Defective bone formation in Krox-20 mutant mice

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SUMMARY

Endochondral ossification is the prevalent mode of vertebrate skeleton formation; it starts during embryogenesis when cartilage models of long bones develop central regions of hypertrophy which are replaced by bony trabeculae and bone marrow. Although several transcription factors have been implicated in pattern formation in the limbs and axial skeleton, little is known about the transcriptional regulations involved in bone formation. We have created a null allele in the mouse Krox-20 gene, which encodes a zinc finger transcription factor, by in frame insertion of the E. coli lacZ gene and shown that hindbrain segmentation and peripheral nerve myelination are affected in Krox-20−/− embryos. We report here that Krox-20 is also activated in a subpopulation of growth plate hypertrophic chondrocytes and in differentiating osteoblasts and that its disruption severely affects endochondral ossification. Krox-20−/− mice develop skeletal abnormalities including a reduced length and thickness of newly formed bones, a drastic reduction of calcified trabeculae and severe porosity. The periosteal component to bone formation and calcification does not appear to be affected in the homozygous mutant suggesting that the major role for Krox-20 is to be found in the control of the hypertrophic chondrocyte-osteoblast interactions leading to endosteal bone formation.

Key words: bone formation, gene disruption, Krox-20, transcriptional control, mouse

INTRODUCTION

Bone morphogenesis is a complex process, which results from the regulated proliferation, migration, condensation and differentiation of progenitor cells deriving from the neural crest, the sclerotome and the lateral plate mesoderm. During skeletogenesis, mesenchymal cells aggregate and give rise to condensations which then differentiate leading to the generation of mature bone or cartilage (Erlbacher et al., 1995). It is possible to distinguish between two major modes of bone development: intramembranous and endochondral. The first one involves the direct differentiation of precursor cells into osteocytes (bone-forming osteoblasts) and is typical of the flat bones of the skull. The second one is characterised by the gradual conversion of an initial cartilage model into bone. This occurs by the concomitant deposition of bone matrix in a cylinder at the periphery of the diaphysis by perichondral osteoblasts (periosteal bone) and within the cartilage scaffold by osteo-progenitor cells (endosteal bone). This last population invades the cartilage model together with bone marrow cells during the neovascularisation process that leads to the formation of the bone marrow cavity. The process of endosteal bone formation is responsible for the generation of bony trabeculae and of the inner surface of the cavity of most long bones.

Replacement of cartilaginous models by bony tissue involves an orderly sequence of chondrocyte differentiation, which leads to the formation of calcified hypertrophic cartilage followed by the death or further maturation of chondrocytes to osteoblast-like cells. Although most endochondral bone-forming cells derive from marrow stromal cells (Pechak et al., 1986; Beresford, 1989), the possibility of an additional differentiation of hypertrophic chondrocytes to “osteoblast-like” cells has been frequently suggested in the literature (Thesingh and Scherft, 1986; Weiss et al., 1987; Yoshioka and Yagi, 1988; Thesingh et al., 1991; Galotto et al., 1994). This hypothesis was recently supported by the osteogenic differentiation observed in long-term cultures of chondrocytes (Closs et al., 1990; Strauss et al., 1990; Descalzi-Cancedda et al., 1992; Roach, 1992; Gentili et al., 1993) and by cartilage transplantation experiments (Moskalewski and Malejczyk, 1989).

Tissue recombination assays have shown the critical importance of inductive events for the process of skeletogenesis. For example, communication between the apical ectodermal ridge and the mesodermal progress zone is required for the generation of limb skeleton (Morgan and Tabin, 1994), while formation of the axial skeleton relies on molecular signalling between the notochord and sclerotomal cells (Fan and Tessier-Lavigne, 1994). Factors, such as fibroblast growth factor (FGF), retinoic acid and sonic hedgehog, have been implicated as soluble mediators of these inductive interactions (Laure et al., 1994; Niswander et al., 1994; Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). It has been proposed that these soluble factors
might regulate skeletal form by establishing patterns of expression of transcriptional factors (Dollé et al., 1993; Lauf er et al., 1994). Indeed, it has been shown by targeted inactivation experiments that homeobox-containing genes such as the *msh*-like genes and *MHox* constitute regulators of epithelial-mesenchymal interactions required for skeletal organogenesis (Davidson et al., 1991; Satokata and Maas, 1994; Martin et al., 1995). Furthermore, *Hox* genes have been demonstrated to play a central role in the determination of the anatomic identity of single skeletal elements (Morgan and Tabin, 1993; Krumlauf, 1994). However, little is known specifically about the transcriptional regulation of the replacement of cartilaginous models by bony tissue. Here we present evidence for the involvement of the transcription factor Krox-20 in this latter process.

