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Pax1 and Pax9 activate *Bapx1* to induce chondrogenic differentiation in the sclerotome

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SUMMARY

We have previously shown that the paired-box transcription factors Pax1 and Pax9 synergistically act in the proper formation of the vertebral column. Nevertheless, downstream events of the Pax1/Pax9 action and their target genes remain to be elucidated. We show, by analyzing *Pax1;Pax9* double mutant mice, that expression of *Bapx1* in the sclerotome requires the presence of Pax1 and Pax9, in a gene dose-dependent manner. By using a retroviral system to overexpress *Pax1* in chick presomitic mesoderm explants, we show that Pax1 can substitute for Shh in inducing *Bapx1* expression and in initiating chondrogenic differentiation. Furthermore, we demonstrate that Pax1 and Pax9 can transactivate regulatory sequences in the *Bapx1* promoter and that they physically interact with the *Bapx1* promoter region. These results strongly suggest that *Bapx1* is a direct target of Pax1 and Pax9. Together, we conclude that Pax1 and Pax9 are required and sufficient for the chondrogenic differentiation of sclerotomal cells.

Key words: Pax1, Pax9, Bapx1, Shh, Sclerotome, Chondrogenesis

INTRODUCTION

The axial skeleton of vertebrate organisms originates from somites, which are paraxial mesoderm structures that lie on either side of the neural tube and the notochord. Soon after their formation, the epithelial somites start to differentiate in response to signals from surrounding tissues. The ventromedial region of the somites de-epithelializes to form the mesenchymal sclerotome, whereas the dorsolateral part remains epithelial and forms the dermomyotome. Sclerotomal cells migrate towards the notochord where they condense to form mesenchymal prevertebrae. Sclerotomal cells located around the notochord contribute to the vertebral bodies and intervertebral discs of the future vertebral column. The lateral sclerotome participates in the formation of the ribs and neural arches. After forming mesenchymal condensations, sclerotomal cells differentiate into chondroblasts to form the cartilagous anlagen of the vertebral column, which is subsequently replaced by bony tissues through endochondral ossification (Brand-Saberi and Christ, 2000).

Pax1 and Pax9 play a critical role in the formation of the axial skeleton. They belong to the Pax family of transcription factors, characterized by the presence of a conserved DNA binding domain: the paired box. Pax genes were originally identified in *Drosophila*, but later they have been found in numerous organisms where they play important roles in embryonic patterning and organogenesis. Among them, Pax1 and Pax9 constitute a paralogous group characterized by the presence of the paired-box and the octapeptide domain, and the absence of a homeobox (Strachan and Read, 1994).

Pax1 and *Pax9* show a similar but not identical expression pattern during mouse development. They are expressed in the developing sclerotome, where no other Pax genes are expressed. *Pax1* is first expressed in nascent somites shortly before de-epithelialization, by cells located in the ventromedial part, which marks the emerging sclerotome population. *Pax1* is initially expressed in all sclerotomal cells, but later its expression becomes stronger in the posterior ventromedial compartment. Subsequently, *Pax9* is expressed in the posterior ventrolateral compartment of the sclerotome. Thus, *Pax1* is predominantly expressed in the region of the future vertebral bodies and intervertebral discs, whereas the *Pax9* expression domain extends more laterally in the region of the future neural arches and the proximal part of the ribs (Deutsch et al., 1988; Neubüser et al., 1995).

Pax1 is required for the proper formation of ventral structures of the vertebral column (Balling et al., 1988; Wallin et al., 1994; Wilm et al., 1998). Although *Pax9*-deficient mutant mice have no apparent malformations in the axial skeleton (Peters et al., 1998), *Pax1*;*Pax9*-double mutants show a dramatic increase in the severity of the vertebral defects when compared with *Pax1*-deficient mutants. In the absence of both

Pax1 and *Pax9*, the ventromedial structures of the vertebral column are not formed (Peters et al., 1999). Thus, there is a functional redundancy between the two Pax genes during vertebral column development. The sclerotomal cells of embryos deficient for both *Pax1* and *Pax9* do not properly condense around the notochord and cannot initiate chondrogenesis. There is a decrease in the proliferation rate of the sclerotomal population in *Pax1*;*Pax9*-double mutants, followed by an increase in apoptosis (Peters et al., 1999).

The secreted molecule Sonic hedgehog (Shh), which is produced by the notochord and the floor plate, plays pivotal roles in the dorsoventral patterning of somites and in the survival of sclerotomal cells (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Chiang et al., 1996). Sclerotomal expression of several genes including *Pax1* and *Pax9* is known to be dependent on Shh (Brand-Saberi et al., 1993; Dietrich et al., 1993; Koseki et al., 1993; Neubüser et al., 1995).

Bapx1 (also known as *Nkx3-2*) and *Nkx3-1* genes are the mouse homologs of *Drosophila Nk-3*/*bagpipe* (*bap*), a mesodermal gene essential for the formation of the visceral musculature (Azpiazu and Frasch, 1993). They are expressed in the sclerotome (Tribioli et al., 1997; Tanaka et al., 1999; Kos et al., 1998) in a Shh-dependent manner (Murtaugh et al., 2001; Kos et al., 1998). *Nkx3-1*-deficient mice show no sclerotomal defects (Bhatia-Gaur et al., 1999; Schneider et al., 2000). Interestingly, mouse mutants lacking *Bapx1* show a vertebral phenotype strikingly similar to that of mice mutant for both *Pax1* and *Pax9* (Lettice et al., 1999; Tribioli and Lufkin, 1999; Akazawa et al., 2000). Overexpression experiments in the chick have recently demonstrated the role of *Bapx1* in chondrogenic differentiation of sclerotomal cells in response to Shh (Murtaugh et al., 2001). As neither *Pax1* nor *Pax9* expression is altered in *Bapx1* mutants (Lettice et al., 1999; Tribioli and Lufkin, 1999; Akazawa et al., 2000), the possibility exists that *Bapx1* might be a downstream target of *Pax1* and *Pax9*.

In this study, this possibility was tested by a combination of genetic and molecular approaches. We show that *Bapx1* expression in the sclerotome is dependent on the two Pax genes. We also show that *Pax1* and *Pax9* are not only necessary, but also sufficient for *Bapx1* expression and for the induction of sclerotome chondrogenesis. Furthermore, we have obtained strong evidence that *Bapx1* is a direct target of *Pax1* and *Pax9* in the sclerotome. Taken together, we conclude that *Pax1* and *Pax9*, as main mediators of Shh signaling, are essential and sufficient for the induction of sclerotome chondrogenesis.

MATERIALS AND METHODS

In situ hybridization

Pax1;Pax9 mutant embryos were generated and genotyped as previously described (Peters et al., 1999). Paraffin wax embedded sections and whole-mount in situ hybridization was performed as described (Neubüser et al., 1995). An *Nkx3-1* probe was obtained by RT-PCR on mouse genomic DNA with the following primers, 5′-CACAGTGGCTGATGTCAAGG-3′ and 5′-AAGCATGAACGGAGAGGTCC-3′, to amplify the 3′UTR (position 793 to 1704) (Sciavolino et al., 1997). *Meox2* (Candia et al., 1992), *Bapx1* (Lettice et al., 2001) and *Nkx3-1* RNA probes were labeled with digoxigenin and developed with BM-purple (Roche). *Meox1* (Candia et al., 1992) RNA probe was labeled with fluorescein and developed with Fast Red (Roche). No signal was obtained with sense probes in all cases (not shown).

