Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior–posterior axis is specified by mesoderm

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Summary

It is rapidly becoming accepted that the vertebrate neural tube, in particular the hindbrain, develops into a segmented structure. After segment formation, cells in the neural tube do not cross segmental boundaries, and segment-specific gene expression is observed. However, it is not known what positional cues instruct the neural tube to express genes in this restricted manner. We have cloned a murine homeobox-containing gene, Hox-2.9, whose expression in the neural tube at E9.5 is restricted to a segment of the hindbrain known as rhombomere 4. A study of its expression pattern earlier in development revealed that prior to the start of neurulation (E7.5) Hox-2.9 is expressed within and posterior to the embryonic mesoderm that will participate in hindbrain formation. With the onset of neurulation, expression then becomes detectable in the neural plate as well, but only in the part that overlies the Hox-2.9-expressing mesoderm; it is not detected in the more anterior neuroectoderm that will form the future midbrain and forebrain. On the basis of these findings, we propose that the mesoderm is providing cues that serve to instruct the overlying neuroectoderm with respect to its position along the anteroposterior axis and that Hox-2.9 participates in or reflects this process. As neurulation continues and individual segments form, a second phase of expression is detected in the neural tube in which high levels of Hox-2.9 transcripts become restricted to rhombomere 4. Hox-2.9 expression is also detected in the developing branchial arch units of the hindbrain region, in a pattern that suggests to us that here, too, mesoderm is providing a localized signal that induces Hox-2.9 expression, in this case in endoderm of the pharynx and in superficial ectoderm. In general, we interpret the expression patterns of Hox-2.9 in the hindbrain region as suggesting that the specific mechanisms of pattern formation in mammals are fundamentally similar to those of amphibians and avians – i.e. anteroposterior positional information is acquired by mesoderm, mesoderm induces positional values within (neuro-) ectoderm and endoderm, and both events occur within a restricted window of time.

Key words: mouse embryo, Hox-2.9, expression, axis, mesoderm, positional information.

Introduction

Despite extensive differences in their adult forms, frogs, chickens, mice, and other vertebrates employ similar strategies to establish the basic body plan (Balinsky, 1981; Slack, 1983). The first step is mesoderm formation; these cells initially appear between apposed layers of ectoderm and endoderm at the prospective posterior end of the embryo and then become widely distributed as the primary germ layers undergo a series of coordinated movements known as gastrulation. The next major event – induction of the neural plate (the start of neurulation) – has been shown in amphibians and avians to be mediated by a subset of the mesoderm (dorsal mesoderm – future notochord, somite, and head mesoderm), which interacts with nearby ectoderm (Spemann, 1938; Cooke, 1985; Hamburger, 1988; Jones and Woodland, 1989). This induction is temporally restricted; shortly after neurulation takes place, uninduced ectoderm loses its ability (competence) to respond to mesoderm and differentiates into epidermis (Albers, 1987; Jones and Woodland, 1989). Finally, the dorsal mesoderm itself becomes subdivided as it moves toward the prospective head region and appears to mediate specification of regional identities along the anterior–posterior (A–P) axis in adjacent neural plate and endoderm (Mangold, 1933; Okada, 1957; Toivonen and Saxen, 1968; Balinsky, 1981; Slack, 1983; Ruiz i Altaba and Melton, 1989; Sive et al. 1989).

In mammalian embryos, relatively little is known about how these processes occur (reviewed by Bedd-
ingston, 1983, 1986). As in *Xenopus* and chicken, the information for specification of A–P regional identities must reside in at least one of the germ layers, since at the end of gastrulation, pieces of the embryo containing all three germ layers can be shown to differentiate in *vitro* into structures characteristic of the region along the A–P axis from which they were originally explanted (Snow, 1981). However, as is the case for the other vertebrates, in mouse this information does not appear to reside in the ectoderm, since ectoderm grafts transplanted to different regions of the embryo (heterotopic grafts) adopt cellular fates typical of the new location, rather than of the site from which they were explanted; thus individual cell fates in the ectoderm do not seem to have been determined at this time (Beddington, 1982). Furthermore, in a few specific cases, acquisition of positional information by ectoderm and endoderm has been shown to be dependent on the presence and/or type of mesoderm (Wessler and Rutter, 1969; Snow, 1981); thus in mammals, mesoderm is likely to carry out a role similar to the one it plays in amphibian and avian embryos.

With respect to timing of positional specification in the germ layers, fate maps, although relatively imprecise, suggest that establishment of A–P regional identities in mammalian embryos takes place during late gastrulation/early neurulation. Before then, the tissues that presumably interact to provide positional information are not juxtaposed and thus require extensive rearrangement if they are to participate in short range inductions (Lawson and Pedersen, 1987; Tam and Beddington, 1987; Tam, 1989).

Unfortunately, many of the experimental approaches that have yielded important insights into the mechanisms of amphibian and avian embryogenesis have not been technically feasible in mice. An alternative means of investigating specification of A–P positional identities is to study genes that control this process or at least that serve as markers for it. In *Drosophila*, a cluster of homeobox-containing genes, known as the *Antp*-like HOM gene family, are differentially expressed along the A–P axis and determine individual segmental identities (Akam, 1989; Lewis, 1989). Homologous clusters of genes are found in vertebrates differentially expressed along the A–P axis, suggesting that, as in *Drosophila*, they may participate in specification of positional identities (Graham et al. 1989; Duboule and Döllé, 1989).

In *Xenopus*, the observation that one of these genes, *XIIHBox-1*, is expressed in mesoderm and neuroectoderm in regions that share the same anterior and posterior boundaries (Oliver et al. 1988) led to the proposal that position-specific determination might occur across germ layers (De Robertis et al. 1989). In the mouse, this type of pattern has been difficult to demonstrate; the four clusters of genes (known as Hox-1, -2, -3, and -4; Duboule et al. 1990) have been studied most intensively during mid-gestation (E12.5), and although expression of each of the genes begins at a different point along the A–P axis, the anterior boundaries of expression observed for an individual gene are not aligned (in register) when derivatives of different germ layers (neuroectoderm and neural crest, somitic and lateral mesoderm, and endoderm) are compared (Holland, 1988; Holland and Hogan, 1988). However, since these tissues continue to undergo relative displacement after gastrulation, the discordant A–P boundaries of Hox gene expression observed during mid-gestation might evolve from an expression pattern that is coordinate earlier in development, at the time when ectoderm and endoderm acquire A–P positional identities (Hogan et al. 1985; De Robertis et al. 1989).