The *Krox-20* gene was originally identified as a serum response immediate-early gene which encodes a protein with three C2H2-type zinc fingers (Chavrier et al., 1988a,b; Gilardi et al., 1991). The Krox-20 protein was subsequently shown to bind to a specific DNA sequence and to constitute a transcription factor (Chavrier et al., 1990; Nardelli et al., 1991; Vesque and Charnay, 1992). During embryogenesis, *Krox-20* is first expressed in two prospective hindbrain segments, the rhombomeres 3 and 5, (Wilkinson et al., 1989) and its product has been shown to constitute a direct transcriptional activator of two *Hox* genes within these rhombomeres (Sham et al., 1993; Nonchev et al., unpublished data). The important role of *Krox-20* in the developing hindbrain was confirmed by targeted inactivation, which resulted in a partial or total deletion of rhombomeres 3 and 5 (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). In our case, the creation of a null allele of *Krox-20* involved the in frame insertion of the *E. coli lacZ* coding sequence, resulting in the synthesis of a Krox-20/β-galactosidase hybrid protein which retained β-galactosidase activity (Schneider-Maunoury et al., 1993). This allowed the detailed analysis of the *Krox-20* expression pattern and revealed its additional role in Schwann cells, where its disruption resulted in a block in differentiation, thus preventing myelination in the peripheral nervous system (Topilko et al., 1994).

We have now pursued the analysis of *Krox-20* expression and function. We here demonstrate that the gene is active both in hypertrophic chondrocytes and osteoblasts/osteocytes and that its inactivation results in a defect in endochondral ossification.

**MATERIALS AND METHODS**

**lacZ expression patterns and histological analyses**

Postimplantation embryos were recovered at the appropriate stages, considering the day of the plug as day 0.5 of development. For *lacZ* expression pattern analysis, embryos were fixed for 15 minutes in PBS containing 2% PFA and 0.2% glutaraldehyde. X-gal staining was performed as described (Sham et al., 1993). At 16.5 dpc and later stages embryos were fixed and stained by perfusion through the umbilical vein. X-gal-stained embryos were either embedded in paraffin and sectioned (10 µm serial sections) or dehydrated and clarified in a benzyl-benzoate/benzyl alcohol solution 2:1 to reveal the staining of inner structures. In some experiments, the sections were counterstained with cresyl violet or with cosin-hematoxilin.

**Antibodies and immunohistochemistry**

To determine the tissue localisation of Krox-20, we used a rabbit antiserum (539A) raised against a Krox-20 fusion protein whose binding properties have been previously described (Vesque and Charnay, 1992). Staining with the anti-Krox-20 polyclonal antibody was performed on frozen sections (10 µm) of wild-type whole embryos or dissected tissues fixed in 2.5% paraformaldehyde for 1 hour and equilibrated in 30% sucrose. Sections were treated sequentially with affinity purified antibody diluted in blocking solution (10 µg ml⁻¹ in PBS, 5% foetal calf serum (FCS); overnight) and peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham, 10 µg ml⁻¹ in PBS, 5% FCS; 2 hours); peroxidase activity was revealed with dianaminobenzidine with standard techniques. Control sections were stained with preimmune serum of the same rabbit.