Retroviral vectors

pRCAS(A)-Pax1

The coding region of mouse *Pax1* was excised from pPax1 (see below) with *Nco*I/*Eco*RI and subcloned in *Nco*I/*Eco*RI-digested pSlax13 shuttle vector (a gift from C. Tabin) (Logan and Tabin, 1998) to create pSlax-Pax1. To engineer an HA N-terminal epitope, the following two oligonucleotides were annealed to create an HA linker with *Nco*I cohesive ends: 5′-CATGTACCCATACGATGTTCCAGATTACGCT-GG-3′ and 5′-CATGCCAGCGTAATCTGGAACATCGTATGGGTA-3′. This HA linker was ligated to *Nco*I linearized pSlax-Pax1 to generate a HA-Pax1 fragment. This fragment was excised from pSlax13 with *Cla*I and subcloned into *Cla*I-digested pRCASBP(A) (Hughes et al., 1987). The identity of the final product was confirmed by sequencing. Western blot on RCAS-A-Pax1 infected chick embryo fibroblasts (CEF) with HA11 antibody (BAbCO) against the HA epitope confirmed expression of HA-Pax1 protein (not shown). Immunocytochemistry analysis on RCAS-Pax1-infected CEF showed nuclear localization of HA-Pax1 (not shown), as expected for its role as transcription factor. The control vector pRCAS(A)-AP contains a human placental alkaline phosphatase cDNA (a gift from C. Tabin) (Fekete and Cepko, 1993).

Explant cultures, viral infection and RT-PCR

Fertile chicken White Leghorn eggs were obtained from Needle farm, Hertfordshire, UK. Chick presomitic mesoderm from HH10 stage embryos were isolated and embedded in collagen gels as described (Münsterberg et al., 1995). We used the semi-defined serum-free culture medium as described (Murtaugh et al., 1999) with or without Shh (Ontogeny) supplement at 500 ng/ml. Retroviral infection was performed as described (Maroto et al., 1997) with 4×10^4 cfu of RCAS-Pax1 and 2×10^5 cfu of RCAS-AP. Explants were harvested on the fifth day and processed for RT-PCR.

RT-PCR was carried out essentially as described (Münsterberg et al., 1995), except that 10% DMSO was included in the RT reaction mixture. Amplification of chick *Bapx1* was performed in the presence of 5% formamide at annealing temperature 65°C with primers 5′-GCTCCCGCGCCGCCTTCTCC-3′ and 5′-GGCGGCCGCGGCA-CAGGACAG-3′. The specificity of the amplified product was confirmed by subcloning and sequencing. Aggrecan was amplified as described (Murtaugh et al., 1999) at annealing temperature 50°C. Mouse *Pax1* was amplified at annealing temperature 60°C with primers 5′-GCTGCCTACTCCCCCAAGA-3′ and 5′-CGCTGTA-TACTCCGTGCTG-3′.

Plasmid construction for transactivation assays

Expression plasmids

pPax1 and pPax9 contain mouse *Pax1*- and *Pax9*-coding sequences, respectively, under control of the CMV promoter cloned in pcDNA3 (Invitrogen). To generate pPax1, the coding region of mouse Pax1 was amplified by RT-PCR from RNA isolated from E11.5 mouse embryos, with primers 5′-CGTTCCATGGAGCAGACGTACGGC-3′ and 5′-GTAGAATTCCTCTGAACCGGGCTGTGGCTC-3′, which contain *Nco*I and *Eco*RI sites, respectively. The PCR product was cloned in pCR2.1-TOPO vector. The insert was excised with *Eco*RI, subcloned in *Eco*RI-digested pcDNA3 vector, checked for correct orientation and sequenced. pPax9 was a generous gift from J. Gerber.

Reporter plasmids

p5.3Bp-luc, p2.8Bp-luc, p1.9Nk-luc, p0.9Bp-luc, p0.7Bp-luc and p0.3Bp-luc contain genomic sequences of the mouse *Bapx1* gene [5′ end positions at –5285, –2762, –1947, –880, –748 and –270, respectively; and 3′ end +109 (+1 is the first nucleotide in the

published cDNA sequence with GenBank Accession Number U87957)], cloned into pGL3-Basic vector (Promega) with the firefly luciferase gene as reporter. To generate p5.3Bp-luc, a 7 kb *Bapx1* genomic clone based on pBluescriptII SK (Lettice et al., 1999) was partially digested with *Kpn*I/*Bam*HI, and the corresponding fragment was subcloned into *Kpn*I/*Bgl*II digested pGL3-Basic. The 3′ end of the insert in the final construct was confirmed to correspond to position +109 by sequencing. p2.8Bp-luc was obtained by excising *Kpn*I/*Bgl*II fragment from p5.3Bp-luc. p1.9Bp-luc, p0.9Bp-luc, p0.7Bp-luc and p0.3Bp-luc were generated by controlled treatment with exonuclease III after *Kpn*I/*Pml*I digestion of p5.3Bp-luc (Erase-a-Base, Promega). To generate intron constructs pBpIA-luc and pBpIB-luc, which contain the intron segment in forward and reverse orientation, respectively, a 1.3 kb *Bapx1* intron segment obtained as a *Rsr*II/*Mlu*I fragment from the 7 kb *Bapx1* genome clone was subcloned into *Mlu*I digested pGL3-Promoter vector (Promega) with the SV40 promoter to direct firefly luciferase expression. Integrity of all plasmids was confirmed by sequencing. pRL-SV40 vector (Promega) contains the reporter *Renilla* luciferase gene upstream of SV40 early enhancer/promoter, and was used to normalize the transfection efficiency among different experiments.

Transient transfection assays

Mouse embryonic fibroblasts NIH3T3 or monkey kidney COS-7 cells were plated into six-well plates in DMEM medium (GibcoBRL) supplemented with 10% fetal calf serum (GibcoBRL). When cells reached about 35% confluency, the DNA was transfected with Lipofectamine Plus reagent (Invitrogen). In each experiment, 0.5 μg of firefly reporter plasmid was co-transfected with different amounts of pPax1, pPax9 or pcDNA3 control vector. The total amount of transfected DNA was made equal in each experiment by completing with pcDNA3. In addition, 20 ng of pRL-SV40 vector was always co-transfected for normalization. Forty-two hours after transfection, at about 100% confluence, cell extracts were collected and firefly and *Renilla* luciferase activities were measured (Dual-Luciferase Reporter Assay, Promega). Firefly luciferase activity in each sample was normalized to *Renilla* luciferase to correct for variations in transfection efficiency. For each assay, two to nine experiments were performed in duplicate.

Sequence analysis

Mouse and human genome sequences including *Bapx1* used for comparison are taken from NW_000229 and AF009802, respectively. Percent Identity Plot (PIP), TFSEARCH and MatInspector analyses were carried out with programs available at the websites of The Pennsylvania State University (http://bio.cse.psu.edu), Computational Biology Research Center (http://www.cbrc.jp) and Genomatix (http://www.genomatix.de), respectively.