We have cloned and studied a gene designated Hox-2.9. Expression of this gene has been previously described during late neurulation (E8.5), when it is most prominently observed in rhombomere 4 of the hindbrain (Murphy et al. 1989; Wilkinson et al. 1989). These data have been interpreted as providing evidence that specification of positional information in the neural tube occurs after differentiation of the central nervous system. In contrast, we have obtained results on the expression pattern of this gene at earlier times that suggest that positional information in the neural tube is acquired earlier, by the onset of neurulation. Furthermore, we propose that our data support the hypothesis that mesoderm directs the acquisition of positional information by both ectoderm and endoderm. We discuss the Hox-2.9 expression pattern in the context of cellular determination known or hypothesized to occur during gastrulation and early neurulation, and in relationship to the movements of the germ layers and their derivatives during this time.

### Materials and methods

#### Embryos

Mouse embryos at various stages of gestation were obtained by mating random bred ICR animals (Simenson Laboratories, Gilroy, CA). The day on which the vaginal plug was detected was considered 0.5 days of gestation (E0.5).

#### Homeobox amplification using degenerate oligonucleotide primers

Mouse genomic DNA (50 ng) and primers (25 pmols each) in 50 μl of PCR cocktail (10% [vol/vol] dimethyl sulfoxide/1× *Taq* polymerase buffer [New England Biolabs]/each dNTP at 1.5 mM) were denatured (5 min, 95°C) and cooled to 72°C. *Taq* Polymerase (2.5 U, Perkin-Elmer-Cetus) was added and the mixture was overlaid with 30 μl of mineral oil (Sigma). The reaction was run using the following program (94°C, 40 s; 40°C, 1 min; 72°C, 1 min), followed by a 15 min final extension at 72°C. Primer sequences: ELEKEF: 5'-GAdCTzGxAzGAdTT-3'; WFQNNRrc (reverse complement of WFQNNR): 5'-CzGzCGzTzTTzTGzXzAACCz-3', where x=A/G, y=C/T, and z=A/G/C/T.

Hox-2.9 cDNA 3'-end amplification

1 μg of E12.5 embryo poly(A)⁺ RNA (Shackleford and Varmus, 1987) in 16.5 μl of water was reverse transcribed by heating at 65°C for 3 min, rapidly cooling on ice, adding 2 μl of 10× *RT* buffer (1× *RT* buffer is 50 mM Tris–HCl, pH 8.15
at 41°C/ 6 mM MgCl₂/ 40 mM KCl/ 1 mM dithiothreitol/ each dNTP at 1.5 mM [Pharmacia], 0.25 μl (10 units) of RNasin (Promega Biotech), 0.5 μl of (dT)₁₀-adaptor primer (1 μg/ml⁻¹, [Frohman et al. 1988]), and 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL), and incubating for 2 h at 41°C. The reaction mixture was diluted to 1 ml with TE (10 mM Tris–HCl, pH 7.5/1 mM EDTA) and stored at 4°C. 5 μl aliquots of the cDNA pool were amplified using the PCR conditions described above, except that the annealing temperature was increased to 55°C, and a Hox-2.9-specific primer (bp 658-675, Fig. 2A) and an adaptor primer (Frohman et al. 1988) were substituted for primers ELEKEF and WFQNRRrc.

**Hox-2.9 cDNA 5’ end amplification**

1 μg of E10.5 poly(A)⁺ RNA was reverse transcribed as described above except for the addition of 20 μCi ³²P-dCTP (5000 Ci/mmol⁻¹, New England Nuclear) and the substitution of 20 pmol of a Hox-2.9-specific primer (bp 886-870, Fig. 2A) for (dT)₁₀-adaptor. Excess primer was removed and the first strand products were tailed with dATP residues as previously described (Frohman, 1990). The reaction mixture was diluted to 100 μl in TE and 5 μl aliquots were amplified as above, except for the substitution of (dT)₁₀-adaptor primer (10 pmol), adaptor primer (25 pmol), and a second Hox-2.9-specific primer (bp 858-842) (25 pmol).

**Southern and Northern blot analyses**

PCR products and mouse genomic DNA restriction fragments were separated by electrophoresis (1% agarose gel containing 0.5 μg/ml⁻¹ ethidium bromide), transferred to Genescreen (New England Nuclear) and hybridized with a ³²P-labeled probe (Bethesda Research Labs nick translation kit). Recombinant inbred mouse strain DNAs were purchased from Jackson Laboratories (Bar Harbor, ME). Northern blot analyses were carried out as previously described (Joyner et al. 1985).

**Cloning and sequencing of amplification products**

Genomic DNA or cDNA amplification products were transferred into TE using spin column chromatography (Maniatis et al. 1980, pp 466-467) and separated by electrophoresis. In some cases, cDNA amplification products were digested with restriction enzymes that cleaved sites in the antisense strand and the products were used to obtain new members of the murine homeobox-containing gene family. The alignment of the homeodomain of 20 such genes and their *Drosophila* HOM counterparts demonstrates that two short helical regions (homeodomain amino acids [aa] 15-20 and 22-24) are perfectly conserved in all members of the family, whereas the remainder of the domain is quite variable (Fig. 1). Degenerate oligonucleotide primers (designated ELEKEF and WFQNRRrc) representing DNA sequences that encode aa in the conserved regions were employed to amplify the homeobox fragment between the primers.

In an attempt to identify fragments containing previously unknown *Hox* homeoboxes, the amplification products were examined by Southern blot analysis using a probe for the *Drosophila* zen gene. This probe was chosen because no mammalian homolog of this gene, which contains a HOM-like homeobox (Rushlow et al. 1987), had yet been identified, and because we found that four mouse genomic fragments could be detected by Southern blot hybridization using this probe under conditions of reduced stringency, suggesting that *zen* sequences might have been conserved during evolution (data not shown). Amplifi-
Fig. 1. PCR-based strategy for cloning of mouse homeoboxes. Alignment of the homeodomain sequences of the *Drosophila* HOM (bold) and mouse *Hox* genes (Duboule and Dollé, 1989; Graham et al. 1989) shows two regions of perfect sequence conservation (shaded columns). Degenerate oligonucleotide primers representing these conserved sequences (designated ELEKEF and the reverse complement of WFQNRR) were employed for amplification of new murine *Hox* homeobox fragments. The shaded bar below illustrates the anticipated amplification product.

