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# Structure and expression pattern of the murine *Hox-3.2* gene

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## Summary

**The murine homeobox-containing gene *Hox-3.2* is the most 5′ member of the *Hox-3* complex on chromosome 15 isolated to date. Conceptual translation of the longest ORF gives a protein of 260 amino acids lacking the conserved hexapeptide found in most homeobox genes. Northern analysis detects three transcripts of 1.5, 1.9 and 3.2 kb in day 9 to 15 p.c. embryos. As early as day 8.5 p.c., transcripts can be detected in the posterior part of the embryo by *in situ* hybridization. At this developmental stage no or only very weak expression is visible in the neural plate. At day 10.5 *Hox-3.2* is detected in the ventral part of the neural tube with a sharp anterior boundary at the level of the third thoracic prevertebra. This anterior boundary remains at day 12.5 and day 14.5. In contrast to *Hox-3.1*, *Hox-3.2* is not expressed in the dorsal horns containing the sensory neurons at day 14.5 p.c. *Hox-3.2* transcripts are also detected in the posterior prevertebrae, the hindlimb buds and the cortex of the developing kidney. Unlike *Hox-1.4* and *Hox-1.3* and their paralogs, *Hox-3.2*, *-2.5* and *-4.4 (5.2)* show strikingly different anterior boundaries of expression in the CNS and prevertebrae.**

Key words: *in situ* hybridization, homeobox, *Hox-3*, mouse embryogenesis, paralogous genes.

## Introduction

The homeobox is a highly conserved 180 bp sequence, which was first described in genes controlling pattern formation in *Drosophila melanogaster* (McGinnis *et al.* 1984; Scott and Weiner, 1984; reviewed by Akam, 1987 and Gehring, 1987). This 180 bp sequence normally located close to the *C*-terminus of the protein, encodes a 60 amino acid homeodomain containing a helix-turn-helix motif that functions as a sequence-specific DNA-binding domain (Desplan *et al.* 1985; Laughon and Scott, 1984; Fainsod *et al.* 1986; Cho *et al.* 1988; Hoey and Levine, 1988; Otting *et al.* 1988; Qian *et al.* 1989).

To date, more than 30 murine *ho*meob*ox* genes (*Hox* genes) have been isolated due to their homology to the *Antennapedia* (*Antp*) homeobox or other members of this highly conserved gene family (for review see Kessel and Gruss, 1990). Many of these genes are arranged in clusters, in *Drosophila* as well as in vertebrates (for review see Akam, 1989). Four clusters have been identified in the mouse genome, the *Hox-1* cluster on chromosome 6 (Colberg-Poley *et al.* 1985*a*; Duboule *et al.* 1986), the *Hox-2* cluster on chromosome 11 (Hart *et al.* 1985; Graham *et al.* 1988), the *Hox-3* cluster on chromosome 15 (Awgulewitsch *et al.* 1986; Breier *et al.* 1988) and the *Hox-4* cluster on chromosome 2 (Duboule and Dollé, 1989; the nomenclature for the *Hox-5* cluster has been recently updated and the cluster was renamed as *Hox-4*; *Hox-5.1*=*Hox-4.2*, *Hox-5.2*=*Hox-4.4*, *Hox-5.3*=*Hox-4.5*; see Duboule *et al.* 1990; we will use the new nomenclature, but give the old names in brackets). These clusters can be aligned on the basis of homologies between the homeobox sequences. The identical order of paralogous genes (Schughart *et al.* 1988) in all four clusters suggests that they emerged by complete or partial duplication of a common ancestral cluster (Kappen *et al.* 1989). Moreover, alignment can be extended to the *Drosophila ANT-C* and *BX-C* complexes (Gaunt *et al.* 1988; Duboule and Dollé, 1989; Graham *et al.* 1989). A strong correlation exists between the position of a gene within the cluster and its expression pattern along the anteroposterior (A–P) axis both in *Drosophila* and vertebrates (Akam, 1989). The more 3′ a gene is located in the cluster, the more anterior is its border of expression in the developing embryo. In the mouse, this is most obvious in the central nervous system, where distinct anterior boundaries of expression are exhibited (Gaunt *et al.* 1988; Duboule and Dollé, 1989; Graham *et al.* 1989). Recently it has been reported that the paralogous genes, *Hox-1.4*, *-2.6* and *-4.2 (5.1)* as well as *Hox-1.3*, *-2.1* and *-3.4* display similiar anteroposterior boundaries of expression in the developing CNS and prevertebrae, although some tissue-specific differences exist (Gaunt *et al.* 1989; 1990). Since these genes are found toward the 3′end of the cluster, it was of interest to determine whether paralogous genes at the 5′end of the cluster also show similar anterior boundaries of expression.

Two recent reports have highlighted the potential role of homeobox genes in specifiying positional information in the developing vertebrate embryo. The

*Xenopus XlHbox 1* gene encodes two transcripts that produce 'long' and 'short' proteins. These proteins are expressed in the cervical region of *Xenopus* embryos. When antibodies to the 'long' protein were injected into single-cell embryos, the anterior spinal cord was transformed into a hindbrain-like structure. The long and short proteins appear to act antagonistically, since after injection of mRNA for the short protein into the embryo a similar phenotype is observed (Wright *et al.* 1989). Ectopic expression of the murine *Hox-1.1* gene in transgenic mice results in cranofacial abnormalities and variations of cervical vertebrae (Balling *et al.* 1989; Kessel *et al.* 1990). It occurs that both ectoderm and mesoderm derivatives are affected: cranial neural crest cells and the first cervical somites. Specifically the derivatives of the first cervical somite are altered in these mice, resulting in the manifestation of an extra vertebra (Kessel *et al.* 1990). Thus the additional presence of *Hox-1.1* interferes with embryogenesis in a disruptive and reprogramming fashion. These results suggest that altered homeobox gene expression in vertebrates can lead to transformations very similar to the homeotic transformations observed in *Drosophila* mutants. This indicates an important role of these genes in vertebrate pattern formation.

In this study we present a detailed analysis of the mouse *Hox-3.2* gene and its expression during murine development. *Hox-3.2* is the most 5′ gene in the *Hox-3* cluster isolated to date and is located approximately 8 kb upstream of *Hox-3.1* (Breier *et al.* 1988; Le Mouellic *et al.* 1988). Using Northern and *in situ* hybridization the temporal and regional pattern of *Hox-3.2* expression was analysed during mouse development from day 8.5 to day 16.5 of gestation. Transcripts were detected in the posterior part of the neural tube starting at the level of the third thoracic prevertebra (T3), in prevertebrae posterior to T9, the proximal part of the hindlimb buds and the developing kidney, which continues to express *Hox-3.2* in the adult tissue. The restricted pattern of *Hox-3.2* expression during this time is consistent with *Hox-3.2* specifying positional information in the mouse embryo. This study also reveals that important differences exist in the expression of paralogous genes and in the expression of genes within each gene cluster.

## Materials and methods

### *cDNA library screening*

Approximately $1.5\times10^6$ clones of a λgt10 cDNA library prepared from 8.5 day p.c. mouse embryo RNA (Fahrner *et al.* 1987) were screened under high stringency conditions by hybridization using a random oligo-labelled (Feinberg and Vogelstein, 1983) 180 bp genomic fragment containing the first 154 bp of the *Hox-3.2* homeobox (Breier *et al.* 1988). Hybridization conditions were as follows: 6×SSC, 0.5% SDS, 5×Denhardts, 0.1 mg ml$^{-1}$ denatured salmon sperm DNA, 65°C. Filters were washed in 2×SSC, 1% SDS for 30 min followed by two further washes for 30 min in 0.1×SSC, 0.1% SDS at 65°C.

### *DNA sequence analysis*

Subclones of the *Hox-3.2* cDNA and overlapping subclones of the genomic DNA were generated both in M13 mp18 and M13 mp19. The nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.* 1977) using an M13 sequencing kit (Sequenase, US Biochemicals).

### *Isolation of cells, tissues, embryos and RNA*

PCC7 cells were grown as monolayers in Dulbecco's modified Eagles media supplemented with 10% foetal calf serum. Cells were either induced to differentiate into neuroepithelium by treating them with retinoic acid alone ($5\times10^{-7}$ M) or into neuronal-like cells by treating them with retinoic acid and dibutyryl cAMP ($10^{-3}$ M). Six days after initiating treatment cells were analyzed for the presence of neuroepithelial- or neuronal-like cells.

Embryos for Northern and *in situ* analysis were obtained from natural matings of female NMRI mice. The day of the vaginal plug was designated as day 0 p.c. Tissues were isolated from adult NMRI mice. Total RNA was isolated by homogenizing cells, tissues or embryos in guanidinium thiocyanate and poly $(A)^+$ RNA was obtained after elution from oligo (dT)-cellulose columns according to Ausubel *et al.* (1989).

### *Northern blotting*

Samples containing 10 μg of poly $(A)^+$ RNA were denatured at 68°C, separated on 1% agarose–formaldehyde gels and transferred to Hybond N (Amersham) nylon membranes using 10×SSC. Nucleic acids were crosslinked under 309 nm UV light and the filters were then hybridized under high stringency conditions. Hybridization conditions were as follows: 7.5% dextran sulfate, 5×SSC, 5×Denhardts, 50% deionized formamide (FA), 1% SDS, 10 mM Tris–HCl pH 7.5, 0.1 mM sodium pyrophosphate, 0.1 mM ATP and 0.1 mg ml$^{-1}$ denatured salmon sperm DNA. Filters were hybridized at 42°C for 16 h. Filters were washed in 2×SSC, 1% SDS at 65°C followed by 0.1×SSC, 0.1% SDS, 65°C for 30 min.

