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# The role of *Bapx1* (*Nkx3.2*) in the development and evolution of the axial skeleton

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ABSTRACT

The bagpipe-related homeobox-containing genes are members of the NK family. *bagpipe* (*bap*) was first identified in Drosophila and there are three different bagpipe-related genes in vertebrates. Only two of these are found in mammals, the *Nkx3.1* and the *Bapx1* (*Nkx3.2*) gene. The targeted mutation in the mouse *Bapx1* gene shows a vertebral phenotype in which the ventromedial elements are lacking; these are the centra and the intervertebral discs. In addition, a region of gastric mesenchyme is abnormal. This mesenchyme surrounds the posterior region of the presumptive stomach and duodenum, and in the mutant fails to support normal development of the spleen. In Drosophila, *bagpipe* has a role in gut mesoderm and the mutant embryos have no midgut musculature. Thus *bap* related genes in mouse and *Drosophila* have roles in patterning gut mesoderm; however, neither of the mammalian genes has a discernible role in the gut musculature. In contrast, both mammalian genes have roles in developmental processes that have appeared recently in evolution. The *Bapx1* gene found in fish, amphibians, birds and mammals appears to have derived vertebrate specific functions sometime after the split between the jawless fish and gnathostomes.

*Key words*: *Bapx1*; axial skeleton; *Nkx3.1*; development; evolution.

## INTRODUCTION

The mammalian *Bapx1* gene (Tribioli & Luifkin, 1997; Tribioli et al. 1997) (also referred to as *Nkx3.2*) contains a homeobox and belongs to the NK family of developmental genes first identified in *Drosophila* (Kim & Nirenberg, 1989). This gene family was initially described as being comprised of 3 closely related members: the NK2, 3 and 4 genes responsible for the *ventral nervous system defective* (*vnd*), *bagpipe* (*bap*) and the *tinman* (*tin*) phenotypes respectively (reviewed in Harvey, 1996). The *bap* and *tin* genes are now known to comprise a cluster of related homeobox containing genes (referred to as the 93DE cluster) which includes *slouch* (*slou*), *ladybird* (*lbe and lbl*) and *C15* (for review see Jagla et al. 2001). *Bapx1* is most similar to the *Drosophila bap* (*Nkx3*) gene and along with closely related vertebrate genes in chick (Schneider et al. 1999), amphibian (both *Xenopus* [Newman et al. 1997] and urodele [Nicolas et al. 1999]) and fish (C. Kimmel, this volume), appear to form a distinct subgroup within the NK family.

In *Drosophila*, *bap* is required for the specification of the visceral mesoderm during midgut musculature formation (Azpiazu & Frasch, 1993). Genetic lesions within the *Drosophila bap* gene show a reduction or deletion in the visceral musculature. The role of *bap* in gut musculature and the expression of *Bapx1* in splanchnic mesoderm surrounding the gut led to the suggestion that the *Drosophila* and mouse genes may have similar roles during gut development (Tribioli et al. 1997). Analysis of *Bapx1* mutant mice does not support a similar role and in contrast, suggests that *Bapx1* has acquired novel functions during vertebrate evolution.

## RESULTS AND DISCUSSION

### Nkx3 *genes in vertebrates*

To determine the number of *Nkx3*-like genes in mouse, we did a large genomic screen using the *Bapx1* homeobox region as probe. In addition a conserved

