The EMBO Journal vol.9 no.6 pp.1839–1852, 1990

A murine *even-skipped* homologue, *Evx 1*, is expressed during early embryogenesis and neurogenesis in a biphasic manner

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Communicated by P.Gruss

Using the *Drosophila even-skipped (eve)* homeobox as a probe, we have isolated two murine genes, *Evx 1* and *Evx 2*, from a genomic library. *Evx 1, Evx 2, eve* and the Xenopus *Xhox-3* constitute a family of related genes based on similar homeodomain sequences. In addition, *Evx 1* and *Evx 2* share extended amino acid conservation outside of the homeobox. The *Evx 1* protein consists of 416 amino acids as deduced from the longest open reading frame of *Evx 1* cDNAs. *Evx 1* is located 3.7 cM from the Hox 5 locus on mouse chromosome 2. It is expressed in undifferentiated F9 stem cells but not in cells differentiated with retinoic acid and cAMP. During embryogenesis, *Evx 1* shows a biphasic expression pattern. From days 7 to 9 p.c. *Evx 1* expression emerges at the posterior end of the embryo within the primitive ectoderm, and later in the mesoderm and neuroectoderm. From days 10 to 12.5 p.c. *Evx 1* transcripts are restricted to specific cells within the neural tube and hindbrain along their entire lengths and coincides temporally, as well as spatially, with maturation of early forming interneurons, possibly commissural interneurons. The early and late transcription pattern is compatible with a role of *Evx 1* in specifying posterior positional information along the embryonic axis similar to the *Xenopus Xhox-3* and in specifying neuronal cell fates within the differentiating neural tube in analogy to *eve* in the embryonic central nervous system of *Drosophila*, respectively.

Key words: even-skipped/homeobox/primitive streak stage/neurogenesis/mouse embryogenesis

Introduction

The identification of developmental control genes in *Drosophila* laid the foundation for investigating the molecular mechanisms of embryonic development. By interacting in a hierarchical and combinatorial manner, many of these genes, classified as maternal effect, gap, pair-rule, segment polarity and segment identity genes, establish the metameric body plan from a fertilized egg (see Akan, 1987; Ingham, 1988 for reviews). A large number of their gene products share protein domains such as the zinc-finger motif (Rosenberg *et al.*, 1986; Tautz *et al.*, 1987), the paired box (Bopp *et al.*, 1986) and the homeobox (McGinnis *et al.*, 1984; Scott and Weiner, 1984). *Drosophila* homeoboxes belong to different classes known as *Antennapedia (Antp), bicoid (bcd), caudal (cad), engrailed (en), even-skipped (eve), muscle segment (msh)* and *paired (prd)* type homeoboxes based on their relative degrees of amino acid and nucleic acid homology (see Scott *et al.*, 1989 for review; Robert *et al.*, 1989). On the molecular level homeobox proteins bind DNA and activate or repress transcription *in vitro,* suggesting that they also control development at the transcriptional level in the organism (Hoey and Levine, 1988; Hoey *et al.*, 1988; Biggin and Tjian, 1989: Dearolf *et al.*, 1989).

In contrast to *Drosophila*, little is known about the molecular mechanisms controlling vertebrate morphogenesis. Zinc finger (see Klug and Rhodes, 1987 for review), paired box (Deutsch *et al.*, 1988; Dressler *et al.*, 1988; Burri *et al.*, 1989; Dressler *et al.*, 1990; Walther, Goulding and Gruss in preparation) and homeobox (see Dressler and Gruss, 1988; Holland and Hogan, 1988; Scott *et al.*, 1989; Wright *et al.*, 1989 for reviews; Duprey *et al.*, 1989; Robert *et al.*, 1989; Goulding, Walther, and Gruss, in preparation) genes with high sequence homology to the respective *Drosophila* conserved domains have also been identified in the genomes of chick, frog, mouse and man and show specific expression patterns during embryogenesis. *En*-homeobox genes, for example, are expressed during neurogenesis in species of different phyla (Patel *et al.*, 1989b). Mouse *Antp* type homeobox genes show striking similarities to the genes of the *Drosophila Antennapedia* (*ANT-C*) and *Bithorax* (*BX-C*) complexes with respect to their clustered organization and their expression patterns along the antero-posterior axis, suggesting that some roles of these genes during development have been conserved (Gaunt *et al.*, 1988; Duboule and Dollé, 1989; Graham *et al.*, 1989). Studies in *Xenopus* and mouse support that homologues of *Drosophila* regulatory genes represent tools to unravel molecular mechanisms of vertebrate development. Manipulation of the expression pattern of certain homeobox genes could be correlated with specific developmental abnormalities (Harvey and Melton, 1988; Balling *et al.*, 1989; Kessel *et al.*, 1990; Ruiz i Altaba and Melton, 1989b,c; Wolgemouth *et al.*, 1989). Also, in the mouse, a point mutation in the paired box of the *Pax 1* gene is likely to cause the developmental mutant phenotype *undulated* (Balling *et al.*, 1988).

Until now, murine homologues from all *Drosophila* homeobox classes have been described, with the exception of *bcd* and *eve* type homeoboxes. In *Drosophila, eve* shows two distinct expression patterns during development. As a primary pair rule gene *eve* plays a key role in the regulation of the segmentation process since complete loss of *eve* function causes an asegmental phenotype (Nüsslein-Volhard *et al.*, 1985). *Eve* is involved in transforming the broad spatial, aperiodic expression patterns of the gap genes into a system of precise periodic expression patterns of the pair rule and segment polarity genes. The periodic seven stripe expression ('pair rule') pattern of *eve* provides the earliest periodic values in the developing embryo and thus the basis for the metameric organization of the body plan (Frasch *et al.*, 1988; Hoey and Levine, 1988; Hoey *et al.*, 1988; In-

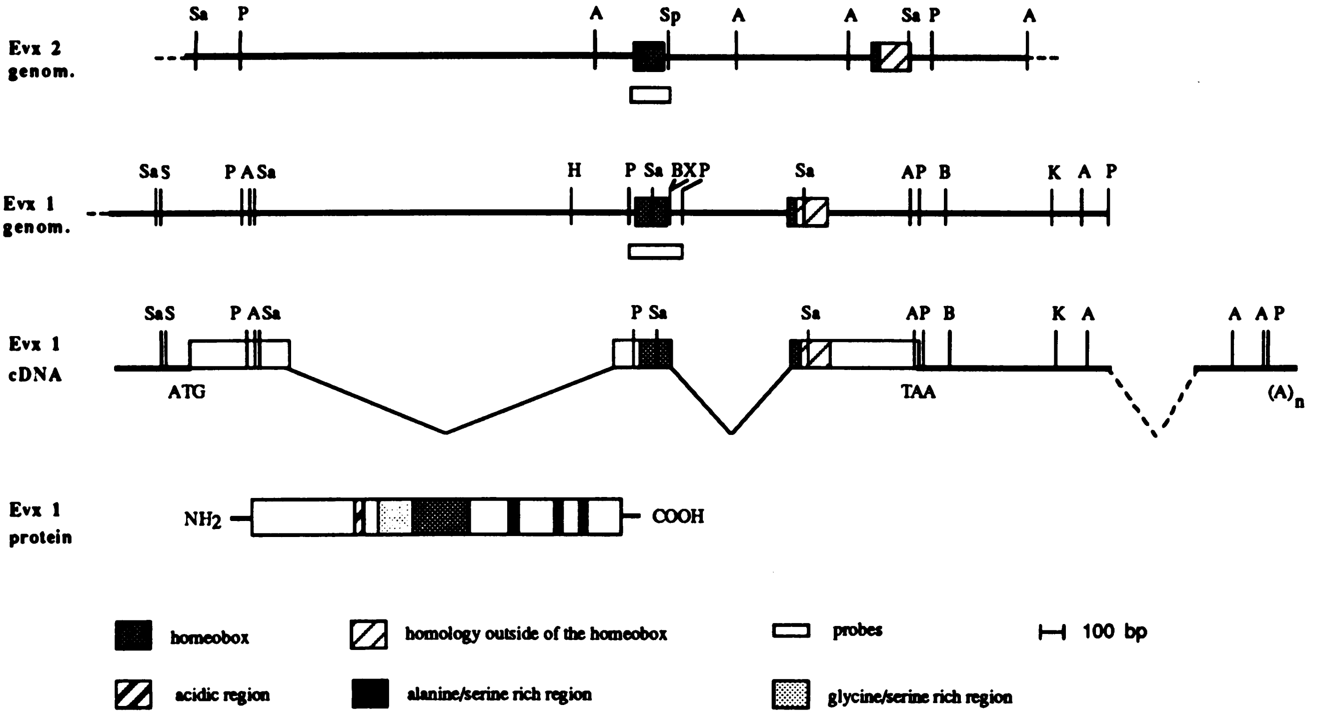

Fig. 1. Genomic organization of *Evx 1* and *Evx 2* of the regions containing the homeoboxes, cDNA map and predicted protein structure of *Evx 1*. Both genes are orientated with respect to transcription from 5′ to 3′. In *Evx 2* additional *Pst*I and *Sac*II sites are present near the indicated positions. Probes for Southern, Northern and *in situ* analysis are generated from either a 165 bp *Hae*II–*Sph*I fragment of *Evx 2* or from a 226 bp *Pst*I–*Pst*I fragment of *Evx 1*. Only the coding region of the cDNA is boxed. Abbreviations for restriction enzymes: A, *Ava*II; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*II; Sp, *Sph*I; X, *Xho*I.

gham *et al.*, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989). During neurogenesis, *eve* is expressed in distinct neurons of the embryonic central nervous system (CNS) (MacDonald *et al.*, 1986; Frasch *et al.*, 1987). *Eve*$^-$ mutants show neuronal transformations and aberrant axon morphologies (Doe *et al.*, 1988).

Here we describe the isolation of two distinct murine *eve*-related genes which we called *Evx 1* and *Evx 2* (*ev*en-skipped homeob*ox*). *Evx 1* and *Evx 2* share extended sequence homology immediately up- and downstream of the homeobox. *Evx 1* maps to mouse chromosome 2. *Evx 1* transcripts disappear after differentiation of F9 stem cells and thus are regulated differently than most other murine homeobox genes. During embryogenesis, *Evx 1* shows a biphasic expression pattern. In early post-implantation stages, *Evx 1* transcripts are detected in the primitive ectoderm, neuroectoderm and mesoderm at the posterior end of the embryo. Later in development, *Evx 1* expression is restricted to subsets of cells within the developing neural tube along its entire axis. The distribution patterns of *Evx 1* transcripts indicate a function of *Evx 1* in specifying posterior positional information at the primitive streak stage and neuronal identities during early neuroblast differentiation.

