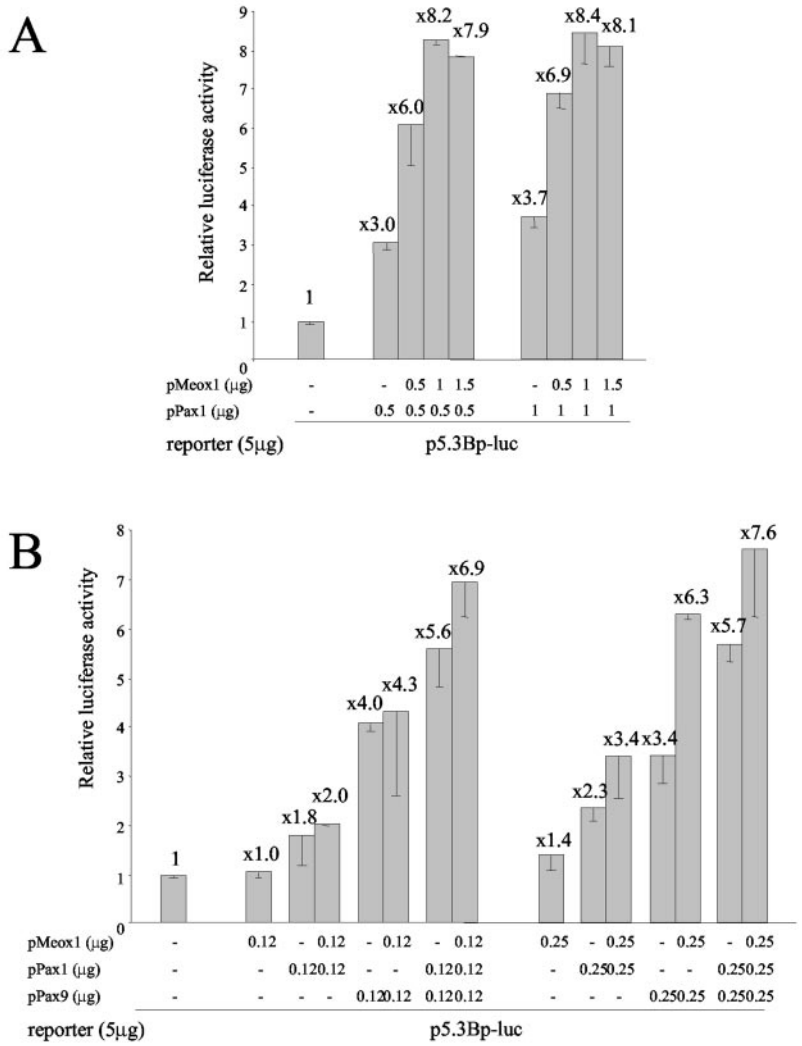

FIG. 3. Meox1, Pax1, and Pax9 contribute to maximal *Bapx1* promoter activity. The promoter activities of the p5.3Bp-luc construct alone and cotransfected with the indicated amounts of expression plasmids for Meox1 (pMeox1), Pax1 (pPax1), and Pax9 (pPax9) are shown. Numbers above the bars indicate induction over the basal p5.3Bp-luc promoter activity. Error bars indicate the standard deviation.

of about 5.5 kb of the mouse *Bapx1* promoter followed by the luciferase reporter gene (Fig. 2A). We have previously shown that this construct is transactivated by Pax1 and Pax9 (21). p5.3Bp-luc was cotransfected with increasing amounts of the Meox1 expression vector pMeox1 in NIH 3T3 cells. Small amounts of pMeox1 (0.12 and 0.25 μg) had no significant effect on the *Bapx1* promoter activity, but larger amounts of pMeox1 (0.5, 1, and 1.5 μg) induced a dose-dependent activation of the *Bapx1* promoter, up to fourfold when 1.5 μg of pMeox1 was employed (Fig. 2B). This transactivation effect of Meox1 was specific for *Bapx1* regulatory sequences, as Meox1 did not activate the control vector pGL3-Basic or pGL3-Promoter, a reporter vector containing an SV40 promoter upstream of the luciferase gene (Fig. 2B).

Maximal *Bapx1* promoter activity is reached in the presence of Pax1, Pax9, and Meox1. The Meox1 and Pax1 proteins can interact in the yeast two-hybrid system as well as in a pulldown assay (22). Furthermore, we have shown that the sclerotomal transcription factors Pax1 and Pax9 can transactivate the *Bapx1* promoter (21). Therefore, we asked whether the Pax1 and Pax9 proteins could cooperate with Meox1 in activating the *Bapx1* promoter in transient-transfection assays. Pax1 alone increased *Bapx1* promoter activity around threefold (21) (Fig. 3A). Interestingly, cotransfection of the p5.3Bp-luc plasmid with increasing amounts of the Meox1 expression vector (0.5, 1, and 1.5 μg) together with the Pax1 expression vector (0.5 or 1 μg) further stimulated the promoter activity eightfold over the basal level (Fig. 3A). The activation levels were similar with 1 or with 1.5 μg of Meox1 expression vector together with Pax1, most probably due to saturation of the reporter activity (Fig. 3A). We next tested the ability of Pax9 to modulate *Bapx1* transactivation in combination with Pax1 and Meox1. To avoid saturation effects, we employed small amounts of the expression vectors. As shown in Fig. 3B, even with a low dose of the expression vectors, maximal *Bapx1* promoter activity was achieved in the presence of the three factors Pax1, Pax9, and Meox1.

