Expression and function of *Pax 1* during development of the pectoral girdle

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**SUMMARY**

*Pax 1* is a member of the paired-box containing gene family. Expression has previously been observed in the developing sclerotomes and later in the anlagen of the intervertebral discs. Analysis of *Pax 1*-deficient *undulated* mice revealed an important role for this gene in the development of the axial skeleton, in which *Pax 1* apparently functions as a mediator of notochordal signals during sclerotome differentiation. Here we demonstrate that *Pax 1* is also transiently expressed in the developing limb buds. A comparative phenotypic analysis of different *undulated* alleles shows that this expression is of functional significance. In mice that are mutant for the *Pax 1* gene severe developmental abnormalities are found in the pectoral girdle. These include fusions of skeletal elements which would normally remain separate, and failures in the differentiation of blastemas into cartilaginous structures. Although *Pax 1* is also expressed in the developing hindlimb buds and Wollfian ridge, no malformations could be detected in the corresponding regions of *Pax 1* mutant mice. These findings show that, in addition to its role in the developing vertebral column, *Pax 1* has an important function in the development of parts of the appendicular skeleton.

Key words: *Pax 1*, *undulated*, limb bud, pectoral girdle, acromion, bone morphology

**INTRODUCTION**

The developing limb bud has attracted great interest in recent years, particularly as a model system for the study of vertebrate pattern formation. Much of our knowledge in this area is based on the chick embryo, in which limb development can readily be observed and manipulated in ovo. Classical experiments documenting the effects of surgical manipulation and other treatments have led to the discovery that certain regions of the limb bud have endogenous patterning and growth promoting properties. These include the zone of polarizing activity (ZPA) in the posterior proximal limb bud, which affects polarity in the anteroposterior axis (Saunders and Gasseling, 1968; Balcuns et al., 1970), and the apical ectodermal ridge (AER), which is essential for limb bud outgrowth and proximodistal patterning (Saunders, 1948). The region-specific effects of the AER, ZPA and other limb bud tissues on cell proliferation and differentiation (reviewed by Tabin, 1991) have prompted efforts to identify the genes and molecules that are involved in cell-cell communication in the developing limb, and to work out the mechanisms by which positional information is specified and interpreted. Molecules that have been implicated as endogenous signals in the developing limb bud include retinoic acid (Tickle et al., 1982), which can induce ectopic ZPA activity in anterior limb bud mesenchyme (Summerbell and Harvey, 1983; Wanek et al., 1991), the product of the *sonic hedgehog* gene, which is produced in the ZPA and mediates ZPA activity (Riddle et al., 1993), and growth factors such as bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-4 (FGF-4), which affect outgrowth and patterning of the limb (Niswander and Martin, 1993). Many aspects of limb development appear to be regulated by homeobox-containing genes (reviewed by Morgan and Tabin, 1993). These include members of the *Hox* family, some of which may play important roles in the specification of positional information. Evidence for this has come from the analysis of *Hox* gene expression patterns, and of limb patterning defects caused by targeted mis-expression or disruption of *Hox* genes (Yokouchi et al., 1991; Morgan et al., 1992; Dollé et al., 1993; Haack and Gruss, 1993). Many of the classical studies on limb patterning and development have concentrated on the formation of the appendicular skeleton, especially the bones between the stylopod (femur or humerus) and the digits. Much less is known about the origin and development of the more proximal elements that constitute the pectoral and pelvic girdles. There are a number of pragmatic reasons for this. Firstly, the most proximal components of the appendicular skeleton are probably specified earlier in development than the more distal structures (Saunders, 1948), and they are situated more medially within the embryo. The girdle regions are therefore much less amenable to the surgical techniques that have been used to investigate the development of structures that form within the limb bud proper. Also, unlike the bones of the distal limb, the girdle bones are not conveniently organized within a single plane. Whereas the essential features of the skeletal pattern in the distal limb can be described in terms of the anteroposterior
and proximodistal axes, three dimensions must be invoked to describe the topography of the limb girdles. Despite the scarcity of experimental data on this subject (Yander and Searls, 1980), there is some evidence to suggest that the mechanisms controlling pattern and histogenesis in the limb girdles may be similar to those operating on more distal structures. For example, retinoic-acid-soaked beads implanted in the chick wing bud have been shown to affect patterning in both the distal wing skeleton and the pectoral girdle (Oliver et al., 1990). The ongoing molecular analysis of limb bud patterning and morphogenesis may therefore provide insight into the development of other less tractable parts of the skeleton.

_Pax_1 belongs to a family of genes (reviewed by Noll, 1993) that contain a conserved sequence motif known as the paired-box. The significant roles of _Pax_ genes in vertebrate development are underscored by their association with a number of murine and human developmental disorders (reviewed by Chalepaksis et al., 1993). _Pax_1 is expressed in the somites and developing axial skeleton of the mouse embryo (Deutsch et al., 1988), where it is important for the formation of the vertebral column. This is illustrated by the severe phenotypic abnormalities that have been found in the vertebrae and intervertebral discs of _undulated_ mice, in which the _Pax_1 gene is mutated (Grüneberg, 1950; Balling et al., 1988; Wallin et al., 1994). In the present paper, we show that _Pax_1 is also expressed in the limb buds of the mouse embryo, and describe the distribution of _Pax_1 transcripts in relation to the developing appendicular skeleton. We have also analysed the phenotypes of mice carrying various combinations of three different _undulated_ mutant alleles, and show that these include a range of abnormalities in the pectoral girdle. All of the mutant alleles affect the development of a particular structure called the acromion, which normally forms from mesenchyme within the domain of _Pax_1 expression in the forelimb bud. The pattern of _Pax_1 expression in wild-type forelimb buds, and the nature of the abnormalities found in the shoulder region of _undulated_ mutants argue that this gene plays an important part in determining the morphology of the mouse pectoral girdle, and suggest that the limb bud may be a useful system in which to investigate both the regulation of _Pax_1 and its function in the development of skeletogenic mesenchyme.