Results

Isolation of murine eve homologous genes

In order to isolate mouse genes related to the *Drosophila eve* homeobox gene, a 210 bp *Hin*fI–*Bst*NI fragment of the *Drosophila* coding sequence, containing the homeobox, was used to screen a genomic mouse liver library (Clontech). Two recombinant λ clones were identified each containing an *eve* type homeobox and covering ~15 kb of genomic sequence. Restriction enzyme analysis indicates that two different genes have been isolated which we called *Evx 1* and *Evx 2* (Figure 1). The homeobox probes hybridize to unique bands on genomic Southern blots, further excluding cross-hybridization to each other (not shown). A 226 bp *Pst*I–*Pst*I fragment of the *Evx 1* clone was then used to screen a day 8.5 p.c. embryonic cDNA library (Fahrner *et al.*, 1987). Eight overlapping clones with insert sizes of 2–3 kb could be isolated.

Both Evx homeoboxes are interrupted by introns of 543 bp (*Evx 1*) and 925 bp (*Evx 2*), respectively, just within the recognition helix between amino acid positions 46 and 47 (Figures 1, 2A and 3). Splice junction sites are nearly identical to the consensus sequences defined by Shapiro and Senapathy (1987). The genes isolated here are quite distinct from all other known mouse homeoboxes. Together with the *Xenopus Xhox-3* and the *Drosophila eve* gene they constitute a separate homeobox subfamily (Figures 2A and 3). The *Evx 1* homeodomain is identical to the published *Xhox-3* sequence on the amino acid level, whereas *Evx 2* differs only in the first position, where a valine substitutes a methionine (Figures 2A and 3). Of the nucleotides, 82.5% are identical between *Evx 1* and *Evx 2* (Figure 2). *Evx 1* and *Evx 2* share 76.5 and 78.7% homology with the *Drosophila eve* homeobox on the nucleic acid and 86.9 and 88.5% on the amino acid level. Four out of eight (*Evx1*) or seven (*Evx 2*) amino acid exchanges are conservative according to Schwartz and Dayhoff (1979) (Figures 2 and 3). Positions which are nearly invariant in all known homeobox classes (Scott *et al.*, 1989) are also highly conserved in *Evx 1* and *Evx 2* (Figures 2 and 3).

Both mouse genes share homologies in the regions immediately upstream and downstream of the homeobox: 73%

```
A
Evx 1    ATG CGC CGA TAC CGC ACG GCC TTT ACC CGG GAG CAG ATT GCA AGG CTG GAG AAA GAG TTC TAC AGG GAG AAC TAC GTT TCA AGA CCG CGG AGA
Evx 2    G-- --G --- --- --T --- --A --C --- --C --A --- --C --G C-C --- --- --- --- --- --- --- --- --- --- --G --T C-G --C --C C-G
eve      G-A --- --C --T --- --C --- --C --- --T --C --- C-G -GT C-C T-- --- --G --- --- --- -A- --- --- --- --G --C C-T --C --T C-C

Evx 1    TGC GAA CTG GCA GCA GCC TTA AAC CTT CCT GAA ACT ACC ATC AAG GTG TGG TTT CAG AAC CGG CGC ATG AAG GAC AAG CGT CAG CGG CTG GCC
Evx 2    --- --G --- --C --T --A C-C --- --G --C --- --C --- --- --- --- --- --C --- --- --- --- --- --- --- --A --G --- --C --- ---
eve      --- --- --- --C --C CAG C-G --- --C --G --G -GC --G --- --- --- --- --C --- --- --C --- --- --- --- --- --- --- A-- A-C ---

Evx 1    Met Arg Arg Tyr Arg Thr Ala Phe Thr Arg Glu Gln Ile Ala Arg Leu Glu Lys Glu Phe Tyr Arg Glu Asn Tyr Val Ser Arg Pro Arg Arg
Evx 2    Val --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
eve      Val --- --- --- --- --- --- --- --- --- Asp --- Leu Gly --- --- --- --- --- --- --- Lys --- --- --- --- --- --- --- --- ---
Xhox-3   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

Evx 1    Cys Glu Leu Ala Ala Ala Leu Asn Leu Pro Glu Thr Thr Ile Lys▼Val Trp Phe Gln Asn Arg Arg Met Lys Asp Lys Arg Gln Arg Leu Ala
Evx 2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---▼--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
eve      --- --- --- --- --- Gln --- --- --- --- --- Ser --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Ile ---
Xhox-3   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---▼--- --- --- --- --- --- --- --- --- --- --- --- --- ---

B
Evx 1    GAC CAG [HOMEOBOX] ATG ACG TGG CCG CAC CCG GCC GAC CCT GCC TTC TAC ACC TAC ATG ATG AGC CAC GCG GCG GCC GCG GGC GGC
Evx 2    --T --- [        ] --- T-- --- --- --T --A --- --- --C AG- --- --- --- --- --- --- -CA --- --- --- --- A-C --A A--

Evx 1    CTG CCC TAC CCC TTC CCG TCG CAC CTG CCC CTG CCC TAC TAC TCG CCC GTG GGC CTG GGC GCC GCG TCC GCC GCC TCG GCC GCC
Evx 2    --A --- --- --T --T -AC --- --- G-- --G --- -A- --- --- C-- -A- --- --- G-C AC- --G --T G-A --A --G G-C --A ---

Evx 1    Asp Gln [HOMEOBOX] Met Thr Trp Pro His Pro Ala Asp Pro Ala Phe Tyr Thr Tyr Met Met Ser His Ala Ala Ala Ala Gly Gly
Evx 2    --- --- [        ] --- Ser --- --- --- --- --- --- --- Ser --- --- --- --- --- --- Thr --- --- --- --- Thr --- Ser

Evx 1    Leu Pro Tyr Pro Phe Pro Ser His Leu Pro Leu Pro Tyr Tyr Ser Pro Val Gly Leu Gly Ala Ala Ser Ala Ala Ser Ala Ala
Evx 2    --- --- --- --- --- His --- --- Val --- --- His --- --- Pro His --- --- Val Thr --- --- Ala --- --- Ala --- ---
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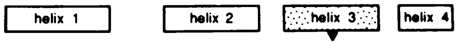
Fig. 2. Homeobox sequence comparison of *Evx 1*, *Evx 2*, *eve* and *Xhox-3*. Horizontal lines indicate identity with *Evx 1*. (**A**) Nucleic acid sequences (upper part) and deduced amino acid sequences (lower part) are shown. *Eve* sequences start with nucleotide number 306 and amino acid position 70 as defined by Frasch *et al.* (1987). *Xhox-3* sequence is from Ruiz i Altaba and Melton (1989a). Arrows show position of the intron within the homeodomains of *Evx 1*, *Evx 2* and *Xhox-3*. (**B**) Extended *Evx* homology outside of the homeobox. Nucleic acid and amino acid sequences of *Evx 1* and *Evx 2* immediately up- and downstream of the homeobox are compared.

of the amino acids and 77.6% of the nucleotides are identical in the region 3′ of the homeobox, coding for 52 amino acids. Flanking the 5′ end of the homeobox, a dipeptide (Asp–Gln) is present in both genes (Figure 2B). Sequence similarities to the corresponding regions of the *Drosophila* gene are limited to 4 amino acids adjacent to the 3′ end of the homeobox (compare *Drosophila*: Val–Ala–Trp–Pro with *Evx 1/2*: Met–The/Ser–Trp–Pro in Figure 2B).

Figure 4 shows the sequence of a 2886 bp cDNA which covers the entire coding sequence of the *Evx 1* protein. This cDNA is probably not full length but lacks ~300 bp at the 5′ end since the full length of the transcript is 3.2 kb as judged from Northern blot hybridization experiments (see below). All cDNAs so far analysed have their 5′ ends upstream of the TAG stop codon in front of the presumable translation start site. Comparison of *Evx 1* genomic and cDNA sequences indicates the presence of two other introns (Figures 1 and 4). One separates a region which encodes glycines and serines with a frequency of 52.6% from the remaining upstream sequence, the other is located in the untranslated 3′ region. The longest open reading frame (ORF) codes for a protein of 416 amino acids and shows extensive structural similarities to the *Xenopus Xhox-3* transcript along its entire length (A. Ruiz i Altaba and D.A.Melton, personal communication). As in *eve* and *Xhox-3*, the homeobox lies



```
Evx 1    MRRYRTAFTREQIARLEKEFYRENYVSRPRRCELAAALNLPETTIKVWFQNRRMKDKRQRL
Evx 2    V------------------------------------------------------------
Xhox-3   ------------------------------------------------------------
eve      V---------D-LG-------K--------------Q-----S-----------------I
ant      RK-G-QTY--Y-TLE-----HFNR-LT-R--I-I-H--C-T-RQ--I--------W-KENK
bcd      P--T--T--SS---E--QH-LQGR-LTA--LAD-S-K-A-GTAQV-I--K---RRH-I-SD
cad      KDK--VVY-DF-RLE----YCTSR-ITIR-KS---QT-S-S-RQV-I------A-ERTSNK
en       EK-P----SS--L---KR--NENR-LTER--QQ-SSE-G-N-AQ--I----K-A-I-KSTG
msh      N-KP--P--TQ-LLS---K-REKQ-L-IAE-A-FSSS-R-T--QV-I------A-A--LQE
prd      Q--C--T-SAS-LDE--RA-E-TQ-PDIYT-E---QRT--T-AR-Q---S---ARLRK-HT
Oct-2    R-KK--SIETNVRFA---S-LANQKPTSEEILLI-EQ-HMEKEV-R---C---Q-E--INP
```

Fig. 3. Alignment of the *Evx 1* and *Evx 2* homeoboxes of different classes. One member of all so far identified homeobox classes of *Drosophila* (*Ant*, *bcd*, *cad*, *en*, *eve*, *msh*, *prd*), a member of the Pou homeobox family (*Oct-2*), *Xhox-3*, *Evx 1* and *Evx 2* have been compared with each other with respect to the amino acid sequence. Amino acids are in single letter code. The *Evx 1* sequence is given in the top line and only residues diverging from the *Evx 1* sequence are shown in the following lines. The arrow indicates the position of the intron in *Evx 1*, *Evx 2* and *Xhox-3*. Positions of α helices (Otting *et al.*, 1988) within the homeodomain are drawn at the top. The putative DNA recognition helix is stippled.