Identification of the *lab*-like homeobox as a fragment of a gene likely to be in the *Hox-2* complex was accomplished by standard recombinant inbred (RI) strain analysis (Taylor, 1981). Using the cloned 450bp genomic fragment as a probe, a SstI restriction fragment length variant between AKR/J and DBA/2J was identified, and the strain distribution pattern of this marker in 24 AKXD RI strains was determined by Southern blot analysis. Comparison of these results (not shown) with data reported by Hart et al. (1988) showed that recombination between the *lab*-like homeobox and the *Hox-2.1* gene occurred in only 1/24 RI strains tested (strain 15). Using the method of Silver (1985) for estimating confidence intervals for linkage...
estimates based on RI strain analysis, these results place the lab-like homeobox within 1.1 cM of Hox-2.1 on chromosome 11 (95% confidence intervals of 0.03 to 7.7 cM). Confirmation that the lab-like homeobox gene is part of the Hox-2 complex, and designation of the gene as ‘Hox-2.9,’ comes from a comparison of its sequence with the recently published sequence of the Hox-21 gene, the human homolog of the mouse Hox-2.9 gene (Acampora et al. 1989) and with unpublished sequences of the mouse Hox-2.9 homeobox (R. Krumlauf, personal communication).

Analysis of Hox-2.9 cDNAs

We obtained cDNAs for Hox-2.9 using a PCR strategy (Rapid Amplification of cDNA Ends; RACE) that directs the amplification of cDNAs from an arbitrary point in the transcription unit to the ends of the message (Frohman et al. 1988; Frohman and Martin, 1989). Subsequent amplification of genomic DNA using primers derived from the cDNA sequence demonstrated that the Hox-2.9 gene, like many other members of the Hox family, is composed of two exons and is spliced just upstream of the homeobox (data not shown). The overlapping cDNAs cloned represent 1526 bp of mRNA sequence containing a 95 bp 5′ untranslated (UT) region, an 891 bp open reading frame encoding the lab-like homeobox, and a 540 bp 3′ UT region (Fig. 2A). Since Northern blot analysis (below) suggests that the message size is ~2100 nt, presumably including ~200 nt from a poly-A tail, it is likely that ~400 nt of additional sequence exists. Much of this may be 3′ of the Hox-2.9 amplification we cloned, since the absence of a consensus polyadenylation signal sequence (Wickens and Stephenson, 1984) suggests that the entire 3′ UT region was not obtained.

Sequence analysis revealed that the Hox-2.9 homeodomain is most similar to those of Drosophila lab (83% aa identity) and mouse Hox-1.6 (87% aa identity; Baron et al. 1987; LaRosa and Gudas, 1988; see Fig. 2B). Conserved coding sequence is also found at the 3′ end of the first exon (where a hexapeptide conserved in many Hox genes is encoded) and to a lesser extent in the second exon in the region immediately 5′ of the homeodomain. Additional conservation with Hox-1.6 is found in the presumed translation initiation region.

Hox-2.9 and Hox-1.6 cDNAs both encode two potential translation start sites in frame with the homeodomain, separated by 4 aa. LaRosa and Gudas (1988) have proposed that translation of Hox-1.6 is initiated at the downstream site, based on comparison of the adjacent nucleotide sequences with consensus sequences for translation initiation (Kozak, 1987). Beginning at the comparable site, the Hox-2.9 sequence encodes Met-Ser-Ser, a sequence frequently found at the amino termini of homeodomain proteins (Duboule et al. 1988), suggesting that in both genes, translation initiates at the downstream site. Moreover, a short ORF (37 aa) starting with a Met but out of frame with the homeodomain begins at nt 1 of the cDNA sequence and terminates at a TGA stop codon, the first two nucleotides of which constitute part of the second Hox-2.9 potential translation start site. Sequences encoding small peptides have similarly been found in the 5′ UT regions of many homeobox-containing genes (Breier et al. 1988) and proto-oncogene (Kozak, 1987) transcripts; a role for these sequences in control of translation initiation has been proposed (Kessel and Gruss, 1988). On the other hand, since the coding sequences upstream of the second site are conserved, and acceptable translation initiation consensus sequences (Kozak, 1987) are found at the upstream site in both genes, translation may instead be initiated at the upstream site.

LaRosa and Gudas (1988) reported that a significant fraction of Hox-1.6 transcripts have been spliced to remove 203 nt from the first exon, resulting in a frame-shift upstream of the homeobox sequence. However, the splice sites present in Hox-1.6 are not conserved in Hox-2.9, and PCR amplification of reverse-transcribed E10.5 mRNA with oligonucleotide primers flanking the potentially spliced region yields only a single Hox-2.9 product (data not shown), suggesting that similar alternate splicing of the Hox-2.9 gene does not occur.

Analysis of a partial homeobox-like sequence found in the Hox-2.9 intron

As noted above, one of the Hox-2.9 amplification products initially isolated was a 450 bp fragment. Sequence analysis revealed the unexpected finding that it contained the same primer (WFQNRR-rc) at both ends. At one end is the Hox-2.9 homeobox; at the other end (which terminates in the Hox-2.9 intron) but in the opposite orientation, we found a partial and very divergent homeobox sequence (Fig. 3). We have been unable to determine, however, whether this sequence represents part of a functional homeodomain protein encoded by the antisense strand of the Hox-2.9 gene. Although it encodes several of the aa characteristic of homeodomain sequences, one highly conserved aa (position 51) is absent. On the other hand, increasingly divergent homeodomains are being reported. The fact that the homeodomain is incomplete is also not decisive, since several homeobox-containing genes contain splice sites within the homeobox, and potential splice acceptor sites are present that would allow the stop codon encoded at the equivalent of position 31 in the homeodomain to be bypassed. However, whether this sequence is ever transcribed remains unresolved, since we were not able to detect messages containing this sequence by Northern blot analysis of embryo RNA (E10.5–E17.5), and we have been unable to produce 3′ end cDNAs containing this sequence using the RACE protocol (data not shown).

