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Expression of four zebrafish *wnt*-related genes during embryogenesis

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Summary

The *wnt* gene family codes for a group of cysteine-rich, secreted proteins, which are differentially expressed in the developing embryo and are possibly involved in cellular communication. Here, we describe the polymerase chain reaction based cloning and embryonic expression patterns of four zebrafish *wnt*-related sequences; *wnt[a]*, *wnt[b]*, *wnt[c]* and *wnt[d]*. One of these genes, *wnt[a]*, is a potential homologue of murine *Wnt-3*, while the other three genes most likely represent new members of the vertebrate *wnt* gene family. In zebrafish embryos, transcripts of *wnt[a]* are confined to the dorsal diencephalon, the dorsal midbrain, the rhombic lips and the dorsal portions of the spinal cord. *wnt[b]* is expressed in the tail bud and at considerably lower levels in the mesoderm of the head. *wnt[c]* transcripts are present within the diencephalon and the posterior midbrain whereas *wnt[d]* shows a surprisingly similar expression pattern to zebrafish *wnt-1*. By analogy to *wnt-1*, it is likely that the members of the zebrafish *wnt* gene family play an important role in cell-to-cell signalling during pattern formation in the neural tube and the tail bud.

Key words: diencephalon, embryogenesis, midbrain, spinal cord, tail bud, *wnt*-related genes, zebrafish.

Introduction

The proto-oncogene *int-1* (now renamed *Wnt-1*; Nusse et al., 1991) was discovered by the analysis of integration sites of mouse mammary tumor virus (MMTV) in virus-induced mammary tumors (Nusse and Varmus, 1982; for review, see Nusse, 1988). A link between tumor biology and developmental biology was established when Jakobovits et al. (1986) showed that the *Wnt-1* gene was transcribed in mice between days 8 and 13 of development. Shortly thereafter, Wilkinson et al. (1987) presented an extensive series of in situ hybridization experiments demonstrating that *Wnt-1* expression is restricted within the developing central nervous system (CNS). Recently, a crucial role for *Wnt-1* in murine CNS development has been demonstrated by targeted gene disruption (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Interestingly, the gene disruption did not affect the development of all regions in the CNS that express the *Wnt-1* gene. One reason could be a redundancy of *wnt* genes enabling another gene to substitute partly for the loss of *Wnt-1* functions.

In 1987, Rijsewijk et al. showed that the *Drosophila* homolog of *Wnt-1* is the segment polarity gene *wingless*, a gene required for the proper formation of individual segments. Since then, the identification of genes related to *Wnt-1* demonstrated the existence of a large gene family comprising at least 10 members in mouse (Wainwright et al., 1988; McMahon and McMahon, 1989; Roelink et al., 1990; Gavin et al., 1990; Nusse et al., 1991), six members in *Xenopus* (Noordmeer et al., 1989; Christian et al., 1991a,b) and three members in humans. The deduced amino acid sequence homologies of different wnt proteins vary within species from 40% to 87% identity. The predicted relative molecular masses of the proteins range from 39 to 42 kDa and they contain an amino-terminal hydrophobic leader sequence, conserved cysteine residues and several potential N-linked glycosylation sites.

Biochemical studies of the murine Wnt-1 protein show that it is processed and glycosylated (Brown et al., 1987, Papkoff et al., 1987) and enters the secretory pathway (Papkoff et al., 1987; Papkoff, 1989; Bradley and Brown, 1990; Papkoff and Schryver, 1990), but remains associated with the cell surface or the extracellular matrix (Bradley and Brown, 1990; Papkoff and Schryver, 1990). It therefore seems likely that wnt proteins are secreted and play a role in short range cell-to-cell communication. Consistent with this notion, the *Drosophila* wingless protein affects the fates of neighboring, engrailed-expressing cells (Bejsovec and Martinez-Arias, 1991).

The expression of the different vertebrate *wnt* transcripts during embryogenesis show, in most cases, highly restricted patterns in the CNS and in mesodermal tissues (e.g. see Wilkinson et al., 1987; McMahon and McMahon, 1989; Roelink and Nusse, 1991). The transcripts of murine *Wnt-*

5A, however, correlate at early developmental stages to a posterior position in the embryo rather than to specific tissues (Gavin et al., 1990) and the expression of the most divergent member of the gene family, *Xenopus Xwnt-8* (40% identity to *Xenopus Xwnt-1*), is primarily confined to the ventral mesoderm of the gastrulating embryo (Christian et al., 1991b).

In this study, we report the characterization of four zebrafish *wnt* clones; *wnt[a]*, *wnt[b]*, *wnt[c]* and *wnt*[*d*], and an analysis of their spatial and temporal expression during embryogenesis.

Materials and methods

Isolation of zebrafish wnt *sequences by PCR*

The PCR (polymerase chain reaction) reaction was performed on 0.5 μg zebrafish genomic DNA using the following oligonucleotides:

wnt-x 5′-dCAG/AGATGC/TAAGATGC/TCAC/TGG-3′;
wnt-y 5′-dCAG/ACACCAA/GTGA/GAANNNG/ACA-3′.

The reaction conditions were: 30 cycles with denaturation for 1 minute at 94˚C, annealing for 1 minute at 58˚C, and extension for 2 minutes at 72˚C using Taq DNA polymerase (Perkin-Elmer-Cetus) according to the manufacturer's instructions. PCR products were run on a low-melting-point agarose gel and reamplified (20 cycles: denaturation for 1 minute at 94˚C, annealing for 1 minute at 55˚C, extension for 2 minutes at 72˚C) in the presence of kinased oligonucleotides. To subclone the PCR fragments, the reaction mix was extracted with chloroform and blunt ends were created with Klenow polymerase (Sambrook et al., 1989). Then the PCR fragments were again run on a low-melting-point agarose gel and the bands were excised, heated for 10 minutes at 70˚C and ligated (in 70 mM Tris-HCl, pH 7.5, 20 mM dithiothreitol, 2 mM $MgCl_2$ and 0.5 mM ATP in the presence of the agarose) at room temperature into the *Sma*I site of the Bluescript M13 phagemid (Stratagene).

DNA sequencing and analysis

The PCR inserts were sequenced on both strands after subcloning in M13mp18 and M13mp19 vectors using Sequenase (US Biochemicals). DNA sequences and derived amino acid sequences were analyzed on a VAX/VMS computer using the GCG software package (version 7.0; Devereux et al., 1984).

In situ hybridization on tissue sections

In situ hybridization and autoradiography were performed as described by Krauss et al. (1991b) with the following changes: the ^{35}S-labelled DNA probes were made from the entire subcloned PCR fragments of clones *wnt*[*a*], *wnt*[*b*], *wnt*[*c*] and *wnt*[*d*]. The hybridization and washing steps were carried out at 45˚C and the sections were exposed for 14 days. Photomicrographs were taken using a Nikon Microphot FXA photomicroscope.