```
   1  CCTCCTTTCATCTTCACTGTGGCAGACGTTTCTATTTATCCACTTGCGTTCGCCGAGTGGCGTCACCAGCGGTACTGTAATGACGATTGCAGCAGGAGGATGACAGCTTAGAAAGAAGAG
 121  GGCAATGGGGCTTCCTCCCAGAGGCGGTGCGGCACAGAGGAGCGCTCGCATCACAAGGTGACCCTAGCTCCCCACTGCCATCTCCGCGGTCGCCGTCGACACGGGCCTGGGGCTACCCGC
 241  CGCTGGCCTTGTCGCCTTAGCTCCTCTTCTCAGCCAAGATCCCAGGGAGCCTGGGATTAGGAGCTTACTTGGGGGTTTTCCCCCTCCCCCCCTCTGGAGAGTCCGGGGATGGAGAGCCGA
                                                                                                                      MetGluSerArg

 361  AAGGACATGGTTATGTTTCTGGATGGGGGTCAGCTTGGCACTCTGGTTGGTAAGAGGGTCTCTAATTTGTCCGAAGCCGTGAGCAGCCCGCTGCCTGAACCGCCAGAGAAGATGGTGCCC
   5  LysAspMetValMetPheLeuAspGlyGlyGlnLeuGlyThrLeuValGlyLysArgValSerAsnLeuSerGluAlaValSerSerProLeuProGluProProGluLysMetValPro

 481  CACGGTTGCCTGAGCCCGCGAGCCGGCCCTCCGACTTCCCGGGAGCGTGGCGGGGGAGGCCAGGAGGAGGAGCCGGTCGATGGACTAGCAGGCAGTGCTGCAGGGCTGGGCGCCGAGCCA
  45  HisGlyCysLeuSerProArgAlaGlyProProThrSerArgGluArgGlyGlyGlyGlyGlnGluGluGluProValAspGlyLeuAlaGlySerAlaAlaGlyLeuGlyAlaGluPro

 601  CGGTCTGCTGGAGCGGCCATGCTTGGCCCGGGACCCCCAGTCCCCTCCGCGGACAGCCTCTCTGGCCAAGGGCAACCTAGTAGCTCAGACACCGAATCGGATTTCTATGAAGAAATCGAG
  85  ArgSerAlaGlyAlaAlaMetLeuGlyProGlyProProValProSerAlaAspSerLeuSerGlyGlnGlyGlnProSerSerSerAspThrGluSerAspPheTyrGluGluIleGlu
                                                            ▼
 721  GTGAGCTGCACCCCAGACTGCGCCACCGGGAACGCCGAGTACCAGCACAGCAAAGCGCCAGGCTCCGATGCTCTGGGTAGCAGTCCTACCAGTGGCAGCGAGGCCCCCAAGAGTAACGGT
 125  ValSerCysThrProAspCysAlaThrGlyAsnAlaGluTyrGlnHisSerLysAlaProGlySerAspAlaLeuGlySerSerProThrSerGlySerGluAlaProLysSerAsnGly

 841  GGCAGCGGCGGCAGCGGCTCTCAAGGCACCCTGGCCTGCAGCGCCAGTGACCAGATGCGCCGATACCGCACGGCCTTTACCCGGGAGCAGATTGCAAGGCTGGAGAAAGAGTTCTACAGG
 165  GlySerGlyGlySerGlySerGlnGlyThrLeuAlaCysSerAlaSerAspGlnMetArgArgTyrArgThrAlaPheThrArgGluGlnIleAlaArgLeuGluLysGluPheTyrArg
                                                                              ▼
 961  GAGAACTACGTTTCAAGACCGCGGAGATGCGAACTGGCAGCAGCCTTAAACCTTCCTGAAACTACCATCAAGGTGTGGTTTCAGAACCGGCGCATGAAGGACAAGCGTCAGCGGCTGGCC
 205  GluAsnTyrValSerArgProArgArgCysGluLeuAlaAlaAlaLeuAsnLeuProGluThrThrIleLysValTrpPheGlnAsnArgArgMetLysAspLysArgGlnArgLeuAla

1081  ATGACGTGGCCGCACCCGGCCGACCCTGCCTTCTACACCTACATGATGAGCCACGCGGCGGCCGCGGGCGGCCTGCCCTACCCCTTCCCGTCGCACCTGCCCCTGCCCTACTACTCGCCC
 245  MetThrTrpProHisProAlaAspProAlaPheTyrThrTyrMetMetSerHisAlaAlaAlaAlaGlyGlyLeuProTyrProPheProSerHisLeuProLeuProTyrTyrSerPro

1201  GTGGGCCTGGGCGCCGCGTCCGCCGCCTCGGCCGCCGCCTCGCCCTTCAGCGGCCCCCTGCGCCCGCTCGACACCTTCCGCGTGCTCTCGCAGCCCTACCCACGGCCCGAACTGCTGTGC
 285  ValGlyLeuGlyAlaAlaSerAlaAlaSerAlaAlaAlaSerProPheSerGlyProLeuArgProLeuAspThrPheArgValLeuSerGlnProTyrProArgProGluLeuLeuCys

1321  GCCTTCCGCCACCCGCCGCTCTATCCCGGCCCGGCGCACGGACTCGGCGCCTCGGCCGCCGCCGCCGCTGCCGCCGGGCCCTGCTCCTGCCTCGCCTGCCACAGCGGCCCGGCCAACGGG
 325  AlaPheArgHisProProLeuTyrProGlyProAlaHisGlyLeuGlyAlaSerAlaAlaAlaAlaAlaAlaAlaGlyProCysSerCysLeuAlaCysHisSerGlyProAlaAsnGly

1441  CTGGCGCCCAGGGCCGCCGCCGCTGCCGCCGCCTCAGACTTCACTTGTGCCTCCACCTCCCGCTCGGACTCCTTCCTCACGTTCGCCCCCTCTGTGCTCAGCAAGGCCTCCTCCGTGGCG
 365  LeuAlaProArgAlaAlaAlaAlaAlaAlaAlaSerAspPheThrCysAlaSerThrSerArgSerAspSerPheLeuThrPheAlaProSerValLeuSerLysAlaSerSerValAla

1561  GCGTTGGACCAGAGAGAGGAGGTGCCCCTCACCAGATAAGGGGGCGCCCTCGGGGCTGCCGACTGCAGGATGGACGTCGTGGGGTGGAGGTCGGGGGAGCCTTCGGGGCTCCTCCAGCAT
 405  AlaLeuAspGlnArgGluGluValProLeuThrArg