Expression of Hox-2.9 during embryogenesis

Northern blot analysis of Hox-2.9 expression

A probe derived from the 3′ end of the Hox-2.9 cDNA (probe 1, Fig. 3) was used to examine the temporal pattern of Hox-2.9 expression during embryogenesis (Fig. 4). A 2.1 kb transcript is detected at very low levels in undifferentiated PSA-1 teratocarcinoma stem
Fig. 2. Hox-2.9 sequence and comparison with other murine Hox genes. (A) Sequence of a Hox-2.9 cDNA clone. Features to be noted: the homeobox (shaded box), the conserved hexapeptide (half box, bp 618-635); regions of amino acid identity with mouse Hox-1.6 and Drosophila lab (solid underlining); regions of identity with Hox-1.6 only (dashed underlining); location of splice site (triangle). (B) Comparison of the homeodomain sequences in murine lab-like and other Hox gene sub-families. Above are shown the homeodomain sequences of the Drosophila Antp gene and a representative member of each Hox gene sub-family (see Fig. 1). Amino acid residues that are the same in all subfamily members but different from those found in Antp are named using the gene and a representative unique to this lab subfamily gene. Residues vary within the subfamily are indicated by an asterisk (*) . Below (boxed sequences) are shown the homeodomain sequences of the two murine lab-like genes and the Drosophila lab gene. Residues unique to this lab subfamily are shaded.
Expression of Hox-2.9 is a marker of A–P position in the mesoderm during late gastrulation

By E7.5 (late gastrulation), the embryonic ectodermal subpopulations fated to form the forebrain, midbrain, hindbrain, and spinal cord are found in the correct cranio-caudal order along the A–P axis (Tam, 1989; see schematic drawing in Fig. 5). Precursor cells for other ectodermal derivatives (surface ectoderm, placodes, and neural crest) are found in the posterior–proximal region of the embryo, but evidence for their regionalization along the A–P axis is lacking (Tam, 1989). The paraxial mesoderm (termed somitomeres 1–4) and the lateral mesoderm destined for the parts of the head containing the forebrain, midbrain, and the anterior portion of the hindbrain (metencephalon) have emerged from the primitive streak and underlie the cranial ectoderm (Meier and Tam, 1982), whereas that fated for the posterior hindbrain (myelencephalon; rhombomeres) region is still separating from the primitive streak (Meier and Tam, 1982; Tam and Beddington, 1987). The remainder of the prospective mesoderm is located in or near the primitive streak, which extends to the distal tip of the embryo. The anterior end of the primitive streak is delineated by an indentation, the archenteron, which is thought to be analogous to Hensen’s node in the chick embryo (Tam and Beddington, 1987; Tam, 1989).

At E7.5, Hox-2.9 transcripts are detected along the length of the primitive streak and in the mesoderm in the posterior half of the embryo, but no Hox-2.9...
expression is observed in the anterior half of the embryo (Fig. 5A). Thus, even at this early stage, Hox-2.9 is a marker for A–P position in the mesoderm, since it is expressed in the mesoderm that will become associated with the posterior hindbrain and more posterior structures (located at this time just anterior/lateral to [and in] the primitive streak), but is not expressed in mesoderm destined for more anterior regions of the head. Hox-2.9 transcripts are also not detected in embryonic ectoderm outside the primitive streak (Fig. 5B, C). Thus, the ectoderm that will develop into the Hox-2.9-expressing neuroectoderm does not yet express Hox-2.9, whereas the mesoderm that will come to underlie it does.

**Hox-2.9 expression is first detected in neuroectoderm during early neurulation.**

Neurulation commences at E7.75–E8 (pre-somite stages), when the neural plate rises above the ectodermal sheet and constrictions appear that delineate the boundaries of the seven 'neuromeres' that will develop into the forebrain, midbrain, and hindbrain (metencephalon and rhombencephalon). By this time, approximately 8–10 cuboidal blocks of paraxial mesoderm (somitomeres; Meier and Tam, 1982) stretch along the A–P axis. The first seven somitomeres underlie the neuromeres, but whether there is a precise one-to-one physical or functional relationship between them is controversial (Keynes and Lumsden, 1990). These seven somitomeres never condense into somites, whereas the remaining somitomeres (presomitic mesoderm), bounded posteriorly by the archenteron, begin to condense into cranial somites around E8 (Rugh, 1968) as more somitomeres emerge from the primitive streak.

During early neurulation, Hox-2.9 transcripts are still detected in abundance along the length of the primitive streak (Fig. 6A, B). In contrast to observations at E7.5, however, expression is now found, albeit at lower levels, in mesoderm anterior of the archenteron, presumably as a consequence of an anterior displacement, relative to the primitive streak, of mesodermal cells that began expressing Hox-2.9 earlier. As before, transcripts are not detected in the region anterior of the prospective hindbrain. However, transcripts are now detected in ectodermal cells anterior of (Fig. 6A, B) and lateral to (Fig. 6C, D; contrast with Fig. 5B, C) the primitive streak, although only in cells at or posterior to the leading edge of Hox-2.9-expressing mesoderm. Furthermore, within the Hox-2.9-positive neuroectoderm anterior of the archenteron there is variation in the level of expression. Expression is highest at the rostral end of this region (Fig. 6A, B), in a stripe about 8–12 cell diameters in length along the A–P axis in a caudal part of the head fold. From review of numerous sections, this region appears to be located within hindbrain neuromere B, in the region that will give rise to rhombomere 4 (pro-rhombomere 4). Moreover, transcripts are not detected in the neuroectoderm anterior of neuromere B. Thus, the observed lack of expression in prospective neuroectoderm anterior of Hox-2.9-expressing mesoderm and the finding that expression in mesoderm precedes that in ectoderm suggests that Hox-2.9 expression in ectoderm may result from an inductive signal provided by underlying mesoderm.

**Coordinate expression of Hox-2.9 in the three germ layers during branchial arch unit formation.**

In the posterior part of the embryo at E8.5 (Fig. 7), transcripts are found along the length of the neural plate and primitive streak/tail bud, but are not detected in more ventral structures. In particular, posterior lateral plate mesoderm and endoderm, which form the extraembryonic vessels and hindgut, do not contain detectable levels of Hox-2.9 mRNA (Fig. 7C, D; data not shown). Anterior of the primitive streak, expression is detected in mesoderm and the neural tube at lower levels than in the primitive streak (Fig. 7A–F). As the rostral end of the presomitic mesoderm condenses into somites, expression of Hox-2.9 abruptly ceases in the paraxial and intermediate mesoderm, although it is still detectable in the lateral plate mesoderm (Fig. 7E, F).