### In situ *hybridization*

Radioactive probes were transcribed *in vitro* from linearized plasmid containing the first 361 bp of the *Hox-3.2* cDNA using 0.1 mCi $^{35}$S-UTP and 0.1 mCi $^{35}$S-CTP and T3 or T7 polymerases (Promega Biotech.). After DNase I digestion, probes were precipitated with 10% trichloracetic acid and collected on nitrocellulose filters (Millipore). Probes were eluted from the filters in 50 mM EDTA pH 8, 0.1% SDS at 65°C for 30 min. Following ethanol precipitation probes were partially degraded with 0.2 M NaOH on ice for 30 min, neutralized with 1 M acetic acid and ethanol precipitated. Finally, the probes were resuspended in 50% FA, 10 mM DTT. Sections (8 μm) were prepared and hybridized as described (Hogan *et al.* 1986; Dony and Gruss, 1987). Sections were cut with a cryostat and transferred to subbed slides. Sections were quickly dried at 50°C, fixed in 4% paraformaldehyde (PFA) and dehydrated in a graded ethanol series. Slides were stored at −20°C until the day of hybridization. Prior to hybridization, slides were dipped in distilled water and incubated for 30 min in 2×SSC at 70°C. After a second rinse with water, slides were digested with 0.125 mg ml$^{-1}$ pronase for 10 min at RT. Digestion was stopped in 0.2% glycine for 30 s. Slides were rinsed in PBS and refixed in 4% PFA for 20 min, rinsed again in PBS and acetylated for 10 min in 0.1 M triethanolamine with 1/400 volume of acetic acid. After rinsing slides in PBS, sections were dehydrated in

graded ethanol and air dried for 1–2 h. The probe was diluted to a final concentration of $5\times10^6$ cts $min^{-1}$ $ml^{-1}$ in a buffer containing 50% FA, 2×SSC, 10 mM Tris, 10 mM $NaPO_4$ pH 6.8, 5 mM EDTA, 10% dextran sulphate, 150 μg tRNA $ml^{-1}$, 150 μg denatured salmon sperm DNA, 0.1 mM UTP, 10 μM S-ATP, 1 mM ADPβS, 10 mM DTT, and 10 mM β-mercaptoethanol. The hybridization mix was boiled for 2 min, applied directly onto sections and covered with siliconized cover slips. Slides were hybridized overnight in a humid chamber at 48°C. Following hybridization slides were washed for 2–4 h in 50% FA, 2×SSC and 10 mM β-mercaptoethanol at 37°C followed by RNase digestion. A second wash in 50% FA, 2×SSC, 10 mM β-mercaptoethanol was done overnight followed by dehydration through a graded ethanol series. Slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with water) and exposed for 8–12 days at 4°C. Development was done at RT for 3 min in Kodak D-19, followed by 1 min in 1% acetic acid and 3 min in 30% sodium thiosulphate. After repeated washes in distilled water slides were stained with Giemsa, dried and coverslips applied. Photomicrographs were taken using a Leitz Labovert bright-field/dark-field microscope.

### In vitro *transcription and translation*

Plasmid containing the complete *Hox-3.2* cDNA was linearized and sense and antisense strands were synthesized with either T3 or T7 polymerase (Promega). The reaction conditions were 40 mM Tris–HCl pH 8, 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine, 5 mM DTT, 2 mM dNTPs, 2 mM $m^7G(5')ppp5'G$, 2 μg DNA template and 2 μl enzyme. Approximately 1/50 of the RNA template was then translated in a rabbit reticulocyte lysate according to the manufacturer's specifications (Promega) using [$^{35}$S]methionine (Amersham) to label the protein.

## Results

### *Characterization of the* Hox-3.2 *cDNA and its genomic structure*

The characterization of the murine *Hox-3.2* homeobox has been recently reported (Breier *et al.* 1988). To obtain *Hox-3.2* cDNAs, a 180 bp probe, containing the first 154 bp of the *Hox-3.2* homeobox was used to screen a λ gt10 cDNA library, prepared from 8.5 days p.c. mouse embryo poly $(A)^+$ RNA (Fahrner *et al.* 1987). Hybridization under high stringency conditions led to the isolation of several positive cDNA clones. The complete nucleotide sequence of the longest cDNA (1450 bp) is shown in Fig. 1A. This cDNA contains a typical polyadenylation signal at position 1412 (AATAAA; Birnstiel *et al.* 1985) and the longest open reading frame (ORF) consists of 791 bp (nucleotide position 337–1128).

Three ATG codons (nucleotide position 349, 625 and 772) were found in frame with the homeobox. The surrounding nucleotide sequence of all three matches only poorly to the consensus sequence established for eukaryotic translational start (CCACCAUGG; Kozak, 1986). In such cases the ATG codon that lies closest to the 5'end should be favoured (Kozak, 1978, 1983). Use of the first ATG would produce a protein consisting of 260 amino acids, containing the homeodomain close to its carboxyl terminus. The close proximity of the homeodomain to the 3' end of the protein has been reported also for other vertebrate homeo proteins (Kessel *et al.* 1987; Krumlauf *et al.* 1987; Fibi *et al.* 1988; Odenwald *et al.* 1987; Breier *et al.* 1988; Bogarad *et al.* 1989; Rubin *et al.* 1987; Meijlink *et al.* 1987; Schughart *et al.* 1988).

A cosmid clone (kindly provided by B. Herrmann) containing genomic sequences from the *Hox-3* cluster was used to establish the primary structure of the *Hox-3.2* gene. The *Hox-3.2* homeobox is located about 8 kb upstream of the *Hox-3.1* homeobox. Digestion of this cosmid with a variety of restriction enzymes and subsequent hybridization under high stringency conditions either with the *Hox-3.2* homeobox-probe or 5' probes of the cDNA (probe 1: nucleotides 1–361; probe 2: nucleotides 637–905) identified *Hox-3.2* coding sequences. The structure of the genomic region encoding the 1.45 kb *Hox-3.2* cDNA is shown in Fig. 1B. Comparison of homologous fragments derived from the cDNA and genomic clones identified a single intron of approximately 1.7 kb in the *Hox-3.2* coding sequence. The fragments surrounding the splice junctions were isolated (the 700 bp *Apa*I–*Apa*I fragment at the 5'splice junction and the 800 bp *Bam*HI–*Bam*HI fragment at the 3'splice junction), subcloned and sequenced. The sequences at the 5' splice site (AG/TAAGTT) and the 3'splice site (TTTGTCCCTCCAG/GT) are in good agreement with the splice consensus (5': AG/$GT^A{}_G$AGT; 3': $^T{}_C{}^T{}_C$TT$^T{}_C{}^T{}_C{}^T{}_C{}^T{}_C{}^T{}_C$NCAG/G; Shapiro and Senapathy, 1987). Thus, the *Hox-3.2* gene consists of two exons of approximately 900 and 550 bp separated by an intron of 1.7 kb. The first exon encodes at least 350 bp of 5'untranslated sequence and 179 amino acids, whilst the second exon encodes 81 amino acids including the homeodomain and a 3'untranslated region of 300 bp.

Unlike the *Hox-4 (-5)* cluster, where *Hox-4.5 (-5.3)* is located about 6 kb upstream of *Hox-4.4 (-5.2)*, the paralog of *Hox-3.2*, no additional homeobox could be detected within 20 kb upstream of *Hox-3.2* by using *Hox-3.1*, *Hox-3.2*, *Hox-1.1* or *Antp* homeobox probes and low stringency hybridization conditions (Duboule and Dollé, 1989). This indicates that loss of such a gene from the *Hox-3* cluster may have occurred after duplication from the ancestral complex or alternatively that the *Hox-3* cluster arose by partial duplication.

### *The predicted* Hox-3.2 *protein*

The putative *Hox-3.2* protein, as deduced by conceptual translation of cDNA sequences encodes a protein of 260 amino acids, with a predicted relative molecular mass of $29.3\times10^3$. In order to confirm this, the complete *Hox-3.2* cDNA was transcribed and translated *in vitro* using [$^{35}$S]methionine to label the protein. The resulting protein mix was analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The observed relative molecular mass was approximately $33\times10^3$ (Fig. 2). Since homeobox-containing genes migrate in general slower than expected (Kessel *et al.* 1987), the obtained relative molecular mass is consistent with the use of the first ATG codon.