1681  CCCACCTGTCACCTCTCTTCTCAGATGCACAGGGGAAAGGAGGTGGAGGTGGGGGGGGGACACCATAAAGGACTCCGCCTAGAGGATCCAGGAGCCCCAGAAGCGACATTGGCTGCTTAG
1801  GAGCAGGAATGGGGTGGGAAAGCGTCCCCTGGGGTGGCCAGGCCTTGCCATTCTCCACCGAAACCACCACTCTATCAGAGGTCGAGGCCTTGGTTCAACAGCTACCCGGGAACAACCCAC
1921  TCCCAAGCCCCTCGTACCCTTCAACTGGGCCCCCACTCTCCTGTCAAGGGCAAGAGCTGCGAAGGGAACTCGCTGTTTCTTGGAACAAAATGCTGTGTATGCAGAGCAGGTAGAAATTAA
2041  TCTTCACCAGCTTTTCCAACGCATGGCAGGGGGCTTGTTGATGGCAACATCCCAGCCATTTAGGGGAGAGATGATTTACTGCTAGGGAGATGCTTGCCACTTGGCGAGGAACCTGGGAGC
2161  CGGCTCACCTTCCCAGGGCCTCAACTACACAGCATCTGGGGAGTGGTACCCTCTACCAGCCACTGACTTCTTTCCCTATTTCTGGCTTTGTGACATCTCACGACCCGACCTGTGCAGCTC
2281  CAGCCTTCTCCCTTTCCGGTTTGCATTGGCAGGGAAGGGGCAGGGGCTCCAGGGTCCCTAGGCAGCTTCTGTGGGGCGAACCTCTTCTCCCTTAACCCAGCACACAGCCTGATTAGCAAG
                                                       ▼
2401  TGATGGGTGAGGAGGGGTTTTTGAATGTTGAATGTATGTATAATAATGATCACCACTCTGCTGGGCCACCAAGCCCGGAGCTGCTGAGCCGGTCTAACAAGGCGGCCTGGGAAGAGCTTA
2521  GGGAACGGAGACTTCTTACATTCTTCTCTCATTGTCTCCCCAAATTGCCACAGGGCCTTGGCTTTCAGCTGCCAGTACAAACCTTCAGCGCCTCTGGAGGACCCTGTCTCTCCCCTTCAC
2641  TGGGGTTTATTGGGGAGCCACTTAGGAACTCCTGTACTCATCCTTATAGCCAGTGTTTGAGGAAAAGGACAACTTCAATCATCCAATGCAGGCTTCCCTCCACTGGGAGGAAGTGGTCCT
2761  TCCTGCAGGGAATGAATTTGGTTTGGTTTGGGGTTTTCCTTTGCAGCCCAAAGAATTTGCTGTTATGATTTGTTAACCATATTGCAATAAAAGCTGAACATGATTCTTACTTTAGCAAAA
2881  AAAAAA
```

Fig. 4. cDNA sequence and predicted protein sequence of *Evx 1*. The nucleotide sequence shown is from a single cDNA clone. Numbers to the left count the bases of the cDNA and the amino acids of the protein. The homeobox is in bold letters. The acidic region is double underlined, the region of the extended homology to *Evx 2* and the polyadenylation signal are underlined. Triangles indicate positions of introns. Stop codons flanking the ORF are dotted underlined.

at the more amino-terminal region of the *Evx 1* protein. This is in contrast to all mouse homeobox genes so far studied, where it is positioned at the more carboxy-terminus (Wright *et al.*, 1986; Joyner and Martin, 1987; Duprey *et al.*, 1989). In addition to the glycine/serine rich region and the homeodomain, a highly acidic region of 10 amino acids is located at positions 114–124 and three repeats rich in alanine and serine are located in the region carboxy-terminal of the homeobox. Similar repeats of various length encoding alanine have also been reported in *eve* and other developmentally important *Drosophila* genes such as *en*, *Notch* and *cad* (Poole *et al.*, 1985; MacDonald *et al.*, 1986; Wharton *et al.*, 1986; Frasch *et al.*, 1987).

The Evx 1 gene maps to mouse chromosome 2

In order to determine the chromosomal localization of the *Evx 1* gene, we have used the inter-species backcross system (Guenet, 1986). Genomic Southern blot hybridization of DNA derived from the laboratory inbred strains *Mus musculus* C57BL/6 and *M.spretus* line SPE/PAS showed a restriction fragment length polymorphism (RFLP) upon *Sau* 3A digestion. The probe used for hybridization was a 226 bp *Pst*I–*Pst*I fragment which lacks the most highly conserved 3′ end of the recognition helix. It detects an 800 bp fragment in the *M.musculus* and a 500 bp fragment in the *M.spretus* strain. This RFLP was utilized to determine the segregation of *Evx 1* alleles from a panel of 27 back-cross progeny of (C57BL/6×SPE/PA) F_1 females and C57BL/6 males. The results were then compared to the segregation pattern of all other genes analysed in the same cross. *Evx 1* is closely linked to the Hox 5 cluster (Featherstone *et al.*, 1988; Duboule and Dolle, 1989; Siracusa *et al.*, 1990), as it shows 96.3% co-segregation with the Hox 5 locus. Thus, the linkage distance between *Evx 1* and Hox 5 is ~3.7±3.6 cM.

Evx 1 is expressed during mouse embryogenesis and in undifferentiated F9 stem cells

The expression of *Evx 1* was analysed in diverse adult tissues and in embryos from days 9.5 to 17.5 p.c. by Northern blot hybridization to polyadenylated RNA using the *Pst*I fragment (see Fig. 1). No transcript could be detected in brain,

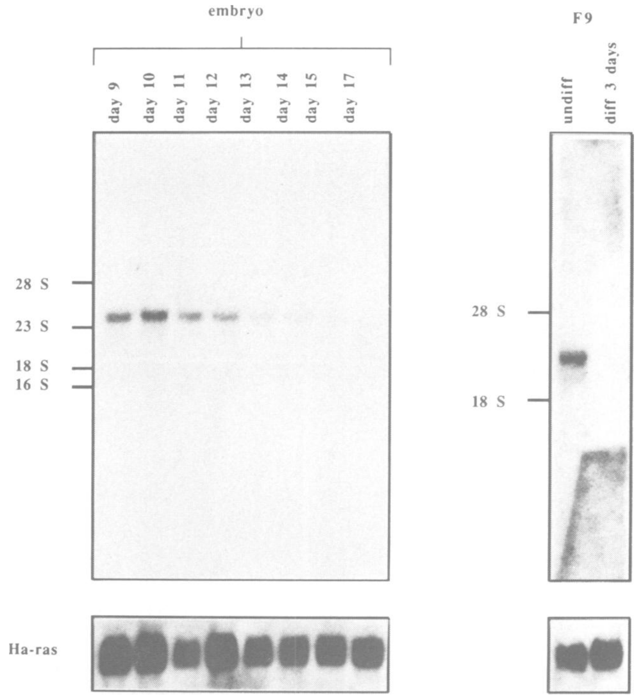


Fig. 5. Expression of *Evx 1* in total embryonic tissue and in the embryonal carcinoma cell line F9 detected by Northern blot hybridization. Polyadenylated RNA of the embryonic stages (15 μg) indicated and of F9 stem cells as well as 3 days differentiated cells (10^{-7} M RA, 10^{-3} M cAMP; each 10 μg) were separated on 1% formaldehyde/MOPS agarose gels and blotted. Blots were hybridized under high stringency conditions with a *Pst*I–*Pst*I fragment containing the 5′ regions of the *Evx 1* homeobox.

heart, intestine, liver, lung, kidney, spleen, testis, stomach, thymus, uterus and salivary gland (data not shown). However the probe detects a single transcript of ~3.2 kb present during mouse embryogenesis (Figure 5). *Evx 1* transcripts are present in embryos of day 9 p.c., accumulate from days 10 to 12.5 p.c. and decrease strongly thereafter. Isolation of eight cDNA clones from a day 8.5 p.c. embryonic cDNA library (Fahrner and Hogan, 1985) and detection of *Evx 1* transcripts in days 7–9 p.c. embryos by *in situ* analysis (see below) demonstrate that *Evx 1* is expressed at earlier stages than examined by Northern blot analysis.

In the mouse teratocarcinoma F9 cell system, which is believed to model cellular differentiation and early embryonic development (Martin, 1980; Hogan *et al.*, 1983), a single *Evx 1* transcript of about the same size (3.2 kb) as found in embryos is detected specifically in undifferentiated stem cells (Figure 5). The transcript disappears upon differentiation into parietal endoderm after administration of retinoic acid and cAMP (Figure 5), as soon as 24 h thereafter (not shown). However, no expression could be seen in undifferentiated P19 cells (not shown).

Evx 1 exhibits a biphasic expression pattern during embryogenesis

In situ hybridization analysis of sagittal and transverse sections of mouse embryos from days 7–12 p.c. was performed with an antisense RNA probe from the 5′ part of the *Evx 1* homeobox, transcribed from the *Pst*I–*Pst*I fragment already used in the Southern and Northern blot hybridization experiments. As a control, adjacent sections were hybridized with a RNA sense probe of the same fragment and no specific signals were detected (not shown). Two phases of *Evx 1* expression with distinct patterns can be discerned; an early expression from days 7–9 p.c. and a late transcription from days 10–12.5 p.c. No expression is detected using the *in situ* analysis between day 9 and 10 p.c. (data not shown).

In sagittal sections of early to mid-primitive streak embryos, before closure of the amnion, a weak signal can be detected in the posterior region of the embryo (Figure 6A i and ii). The signal mainly lies over ectodermal cells but could also be due to, at least in part, some mesodermal cells which are continuously forming by ingression through the primitive streak at this stage. At about half a day later when the amniotic cavity is sealed off and allantois, foregut pocket and the anterior neural plate appear this signal increases (Figure 6A iii and iv). It can now definitively be assigned to ectoderm and mesoderm of the post to mid primitive streak region. Sagittal sections of day 8.5 to nearly 9 p.c. embryos show strong *Evx 1* hybridization signals in the posterior neuroectoderm and the underlying mesoderm (Figure 6A v–viii). The anterior boundary of *Evx 1* expression in neuroectoderm and mesoderm is posterior to the last somite (see arrow in Figure 6A v and vii). Thus, the early phase of *Evx 1* expression remains localized at the caudal end of the embryo and is therefore different from the Hox genes which although initially expressed caudally exhibit the activity later more rostrally. The region of mesodermal *Evx 1* expression is posteriorly displaced with respect to *Evx 1* expression in the neuroectoderm. This is confirmed by cross-sections of the posterior part of the embryo (Figure 6B). Seemingly, expression in the mesoderm is more intense the more posterior the section, whereas in the ectoderm it shows the same intensities (Figure 6B, 1–4).