Electrophoretic mobility shift assay (EMSA)

Pax1, Pax9 and luciferase (control) proteins were synthesized by TNT-coupled wheatgerm extract system (Promega). EMSA was performed basically as previously described (Hennighausen and Lubon, 1987). Protein translation extracts (2 μl) were incubated at room temperature for 15 minutes with 5×10^4 cpm of each corresponding ^{32}P-labeled double stranded oligonucleotide and 1 μg of poly(dI-dC) in 15 μl of 20 mM HEPES pH 7.9, 60 mM KCl, 10% glycerol, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.1% bovine serum albumin. The reaction mixture was analyzed by electrophoresis and visualized by autoradiography. In competition assays, a 250 or 500-fold excess of cold double-stranded oligonucleotide was pre-incubated at room temperature for 15 minutes before the addition of the labeled oligonucleotide. In reactions including antibodies, 1 μl (2 μg) of anti-Pax1 goat polyclonal antibody (M-19, Santa Cruz Biotechnology) or 3 μl (1.2 μg) of goat anti-mouse antibody as control (115-035-068, Jackson Immuno Research Laboratories) was added and incubated for 20 minutes at room temperature before the addition of the probe. The oligonucleotides B4, B5, S4, S1 and S2 from the *Bapx1* promoter region are as shown in Fig. 7B. The *Drosophila*-derived oligonucleotides e5-5 and e5-3 correspond to the oligonucleotides 5 and 3, respectively, described elsewhere (Chalepakis et al., 1991).

RESULTS

Bapx1 expression in the sclerotome requires *Pax1/Pax9*

In order to identify target genes of *Pax1/Pax9* in the sclerotome, expression of potential candidates that show overlapping expression domains with these Pax genes in somites has been analyzed by whole-mount in situ hybridization in mutant embryos deficient in *Pax1* and *Pax9*.

Meox1 and *Meox2* (also known as *Mox1* and *Mox2*, respectively) constitute a subfamily of homeobox-containing genes that are expressed in the sclerotome (Candia et al., 1992). *Meox1* plays a critical role in axial skeleton development (Stamataki et al., 2001), while *Meox2* is dispensable for the formation of the vertebral column (Mankoo et al., 1999). As shown in Fig. 1, somitic expression of neither *Meox1* (Fig. 1A,B) nor *Meox2* (Fig. 1C,D) is altered in the absence of both *Pax1* and *Pax9*. In single *Pax1* or *Pax9* homozygous mutant embryos, expression of *Meox1* and *Meox2* is also not affected (not shown).

We then examined expression of *Nkx3-1* and *Bapx1* in

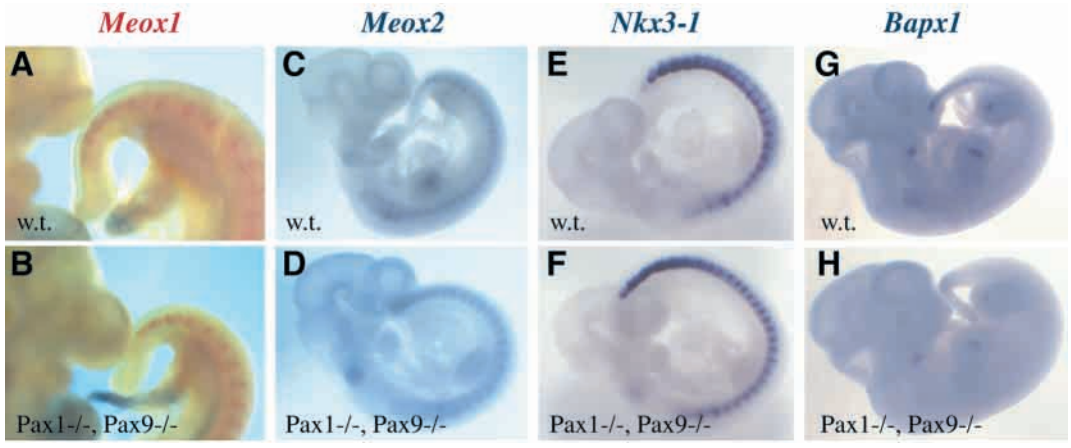

Fig. 1. Expression of sclerotomal genes in *Pax1;Pax9* mutant embryos. Whole-mount in situ hybridization for *Meox1* (A,B; E10.5), *Meox2* (C,D; E10.5), *Nkx3-1* (E,F; E9.5) and *Bapx1* (G,H; E11.5) on wild type (w.t.; A,C,E,G) or *Pax1*$^{-/-}$, *Pax9*$^{-/-}$ (B,D,F,H) mouse embryos.

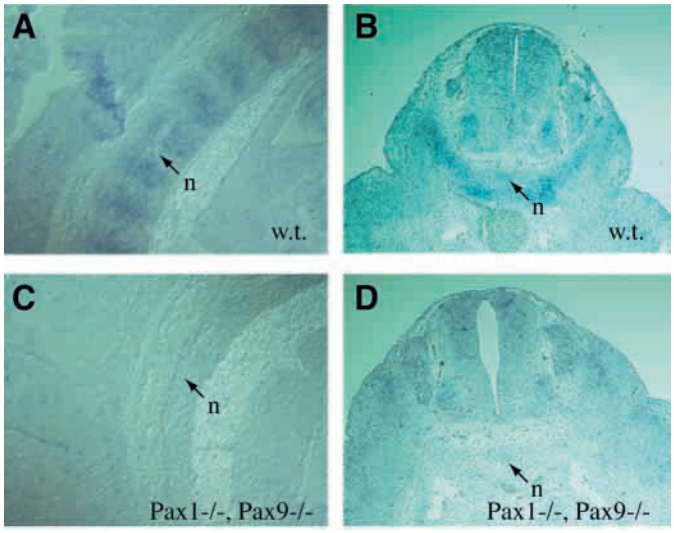

Fig. 2. Absence of *Bapx1* expression in the sclerotome of *Pax1*;*Pax9*-deficient embryos. In situ hybridization for *Bapx1* on sagittal (A,C) and transverse (B,D) sections of wild type (w.t.; A,B) or $Pax1^{-/-}$, $Pax9^{-/-}$ (C,D) E11.5 mouse embryos at the lumbar level. The arrows indicate the notochord (n). Note that in the mutant embryo (C,D) loose mesenchyme of sclerotomal cells around the notochord lack *Bapx1* expression.

Pax1;Pax9 mutants. *Nkx3-1* expression in the sclerotome of *Pax1;Pax9*-double homozygous embryos remains unchanged (Fig. 1E,F). Normal expression pattern is observed in single *Pax1* or *Pax9* mutant embryos, as well as in *Pax1;Pax9* mutant embryos with other genotype combinations (not shown). Interestingly, *Bapx1* expression in the sclerotome is undetectable in the absence of both *Pax1* and *Pax9* (Fig. 1G,H), whereas *Bapx1* expression in other domains like the mandibular region of the first branchial arch, mesenchyme of the limb buds (see Fig. 1H) or splanchnic mesoderm (not shown) is not affected. We did not detect *Bapx1* transcripts even in the youngest somites of the double mutants. The lack of *Bapx1* expression in the *Pax1*;*Pax9*-deficient sclerotome was confirmed by in situ hybridization on sagittal and transverse sections (Fig. 2). It is noteworthy that sclerotomal cells that express *Meox1*, *Meox2* and *Nkx3-1* are present in the double mutants. This indicates that the loss of *Bapx1* expression is not a secondary consequence of lack of sclerotomal cells, but it is rather a very early molecular defect caused by the absence of the sclerotomal Pax genes.