For double labelling of β-galactosidase activity and collagen type X or osteocalcin, 9 dpc (days post natal) mice were first stained with X-gal by perfusion, embedded in paraffin and sectioned (8 µm). The deparaffinized sections were incubated overnight with the appropriate dilution of anti-collagen type X or anti-osteocalcin affinity-purified antibodies (Kirsch and Von Der Mark, 1991) followed by biotinylated anti-rabbit second antibody and phycoerythrin-conjugated streptavidin (Biomedia, CA) and observed with a Zeiss Axiosphot

![Fig. 1. Expression of Krox-20/lacZ in the developing skeleton.](image-url)
microscope equipped with the appropriate filters. The orange-yellow signal of phycoerythrin, which is easily distinguished from the green autofluorescence of calcified regions of the section, was enhanced by digital treatment of the images.

Skeletal preparations
Double staining of the skeleton with alcian blue and alizarin red was performed as described elsewhere (Wallin et al., 1994).

RESULTS

Krox-20 is expressed in sites of endochondral bone formation

Previous analysis of heterozygous Krox-20 mutant mice has shown that the expression pattern of the Krox-20/lacZ hybrid gene in the developing hindbrain and in the peripheral nervous system faithfully reproduces that of the normal gene (Schneider-Maunoury et al., 1993; Topilko et al., 1994). Further analysis of Krox-20/lacZ expression revealed that the gene was also expressed in whisker and hair follicles as well as in all sites of endochondral bone formation and no qualitative differences in β-galactosidase activity were observed between heterozygotes and homozygotes up to 17.5 dpc, although the activity level was higher in homozygotes (Figs 1, 2D-E and data not shown). In the skeleton, β-galactosidase activity was first detected in the central region of the cartilage model of long bones around 14.5 day post coitum (dpc) (Fig. 1A). At this stage, expression was confined to a population of chondrocytes located at the periphery of the diaphyseal part of the cartilage model with no labelling of the more centrally located chondrocytes or of the epiphyseal regions (Fig. 2D). At later stages of development, β-galactosidase activity appeared progressively in all bones undergoing endochondral ossification in a temporal sequence similar to that described for the initiation of ossification (Kaufman, 1992) (Fig. 1B). In bones formed by intramembranous ossification, such as those of the skull, β-galactosidase activity was not detected during embryonic development (Fig. 1C) and appeared only perinatally in differentiating bone cells (data not shown). The only bone of the skull to express Krox-20/lacZ during embryogenesis was the supraccipital bone, which has an endochondral origin (Fig. 1C). Around 17 dpc, X-gal staining was observed in hypertrophic cartilage cells located in a cylinder at the periphery of the diaphysis (Fig. 1D,E) and in the most differentiated layer of hypertrophic cartilage in the growth plate (Fig. 2E). At later stages of development, Krox-20/lacZ expression continued to be detected in the cartilage at the periphery of the growth plate and in calcified hypertrophic chondrocytes at the border between the hypertrophic cartilage and the primary spongiosa (Fig. 4A). To confirm that the pattern of distribution of β-galactosidase activity in the developing bone did reflect the expression of Krox-20, sagittal sections of wild-type 16.5 dpc embryos were stained with an anti-Krox-20 polyclonal antibody. This led to a nuclear staining consistent with the location of the cytoplasmic enzymatic activity observed in the Krox-20+/− embryos (Fig. 2A-C).

During bone differentiation, β-galactosidase activity was
also observed in most periosteal osteoblasts (Fig. 2F) and osteocytes (Fig. 2G) and in osteoblasts participating in endochondral ossification (Fig. 2H). β-galactosidase activity could be detected in osteoblast only as they differentiated into osteocytes and began to deposit a bony matrix. Our data suggests that most osteocytes did express Krox-20/lacZ. However, at the present level of our analysis, we do not know whether all of them were positive.

**Skeletal alterations in Krox-20<sup>−/−</sup> mice**

The observation of the expression of Krox-20 in regions of endochondral ossification led us to examine skeletal development in Krox-20<sup>−/−</sup> mice in greater detail. As reported previously, the homozygous Krox-20 mutation is not lethal before birth (Schneider-Maunoury et al., 1993). Newborn mutant pups are of a similar size as their littermates; about two thirds of them die within the first 48 hours after birth and most of the others die around the end of the second week. These animals gain weight more slowly than their littermates and are smaller in size (Fig. 3A,B,D).