Results

Isolation of four zebrafish wnt *clones by PCR*

On the basis of a comparison between murine int-1 (Wnt-1),irp (Wnt-2) and *Drosophila* wingless (wg) (taken from Wainwright et al., 1988), two sets of degenerate oligonucleotide primers were designed. The primers were chosen from short, highly conserved amino acid motifs within the C-terminal portion of the wnt proteins. The 5′ set encompassed the amino acid sequence QECKCHG (comprising amino acid positions 237-243 of *Drosophila* wg; Fig.1A), whereas the 3′ set encompassed the sequence CXFHWCC (comprising amino acid positions 453-459 of *Drosophila* wg; Fig.1A). Using the two primers for a PCR amplification with an annealing temperature of 55˚C on genomic DNA, two major bands of approximately 380 and 400 base pairs and a ladder of different-sized minor bands were generated (Fig.1B). However, after increasing the annealing temperature to 58˚C, only the two major bands remained (Fig. 1B). The two bands were subcloned, and a subsequent digestion with *Xba*I/*Sal*I and *Rsa*I revealed several classes of different clones (not shown).

To identify clones with spatially restricted expression patterns during early zebrafish development, we performed in situ hybridizations with 10 independent clones on 16 hour zebrafish embryos (not shown). Four of the 10 clones showed a distinct expression pattern (for summary, see Fig.8). These clones were sequenced and the deduced amino acid sequences were compared with the published murine (see Gavin et al., 1990) and *Xenopus* (Christian et al., 1991a) wnt sequences (Fig.2A-D). On the basis of this comparison, the zebrafish wnt sequences were designated wnt*[a]*, wnt*[b]*, wnt*[c]* and wnt[d] (Fig.2). Owing to some exonuclease activity during the subcloning at room temperature, clone wnt*[b]* and clone wnt*[c]* are truncated at their N-terminal and COOH-terminal ends, respectively.

The comparison of the derived amino acid sequences of the zebrafish clones to murine and *Xenopus* wnt proteins indicate several relationships. wnt*[a]* shares more homology with murine Wnt-3 (Roelink and Nusse, 1991) and *Xenopus* Wnt-3 (Christian et al., 1991a) than with all other related proteins (90.1% identity; Fig. 2A). Similarly, clone wnt*[b]* seems closely related to murine Wnt-5a and Wnt-5B (89.2% and 87.2% identity, respectively; identity to *Xenopus* Xwnt-5A: 83.3%; Fig.2B). However, considering also the expression pattern of *wnt[b]* (Fig.5A-E), it is questionable whether this gene is a direct homologue to either of the murine *Wnt-5* genes (see Gavin et al., 1990 and Christian et al., 1991a,b). wnt*[c]*, although showing highest homology to murine Wnt-7B (72.2% sequence identity; Fig.2C; Gavin et al., 1990), does not seem to have an identified homologue in other species. Surprisingly, we also obtained a clone that exhibits a similar expression pattern as *wnt-1* (Fig.7A-D) but shows only 43% identity to zebrafish wnt-1 (Molven et al., 1991) in its derived amino acid sequence (Fig. 2D). Apart from *Xenopus* Xwnt-8, wnt[d] is the most divergent member of the wnt family described so far (see Fig. 2E).

As expected for putative signalling peptides of the wnt family, all cysteine residues throughout the deduced sequences of the four zebrafish clones are absolutely conserved (Fig.2A-D). Furthermore, we find a highly conserved putative N-linked glycosylation site, which is encoded by all four zebrafish *wnt* genes (e.g. at position 91-93 of wnt*[a]*; see Fig.2A) and the compared mouse (Gavin et al., 1990) and *Xenopus* (Christian et al., 1991a,b) sequences (Fig.2A-D; in this context it might be noteworthy that only

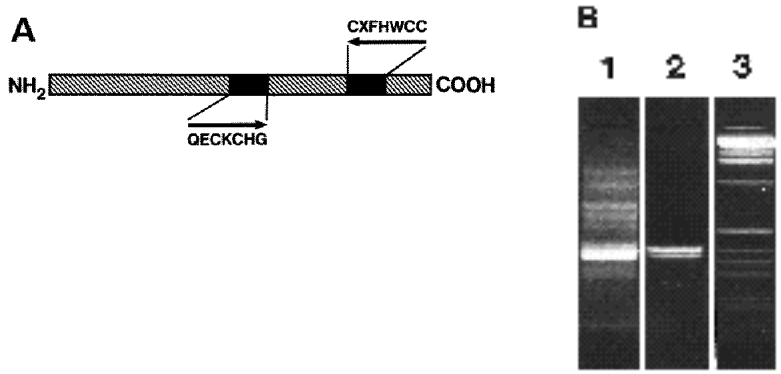

Fig. 1. PCR strategy for cloning of zebrafish *wnt*-related sequences. (A) Scheme representing the conserved amino acid regions within wnt-1 selected for construction of degenerate oligonucleotides. X indicates an unspecified amino acid. (B) PCR products obtained at different annealing temperatures and separated on a 1.6% agarose gel: annealing at 55˚C (lane 1); annealing at 58˚C (lane 2); size marker (1 kb ladder; lane 3).

the *Xenopus* Xwnt-8 sequence does not share this glycosylation site; see Christian et al., 1991b). A second putative N-linked glycosylation site is shared by the members of the "Wnt-5 subfamily" and *Xenopus* Xwnt-8 (position 70-72 of wnt*[b]*; see Fig.2B).

To determine the spatial and temporal expression of the four zebrafish *wnt* genes in the course of development, we performed in situ hybridizations on serial transverse and sagittal tissue sections of embryos at 12 hours, 16 hours and 24 hours of development.

