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A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm

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Summary

Pattern formation in the hindbrain and paraxial mesoderm of vertebrates occurs by the formation of a series of repeated segments. These processes of segmentation appear different at the morphological level, since hindbrain segments, the rhombomeres, form by the subdivision of the neural epithelium into compartments, whereas the mesodermal somites form by the sequential aggregation of mesenchymal cells into epithelial balls. Previous studies have implicated genes encoding transcription factors in the development of hindbrain segments, but nothing is known of genes involved in the formation of somites. Cellular interactions and signal transduction must be an important aspect of hindbrain segmentation, so we have screened for tyrosine kinases expressed in rhombomere-restricted patterns in the developing mouse embryo. We have identified a receptor protein tyrosine kinase, *Sek*, that has high relative levels of expression in rhombomeres 3 and 5. This alternating pattern is established coincidentally, both spatially and temporally, with the expression of *Krox-20*, a zinc-finger gene expressed prior to the morphological formation of rhombomeres. In addition, *Sek* expression occurs in several other developing tissues, including a dynamic regulation in the developing forebrain, spinal cord, early mesoderm and anterior presomitic mesoderm (segmental plate). The latter expression occurs in two stripes that correlate with, and presage, the formation of somites. *Sek* expression initially occurs throughout the presumptive somite, then becomes restricted anteriorly, and finally is down-regulated as the definitive somite is formed. These data suggest that despite the morphological differences in the segmentation of the hindbrain and mesoderm, *Sek* is involved in the segmental patterning of both of these tissues.

Key words: segmentation, rhombomeres, somites, mesoderm, CNS development, neural crest, receptor protein tyrosine kinase, cell-cell interactions.

Introduction

The subdivision of tissues into a series of segments, each of which generates a homologous structure, is a mechanism of pattern formation found in many animal phyla. Genetic screens and molecular cloning have enabled considerable advances in our understanding of the process of segmentation in the early *Drosophila melanogaster* embryo (Akam, 1987; Ingham and Martinez-Arias, 1992). In contrast, little is known of molecular mechanisms of segmentation in vertebrate tissues.

The most conspicuous segmentation in vertebrate embryos is seen in the somites, pairs of epithelial balls that form sequentially from anterior-to-posterior by the condensation of mesenchymal paraxial mesoderm, initially into loose aggregations of cells, somitomeres (Meier, 1979; Tam et al., 1982; reviewed by Jacobson, 1988), and then into definitive epithelia. The formation of the somite epithelium is believed to involve changes in cell-cell adhesion (reviewed by Keynes and Stern, 1988), but nothing is known of how this is controlled or how the repeated pattern of somites is generated, although there is circumstantial evidence that it might be linked to the cell cycle (Primmett et al., 1989).

Recent studies have shown that segmentation also occurs in a specific region of the developing central nervous system (CNS), the hindbrain, where early in its development a series of constrictions and bulges, the rhombomeres, appear. This segmentation is morphologically different from that in mesoderm: whereas somites form sequentially by the aggregation of mesenchymal cells, rhombomeres are formed in a non-sequential manner in a pre-existing epithelium (Vaage, 1969). Clonal analysis in chick embryos has shown that as each rhombomere boundary forms, cell

movement across it is restricted and thus the hindbrain becomes subdivided into at least five compartments corresponding to rhombomeres r2-r6 (Fraser et al., 1990). Subsequently, neuronal development occurs in a pattern that correlates with, and presumably derives from, this segmentation; each of the branchial motor nerves arises from adjacent pairs of rhombomeres, the Vth nerve from r2 and r3, the VIIth nerve from r4 and r5, and the IXth nerve from r6 and r7 (Lumsden and Keynes, 1989). Clues regarding the cellular basis of the formation of hindbrain segments have come from grafting experiments showing that the juxtaposition of r3 and r5 does not lead to the formation of a morphological boundary, but when either of these is grafted adjacent to any even-numbered rhombomere a new boundary is always generated (Guthrie and Lumsden, 1991). These data suggest that an alternation in cellular properties, such as cell adhesion, underlies the restriction of cell movement across rhombomere boundaries.

Several genes encoding potential transcription factors have been implicated in the segmental patterning of the hindbrain by their rhombomere-restricted expression patterns. Expression of the zinc finger gene *Krox-20* is restricted to r3 and r5 (Wilkinson et al., 1989a,b) and the establishment of this alternating pattern prior to lineage restriction in the chick hindbrain is consistent with an early role, possibly in the formation of rhombomeres (Nieto et al., 1991). In addition, the domains of anteriorly-expressed *Hox* homeobox genes correlate with rhombomere boundaries (Murphy et al., 1989; Wilkinson et al., 1989b; Sundin and Eichele, 1990) and with hindbrain neural crest (Hunt et al., 1991a,b) that originates from specific rhombomeres (Lumsden et al., 1991), implicating these genes in the determination of segment phenotype. Finally, several retinoic acid receptors (Ruberte et al., 1991) and binding proteins (Maden et al., 1991, 1992) have been found to have segment-restricted expression, perhaps reflecting the proposed role of retinoic acid in regulating *Hox* gene expression.