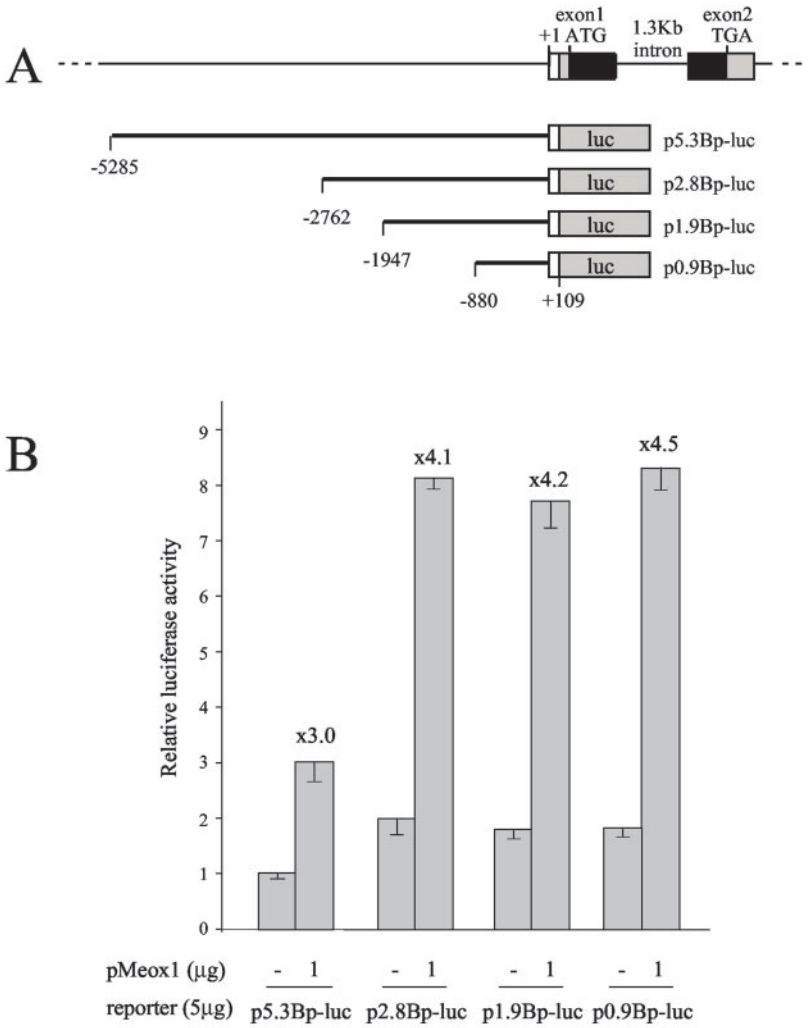

FIG. 4. Meox1 transactivation activity on *Bapx1* promoter deletion constructs. (A) Scheme of the *Bapx1* gene and of the promoter deletion constructs used in transient-transfection assays. (B) Luciferase activity driven by the *Bapx1* promoter deletion plasmids alone or cotransfected with 1 μg of the Meox1 expression plasmid pMeox1. Numbers above the bars indicate induction over the basal activity of the same construct in the absence of Meox1. Error bars indicate the standard deviation.

Meox1 directly binds the *Bapx1* promoter. Consensus sequences for Meox1 binding to DNA have not been characterized so far. Thus, to identify the region of the *Bapx1* promoter responsible for the Meox1-induced transactivation, we performed transient-transfection experiments with a series of deletion constructs of the *Bapx1* promoter (Fig. 4A). The removal of the interval between positions −5285 and −2762 of the *Bapx1* promoter led to an increase (from 3.0- to 4.1-fold) in the Meox1-induced activation of the *Bapx1* promoter, suggesting that this deleted interval contains elements that negatively modulate Meox1 activity (Fig. 4B). Further deletions, up to position −880, did not alter the Meox1 transactivation properties (Fig. 4B). This suggests that the Meox1-responsive element(s) can be located between positions −880 and +109 of the *Bapx1* gene.