Expression of wnt[a] and wnt[b] in the tail bud

At 12 hours of development, *wnt[a]* expression is seen within a circumscribed region in the tail of the embryo that includes both ectodermal and mesodermal tissues (Fig. 3A,B). As in the case of *wnt[a]*, the expression of *wnt[b]* is detected within the tail of the embryo at the same developmental stage (Fig. 5A,B). The *wnt[b]* signal in this area extends more anteriorly compared to *wnt[a]*. Higher levels of *wnt[a]* and *wnt[b]* expression were detected in the zebrafish tail at 16 hours of development (Figs 3C,D;5C,D;8). Neither transcript showed any tissue-specific localization in the tail bud, but rather correlated with posterior positions. Whereas *wnt[a]* transcripts are restricted to the tip of the tail bud (Fig. 3C,D), *wnt[b]* transcripts include the area of *wnt[a]* expression but extend in a graded fashion further anteriorly (Fig. 5C,D). In addition, a considerably weaker staining with the *wnt[b]* probe is detected in the tissue underlying the midbrain ventrally (not shown). By 24 hours of development, high levels of *wnt[b]* transcripts were apparent within the tail bud in tissues such as the neuroectoderm and the somitic mesoderm (Fig. 5E,F), whereas *wnt*[*a*] transcripts were no longer detected within this area (not shown). Interestingly, the spatial distribution of the *wnt[b]* transcripts does not coincide with the expression patterns reported for the murine genes that show the highest homology to *wnt[b]* (Gavin et al., 1990). Unlike *wnt[b]*, murine *Wnt-5b* is transcribed uniformly. Murine *Wnt-5a* is expressed in the tail bud, but transcripts of this gene are also seen in the ventral portion of the entire midbrain, the hindbrain and the spinal cord (Gavin et al., 1990), regions that do not express *wnt[b]* (see Fig. 5C,D).

Expression of wnt[a] in the neural keel

Within the neural keel (in teleost fishes, the neural tube is preceded by a solid cord of ectodermal cells, the "neural keel"), *wnt[a]* expression is first detected at approximately 12 hours. We found a weak staining localized in the dorsal portion of the presumptive midbrain (Fig.3A,B). No expression was detected in the forebrain and in the rostral region of the hindbrain that will give rise to the cerebellum. However, dorsally located transcripts extended continuously from the more caudal portions of the hindbrain to the spinal cord (Fig. 3A,B). Transverse sections show that the positive cells appear as a semi-circle along the dorsal wall of the hindbrain (Fig.4A,B). At 16 hours of development, the expression pattern of *wnt[a]* seems to be comparable to the pattern described for the murine *Wnt-3* transcripts (Roelink and Nusse, 1991). The *wnt[a]* expression is confined to the dorsal portion of the midbrain and the developing diencephalon, where it shows a rostral boundary that divides the diencephalon vertically at the position of the presumptive epithalamus (Figs 3C,D; 8; for transverse sections, see Fig. 4C,D). The caudal boundary of the *wnt*[*a*] expression within the presumptive brain marks a narrow band along the junction between the midbrain and the cerebellum (Figs 3C,D; 4C,D; 8), which overlaps with the posterior border of the *wnt-1* expression (Molven et al., 1991). No *wnt[a]* signal is seen within the presumptive cerebellum, but the expression reappears as a band over the rhombic lips and continues in the roof plate of the spinal cord. The expression pattern of *wnt[a]* remains basically unaltered until 24 hours of development, when morphological subdivisions of the neural tube are more apparent (Figs 3E,F; 4E,F). At 36 hours of development, we did not detect *wnt[a]* transcripts in the rostral brain (not shown), and only the dorsal band in the hindbrain and spinal cord continued to express the gene (Fig. 3G,H; for cross-sections through the spinal cord see Fig. 4G,H).

Expression of wnt[c] in the neural keel

wnt[c] does not show any similarity to the expression patterns reported for other members of the vertebrate *wnt* gene family. At 12 hours of development, no transcripts of the gene could be detected with in situ hybridization on tissue sections (Fig. 6E,F). At 16 hours, transcripts of the gene are observed exclusively in the neural keel where they are restricted to two areas: the rostral portion of the diencephalon and a triangular shaped dorsocaudal portion of the midbrain (Figs 6A,B; 8). As seen from transverse sections in Fig. 6G,H, *wnt[c]* expression is distributed throughout the entire wall of this sector of the neural tube. The level

of transcripts decreases significantly at 24 hours and no signals could be detected in tissue sections derived from 36 hour embryos. As seen in Fig. 6C and D, the posterior boundary of the *wnt[c]*-expressing area in the diencephalon is located in front of the epiphysis and, judging from in situ hybridizations on parallel sections (not shown), is slightly

A

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                                                                                  70
Zwnt[a]  LSGScEVKTcWWSQPDFRVIGDYMKDKYDSASEMVVEKHRESRGWVETLWPKYPYYKPPTETDLVYYESS
Xwnt-3   ........................L.............Q...........R...TFFR....R..I.....
Mwnt-3   ............A.....A...FL.............................RA..ALF.....R......N.
Mwnt-3a  ..................T...FL.............................R.R.T.F.V...R......A.

                                                                 121
Zwnt[a]  PNFcEPNPETGSFGTRDRTcNLTSHGIDGcDLLccGRGHNTRTEKRKEKcH
Xwnt-3   ...................V..VS.............................          90.1 (96.7)
Mwnt-3   ...................V..V..............................          90.1 (95.9)
Mwnt-3a  ...................VS.................A...R.R....          87.6 (96.7)
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B

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                                                                                  70
Zwnt[b]  VSGScSLKTcWLQLADFRRVGEFLKEKYDSAAAMRINRRGKLELVNNRFNPPTGEDLVYIDPSPDYcLRN
Mwnt-5b  ..............E..K..DR.............T.Q.....A.S...Q..P.....V.........
Mwnt-5a  ..................K..DA............L.S....VQ..S...S..TQ...........V..
Xwnt-5a  ..........Y.......-..DL........S..KL.T....VQ...K..S..MN...........VH.

                                                  102
Zwnt[b]  ETTGSLGTQGRLcNKTSEGMDGcELMccGRGY
Mwnt-5b  ..............................          89.2 (94.1)
Mwnt-5a  .S............................          87.2 (95.1)
Xwnt-5a  .S............................          83.3 (95.1)
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C

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                                                                                  70
Zwnt[c]  TKTcWTTLPKFREIGYVLKERYTTALEVEAVRATRFRQPSFLRLKQSRGYIKPTDTDLVFLERSPNYcEE
Mwnt-7b  .............V.HL...K.NA.VQ..V...S.L...T...I..L.S.Q..ME....YI.K.......
Mwnt-7a  .........Q...L.....DK.NE.VH..P...S.NKR.T..KI.KPLS.R..M.....YI.L.......

                                                                 115
Zwnt[c]  DTVTGSAGTRGRLcNHTSPLTDGcNLMccGRGHNTHQYTRVWQcN
Mwnt-7b  .AA...V..Q....R...GA...DT......Y......K.....          72.2 (88.7)
Mwnt-7a  .P....V..Q..A..K.A.QAS..D.......Y.....A......          67.8 (85.2)
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D

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                                                                                  70
Zwnt[d]  TSGScQFKTcWYVSPEPRLVGSLLREKFLTAIFINSQNKNNGVFNSR-TGGSTGSDPLRGQRRRSISREL
Zwnt-1   M....TVR...MRL.S.....DY.KDR.DG.SRVVYA..GSNRASH.--ADPRHLE.ENPAHKLPS..D.
Xwnt-1   M....SL....MRL.P..S..DA.KDR.DG.SKVTYS.NGSNRWG..--SDPPHLE.ENPTHALPS.QD.
Mwnt-1   M....TVR...MRL.TL.A..DV..DR.DG.SRVLYG.RGSNRA-...--AELLRLE.EDPAHKPPSPHD.