When we analyzed *Pax1*-single homozygous mutant embryos, we invariably observed a decrease in the intensity of *Bapx1* staining in the sclerotome, when compared with wild-type littermates (Fig. 3A,B,E,F). However, *Bapx1* expression in the sclerotome of *Pax9*-single deficient embryos did not show significant changes (Fig. 3C,D). When only one functional copy of *Pax1* is present ($Pax1^{+/-}$, $Pax9^{-/-}$), the level of *Bapx1* expression is considerably reduced (compare Fig. 1G with Fig. 1E). *Bapx1* expression is even weaker, when only one functional *Pax9* gene copy is present ($Pax1^{-/-}$, $Pax9^{+/-}$; Fig. 1H). These results indicate that somitic *Bapx1* expression is dependent on the presence of *Pax1* and *Pax9* in a dose-dependent manner, with *Pax1* having a stronger role than *Pax9*.

In summary, these data indicate that *Meox1*, *Meox2* and *Nkx3-1* expression in the sclerotome is not dependent on the *Pax1*/*Pax9* activity. They may act either upstream, or in different regulatory pathways. However, somitic expression of *Bapx1* requires *Pax1* and *Pax9*.

Pax1 can substitute for Shh in the induction of *Bapx1* expression and sclerotome chondrogenesis

In order to assess the potential of Pax1 and Pax9 to activate *Bapx1*, we have employed a retroviral system to overexpress *Pax1* in explants of chick presomitic mesoderm (PSM). When the explants were grown in the absence of Shh, we did not detect *Bapx1* expression (Fig. 4A, lane 1). However, we observed *Bapx1* induction when the explants were exposed to Shh (Fig. 4A, lane 3), as previously reported (Murtaugh et al., 2001). Because one of the early effects of Shh is the induction of *Pax1* (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Münsterberg et al., 1995; Murtaugh et al., 1999), there is the possibility that Shh acts through *Pax1* to activate *Bapx1* expression. Interestingly, we observed that overexpression of mouse Pax1 in chick PSM explants cultured in medium with Shh led to a significant increase of the *Bapx1* expression levels

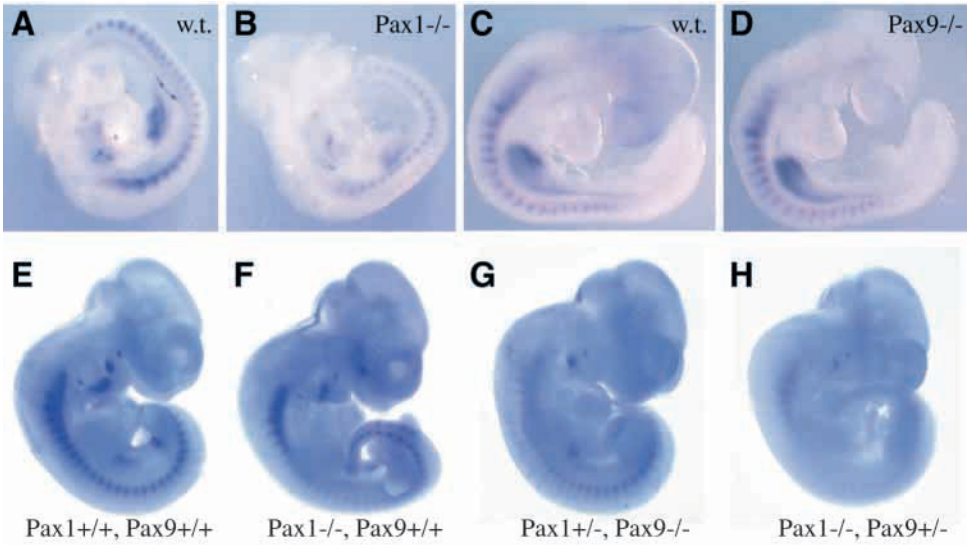

Fig. 3. *Bapx1* expression level depends on the *Pax1*/*Pax9* gene dosage. Whole-mount in situ hybridization for *Bapx1* on E9.5 (A-D) or E10.5 (E-H) mouse embryos. Genotypes are indicated on top of each panel. w.t., wild type. Note the decrease in *Bapx1* staining intensity, as fewer functional copies of *Pax1* or *Pax9* are present.

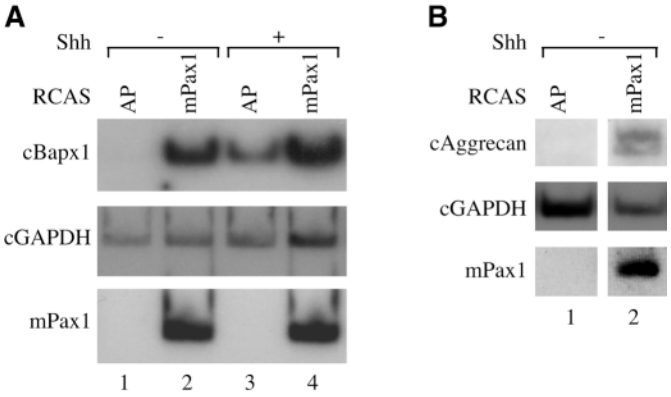

Fig. 4. *Pax1* induces *Bapx1* expression and chondrogenesis in explants of chick PSM. Explants of PSM isolated from stage HH10 chick embryos were infected with the control RCAS-A-AP retrovirus (AP; A, lanes 1,3; B, lane 1) or with the RCAS-A-Pax1 retrovirus overexpressing mouse Pax1 (A, lanes 2,4; B, lane 2), and cultured in the absence (A, lanes 1,2; B) or the presence (A, lanes 3,4) of 500 ng/ml Shh. After 5 days of culture, explants were harvested and analyzed by RT-PCR. The PCR products for chick *Bapx1*, aggrecan, *Gapdh* and mouse *Pax1* are shown.

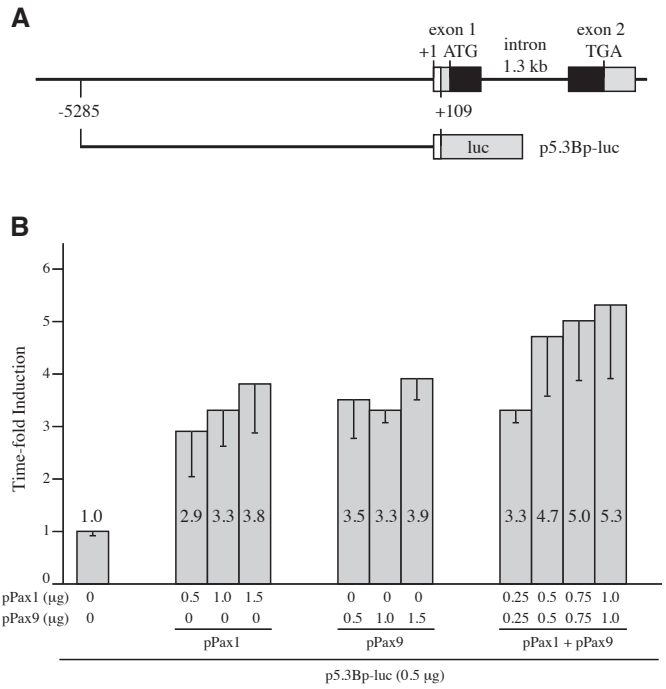

Fig. 5. *Pax1* and *Pax9* can transactivate *Bapx1* promoter. (A) Scheme of the *Bapx1* genomic structure and of the p5.3Bp-luc reporter plasmid. The boxes indicate exons of *Bapx1* (top) or the luciferase (luc) reporter (bottom). The coding regions of Bapx1 are shown by the black boxes, together with the first ATG codon (position +277) and the stop codon (TGA) in exon 2. The p5.3Bp-luc plasmid contains 5.4-kb genomic sequences from the *Bapx1* promoter region and a part of 5′ UTR (–5285/+109) upstream of the luciferase reporter. (B) Promoter activities of the p5.3Bp-luc construct alone or co-transfected with the indicated amounts of expression plasmids for Pax1 (pPax1) or Pax9 (pPax9), or both. Numbers inside or above the bars indicate time fold induction with respect to the basal p5.3Bp-luc promoter activity. Lines in the bars indicate s.d.