To determine the position of this element(s), we performed EMSAs, employing as labeled probes three large overlapping DNA fragments covering the complete −880 to +109 region of the Bapx1 promoter (fragments A, B, and C, as shown in Fig. 5A). In these assays, the in vitro-synthesized Meox1 protein formed a retarded complex with DNA fragment C (Fig. 5B), but no retarded bands were detected when the A or B fragment was used as the probe (not shown). Comparison of the sequence of the mouse *Bapx1* promoter with that of its human counterpart (Blast 2 sequences, NCBI analysis) identified two stretches with a high percentage of sequence identity in the C fragment (shaded in Fig. 6A), between positions −839 and −780 (81% identity) and between positions −751 and −702 (84% identity). In addition, several putative consensus motifs for transcription factors of the homeodomain type (underlined in Fig. 6A) were detected after analysis with the MatInspector program (Genomatix, http://www.genomatix.de). On this basis, we selected oligonucleotides B4, B5, and B6 (Fig. 6A) for further EMSA experiments. When these labeled oligonucleotides were incubated with Meox1 protein, a specific retarded complex was observed only with the B5 oligonucleotide (Fig.

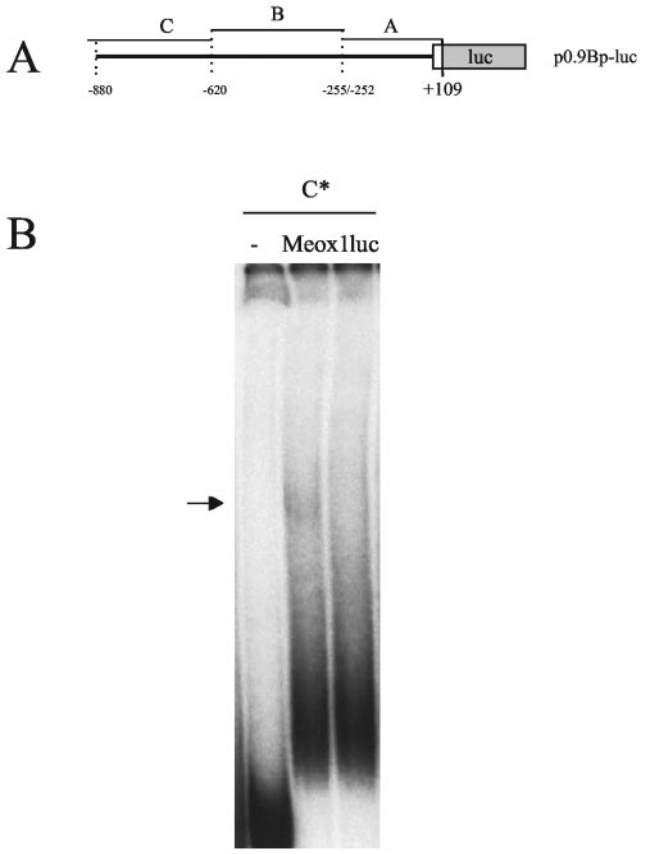

FIG. 5. Meox1 binds to sequences of the *Bapx1* promoter. (A) Scheme of the DNA fragments of the *Bapx1* promoter employed in EMSAs. (B) EMSA. DNA fragment C was labeled and incubated with in vitro-translated Meox1 protein (Meox1), luciferase protein as a control (luc), or no protein (−), as indicated. The arrow points to a retarded complex formed in the presence of Meox1.

6B). The specificity of this complex was further confirmed by the addition of an anti-Meox1 antibody to the binding reaction, which induced the formation of a supershifted band together with a strong decrease in the intensity of the Meox1-B5 retarded complex (Fig. 6C and D).

As expected from the specific interaction, the Meox1-B5 complex was eliminated by an excess of unlabeled B5 oligonucleotide but not by an excess of unlabeled B4 and B6 oligonucleotides (Fig. 6D). Interestingly, oligonucleotide B7, comprising the 5′ part of B5 (Fig. 6A), did compete for B5 binding, whereas oligonucleotide B8, comprising the 3′ part of B5, did not (Fig. 6D). This result narrowed the Meox1 binding site to the B7 region, located between positions −840 and −810 of the *Bapx1* promoter. Indeed, Meox1 bound the labeled B7 oligonucleotide, and the anti-Meox1 antibody induced the disappearance of the specific retarded complex concomitant with the appearance of a supershifted band (Fig. 6E). Similarly, we performed an EMSA with in vitro-synthesized Meox2 protein and with anti-Meox2 antibody, which indicated that Meox2 can also bind to the B7 sequence of the mouse *Bapx1* gene (Fig. 6E).

To further characterize the Meox binding site, we performed competition experiments with an excess of mutated oligonucleotides in the B7 region (Fig. 7A). While oligonucleotide B7 harboring mutations in m1 and m4 competed for Meox1 binding (Fig. 7B), oligonucleotide B7 with mutations in m2 or m3 did not compete for binding (Fig. 7B), indicating that both the m2 and m3 sites are important for Meox1 binding. Accordingly, oligonucleotides with different mutations including either m2 or m3 (m2/3 and m2/3/4) did not compete (Fig. 7B). Similar results were obtained when the labeled oligonucleotide employed for binding and competition assays was B7 instead of B5 (Fig. 7C). These results demonstrate that Meox1 can bind in vitro to the TAATTA sequence located at the −827 position of the *Bapx1* promoter.