**A** Hox-3.2 cDNA

```
   1  CAC ACG GTC AAG GGG GGG GGG TCC CTG ATG AGA TAT GAT TCG TCC
  46  GGG GAG GGG GGG CTG TCT GGC GCC TTG GGT CCA GAG TTG CAA ATC
  91  GCC AGG AAA ACG CAG GTC GCG GGG ATC AGC AAA GAG AAG GGG GCG
 136  GTA AAG GGG GGA AAA AGC AAG GGG GAA AAA AAG CCC CTG CGC ATT
 181  GAT CCG CGC CGT ATT TTT GGG TAA ATA CGA TCA CGT GGG GGC TGG
 226  GGA ACC AAT GAG CTG CCG GGA AAA GGC TGG AAA AAT AAT TAC CTG
 271  CCT TGA TTG TTC TGT GAG CAG ATA AAA AGT ACA TAT ACA GTT CAT

 316  ACA ATA ATC TTA TGT ATG TAA AAC CCT GTT ACG ATG TCG GCG ACG
                                                Met Ser Ala Thr
          Apa I
 361  GGG CCC ATC AGT AAC TAT TAC GTG GAC TCG CTC ATC TCT CAC GAC
      Gly Pro Ile Ser Asn Tyr Tyr Val Asp Ser Leu Ile Ser His Asp

 406  AAT GAA GAC CTC CTA GCG TCC AGG TTT CCG GCC ACC GGG GCT CAC
      Asn Glu Asp Leu Leu Ala Ser Arg Phe Pro Ala Thr Gly Ala His

 451  CCT GCC GCC GCC AGA CCC AGC GGC TTG GTG CCG GAC TGT AGC GAT
      Pro Ala Ala Ala Arg Pro Ser Gly Leu Val Pro Asp Cys Ser Asp

 496  TTT CCG TCC TGT AGC TTC GCG CCC AAG CCG GCT GTA TTC AGT ACG
      Phe Pro Ser Cys Ser Phe Ala Pro Lys Pro Ala Val Phe Ser Thr

 541  TCG TGG GCG CCG GTG CCC TCG CAG TCG TCT GTG GTC TAT CAC CCT
      Ser Trp Ala Pro Val Pro Ser Gln Ser Ser Val Val Tyr His Pro

 586  TAC GGC CCC CAG CCC CAC CTC GGC GCC GAC ACG CGC TAC ATG CGG
      Tyr Gly Pro Gln Pro His Leu Gly Ala Asp Thr Arg Tyr Met Arg
             Xho I
 631  ACT TGG CTC GAG CCG CTG TCC GGC GCC GTC TCC TTC CCC AGC TTC
      Thr Trp Leu Glu Pro Leu Ser Gly Ala Val Ser Phe Pro Ser Phe

 676  CCG GCC GGG GGC CGT CAC TAC GCC CTC AAG CCC GAC GCC TAC CCG
      Pro Ala Gly Gly Arg His Tyr Ala Leu Lys Pro Asp Ala Tyr Pro

 721  GGG CGC CGC GCC GAC TGC GGC CCG GGC GAC GGC CGC AGC TAC CCG
      Gly Arg Arg Ala Asp Cys Gly Pro Gly Asp Gly Arg Ser Tyr Pro

 766  GAC TAC ATG TAC GGC TCG CCC GGG GAA CTG CGC GAC CGC GCC CCG
      Asp Tyr Met Tyr Gly Ser Pro Gly Glu Leu Arg Asp Arg Ala Pro

 811  CAG ACG CTG CCC TCG CCC GAG GCG GAC GCG CTG GCC GGC AGC AAG
      Gln Thr Leu Pro Ser Pro Glu Ala Asp Ala Leu Ala Gly Ser Lys
                                            ▼
 856  CAC AAA GAG GAG AAG GCC GAC CTG GAC CCT AGC AAC CCC GTG GCC
      His Lys Glu Glu Lys Ala Asp Leu Asp Pro Ser Asn Pro Val Ala
          Bam HI
 901  AAC TGG ATC CAC GCC CGT TCC ACA AGG AAG AAG CGC TGC CCC TAC
      Asn Trp Ile His Ala Arg Ser Thr Arg Lys Lys Arg Cys Pro Tyr

 946  ACC AAG TAC CAG ACG CTG GAA CTG GAG AAG GAG TTT CTC TTC AAT
      Thr Lys Tyr Gln Thr Leu Glu Leu Glu Lys Glu Phe Leu Phe Asn

 991  ATG TAT TTA ACC AGG GAC CGT CGG TAC GAG GTG GCC CGT GTT CTC
      Met Tyr Leu Thr Arg Asp Arg Arg Tyr Glu Val Ala Arg Val Leu

1036  AAT CTC ACT GAG CGG CAG GTC AAA ATC TGG TTT CAG AAC CGG AGG
      Asn Leu Thr Glu Arg Gln Val Lys Ile Trp Phe Gln Asn Arg Arg

1081  ATG AAG ATG AAA AAG ATG AAT AAA GAG AAA ACC GAC AAG GAA CAA
      Met Lys Met Lys Lys Met Asn Lys Glu Lys Thr Asp Lys Glu Gln

1126  TCC TAA GCC CTG CCC CAG ACT GCT GCC TCG GCA CAG CCA AGG GAA
      Ser End

1171  ACA CAA AAA CCC CCA CAA AAA ATG CCC CAA CCC AGG CGG GAG AAA
1216  GCA CGA AAA GAA AAG GAA AGA ACA AGA TAG AGA AAA GCC CAC CGT
1261  CTT AAA AAG AAA AGA AAA AAA AGG AAG GGG AAA AAT GCA AAC TCT
1306  TGC GAT GTG GGA GGG TTA AGT GTT GAG AAA TTG GTG TTT AGA GTT
1351  AGT TCT ATC CAT CGA GGA GGA GGC AGG AGA GAA ACT GCG TTC TCT
1396  TCC CCA GCG CAA CTG AAA TAA ATG ACA CAC ACA AAT GTG AAA AAA
1441  AAA AAA AAA
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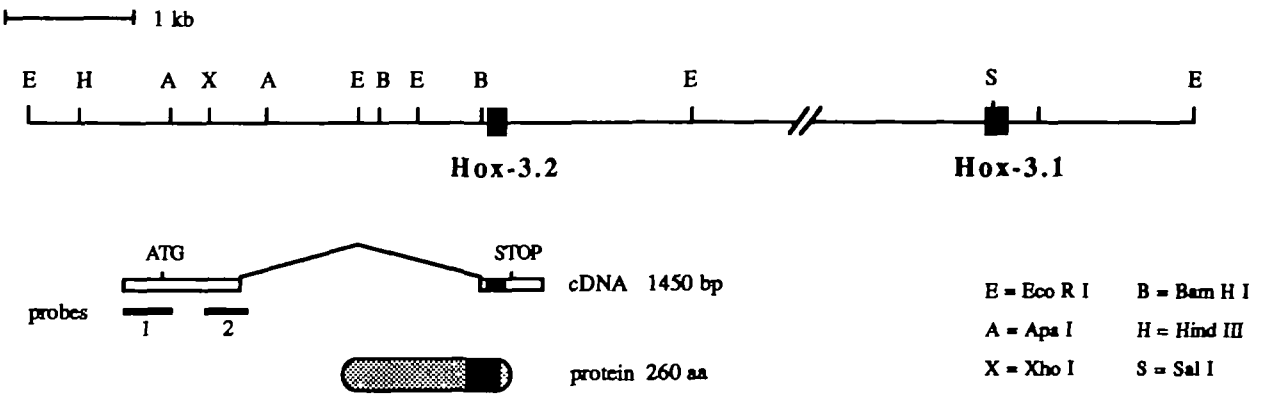
**Fig. 1.** (A) *Hox-3.2* cDNA sequence and putative primary structure of the *Hox-3.2* protein. The last STOP codon (TAA) before the translational start site and the polyadenylation signal (AATAAA) are underlined. The homeodomain is boxed. Restriction sites used for isolating probes are given (nucleotide 1 – *Apa*I=probe 1, *Xho*I–*Bam*HI=probe 2; compare Fig. 1B). (B) Genomic structure of the *Hox-3.2* gene. The distance between the *Hox-3.1* and *Hox-3.2* homeobox is approximately 8 kb. Homeobox sequences are indicated as black boxes. The *Hox-3.2* transcription unit consists of two exons interrupted by a 1.7 kb intron 5′ of the homeobox. Probes 1 and 2 used for *in situ* hybridization are marked by bold lines. Some restriction sites are given (compare Fig. 1A).

**B**



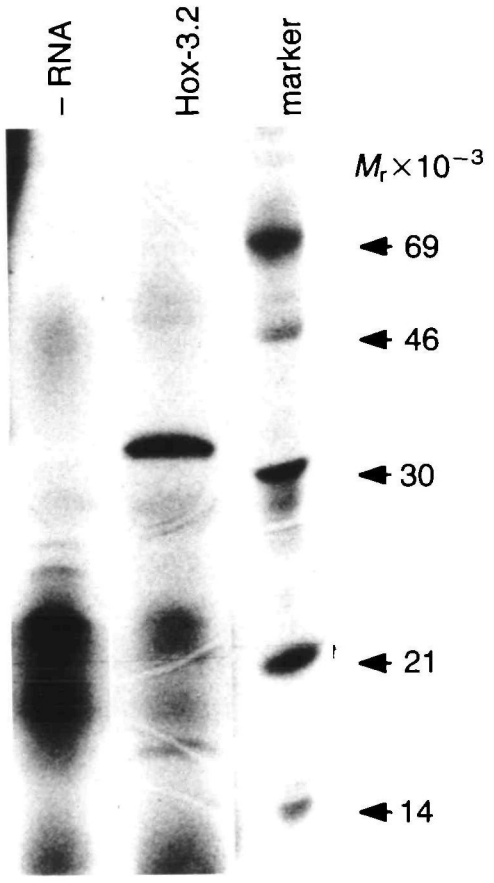



**Fig. 2.** *In vitro* translated *Hox-3.2* protein. The complete cDNA sequence was transcribed and translated *in vitro* in a rabbit reticulocyte lysate using [$^{35}$S]methionine to label the protein. The first lane shows the control reaction without RNA, lane 2 the *Hox-3.2* protein and lane 3 the marker (Rainbow mol. wt marker, Amersham).

The *Hox-3.2* homeodomain shows the highest homology to *Hox-1.7* (Rubin *et al.* 1987), *Hox-2.5* (Bogarad *et al.* 1989) and *Hox-4.4* (*-5.2*) (Dollé and Duboule, 1989), the paralogs of *Hox-3.2* (Duboule and Dollé). The homology to *Hox-1.7* and *Hox-2.5* extends up to the splice site (9/11 amino acids) with the position of the splice site being conserved as well. *Hox-3.2* lacks the conserved hexapeptide (IleTyrProTrpMetArg), which is usually found upstream of the splice site in the exon preceding the homeobox-containing exon (Baron *et al.* 1987; Kessel *et al.* 1987; Krumlauf *et al.* 1987; Meijlink *et al.* 1987; Odenwald *et al.* 1987; Breier *et al.* 1988; Fibi *et al.* 1988; Le Mouellic *et al.* 1988; Schughart *et al.* 1988). The hexapeptide is also absent in the paralogs of *Hox-3.2*, *Hox-1.7* (Rubin *et al.* 1987) and *Hox-2.5* (Bogarad *et al.* 1989) and in the homologous gene of *Hox-3.2* in *Xenopus*, *XlHbox 6* (Sharpe *et al.* 1987).