The establishment and specification of hindbrain segments must involve a cascade of interactions and understanding of this process will require the identification of further potential components. In the current absence of systematic genetic approaches to screen for such genes, the strongest criterion we have for implicating genes in hindbrain segmentation is through their expression patterns. The formation of rhombomeres must involve cellular interactions, but thus far no genes encoding cell-cell signals or components of signal transduction cascades have been implicated in hindbrain segmentation. Promising candidates might be found among the receptor protein tyrosine kinases, since genetic analysis has revealed critical roles of members of this superfamily in pattern formation in the early *Drosophila* embryo and eye and in the development of a number of vertebrate tissues (reviewed by Pawson and Bernstein, 1990). We therefore set out to clone tyrosine kinase sequences from cDNA isolated from the mouse hindbrain and to screen these for segment-restricted expression by in situ hybridisation (Gilardi-Hebenstreit et al., 1992). Here, we describe the expression pattern of a putative receptor tyrosine kinase, *Sek*, that is expressed in alternating rhombomeres, r3 and r5, of the mouse hindbrain. *Sek* expression occurs early during hindbrain segmentation, at the time that *Krox-20* is up-regulated. Unexpectedly, we also find expression of *Sek* in two stripes that correlate with prospective somites in mesenchymal mesoderm. In addition, we find expression in several other developing tissues - the forebrain, spinal cord, notochord, and neural crest - in patterns that do not correlate with segmentation. These findings suggest that the *Sek* gene has multiple roles in embryogenesis, that include the segmental patterning of both the hindbrain and mesoderm.

Materials and methods

In situ hybridisation analysis with radiolabelled probes

In situ hybridisation with ^{35}S-labelled RNA probes and washing at high stringency was carried out as described (Wilkinson and Green, 1990). *Sek* probe corresponding to residues 1848-2263 of *Sek* cDNA which specifically detects an approximately 7 kb transcript on RNA blots (Gilardi-Hebenstreit et al., 1992) was used for routine analysis. Probes corresponding to three other regions of *Sek* cDNA (Fig. 1) also detected the expression pattern described here. The *Krox-20* probe is as described by Nieto et al. (1991).

Whole-mount in situ hybridisation

The synthesis of digoxigenin-labelled probe and whole-mount in situ hybridisation with the *Sek* sequences described above was carried out as described in detail elsewhere (Wilkinson, 1992). Briefly, this involved the overnight fixation of embryos in 4% paraformaldehyde in PBS, following by washing twice for 5 minutes with PBS, 0.1% Tween-20 (PBTw). Embryos were (1) dehydrated through 25%, 50% and 70% methanol in PBTw, and then 100% methanol, (2) rehydrated through this series in reverse and (3) washed twice for 5 minutes in PBTw. They were then bleached for 1 hour in 6% H_2O_2 in PBTw, washed three times for 5 minutes with PBTw and treated with 10 μg/ml Proteinase K for 10 minutes. Following this, the embryos were washed with PBTw and post-fixed with 4% paraformaldehyde, 0.2% glutaraldehyde. Prehybridisation was carried out for 2-3 hours at 70˚C in 50% formamide, 5× SSC, pH 4.5, 1% SDS, 50 μg/ml heparin and 50 μg/ml yeast RNA. Hybridisation in the latter solution containing 1 μg/ml digoxigenin-labelled RNA probe was carried out overnight at 70˚C. The embryos were then washed for 30 minutes at 70˚C in 50% formamide, 5× SSC, pH 4.5, three times for 5 minutes with 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20 and then treated for 1 hour with 100 μg/ml RNAase A in this buffer. Embryos were then washed in 50% formamide, 2× SSC at 65˚C for 1 hour. Finally, preblocking of the embryos with serum, incubation with anti-digoxigenin antibody-alkaline phosphatase (Boehringer), washing and colour development was as described (Wilkinson, 1992).