To prove that the identified TAATTA sequence is functionally important, we analyzed the luciferase activity driven by p0.7Bp-luc, a reporter construct that lacked the detected Meox1 binding site (Fig. 8A). As shown in Fig. 8B, removal of the sequence between −880 and −748 led to a twofold decrease in the capacity of Meox1 to transactivate the *Bapx1* promoter. This demonstrates that the Meox1-responsive element has functional relevance in the activation of the *Bapx1* promoter.

Conservation of Meox1 binding to the *Bapx1* promoter. To confirm in vivo the observation that Meox1 binds in vitro to the *Bapx1* promoter, we performed ChIP experiments on the human Hek293 cell line transfected with the Meox1 expression vector pMeox1. First, we cotransfected pMeox1 together with the p0.9Bp-luc plasmid, which contained sequences of the mouse *Bapx1* promoter from positions −880 to +109, including the Meox1 binding motif. After immunoprecipitation of the cross-linked material with an anti-Meox1 antibody and PCR with primers F1 and R1 (Fig. 6A), we obtained a band corresponding to sequences of the mouse *Bapx1* gene (Fig. 9A), indicating that Meox1 was bound to the mouse *Bapx1* promoter.

To assess if Meox1 was also able to bind to the endogenous *BAPX1* promoter, we performed the ChIP assay on Hek293 cells transfected only with the Meox1-expressing vector. After immunoprecipitation with the anti-Meox1 antibody, we obtained an amplified PCR band corresponding to the human *BAPX1* promoter (Fig. 9B), confirming that Meox1 binds to the human *BAPX1* gene in a chromatin environment. The specificity of the experiments was supported by the lack of *Bapx1* and *BAPX1* amplification when either extracts of mock-transfected cells or preimmune serum was employed for the immunoprecipitation reaction (Fig. 9). As an additional control, we tried to amplify regions of other promoters containing homeobox binding sites. For this purpose, we selected the *TRP-1* and *TRP-2* genes, which code for enzymes involved in melanin biosynthesis and whose promoter regions contain binding sites for the homeodomain-containing transcription factor Otx2 (15, 23). In no case were specific bands amplified (Fig. 9 and data not shown), confirming the specificity of the Meox1 interaction with the *Bapx1* or *BAPX1* promoter. These results prove that Meox1 binds in vivo to the *Bapx1* regulatory sequences and additionally suggest that the regulation of *Bapx1* by Meox1 is conserved between mice and humans through evolution.

DISCUSSION

Expression studies and functional genetic analysis have shown that the two *Meox* genes, *Meox1* and *Meox2*, are essen-