The homeodomain of *Hox-3.2* and its paralogs are slightly more similar to the *Drosophila Abd-B* (43/60 amino acids; Regulewski *et al.* 1985; Celniker *et al.* 1989) than the *Antp* homeodomain (42/60 amino acids). However, when the sequences are aligned, it becomes clear that the *Abd-B* and *Hox-3.2* paralogous homeodomains are clearly distinct from the *Antp* homeodomain (Duboule and Dollé, 1989).

*Northern analysis*

The expression pattern of *Hox-3.2* during mouse development was analysed by Northern blot analysis of poly (A)$^+$ RNA from teratocarcinoma cell lines, mouse embryos and adult mouse tissues. The same probe used for cDNA isolation was utilized to detect *Hox-3.2* transcripts. This probe detected a single band in Southern blot analysis of mouse genomic DNA under high stringency conditions excluding cross-hybridization to other homeobox genes (data not shown).

Fig. 3A shows the hybridization under stringent conditions of the *Hox-3.2* probe to RNA from day 9–16 p.c. embryos. Two major transcripts of 1.5 and 1.9 kb and a minor transcript of 3.2 kb were detected. Transcripts of *Hox-3.2* are most abundant in day 10–12 p.c. embryos. Later than day 13 of gestation, expression decreases sharply and by day 16 no *Hox-3.2* mRNA was detected by Northern blot analysis. Weak expression was observed in day 9 p.c. embryos and in *in situ* hybridization of 8.5 day embryos. These results, as well as the isolation of *Hox-3.2* cDNAs from an 8.5 day p.c. embryonic library, demonstrate that *Hox-3.2* is expressed as early as day 8.5 of embryogenesis. The 5′ cDNA-probe 1 (nucleotide 1–361) detects only the two major transcripts, indicating that the 3.2 kb transcript is probably the result of alternative splicing. However, we have isolated an additional *Hox-3.2* cDNA where the polyadenylation signal at position 1412 was not utilized and continued for another 450 bp at the 3′ end (data not shown). This suggests that the 1.5 and 1.9 kb transcripts may result from poly-A variation instead of alternative splicing.

We have also examined poly (A)$^+$ RNA derived from a variety of adult mouse tissues including lung, liver, kidney, heart, brain, testis, ovary, intestine and spleen for *Hox-3.2* expression. The only adult tissue that expressed the *Hox-3.2* gene was kidney, where a homeobox probe detected only the two major transcripts of 1.5 and 1.9 kb (data not shown).

The expression of *Hox-3.2* was analysed in the murine teratocarcinoma cell line F9. F9 EC stem cells can be differentiated *in vitro* into parietal endoderm by treatment with retinoic acid (RA) and dibutyryl cyclic AMP (cAMP). Unlike many other homeobox genes including *Hox-1.3* and *Hox-3.1* (Breier *et al.* 1986; Fibi *et al.* 1988), *Hox-3.2* is neither expressed in F9 stem cells nor in F9 cells differentiated for 1 to 4 days (data not shown).

While RNA of undifferentiated PCC 7 cells contained no detectable *Hox-3.2* transcripts even after prolonged exposure of the Northern blot (data not shown), three *Hox-3.2* transcripts were observed in RNA from PCC 7 cells differentiated either into neuroepithelial-like cells by RA treatment or into neuron-like cells in the presence of RA and cAMP (Fig. 3B) using the *Hox-3.2* homeobox probe. These

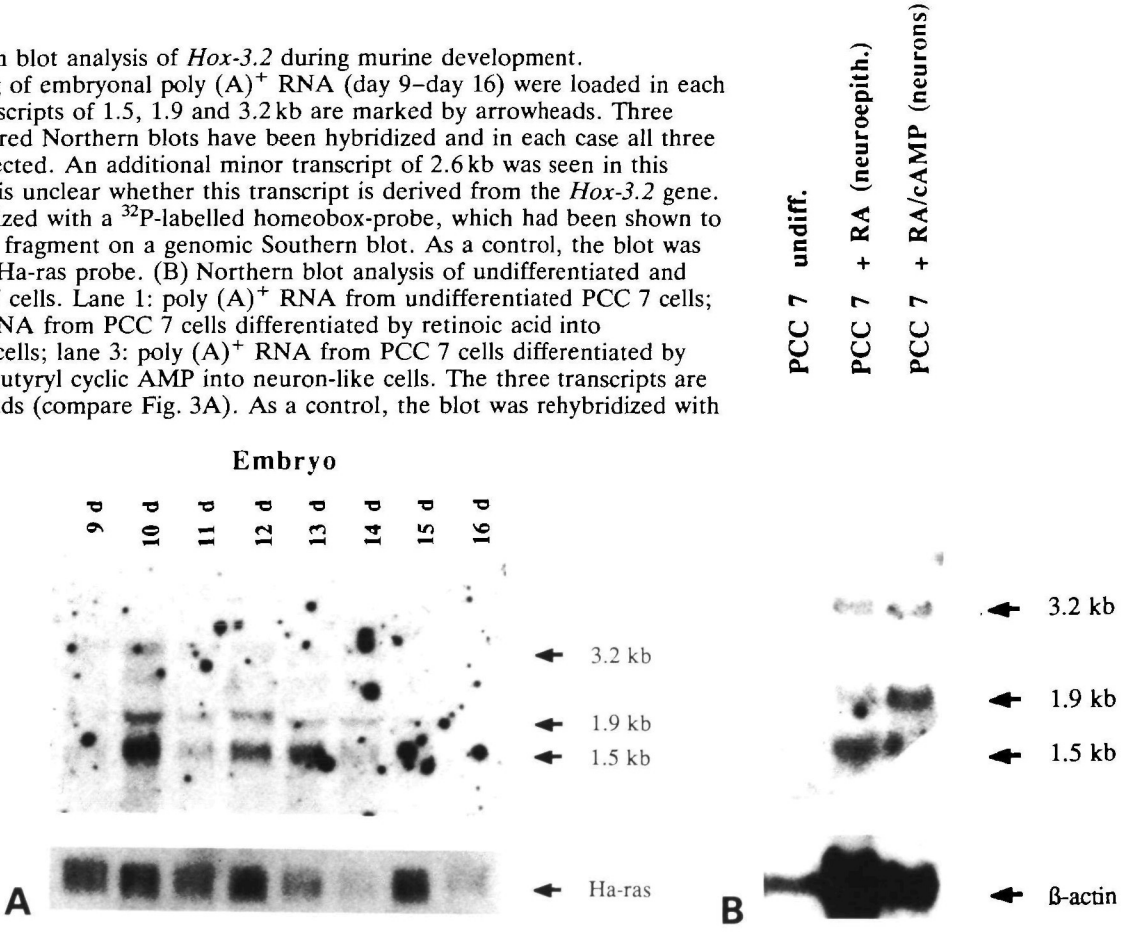



**Fig. 3.** (A) Northern blot analysis of *Hox-3.2* during murine development. Approximately 10 $\mu$g of embryonal poly $(A)^+$ RNA (day 9–day 16) were loaded in each lane. The three transcripts of 1.5, 1.9 and 3.2 kb are marked by arrowheads. Three independently prepared Northern blots have been hybridized and in each case all three transcripts were detected. An additional minor transcript of 2.6 kb was seen in this hybridization and it is unclear whether this transcript is derived from the *Hox-3.2* gene. The blot was hybridized with a $^{32}$P-labelled homeobox-probe, which had been shown to hybridize to a single fragment on a genomic Southern blot. As a control, the blot was rehybridized with a Ha-ras probe. (B) Northern blot analysis of undifferentiated and differentiated PCC 7 cells. Lane 1: poly $(A)^+$ RNA from undifferentiated PCC 7 cells; lane 2: poly $(A)^+$ RNA from PCC 7 cells differentiated by retinoic acid into neuroepithelial-like cells; lane 3: poly $(A)^+$ RNA from PCC 7 cells differentiated by retinoic acid and dibutyryl cyclic AMP into neuron-like cells. The three transcripts are marked by arrowheads (compare Fig. 3A). As a control, the blot was rehybridized with a $\beta$-actin probe.

three transcripts were of the same size as those detected in the mouse embryo. Interestingly, expression of the *Hox-3.2* gene was not seen in PC 12 cells, which can be also differentiated into neuron-like cells in the presence of NGF (data not shown).

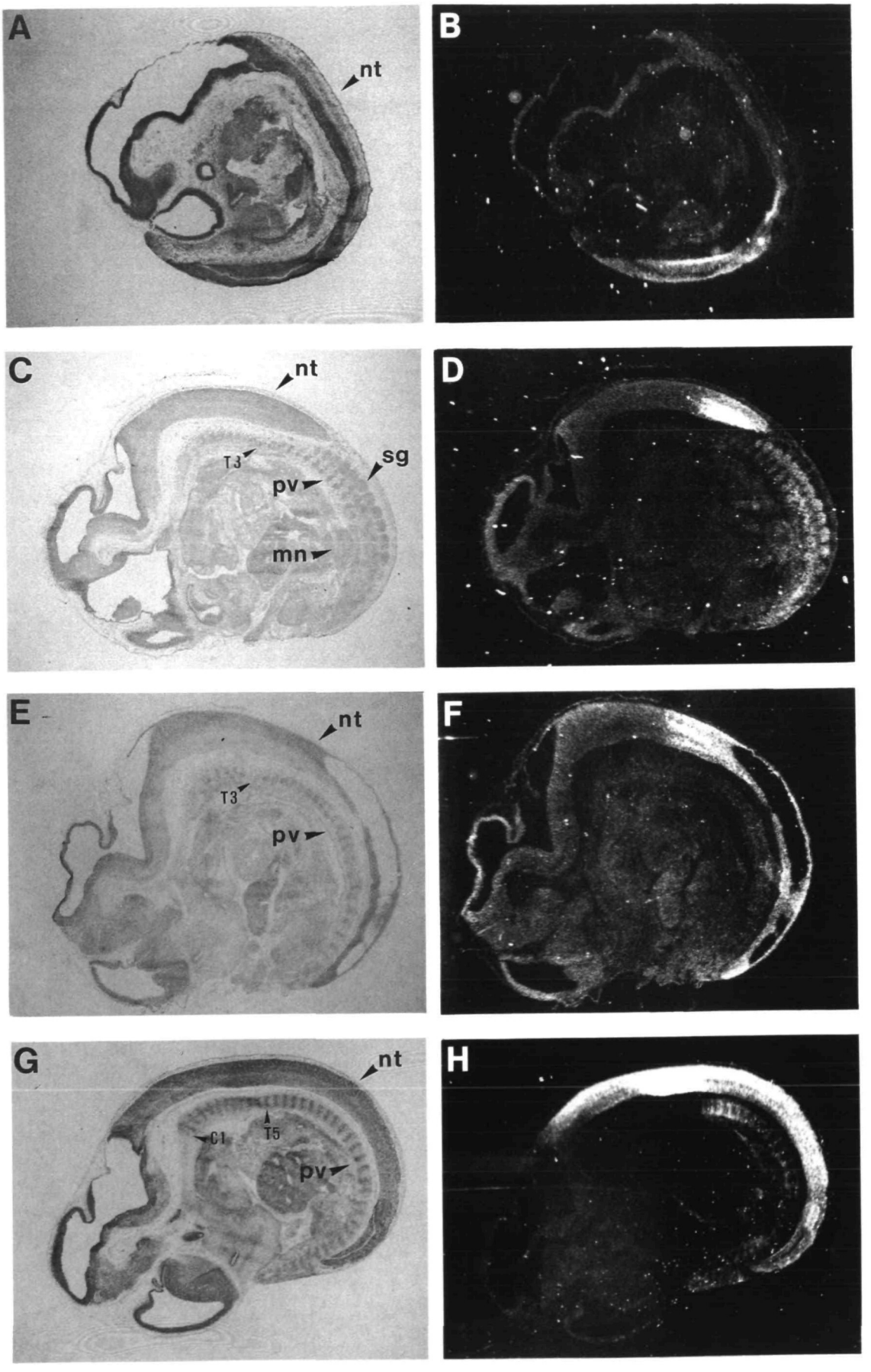
### In situ *hybridization*

In order to identify the spatial pattern of *Hox-3.2* expression during embryogenesis, cryosections of day 8.5 to 16.5 p.c. embryos were hybridized with $^{35}$S-labelled RNA probes generated from linearized plasmid containing the first 361 bp of the *Hox-3.2* cDNA (probe 1) or nucleotides 637–905 (probe 2). The two probes detected the same expression pattern, although probe 1 gave stronger signals. The specificity of the described results was controlled with *Hox-3.2* sense probes which did not detect any specific signal (data not shown).

Expression was first detectable in 8.5 day embryos (Fig. 5A/B). At this stage the neural plate is still open throughout the posterior part of the embryo and consists only of undifferentiated neuroepithelial cells. There is little, if any, expression visible in cells of the neural plate, but a signal was seen at the base of the allantois.