Results

Screening for segment-restricted tyrosine kinases

The strategy that we used to search for tyrosine kinases with segmental expression was to clone kinase sequences from hindbrain cDNA and to screen these by in situ hybridisation (Gilardi-Hebenstreit et al., 1992). Putative kinase sequences were PCR-amplified from cDNA prepared from micro-dissected 9.5-day mouse embryo hindbrains by the use of redundant oligonucleotides that correspond to conserved regions of the catalytic domain (Wilks, 1989). These

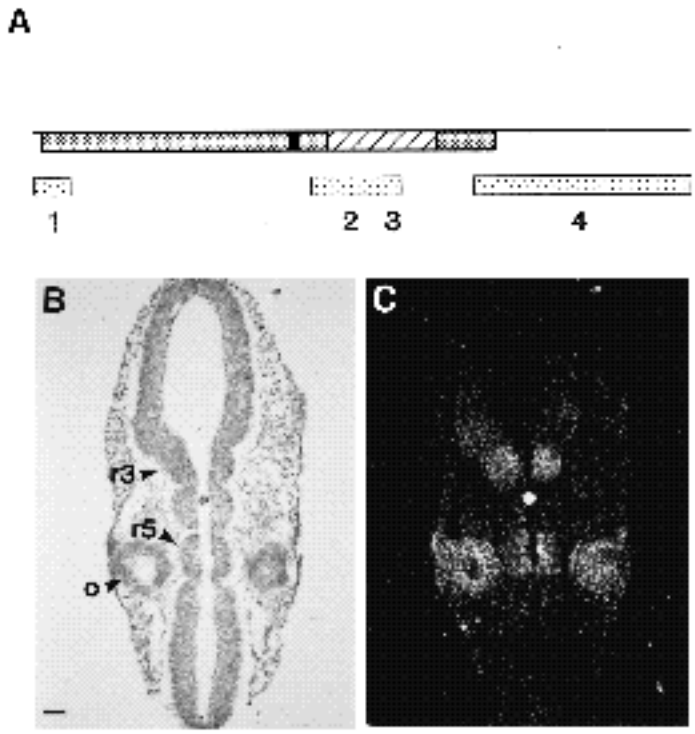


Fig. 1. Structure of *Sek* and expression in hindbrain segments. (A) Diagram of *Sek* transcript based on sequence of cDNA clones (Gilardi-Hebenstreit et al., 1992). The upper part represents 4242 bp of *Sek* cDNA that encompasses the open reading frame, indicated by the bar. The black box represents the putative transmembrane sequences and the hatched box the cytoplasmic kinase domain. Below, the *Sek* probes used for in situ hybridisation analysis are depicted. The probes correspond to the following residues of *Sek* cDNA: probe 1, residues 1-305; probe 2, residues 1848-2263; probe 3, residues 2298-2472; probe 4, residues 2782-4242 (Gilardi-Hebenstreit et al., 1992). (B) Bright-field, (C) dark-field, in situ hybridisation of a coronal section of the 9-day mouse embryo hindbrain with probe 2 revealing expression of *Sek* in rhombomeres r3 and r5 and the otocyst (o). Lower levels of expression are found in r2 and r6. The same expression pattern (not shown) was observed using 5′ sequences (probe 1), the PCR-derived fragment of the kinase domain (probe 3) and 3′ sequences (probe 4). The bar indicates 50 μm.

were ligated into plasmid vector and 11 clones found to encode distinct kinase sequences were screened for segmental expression by in situ hybridisation of radiolabelled probes to coronal sections of 9- to 9.5-day mouse embryo hindbrains. Through this strategy we identified one clone that detects RNA expressed in a segment-restricted pattern, with high relative levels of transcripts in alternating rhombomeres (r), r3 and r5, and also in the otocyst (data not shown). This expression pattern is also revealed by probes from other regions of this gene obtained from cDNA clones (Fig. 1). Based on the identification of this gene as a segmentally-expressed kinase, we designate it *Sek*. DNA sequence analysis of the complete coding region of *Sek* indicates that it encodes a putative receptor PTK of the *Eph* family (Gilardi-Hebenstreit et al., 1992).

In situ hybridisation studies indicated that *Sek* expression occurred not only in the hindbrain, but also in a dynamic pattern in other regions of the CNS, and in mesoderm derivatives and in neural crest. Below we present a detailed analysis of *Sek* expression in these various tissues in comparison with appropriate markers. We describe *Sek* expression first in the developing CNS, then in early mesoderm and its derivatives, and finally in neural crest and other tissues.