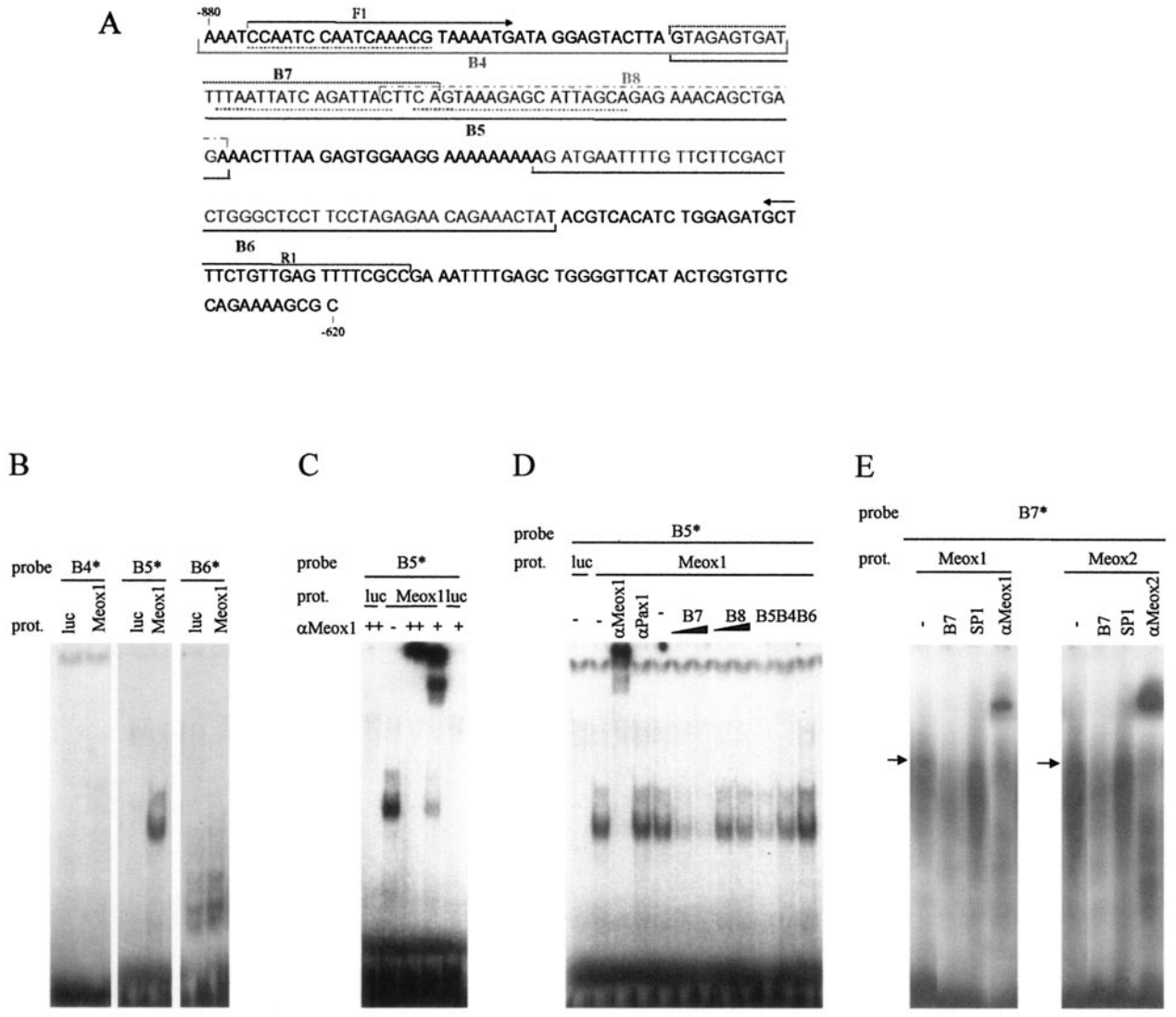

FIG. 6. Localization of the Meox1 binding motif in the *Bapx1* promoter. (A) Sequence of fragment C (interval between −880 and −620). The two shaded segments (between positions −839 and −780 and between positions −751 and −702) are areas of a high percentage of sequence identity with the human gene (81 and 84%, respectively). Underlined sequences show putative binding sites for other homeodomain transcription factors. The different oligonucleotides employed in EMSAs (B4, B5, B6, B7, and B8) are indicated by brackets (lines and dots). (B to E) Results of EMSA experiments. The indicated labeled oligonucleotides (probe, marked with an asterisk) were incubated with the in vitro-translated Meox1 protein, Meox2 protein, or luciferase protein as a control (luc). When indicated (C to E), anti-Meox1 antibody (αMeox1), anti-Meox2 antibody (αMeox2), or anti-Pax1 antibody as a control (αPax1) was included in the binding reaction. In the lanes marked ++ (C), the anti-Meox1 antibody was five times more concentrated than in those marked +. Molar excesses (250-fold for B5, B4, B6, and SP1; 500-fold for B7 and B8) of unlabeled oligonucleotides B7, B8, B5, B4, and B6 or the control SP1 were added as competitors when shown (D and E). (B) Observe that specific complexes were formed with the B5 oligonucleotide but not with B4 or B6. (C and D) An anti-Meox1 antibody (αMeox1) induced a supershifted band and eliminated the Meox1-B5 retarded complex, whereas an anti-Pax1 control antibody did not. (D) Note also that B7 but not B8 specifically eliminated Meox1 binding. (E) Meox1 and Meox2 bound specifically to the B7 oligonucleotide.

tial for proper sclerotome differentiation and for normal morphogenesis of the axial skeleton (3, 14). The present study provides a novel insight into the molecular mechanisms by which Meox proteins exert their function. We have shown, by analysis of mutant mice, that the Meox proteins are required for sclerotomal expression of the *Bapx1* gene, which in turn is involved in further sclerotome differentiation and chondrogenesis. Mice deficient in both Meox proteins lack an axial skeleton due to a block in the proper differentiation of the sclerotome. Nevertheless, sclerotomal cells are initially specified, as demonstrated by the detection, albeit at lower levels, of transcripts for the sclerotomal markers *Pax9* and *Foxc2* (14). Thus, the absence of *Bapx1* expression in *Meox1*; *Meox2* double mutant animals cannot be explained by the lack of specification of the sclerotomal population but must be a direct consequence of the absence of *Meox* gene function in the scle-