In sagittal sections of day 10–12 p.c. embryos, *Evx 1* expression appears as a narrow band along the entire neural tube with an anterior border at the level of the rhombencephalic isthmus (Figures 7 and 8). The stripe of expression is situated in the more ventral part of the spinal cord. The analysis of adjacent sagittal sections shows that the signal disappears when leaving the area near the medial plane, indicating that only a few cells in this plane express the gene at high levels. This is particularly striking for the day 10 p.c. embryo (Figures 7A–F and 8A and B) where the neural tube mainly consists of ventricular cells. In addition, faint expression is detected in regions ventral to the intense signal within the neural tube of day 11.5 p.c. embryos (see area between arrows in Figure 8D). The signal first appears at the thoracic level and extends up to the anterior border. The same pattern of expression is observed in the spinal cord of day 12 p.c. embryos. At this stage, another diffuse signal of the same intensity appears in dorsal regions of the spinal cord at the cervical to anterior rhombencephalic level (Figure 8E and F). No expression is detected outside of the neural tube in any other tissue or region of the developing embryo.

The distribution of *Evx 1* expressing cells in the transverse plane of the neural tube of a day 12.5 p.c. embryo at different levels along the caudal to rostral axis from tail to hindbrain is shown in Figure 9. Within a single embryo, different antero-posterior levels of the neural tube correspond to different stages of development due to the rostro-caudal and ventro-dorsal gradient of maturation, proliferation and neuroblast migration (Hamburger, 1948; Fujita, 1964; Smart, 1972; Nornes and Das, 1974; Nornes and Carry,

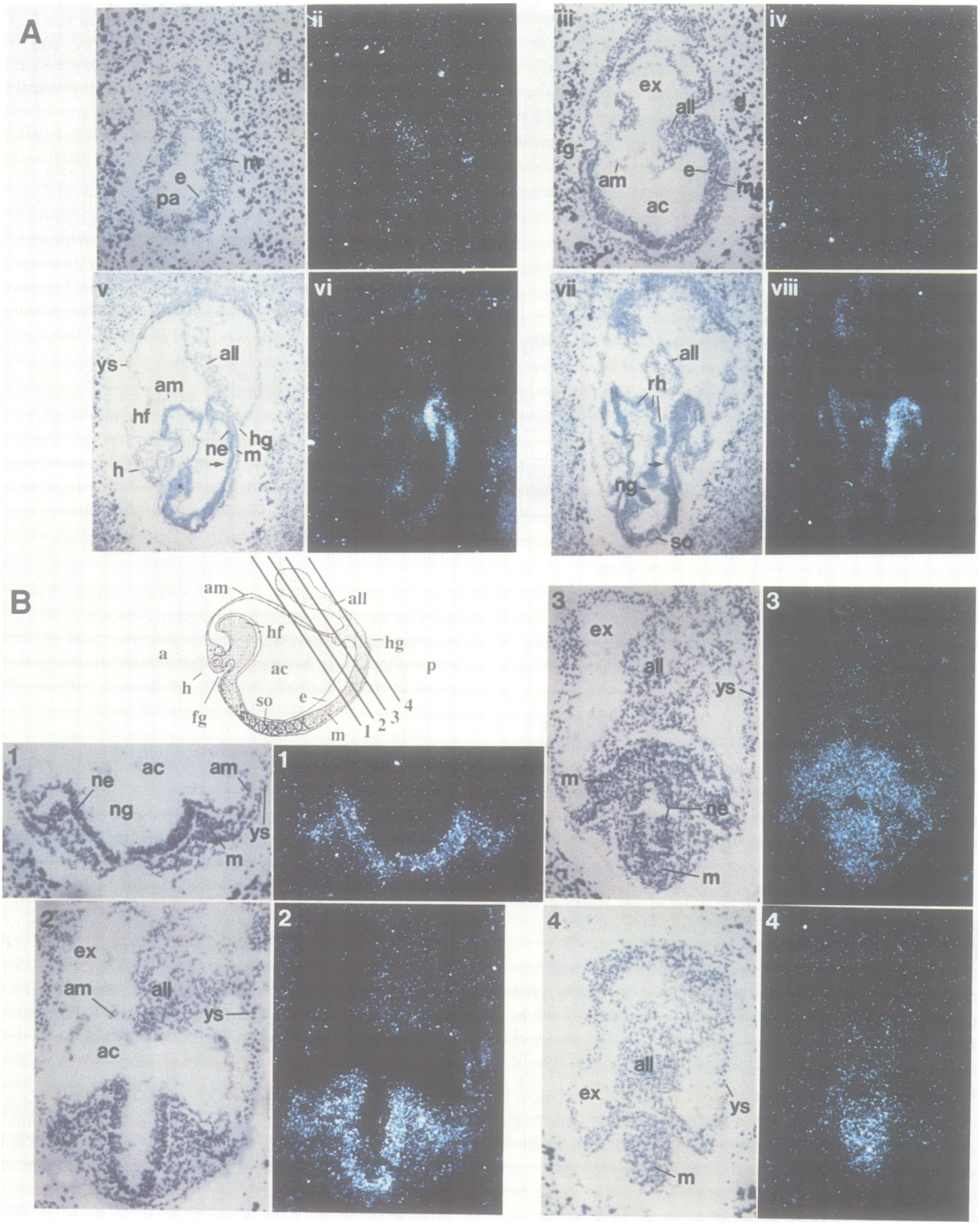


Fig. 6. Expression of *Evx 1* in day 7, 7.5, 8.5 and 9 p.c. embryos. Anterior is always to the left, posterior to the right. (**A**) *In situ* hybridization to sagittal section of day 7 p.c. (i and ii), 7.5 p.c. (iii and iv), 8.5 p.c. (v and vi) and 9 p.c. (vii and viii) embryos. Photographs (i, iii, v and vii) represent brightfield photographs and (ii, iv, vi and viii) are the corresponding darkfield photographs, respectively. The arrows in (v) and (vii) mark the anterior limit of *Evx 1* expression. (**B**) *In situ* hybridization to cross-sections of day 8.5 p.c. embryos. The schematic drawing indicates the plane of the sections (1, 2, 3 and 4)). Brightfield and darkfield images of each section are shown side by side. Abbreviations: a, anterior; ac, amniotic cavity; all, allantois; am, amnion; d, deciduum; e, ectoderm; ex, exocoelom; fg, foregut pocket; h, heart region; hf, headfold; hg, hindgut pocket; m, mesoderm; ne, neuroectoderm; ng, neural groove; p, posterior; pa, proamniotic cavity; rh, rhombomeres; so, somite; ys, yolk sac.

1978; Altman and Bayer, 1984). *Evx 1* expression successively appears in three bilaterally symmetrical regions in the neural tube. In the tail, *Evx 1* expression becomes visible as two dots just ventral to the sulcus limitans within the neuroepithelium and remains restricted to this region even when the intermediate zone has begun to form (Figure 9B and D). The signal is limited to a cluster of 30–40 cells and does not extend to the ventricular surface. This signal increases in strength and stays at the same position within various developmental stages (Figure 9F, H, K and M). The labelled area never expands to the lumen and remains nearly constant in its dorso-ventral expansion, but spreads out radially to the margin of the ventricular zone, seemingly not crossing the border to the intermediate zone.

A second area of *Evx 1* expression is first detectable at lower thoracic spinal cord levels of the day 12.5 p.c. embryo and continues anteriorly (Figure 9F, H, K and M). The signal is situated in the ventral part of the intermediate zone between the developing motor column and the margin of the intermediate zone. It corresponds to the faint signal described in the sagittal sections (Figures 7H and K and 8D and F) and proves to be less intense probably because of the looser packing of cells in the mantle than in the intermediate zone.

Evx 1 expression in the dorsal region of the neural tube is first detected in sections from the cervical level of the day 12.5 p.c. embryo (Figure 9H). The signal extends within the intermediate zone along the neuroepithelium border to the level of the first *Evx 1* and shows the same intensity as in the ventral intermediate zone. The signal corresponds to the weak signal seen in dorsal regions of the neural tube at the cervical level of sagittal sections (Figures 7K and 8F).

In day 10 p.c. embryos, transverse sections along the entire length of the neural tube show the same *Evx 1* expression pattern as seen in the tail of day 12 p.c. embryos.

In day 11 p.c. embryos, *Evx 1* expressing cells are also detected in the ventral intermediate zone. The expression pattern in cervical regions of day 11 p.c. embryos (not shown) corresponds to the pattern at the thoracical level of day 12 p.c. embryos (Figure 9F). Expression in dorsal regions of the intermediate zone cannot be detected before day 12 p.c. The *Evx 1* expression pattern according to the degree of maturation of the spinal cord is summarized in Figure 10.

Discussion

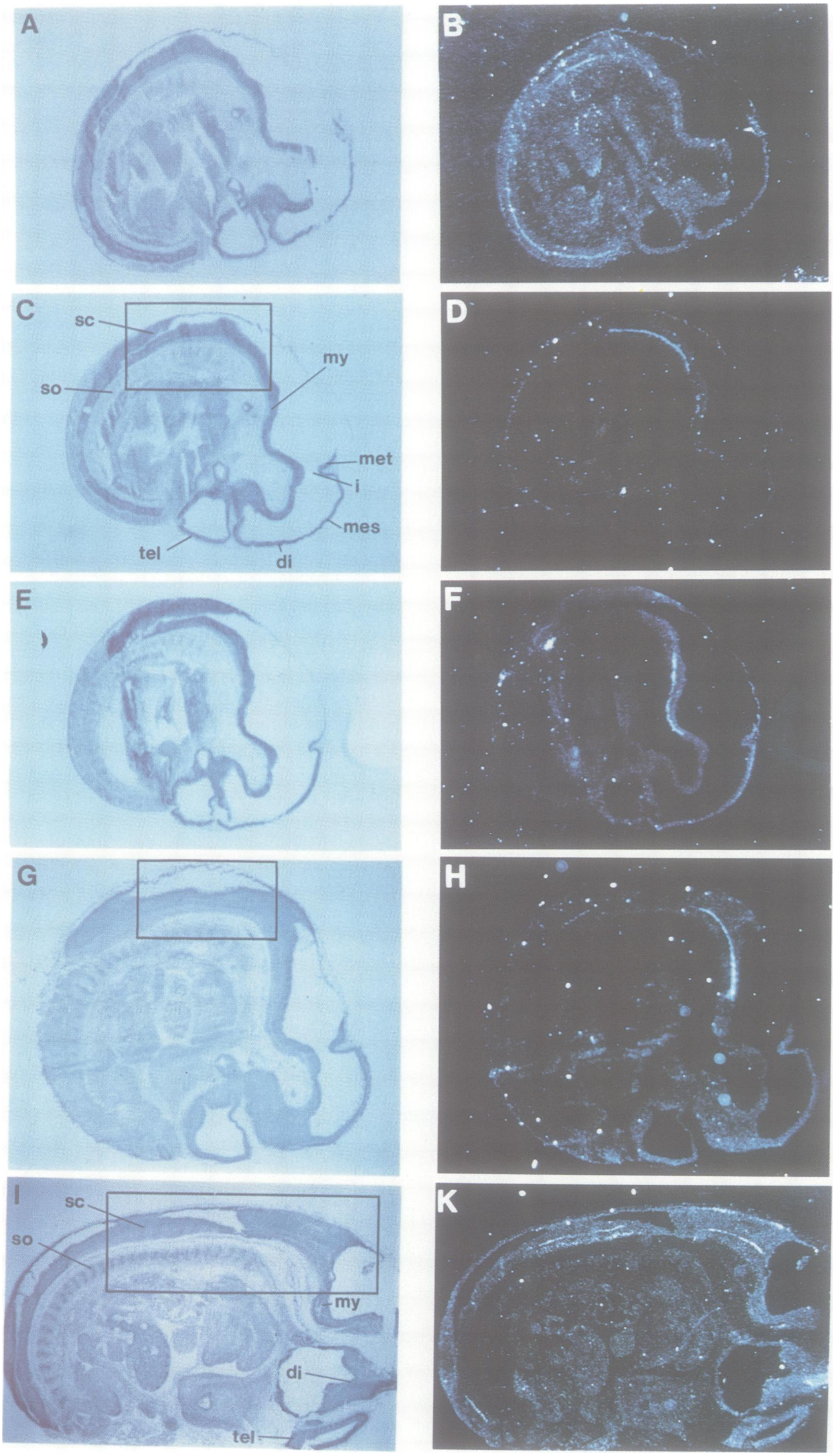
Two eve related genes are present in the mouse genome

We have isolated two murine genes with high sequence homology to the *Drosophila eve* homeobox (MacDonald *et al.*, 1986; Frasch *et al.*, 1987) and to the *Xenopus Xhox-3* gene (Ruiz i Altaba and Melton, 1989a). Only two nonconservative amino acid exchanges within the homeodomain (Gly to Ala and Gln to Ala at position 14 and 37, respectively) occurred between *Drosophila* and mouse. As these exchanges are located outside of the recognition helix the proteins might have the same DNA-binding sequence, especially since the specificity of the *eve* DNA-binding domain is directly dependent on the homeodomain, rather than on its protein context (Hoey *et al.*, 1989). Moreover, that vertebrates do not only have conserved homeodomains but also target sequences of *Drosophila* is supported by *in vitro* studies of the *Xenopus* homeobox gene *XlHbox 1* (Cho *et al.*, 1988).

The Evx sequences are more similar to each other and to the *Xenopus Xhox-3* than to the *Drosophila* gene. In addition, *Xhox-3* has an intron at exactly the same position within the homeodomain as the mouse genes and a second *eve* related gene seems to exist also in the frog (Ruiz i Altaba and Melton, 1989a). Thus, similar to the *Antp* and *en* like homeobox genes vertebrate *eve* like genes might have been duplicated during evolution from a common ancestral gene (Duboule and Dollé, 1989; Graham *et al.*, 1989; Patel *et al.*, 1989b; Schughart *et al.*, 1989).

Evx 1 expression during gastrulation