Establishment of segmental Sek *expression in the hindbrain*

Based on the finding that *Sek* is expressed at high relative levels in r3 and r5, it was pertinent to examine when this alternating expression became established during the segmentation of the hindbrain. To assess this, we analysed the timing and spatial restriction of its expression relative to the *Krox-20* gene; the latter provides a particularly useful marker since, like *Sek*, it is expressed in r3 and r5, and the domains of *Krox-20* expression are detected well in advance of morphological segmentation (Wilkinson et al., 1989a,b; Nieto et al., 1991). In the 7.75-day (0-somite) mouse embryo, *Krox-20* expression occurs in a single narrow stripe that corresponds to prospective r3, whereas *Sek* expression occurs in a relatively broad domain in the hindbrain (Fig. 2A-C). Higher levels of *Sek* transcripts are found coincident with the domain of *Krox-20* expression, and lower levels extend to a boundary in the posterior hindbrain. A similar expression pattern is seen in the 4-somite embryo (Fig. 2D-I), but by the 8-somite stage high relative levels of *Sek* expression are found in two stripes that coincide with the domains of *Krox-20* expression in prospective r3 and r5 (Fig. 2J-L). This coincidence of *Krox-20* and high relative levels of *Sek* expression is also seen in the dorsoventral plane in transverse sections (Fig. 3A-F). However, unlike *Krox-20*, lower-level expression of *Sek* occurs in prospective r2, r4 and r6 (Fig. 2J,K). This alternating expression of *Sek* is maintained following the morphological appearance of rhombomeres, when high-level expression occurs in r3 and r5, and lower-level expression in r2 and r6 (Fig. 2M-O). Subsequently, expression of *Sek* is down-regulated, initially in r5 and then in r3 (compare 9 day-embryo in Fig. 1A,B with the 9.5 day-embryo in Fig. 2M,N); this is different from the situation with *Krox-20* which is down-regulated in r3 prior to r5 (Fig. 2O, and Wilkinson et al., 1989b). A distinct pattern in the down-regulation of these genes is also seen at this stage by the analysis of transverse sections which reveal expression of *Sek* throughout r3 and r5 (Fig. 4A,B,D,E,G,H), whereas *Krox-20* expression has been down-regulated in the intermediate region of r3 (Fig. 4F), but not r5 (Fig. 4I). Overall, these data indicate that *Sek* is expressed in the hindbrain at the time that *Krox-20* expression is up-regulated and that the alternating domains of *Sek* expression appear prior to the formation of rhombomeres.

Expression of Sek *in the forebrain*

At 7.75 days of development (0-somite embryo) *Sek* expression in the neural plate occurs only in the hindbrain (Figs 2A,B, 5A,B). However, by the 2-somite stage, *Sek* transcripts are detected in a domain in the anterior neural plate (Fig. 5C,D), and a similar expression pattern is observed in the 4-somite (Fig. 5E,F), 6-somite (Fig. 5G,H)