(Fig. 4A, lane 4, compare with lane 3), indicating that a high dose of Pax1 can enhance the effect of Shh on the induction of *Bapx1*. Furthermore, exogenous expression of Pax1 was sufficient to induce *Bapx1*, even in the absence of Shh (Fig. 4A, lane 2).

To further explore the role of Pax1 in sclerotome differentiation, we have analyzed expression of the early chondrocyte marker *aggrecan* in this in vitro system. It has previously been reported that *aggrecan* is induced by Shh treatment (Murtaugh et al., 1999), or by overexpression of Bapx1 (Murtaugh et al., 2001) in chick PSM explants. Consistent with these published observations, as well as our finding that Pax1 alone can induce *Bapx1*, we have observed that overexpression of Pax1, in the absence of Shh, promotes aggrecan expression (Fig. 4B, lane 2).

Pax1 and Pax9 can transactivate *Bapx1* promoter

Our data suggest that Pax1 and Pax9 may directly activate *Bapx1* expression. To test this hypothesis, we analyzed putative *Bapx1* regulatory sequences for their response to transactivation by Pax1 and Pax9 in transient transfection assays. The genomic structure of *Bapx1*, like other *NK* family members, is simple, consisting of two exons and a single intron of 1.3 kb. Thus, as first approximation we have analyzed the intron as well as a 5.4 kb upstream segment (Fig. 5A). We first tested if the 1.3 kb intron can act as an enhancer regulated by Pax1/Pax9. No specific effect is observed when Pax1 and/or Pax9 expression plasmids are co-transfected with a reporter plasmid containing the 1.3 kb intron in forward or reverse orientation upstream of the SV40 promoter (constructs pBpIA-luc and pBpIB-luc, see Materials and Methods), in NIH3T3 and COS7 (data not shown).

We next tested the 5′ region of *Bapx1* with the plasmid p5.3Bp-luc that contains the 5.4 kb fragment, including a part of 5′UTR and the putative promoter region of *Bapx1* (positions –5285 to +109, with position +1 being the published 5′ end of *Bapx1* exon 1) (Fig. 5A). This fragment possesses a basal promoter activity, as it can drive luciferase expression in NIH3T3 cells (22% of SV40 promoter activity, data not shown). Interestingly, when p5.3Bp-luc is co-transfected with the Pax1-expression plasmid, there is a dose-dependent increase in the promoter activity, from 2.9 times induction (0.5 μg of the Pax1-expression plasmid) to 3.8 times induction (1.5 μg) (Fig. 5B). Co-transfection with the Pax9 expression plasmid also induces a significant increase in the *Bapx1* promoter activity up to 3.9 times (Fig. 5B). The maximum transactivation effect is reached when both Pax1 and Pax9 plasmids are co-transfected. This transactivation effect is specific for the 5.4 kb fragment, as neither Pax1 nor Pax9, or both in combination, can activate the control vector pGL3-Basic (see Fig. 6B and data not shown). Similar transactivation properties are also observed in COS7 cells (not shown).

To further narrow down the region(s) responsible for the transactivation activity of Pax1/Pax9, we generated a series of 5′ deletion constructs, as schematized in Fig. 6A, and analyzed their response to transactivation by Pax1 and/or Pax9. Co-transfection of p2.8Bp-luc (–2762 to +109) with the Pax1 and/or Pax9 expression plasmids leads to a significant increase in the promoter activity of up to about eight times, when both expression plasmids are co-transfected (Fig. 6B). Similar

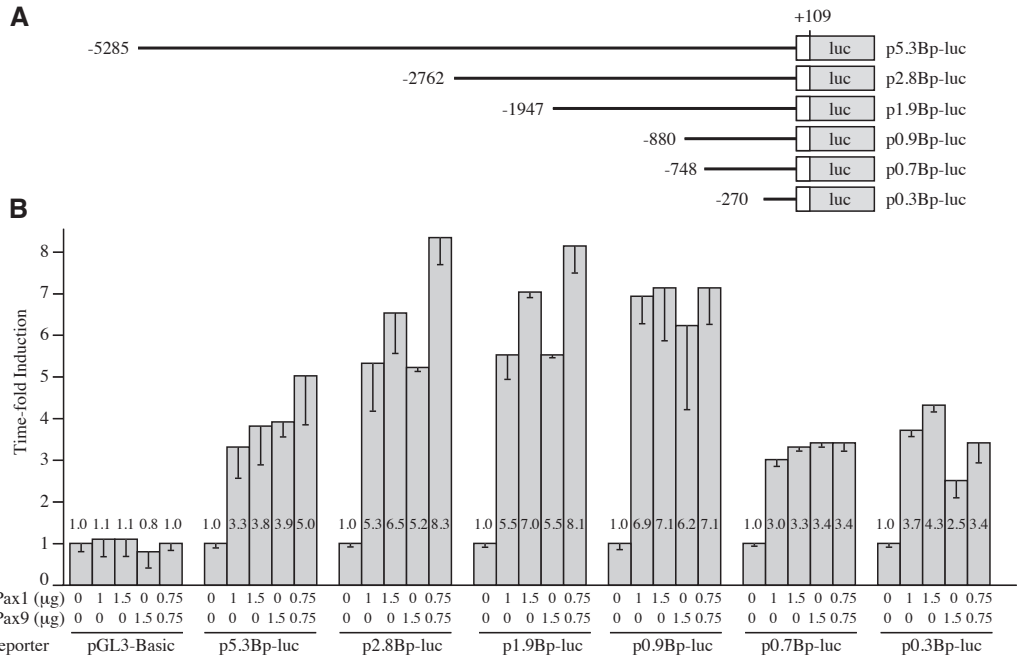

Fig. 6. *Pax1* and *Pax9* transactivation activity on different *Bapx1* promoter deletion constructs. (A) *Bapx1* promoter deletion plasmids used in transient transfection assays. (B) Luciferase activity driven by the *Bapx1* promoter deletion constructs or control vector (pGL3-Basic), alone or co-transfected with the indicated amounts of expression plasmids for Pax1 (pPax1) or Pax9 (pPax9), or both. Numbers inside or above the bars indicate the time fold induction with respect to the basal activity of the same construct in the absence of transfected Pax1 and Pax9. Lines in the bars indicate s.d.

activities are observed for p1.9Bp-luc (–1947 to +109) and p0.9Bp-luc (–880 to +109) (Fig. 6B). Interestingly, when additional 5′ 132-bp sequences in p0.9Bp-luc are deleted (p0.7Bp-luc; –748 to +109), the activation properties of Pax1/Pax9 significantly drops by more than half, but still with 3.0- to 3.4-fold transactivation capacity. The shortest segment tested in p0.3Bp-luc (–270 to +109) is similarly activated by Pax1/Pax9 (Fig. 6B). These data indicate that the region between –880 and +109 contains *cis*-regulatory elements responsible for Pax1/Pax9-induced transactivation.