The skeleton of Krox-20<sup>−/−</sup> mice was found to be much more transparent to X-rays than that of wild-type littermates indicating a strong calcium deficiency (Fig. 3A,B). Long bones of Krox-20<sup>−/−</sup> mutant mice were thinner and shorter than those of their littermates and very poorly stained by alizarin (Fig. 3D); two weeks after birth, the tibia, femur and radius of Krox-20<sup>−/−</sup> mice were 24±5% (n=7) shorter than those of Krox-20<sup>+/+</sup> littermates; skull bones were much less affected by the mutation (only 6±2% reduction, n=4). Most bones were very porous; this was more evident in bones where the periosteal contribution to ossification is less prominent such as the vertebrae and the bones of the wrist (Fig. 3C). The mandible, which has a larger endochondral contribution to the ossification of its basal aspect than to that of its temporal aspect, was deformed, having a more pronounced reduction in size in the base (Fig. 3E-G).

**Histological defects in bones from Krox-20 homozygous mutants**

Histological analysis of bones from 9 day postnatal (dpn) Krox-20<sup>−/−</sup> mutant mice revealed a lesion characterised by a compression of the hypertrophic growth plate, a very strong reduction in the number and length of calcified trabeculae (Fig. 4C-F) and abnormalities in the endosteal part of the diaphysal bone (Fig. 4G-H). At birth, the growth plates of homozygous mutant bones, where a bone marrow cavity had formed, terminated abruptly with a layer of calcified hypertrophic cartilage and no trabecular bone formation (Fig. 4A,B). At later stages, clumps of β-galactosidase-expressing cells, possibly osteoblasts, were often seen in the proximity of, but not in contact with, the growth plate (Figs 4D,F, 5B) as if a direct contact between fully differentiated cartilage and osteoblasts could not be established in mutant animals. In Krox-20<sup>+/−</sup> mice, β-galactosidase activity was strongly reduced in hypertrophic cartilage cells after the beginning of trabeculae formation (Fig. 4C) and persisted only in cartilage cells located at the periphery of the growth plate (Fig. 4E). In contrast, in Krox-20<sup>−/−</sup> mice, Krox-20/lacZ-positive cells continued to be found at the border between the hypertrophic cartilage and the bone marrow cavity (Fig. 4D,F), suggesting that Krox-20 might be expressed at a very late stage of cartilage differentiation and raising the possibility that the mutation could result in a block at this stage, thus preventing Krox-20 down-regulation.

**Bone differentiation markers in Krox-20<sup>−/−</sup> mutant bones**

In order to better define the stage of bone formation affected by
the *Krox-20* mutation, we have analysed the expression of molecular markers of cartilage and bone differentiation. Collagen type X is a matrix molecule expressed only by hypertrophic chondrocytes at an advanced stage of differentiation (Jacenko et al., 1991; Kirsch and Von Der Mark, 1991). Although the lesions observed in *Krox-20*−/− mice resemble closely those observed in transgenic mice in which the function of collagen type X had been altered by the expression of a truncated form of the protein (Jacenko et al., 1993), we did not observe any obvious alteration in the pattern of expression of this gene (Fig. 5A,B). This is not surprising since counterstaining of the same sections for β-galactosidase activity showed that *Krox-20/lacZ* positive cells (arrows in Fig. 5A,B) are located in a layer of chondrocytes closer to the bone marrow than collagen type X-positive chondrocytes. Staining with anti-osteocalcin, an early marker of bone differentiation (Mackowiak et al., 1992), revealed a very strong reduction in the number of osteocalcin-positive cells in the endosteal part of mutant bones (Fig. 5C,D). The few endosteal osteocalcin-positive cells were not linked to the growth plate nor did they form bony trabeculae, but were clustered in isolated clumps (Fig. 5D). Periosteal bone was strongly osteocalcin-positive in the *Krox-20*−/− mice as in their control littermates (Fig. 5C,D), suggesting that bone formation by periosteal osteoblasts was not affected by the *Krox-20* mutation.