In the anterior part of the embryo, the Hox-2.9 expression pattern has become considerably more
complex; it is best appreciated in the context of the branchial arch units, repeated structures homologous to the gill apparatus in fish, which are beginning to be organized at this time (Rugh, 1968; Theiler, 1989; see Fig. 8 schematic). The dorsal part of each unit contains a hindbrain neuromere, presumptive muscle (paraxial mesoderm), a cranial motor nerve and ganglia that innervate the muscle, and dorsolateral neural crest. The ventral part contains a protrusion of neural crest (branchial arch), an outpouching of the foregut (branchial or pharyngeal pouch), and involuting surface ectoderm (branchial cleft). For example, the second branchial arch unit contains hindbrain neuromere B (pro-rhombomeres 4 and 5), prospective jaw-opening muscles, the VII cranial ganglion (facial) and cranial nerve VII, the hyoid branchial arch, the caudal aspects of the first pharyngeal pouch and branchial cleft, and the rostral aspects of the second pharyngeal pouch and branchial cleft.

Hox-2.9 is expressed at two locations within the region containing the branchial arch units. In the dorsal aspect of the second branchial arch unit, Hox-2.9 transcripts are detected in hindbrain neuroectoderm (including floor plate cells; data not shown), restricted to a region, anterior of the otic invagination, that will give rise to rhombomere 4 at E9.5 (Fig. 8A). Hox-2.9 is also expressed in neural crest and in 'thin' ectoderm just lateral to the neural tube (Fig. 7A, B; Fig. 8B–E). Hox-2.9 is not a marker for all second arch neural crest, since only dorsal neural crest, most of which escaped recently from the neural tube, expresses Hox-2.9, whereas older neural crest, which has mostly migrated ventrally to the second branchial arch (Verwoerd and van Oostrom, 1979; Nichols, 1987), does not (Fig. 8D, E). Expression levels of Hox-2.9 in second arch cranial (paraxial) mesoderm were greatly decreased when compared to the levels observed at E8, but were above background.

The other location in which Hox-2.9 is expressed is the ventral aspect of the third branchial arch unit,
Fig. 6. Expression of Hox-2.9 at E7.75 and E8. (A, B) In situ hybridization of the Hox-2.9 probe to a sagittal section of a mouse embryo at E7.75. The approximate boundaries of the prospective forebrain (fb), midbrain (mb), metencephalon (mt) and hindbrain neuromeres A (N_A) and B (N_B) are indicated in A. The arrows in B indicate the borders of the region in the prospective hindbrain with elevated Hox-2.9 expression. The upper arrow also indicates the anterior limit of Hox 2.9 expression detected in the cranial mesoderm. (C, D) In situ hybridization of the Hox-2.9 probe to a transverse section of a mouse embryo at E8. Additional abbreviations: ar, archenteron; ec, ectoderm; fg, foregut pocket; me, mesoderm; np, neural plate; ps, primitive streak. Mag. ~200x.

Fig. 7. Expression of Hox-2.9 in the posterior part of the embryo at E8.5. The schematic diagram depicts a near-sagittal section of a mouse embryo at ~E8.5. The thick lines represent the planes of the sections in C and E. (A, B) In situ hybridization of the Hox-2.9 probe to a section that is sagittal in the anterior and posterior regions and, because the embryo is "turning," is near-transverse in the middle of the embryo (mag. ~50x). In the posterior region, the highest level of Hox-2.9 expression is detected in the tail bud (tb). The rectangle in B delineates a region in the anterior part of the embryo shown at higher magnification in Fig. 8 B, C. (C, D) A near-transverse section (mag. ~200x) through the posterior end of the pre-somatic mesoderm (psm). (E, F) A near-transverse section (mag. ~200x) angled through the anterior end of the pre-somatic mesoderm and the most recently condensed somite (so), showing the lack of Hox-2.9 expression in the somite and intermediate mesoderm (region between vertical arrows), but relatively high levels of expression in the neural tube, presomatic mesoderm and lateral plate mesoderm (lpm). Additional abbreviations: fb, forebrain; N_A, neuromere A; N_B, neuromere B; N_C, neuromere C; ng, neural groove; nt, neural tube; xv, extra-embryonic vessel.
where transcripts are detected in several adjacent tissues: in lateral mesoderm, surface ectoderm in the caudal half of the second branchial cleft, and endoderm in the caudal half of the second pharyngeal pouch and the wall of the foregut posterior to the pouch (Figs 7A, B; 8B, C). The rostral limit of expression in all three juxtaposed germ layers, mesoderm, ectoderm, and endoderm, appears to be at the same A–P level. This
expression pattern suggests to us that there is coordinate regulation of Hox-2.9 expression in multiple germ layers, as discussed below.

Segment specific expression of Hox-2.9 in the neural tube
By E9.5, further development of the dorsal part of the
Fig. 9. Hox-2.9 expression at E9.5. (A, B) A near-sagittal section through an embryo at E9.5 (mag. ~25×), showing abundant expression of Hox-2.9 in rhombomere 4 (r4), endoderm of the pharynx (ph) and lateral plate mesoderm (lpm). (C, D) A near transverse section through rhombomere 4 (mag. ~200×) illustrates the lack of detectable Hox-2.9 expression in the cells of the floor plate (fp). (E) A frontal section showing the branchial arch (BA) unit region (mag. ~75×). The rectangle delineates the region shown in F (mag. ~150×). Hox-2.9 expression is detected in surface ectoderm (se) of the 3rd branchial cleft, endoderm of the 3rd pharyngeal pouch (3rd pp) and lateral mesoderm. Additional abbreviation: ht, heart.

branchial arch unit has taken place; here, the region encompassing neuromere B has developed into the myelencephalon rhombomeres 4 and 5, and expression of Hox-2.9 is found limited to rhombomere 4 (Fig. 9A–D), one segment anterior to the otic vesicle, as previously described (Murphy et al. 1989; Wilkinson et al. 1989). Expression is no longer detectable in floor plate cells of the neural tube (Fig. 9C, D), which raises the possibility that regulation of the cells of the floor plate is distinct from that of cells in the walls of the neural tube. It has been suggested that these two populations of cells arise from distinct regions of the epiblast in the chicken (Smith and Schoenwolf, 1989).