Comparative sequence analysis of the *Bapx1* promoter region

Sequence comparison between 8550 bp mouse and 8600 bp human genomic regions encompassing the *Bapx1* gene shows a significant degree of similarities even outside the coding sequences (Fig. 7A). In the region between –880 and +109, overall sequence conservation is as high as 72% between the two species. Most of conserved noncoding sequences (CNS) as revealed by PIP analysis (Schwartz et al., 2000) are located in this interval (Fig. 7A). Two prominent CNS segments are found in the regions between –543 and –408 (136 nucleotides, 78%) and between +92 and +198 (107 nucleotides, 82%). The interval between –880 and –748 (boxed in green in Fig. 7A, and marked in green in Fig. 7B), which is important for Pax1/Pax9-responsiveness, also includes two short CNS segments (69 nucleotides, 80% and 69 nucleotides, 78%) that are separated by a single-base gap. These data suggest that most of important *cis*-regulatory elements of *Bapx1* reside in the region between –880 and +109.

Pax1 and Pax9 directly interact with *Bapx1* promoter sequences

In order to ascertain whether Pax1 and Pax9 directly activate *Bapx1* via binding to its *cis*-regulatory sequences, we performed electrophoretic mobility shift assays (EMSA) with in vitro translated Pax1 and Pax9 proteins and a series of labeled oligonucleotides from the region between –880 and –748. When EMSA was performed with the labeled oligonucleotides B5 or S4 (Fig. 7B) and Pax1 protein, we did not detect any retarded band (not shown). Interestingly, when we incubated the labeled oligonucleotide B4 (Fig. 7B) with Pax1 protein, we detected two retarded protein-DNA complexes (Fig. 7C, lane 1). These bands represent the complex of Pax1 protein with B4, as they did not appear when an in vitro synthesized control protein was employed (Fig. 7C, lane 6), and as they were specifically competed by an excess of the cold e5-5 oligonucleotide that has previously been shown to bind Pax1 (Fig. 7C, lanes 2, 3); but not by e5-3, a mutated sequence that does not bind Pax1 (Fig. 7C, lanes 4, 5). The lower band could be due to partial Pax1 synthesis or to a Pax1 degradation product. The binding of the larger form of Pax1 to B4 was specifically blocked when an anti-Pax1 antibody was included in the binding reaction (Fig. 7D, lane 4), confirming that the bound protein is Pax1.

By competition experiments with the oligonucleotides S1 and S2, located in the 5′ or 3′ part of B4, respectively (see Fig. 7B), we could narrow down the binding region of Pax1 to positions –880 to –844, as an excess of the cold S1 specifically competed Pax1 binding to B4, whereas S2 did not (Fig. 7E, lanes 4, 5 and lanes 6, 7). Furthermore, by labeling S1 and S2, we confirmed that Pax1 binds to S1, but not to S2 (Fig. 7F, lanes 2, 5). Similarly, we did not detect any retarded band when we incubated Pax9 protein with the labeled B5, S4 and S2 oligonucleotides (Fig. 7F, lane 6, and data not shown), but we detected a weak, but specific, band when Pax9 was incubated with B4 and S1 (Fig. 7F, lane 3, and data not shown).