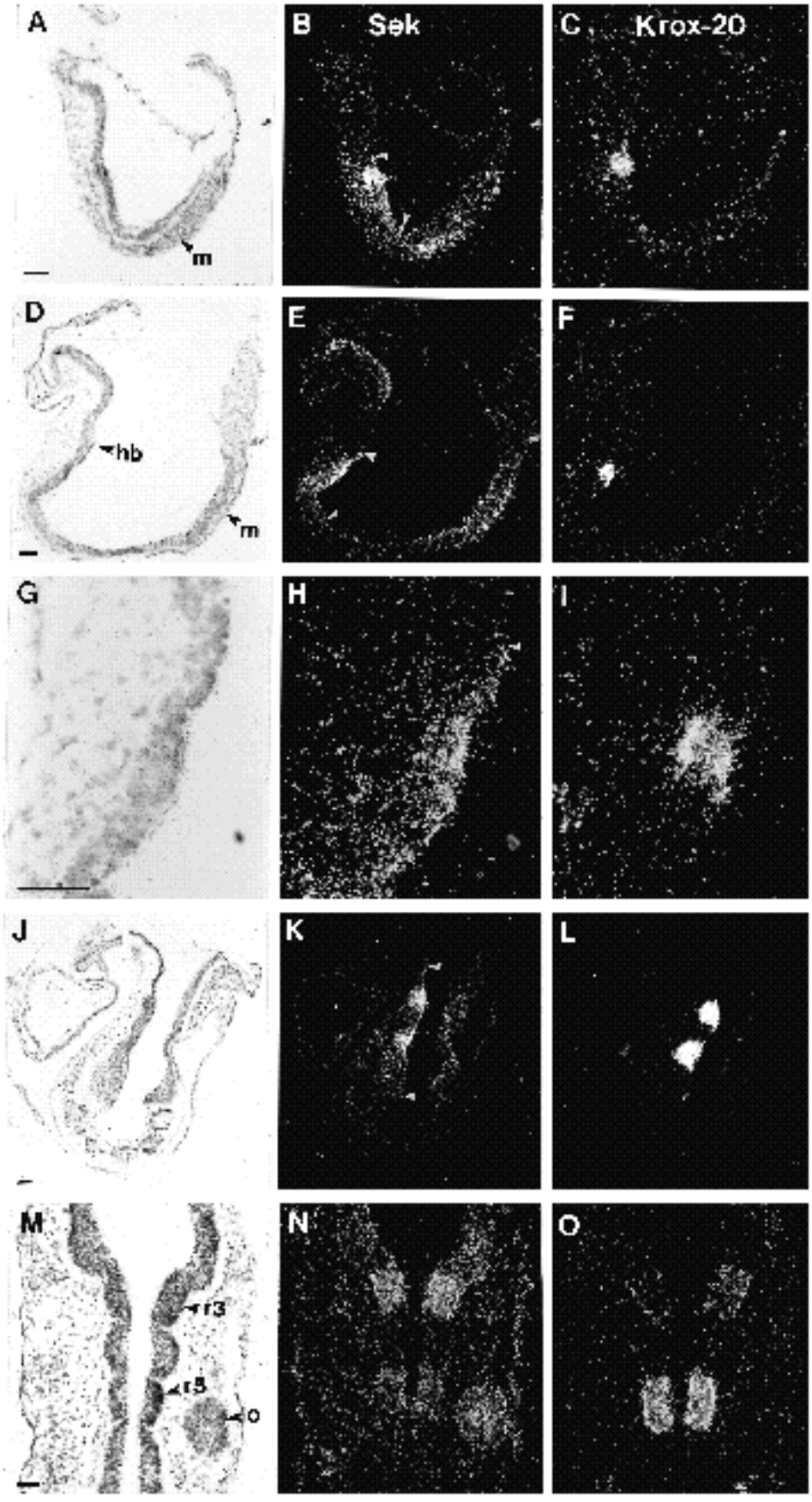


Fig. 2. Establishment of segmental *Sek* expression in the hindbrain. The establishment of *Sek* gene expression in the hindbrain was examined by in situ hybridisation analysis of sections of neurula-stage (7.75- to 9.5-day) mouse embryos. Rhombomeres are not morphologically apparent at early stages, so *Krox-20* gene expression was used as a molecular marker of prospective r3 and r5. (A-C) 0-somite (7.75-day) embryo; (D-I) 4-somite (8-day) embryo; (J-L) 8-somite (8.5-day) embryo; (M-O) 9.5-day embryo. A-L are longitudinal sections and M-O are coronal sections through the hindbrain. G-I are higher magnification photographs of the embryo shown in D-F to show the anterior limit of expression. In each row, the left-hand panel is a bright-field photograph, the centre panel a dark-field photograph of this section hybridised with *Sek* probe, and the right-hand panel a dark-field photograph of an adjacent section hybridised with *Krox-20* probe. The embryo shown in M-O is older than that shown in Fig. 1 and by this stage down-regulation of *Sek* and *Krox-20* expression is occurring, in r5 and in r3, respectively. Similar results were obtained in whole mount with non-radioactive probes (see Fig. 7), but this method could not be used on tissue sections to compare *Sek* and *Krox-20* expression directly. hb, hindbrain; m, mesoderm; o, otocyst; r, rhombomere. The white arrowheads indicate the limits of expression of *Sek* in the hindbrain. The bar indicates 50 μm.