**MATERIALS AND METHODS**

**Mice**

_undulated_ (un) mice were purchased from the Jackson laboratory. _Undulated-short tail_ (Un) and _undulated-extensive_ (une) mutant mice were kindly provided by Dr A.M. Malashenko, Krosnogors, Russia and Dr J.L. Cruickshank, Leeds, England, respectively. These mutants were backcrossed onto the C57BL/6 strain. N1 to N5 generations were used for analysis. Embryos were recovered on days 12.5 to 18.5 post coitum (pc) where day 0.5 was 12 noon on the day of detection of the vaginal plug.

Methods for genotype determination and the preparation of stained skeletal whole mounts were as described in Wallin et al. (1994).

**In situ hybridization**

Wild-type (CBA/Ca) mouse embryos were collected between 10 and 16.5 dpc. In order to study the development of the appendicular skeleton in detail between 11.5 and 12.5 dpc, embryos in this age range were classified according to the limb bud staging system of Wanek et al. (1989). They were found to span five stages of limb bud development. Forelimb buds ranged between stages 5 and 9, and hindlimb buds between stages 4 and 8. After dissection embryos were fixed overnight at 4°C in 4% (w/v) paraformaldehyde in phosphate-buffered saline, dehydrated and embedded in paraffin wax. They were then processed according to the procedure of Wilkinson and Green (1990). 35S-labelled antisense cRNA probes for _Pax_1 were generated either from a _HinII-SacI_ paired-box containing fragment as described by Deutsch et al. (1988) or from the 3′ untranslated region of the _Pax_1 cDNA clone CX10 (Chalepaksis et al., 1991). The latter probe was synthesized from CX10 DNA digested with _Ksp632I_ and transcribed with T7 RNA polymerase. Both probes gave the same in situ hybridization pattern. Probes for type II collagen RNA were synthesized from a plasmid (pEXII) that contains most of exon II from the murine alpha-I(II) collagen gene (Metsärinta et al., 1991). This region was cloned by PCR amplification of BALB/c genomic DNA using oligonucleotide primers 5′-CCG GAA TTC ACT GGC AGT GGC GAG TTC AGC-3′ and 5′-CCA AGC TTA GGA GGC TGC CAG CTG TCT GC-3′. The amplified PCR product was digested with HindIII and EcoRI and cloned into Bluescribe (Stratagene). A 35S-labelled antisense probe was obtained by T7 RNA polymerase transcription of HindIII digested pEXII DNA. Antisense _Hox_6 probes were synthesized by HindIII digestion and SP6 transcription of a plasmid designated pGEM4/500 (Sharpe et al., 1988) which was a gift from P. Sharpe of the University of Manchester. Since this probe contained homeobox sequences, it is possible that it may cross-hybridize with transcripts from other _Hox_ genes in addition to those from _Hox_6.

**RESULTS**

The distribution of _Pax_1 RNA in the developing limb and girdle regions of the mouse embryo was investigated by in situ hybridization using 35S-labelled antisense cRNA probes. In addition to _Pax_1, a probe for expression of collagen II (type II collagen) RNA was used to label regions of chondrogenic mesenchyme (Swalla et al., 1988; Cheah et al., 1991). Since the proximal limb bud region of the mouse embryo has not been accurately fate mapped, the collagen II marker was a valuable aid in achieving one of the main aims of this study, which was to establish the anatomical relationship between domains of _Pax_1 expression in the limb buds and the developing pectoral and pelvic girdles.