Hox-2.9 expression is no longer detected in cranial mesenchyme, but neural crest expression is similar to that seen at E8.5. Thus, expression continues to be detected in the dorsal aspect of the second branchial arch unit. In contrast, Hox-2.9 transcripts are no longer detected in the ventral aspect of the third branchial arch unit, but are now observed in the fourth. Transcripts are present in abundance in the caudal half of the third branchial cleft (surface ectoderm), in the caudal half of the third pharyngeal pouch and in the surrounding walls of the pharynx, posterior to the developing thyroid, but anterior to the lung bud (Fig. 9A, B, E, F). Expression is also detected at lower levels in lateral plate mesoderm (gut-associated mesoderm) near the third pouch. On the basis of the studies of Noden (1988), we think that this mesoderm is likely to have descended from the lateral plate mesoderm that expressed Hox-2.9 at E8. Expression in more posterior tissues of endodermal origin is seen at low levels. Hox-2.9 transcripts are also still detectable in the tail bud (data not shown).

At E10.5, expression is largely restricted to the 4th
rhombomere, where it is found in both germinal and intermediate layers of the neural tube (data not shown). Very low levels can be detected in the endoderm and mesoderm structures that were positive at E9.5, but not in the neural crest.

Discussion

In Drosophila, the segmented body plan develops from embryonic compartments whose unique A–P positional identities are specified by the HOM genes. In vertebrates, too, the body plan is basically segmented (i.e. it is composed of repeated regions containing derivatives of multiple germ layers), but the molecular mechanism by which this pattern arises is unknown. The finding that the Hox genes in vertebrates are evolutionarily related to the HOM genes and like them are expressed in overlapping but distinct A–P domains, whose limits in some cases appear to coincide with segmental boundaries (Gaunt, 1988; Duboule and Dollé, 1989; Graham et al. 1989; Wilkinson et al. 1989; Keynes and Lumsden, 1990), raised the possibility that there might be more extensive similarities between the basic mechanisms of segment development in vertebrates and invertebrates than had been previously appreciated.

An important question in the study of segment development is how the unique A–P positional identity of a given segment is conferred on all its constituents. In Drosophila, this is achieved by differential expression of the HOM genes in compartments before the primary germ layers are established; thus the same A–P positional identity is acquired by all the cells in the compartment. Because gastrulation in Drosophila does not involve major shifts in the relative A–P positions of the germ layers, HOM gene expression patterns and thus positional identities are maintained roughly 'in register' in the germ layers through later development. In vertebrates, in contrast, A–P positional specification takes place after the three primary germ layer lineages have been set aside and after adjacent cells fated to enter different germ layers have undergone extensive relative displacement as a result of the events of gastrulation; thus in principle, the analogy with Drosophila is of limited applicability.

How then are A–P segmental identities established in vertebrates? It seems unlikely that they are determined independently in each germ layer, since much evidence has suggested that A–P positional values in vertebrate ectoderm and endoderm are acquired through short-range interactions with patterned mesoderm (Mangold, 1933; Hogan et al. 1985; Hamburger, 1988). However, such experiments have not had sufficient resolving power to determine whether the signals provided by mesoderm simply establish a crude pattern (dividing the embryo into forebrain, midbrain, hindbrain, and spinal cord) or whether in fact they confer positional identities to ectoderm and endoderm at each segmental level.

To the extent that the Hox genes reflect specification of A–P identities, a study of their expression patterns can provide some insights into the mechanisms by which positional information might be acquired through inductive interactions during vertebrate development. For example, contiguous expression of a particular Hox gene in different germ layers ('in-register' expression) would suggest that the same gene is used in different germ layers to mark segment identity and would be consistent with mesodermal specification of segment identity directly across germ layers. An 'out-of-register' or seemingly uncoordinated pattern would be more difficult to interpret, but would be observed if segment identity in different germ layers was specified by different Hox genes. Alternatively, an out-of-register pattern would be observed even if a particular Hox gene marks or mediates acquisition of the same A–P segmental identity in different germ layers, if inductive mechanisms involving short-range but not contiguous interactions (see Sive and Weintraub, 1989) operate to produce a situation in which cells in different germ layers that have the same segmental identity are not contiguous.

In fact, most reports of Hox gene expression in mid-gestation mouse embryos describe an out-of-register pattern. For example, transcripts from a given Hox gene are detected more anteriorly in the neuroectoderm than in the mesoderm at E10.5–E12.5 (Duboule and Dollé, 1989; Graham et al. 1989; Gaunt et al. 1989). In contrast, Oliver et al. (1988) reported that in Xenopus embryos, contiguous (in register) Hox gene expression is observed in different germ layers. This suggests that in Xenopus positional identities in different germ layers within a developing segmental unit are established through short-range inductive interactions and that the same Hox gene marks or mediates the establishment of a given segmental identity in different germ layers. A means of reconciling the observed differences between the murine and amphibian expression patterns was suggested by De Robertis et al. (1989). They proposed that the Hox gene expression patterns observed in mouse embryos at late stages of neurulation or during organogenesis were out of register not because they were initially established in that manner, but rather because the relative positions of the germ layers change between the time when regional identities are established (in register) and the time when the observations were made (Snow, 1981; Beddington, 1982; Chan and Tam, 1986; Lawson and Pedersen, 1987; Noden, 1988; Tam, 1989).

With this in mind, we have examined Hox-2.9 expression from late-gastrulation to mid-gestation and describe here the first clear example of a Hox gene whose expression pattern in mouse embryos is consistent with the hypothesis that induction of equivalent A–P positional values does occur among adjacent cells of different lineages in all vertebrates.

Hox 2.9 as a marker of A–P positional values in mesoderm and neuroectoderm during gastrulation and neurulation

In amphibians and chickens, mesoderm appears to
acquire its A–P positional information during the process of involution through the blastopore lip or after ingestion through the primitive streak, respectively (Kiency et al. 1972; Slack, 1983; Hamburger, 1988; Durston et al. 1989). In mice, there is little direct evidence of how or when positional information is acquired by the mesoderm. Indirect evidence suggests that mesodermal precursors acquire some measure of positional information with respect to their eventual location along the dorsoventral axis by the time they have reached the primitive streak (see diagram, Fig. 5; Snow, 1981; Tam and Beddington, 1987). However, A–P positional information does not appear to be specified until the mesoderm emerges from the primitive streak (Snow, 1981; Tam and Meier, 1982; Tam and Beddington, 1987).