                                                                 127
Zwnt[d]  VYFEKSPDFcDREPAVDSLGTQGRIcNKSSPGMDGcGSLccGRGHNILKQARSERcH
Zwnt-1   .......N..SYNGKTGTH..S..T..S...AL...EL......YKTRMEQVT....          42.9 (60.3)
Xwnt-1   .......N..SPSEKNGTP..T.....ST.L.L...EL......YRS.AEKVT....          42.9 (57.9)
Mwnt-1   .......N..TYSGRLGTA..A..A..S...AL...EL.......RTRT.RVT...N          41.3 (59.5)
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E

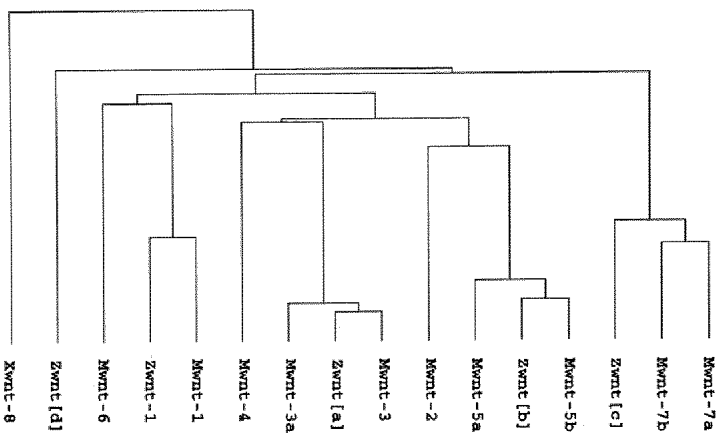

Fig. 2. (A-D) Comparison of the gene-derived amino acid sequences of the zebrafish wnt clones to representative members of the mouse and *Xenopus* wnt family. The conserved cysteine residues (small, bold lettering) and potential N-linked glycosylations sites (underlined) as well as chemically similar amino acid residues (bold) are indicated. The percentage identity of the zebrafish sequences to the murine and *Xenopus* sequences (as well as wnt[d] to wnt-1) are indicated at the right margin of each panel with the percentage similarity (after including changes to chemically similar amino acids scored according to Johansen et al. (1989) shown in parenthesis. (E) A dendrogram showing the result of a multialignment of the 10 different murine wnt proteins, *Xenopus* Xwnt-8 and the five zebrafish wnt protein sequences. The positions used in the alignment corresponds to positions 225 to 349 of wnt-1. The length of the vertical components in the figure indicate sequence divergence. To distinguish wnt proteins from different species, a Z has been added in front of the zebrafish sequences, a M stands for murine and a X for *Xenopus* sequences.

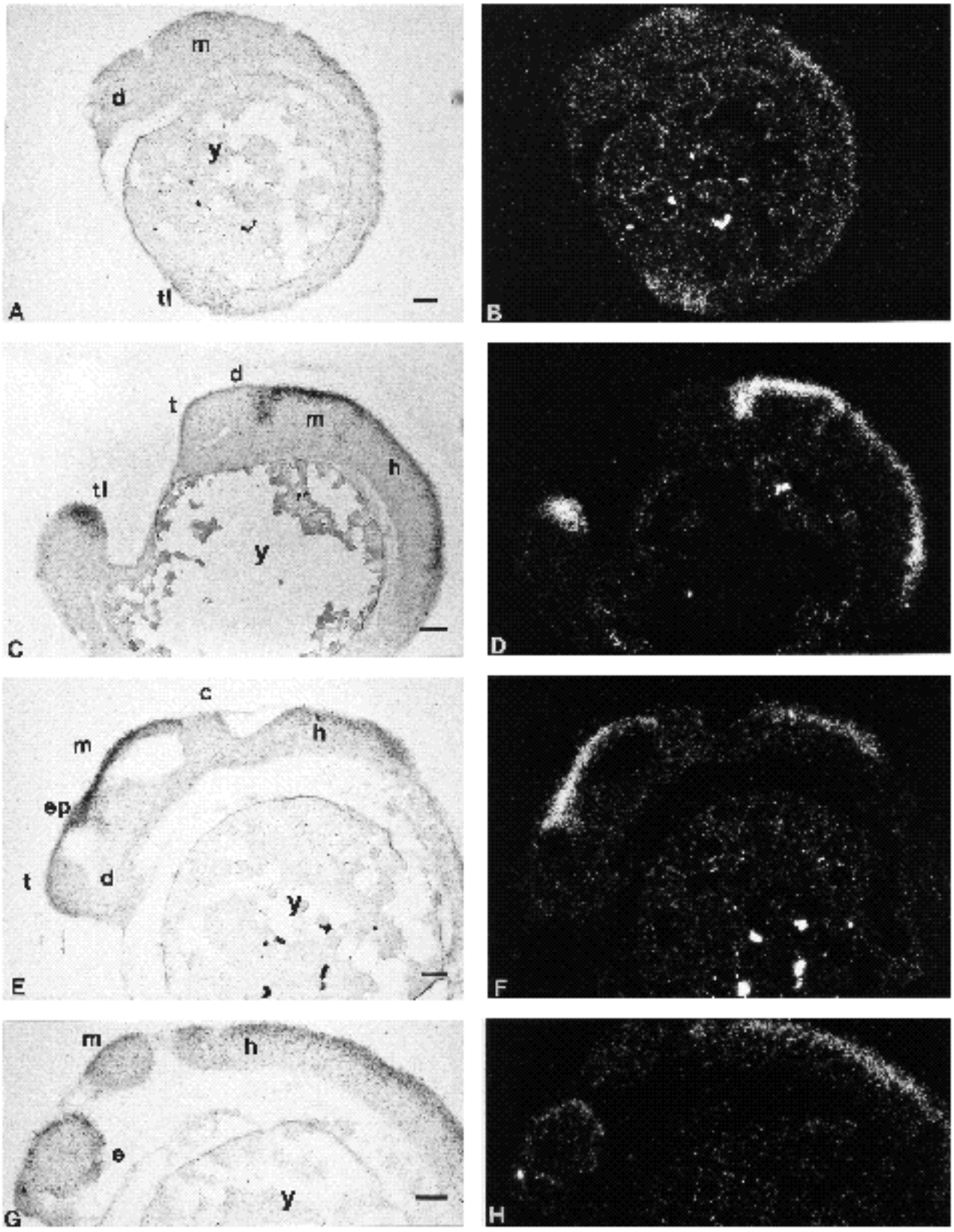

Fig. 3. Localization of *wnt*[*a*] transcripts by in situ hybridization on tissue sections of zebrafish embryos at different developmental stages. Sagittal sections are shown for embryos after 12 hours (A,B), 16 hours (C,D), 24 hours (E,F) and 36 hours (G,H) of development. Bright-field (A,C,E,G) and dark-field (B,D,F,H) images are shown side by side. The embryos are oriented with their anterior end to the left. The embryo in C,D is slightly turned. Abbreviations: c, cerebellum; d, diencephalon; e, eye; ep, epiphysis; h, hindbrain; m, midbrain; t, telencephalon; tl, tail; y, yolk. Bars, 50 μm.