**Pax_1 expression in the limb buds between 10.0 and 10.5 dpc**

_Pax_1 hybridization signal was first detected in the forelimb bud at approximately 10.0 dpc (not shown). At this stage the expression domain included mesenchyme occupying the anterior proximal region of the forelimb bud and a small outward bulge that is present at the base of the bud at this stage (Kaufman, 1992). The signal was most intense in the dorsal anterior part of the bud, but overall expression here was weaker than in the sclerotomes. A similar expression domain was observed in the forelimb bud at 10.5 dpc (Fig. 1A, A′) at which stage the level of expression was similar to that in the sclerotomes. Again the greatest signal intensity was found anterodorsally where the bud mesenchyme was in contact with the ectoderm. This ectoderm was negative for _Pax_1. Alternate serial sections probed for collagen II transcripts showed that the _Pax_1 expression domain was situated immediately lateral to a large patch of chondrogenic mesenchyme corresponding to the early scapulo-humeral blastema (Fig. 1B). The medial
Fig. 1. Expression of Pax 1 in mouse limb buds at 10.5 dpc. Frontal (A,A',B) and sagittal (C,C') sections of mouse embryos were probed for Pax 1 and type II collagen transcripts to show the pattern of Pax 1 expression in relation to the blastemal appendicular skeleton. A and A' show a section probed for Pax 1, photographed under bright-field and dark-ground illumination. B is a dark-ground picture of a nearby section from the same embryo, probed for collagen II. Pax 1 is expressed in the anterior proximal mesenchyme of the forelimb bud, shown here on the left side of the embryo, in a domain that is immediately adjacent to a patch of collagen II expression corresponding to the scapulo-humeral blastema. Pax 1 is also expressed in the anterior proximal hindlimb bud and lateral mesoderm in the flank (A',C'). Autoradiographic exposure times were 17 days (A,A'); 10 days (B); 4 days (C,C'). Bar, 0.5 mm. D shows the structure of the adult mouse scapula (adapted from Grüneberg, 1950). On the left is an anterior view of the left scapula and on the right is a lateral view of the right scapula. Dorsal is to the left. In the adult mouse the flat medial surfaces of the scapula blades face each other. The spina and acromion are on the lateral surface, with the acromion pointing ventrally. a, acromion; b, scapula blade; c, coracoid process; fl, forelimb bud; hl, hindlimb bud; lr, lateral (Wolffian) ridge; n, neural tube; s, sclerotome. sp, spina scapula.
Fig. 2. *Pax 1* expression in the pectoral girdle at 11.5-14.5 dpc. Frontal sections through the pectoral region were probed for expression of *Pax 1* or collagen II (indicated by c in lower right corner). Anterior is towards the top. At stage 5 (top row) *Pax 1* is expressed in a discrete portion of relatively uncondensed mesenchyme within the anterior proximal forelimb bud (A). Collagen II expression marks the position of the scapulo-humeral blastema (B,B'). The plane of these sections is tilted so that the bud on the left is seen at a more ventral level than the bud on the right. The arrow in B shows the point where the left principal limb bud vein branches from the left cardinal vein. Sections in the second row are from two different dorsoventral levels at forelimb stage 7. Medialis to the left and lateral to the right. At the more dorsal level (C,D), strong collagen II expression is seen in the blastemal scapula blade and spina. This region corresponds to the dorsal limit of the *Pax 1* expression domain (D). Much stronger and more extensive *Pax 1* expression is found at the more ventral level shown in E-F'. At this stage the distal end of the acromion blastema appears in cross section as a small, round condensation with weak collagen II expression (E,E'). *Pax 1* expression is concentrated in and around this condensation (F,F'). The patch of signal in the lower part of the limb bud corresponds to the anterior limit of *Pax 1* expression in the lateral trunk. The third row shows sections from two different dorsoventral levels at forelimb stage 9. Lateral is to the left and medial to the right. In G, strong collagen II expression is shown in the scapula and the dorsal (proximal) part of the acromion. H and H' show that *Pax 1* is not expressed in this part of the acromion, and only weak traces of signal appear in the surrounding area. The distal end of the acromion is histologically less mature and exhibits only weak expression of collagen II (J), but strong expression of *Pax 1* (K). The *Pax 1* expression domain extends ventrally into the developing shoulder joint, shown at 14.5 dpc in L and L'. Signal is distributed around and between the ends of the acromion and clavicle, but not the coracoid process. a, acromion; c, coracoid; cl, clavicle; cv, left cardinal vein; hu, humerus; s, sternum; sc, scapula blade; sp, spina scapula. Autoradiographic exposure times were 4 days (B, B'); 6 days (A); 9 days (C,E,E',G,J); 10 days (L,L'); 16 days (D,F,F',H,H',K). Bar, 0.25 mm.
Fig. 3. Expression of *Pax 1* in the developing pelvic region. Transverse sections of the pelvic region were probed for expression of *Pax 1* and collagen II (B, D). At hindlimb stage 6 (A-B), *Pax 1* is expressed strongly in mesoderm at the anterior end of the ileum blastema. At stage 8 (C-D), there is strong *Pax 1* expression ventrolateral to the pubis blastema, and weaker signal within the abdominal wall (C'). At 14.5 dpc (E, E'), *Pax 1* expression is localized around the ischium and pubis anlagen and certain blood vessels in the lower abdominal wall. Autoradiographic exposure times were 9 days for the collagen II probe and 16 days for the *Pax 1* probe. Bar, 0.25 mm. F shows the lateral aspect of the left half of the adult mouse pelvic girdle (adapted from Grüneberg, 1963). Dorsal is to the top and anterior to the left. a, acetabulum; f, femur; g, urogenital ridge; il, ileum; is, ischium; m, metanephros; p, pubis; r, ischial ramus; w, abdominal wall.
border of the Pax 1 domain overlapped slightly with this region. Lower levels of Pax 1 expression were detected in the anterior proximal mesenchyme of the hindlimb bud and a longitudinal band of mesenchyme in the lateral region of the trunk (Fig. 1A′,C,C′). We believe this region corresponds to the Wolffian ridge, from which the limb buds originally grow out (Searls and Janners, 1971; Kaufman, 1992).

Since there are few anatomical features within the limb bud mesenchyme at this stage, we also probed for the expression of Hoxc 6 as a spatial marker. Hoxc 6 RNA (Sharpe, 1991) and protein (Oliver et al., 1988) are expressed in the anterior proximal region of the forelimb bud. We detected hybridization in the flank, forelimb and hindlimb buds that completely encompassed the corresponding domains of Pax 1 expression (data not shown). Since Hoxc 6 protein is reported not to be expressed in the hindlimb bud (Oliver et al., 1988), and our probe included the Hoxc 6 homeobox, the signal that we detected in this region may represent cross-hybridization with transcripts from other Hox genes. However, in recent experiments by Jegalian et al. (1992), a lacZ reporter gene under the control of Hoxc 6 regulatory sequences was found to be expressed in both the forelimb and hindlimb buds of transgenic mouse embryos. This could indicate that Hoxc 6 is transcribed but not translated in the hindlimb buds, or that Hoxc 6 protein is undetectable in this region for technical reasons.