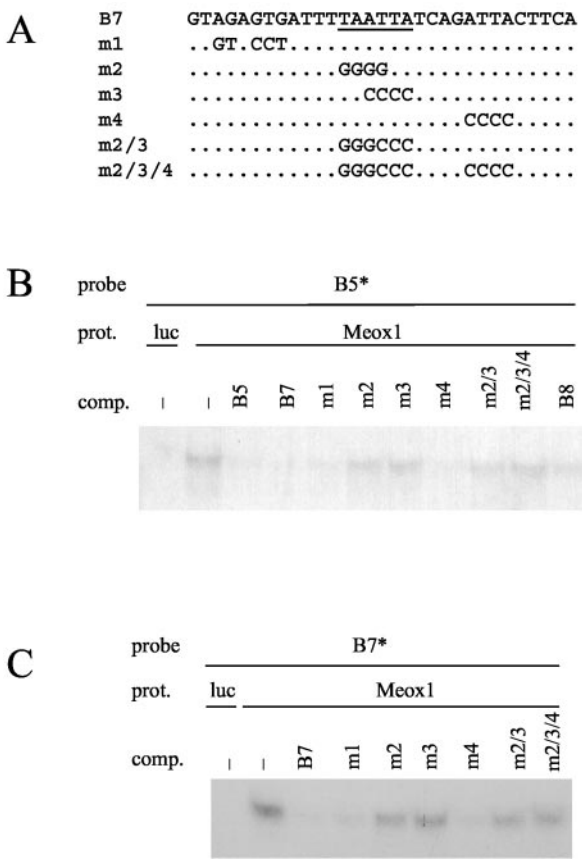

FIG. 7. Meox1 binds the TAATTA motif of the *Bapx1* promoter. (A) Scheme of the mutations introduced in the B7 oligonucleotide for competition in EMSAs. (B and C) EMSAs. The labeled B5 and B7 oligonucleotides (probe, marked with an asterisk) were incubated with the in vitro-translated Meox1 protein or luciferase protein as a control (luc). A 500-fold molar excess of the indicated unlabeled oligonucleotides was included in the binding reaction mixture as competitors (comp.). Note that the m2 and m3 mutations, located in the TAATTA motif, abolished the capacity to compete for Meox1 binding.

rotome. In line with this idea, we have demonstrated that Meox1 can activate *Bapx1* expression in cell lines by directly binding to its promoter.

To our knowledge, this is the first study in which a direct target of Meox1 has been identified. Furthermore, we provide the first characterization of a DNA binding motif for the homeodomain transcription factor Meox1. The core motif, TAATTA, is a palindromic sequence composed of two overlapping consensus homeobox sites, TAAT, arranged in a sequential array and in inverted orientation. This core motif is flanked by a thymidine at each side (T*TAATTA*T) in the mouse *Bapx1* promoter. Whether these adjacent sequences add further specificity still needs to be determined. The palindromic sequence T*TAATTA*A is present in a similar position in the human *BAPX1* promoter, suggesting a possible conservation of the Meox1-mediated regulation of *BAPX1* in humans. This assumption is supported by the ChIP data, which prove that Meox1 can bind to the endogenous human *BAPX1* promoter. Interestingly, the motif that we identified resembles the one characterized for the homeodomain protein Cart-1, which is expressed in tissues undergoing chondrogenesis (29). The Cart-1 binding motif consists of two inverted TAAT sequences separated by three or four nucleotides (2). Computer searches for the identified Meox1 binding motif in the available genomic databases may help to identify additional target genes.

We characterized only one Meox1 binding site on the *Bapx1* promoter. Deletion of this site diminished but did not totally abolish the Meox1 activation properties (Fig. 8), indicating that additional Meox1-responsive elements could be located downstream of position −748. However, a computer search of this region identified only one homeodomain binding core motif, TAAT, at position +8 (GATAATCG), which is unlikely to play a transcriptional role. Thus, another unrelated or divergent Meox1 binding motif(s) might be responsible for this activity, or transactivation by Meox1 might be indirect, through an intermediate cofactor or through the activation of different transcription factors.

Similar to *Meox1* and *Bapx1*, *Meox2* is also expressed in the sclerotome of somites (3, 4). The recent analysis of double homozygous mutant mice lacking both Meox1 and Meox2 indicated that Meox2, in concert with Meox1, plays an essential role in axial skeletal development (14). Thus, the lack of vertebral defects in mutant mice lacking *Meox2* (13) is possibly due to functional redundancy between *Meox1* and *Meox2*. The homeodomains of Meox1 and Meox2 are basically identical in sequence, suggesting that they very likely have the same DNA-binding sites (3). Accordingly, in EMSAs, Meox2 bound to the same sequences of the *Bapx1* gene as Meox1 did (Fig. 6E). In addition, we have shown that a single *Meox2* allele is sufficient to maintain some level of *Bapx1* expression in the sclerotome (Fig. 1C). These data suggest that Meox2, in addition to Meox1, could directly activate transcription of the *Bapx1* gene.