By 10.5 days of development the neural folds have closed and consists mainly of a ventricular layer containing mitotically active cells. It is in the ventral region that the first cells start to migrate out into the newly formed mantle layer after they have undergone their last mitosis (Nornes and Carry, 1978; Wentworth, 1984*a*,*b*; Altman and Bayer, 1984). Expression of *Hox-3.2* was clearly restricted to this ventral part of the neural tube on transverse sections (Fig. 5C/D) and hybridization of a sagittal section of 10.5 day embryos exhibited the same pattern. Only the ventral part of the neural tube is positive for *Hox-3.2* expression with an anterior boundary at the level of the third thoracic prevertebra (Fig. 4A/B). It is difficult to ascertain whether *Hox-3.2* is expressed in spinal ganglia and

**Fig. 4.** Anterior–posterior boundaries of expression of *Hox-3.2* and *Hox-3.1* in day 10.5 and day 12.5 p.c. mouse embryos. (A,C,E,G) bright-field images; (B,D,F,H) dark-field images of (A,C,E,G). (A,B) Sagittal section of a day 10.5 p.c. embryo hybridized with a *Hox-3.2* antisense RNA probe. (C,D) Parasagittal section of a day 12.5 embryo hybridized with a *Hox-3.2* probe. (E,F) Sagittal section of the same day 12.5 p.c embryo hybridized with a *Hox-3.2* probe. (G,H) Sagittal section of a day 12.5 p.c. embryo hybridized with a *Hox-3.1* probe. mn=metanephros, nt=neural tube, sg=spinal ganglia, pv=prevertebrae. C1=first cervical, T3=third thoracic and T6=sixth thoracic prevertebra. C1 and T3 mark the anterior boundaries of expression of *Hox-3.1* and *Hox-3.2* in the neural tube.


A
nt
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prevertebrae at this time since expression in these structures is very weak; however, it is clearly expressed at day 12.5 in both spinal ganglia and prevertebrae.

In day 12.5 embryos, *Hox-3.2* transcripts were detected in the neural tube (Fig. 4E/F), the spinal ganglia, prevertebrae (Fig. 4C/D) and the proximal part of the hindlimb bud (not shown), while expression in the forelimbs has not been studied. Weak expression was also observed in the area where the metanephric tubules and metanephric ducts develop. As on day 10.5, expression in the neural tube was restricted to the posterior regions of the embryo and the sharp anterior boundary at the level of the third thoracic prevertebra was maintained. Transverse sections demonstrate that the signal in the neural tube was again seen only in postmitotic cells, including the mantle layer, marginal layer and the ventral horns, which contain the motor neurons (Fig. 5E/F). As with day 8.5 and day 10.5 embryos, the ependymal layer was negative for *Hox-3.2* expression. In prevertebrae and spinal ganglia, the signal intensity was much lower than in the neural tube. The anterior boundary of expression in prevertebrae was difficult to define, since it was not as sharp as in the neural tube. Expression was more caudal, starting approximately at the ninth thoracic prevertebra.

By day 14.5 the dorsal horns containing sensory neurons have formed. The lumen of the neural tube is surrounded by a very thin ependymal layer with the neural tube consisting in the main of intermediate and marginal layers. Expression is very strong in these two outer layers of the neural tube, while the ependymal layer remains negative. Interestingly, the dorsal horns were clearly negative for *Hox-3.2* expression and in the ventral horns expression was also considerably reduced compared to the mantle layer (Fig. 5G/H). The anterior boundary of expression was still maintained at the same level as in day 10.5 and day 12.5 embryos (data not shown).

These results were compared with the expression pattern of the next 3′ gene of the *Hox-3* cluster, *Hox-3.1*. In contrast to *Hox-3.2*, *Hox-3.1* is expressed strongly in the dorsal horns at day 14.5 (Fig. 5I/K), while the expression pattern on transverse sections at earlier stages of development is very similar to *Hox-3.2* (Breier *et al.* 1988; Le Mouellic *et al.* 1988). There were also important differences in the expression pattern along the A–P axis, where *Hox-3.1* was expressed up to the first cervical vertebra in spinal cord and in the thoracic prevertebrae T5–T10 (Fig. 4G/H).

As mentioned above, *Hox-3.2* is also expressed in the developing kidney starting at day 12.5. At day 14.5 and 16.5 expression is mainly found in the cortex, whereas in the mesenchyme the signal is considerably reduced (Fig. 6). Another strong positive signal, which could not be located unequivocally, was found close to the developing kidney at day 14.5 and appears to be either the ureter, umbilical cord or part of the genital ridge.

Thus, *Hox-3.2* expression was found in mesoderm- and ectoderm-derived structures, but not in endoderm. Expression is first seen in mesoderm as early as day 8.5 and in ectoderm after the ventral horns start to form in the neural tube at day 9.5 (not shown). Kidney is the only adult organ where expression could be detected.

## Discussion

This study describes the primary structure of the *Hox-3.2* cDNA and the expression pattern of the *Hox-3.2* gene in murine embryogenesis. The *Hox-3.2* gene represents the most 5′ member of the *Hox-3* complex on mouse chromosome 15 identified to date. Although in the *Hox-4 (-5)* cluster the *Hox-4.5 (-5.3)* gene lies approximately 6 kb upstream of the paralog of *Hox-3.2*, namely *Hox-4.4 (-5.2)* (Duboule and Dollé, 1989), low stringency hybridization did not reveal any conserved homeobox sequence within the next 20 kb upstream of the *Hox-3.2* gene, while the paralog of *Hox-4.5 (-5.3)* in the *Hox-1* cluster, *Hox-1.8* was identified under the same conditions (Haack *et al.* in preparation). Thus, it seems that the paralogs of *Hox-1.8* and *-4.5 (-5.3)* appear to be absent from both the *Hox-3* and *Hox-2* clusters (Graham *et al.* 1989; this study). In this respect, the organization of the murine *Hox* gene clusters is very similar to the situation observed in human (Acampora *et al.* 1989), where only the human homologs of *Hox-1.8* and *Hox-4.5 (-5.3)* are present. Further upstream two additional homeobox genes have been described in the human *Hox-3* cluster suggesting that homologous genes are also present in mouse (Acampora *et al.* 1989).