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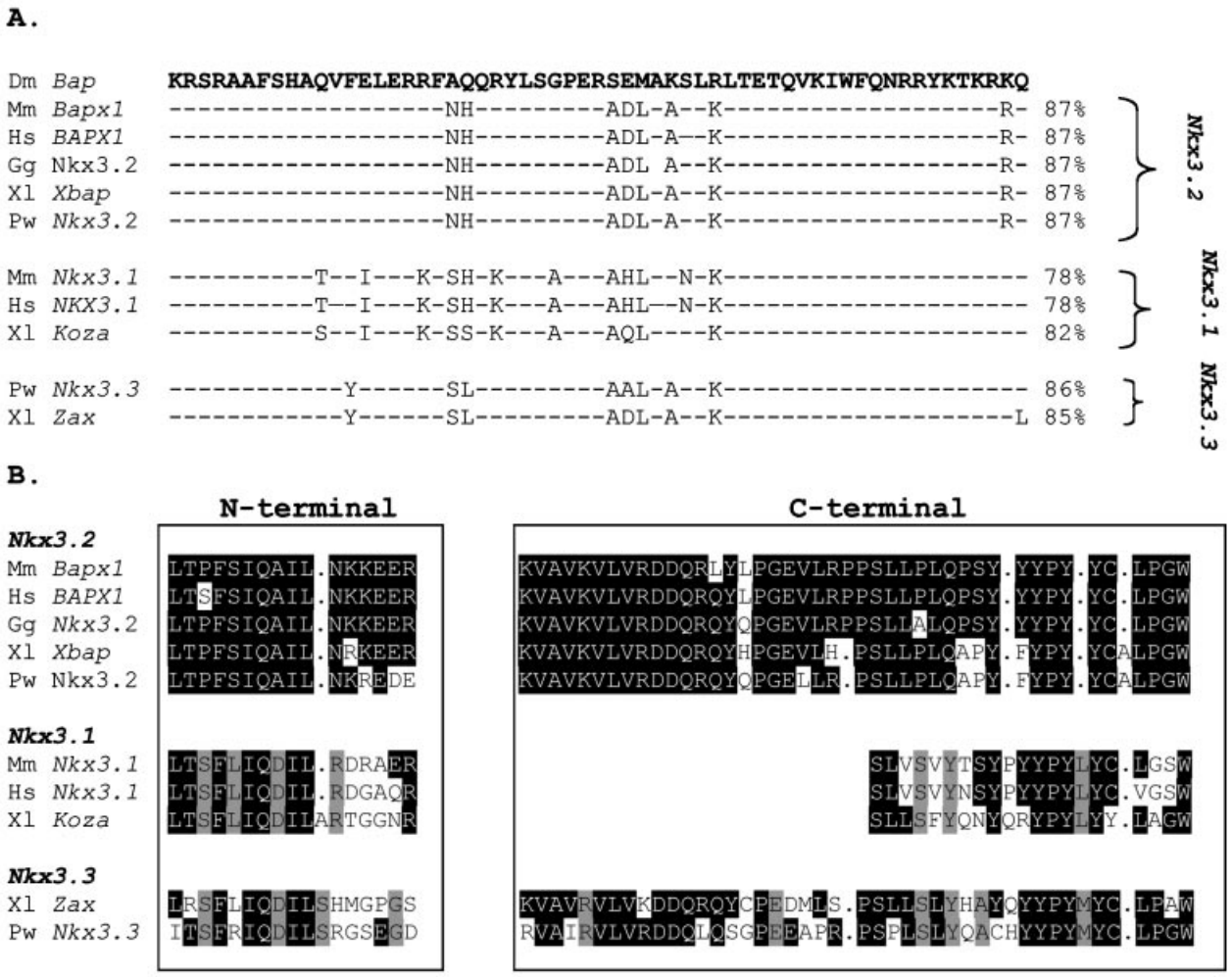


Fig. 1. Comparison of the amino acid sequences of the vertebrate Nkx3 genes shows 3 sequences subgroups. (*A*) Lineup of the homeodomains of the vertebrate Nkx3 genes in comparison to the *Drosophila* bagpipe (Dm *bap*) homeobox (top line). Clustering of the sequences into three subgroups is evident. The subgroups are *Nkx3.2* (at the top), *Nkx3.1* and *Nkx3.3*. The percentage identity with *bap* is shown to the right hand side of each sequence. Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Hs, human; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Pw, *Pleudeles waltl* (a urodele amphian). (*B*) Regions of additional sequence similarity in the Nkx3 genes. The N-terminal box and C-terminal box are located to their respective sides of the homeodomain. The identical amino acids among all three subgroups are denoted by shading in black. Grey shading represents amino acids conserved within subgroups. The similarities with the N- and C-terminal boxes further support the classification of the subgroups. *Nkx3.1* genes do not show any appreciable similarity to *Nkx3.1* and *Nkx3.3* in the 5′ half of the C-terminal box.