DISCUSSION

Previous studies have shown that *Bapx1* mediates Shh

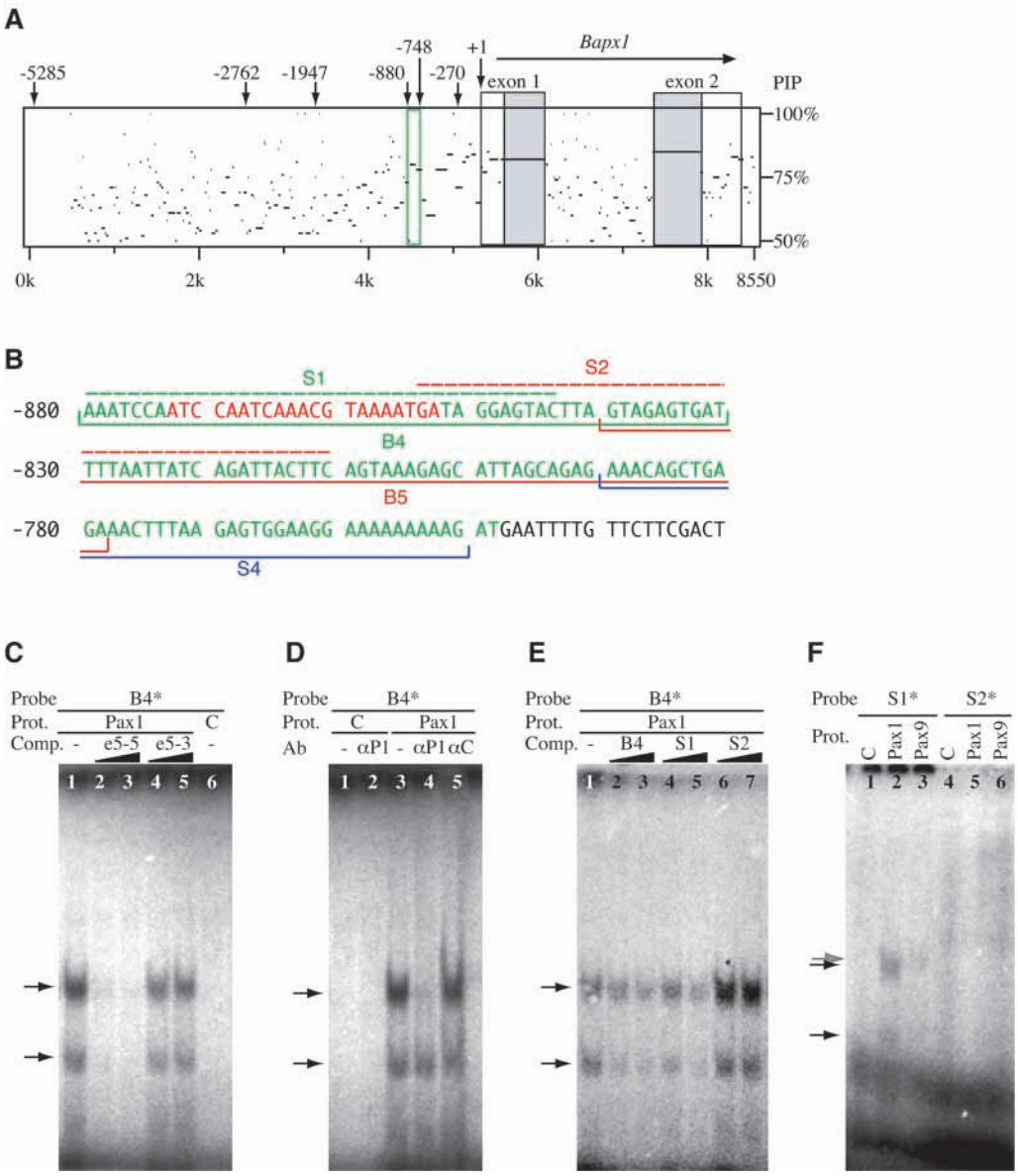

Fig. 7. Pax1 and Pax9 bind to regulatory sequences of the *Bapx1* promoter. (A) PIP analysis of an 8550 bp mouse *Bapx1* genome sequence when compared with an 8600 bp human BAPX1 sequence. Exons are outlined in black and coding parts are gray. Numbers at the top indicate nucleotide positions as defined in the text, and they correspond to 5′ ends of intervals included in the series of deletion constructs used in the transactivation assay. The interval between –880 and –748 is outlined in green. PIP scores in % are shown on the right, with 50% identity at the bottom level. Note the presence of several CNS segments outside the coding region. Many of them are located in the interval between –880 and +109. (B) Sequence of the interval between –880 and –731. The different oligonucleotides (S1, S2, B4, B5 and S4) employed in EMSA assays are indicated by lines. Sequence in green (–880 to –748) corresponds to the segment included in the plasmid p0.9Bp-luc, but not in the plasmid p0.7Bp-luc. A potential Pax6-binding site predicted by TFSEARCH and MatInspector is in the region of S1 and indicated in red. (C-F) Results of EMSA experiments. The indicated labeled oligonucleotides (Probe, marked with an asterisk) were incubated with the in vitro translated protein (Prot.) Pax1, Pax9 or luciferase as a control (C), separated and visualized as described in the Materials and Methods. The specific Pax1 (black arrows) and Pax9 (gray arrow in F) complexes are indicated. (C) Pax1 binds to oligonucleotide B4. Cold oligonucleotides e5-5 or e5-3 were added at 250-fold (lanes 2, 4) or 500-fold (lanes 3, 5) molar excess, as indicated. e5-5 specifically competes with B4 for Pax1 binding, whereas mutated e5-3 does not. (D) Specific inhibition of Pax1-B4 complex formation by anti-Pax1 antibody. The indicated antibodies were incubated in the binding reactions. αP1, anti-Pax1 goat antibody; αC, anti-mouse goat antibody, used as control. The anti-Pax1 antibody abrogates the formation of the larger complex. (E) Competition experiments employing a 250-fold (lanes 2, 4, 6) or 500-fold (lanes 3, 5, 7) molar excess of cold oligonucleotides B4, S1 or S2. Note that oligonucleotides B4 and S1 compete with B4 for Pax1 binding, whereas oligonucleotide S2 does not. (F) Binding of Pax1 and Pax9 to oligonucleotide S1. S1 or S2 labeled oligonucleotides were incubated with Pax1, Pax9 or luciferase control protein as indicated. Both Pax1 and Pax9 interact with S1 oligonucleotide, but not with S2 oligonucleotide.

signaling to induce chondrogenic differentiation in the sclerotome. However, how the Shh signal leads to *Bapx1* activation remains unclear. In the present study, we have provided evidence that Pax1 and Pax9 are not only required, but also sufficient to induce *Bapx1* expression and sclerotome chondrogenesis. This indicates that Shh signaling indirectly activates *Bapx1* via *Pax1* and *Pax9*. Furthermore, our results have shown that Pax1 and Pax9 can transactivate the *Bapx1* promoter and that both Pax proteins can bind in vitro to a specific region of the promoter, strongly suggesting that *Bapx1* is a direct target of the two Pax genes. Striking similarity in the axial skeleton defects between *Bapx1*- and *Pax1;Pax9*-deficient mice strongly suggests that Bapx1 is a main downstream effector of Pax1 and Pax9. Therefore, in order to understand the mechanisms involved in sclerotome chondrogenesis at the molecular and cellular levels, it will be important to identify targets of Bapx1 in the sclerotome.

Despite the similarity in the expression patterns between *Pax1*/*Pax9* and *Bapx1* in the early phase of sclerotome development, their expression profiles significantly differ at the

later stages. Once chondrogenesis has started, both *Pax1* and *Pax9* are rapidly downregulated, while *Bapx1* expression is maintained in chondrocytes even after *Pax1*/*Pax9* expression diminishes (Tribioli et al., 1997; Murtaugh et al., 2001). This observation suggests that *Pax1*/*Pax9* may be required only for the initiation of *Bapx1* expression, but not for its maintenance. Accordingly, it has recently been proposed that an auto-regulatory loop between Bapx1 and Sox9 maintains expression of both in sclerotome derivatives (Zeng et al., 2002). As expression of *Sox9* is initiated in young somites of *Pax1;Pax9*-deficient embryos (Peters et al., 1999), induction of *Sox9* expression in sclerotome cells does not appear to be dependent on Pax1 and Pax9, and probably not on Bapx1. How sclerotomal *Sox9* expression is initiated remains to be elucidated.

A conserved regulatory pathway

The *Drosophila* homolog of *Bapx1*, *bagpipe* (*bap*), is expressed in a subset of dorsal mesodermal cells and in the absence of the *bap* function, the development of the visceral musculature is disrupted (Azpiazu and Frasch, 1993). As *Bapx1* mutant mice show no defects in the formation of the gut musculature, it has been proposed that there is no equivalent function for the mouse and *Drosophila* genes (Lettice et al., 1999). Nevertheless, there could be still common regulatory mechanisms. In *Drosophila* embryos, the dorsal mesoderm homeobox gene *tinman* (*tin*), in combination with *decapentaplegic* (*dpp*), activates *bap* expression (Azpiazu and Frasch, 1993; Staehling-Hampton et al., 1994). The zinc-finger transcription factor *schnurri* (*shn*) has been proposed to mediate *dpp*-mediated *bap* activation (Staehling-Hampton et al., 1995). In addition, the ectodermal segmental regulators *hedgehog* (*hh*), *wingless* (*wg*) and *sloppy paired* (*slp*) restrict *bap* to segmental clusters of cells within the dorsal mesoderm, with *hh* having a positive and *wg* and *slp* a negative effect on *bap* regulation (Azpiazu et al., 1996; Riechmann et al., 1997; Lee and Frasch, 2000). However, *wg* and *hh* signals are not sufficient to mediate normal mesoderm segmentation and *bap* activation (Azpiazu et al., 1996). It is postulated that as yet unidentified genes, expressed in stripped pattern in the early mesoderm, are responsible for early *bap* segmental expression, and that *pox meso* (*poxm*) might fulfill those conditions (Azpiazu et al., 1996). *poxm* is the *Drosophila paired* gene that is most related to *Pax1*/*Pax9*, as it is expressed in mesoderm and lacks a homeodomain (Bopp et al., 1989). Thus, the regulatory pathway involving positive regulation of *Bapx1* by *Pax1*/*Pax9* in mesodermal tissues could be conserved through evolution.

Pax1 and *Pax9* in sclerotome chondrogenesis

It is proposed that Shh alters the competence of somitic cells to activate the chondrogenic differentiation program in response to subsequent BMP signals, and that Shh induces the expression of still unknown chondrogenic 'competence factors' (Murtaugh et al., 1999). Recently, it has been shown that *Bapx1* is one of such competence factors, as it is induced by Shh, and that overexpression of *Bapx1* in chick PSM mimics the chondrogenic effects of Shh (Murtaugh et al., 2001). Misexpression of Bapx1 in vivo through retroviral infection in the chick embryo leads to an expansion of the axial skeleton with thickened and often fused neural arches, and an ectopic eighth 'riblet' (Murtaugh et al., 2001). In the present study, we show that Pax1 also possesses chondrogenic properties, as it can induce aggrecan expression in cultures of chick PSM (Fig. 4B). Therefore, Pax1 can also be regarded as one of the competence factors.