**Pax 1 expression during early development of the pectoral girdle**

In order to relate the Pax 1 expression domain in the forelimb bud to the developing appendicular skeleton, alternate frontal sections from embryos between 11.5 and 14.5 dpc were probed for Pax 1 and collagen II transcripts. A detailed inspection of collagen II expression during the period 11.5-12.5 dpc (forelimb stages 5-9) enabled us to observe the early development of the scapula, and particularly the acromion. In the adult mouse, this is a thin bony process on the lateral side of the scapula, extending from the ventral end of the spina scapula to the shoulder joint (see Fig. 1D).

**Fig. 4.** Expression of Pax 1 in distal regions of the limb. Sections through the hindlimbs of mouse embryos at 14.5 dpc showing patches of cells expressing Pax 1 in the knee joint (A,A′), the tarsal region (B,B′) and between the digits (C,C′). Autoradiographic exposure times were 16 days for A,A′; 10 days for B-C′. Bar, 0.25 mm. is, ischium; lf, lateral condyle of femur; t, tibia.
The main body of the scapula and the humerus appeared to arise from a single large mesenchymal mass in which collagen II RNA was detected from 10.5 dpc onwards (Fig. 1B). The level of collagen II expression in the centre of this mass increased dramatically as the cells condensed to form the blastemal scapula and humerus. With the aid of this marker, the basic shape of these two elements could be discerned at forelimb stage 5 (Fig. 2B'), and finer details of their morphology gradually became apparent during subsequent stages. The first vestige of the acromion was visible at stage 6 (not shown) as a short spur of condensing mesenchyme cells that stuck out laterally from the spina scapula and exhibited weak expression of collagen II. The length of this outgrowth had increased by stage 7 but its level of collagen II expression was still very low compared to the neighbouring scapula blade and spina (Fig. 2C,E'). By stage 9 collagen II was expressed strongly in the precartilaginous proximal end of the acromion (Fig. 2G) but weakly in its distal (ventral) extremity, where the cells were still undergoing condensation (Fig. 2J).

Pax 1 expression in stage 5 forelimb buds (Fig. 2A) appeared much as it had at 10.5 dpc. By now the cells in the scapula blastema were more densely packed than the adjacent Pax 1-expressing cells, which occupied a block of lateral mesenchyme that lay along the ventral half of the scapula blastema and extended beyond the prospective coracoid process. By stage 7, cells at the centre of the Pax 1 domain had lost their homogeneous uncondensed appearance. Comparison with the collagen II marker revealed that the most intense Pax 1 hybridization signal was localized over a small focus of condensing mesenchyme that corresponded to the distal end of the acromion blastema (Fig. 2E-F'). At stage 9, in complete contrast to collagen II, Pax 1 expression was very weak in the proximal end of the acromion blastema, and strong in the newly condensed distal end (Fig. 2H,K). Our interpretation of

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**Fig. 5.** Comparison of scapula-clavicle phenotypes at the new-born stage. Skeletal clearance preparations of the following genotypes are shown: +/+ (A), Un+/+ (B), Un+/Un+ (C), un/un (D), un/ure (E), un/ure (F), Un+/un (G) and Un+/ure (H). In I the principal skeletal elements are depicted schematically. In the Pax 1 null mutant, Un+/Un+, the acromion is completely fused to the blade of the scapula and also slightly shortened (C). The heterozygous Un+/+ mice are phenotypically abnormal as well. The spine of the scapula is longer but here the fusion between the acromion and the scapula blade in not complete (B). Note also the strong phenotypic similarity between the un/un, un/ure and un/ure skeletons in D-F. The acromion is completely missing. The phenotypes of the Un+/un and Un+/ure compound heterozygotes resemble that of Un+/Un+ mice, but in addition, almost invariably, the lateral end of the clavicle is fused to the scapula between the acromion and the coracoid process (G,H).
these observations was that the acromion was not composed of
cells from the main body of the scapula blastema, but that it
formed somewhat later by the gradual condensation of Pax I-
expressing mesenchyme cells onto the ventral end of the spina
scapula. The resulting finger-like process subsequently
underwent chondrification from its proximal to its distal end,
with the concomitant downregulation of Pax I expression.

The Pax I expression domain also extended ventrally into
the region of the prospective shoulder joint, where strongly
positive cells were localized in the interzonal region between
the distal end of the acromion, the lateral end of the clavicle,
and the anterior surfaces of the coracoid process and the head
of the humerus (Fig. 2K,L,L'). From their position, we deduce
that these cells could give rise to articular cartilage, or to the
dense connective tissue of the joint capsule.

**Pax 1 expression in the developing pelvic region**

Transverse sections through the pelvic regions of mouse
embryos at hindlimb stages 6 (11.5 dpc) and 8 (12.5 dpc) were
probed for expression of Pax I and collagen II transcripts.
The structure of the adult pelvis is shown in Fig. 3F. At stage 6, the
early blastemas of the ischium, ilium and pubis could just be
visualized by collagen II hybridization. A region of strong Pax
I expression in the anterior proximal mesenchyme of the
hindlimb bud was found directly anterior to a patch of weak
collagen II expression corresponding to the ilium blastema (Fig.
3A-B). A small patch of Pax I expression was also found at the
posterior side of the ischial ramus, and at weaker levels in the
lateral mesoderm of the trunk (not shown). At stage 8, strong
Pax I expression was localized in a small area of tissue adjacent
and lateral to the chondrifying pubis blastema and weaker
expression was present within the abdominal wall (Fig. 3C-D).
At 14.5 dpc, Pax I expression was found in tissues surrounding
the ischium, and in small patches of tissue lateral and ventral to
the pubis (Fig. 3E-E'). The changing distribution of Pax I tran-
scripts in this region of the body suggested that Pax I-express-
ing cells from the hindlimb bud may contribute to parts of the
ileum, pubis and the ischium, or to connective tissues around
these structures. Cells from the trunk lateral mesoderm appeared
to express a lower level of Pax I from stage 6 onwards, and may
contribute to tissues in the abdominal wall.