region 3′ of the homeobox (shown in Fig. 1*C*) was also used. Only the *Bapx1* and *Nkx3.1* genes were isolated. Several non *bap Nkx2*-like genes were selected using the homeobox region as probe due to cross-similarities within these subgroups. In addition, screening the Human Genome Database, the mouse genome (HTGS), and EST databases (dbEST), only *Bapx1* and *Nkx3.1* genes were found. Thus the data supports only 2 *bagpipe*-related genes in mouse and man. This is further supported by a recent study by Pollard & Holland (2000). They conclude that the *Nkx1*, *3*, and *4* genes occupy distinct locations within a large primordial chromosomal cluster containing a number of homeobox-containing genes, termed the NKL cluster. In both mouse and human, 2 clusters are predicted which contain *Nkx3* related genes; the clusters are a result of chromosomal duplications events that occurred during vertebrate evolution. The *bap*-related genes that reside in these syntenic linkage groups are *Bapx1* and *Nkx3.1*.

*Nkx3* related genes have been isolated from a number of vertebrates, and we have grouped these by overall sequence similarity (Fig. 1). Analysis of the homeobox sequence, the most highly conserved domain within the NK gene family, shows that the *Nkx3* genes cluster into three subgroups (Fig. 1*A*). The *Nkx3.2* subgroup of genes displays marginally higher sequence similarity to Drosophila *bap*. Outside the homeodomain, the subgroups are divergent suggesting derivation from ancient gene duplication

events from a common ancestral gene. Even within the subgroups there has been a high degree of divergence. For example, discounting similarity in the homeodomain, the 2 mammalian *Nkx3.1* proteins show 40 % amino acid nonidentity. Multiple sequence alignments (pileup in the GCG suite of programs) reveal two other conserved domains in the remainder of the proteins that distinguish the vertebrate *Nkx3* genes. These are an N-terminal box, encompassing the TN domain (Harvey, 1996) found in other NK genes, and a longer C-terminal box encompassing part of the NK2-SD domain. These are distinctly different from other vertebrate NK genes and further distinguish the subgroups of *Nkx3* genes (Fig. 1 *B*).

Interestingly, homologues of *Nkx3.3* have not been reported in birds and were not found in mammals. *Xenopus laevis* contains a number of novel genes due to a tetraploidy event in the recent ancestry of that species. *Nkx3.3* did not arise from such an event, since the gene appears in the urodele *Pleurodeles waltl* (Nicolas et al. 1999). Thus *Nkx3.3* either arose as a unique amphibian member to the *Nkx3* class or was lost later in the evolutionary lineage to mammals. The *Nkx3.3* gene in *Xenopus* called zampogna (*Zax*) (an Italian bagpipe; Newman & Kreig, 1999) is expressed in the muscle layer of the forming midgut and in the pharyngeal endoderm. It is interesting to speculate that the *Nkx3.3* gene provides the primordial function related to the role of Drosophila *bap* in specification of gut musculature.

### *Expression of* Bapx1 *in development*

*Bapx1* expression in the mouse (Tribioli et al. 1997) is detected initially at E8.5 in the splanchnic mesoderm adjacent to the prospective gut endoderm and in the sclerotomal portion of the somites. At E10.5 *Bapx1* is detected in limb mesenchyme and the first branchial arch that becomes restricted to the precursor of Meckel's cartilage. At the time that *Pax*1 is first seen in the sclerotome *Bapx1* expression turns on. By E9.5 *Bapx1* is evident in the sclerotome (Fig. 2 *A*), and (by E10.5) *Bapx1* is expressed in migrating sclerotome that moves toward and surrounds the notochord (Fig. 2 *B*).