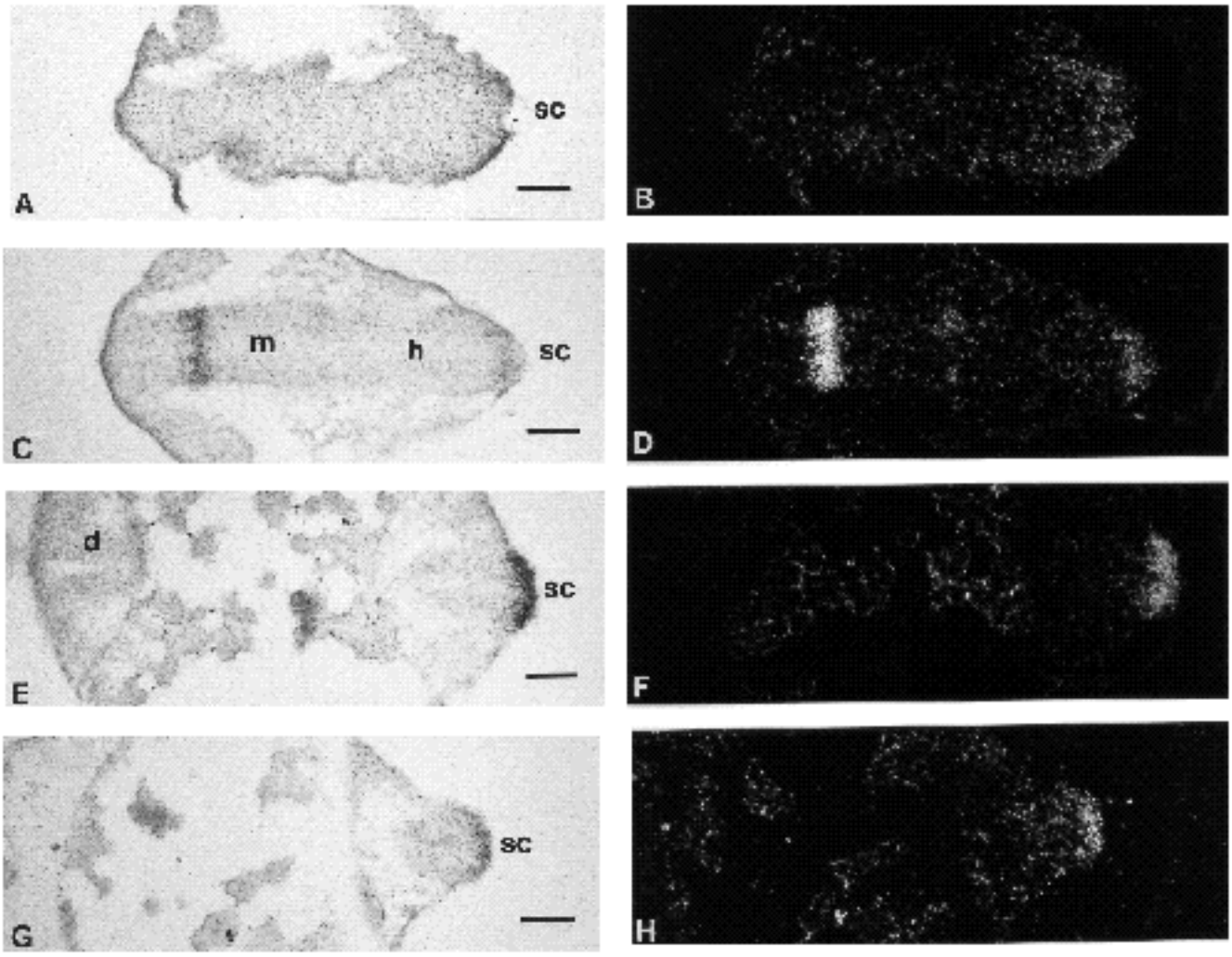

Fig. 4. Distribution of *wnt*[*a*] transcripts in cross sections of embryos at 12 hours (A,B), 16 hours (C,D), 24 hours (E,F) and 36 hours (G,H) of development. The embryos are oriented with the anterior end to the left. (A,C,E,G) Bright-field micrographs; (B,D,F,H) dark-field micrographs. Abbreviations: d, diencephalon; h, hindbrain; m, midbrain; sc, spinal cord. Bars: 50 μm.

overlapping or directly adjacent to the anterior boundary of the *wnt[a]*-expressing area. Rostrally, the *wnt[c]*-expressing area includes the anterior part of the presumptive thalamus. No signal is detected in the hypothalamic nuclei and the optic stalk (Fig.6A-D).

Expression of wnt[d] in the neural keel

wnt[d] shows an expression pattern that is similar to the pattern described for zebrafish *wnt-1* (Molven et al., 1991) for 18-hour embryos. As in the case of *wnt-1*, transcripts of *wnt*[*d*] are confined at 16 hours to the dorsal midline of the midbrain, the hindbrain and the spinal cord (Figs 7A-D; 8). Signal is also seen in a transverse band at the boundary between midbrain and hindbrain and underneath the anlage of the epiphysis (Figs. 7A-D; 8). Analogous to *wnt-1*, we find a gap of *wnt*[*d*] expression in the presumptive cerebellum (Fig. 7A-D). To confirm these rather surprising results, we performed three independent in situ hybridizations with independently prepared ^{35}S-labeled probes and used diagnostic restriction digestions each time to rule out any mixup of probe DNA.