**Pax 1 expression in the limbs**

In addition to the major sites of expression in the developing
pectoral and pelvic regions, we also detected Pax I transcripts
in more distal parts of the limbs from 12.5 dpc. These were
localized within the elbow, knee, wrist and ankle joints, and
between the digits. Expression in these regions was maximal
at 14.5 dpc (Fig. 4), after which transcript levels in these areas
diminished.

**Phenotypic abnormalities of the pectoral girdle in
Pax 1 mutant mice**

Investigation of the role of Pax I in skeletal development has
been greatly facilitated by the availability of three different Pax
I mutants in the mouse. These are the recessive alleles
undulated (un) and undulated-extensive (unex) (Wright, 1947;
Blandova and Egorov, 1975), and the semidominant
Undulated-short tail (Uns) (Wallace, 1985). Mutations in the
Pax I gene have been demonstrated for all three alleles. In un
there is a point mutation in the paired-box of Pax I, while unex
and Uns are both deletion mutants. The fifth exon of the Pax
I gene, which includes carboxy-terminal coding sequence, has
been lost in unex, and the entire locus is deleted in Uns (Balling
et al., 1988; Wallin et al., 1993; 1994; Dietrich et al., unpub-
lished data).

Skeletal phenotypes of the different undulated alleles were analyzed in cleared whole-mount preparations stained with
Alcian blue and alizarin red. Since one of the genotypes that we examined (Uns/Uns) is lethal within a few hours of birth,
most of our phenotypic comparisons were done at the newborn
stage, when the salient anatomical features of the mouse
skeleton can readily be observed. We also followed the
embryonic development of the phenotypes in embryos
collected at 12.5, 13.5, 15.5 and 17.5 dpc. 12.5 dpc is the
earliest stage at which limb skeletal elements can be stained
with Alcian blue.

As already described by Grüneberg (1950), the acromion
process of the scapula is missing in un homozygotes (Fig. 5D).
This structure and adjacent parts of the spina scapula appar-
ently fail to chondrify and subsequently to ossify, but a
remnant is present as a ligament, which now attaches to the
lateral end of the clavicle. In unex/unex mice the skeletal
phenotype was very similar to that in un/un mice, as was the
case in unex/unex compound heterozygotes (Fig. 5E,F). Very
rarely, we noted some unex/unex mutant mice with a more or
less normal acromion, which suggests that the manifestation of
this abnormality is under the influence of modifier genes
(Grüneberg, 1950). The pelvic girdles of un and unex mutants
were phenotypically normal.

A slight abnormality of the pectoral girdle could be seen in
mice that were heterozygous for the semidominant mutant Uns,
in which the Pax I gene is completely deleted (Wallin et al.,
1994). The acromion appeared to contact the scapula over a
longer distance, so that the space between the acromion
process and the blade of the scapula was reduced (Fig. 5B). In
homozygous Uns/Uns mice the acromion had apparently
fused with the central portion of the scapula and the coracoid
process. The compound heterozygotes, Uns/un and Unsex/unex,
displayed a very similar phenotype to Uns/Uns, but in addition
the clavicle was almost invariably fused with the scapula (Fig.
5G,H).

Examination of undulated embryos revealed that all of the
characteristic abnormalities were already evident at the carti-
laginous stage of skeletal development (Fig. 6). In 13.5 dpc
un/un embryos, the acromion anlage was severely reduced and
appeared to become incorporated into the spina scapula (Fig.
5B,F). The acromion anlage was also short in Unsex/un
and Unsex/Uns embryos, and appeared to be attached to a more
ventral part of the scapula (Fig. 5C,D). Although the principal
abnormalities could already be seen at the cartilaginous stage,
the scapula-clavicle fusion in the compound heterozygote
Unsex/un was a later event as these bones first arise as separate
elements (Fig. 5C).

In contrast to the pectoral girdle, we have been unable to
detect any abnormalities in the pelvic girdle or in other parts
of the limbs. To look for more subtle phenotypic changes, we
have performed a thorough histological analysis of the mutants
at 18.5 dpc. Special attention was paid to the formation of limb
joints and tendons. The development of these structures
appeared anatomically and histologically normal (data not
shown).
DISCUSSION

Pax 1 is a member of the paired-box (Pax) gene family (reviewed by Chalepakis, 1993; Noll, 1993) isolated on the basis of sequence similarity to Drosophila pair-rule and segment polarity genes (Bopp et al., 1986; Deutsch et al., 1988). It is expressed in somites, where transcripts and protein are detectable from 8.5 and 9.5 dpc respectively, later becoming localized in the sclerotomes and intervertebral discs (Deutsch et al., 1988; Wallin et al., 1994). Pax 1 expression in this region is of functional importance, since severe defects have been observed in the vertebral bodies and intervertebral discs of undulated mice, which have mutations in the Pax 1 gene (Grüneberg 1950, 1954; Balling et al., 1988; Wallin et al., 1994). In this report, we have shown that Pax 1 is also expressed in the developing limb bud of the mouse embryo. By following the expression of Pax 1 through successive stages of limb bud development, we have found evidence that Pax 1-expressing cells contribute substantially to parts of the blastemal pectoral and pelvic girdles. A phenotypic analysis of different undulated mutant alleles reveals severe abnormalities in parts of the pectoral girdle that are derived from the Pax 1 expression domain in the forelimb bud. All of the skeletal defects that have been found in undulated mice have therefore been shown to occur within the normal expression domains of Pax 1.