In cotransfection assays, maximal *Bapx1* promoter activity was reached in the presence of both Meox1 and Pax1/Pax9 proteins (Fig. 3), suggesting that Pax1 and Pax9 can collaborate with Meox1 to induce efficient *Bapx1* transcription. This is in agreement with the fact that Pax1 and Pax9 activate the *Bapx1* promoter and are required for proper *Bapx1* expression in the sclerotome (21). EMSAs did not show a supershifted band when we incubated Pax proteins together with Meox1 (data not shown), and therefore we cannot conclude that protein-protein interaction is necessary for this possible cooperation. Nevertheless, two observations support the possibility that a physical interaction between Meox1 and Pax1/Pax9 might be possible and functionally important in vivo. First, binding sites for Pax1/Pax9 (between positions −880 and −844) (21) and for Meox1 (position −827) are in close proximity in the *Bapx1* promoter; second, a biochemical interaction between Meox1 and Pax1 has been reported (22).

The possibility of cooperation between Meox and Pax proteins is also sustained by the striking overlap of Meox expression domains with those of Pax1 or Pax9 during development. In addition to the sclerotome, Meox1 and Pax9 are coexpressed in the neural crest-derived craniofacial mesenchyme and in regions of the tongue (3, 18). Meox1 and Pax1 expression overlaps in the developing sternum and scapula (3, 6, 24). Meox2 and Pax9 are both expressed in the secondary palate (13, 18). Interestingly, about 10% of Meox2 mutant mice have a cleft secondary palate (13), a typical defect of Pax9 mutant mice (18). On the basis of published observations and our

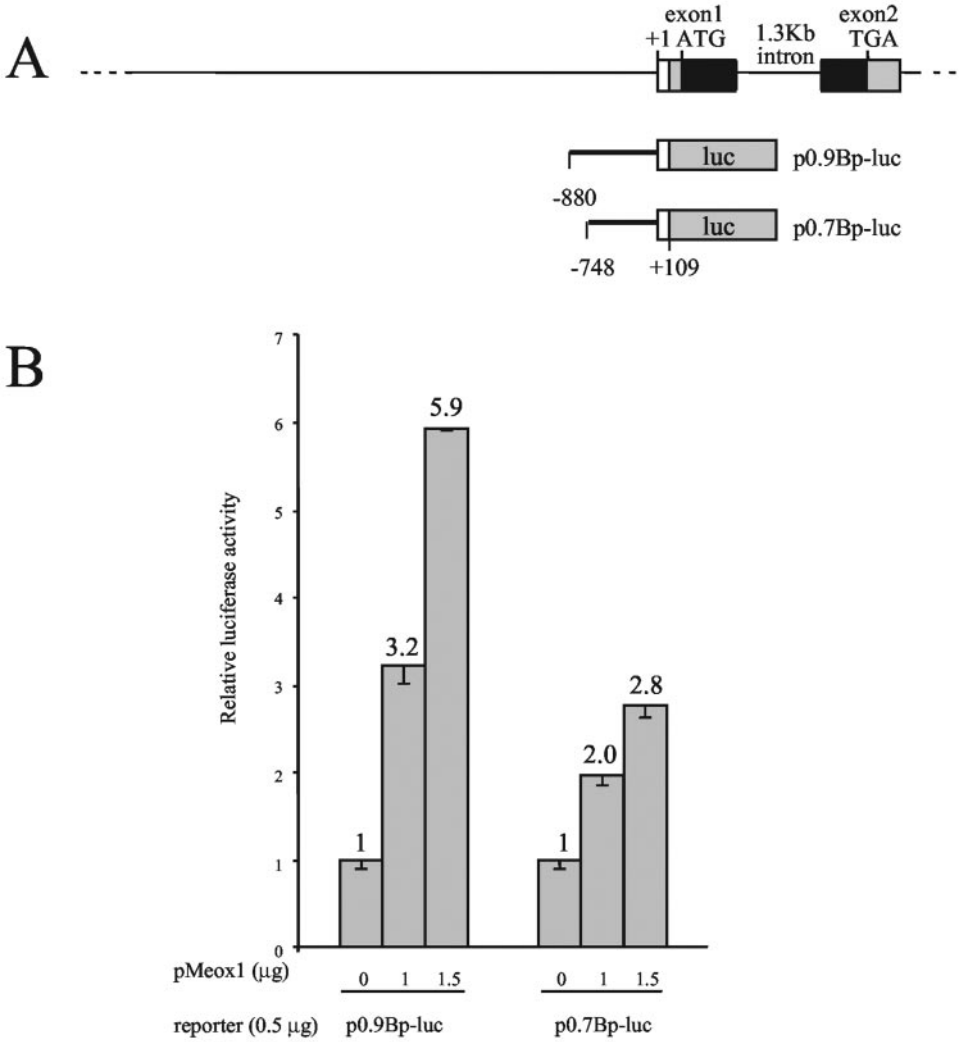

FIG. 8. Meox1 binding motif is required for Meox1-mediated transactivation of *Bapx1*. (A) Scheme of the *Bapx1* promoter constructs used in transient-transfection assays. (B) Luciferase activity driven by the *Bapx1* promoter constructs alone or cotransfected with the indicated amounts of the Meox1 expression plasmid pMeox1. Numbers above the bars indicate induction over the basal activity of the same construct in the absence of Meox1. Error bars indicate the standard deviation. Observe that removal of the segment between positions −880 and −748 induced a twofold decrease in Meox1 transactivating capacity.

findings in the present study, we hypothesize that, during embryo development, depending on the specific tissues and stage, Meox1 and Meox2 proteins cooperate with Pax1 or Pax9 to regulate different target genes, including *Bapx1*. The analysis of compound mutant mice deficient for *Meox1* and *Meox2* and for *Pax1* and *Pax9* should help to clarify whether this interaction is really crucial.