It seems reasonable to assume that once mesoderm acquires position-specification information, changes in gene expression follow. On the basis of the observations reported here, we propose that the Hox-2.9 gene is a good candidate for a marker of differential gene activity established as a consequence of A–P positional specification: it is not expressed in mesoderm of the prospective forebrain, midbrain, or metencephalon regions, but is found in the mesoderm that becomes associated with the prospective hindbrain as this mesoderm is formed (E7.5). Moreover, the anterior limit of Hox-2.9 expression remains within the hindbrain region during the next several stages of development, suggesting that A–P assignments in prospective hindbrain mesoderm are fixed at the time of its formation and are actively maintained during the remainder of gastrulation and neurulation.

Just as differential gene usage should occur in mesoderm that has begun to acquire positional values, the same should be demonstrable for embryonic ectoderm and its derivatives. Since the cells representing the future CNS are arranged in the correct cranio-caudal order before neurulation takes place (i.e. at E7.5; see Tam, 1989), it could be argued that these cells have been instructed with respect to their future lineage and should therefore display differential gene expression. Alternatively, it is possible that these cells are all still equivalent but remain in the same physical order until a later time when positional cues are provided. Evidence for this latter possibility is provided by experiments demonstrating that grafts of prospective hindbrain neuroectoderm adopt the cellular fates of the sites into which they are transplanted; from this it has been concluded that most embryonic ectoderm is not committed to a specific fate at this time (Beddington, 1982). If this view is correct, then one might expect to see no differential expression of genes that mark or mediate A–P specification in the ectoderm until after E7.5. Our observations of Hox-2.9 expression are consistent with this expectation: transcripts cannot be detected in the ectoderm prior to neurulation (i.e. at E7.5), but rather are first observed during early neurulation (E7.75–E8), in a manner that clearly reflects A–P positional assignments in the presumptive CNS. These observations of expression of Hox-2.9 in prospective hindbrain mesoderm followed by coordinate expression of Hox-2.9 in mesoderm and neuroectoderm are most compatible with the simplest model of patterned neural induction, whereby mesoderm gives positional cues to apposed (prospective) neuroectoderm, leading to in register expression of the same Hox gene in both germ layers (De Robertis et al. 1989).

In amphibians, the issue of when A–P positional specification begins in relation to neurulation, i.e. before, during, or subsequent to it, is controversial (Hamburger, 1988). Despite the close temporal proximity of the first detectable Hox-2.9 expression and the onset of neurulation, our data do not allow us to define the precise temporal relationship between ectoderm A–P specification (as reflected by Hox-2.9 expression) and neurulation. Further studies of the expression of Hox-2.9 and early neural-specific markers in pre- and early-neurulation embryos may help to address this issue.

**Extinction of Hox 2.9 expression in cranial mesoderm at the end of neurulation**

As neurulation ends (~E8.5; Rugh, 1968; Chan and Tam, 1986), we find that Hox-2.9 expression becomes greatly reduced in the paraxial mesoderm associated with rhombomere 4, and by E9.5 it is undetectable. If Hox-2.9 expression in the cranial mesoderm early in neurulation reflects the establishment and early maintenance of A–P positional information, then what might be the significance of its extinction at the end of neurulation? One possibility is that this reflects a progression in commitment to a particular positional value, with consequent alterations in gene activity. Another, more intriguing possibility is that it reflects a loss of A–P positional specification by the cranial mesoderm. This view is consistent with the results of studies in the chicken, which have demonstrated that A–P axial identities in hindbrain paraxial mesoderm do not become irreversibly determined; i.e. cranial mesoderm can be respecified by neural crest cells grafted from neighboring regions (Kiency et al. 1972; Noden, 1988). In contrast, trunk paraxial mesoderm cannot be respecified, and thus appears to have become irreversibly determined with respect to its A–P positional identity. In this context it is interesting to note that expression of Hox-1.5 in cranial mesoderm is also extinguished at the end of neurulation (Gaunt, 1988), whereas other Hox genes, whose anterior limits of expression are in the trunk mesoderm, continue to be expressed in that mesoderm throughout mid-embryogenesis.

It is also possible that the observed extinction of Hox gene expression in cranial mesoderm at the end of neurulation reflects changes in the inductive capacity of that tissue. An important feature of the process by which prospective neuroectoderm is instructed by mesoderm is its restriction in time; by the end of neurulation, the neuroectoderm has become committed to specific positional values and no longer appears to respond to positional cues (Hamburger, 1988). Thus, inductive signals from mesoderm are presumably no
longer required to maintain these positional values. On a molecular level, if Hox gene expression in the cranial mesoderm early in neurulation reflects inductive activity as well as establishment and early maintenance of A-P positional information, then the cessation of Hox gene expression by the end of neurulation might signify that this activity ceases with the commitment of the neuroectoderm.

**Segmental expression of Hox-2.9 in late neurogenesis**

Late in neurulation (E8.5–E10.5), the most striking feature of Hox-2.9 expression is its restriction to rhombomere 4 (Murphy et al., 1989; Wilkinson et al., 1989; this report). This expression pattern led Wilkinson et al. (1989) to propose that the expression boundaries of Hox-2.9 are not established until morphological segmentation of the neural tube into neuromeres and assignment of rhombomere identities have taken place. In contrast, we were able to detect Hox-2.9 transcripts in the presumptive posterior hindbrain soon after the beginning of neurulation, before segmentation and rhombomere formation takes place (E7.75–E8), and observed that at E8.5, expression levels in the hindbrain neuroectoderm in the region of pro-rhombomere 4 increase significantly. Interestingly, comparable results were obtained for a chicken gene, Ghox-lab, which contains a lab-like homeodomain (Sundin et al. 1990) and whose expression in the neuroectoderm also becomes restricted to rhombomere 4 (Sundin and Eichele, 1990). At the neural plate stage, prior to the appearance of neuromeres, Ghox-lab is expressed in the neural tube from the primitive streak to an anterior boundary at the level of the future rhombomere 4. Thus for both Hox-2.9 and Ghox-lab, the A-P domain in which they are expressed is established well before morphological segments form in the developing hindbrain.