Early lateral plate *Bapx1* expression has been reported to show left-right asymmetry (Schneider et al. 1999). In chick, expression is highest in the left lateral plate mesoderm and is responsive to left-sided signals such as Nodal, Lefty2 and Shh. In contrast, mouse (E8.5) expression is higher in the right lateral mesodermal plate. Organ asymmetry is reversed in the *inv/inv* mutant mouse and *Bapx1* expression follows, now found higher on the left side. However, by E9.5 this reported asymmetry of expression is lost (Fig. 2 *A*), as seen in the splanchnic mesoderm surrounding the gut. At E10.5, expression of *Bapx1* is seen broadly in the region of the pancreatic mesenchyme, the mesenchyme dorsal to the pancreatc bud associated with spleen development (Fig. 2 *B*, dashed line in *C*). It is not clear what role this early asymmetric expression plays.

### *Role of* Bapx1 *in axial skeletal development*

To determine the function of the *Bapx1* gene, we (Lettice et al. 1999) and others (Tribioli & Lufkin, 1999; Akazawa et al. 2000) targeted mutations into that locus by homologous recombination into mouse ES cells. The mutation is neonatal homozygous lethal, and no mutant newborns survive past the first few hours. The heterozygotes on the whole appear normal except that $\sim 50\,\%$ display tail kinks. At E18.5 the homozygous mutant fetuses appear shorter and squatter (Fig. 3 *A*, *B*). Further observations, which explain the outward stubby appearance, revealed that the axial skeleton is defective, the ventromedial aspects of each vertebra (the centra and the intervertebral discs) being absent (Fig. 3 *C*, *D*). The number of sclerotomal cells surrounding the notochord are markedly reduced, whereas those populating the lateral aspects of the vertebrae appear normal. The lateral prechondrogenic condensations appear normal and the timing of appearance of the multiple ossification centres in all aspects of the vertebra except the ventromedial component is unaffected (Fig. 3 *C*, *D*). Thus a deficiency of *Bapx1* results in specific loss of the midline components of each vertebra.

We examined a number of DNA markers by *in situ* hybridisation to define the developmental processes disrupted by the *Bapx1* mutation (Table). Several other mutations in mouse are known which affect the developing intervertebral bodies of the vertebra. In particular the *Pax1* (Wilm et al. 1998) (originally the undulated [*un*] mutation) and the *Gli2* (Mo et al. 1997) mutations show loss of the intervertebral bodies. Analysis of expression of *Pax1* and *Gli2* in *Bapx1*$-/-$ embryos (E13.5) showed no appreciable affect on the levels (Lettice et al. 1999; Tribioli & Lufkin, 1999). Since the notochord is a source for *Shh* production, we examined the expression of a number of genes responsive to Shh signaling (Table). No abnormal expression was found. Other expression patterns, *Mfh1*, *Mox1*, and *scleraxis*, representing early sclerotomal developmental events were analysed.