Correlation of Pax 1 expression with acromion development

At 10.5 days of normal mouse embryonic development Pax 1 is expressed in mesenchyme within the anterior proximal part of the forelimb and hindlimb buds and in the intervening flank. During later stages, strong expression is found in patches of condensed prechondrogenic mesenchyme corresponding to certain parts of the developing limb girdles. Most significantly, in the pectoral region Pax 1 expression is concentrated in a thin mesenchymal process that will form the acromion, and in cells that are localized within the prospective shoulder joint. These are precisely the sites where abnormalities occur in the pectoral girdles of Pax 1 mutant mice and embryos. No abnormalities have been found in nearby parts of the pectoral girdle such as the medial side of the scapula, or the coracohumeral articulation, where there is no detectable expression of Pax 1 in the embryo. In addition to this spatial correlation, the timing of Pax 1 expression in the shoulder region is compatible with the developmental period

Fig. 6. Abnormalities in the cartilaginous pectoral girdle. Skeletal preparations from 13.5 dpc (A-D) and 17.5 (E-H) dpc embryos of the following genotypes are shown: +/+ (A,E), un/un (B,F), Un+/un (C,G) and Un+/Un+ (D,H). In un/un the acromion anlage is severely reduced, and later gets fully incorporated in the spina scapula (B,F). In the strongly affected genotypes Un+/un and Un+/Un+, the acromion anlage is short as in un/un mice and also displaced almost to the ventral end of the scapula (C,D).
in which defects arise in undulated embryos. The onset of Pax 1 expression in the forelimb bud coincides with an early stage in the condensation of the scapula blastema, preceding the formation of the acromion by at least 24 hours. As development proceeds, the highest level of Pax 1 expression is found in newly condensed mesenchyme cells at the distal end of the lengthening acromion blastema. Expression in the proximal end of the blastema appears to be rapidly downregulated when the cells differentiate as chondrocytes. This time course suggests that Pax 1 function in the developing acromion is executed in the early stages of blastema formation, particularly during mesenchymal condensation. This agrees with observations reported by Grüneberg (1954) and in the present study (Fig. 6) that acromion malformations characteristic of the various undulated genotypes are established before 13.5 dpc. The more prolonged period of Pax 1 expression in the prospective shoulder joint is reflected in the slightly later appearance of scapula/clavicle fusions.

It is notable in this regard that although Pax 1 expression in the limb buds and paraxial mesoderm is not contemporaneous, it is temporally analogous with respect to the development of skeletal tissues in both of these domains. Pax 1 expression can be detected in the somites as early as 8.5 dpc, and persists in sclerotome cells as they subsequently form a continuous mesenchymal sleeve (the perichondral tube) around the notochord (Deutsch et al., 1988; Wallin et al., 1994). When this tissue differentiates into alternating blocks of chondrogenic cells and densely packed mesenchyme, Pax 1 appears to be downregulated in the chondrogenic regions which will eventually give rise to ossified vertebral bodies, but remains high in the dense mesenchymal regions, which will form the intervertebral discs. There are obvious parallels between the pattern of Pax 1 expression here and in the developing acromion and shoulder joint.

Another common feature of the limb bud and paraxial mesoderm is that Pax 1 expression in both of these regions appears to be influenced by neighbouring tissues. Expression of Pax 1 in the paraxial mesoderm depends on a functional notochord. Degeneration or loss of the notochord in various mouse mutants results in the loss of Pax 1 expression in the somites (Dietrich et al., 1993; Koseki et al., 1993), and transplantation of an additional notochord into the paraxial mesoderm of the chick embryo causes an expansion of the Pax 1 expression domain (Brand-Saberi et al., 1993). Based on the expression of Pax 1 in the Danforth’s short tail (Sd) mutant, we have suggested that Pax 1 might function as a mediator of notochordal signals during sclerotome development (Koseki et al., 1993). In the limb bud we have noticed a graded distribution of Pax 1 RNA, with maximal accumulation of transcripts just beneath the ectoderm (Fig. 1A), which could indicate induction of the Pax 1 gene by signals from this tissue. The intriguing possibility that analogous signals may be produced by both the notochord and limb bud ectoderm will be addressed in future investigations.