Recently, attention has been drawn to the fact that the position of a gene within each cluster correlates with its anterior border of expression in the developing embryo (Gaunt *et al.* 1988; Duboule and Dollé, 1989; Graham *et al.* 1989; Dressler and Gruss, 1989). Thus, the more 5′ the position of a gene in the cluster the more posterior its domain of expression in the embryo. This is very similar to the genes of the *Antp* cluster in *Drosophila*, where genes at the 5′ end of the cluster are also expressed more posteriorly (Akam, 1989). The prediction of such a scenario is a posterior pattern of expression for the *Hox-3.2* gene. Our results, showing expression of *Hox-3.2* in the CNS posterior to the level of the third thoracic prevertebra (T3) and in sclerotomes posterior to T9, confirm this suggestion.

### *The* Hox-3.2 *protein contains no hexapeptide*

Analysis of the predicted *Hox-3.2* protein of 260 amino acids revealed a number of interesting features. As with the majority of homeodomain-containing proteins characterized to date, the homeodomain is situated close to the carboxyl terminus. Of the nine amino acids 3′ to the homeodomain, six are highly charged and may also contribute to an interaction with the target DNA. A further highly conserved domain of eleven amino acids is found immediately upstream of the homeodomain in the *Hox-3.2*, *-1.7*, *-2.5* and *XlHbox6* (Sharpe *et al.* 1987) genes but not the *Drosophila* counterpart *Abd-B* (Celniker *et al.* 1989). This domain and the homeodomain are encoded by the second exon of the *Hox 3.2* gene with the 5′ most amino acid forming the intron–exon junction. Outside of this extended

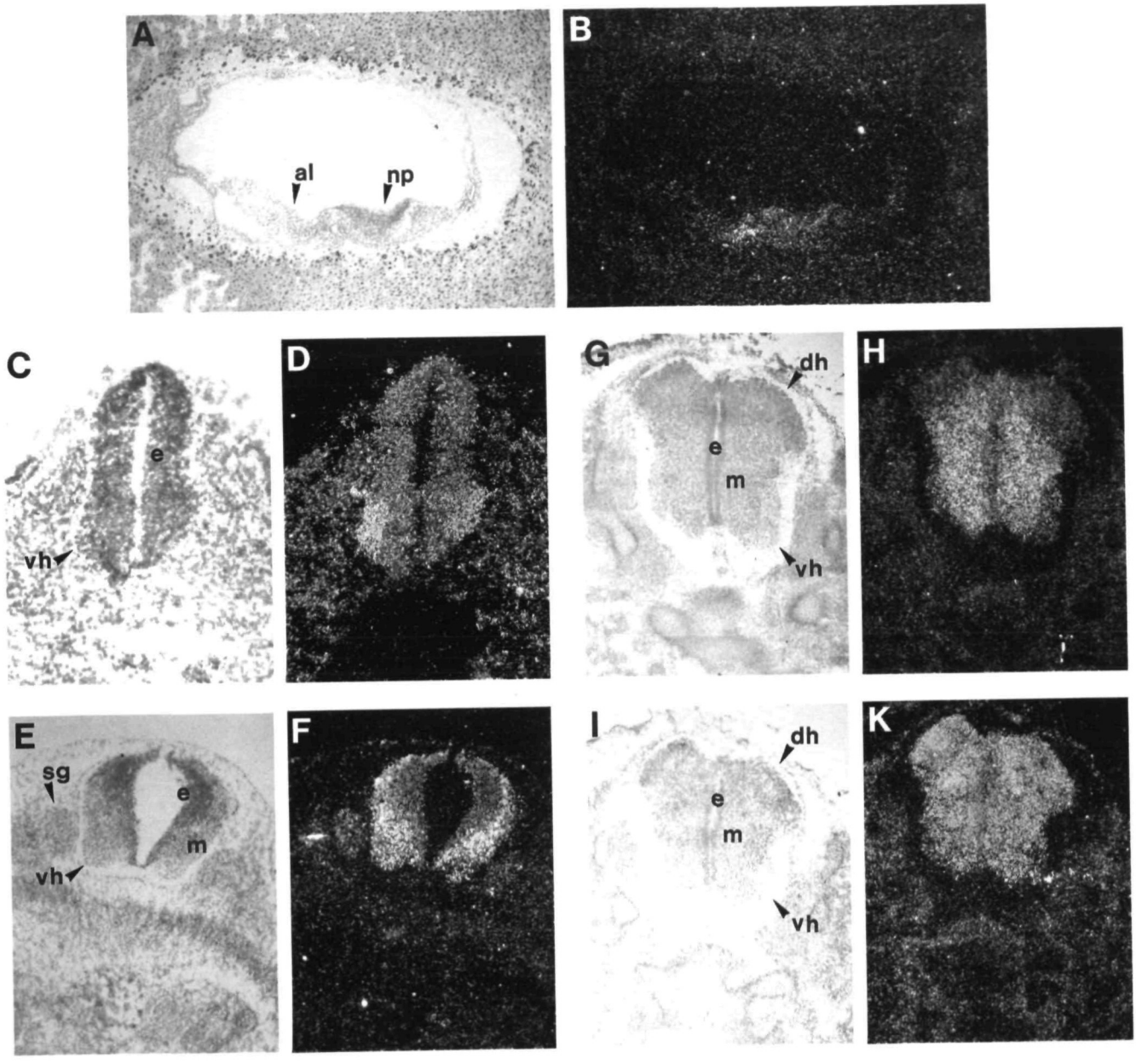


**Fig. 5.** *In situ* hybridization of *Hox-3.2* (A-H) and *Hox-3.1* (I,K) to transverse sections of day 8.5 to day 14.5 p.c. embryos. (A,C,E,G,I) Bright-field images; (B,D,F,H,K) dark-field images of (A,C,E,G,I). (A,B) Section through the posterior part of a day 8.5 p.c. embryo showing allantois and neural plate. (C,D) Section through a day 10.5 p.c. embryo at the level of the hindlimb buds. Formation of the ventral horns has started. (E,F) Section through a day 12.5 p.c. at the level of the 6th to 8th thoracic prevertebra. The ventral horns (containing motor neurons) have formed and the neural tube consists of a still large ependymal layer and the mantle layer. (G,H) Section through a day 14.5 p.c. embryo at the level of the 12th–13th thoracic prevertebra. The ependymal layer is reduced to a very thin layer of cells. The dorsal horns (containing sensory neurons) have formed. (I,K) Nearby section to (G,H) hybridized with a *Hox-3.1* probe. al=allantois, dh=dorsal horns, e=ependymal layer, m=mantle layer, np=neural plate, sg=spinal ganglia, vh=ventral horns.

domain, there appears to be little homology between *Hox-3.2* and its paralogs in mouse and other species. Unfortunately, the entire coding regions of the paralogs have not been reported, precluding a detailed analysis of regions outside these two domains. Nevertheless, the first 13 amino acids of *Hox-1.7* and 50 amino acids of *Hox-2.5* 5′ of the splice site as well as the amino terminal part of *XlHbox6* show no extended conservation. This restricted homology seems to be typical for homologues of the *Drosophila Abd-B* gene, since most murine homologues of other *Drosophila* genes show homologies also in regions outside the homeodomain (Akam, 1989). Interestingly, *Hox-3.2* and the paralogous genes including *XlHbox6* and *Abd-B* contain no hexapeptide, a second highly conserved region besides the homeodomain, encoded by the exon preceding the homeodomain and present in most mouse homeodomain proteins. Other regions homologous between homeodomain proteins are often found at the amino terminus (Kessel *et al.* 1988) and the carboxy terminus, where many homeodomain proteins contain homopolymeric stretches of a single amino acid, e.g. glutamic acid in the *Hox-3.1* protein (Breier *et al.* 1988), glutamine in the *Antennapedia* protein (Schneuwly *et al.* 1986) or alanine in the *even-skipped* protein (MacDonald *et al.* 1986) as well as its murine counterpart *Evx-1* (Bastian and Gruss, 1990). Similar homopolymeric stretches do not occur in the *Hox-3.2* protein. Although the presence of a homeodomain is consistent with *Hox-3.2* functioning as a transcription factor, it is unclear in what context it might act. No sequences similar to strongly activating regions such as acidic or glutamine-rich regions are found in the protein like in many proteins known to activate transcription (Courey *et al.* 1989).

### *Expression in EC cells*

*In vitro* differentiation of F9 EC stem cells by retinoic acid (RA) and dibutyryl cyclic AMP (cAMP) resembles in part the development of the preimplantation embryos between 4 and 5 days of gestation (Martin, 1980; Hogan *et al.* 1981; Strickland, 1981). Most murine homeobox genes can be induced during differentiation of F9 EC stem cells by retinoic acid. Moreover, the genes of the human *Hox-2* cluster are differentially

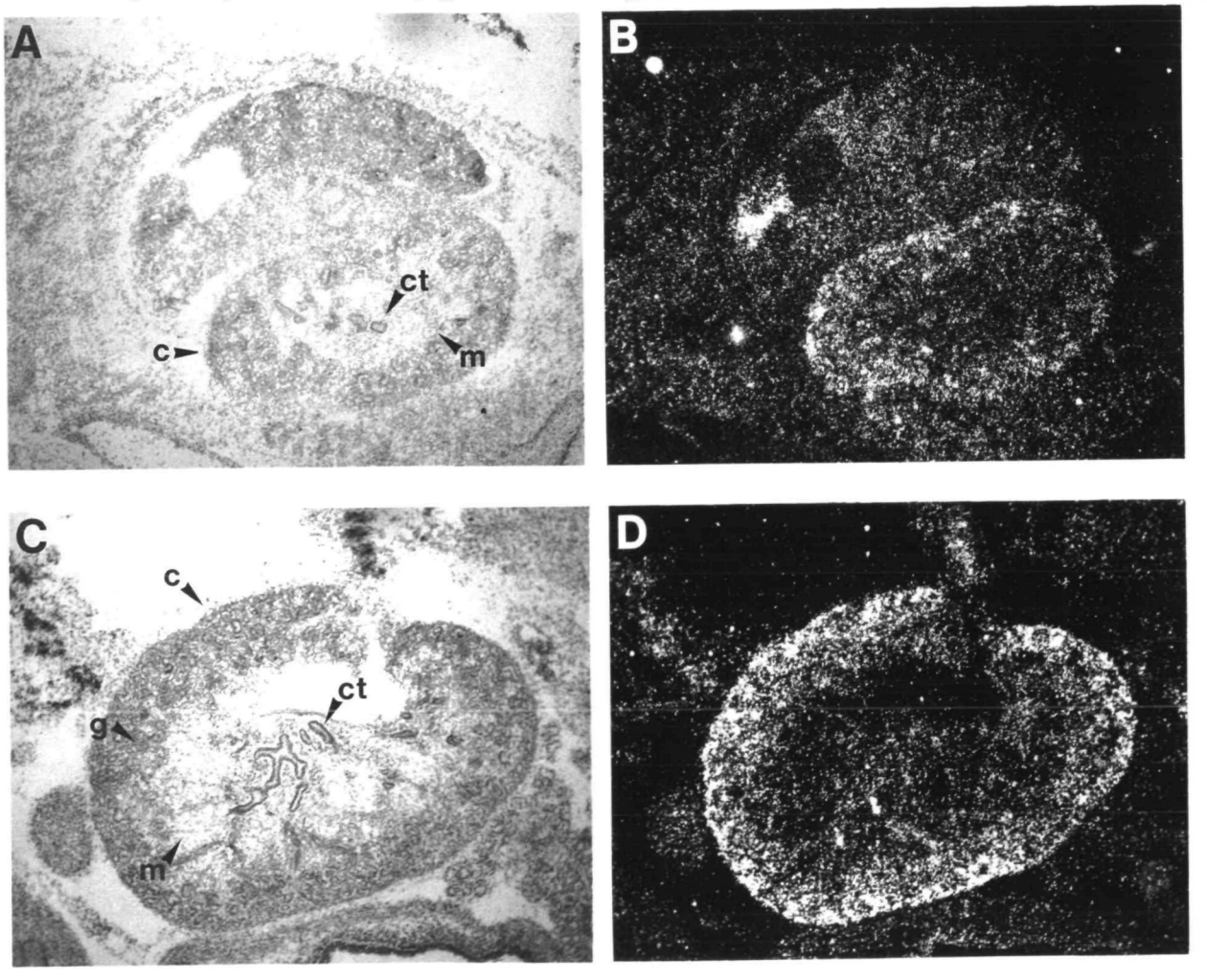