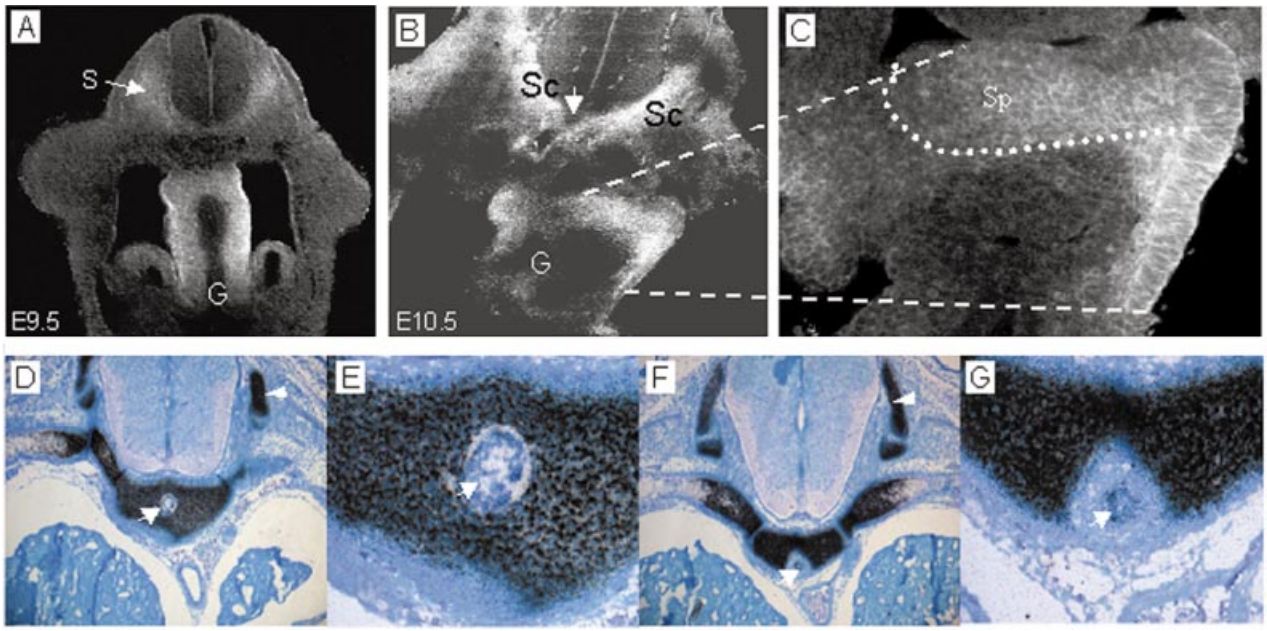


Fig. 2. Gene expression patterns in the developing mouse. (*A–C*) Expression of *Bapx1* in E9.5 (*A*) and E10.5 (*B*, *C*) wildtype mouse embryos. In (*A*), *Bapx1* expression is detected in the sclerotome component of the somite (arrow) and splanchnic mesenchyme surrounding the gut. In (*B*), the sclerotomal cells which are migrating toward the notochord (arrow) express *Bapx1*. Also the gastric mesenchyme expresses *Bapx1* which is shown at a higher magnification in (*C*). The portion of the mesenchyme that will give rise to the spleen is outlined by a dotted line and expresses *Bapx1*. (*D–E*) Transverse sections through E12.5 embryos expressing $\alpha$1 (II) collagen; wildtype, compared with *Bapx1*$-/-$ mutant embryos (*F*, *G*). Expression of $\alpha$1 (II) collagen is shown in the lateral elements (arrowheads, *D* and *F*) of both wildtype and mutant animals. Lack of expression specifically around the notochord (arrows, *E* and *G*) appears as a halo of cells. G, gut; S, somite; Sc, sclerotome, Sp, spleen.

*Mfh1* is of particular interest, since its role is associated with sclerotomal cell proliferation (Winnier et al. 1997). No appreciable differences were seen for the expression of any of these genes. Thus initial patterning of sclerotome occurs normally in the mutants and there appears to be no defect in cell proliferation. Cell proliferation was analysed more directly by BrdU labelling experiments in the *Bapx1* mutants (Tribioli & Lufkin, 1999) and confirmed the above data.

Analysis of differentiation markers, however, showed a contrasting account. Alpha1 (II) collagen (Fig. 3 *D–G*) is expressed during chondrogenesis in a similar vertebral pattern. In the *Bapx1* mutants the expression of alpha1 (II) collagen in the ventromedial elements of each vertebra is missing, whereas expression throughout the remainder of the developing vertebrae are normal. Recently Tribioli & Lufkin (1999) have shown that the migration of sclerotomal cells toward the notochord is unaffected by the *Bapx1* mutation, however there is a high degree of cell death in the cells associated with the notochord. Therefore *Bapx1* is required for directing a subset of sclerotomal cells toward a chondroblastic pathway. In the absence of the gene, the cells migrate but do not survive resulting in the reduction in the cell number of ventromedial cells.