**DISCUSSION**

In this paper, we have analysed the expression pattern of *Krox-20* in the developing skeleton and determined the effects of its inactivation on bone formation. We have shown that *Krox-20* is expressed in three apparently separate lineages of bone-forming cells: chondrocytes, endochondral and perichondral osteoblasts. *Krox-20* expression is first detected at 14.5 dpc in the diaphysis of the cartilage models of long bones. It persists during later development in hypertrophic chondrocytes located at the periphery of the growth plate and at the border with the primary spongiosa. *Krox-20* is also expressed by endosteal and periosteal osteoblasts, but only when they differentiate into osteocytes during the formation of trabecular and periosteal bone; it continues to be transcribed in most osteocytes at least up to 14 dpn. Inactivation of *Krox-20* results in a defect in endochondral bone formation characterised by a compression of the hypertrophic growth plate and a very strong reduction in trabecular bone and in the endochondral component of diaphyseal bone. This defect is shown schematically in Fig. 6. As indicated above, about one third of the *Krox-20* homozygous mutants survive the perinatal period and die at the end of the second week after birth. These animals are smaller than wild-type littermates and, at 14 dpn, their sizes correspond to that of 10-12 dpn controls. We do not know presently whether this smaller...
size is a direct consequence of the defects observed in endochondral ossification. In contrast, these later defects cannot be interpreted themselves as a consequence of a simple growth retardation for the following reasons: (i) abnormal development of the growth plate is detected at birth, when the sizes of homozygous mutant and control pups are very similar; (ii) the histological phenotype observed in the homozygous mutant is never seen in wild-type animals although, as indicated, the mutants can reach the size of 12 dpn wild-type animals.

The formation of trabecular bone involves the interaction of endosteal osteoblasts with the most differentiated layer of hypertrophic chondrocytes in the growth plate. The last known cartilage-specific marker in the process of cartilage differentiation is collagen type X, which is expressed in a layer of the growth plate adjacent to the region of trabecular bone formation (Kirsch and von Der Mark, 1991). As mentioned above, it has been repeatedly suggested that hypertrophic chondrocytes need to undergo a further step of differentiation to “osteoblast-like” cells to create the conditions for endosteal osteoblasts to generate trabecular bone (Thesingh and Scherft, 1986; Weiss et al., 1987; Yoshioka and Yagi, 1988; Thesingh et al., 1991; Galotto et al., 1994). We have observed that, postnatally, Krox-20 is invariably expressed in hypertrophic chondrocytes located in a layer of the growth plate deeper than that containing collagen type X-positive cells. Our data suggest therefore that collagen type X-positive hypertrophic chondrocytes undergo a further step of differentiation characterised by the expression of Krox-20. This gene therefore becomes the last known marker of chondrocyte differentiation. One interpretation of the reduction in trabecular bone amount observed in \textit{Krox-20}\textsuperscript{-/-} mice is therefore that disruption of Krox-20 would prevent the last step of chondrocyte differentiation which would be essential to create the conditions for endosteal bone formation. This might be due to either the alteration of specific genes activated during chondrocyte differentiation or a defect in the asymmetric division of chondrocytes into osteoblast-like cells. This would result in a lesion that shares some similarities with spondylometaphyseal dysplasia or to metaphyseal chondrodysplasia in which chondrocyte differentiation is altered due to a mutation of the collagen type X gene (Jacenko et al., 1993; Gentili et al., 1993).

An alternative, but not exclusive, interpretation of our data would be that the mutation directly affects endochondral osteoblasts. These cells do indeed express Krox-20 as they differentiate into osteocytes to form trabecular bone and inactivation of the gene might alter the properties of these latter cells. The study of in vitro cultures of chondrocytes and/or osteoblasts derived from mutant animals might provide clues in the future to distinguish between these two possibilities.