**Fig. 6.** Expression of *Hox-3.2* in the developing mouse kidney. (A,C) Bright-field image; (B,D) dark-field image of (A,C). (A.B) Section through the developing kidney of a day 14.5 p.c. embryo. (C,D) Section through a 16.5 day p.c. kidney. c=cortex, ct=collecting tubules, g=glomerulus, m=mesenchyme.

activated by RA in the EC cell line NT2/D1 depending on their position in the cluster (Simeone *et al.* 1990), with genes at the 5′ end requiring much higher levels of RA (up to $10^{-5}$ M) for activation than genes in the 3′ part of the cluster ($10^{-8}$ M). Although comparable experiments have not been done for F9 cells, it is interesting that the *Hox-3.2* gene lying in the 5′ region of the cluster is not induced during differentiation of F9 cells, whereas the next 3′ gene, *Hox-3.1*, is activated by RA levels of $5\times10^{-7}$ M in combination with $10^{-3}$ M cAMP (Breier *et al.* 1986). The same is true for the *Hox-1.7* gene, which cannot be induced (Rubin *et al.* 1987) by retinoic acid while the next 3′ member of the cluster *Hox-1.1* is known to be expressed during retinoic-acid-induced F9 cell differentiation into parietal endoderm (Colberg-Poley *et al.* 1986).

It is worth noting that *Hox-3.2* can be induced by retinoic acid in another embryonic carcinoma cell line PCC 7. These cells can be differentiated either into neuroepithelial-like cells by RA or neuron-like cells by RA and cAMP. Both differentiation procedures activate *Hox-3.2*. Thus, although *Hox-3.2* does not respond to RA during differentiation of F9 cells into parietal endoderm, differentiation of PCC7 cells into 'ectodermal' cells induces *Hox-3.2* expression, showing that only one of two retinoic-acid-mediated differentiation pathways involves activation of *Hox-3.2*.

*Temporally and spatially restricted expression during embryogenesis*

Consistent with a role in pattern formation, the *Hox-3.2* gene exhibits a temporally and spatially restricted pattern of expression during embryogenesis. Quantitation by Northern hybridization revealed high levels of *Hox-3.2* expression in mouse embryos from day 10 to 13 of gestation, whereas *Hox-3.2* transcripts are absent in most tissues of the adult organism with the exception of kidney. Although, *Hox-3.2* expression decreases at later stages in gestation, these analyses can be somewhat misleading, since at day 16.5, *Hox-3.2* expression is confined to the spinal cord and cells in the cortex of the developing kidney. Consequently, the very localized expression of *Hox-3.2* in these structures would account for the low levels of transcripts in Northerns of whole embryo RNA.

Many of the known murine homeobox genes encode multiple transcripts during embryogenesis (Odenwald *et al.* 1987; Rubin *et al.* 1987; Bogarad *et al.* 1989). While three transcripts of *Hox-3.2* are found in the developing embryo, only the two major transcripts of 1.5 and 1.9 kb are found in the adult kidney. These tissue-specific differences are also seen with other homeobox genes, e.g. *Hox-2.6*, where the larger 4.1 kb transcript is present in embryo but not in lung, while the 2.4 kb transcript is found in both (Graham *et al.* 1989). It is possible that alternative splicing plays an important part in tissue-specific regulation of many of the vertebrate *Hox* genes.

Delineation of the precise pattern of *Hox-3.2* expression in the mouse embryo was undertaken by *in situ* analysis. Transcripts were first detected at day 8.5 of gestation in the posterior part of the embryo at the base of the allantois. From day 10 onward, expression of *Hox-3.2* was observed in the ventral region of the neural tube, in postmitotic cells that have detached from the lumen of the neural tube and are migrating radially from the ventricular zone. It is here in the ventral horns that the motor neurons are born between day 9 and day 11, followed by relay neurons between day 10 and day 12 and finally sensory neurons in the dorsal horns between day 12.5 and day 14.5 (Wentworth, 1984*a*,*b*; Altman and Bayer, 1984). This pattern of day 10.5 embryos is reflected again in day 12.5 embryos, where expression of *Hox-3.2* is found predominantly in the intermediate zone. This layer of cells consists mainly of migrating and differentiating neuroblasts. Expression also appears in more dorsal regions of the neural tube in cells that will eventually settle in the intermediate grey of the adult spinal cord. This expression pattern persists in the day 14 to day 15 embryos with the dorsal funiculus and dorsal horns showing little or no expression of *Hox-3.2* at a time when the dorsal horn neurons are being generated (Altman and Bayer, 1984). Very little expression is observed in the ventral and lateral funiculi at day 14 to day 15. Expression of *Hox-3.2* throughout neurogenesis exhibits a predominance of expression in ventral regions of the neural tube but it seems unlikely that this pattern merely reflects the earlier maturation of neurons in the ventral neural tube. While expression in the ventral part of the neural tube at day 10.5 and the ventral and lateral regions of the neural tube at day 12.5 coincides with the birth of motor and relay neurons, at later times this expression persists and is absent from regions that will give rise to sensory interneurons. Compared to *Hox-3.1* (Fig. 5I/K), *Hox-3.2* shows a more restricted pattern of expression in the neural tube at day 14.5. *Hox-3.1* at this stage is expressed throughout the intermediate zone in the day 14.5 neural tube including the region of the dorsal horns and substantia gelatinosa. Thus, although *Hox-3.2* transcripts seem to be restricted to postmitotic cells, it is presently unclear what role *Hox-3.2* might play in the differentiation and projection of neurons during neuronal development. Unlike members of the *Pax* gene family (Nornes *et al.* 1990) and *Evx-1* (Bastian and Gruss, 1990), which are expressed in transversely restricted regions, *Hox-3.2* is expressed over a wide transverse region within caudal regions of the neural tube. In this respect, *Hox-3.2* expression in the neural tube appears more consistent with a role in specifying position than neuronal differentiation per se.

