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 presomatic 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 cartilaginous 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 dorsovenetal patterning of somites and in the survival of sclerotomal cells (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Chiang et al., 1996). Sclerotomal survival of sclerotomal cells (Fan and Tessier-Lavigne, 1994; Peters et al., 1999) is essential and sufficient for the induction of sclerotome (Brand-Saberi et al., 1993; Dietrich et al., 1993; Koseki et al., 1993). Interestingly, mouse mutants lacking Pax1 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'-CACAGTGCTGATGTCAAGG-3' and 5'-AACCATGAAACGGAG-AGGCTC-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 Ncol/EcoRI and subcloned in Ncol/EcoRI-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 Ncol cohesive ends: 5'-CATGACATACGATGTCACAGATATTGCTGG-3' and 5'-CATGACACGGAAATCTGGAAATCTGGTA-3'. This HA linker was ligated to Ncol linearized pSlax-Pax1 to generate a Pax1-HA fragment. This fragment was excised from pSlax13 with Clal and subcloned into Clal-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 (BAboCO) 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 presomatic 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'-GCTCCGCGCGCCCTTCTCC-3' and 5'-GCGGCGCGCGCA-CAGAACAG-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'-GCTGCTACTCCCACAAGA-3' and 5'-CGCTGTA-TACTCCGTGCT-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'-CGTTCATGGACAGACTACGGG-3' and 5'-GTGAGATTTCTGACTGGCGCTG-3', which contain Ncol and EcoRI sites, respectively. The PCR product was cloned in pCR2.1-TOPO vector. The insert was excised with EcoRI, subcloned in EcoRI-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 p5.3Bp-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 KpnI/BamHI, and the corresponding fragment was subcloned into KpnI/BglII 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 KpnI/BglII 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 KpnI 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 RsrII/MluI fragment from the 7 kb Bapx1 genome clone was subcloned into MluI 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 order to identify target genes of Bapx1, 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 32P-labeled double stranded oligonucleotide and 1 µg of poly(dI-dC) in 20 mM HEPES pH 7.9, 60 mM KCl, 10% glycerol, 0.1 mM EDTA, 2 mM MgCl2, 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 (Stamatakis 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...
Pax1;Pax9 mutants. Nkx3-1 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.

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.

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. 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. 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
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 drop 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 and Pax9, with a transactivation activity on 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.

**DISCUSSION**

Previous studies have shown that Bapx1 mediates Shh
Activation of Bapx1 by Pax1 and Pax9 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 (Leitice 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; Riehmann 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 has been postulated that as yet unidentified genes, expressed in striped 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.](image-url)
Pax1/Pax9-binding site. Future study will define the nucleotide marked in red in Fig. 7B). This site might turn out to be within the S1 region (positions –873 to –853, nucleotides (Quandt et al., 1995) predicts a potential Pax6 binding site within the promoter 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 PGDFRa 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 TFSSEARCH (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, Paxl 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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