These results, in conjunction with our observations that the anterior limits of Hox-2.9 expression are the same in the neuroectoderm and the paraxial mesoderm, lead us to a substantially different interpretation of the significance of Hox-2.9 expression in the developing CNS than the one suggested by Wilkinson et al. (1989). We propose that the anterior limit of Hox-2.9 expression in the neural plate is initially established during early neurulation, potentially mediated by signalling cues provided by nearby paraxial mesoderm, and that the segmentally restricted increased expression observed later in neurulation represents a second phase of expression in the neural tube. Similar temporal/spatial expression patterns are observed for some *Drosophila* homeobox-containing genes (e.g. *fushi tarazu*), for which a cis-acting element responsive to axial cues regulates production of a small amount of gene product, which then activates a positive-autoregulatory cis-acting element that serves to increase and stabilize expression after the axial cues have been removed (Beachy, 1990). In *Drosophila*, the individual HOM genes are expressed in domains that range from a single segment (e.g. *lab*) to many segments (e.g. *AbdA*); the extent of these domains is determined by simultaneous interactions of many different homeobox-containing genes. Similarly complex regulation presumably takes place in vertebrates, mediated by the increasing number of homeobox-containing, zinc-finger, and growth factor genes that are being found to have expression limits that begin at regional or segmental boundaries in the mesoderm and CNS (Lewis, 1989).

**Expression in the branchial arch units**

The results described here suggest that the expression of Hox-2.9 may also mark or mediate induction of equivalent positional values within the tissues of the developing branchial arch units. There are several theories concerning the origin of these repeated structures. One view, that they represent specialized trunk segments, is based on the observation that the visceral elements of the branchial arch unit (branchial pouches and clefts) are coincident with segmented blocks of mesoderm aligned along the A-P axis (Goodrich, 1930); however, other lines of evidence suggest that the branchial arch unit does not develop in conjunction with the formation of segmented mesoderm but becomes organized only after extensive morphological movements of the mesoderm have taken place (Balinsky, 1981; Romer and Parsons, 1986; Kimmel et al. 1988). One such movement in particular, that of lateral mesoderm, is pertinent to our interpretation of the observed Hox-2.9 expression pattern in the branchial arch units. In the chicken (and presumably in the mouse), lateral mesoderm originally located at the level of the first somite does not remain at the same rostrocaudal level throughout embryogenesis; it migrates ventrally and posteriorly to form laryngeal and gut-associated mesoderm. In contrast, paraxial mesoderm found at the same level remains there, eventually contributing to the bones of the skull at the back of the head (Noden, 1988).

As best as we can determine, the anterior boundaries of Hox-2.9 expression along the A-P axis coincide in paraxial and lateral mesoderm during late gastrulation and early neurulation. By late neurulation (E8.5), however, differences have become apparent: in paraxial (dorsal) mesoderm, weak expression of Hox-2.9 is still detected in the second branchial arch unit (accompanied by intense expression in second arch neural crest and neural tube [rhombomere 4]), whereas Hox-2.9-expressing lateral (ventral) mesoderm extends anteriorly only as far as the third branchial arch unit. We propose that Hox-2.9 is a marker of the same initial A-P positional value in both paraxial and lateral mesoderm, and that the observed difference in the anterior limits of expression of Hox-2.9 in these two tissues is due to posterior displacement of the lateral mesoderm, rather than to independent regulation of Hox-2.9 expression along the A-P axis in paraxial and lateral mesoderm. The finding that one day later, lateral mesoderm expression is found even further posterior, in the fourth branchial arch unit, might result from continued lateral mesoderm displacement.

It is also suggestive that in the ventral aspect of the
branchial arch unit the anterior boundary of Hox-2.9 expression is the same in lateral mesoderm as it is in surface ectoderm and foregut endoderm (in the caudal half of the second branchial cleft and pharyngeal pouch, respectively). Neither of these tissues is thought to be intrinsically segmented in vertebrates (Romer and Parsons, 1986); in fact, evidence has been presented to show that both obtain positional values from closely associated mesoderm (Okada, 1957; Wessler and Rutter, 1969; Richman and Tickle, 1989). We propose that the observed Hox-2.9 expression pattern reflects induction of A-P positional values in the ectoderm and endoderm via signaling cues provided by the lateral mesoderm, much as we hypothesized that Hox-2.9 expression in the neural plate is induced by signals from the paraxial mesoderm. However, it seems likely that the branchial arch unit induction represents a second and later inductive event independent of that proposed for the neural plate, since induction of positional values in the neural plate by paraxial mesoderm probably takes place at a time (E7.5–8) when the prospective branchial arch unit surface ectoderm, foregut endoderm, and lateral plate mesoderm are located in disparate regions of the embryo, according to fate maps that have been reported (Tam and Meier, 1982; Lawson and Pedersen, 1987; Tam, 1989).

Is Hox-2.9 unique among the Hox genes?

We have presented evidence that Hox-2.9 transcripts are found in multiple germ layers, and that during certain periods, i.e. during induction of positional values in the neural plate and during organization of the branchial arch units, there is coordinate in register expression of Hox-2.9 in the different germ layers. These data have led us to propose that Hox-2.9 expression reflects and is potentially involved in the establishment of equivalent positional values in these tissues. At present, it is not possible to determine whether these conclusions can be generalized to other members of the Hox gene family, primarily because there are insufficient data on expression patterns at the relevant times of development. Hox-1.6, the Hox family member most closely related to Hox-2.9, is expressed at the same time and in many of the same tissues as Hox-2.9 (including branchial arch unit endoderm; Duboule and Dollé, 1989; Sundin et al. 1990), but the anterior boundaries of expression have not been well characterized. One other gene, Hox-1.5, has been examined in detail during early neurulation, and appears to have anterior boundaries of expression that coincide in cranial mesoderm and neuroectoderm (Gaunt, 1988); this is consistent with our hypothesis of mesoderm induction of positional values in neuroectoderm and suggests that many Hox genes may have similar general expression patterns, although low levels of expression make analysis difficult.

Because analysis of mRNA localization patterns can provide only correlations between sites of specific gene expression and morphological and inductive events, there is presently no evidence that Hox-2.9 actively participates in the process of positional specification.

Demonstration of a direct relationship between the expression of Hox genes and specification will require experimental manipulation of the embryo. Nonetheless, we believe that the observed correlations between the pattern of expression of Hox-2.9 mRNA and the events that take place during gastrulation are of significance and strengthen the inference that the gastrulation process in mammals is fundamentally similar to that in amphibians and chickens.

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**Note**

The Hox-2.9 sequence described in this paper has been submitted to the EMBL/GenBank Data Libraries under the accession number X53063.

**References**


Hox-2.9 expression in mouse embryos


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