Our *in situ* analysis has revealed initial expression of *Evx 1* in embryos from days 7–9 p.c. *Evx 1* expression originates at the posterior end of the embryo in the region of the posterior to mid primitive streak in both ectoderm and mesoderm. Over the next 1.5–2 days the domain of expression expands in the mesoderm and to the overlying ectoderm but remains restricted to the posterior portion of the fetus. This expression pattern is during the period of gastrulation which starts about half a day earlier. The beginning of gastrulation is characterized by the appearance of the primitive streak which arises at the posterior end of the embryo and defines the antero-posterior axis. As long as the primitive streak persists, until day 9–9.5 p.c., it is proposed to generate primordia of many components of the trunk according to a sequence at which various body levels are laid down and thus establishes the basic body plan of the embryo (Tam, 1984). At around day 10 p.c. the primitive streak is replaced by the tail bud whose histogenetic capacity is restricted to form primarily tissues found in the adult tail. The distinct temporal and spatial distribution of *Evx 1* transcripts may point to a function of *Evx 1* in the mechanism that establishes the antero-posterior axis of the embryo, with *Evx 1* providing posterior positional information at the caudal end of the gastrulating fetus. Maturation of embryonic tissue occurs temporally in an anterior to posterior sequence and embryonic tissue, arising from the primitive streak, is produced in an posterior–anterior manner. Thus, prospective anterior tissue is generated prior to posterior tissue. Therefore, with advancing gastrulation, newly formed tissue receives a more posterior character. The domain of *Evx 1* expression remains restricted to the posterior part of the embryo until the end of gastrulation and its anterior limit is maintained relative to the size of the embryo. The presence as well as the duration of the *Evx 1* signal may define the posterior character of arising and already existing tissue. This concept would also be consistent with the observation that the caudal tissue of the gastrulating embryo becomes progressively restricted in its histogenetic capacity with age: whereas younger tissue has the potential to contribute to adult tissues of cranial and caudal levels, older tissue generates predominantly tissues of caudal levels of the adult body (Tam, 1984). A participation of *Evx 1* in the establishment of the axial polarity in the mouse embryo may further be supported by the data of the *Xenopus Xhox-3* gene. In addition to the striking structural homology between *Evx 1* and *Xhox-3* along the entire coding region, similarities are also seen in the expression pattern of developmentally comparable stages of *Xenopus* and mouse. *Xhox-3* is transiently expressed in the axial mesoderm of gastrula and neurula embryos and the levels of *Xhox-3* mRNA correlate with antero-posterior cell fates (Ruiz i Altaba and Melton, 1989b,c).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Evx 1 transcripts disappear at approximately the same time as the primitive streak. Although the biochemical basis that underlies the processes occurring in the primitive streak are far from clear, from an evolutionary point of view the primitive streak of the mouse may be equivalent to the 'Organizer' region of amphibia. In *Xenopus* mesoderm forming activity, at least in part coming from this region, is thought to be mediated by the release of growth factors such as TGF-β and FGF (Slack *et al.*, 1989; Smith *et al.*, 1989). *Xhox-3* has been shown to response to those factors (Ruiz i Altaba and Melton, 1989c) and this might also be valid for *Evx 1*. Such morphogenetic signals, originating at the posterior end of the mouse embryo, could provide an excellent explanation for the mechanism which establishes the restricted expression pattern of *Evx 1*. Differences in the anterior limits of *Evx 1* expression in the two germ layers (neuroectoderm and mesoderm) may be due to the greater growth of the CNS in relation to the mesoderm as discussed by De Robertis *et al.* (1989). *Xhox-3* has also been suggested to play similar roles in pattern formation along the anteroposterior axis in the tailbud (Ruiz i Altaba and Melton, 1989b). However, we could not detect *Evx 1* expression in the tail bud of the mouse. This may reflect differences in the functional ability between primitive streak and bud with respect to establishing rostrocaudal structures in the mouse as well as differences between the tail bud of mouse and frog, despite otherwise similar histogenetic potentials (Elsdale and Davidson, 1983; Tam, 1984).

Assignment of late Evx 1 to neuronal structures of the CNS

Expression of *Evx 1* in the CNS from day 10–12.5 p.c. shows some characteristic features: expression is restricted to subpopulations of cells in the neural tube and the hindbrain, *Evx 1* expressing cells increase in number in the same measure as neuroblasts mature and the hybridization signals remain in the same area at different stages of development in contrast to the migrating neuroblasts.

The rostral boundary of *Evx 1* expression lies at the border between met- and mesencephalon (the rhombencephalic isthmus) where alar plate, basal plate, roof plate and floor plate of the spinal cord have their anterior ends. In many respects, spinal cord and hindbrain are quite similar in their organization and show the same early histological changes occurring in the wall. Their neuroepithelia are specified to produce different classes of neurons in a mosaic manner (Altman and Bayer, 1984). In the spinal cord and hindbrain, *Evx 1* transcripts are localized in comparable, confined areas in the transverse plane and thus *Evx 1* may be expressed cell-type specific or region specific along the entire lengths of neural tube and rhombencephalon for a few days during development. In the ventricular zone, the *Evx 1* signal is never seen in regions lining the lumen of the neural tube. Since the nuclei of neuroepithelial cells migrate during the cell cycle between the apical and basal part of the ventricular zone and undergo mitosis at the luminar surface (Sauer, 1959; Sidman *et al.*, 1959), these *Evx 1* expressing cells are most likely postmitotic. Thus, they represent early differentiating neurons which are about to migrate radially out of the ventricular zone. However, it cannot be totally excluded that the labelled cells represent proliferative cells which express *Evx 1* transiently during the cell cycle. Cells which have already migrated from the ventricular zone and have undergone their terminal mitosis settle down to form the intermediate zone. Here, *Evx 1* expression appears, transversally arranged, in subpopulations of such differentiating neurons. In the ventral neural tube it is located between the motorneuroblasts and the margin of the ventricular zone, in the dorsal part between the ventricular and the mantle layer dorsally to the signal in the ventricular zone.