Transcripts of *Hox-3.2* are not detected along the entire A–P axis in the neural tube but are restricted to the posterior part caudal to the level of the third thoracic prevertebra. Low expression is seen in the same region in spinal ganglia. In contrast, expression of the next 3′ gene *Hox-3.1* is more restricted in spinal ganglia to levels of T4 to T9 (Breier *et al.* 1988; Le Mouellic *et al.* 1988). On the other hand, the expression domain in the neural tube is much more extended. In this study, using an improved *in situ* hybridization

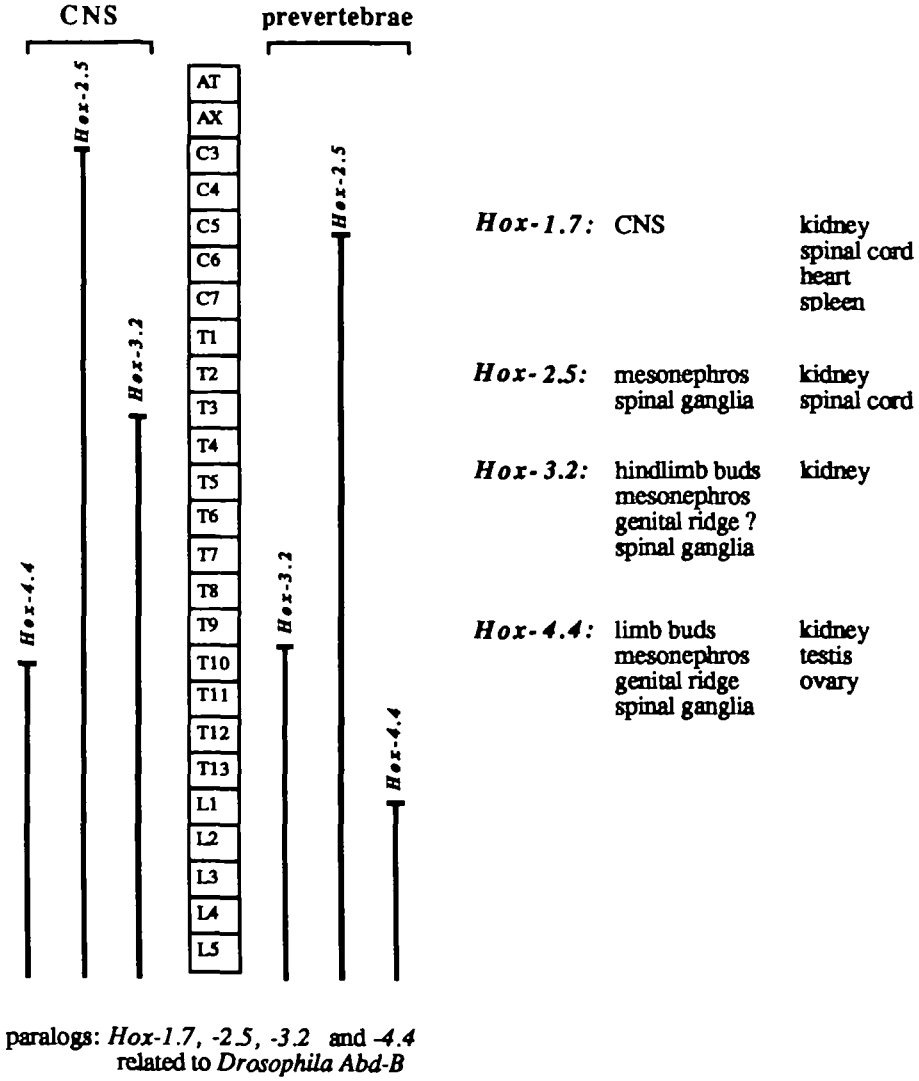


**Fig. 7.** Comparison of the expression pattern of *Hox-3.2* and its paralogs *Hox-1.7*, *Hox-2.5* and *Hox-4.4 (-5.2)*. Expression domains and anterior boundaries of expression in the neural tube and prevertebrae are shown on the left side of the figure. On the right side the expression in developing organs during embryogenesis and in adult mouse tissues are shown (for references see Results).

protocol, transcripts were detectable in the neural tube up to the first cervical prevertebra (Fig. 4G/H), which extends the previously published results to the anterior side (Awgulewitsch *et al.* 1986; Breier *et al.* 1988; Le Mouellic *et al.* 1988). Recently, *Hox-3.1* protein expression was shown to be restricted in the neural tube caudal to the 4th prevertebra (Awgulewitsch and Jacobs, 1990).

As mentioned above, expression of *Hox-3.2* in the mouse starts at 8.5 days of gestation in the region of the allantois. Expression in prevertebrae is best seen at day 12.5 caudal to the 9th thoracic prevertebra but is significantly lower than in the neural tube. This situation is comparable with other homeobox genes, where usually the expression in mesoderm-derived structures is restricted to more posterior domains than in ectoderm-derived structures. However, the relative levels of expression of the *Hox* genes in neuroectoderm and mesoderm varies slightly. For instance *Hox-1.1* expression is stronger in somitic mesoderm than neuroectoderm (Püschel *et al.* 1990). From day 8.5 till day 12.5, it appears that besides the prevertebrae the entire very posterior tail region is weakly positive for *Hox-3.2* expression, a pattern which is also seen with *Hox-3.1*. In the developing kidney transcripts are first seen at day 12.5 in metanephros and at later stages mainly in the cortex. Kidney is also the only adult tissue that expresses all murine paralogs of *Hox-3.2* (Fig. 7).

### *Comparison of the* Hox-3.2 *expression pattern with other homeobox genes*

Two interesting conclusions can be drawn by comparison of the *Hox-3.2* expression pattern with the patterns of other homeobox genes. First, the domains of expression of the paralogous genes in the four clusters, *Hox-1.7*, *-2.5*, *-3.2* and *-4.4 (-5.2)* are substantially different in the CNS and prevertebral column (summarized in Fig. 7). This differs from the findings for *Hox-1.4*, *-2.6*, and *-4.2 (-5.1)* as well as *Hox-1.3*, *-2.1* and *-3.4*, which show not identical but very similar anterior boundaries of expression in the CNS and prevertebral column (Gaunt *et al.* 1989; 1990). Thus, as already suggested by Gaunt and coworkers, paralogs at the 5′ end of the clusters and therefore expressed more caudal in the embryo, may not show similar transcript distributions along the anteroposterior axis.

The second interesting observation is obtained by comparing the expression patterns of homeobox-genes within their clusters. The dorsoventral expression pattern on transverse sections through the neural tube seems to be very similar for genes within the *Hox-2* cluster (Holland and Hogan, 1988; Shughart *et al.* 1988; Graham *et al.* 1988; Bogarad *et al.* 1989). This holds true for the *Hox-3* cluster if one compares *Hox-3.1, Hox-3.2* and *Hox-3.4* at day 12.5 p.c. They all show a similar transcript distribution in transverse sections, where *Hox-3.1* and *Hox-3.4* are very strongly expressed in the mantle layer but considerably weaker in the ependymal layer (Awgulewitsch *et al.* 1986; Breier *et al.* 1988; LeMouellic *et al.* 1988; Gaunt *et al.* 1990). At day 14.5 of gestation, however, *Hox-3.1* and *Hox-3.2* show unique patterns of expression. *Hox-3.1* is expressed abundantly in the dorsal horns of the neural tube, whereas *Hox-3.2* is not or only very weakly expressed in this region. Hence, genes within one cluster do not necessarily show an identical dorsoventral distribution of transcripts. In this respect, it will be very interesting to see which expression pattern *Hox-3.4* and *Hox-3.3* (first described as *Hox-6.1*, Sharpe *et al.* 1988) exhibit at day 14.5 of gestation.

In summary, *Hox-3.2* shows a restricted expression pattern in the posterior part of the developing embryo. Apart from the neural tube, where expression seems to be restricted to postmitotic cells, transcripts are detected in spinal ganglia, prevertebrae and the developing kidney. These results are in agreement with the finding that genes located 5′ in the homeobox gene clusters are expressed more posterior than genes from the 3′ region. *Hox-3.2* belongs to the *Hox-1.7* subfamily. None of the homeobox gene subfamilies described to date vary as drastically in the anteroposterior transcript distribution in CNS and prevertebrae as *Hox-1.7* and its paralogs. Furthermore this subfamily shows some other striking similarities, i.e. highest homology to the *Drosophila Abd-B* gene, lack of the hexapeptide and noninducibility by RA in F9 EC cells. This raises the question of whether this is a unique feature for genes in the 5′ region of the *Hox* clusters. Several genes 5′ to the *Hox-1.7* subfamily have already been isolated in all four clusters either in human or in mouse (for review see Kessel and Gruss, 1990*b*). Thus it will be soon possible to elucidate whether these genes show similar characteristics and like *Hox-3.2* and its paralogs, differ from genes located in the 3′ region of the clusters.

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