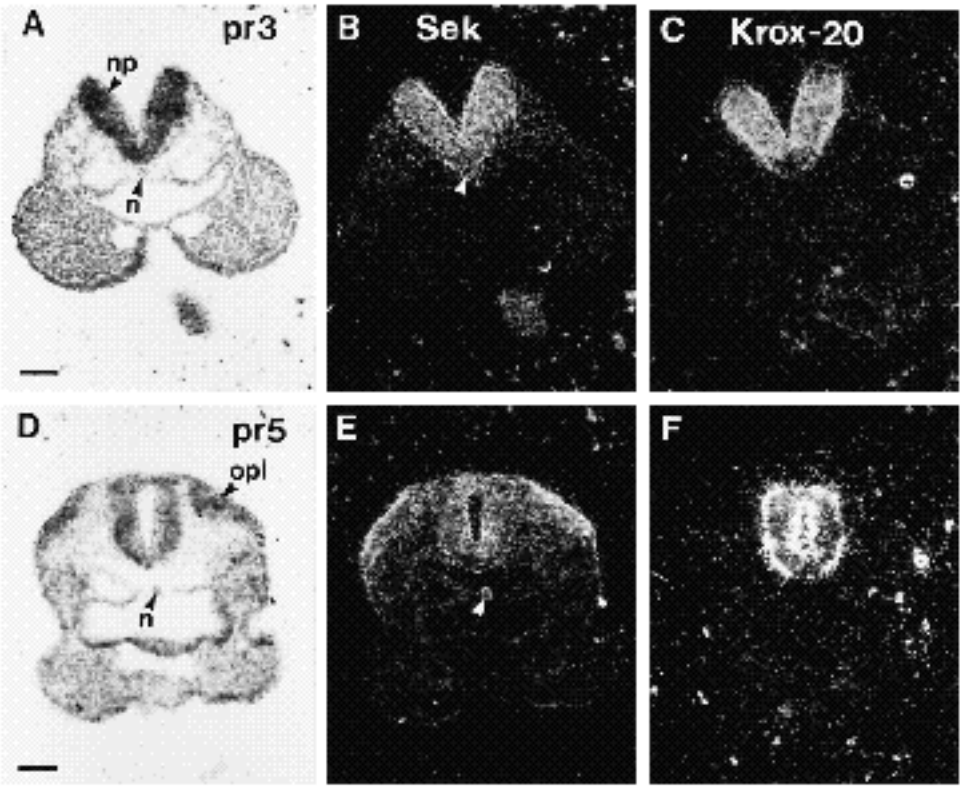


Fig. 3. *Sek* expression in the hindbrain of the 12-somite embryo analysed in the transverse plane. Adjacent transverse sections through presumptive rhombomeres 3 and 5 were hybridised with *Sek* probe or *Krox-20* probe. The left-hand panel (A,D) shows bright-field photographs, the centre panel (B,E) the corresponding dark-field photographs of these sections hybridised with *Sek* probe, and the right-hand panel (C,F) photographs of adjacent sections hybridised with *Krox-20* probe. n, notochord; np, neural plate; opl, otic placode; pr, presumptive rhombomere. The white arrowheads in (B,E) indicate the notochord. The bar indicates 50 μm.

and 8-somite (Fig. 5I,J) embryo. Although it is difficult to precisely correlate the posterior boundary of expression relative to the rapidly changing morphology of the anterior neural plate at early stages, this boundary appears to be in the vicinity of the forebrain/midbrain junction. To examine this, we hybridised adjacent sections of an 8-somite embryo with *Sek* (Fig. 5I) and *wnt-1* (Fig. 5K), which has a midbrain expression domain at this stage (McMahon, 1991). These data indicate that *Sek* expression occurs in the forebrain (and hindbrain) but not the midbrain at this stage. Subsequently, *Sek* expression becomes progressively restricted within the developing forebrain. At the 20-somite stage expression occurs in the telencephalon and the dorsal diencephalon, but does not occur in the ventral diencephalon (Fig. 5L,M). Furthermore, expression does not occur in the developing optic vesicle (Fig. 4A,B). By 12.5 days of development, *Sek* expression has been restricted to specific regions of the telencephalon, with transcripts detected only in the cortical neuroepithelium (the prospective cerebral cortex) and the basal telencephalon (Fig. 5N,O).

Expression of Sek *during the differentiation of the spinal cord*

In contrast to the expression of *Sek* in the forebrain and hindbrain, transcripts are not detected in the spinal cord in the 12-somite embryo (Fig. 6A,B) or at earlier stages. However, at the onset of neurogenesis at 9.5 days of development (25 somites) expression is initiated in the rostral spinal cord and is later modulated in a pattern that reflects the anterior-to-posterior maturation of the neural tube, such that rostral regions are developmentally more advanced than caudal regions. Thus, at 9.5 days low levels of *Sek* expression are detected in the ventral part of the rostral spinal cord (Fig. 6C,D), but not in the caudal spinal cord (Fig. 6E,F). At 10.5 days *Sek* transcripts are detected in the rostral spinal cord in a widespread pattern, with higher levels in ventral and dorsolateral regions (Fig. 6G,H), but in the caudal spinal cord only in its ventral part (Fig. 6I,J). At 12.5 days, this former expression pattern is now found in the caudal spinal cord (not shown), but expression in the rostral spinal cord is restricted to the ventral motor horns and the ventral part of the proliferating ependymal layer (Fig. 6K,L). Overall, these data indicate that *Sek* expression in the spinal cord initially occurs in its ventral part, then spreads to dorsal regions, and finally becomes restricted to ventral regions again.

Sek *expression correlates with segmentation in mesoderm*

Analysis of presomitic gastrulation-stage embryos revealed that in addition to expression in the hindbrain, *Sek* transcripts are found in trunk mesoderm with higher levels in more anterior, mature, regions (Fig. 2A,B). After the initiation of somitogenesis, expression continues to occur in posterior, newly formed mesoderm (Fig. 7A,B,I; see also the sagittal section shown in Fig. 2D), but two stripes of expression are now found in mesoderm immediately caudal to the most recently formed somite, with the anterior stripe narrower than the posterior stripe (Fig. 7A-I). We have analysed >30 embryos from the 3-somite stage (Fig. 7A,B) to the 25-somite stage (Fig. 7E,F) and consistently observed these stripes of expression in the anterior segmental plate. Therefore, the expression of *Sek* is not related to the anterior-posterior level, but rather correlates with the progres-