Can the *Evx 1* expression pattern at this early stage of neural tube development be correlated to differentiating cell types or to known morphogenetic processes? *Evx 1* expression coincides with the development and maturation in the ventro-dorsal and rostro-caudal direction of the spinal cord (Hamburger, 1948; Fujita, 1964; Smart, 1972; Nornes and Das, 1974; Nornes and Carry, 1978; Altman and Bayer, 1984). At around day 9 p.c. most cells of the neural tube are radially oriented ventricular (neuroepithelial) cells, whereas others are in early stages of neuron differentiation, namely ventral root motorneurons, commissural and association (ipsilateral) interneurons (Smart, 1972; Holley, 1982; Altman and Bayer, 1984; Wentworth, 1984). Following development to day 13 p.c., these cells form the early cytoarchitecture of the intermediate zone. The *Evx 1* expression pattern in the intermediate zone is regionally specific for cells generating interneurons. Its temporal and spatial pattern resembles the successive appearance and differentiation of commissural cells within the spinal cord (Holley, 1982; Holley *et al.*, 1982; Wentworth, 1984). Commissurals arise along the entire wall of the spinal cord and hindbrain and generate the earliest axons. Hereby, commissurals continue to differentiate from ventricular cell precursors in the same ventro-dorsal and rostro-caudal manner as the general development of the neural tube. *Evx 1* expression in the ventricular zone at the level just ventral of the sulcus limitans could also belong to commissural cells which appear to be more advanced in their differentiation in this region and therefore may have already reached the stage at which *Evx 1* positive cells in the intermediate zone are thereafter (Wentworth, 1984). Alternatively, they could be assigned to a subpopulation of radially oriented cell bodies belonging to ipsilateral association cells which send their axons through the intermediate zone to the lateral funiculus. These cells, however, are primarily intermingled in the middle to dorsal part of the intermediate zone (Holley *et al.*, 1982).

The restricted pattern of *Evx 1* expression remains essentially constant through several developmental stages. In the ventricular zone, the area of *Evx 1* expression becomes more intense and widened probably due to an increase in depth of the ventricular layer through rapidly occurring cell proliferation, but is limited to this zone although the

Fig. 7. *In situ* hybridization of *Evx 1* to near-midsagittal sections of day 10 to 12 p.c. mouse embryos. Panels (**B**, **D**, **F**, **H** and **K**) are darkfield images of the brightfield views (**A**, **C**, **E**, **G** and **I**) respectively. (A–F) Adjacent sections of a day 10 p.c. embryo showing *Evx 1* expression from tail to isthmus. (F) The anterior border of *Evx 1* expression. (G and H) Day 11 p.c. embryos. (I and K) Day 12 p.c. embryos. Di, mes, met, my, tel are the di-, mes-, met-, myelencephalon, respectively; i, isthmus; sc, spinal cord; so, somite; boxed areas are magnifications shown in Figure 8.

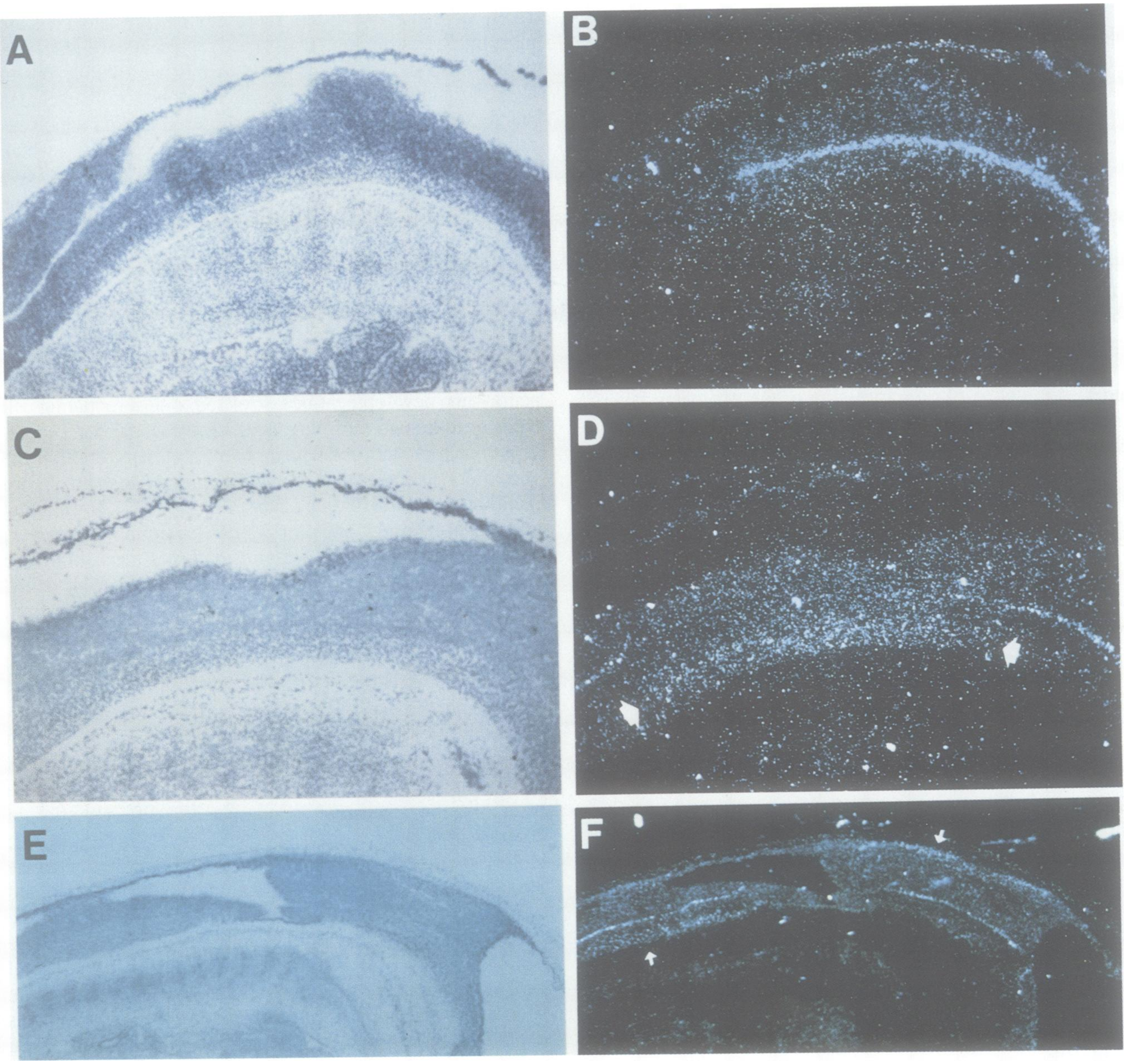

Fig. 8. *In situ* hybridization of *Evx 1* to day 10–12 p.c. embryos. Magnification of boxed areas in Figure 7 are shown. Panels (**B**, **D** and **F**) are darkfield images of brightfield images in (**A**, **C** and **E**), respectively. Anterior is to the right, posterior to the left. (A and B) Day 10 p.c. embryo. (C and D) Day 11 p.c. embryo. The area between arrows in (D) marks weak *Evx 1* expression in the ventral spinal cord. (E, F) day 12 p.c. embryo. Arrows in (F) indicate *Evx 1* expression in the ventral and dorsal spinal cord.

intermediate zone has begun to form. Since early forming neuroblasts are displaced laterally by later forming cells (Nornes and Carry, 1978), *Evx 1* might be required at a certain time during the pattern of neuron differentiation and might be expressed when a specific cell population passes through a specific phase. In this respect, *Evx 1* expression may occur at the time when commissural interneurons generate their axons.

To summarize, the expression pattern of *Evx 1* in the CNS suggests that it is either involved in the mechanism controlling specific neuronal fates and/or more specifically in the process of commissural and ipsilateral axon growth during neuronal differentiation since its expression pattern coincides with the very early appearance of differentiating interneurons. In this respect, *Evx 1* may play a similar role to the *Drosophila eve* during neurogenesis, for *eve* may function in the mechanism controlling cell fate and may regulate genes that control the axonal morphology of at least two of three identified neurons (Doe *et al.*, 1988). *Evx 1* may be useful as an early molecular marker for specific differentiating neuroepithelial cells and/or 'intermediate' cells.

Similarities between murine and Drosophila developmental genes within the CNS

For all genes investigated to date, vertebrate homologues of *Drosophila* developmental control genes, expressed in the embryonic CNS of the fruitfly, are also transcribed in the developing vertebrate CNS (Doe and Scott, 1988; Keynes and Stern, 1988). Recent studies (Lumsden and Keynes, 1989; Murphy *et al.*, 1989; Wilkinson *et al.*, 1989a,b) provide molecular and cellular evidence that the well described rhombomeres of the hindbrain and probably the

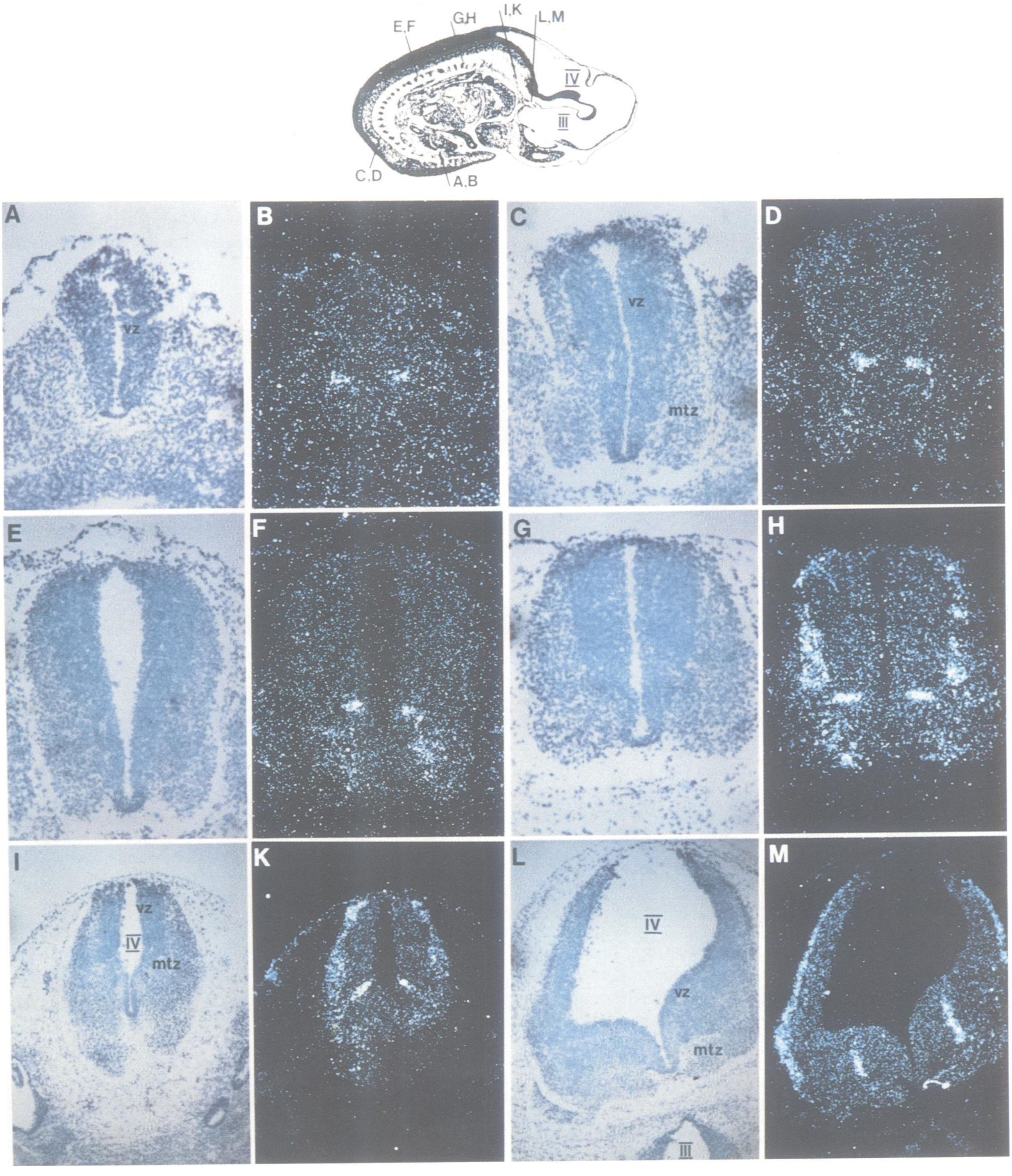

Fig. 9. *In situ* hybridization of *Evx 1* to p.c. 12 day embryos. Transverse sections of the neural tube at various levels from caudal (tail) to rostral (hindbrain). The schematic drawing shows levels of the neural tube sections. Panels (**A**, **C**, **E**, **G**, **I** and **L**) are brightfield images of darkfield images (**B**, **D**, **F**, **H**, **K** and **M**), respectively. Abbreviations: vz, ventricular zone; mtz, mantle zone; IV, fourth ventricle; III, third ventricle.

neuromeres of the spinal cord (Orr, 1887; Streeter, 1908; Neal, 1918; Vaage, 1969; Tuckett *et al.*, 1985; Sakai, 1987) represent segments. Unlike *eve* in the embryonic epidermis of the fruitfly, *Evx 1* appears not to be involved in establishing segmentation of the mouse CNS, since the *in situ* hybridization gives no indication of a segmental ‘pair rule’ pattern of *Evx 1*; not even in the hindbrain of day 8.5–9.5 p.c. embryos when rhombomeres are formed and segmentation is most obvious.

Although the vertebrate CNS might develop on a segmental basis (see above, and Keynes and Stern, 1988), it does not on the other hand function in segmentally independent units but as an integrated apparatus. While in arthropods a pair of ganglia, their appropriate sense organs and

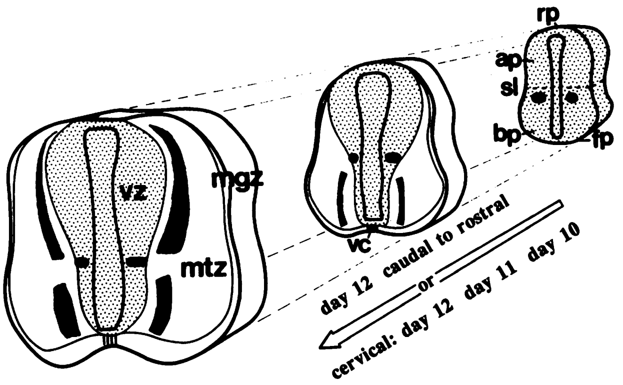

Fig. 10. Schematic illustration showing *Evx 1* expression in sections of the neural tube at different stages of development. Abbreviations: ap, alar plate; bp, basal plate; fp, floor plate; rp, roof plate; sl, sulcus limitans; mtz, mantle zone; mgz, marginal zone; vz, ventricular zone; vc, ventral commissure. Black areas represent *Evx 1* expression.

muscles of a single segment represent such an independent functional unit, in higher vertebrates neural connections within the spinal cord convey information to the brain. Therefore, neurons from different antero-posterior levels of the spinal cord are organized in longitudinal columns to integrate functions to the brain in contrast to the segmentally arranged CNS of lower organisms such as *Drosophila.* Comparison of the expression patterns in the neural tube of the mouse *Evx 1, Pax 2, En 1, Int 1* (Davidson *et al.*, 1986; Wilkinson *et al.*, 1987; Davis and Joyner, 1988; Nornes *et al.*, 1990) and Hox genes (see Holland and Hogan, 1988 for review; Wilkinson *et al.*, 1989a,b) with those of their most closely related counterparts in the embryonic CNS of the fruitfly reveals striking similarities. In *Drosophila en, eve* (Doe and Scott, 1988), *wg* (Patel *et al.*, 1989a) and *pox neuro* (Bopp *et al.*, 1989) are expressed in specific neurons in every segment, whereas homeotic genes are expressed in only some segments of the CNS, with specific patterns (Doe and Scott, 1988). In the mouse, *Evx 1, En 1, Int 1* and *Pax 2* are expressed in transversally restricted regions along the entire neural tube including the hindbrain, whereas Hox genes are transcribed in antero-posterior domains or specific segments within wide transversal areas. In analogy to *Drosophila,* the highly developed vertebrate CNS might use Hox genes for specifying regional CNS differentiation along the antero-posterior axis and *Evx 1*, as well as the other homologs mentioned, for specifying neuronal identities and/or neuronal differentiation processes in the transverse plane along the entire antero-posterior axis. The expression patterns of these genes could therefore reflect longitudinal compartments within the spinal cord. In the mouse, the large number of Hox genes, *Evx 1, En 1, int 1* and *Pax* genes produce a great variety of overlapping expression patterns with different medio-lateral, ventro-dorsal and antero-posterior extensions within the neural tube which could account for the development of the functional diversity of the CNS in higher vertebrates. Thus it is conceivable that the development and establishment of the vertebrate CNS utilizes an evolutionarily conserved regulatory system.