We also tried to overexpress Pax9 in chick PSM using a retroviral construct RCAS(B) (Hughes et al., 1987) engineered to express mouse Pax9 (RCAS-B-Pax9). We observed neither *Bapx1* nor *aggrecan* upregulation when chick PSM explants were exposed to RCAS-B-Pax9 viral supernatant. The lack of positive results with the Pax9 retroviral system could be due to technical problems, such as the low viral titer obtained ($<10^7$ cfu/ml), or the use of a different viral vector (RCAS-B) that might infect sclerotomal cells with lower efficiency. We have occasionally observed that injection of RCAS-B-Pax9 viral supernatant in ovo into the PSM of the chick embryo induces fusion of the proximal part of the ribs, thickening of the neural arches and an ectopic eighth rib (I. R., A. M. and K. I., unpublished). This phenotype is strikingly similar to the one observed by overexpressing *Bapx1* (Murtaugh et al., 2001). Therefore, together with the observed synergistic function between Pax1 and Pax9 in sclerotome development in the mouse and the capacity of Pax9 to transactivate the *Bapx1* promoter, it is conceivable that Pax9 also can activate *Bapx1* and initiate chondrogenesis in vivo.

Activation of *Bapx1* expression by Pax1 and Pax9

The results from our transactivation study and EMSA assay strongly suggest that Pax1 and Pax9 directly activate *Bapx1*. From the transient transfection experiments, we assumed that there are at least two regions responsible for positive regulation by Pax1 and Pax9 in the *Bapx1* promoter region (Fig. 6): the intervals from –880 to –748 and from –270 to +109. We have found that Pax1/Pax9 directly bind to a motif located in the segment between –880 and –844 (Fig. 7). Our analysis failed to find Pax1/Pax9-binding sequences in the interval between –270 and +109. It is notable that transactivation by Pax1 and Pax9 significantly enhanced when the interval between –5285 and –2762 was removed (compare p5.3Bp-luc with p2.8Bp-luc in Fig. 6B). This observation suggests the presence of *cis*-elements that negatively influence on the Pax1/Pax9 transactivation.

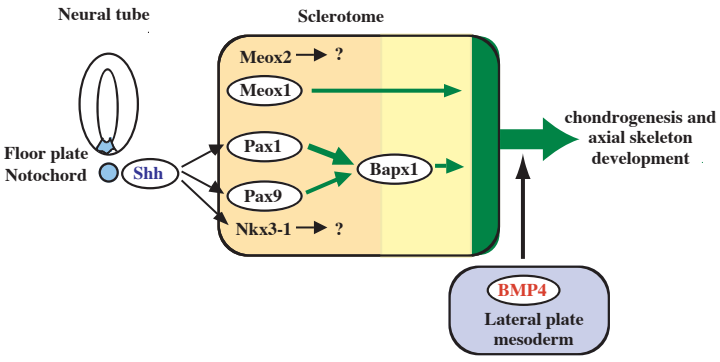

Fig. 8. Model for regulatory pathways involved in sclerotome differentiation. Ovals indicate genes with a proposed role in axial skeletal development. See text for details.

Pax proteins bind to specific DNA sequences through the 128 amino acid long paired domain. The paired domains in Pax1 and Pax9 are almost identical (98%), suggesting that they could bind the same DNA sequences. Indeed, DNA-binding studies have shown that Pax1 and Pax9 can bind the modified e5 motif derived from the *paired* binding sequence in the *Drosophila even-skipped* promoter (Chalepakis et al., 1991; Neubüser et al., 1995). Pax1 also binds in vitro with high affinity to CD19-2 (A-ins) and H2A-17C, two modified sequences identified originally as recognition sites for Pax5 (Adams et al., 1992; Czerny et al., 1993). Similarly, the paired box of zebrafish Pax9 recognizes in vitro with high affinity the original CD19-1 and modified CD19-2 (A-ins) sequences as well as a modified sequence of e5 (Nornes et al., 1996). However, most of these motifs are artificial consensus binding sequences, and none of the genes from which these motifs originate is related to normal expression or function of *Pax1* or *Pax9*. It has been reported that Pax1 can transactivate PDGFRα promoter in transient transfection experiments in some cell lines, but the maximum effect was observed with a mutated Pax1 protein, and the effects were dependent on the cellular context (Joosten et al., 1998). Therefore, *Bapx1* may be the first example of direct targets of Pax1 and Pax9 with a physiological relevance. Furthermore, the sequences contained in the oligonucleotide S1 can be considered as a novel DNA motif for binding of Pax1 and Pax9. In silico analysis by TFSEARCH (Heinemeyer et al., 1998) and MatInspector (Quandt et al., 1995) predicts a potential Pax6 binding site within the S1 region (positions –873 to –853, nucleotides marked in red in Fig. 7B). This site might turn out to be a Pax1/Pax9-binding site. Future study will define the nucleotide motif in the S1 region that is specifically bound by Pax1 and Pax9.

Pax1/Pax9 as main mediators of *Shh* signaling in sclerotome differentiation

Sclerotome differentiation is controlled by a number of molecules and signaling pathways, the hierarchy of which we are beginning to understand. Based on the data from published studies, together with the evidence reported here, we propose a model for sclerotome differentiation as schematized in Fig. 8. Paraxial mesoderm cells experience sequential changes in their responsiveness to specific signals, as they progress through developmental stages in the PSM, in the nascent somites and in the sclerotome. During the transition from PSM to somites (somite formation), paraxial mesoderm cells become competent to respond to Shh. It is proposed that Wnt signaling plays a key role in the establishment of this competence, via differential regulation on Gli genes in paraxial mesoderm cells (Borycki et al., 2000). After somites are formed, Shh plays an essential role in the induction, differentiation and survival of sclerotomal cells, by activating several transcription factors, including *Pax1*, *Pax9, Nkx3-1* and *Bapx1* (thin arrows in Fig. 8). Once sclerotomal cells are specified, some of these transcription factors (circled in Fig. 8; i.e. *Meox1*, *Pax1* and *Pax9*) play a role in further sclerotome differentiation and in chondrogenesis (green arrows in Fig. 8), by activating their downstream targets, including *Bapx1*. Although *Nkx3-1* and *Meox2* are shown to be dispensable for proper axial skeleton formation, their potential roles in the sclerotome still cannot be ruled out. They might have a functional redundancy with their closely related genes *Bapx1* and *Meox1*, respectively. Therefore, whether mice double mutant in *Nkx3-1*;*Bapx1* or in *Meox1*;*Meox2* show synergistic defects is of great interest. Finally, whether the Shh signaling pathway directly activates *Pax1* and *Pax9* is also not known. Shh signaling might indirectly activate *Pax1* and *Pax9,* by activating other transcription factors like *Meox1* and *Meox2*. Analysis of *Pax1* and *Pax9* expression in mice deficient in both *Meox1* and *Meox2* will clarify this point.

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