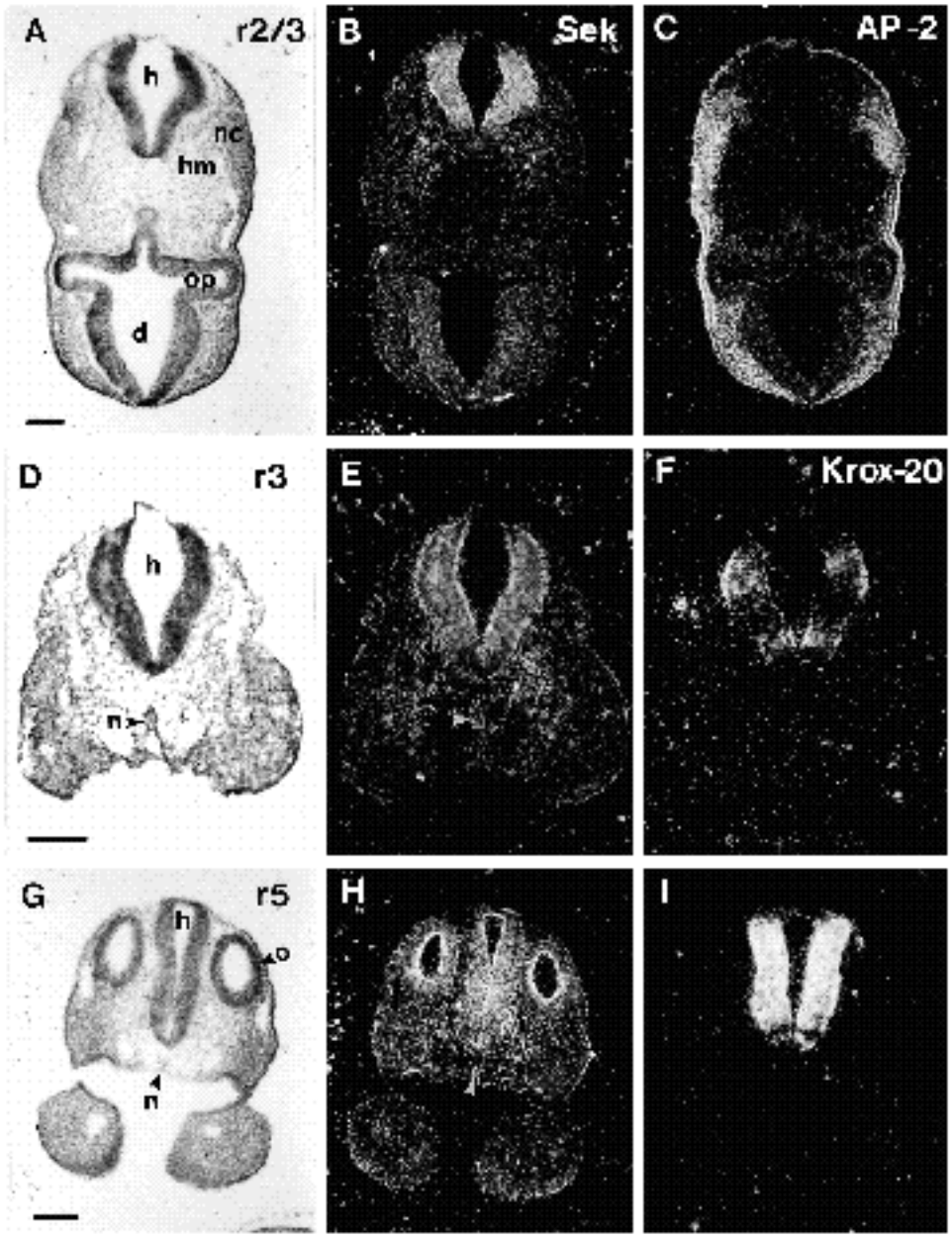
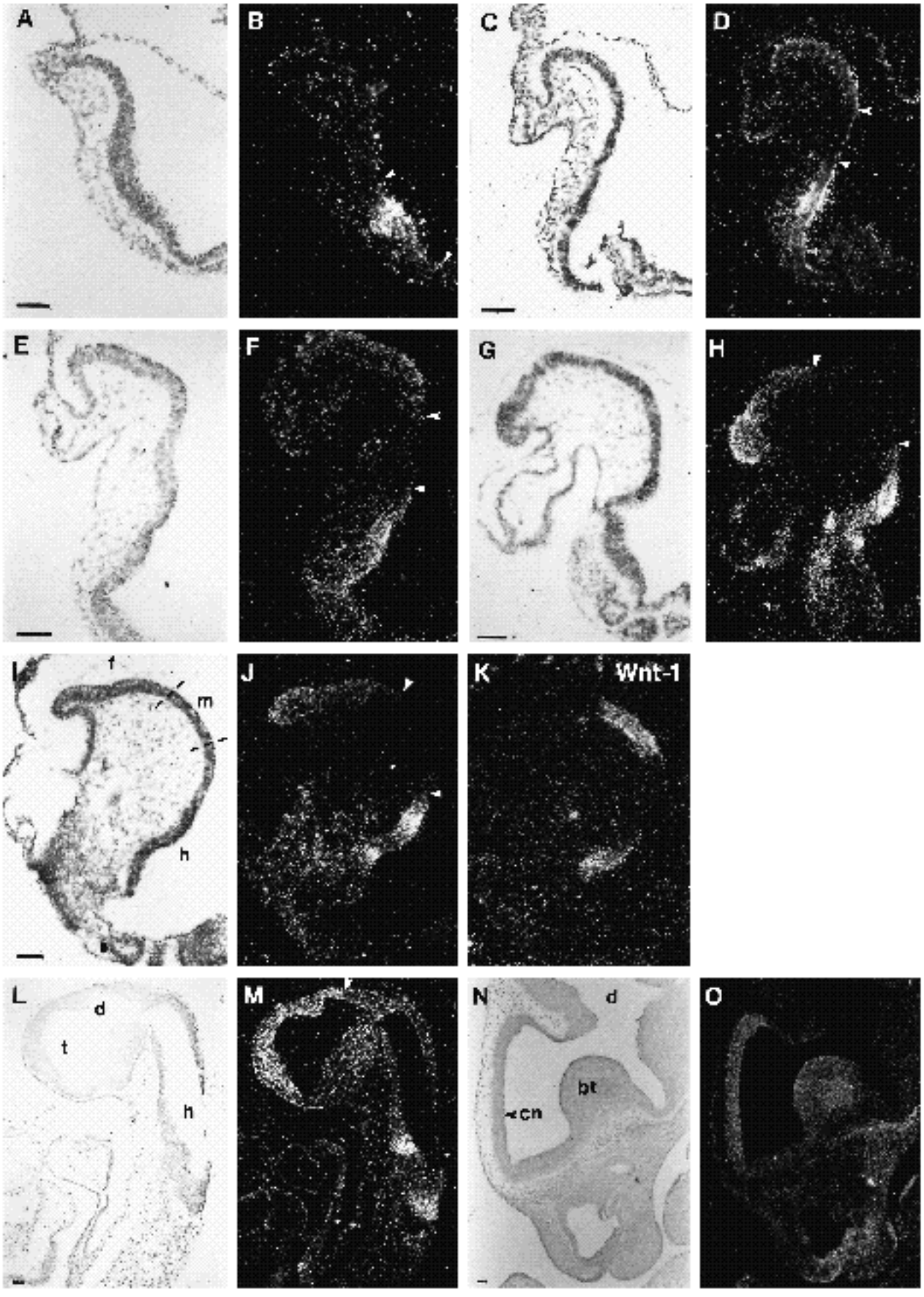