The Evx 1 expression pattern is biphasic

Evx 1 is expressed in a distinct temporal and spatial pattern during embryogenesis from days 7–9 p.c. and from days 10–12.5 p.c., but could not be detected between days 9 and 10 p.c. The first phase of expression can be seen in the ectoderm, mesoderm and neuroectoderm, the second is restricted to ectoderm derived tissue. The later pattern of *Evx 1* cannot be interpreted as an extended pattern of earlier stages, because the appearance of *Evx 1* transcripts and the maturation of the neural tube occur in opposite directions. Therefore we suggest a second, independent induction of *Evx 1* sometime between days 9 and 10 p.c. It might be of evolutionary significance that *eve* and *Xhox-3* also show two different, independent periods of expression: a late expression pattern indicating a function in the developing nervous system and an early pattern which may, in the broadest sense, contribute to the establishment of the embryonic axis. The expression of most *Antp*-like mouse homeobox genes appears sometime during gastrulation and extends continuously to their anterior limits until the midgestation stage. If indeed homeobox genes are setting up positional information, Hox genes may be important in defining antero–posterior regions of the embryonic axis and *Evx 1,* in contrast, may be involved in establishing this antero-posterior axis.

Materials and methods

Genomic and cDNA library screening

Approximately 9×10^5 and 1.2×10^6 clones of a genomic BALB/c mouse liver library (Clontech) and an embryonic λgt 10 C57 BL mouse day 8.5 p.c. cDNA library (Fahrner *et al.*, 1987), respectively, were plated on 24 × 24 cm dishes, transferred to nylon membranes (Amersham) and crosslinked under 309 nm UV light. Genomic filters were hybridized at 65 °C with an oligolabelled (Feinberg and Vogelstein, 1983) *Hin*fI–*Bst*NI fragment of the *eve* cDNA sequence containing the homeobox (Frasch *et al.*, 1988) and washed at room temperature under low stringency conditions (hybridization: 6 × SSC, 0.5% SDS, 5 × Denhardts, 100 μg/ml salmon sperm DNA; washing 2 × SSC, 1% SDS). The cDNA library was probed with an oligolabelled *Pst*I–*Pst*I fragment of the genomic *Evx 1* clone (Figure 1) isolated from the genomic library under more stringent conditions, salt was reduced to 4 × SSC in the hybridization solution and washing was performed at 42 °C.

DNA sequencing

Overlapping M13 subclones of the genomic *Evx 1* and *Evx 2* clones and the *Evx 1* cDNA clone were generated both in M13mp18 and M13mp19. Nucleotide sequences were determined by the dideoxy method (Sanger *et al.*, 1977) using an M13 sequencing kit (Sequenase, US Biochemicals; T7, Pharmacia).

Cells, embryos and RNA isolation

F9 cells (Bernstine *et al.*, 1973) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were induced to differentiate into parietal endoderm by application of 5×10^{-7} M RA and 10^{-3} M cAMP (Strickland *et al.*, 1980). Embryos for Northern blot and *in situ* analysis were obtained from natural matings of female NMRI mice, and midday of the day of the vaginal plug was designated as day 0.5 p.c. Total RNA samples were isolated by homogenizing cells and embryos in guanidinium thiocyanate and polyadenylated RNA was obtained after elution from oligo(dT)–cellulose columns according to Ausubel *et al.*, (1989).

Northern blotting

Polyadenylated RNA samples were electrophoresed through 1% agarose gels in 3.7% formaldehyde and 1 × MOPS buffer (20 mM morpholine propane sulphonic acid, 50 mM Na-acetate, 10 mM EDTA, pH 7.0) and blotted onto nylon membranes with 10 × SSC. Nucleic acids were crosslinked under 309 nm UV light and the filters were then hybridized under high stringency conditions in a sodium phosphate buffer (500 mM NaPi, pH 7.2, 7% SDS, 1 mM EDTA) at 65 °C. Washing was performed with 40 mM NaPi buffer (pH 7.2) containing 1% SDS at 65 °C 3 times for 5 min followed by another incubation of 15 min.

In situ *hybridization and RNA probes*

The protocol used was basically that of Hogan *et al.* (1986), with some modifications, and is essentially as follows. Embryos were removed from the surrounding tissue in cold phosphate buffered saline (PBS), immediately frozen and embedded in OCT medium (Miles Laboratory) using a cold chamber placed on dry ice. Sections were cut at 8 μm and −20 °C in a cryostat, dried onto gelatine-subbed slides for 10 min at 55 °C and fixed in 4% *p*-formaldehyde. For hybridization, sections were pretreated in 2 × SSC for 30 min at 70 °C, subjected to proteinase K treatment (10 min), refixed in 4% *p*-formaldehyde (20 min), acetylated (12 min) and dehydrated at room temperature.

Single-stranded RNA probes were transcribed from a *Pst*I−*Pst*I *Evx1*-fragment cloned into a Bluescript M13 vector using T3 and T7 RNA polymerases. High specific activity RNA was prepared from the coding and non-coding strands of the fragment using [α-^{35}S]UTP and [α-^{35}S]CTP (each > 800 Ci/mmol) for *in vitro* transcription.

Probes were dissolved at a final activity of 5×10^4 c.p.m./μl in hybridization buffer (50% formamide, 10% dextran sulphate, 10 mM Tris, pH 7.5, 10 mM NaPi, pH 6.8, 5 mM EDTA, 10 mM DTT, 10 mM β-mercaptoethanol, 1 mM ADP-β-S, 0.1 mM UTP, 10 μM S-ATP, 2 × SSC, 150 μg/ml salmon sperm DNA, 150 μg/ml yeast tRNA). Approximately 6−10 μl were used, depending on the size of the coverslip. Hybridization was performed in a chamber humidified with 50% formamide, 2 × SSC, 10 mM β-mercaptoethanol overnight at 42 °C. Slides were washed in this buffer (prewarmed at 37 °C) for 2 h, digested with RNase A (20 μg/ml) in 0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol and again washed overnight at 37 °C. Then slides were dehydrated on graded alcohol, immersed in Kodak NTB-2 (diluted 1:1 with water) for autoradiography and exposed for 8−10 days at 4 °C until developed (Kodak D19 developer for 3 min, 1% acetic acid 30 s, 30% $Na_2S_2O_3 \times 5H_2O$ 3 min). Finally, slides were stained with Giemsa for light microscopy.

Acknowledgements

We thank Michael Levine for providing the *eve* cDNA clone, and Karen Fahrner and Brigid Hogan for providing the λgt cDNA library. We thank Rudi Balling for the genomic Southern blots for gene mapping and are very indebted to Jean-Louis Guenet for his help with the chromosomal assignment of the *Evx 1* gene locus. We are also grateful to Howard Nornes for helpful interpretations concerning the expression data, and to Gregory Dressler, Andreas Püschel and Claudia Walther for critical comments on the manuscript. We recently learned that two *Evx* genes have also been isolated in the laboratory of Gail Martin. The proposed nomenclature was accepted by the 'Committee on Standardized Genetic Nomenclature for Mice'. This work was supported by the Max-Planck Society.

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Received on October 26, 1989; revised on February 28, 1990