The fact that *Bapx1* expression is not initiated in the sclerotome of double mutant mice lacking Pax1 and Pax9 where *Meox1* and *Meox2* are expressed normally (21) indicates that the presence of the Meox proteins is not sufficient for *Bapx1* expression in vivo, at least at the beginning of sclerotome differentiation. At later stages, when *Pax1* and *Pax9* expression decreases in some sclerotomal derivatives (e.g., the center of presumptive vertebral bodies), the Meox proteins could contribute to maintaining *Bapx1* expression independently of Pax1 and Pax9, possibly with the help of other factors. Indeed, after the initial induction of *Bapx1*, an autoregulatory loop between Sox9 and Bapx1 that is maintained by bone morphogenetic protein signaling can sustain the sclerotomal expression of *Bapx1* (16, 28). Whether this regulation is achieved in cooperation with Meox1 or Meox2 remains to be established.

The secreted molecule Sonic hedgehog (Shh), produced by the notochord and the floor plate, has been shown to play a pivotal role in the survival of sclerotomal cells (5, 7, 10). Previous reports have shown that Pax1 and Pax9 mediate Shh signaling by activating *Bapx1*, which in turn induces chondrogenic differentiation in the sclerotome (16, 21). In this scenario, *Bapx1* regulation appears to depend upon an Shh-activated pathway, via Pax1 and Pax9, and upon a Meox1/Meox2 pathway. Whether this Meox1/Meox2 pathway is dependent on Shh signaling remains to be elucidated. A possible cross talk between the two pathways may be achieved

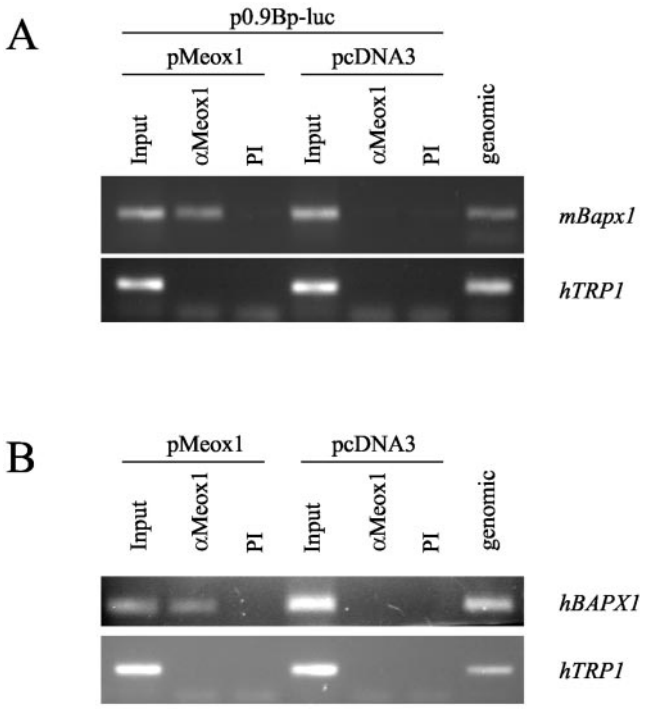

FIG. 9. Meox1 binds to the mouse and human *Bapx1* promoters. ChIP assay on extracts from Hek293 cells transfected with the Meox1 expression plasmid pMeox1 or with plasmid pcDNA3 as a control. (A) Cells were cotransfected with plasmid p0.9Bp-luc, containing sequences of the mouse *Bapx1* promoter. After immunoprecipitation of the cross-linked extracts with an anti-Meox1 antiserum (αMeox1) or with preimmune serum (PI) as a control, the DNA was subjected to PCR with primers that amplify the mouse (*mBapx1*, primers F1 and R1, shown in Fig. 6A) or the human (*hBAPX1*) *Bapx1* promoter or the human *TRP1* promoter as a negative control (*hTRP1*). Input and genomic DNAs are positive controls for the assay. The results show the recruitment of Meox1 to the mouse and human *Bapx1* regulatory sequences in the living cells.

by direct interaction between the Pax and Meox proteins and/or via the direct or indirect regulation of *Pax* genes by the Meox factors, although further analyses are needed to establish these points.

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