Discussion

The identification of genes encoding proteins that share structural homology with Wnt-1 has led to an extensive search for additional members of this family. To date, 11 different *wnt*-related genes are known from studies in mouse, man and *Xenopus* (e.g. see Gavin et al., 1990; Christian et al., 1991a,b; Roelink and Nusse, 1991). On the basis of a PCR strategy on zebrafish genomic DNA, we have identified four members of the zebrafish *wnt* gene family that are differentially expressed during embryonic development. Whereas *wnt[c]* appears to be a novel *wnt* gene with regard to its spatial and temporal expression pattern, we also identified a clone, *wnt*[*d*], which shows a similar expression pattern to *wnt-1*, but shares within the corre-

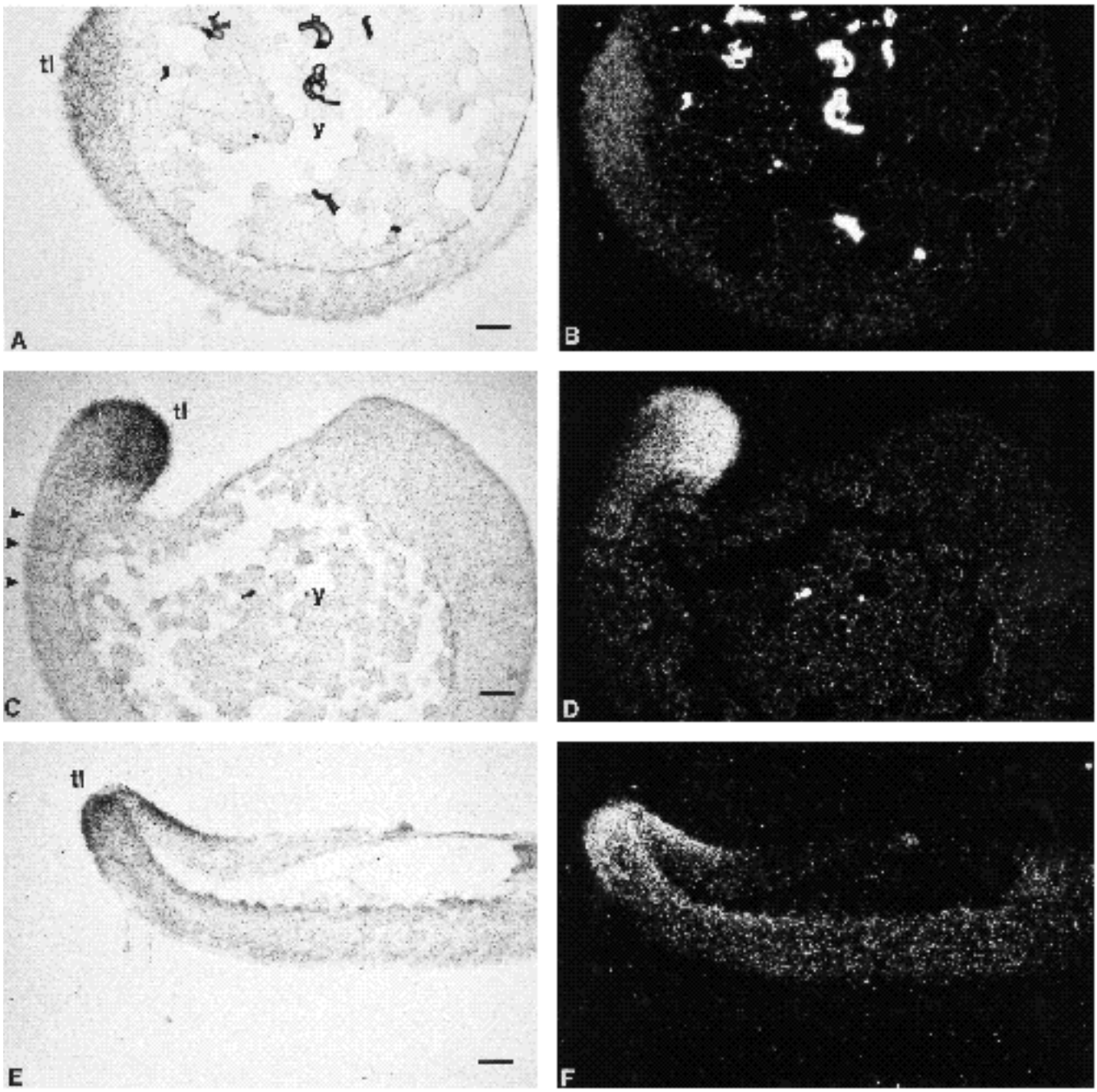

Fig. 5. Expression of *wnt*[*b*] in the tail bud of the developing zebrafish embryo. Sagittal sections through a 12 hour embryo (A,B), a 16 hour embryo (C,D) and a 24 hour embryo (E,F). Bright-field (A,C,E) and dark-field (B,D,F) micrographs are represented side by side. The embryos are oriented with the tail to the left. Observe the high levels of *wnt*[*b*] transcripts, that are localized in the extending tail bud. Abbreviations: tl, tail bud; y, yolk. Bars, 50 μm.

sponding region of the deduced amino acid sequence only 42.9% homology with wnt-1 (Molven et al., 1991).

By analogy to Wnt-1, we assume that the zebrafish wnt-related proteins are able to enter the secretory pathway and thus could be involved in cell-to-cell communication events (Bradley and Brown, 1990; Papkoff and Schryver, 1990). Studies in *Drosophila* have demonstrated that wingless (that shows homology to Wnt-1; Rijsewijk et al., 1987) is an extracellular differentiation factor that is involved in a complex regulatory network, that includes the activities of the segment-polarity genes *engrailed* (Martinez Arias et al., 1988) *patched* and *hedgehog* (Ingham et al., 1991).

In vertebrates, the role of wnt-related proteins remains enigmatic. It has been demonstrated that *Wnt-1* and *engrailed* genes are expressed in the posterior midbrain at early murine development (Wilkinson et al., 1987; Davis and Joyner, 1988), and a recent study by McMahon et al. (1992) demonstrates that *Wnt-1* regulates midbrain devel-