The molecular genetic basis of Pax 1 function in the developing pectoral girdle

In a recent analysis of axial skeleton development in the same undulated mutants that were used here, the phenotypes were interpreted as a graded series of abnormalities which were qualitatively similar, but which differed in their degree of severity (Wallin et al., 1994). The most extreme defects were found in Un+/Un* homozygotes, in which the Pax 1 gene is completely eliminated. This suggested that the un and un* alleles are hypomorphic in character, resulting in Pax 1 function that is reduced in comparison with the wild-type allele. However, the present study has shown that it is more difficult to place the various undulated genotypes in order of phenotypic severity on the basis of abnormalities in the pectoral girdle. We have described three types of defect in this region. These are the failure of the acromion to be resolved as a discrete outgrowth from the scapula blade (in all genotypes that included the deletion Un*), the fusion of the clavicle and the scapula (in Un+/Un* and Un+/Un* compound heterozygotes), and the absence of the acromion or its replacement by a ligament (in un/un and un*/un* homozygotes and un/un* compound heterozygotes). Un+/Un* and Un+/Un* mice exhibit a similar but increasingly severe malformation of the scapula (Fig. 5B,C), presumably reflecting the number of wild-type Pax 1 genes lost in each case. One might predict from this that a genotype consisting of a single hypomorphic allele would produce a phenotype intermediate between those of Un+/+ and Un*/Un*. However, we found that Un+/Un* and Un+/Un* mice had scapula malformations equivalent to those in Un+/Un*, plus fusions of the scapula and clavicle. It seems reasonable to interpret these phenotypes as more severe than, or partially different to, that produced by the null (Un*/Un*) genotype, suggesting that in this context the un and un* mutations have at least some attributes of antimorphic (dominant negative) or neomorphic (novel function) alleles. This could also help to explain why the acromion phenotype of un and un* homozygotes (where it is absent or fails to chondrify) appears to be qualitatively quite different from that of the Un* genotypes.

It is interesting that the un and un* alleles produce such similar phenotypes (Fig. 5D,F), since they involve different lesions in the Pax 1 gene. In un there is a point mutation in the Pax 1 paired-box, leading to the substitution of a single conserved amino acid in the DNA-binding domain of the Pax 1 protein (Balling et al., 1988; Treisman et al., 1991). Functional analysis of wild-type and un Pax 1 proteins has shown that the substituted amino acid in the un Pax 1 paired domain affects its DNA-binding affinity and sequence specificity (Chalepakis et al., 1991). It has also recently been demonstrated that the paired domain consists of two subdomains, which recognize and bind distinct half-sites in target DNA sequences (Czerny et al., 1993). This appears to allow for a degree of flexibility in the range of sites that can be bound by Pax proteins, since divergence from the consensus sequence in one half of a binding site may be tolerated if there is a good match in the other half. Consequently, a mutation in one half of the paired domain, as in un, might alter the binding affinity to some, but not all of the normal Pax 1 binding sites, and could permit binding to additional novel targets. This may provide a molecular explanation for aspects of the pectoral girdle phenotype that are qualitatively different in the un and Un* mutants. While both of these mutations could have similar effects on some or all of the normal downstream targets of Pax 1, as a result of altered DNA-binding efficiency in the case of un and a deficit of protein in Un*, additional abnormalities might be introduced in un by interaction of the mutant protein with unorthodox target genes.

un* involves a deletion that does not include the paired-box,
but removes the most 3’ exon of the Pax 1 gene (Balling et al., 1992). It is not known whether DNA binding or other properties of the encoded protein are affected by this mutation, but it does result in the production of low steady state levels of transcripts (Wallin et al., 1993) and presumably, correspondingly small amounts of unex Pax 1 protein. In this respect it is particularly surprising that the phenotypes of unex mutants resemble those of un rather than Un. It will be necessary to identify the in vivo targets of wild-type and mutant Pax 1 proteins and to carry out further molecular analyses in order to explain fully these observations. Gene dosage, and consequently gene product concentration, are clearly critical factors in the performance of Pax genes in general, and account for the semidominant characteristics of a number of Pax mutant alleles (reviewed in Hill and van Heyningen, 1992). In the present case the importance of gene copy number is illustrated by comparing the pectoral girdle phenotypes of Un+/Unn, Un+/+ and +/+ mice (Fig. 5A-C), which have zero, one and two copies respectively of wild-type Pax 1, and those of Un+/Unr, Unr+/un and un/un (Fig. 5C,G,D), which have zero, one and two copies of the un allele. The effects of gene product concentration can also be observed within a single genotype, by virtue of regional variation in the level of expression in the limb bud. un/un mice occasionally develop a small nodule of cartilage within the distal end of their ligamentous acromion (Grüneberg, 1950), which we can now correlate with the region of maximal Pax 1 expression (Fig. 2). This indicates that at least some of the deleterious effects of the un mutation might be overcome by increasing the level of protein expressed. As already discussed, the phenotype of the Un+/un compound heterozygote predicts other effects of this allele that might not be alleviated by overexpression.

The role of Pax 1 in acromion development

The wild-type expression pattern of Pax 1 in the forelimb bud, and the nature and range of phenotypic abnormalities that we have found in the pectoral girdles of undulated mutants suggest that in vivo targets of wild-type and mutant Pax 1 proteins are likely to include genes that are involved in the growth and/or differentiation of skeletogenic mesenchyme. Abnormal growth of cells within or adjacent to the Pax 1 expression domain could account for all of the mutant characteristics that we have observed. The scapula malformations and scapula/clavicle fusions in the Unn series (Fig. 5) could signify abnormal growth of the scapula blastema, and the loss of the acromion in un and unex homozygotes could result from failure of the acromion blastema to reach a certain minimum size required for chondrification (Grüneberg, 1963). A detailed analysis of the developing axial skeleton in Un+/Unn and Un+/un mutant embryos has revealed severe reductions in the size of the blastemal vertebral bodies and intervertebral discs that would normally form from Pax 1-expressing perichondral cells (Wallin et al., 1994). This suggests that reduced blastemal size is also likely to play a part in the abnormal development of the acromion in these genotypes. Other evidence that Pax 1 may function as a growth regulator has come from cell culture experiments in which the overexpression of Pax 1 led to oncogenic transformation, while a much milder effect was obtained with un-Pax 1 (Maulbecker and Gruss, 1993).

Alternatively, or additionally, Pax 1 might have a role in determining how mesenchymal or blastemal cells differentiate.