Table. *Analysis of gene expression in* Bapx1$-/-$

| |
|---|
| Genes with similar vertebral phenotypes |
| *Pax1* |
| *Gli2* |
| Genes in the *Shh* pathway |
| *Gli2* |
| *Shh* |
| *Ptc* |
| Early sclerotomal markers |
| *Mox1* |
| *Mfh1* |
| *Scleraxis* |
| *BMP7* |
| Cellular proliferation marker |
| *Mfh1* |
| Differentiation markers |
| *Sox9* |
| *a1 (II)-collagen* |

## *Evolution of* Bapx1 *function*

The whole of each vertebra undergoes the process of chondrogenesis. The question, therefore, arises as to why a gene essential to this process only affects a subset of sclerotomal cells, i.e. those that will give rise to each of the centra. First of all, it is apparent that different parts of each vertebra require different signals for their normal development. Both *Pax1* and *Bapx1* participate in production of the intervertebral bodies and are responsive to *Shh* in the notochord

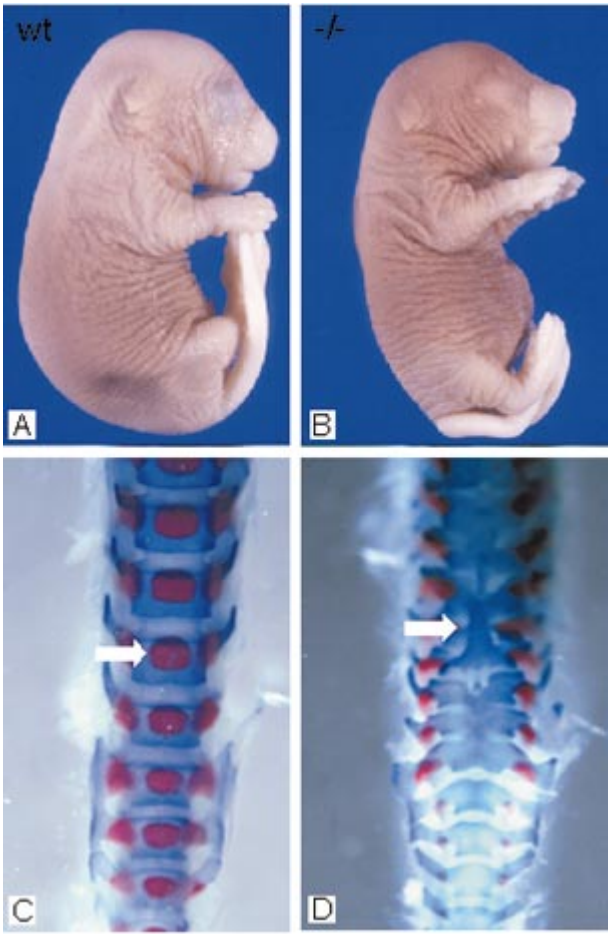


Fig. 3. The phenotypic characteristics of the *Bapx1* mutant fetuses. Top panels compare the outward phenotype of wildtype (*A*) and mutant (*B*) E18.5 embryos. Note that the mutant embryo is squatter and does not show the rounded rump region of the wildtye, and the tail is shorter and bent. The bottom panel shows a ventral view of the vertebral column in E18.5 wildtype (*C*) and mutant (*D*) fetuses. The white arrow in (*C*) indicates the ossification center that will give rise to one of the centra in the wildtype animal. These are missing in the mutant mouse (*D*).