The fact that Krox-20 is expressed simultaneously by two populations of cells, which possibly have different developmental histories and which co-operate in the formation of bony trabeculae, might reflect similar responses to a common inducing signal. Much experimental and genetic evidence suggests that the determination and maintenance of the shape of bones and their relative proportions during growth result from a complex network of local and systemic molecular signals. Some of these mediators have been identified as members of the TGF\textbeta\ superfamily (Kingsley, 1994) and of the FGF family (Rousseau et al., 1994; Peters et al., 1993; Jabs et al., 1994; Muenke et al., 1994) and in other molecules such as the parathyroid hormone-related protein (PTHrP) whose mutation leads to accelerated chondrocyte differentiation and almost complete skeletal ossification at birth (Amizuka et al., 1994; Karaplis et al., 1994). These signals are likely to lead to the expression of specific transcription factors involved in skeletogenesis (Vainio et al., 1993). It will therefore be interesting to analyse whether they are involved in controlling the expression of Krox-20. In any case, the availability of the \textit{Krox-20}\textsuperscript{-/-}\textit{lacZ} marker to monitor the differentiation of osteoprogenitor cells might provide a convenient system to study some aspects of this specific regulatory network.

In \textit{Krox-20}\textsuperscript{-/-} mutant animals, we did not observe any obvious defect in periosteal bone, raising the possibility that the mutation does not affect the differentiation of periosteal osteoblasts which do however express Krox-20 as they become osteocytes. This might reflect the fact that Krox-20 could have different functions.

\textbf{Fig. 5.} Distribution of osteocalcin and collagen type X in \textit{Krox-20}\textsuperscript{+/-} and \textit{Krox-20}\textsuperscript{-/-} mutant bones. Staining with polyclonal antibodies directed against (A,B) collagen type X and (C,D) osteocalcin of the growth plate of the femur from 9 dpn \textit{Krox-20}\textsuperscript{+/-} (A,C) and \textit{Krox-20}\textsuperscript{-/-} (B,D) mice. The positive immunostaining is seen as a orange-yellow signal over a green background of autofluorescence. On the left of each frame is the growth plate and hypertrophic cartilage (hc) and on the right the trabecular bone (tr) and the bone marrow cavity. Sections A and B were simultaneously stained for anti-collagen type X immunoreactivity (orange-yellow) and for the distribution of \beta\-galactosidase activity. \beta\-galactosidase-positive cells could be identified by inspections of the section with phase-contrast optics and are indicated by white arrows; they are always found at the right of the collagen type X-positive region of the growth plate in a layer of hypertrophic cartilage adjacent to the region of trabecular bone formation. Note the reduced number of trabeculae in the \textit{Krox-20}\textsuperscript{-/-} bone; the few endochondral osteoblasts differentiate nevertheless in osteocalcin-positive cells (open arrow in D). Magnification in A,B, \texttimes 420; C,D, \texttimes 90.
in periosteal osteoblasts and in chondrocytes or endosteal osteoblasts. Alternatively, it is possible that the inactivation of Krox-20 is compensated for by the presence of other transcription factors only in certain cellular populations. In this respect, it is interesting to note that Krox-24/Egr-1 (Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988), which encodes a transcription factor closely related to Krox-20, is expressed in areas undergoing bone formation between 14.5 and 17.5 dpc (McMahon et al. 1990). As Krox-20 and Krox-24 bind to identical DNA sequences in vitro (Lemaire et al., 1990), it is possible that they can regulate the same target genes. The two genes could therefore be redundant or subject to compensation mechanisms when the expression of one of them is reduced. The precise identification of the cell types expressing Krox-24/Egr-1 in ossification centres and the analysis of the possible phenotypes associated with a null mutation of this latter gene as well as with the combination of the Krox-20 and Krox-24 mutations should clarify this issue.

Finally, the analysis of Krox-20 function during hindbrain development has led to the demonstration of its involvement in the transcriptional activation of several regulatory genes including Hox genes (Sham et al., 1993; Nonchev et al., unpublished data; Seitanidou et al., unpublished result). It is likely that the hindbrain phenotype resulting from the null mutation originates from the lack of activation of such downstream genes. It is possible that the function of Krox-20 in chondrocytes or osteoblasts is also to control a genetic cascade by the direct transcriptional activation of a set of regulatory genes. Alternatively, Krox-20 could participate in the direct transcriptional control of structural genes specific for the differentiated state. In either case, the identification of the Krox-20 target genes in osteogenic cell types should shed some more light on the molecular mechanisms of bone formation (Erlebacher et al. 1995). In particular, it will be of interest to determine whether some of these targets are conserved between the different systems in which Krox-20 plays a regulatory role.

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