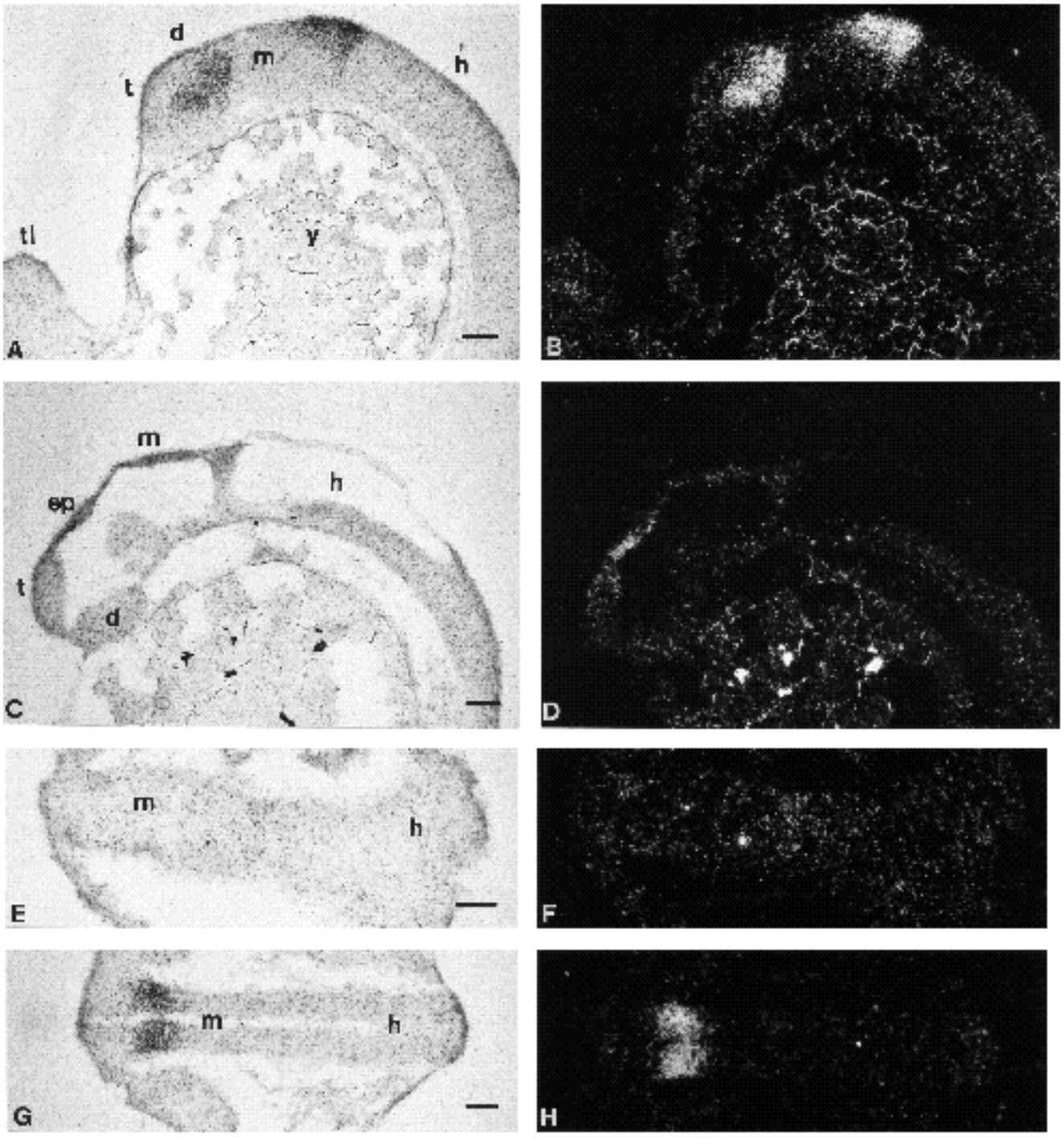

Fig. 6. In situ hybridizations on sections of zebrafish embryos using the *wnt*[*c*] probe. Bright-field (A,C,E,G) and dark-field (B,D,F,H) micrographs of sagittal tissue sections obtained from embryos after 16 hours (A,B) and 24 hours (C,D) of development. The anterior end of the embryo is turned to the left. Transverse sections obtained from embryos after 12 hours (E,F) and 16 hours (G,H) of development. Anterior is to the left. Abbreviations: d, diencephalon, ep, epiphysis; h, hindbrain; m, midbrain; t, telencephalon; tl, tail bud; y, yolk. Bars, 50 μm.

opment by modulating the expression of the murine *engrailed* genes. In zebrafish, we observe that *wnt-1*, *wnt[a]*, *wnt*[*d*], *eng-2* and *pax*[*zf-b*] show overlapping areas of expression in the posterior midbrain (Krauss et al., 1991a,b; unpublished data). It remains to be seen whether these genes interact with each other during the formation of the posterior midbrain.

wnt gene expression in the rostral brain may correlate with the primary subdivisions of the developing rostral brain. In the diencephalon, *wnt[a]* shows a broad stripe of