(Fan & Tessier-Lavigne, 1994; Wallin et al. 1994). In the absence of *Shh* (in the *Shh* mutant mouse), sclerotomal cells appear and initially express *Pax1* at a low level. However, *Pax1* expression never reaches wildtype levels and is lost. Furthermore both *Pax1* and *Bapx1* are induced by SHH in somite culture experiments (Fan & Tessier-Lavigne, 1994, and personal communication). Other genes affecting more lateral aspects of each vertebra are not under the influence of notochordal signals. For example, expression of *Uncx4.1* is not dependent on notochordal signaling but rather requires the Notch/Delta signaling pathway. Deletion of *Uncx4.1*, a paired-like homeobox-containing gene, causes loss of lateral elements such as pedicles, transverse processes and proximal ribs (Leitges et al. 2000; Mansouri et al. 2000). We suggest that sclerotomal cells respond to signals differently depending on their gene expression profile and form vertebral elements adjacent to the signalling source. These discriminating processes specify the individual vertebral components which make up an individual vertebra.

So how did this assemblage of genes and signalling pathways required to form a vertebra come about? The evolutionary history of the early vertebrates suggests that vertebrae evolved in a progression of steps. Individual elements of the vertebra arose at different times in evolution (reviewed in Brand-Saberi & Christ, 2000). In the agnathans such as lamprey and primitive cartilaginous fishes such as the chimera (see Fig. 4), the major support along the anterior posterior axis is the notochord and the fibrous sheath in which it is encased. However, in these primitive fishes there are dorsal cartilaginous elements that protrude from this fibrous sheath that surround the neural tube (reviewed in Brand-Saberi & Christ, 2000). In the selachians (sharks and rays) and the bony fishes the notochord for the first time is surrounded by cartilaginous (in sharks) or bony centra. The dorsal elements presumably gave rise to the neural arches in these fishes. Thus an evolutionary progression is evident in the appearance of elements that compose the vertebrae; the addition of the ventromedial located centra occurred later at a distinct stage in vertebrate evolution. We predict that genes such as *Bapx1* and *Pax1* acquired sclerotomal functions sometime after the split with the agnathans within the lineage of gnathostomes coincident with the appearance of the centra (Fig. 4). A chromosomal duplication event (tetraploidisation) is predicted to have occurred during this evolutionary period (Holland et al. 1994). It seems likely that *Bapx1* function arose during this period as a result of the duplication.

Do the genes acquire additional expression domains in this acquisition of novel function? An indirect argument focuses around the *Pax9* gene. *Pax9* is closely related to *Pax1* and is expressed in sclerotomal cells (and other embryonic regions) overlapping those that express *Pax1*. In mouse, deletion of the *Pax9* gene in combination with *Pax1*$-/-$ shows severe ventromedial defects in the vertebrae (Peters et al. 1999). Therefore *Pax9* is a marker of sclerotomal cells and operates in skeletal development acting in conjunction with *Pax1*. In the agnathan *Lampetra japonica Pax9* is not expressed in the sclerotomal compartment (Ogasawara et al. 2000); however, in teleost *Pax9* marks the sclerotome (Nornes et al. 1996). *Pax9* and presumably *Pax1* and *Bapx1* are recruited from other developmental compartments or