In wild-type embryos, Pax 1-expressing mesenchyme appears to give rise both to cartilage that will later ossify, as in the acromion and vertebral bodies, and to fibrocartilage or other dense connective tissues, as in the shoulder joint and intervertebral discs. In some undulated genotypes, the differentiation of this mesenchyme appears to be skewed towards connective tissue at the expense of osteogenic cartilage. Examples are the development of a ligamentous rather than a bony acromion, and the formation of disproportionately large intervertebral disc anlagen and correspondingly small vertebral body anlagen during differentiation of perichondral mesenchyme (Grüneberg, 1954; Wallin et al., 1994). These effects may indicate that mutations in Pax 1 interfere with the regulation of properties such as matrix production and degradation, cell shape, adhesiveness and junction formation, which are central to the differentiation of mesenchyme-derived tissues.

We have found in the pectoral girdles of undulated mutants that we examined. The first question raised by this observation is whether Pax 1 RNA is translated in all of these sites, and this must be addressed by antibody staining. Regional differences in the severity of Pax 1 mutant phenotypes are also found in the vertebral column, where defects are much more pronounced in the lumbar than the thoracic vertebrae (Grüneberg, 1950; Wallin et al., 1994). These findings suggest that Pax 1 function might be modulated in certain parts of the body by other region-specific factors. One possibility is that some parts of the body may contain other Pax proteins that are able to compensate for the aberrant or reduced amounts of Pax 1 protein in undulated mutants. For example, the Pax 9 gene that has recently been cloned from the human, mouse and chick (Stapleton et al., 1993; Wallin et al., 1993;
A. Ogilvy, personal communication) belongs to the same subclass as Pax 1 (Walther et al., 1991) and is reported to be expressed in some of the same regions of the mouse embryo, including the distal limb (Stapleton et al., 1993; Wallin et al., 1993; R. Balling, unpublished results; A. Ogilvy, personal communication).

An additional reason for regional differences in the expression of undulated phenotypes may be that the role of Pax 1 in skeletal development depends on interactions with other factors that are only present in a subset of Pax 1 expression sites. Obvious candidates for genes that could be involved in such region-specific phenomena are the Hox genes and others that are regulated by them. Members of the Hoxc cluster are interesting in this regard, since they may be involved in the specification of forelimb versus hindlimb differences (reviewed in Morgan and Tabin, 1993). In particular, protein encoded by the Hox 6 gene is not expressed in the hindlimb bud, but is distributed in the anterior proximal region of the forelimb bud in a concentration gradient that overlaps and resembles the graded distribution of Pax 1 transcripts (Oliver et al., 1988). Since Pax 1 mutant phenotypes therefore appear to be restricted to regions where Pax 1 and Hoxc 6 are co-expressed, this raises the intriguing possibility that these two genes interact functionally at some level. It has already been shown that implantation of retinoic-acid-soaked beads or ZPA tissue into the chick wing bud can perturb the expression of Hoxc 6, and lead to malformations of the pectoral girdle which may be analogous to those found in undulated mice (Oliver et al., 1990). In preliminary experiments where Unl was crossed into transgenic mice carrying a Hoxc 6-lacZ reporter gene, no differences were found in the expression of the transgene, suggesting that Hoxc 6 expression does not depend on Pax 1 (J. Wallin, unpublished observations). Similar arguments, invoking Hoxa and Hoxd genes (reviewed in Morgan and Tabin, 1993), might also be applied to account for the apparent absence of undulated abnormalities in the distal limb.

The different effect of Pax 1 mutations on the development of the pectoral and pelvic girdles could also be indicative of fundamental differences in the embryological derivation of these structures. Historically, the axial and appendicular components of the skeleton have been regarded as being separately derived from paraxial (somitic) and somatopleural (lateral plate) mesoderm, respectively. However, there is evidence that in some vertebrate classes this distinction is not so clear cut. Chick-quail transplantation experiments have demonstrated that part of the avian scapula is derived from cervical somites (Chevallier et al., 1977; Beresford, 1983), and somite extirpation experiments indicate that the same may be true in turtles (Burke, 1991). Chevallier et al. (1977) also investigated the development of the chick pelvic girdle, and found no evidence of a somitic contribution. Unfortunately very little is known about the relative contributions of somitic and somatopleural mesoderm to mammalian limb girdles. If the situation in the mouse were found to be analogous to that in the chick, with somite-derived cells contributing to the pectoral but not to the pelvic girdle, this might suggest that cell lineage is an important factor in the execution of Pax 1 function. In this regard it would be particularly interesting to know whether the acromion and scapula blade have separate origins in the early mouse embryo.

This study has suggested several factors that may influence the expression and function of Pax 1 in skeletogenic mesoderm. The distribution of Pax 1 transcripts in early paraxial and limb bud mesenchyme suggests that the gene may respond to notochordal and ectodermal signals, and to spatial cues that could be set by the expression of regulatory genes such as members of the Hox family. Spatial and temporal characteristics of Pax 1 RNA expression in the developing limb buds, and the phenotypes of Pax 1 mutant mice suggest that the function of Pax 1 may depend on a combination of conditions including the absolute concentration of protein produced, the early onset of expression, position in the body (forelimb versus hindlimb, proximal versus distal limb), co-expression of other genes, and the embryonic origin of cells within the expression domain. The availability of a range of murine Pax 1 mutations with known molecular lesions and visible phenotypic effects, and the prospect of exploiting the limb bud as an experimental system, should make it possible to address some of these issues in the future.

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