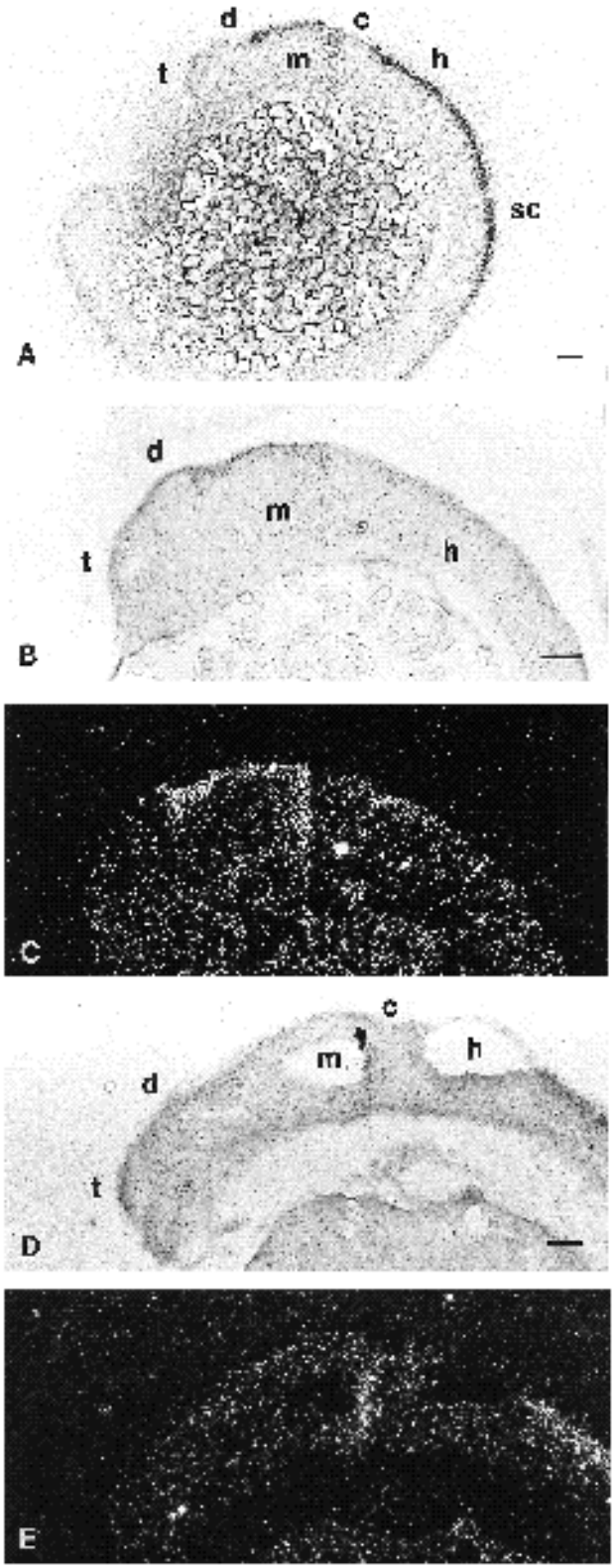

Fig. 7. Micrographs of 16 hour (A-C) and 24 hour (D,E) sagittal zebrafish embryo sections, hybridized with a probe for *wnt*[*d*]. Bright-field (A,B,D) and dark-field (C,E) micrographs are shown. (A) Overview micrograph of a 16 hour embryo. (B,C) Close up of the head region of another 16 hour embryo. Abbreviations: c, cerebellum; d, diencephalon; h, hindbrain; m, midbrain; sc, spinal cord; t, telencephalon; y, yolk. Bars, 50 μm.

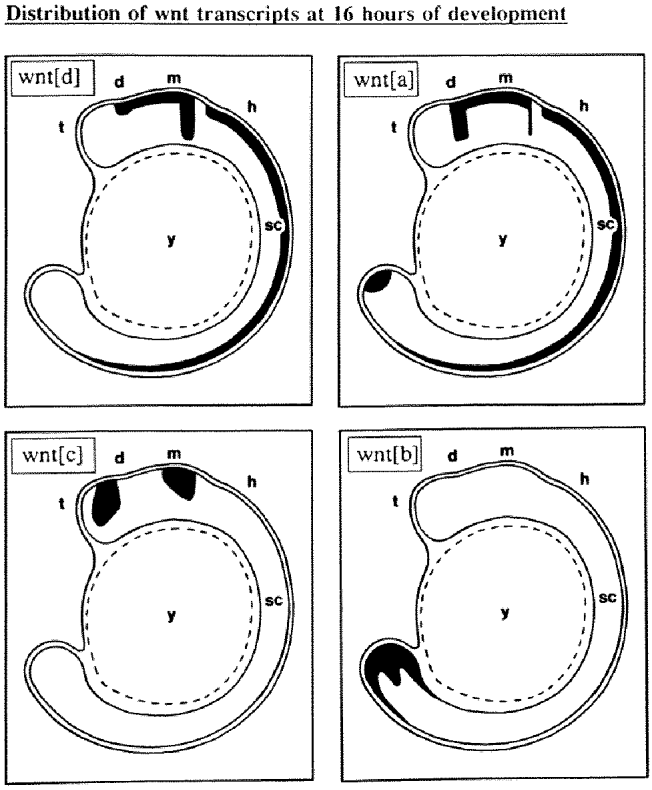

Fig. 8. Schematic illustrations summarizing the spatial expression of the zebrafish *wnt* genes at 16 hours of development in the neural tube and the tail bud. Abbreviations: d, diencephalon; h, hindbrain; m, midbrain; sc, spinal cord; t, telencephalon; y, yolk.

expression confined to the posterior portion of the prospective thalamus. Furthermore, transcripts of *wnt[c]* are seen adjacent to the *wnt[a]*-expressing area in the anterior portion of the thalamus. The earliest known morphological landmark that divides the diencephalon through the thalamus and therefore at the boundary between the two areas expressing *wnt[a]* and *wnt[c]* is an axon tract, the DVDT (dorsoventral diencephalic tract, see Wilson and Easter, 1991). It could be suggested that both *wnt* genes are somehow, in concert with other factors, involved in establishing the boundary between two diencephalic regions. This boundary could later be used as a pathway for the DVDT.

wnt[*d*] transcripts at 16 hours of development show a surprisingly similar expression pattern to *wnt-1* (Molven et al., 1991). Judging from the expression of *wnt*[d] and *wnt-1* genes, it is possible that both gene products may perform similar functions with similar developmental implications. Targeted gene disruption of murine *Wnt-1*, however, leads to severe abnormalities in the midbrain and the cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). It is currently unclear whether a homologue to zebrafish *wnt*[d] is present in mice.

The assumption that some members of the *wnt* gene family are able to substitute at least some of their functions has been substantiated by wnt RNA injections into fertilized *Xenopus* eggs. Olsen et al. (1991) have demonstrated that both Xwnt-1 and Xwnt-8 RNA injections are able to

enhance gap-junctional communications between ventral cells in the *Xenopus* blastula. However, this does not seem to be the case for all members of the *wnt* gene family, as the injection of Xwnt-5A RNA does not show a similar effect. Therefore, Olsen et al. (1991) suggested the existence of multiple receptors for wnt proteins. Consistently, it could be possible that wnt-1 and wnt[d] are involved in different signalling pathways. Alternatively, wnt proteins might display different affinities for the same receptor(s).

It is noteworthy that following *Wnt-1* gene disruption, no mutant phenotype was observed in the spinal cord and in the dorsal portion of the diencephalic regions (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). A likely explanation could be the substitution of Wnt-1 functions by other members of the wnt family. Indeed, Roelink and Nusse (1991) describe two murine *wnt*-related genes that are transcribed within the alar laminae (*Wnt-3*) and the roof plate (*Wnt-3A*). Supporting their data, the expression of the putative zebrafish homologue to *Wnt-3*, *wnt[a]*, is confined to the dorsal midline of the spinal cord and the hindbrain. Furthermore, *wnt*[d] transcripts are seen (with exception of the presumptive cerebellum and the telencephalon) in the dorsal portion of the neural tube.

In the elongating tail bud of the zebrafish embryos, we detected high levels of *wnt[a]* and *wnt[b]* transcripts. Unlike the expression of the zebrafish *wnt* genes in the neural keel, it seems that the expression of *wnt[a]* and *wnt[b]* in the tail is not confined to any particular tissue. We do not know the function of the two transcripts in the tail bud; however it is noteworthy that *wnt[a]* transcripts are detected only at a relatively early developmental stage (12-18 hours), whereas *wnt[b]* transcripts are expressed considerably longer (we detect high levels of expression at 24 hours of development). It could be speculated that *wnt[a]* and *wnt[b]* are involved in establishing or mantaining a specific status of cell differentiation or positional cues (see Gavin et al., 1990).

The similarity of the expression of *wnt-1* and *wnt*[d], the relation between *wnt[a]* and *wnt[c]* in the diencephalon, and the presence of *wnt[a]* and *wnt[b]* in the growing tail bud raise questions that deserve further experimental investigation.

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