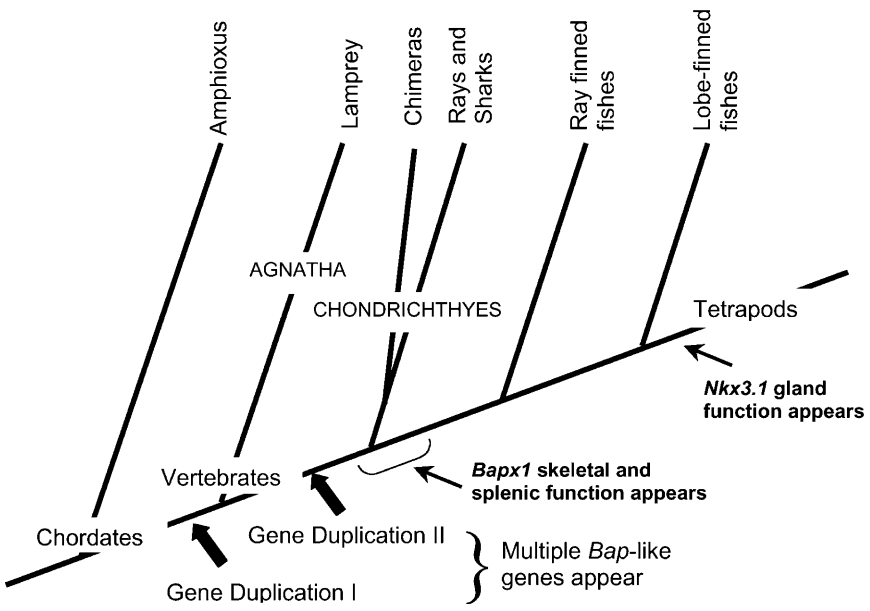


Fig. 4. Predicted evolutionary history of the *Bapx1* and *Nkx3.1* gene functions. The extant species representative of each class is shown at the top of the diagram. The bottom timeline goes from the chordate amphioxus to the appearance of the tetrapods and predicts the points at which gene duplications have been suggested to occur. We predict based on *Bapx1* and *Nkx3.1* functions in mouse at what point *Bapx1* and *Nkx3.1* acquired their functions.

newly appear (as may be the case for *Bapx1*) to participate in this novel developmental function.

The second developmental function for *Bapx1* is the formation of the spleen. *Bapx1* is expressed in spleen mesenchyme (Fig. 2*C*), and in the absence of *Bapx1* the spleen does not appear. This is an early developmental effect; expression of the spleen specific marker *Hox11* (*Tlx1*) is not detected in the mutant embryos (Lettice et al. 1999). The advent of the spleen in vertebrate evolution is not clear. Hildebrand (1988) suggests that cords of spleen tissue exist in lamprey; however, these are associated with the gut submucosa. In selachians, ray-finned fish and all tetrapods the spleen develops external to the gut residing in surrounding dorsal mesentery. We suggest that the *Bapx1* spleen function has arisen as part of genetic network required for reorganisation of the spleen to the periphery of the gut. A crucial stage in spleen evolution may be the repositioning to a location independent of the gut in the gastric mesentery.

### *Evolution of other members of the* Nkx3 *group*

Targeted deletions of *Nkx3.1* in mouse have recently demonstrated the functions of this NK family member (Bhatia-Gaur et al. 1999; Schneider et al. 2000; Tanaka et al. 2000). The mutant phenotype suggests roles in the proliferation of glandular epithelium and in the formation of ducts in the prostate gland and in a minor set of salivary glands called the palatine. The prostate is mammalian specific and salivary glands arose in vertebrates as a requirement once tetrapods became true land-based feeders. Hence, this gene has apparently acquired vertebrate specific functions quite late in vertebrate evolution.

Thus it appears that the *bagpipe*-related genes have undergone a continual acquisition of functions throughout vertebrate evolution. A source for genetic diversity is the process of chromosomal tetraploidisation that is predicted to have occurred twice in vertebrate evolution (Holland et al. 1994) (Fig. 4). These events play an important role in vertebrate evolution particularly for the appearance of vertebrate specific organ systems. Both *Bapx1* and *Nkx3.1* genes were probably generated during these early duplication events (Pollard & Holland, 2000). *Bapx1* may have acquired roles in both spleen and vertebral development at the time of one of these duplication events. *Nkx3.1*, on the other hand, was co-opted later for roles in glandular epithelial development.

Remnants of four NKL clusters (discussed above) are present in the mouse and human genomes; however, only two *Nkx3*-like genes remain suggesting that two were lost. We speculate that the *Nkx3.3* gene identified in amphibian was one of the genes lost in the lineage to mammals.

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