Fig. 4. *Sek* expression in the hindbrain of the 25-somite embryo analysed in the transverse plane. Transverse sections were hybridised with *Sek* probe, and adjacent sections with either AP-2 probe or *Krox-20* probe. The left-hand panel (A,D,G) shows bright-field photographs, the centre panel (B,E,H) the corresponding dark-field photographs of these sections hybridised with *Sek* probe, and the right-hand panel adjacent sections hybridised either with probe for AP-2, a marker of neural crest (C), or *Krox-20* (F,I). In A-C, the plane of section is tilted such that it passes through the diencephalon, whereas D-I are from a different embryo and transect the branchial arches. d, diencephalon; h, hindbrain; hm, head mesoderm; n, notochord; nc, neural crest; o, otocyst; op, optic vesicle; r, rhombomere. The white arrowheads in E,H indicate the notochord. The bar indicates 50 μm.

sive morphogenesis of mesoderm along the body axis. Detailed examination of sections and of whole mounts suggested that the two domains of *Sek* expression correlate with prospective somites (Fig. 7C-H): the anterior domain is in the anterior part of the next somite to form, and the broader posterior stripe corresponds to the next-but-one to condense. Consistent with this, the high level domain of expression is seen to be restricted to paraxial (prospective somitic) mesoderm in transverse sections through the caudal stripe (Fig. 6E,F). Overall, these data indicate that *Sek* expression occurs in early mesoderm, and is then down-regulated, followed by a sharp up-regulation in a pattern that correlates with somitogenesis. This latter expression initially occurs throughout the prospective somite, is then restricted anteriorly and finally is down-regulated as the definitive epithelial somite is formed. This modulation of

Fig. 5. *Sek* expression in the developing forebrain. Longitudinal sections were hybridised with *Sek* probe, and in one experiment adjacent sections were hybridised with probe for *Wnt-1*, which has a midbrain expression domain at this stage. (A,B) 0-somite (7.75-day) embryo; (C,D) 2-somite embryo; (E,F) 4-somite embryo; (G,H) 6-somite embryo; (I-K) 8-somite embryo; (L,M) 20-somite (9-day) embryo; (N,O) 12.5-day embryo. In each row, a bright-field photograph is to the left of the corresponding dark-field photograph of the section hybridised with *Sek* probe (B,D,F,H,J,M,O) or an adjacent section hybridised with *Wnt-1* probe (K). bt, basal telencephalon; cn, cortical neuroepithelium; d, diencephalon; f, forebrain; h, hindbrain; m, midbrain; t, telencephalon. The small arrowheads indicate limits of *Sek* expression in the hindbrain, and the larger arrowheads the posterior limit of expression in the anterior neural epithelium. The bar indicates 50 μm.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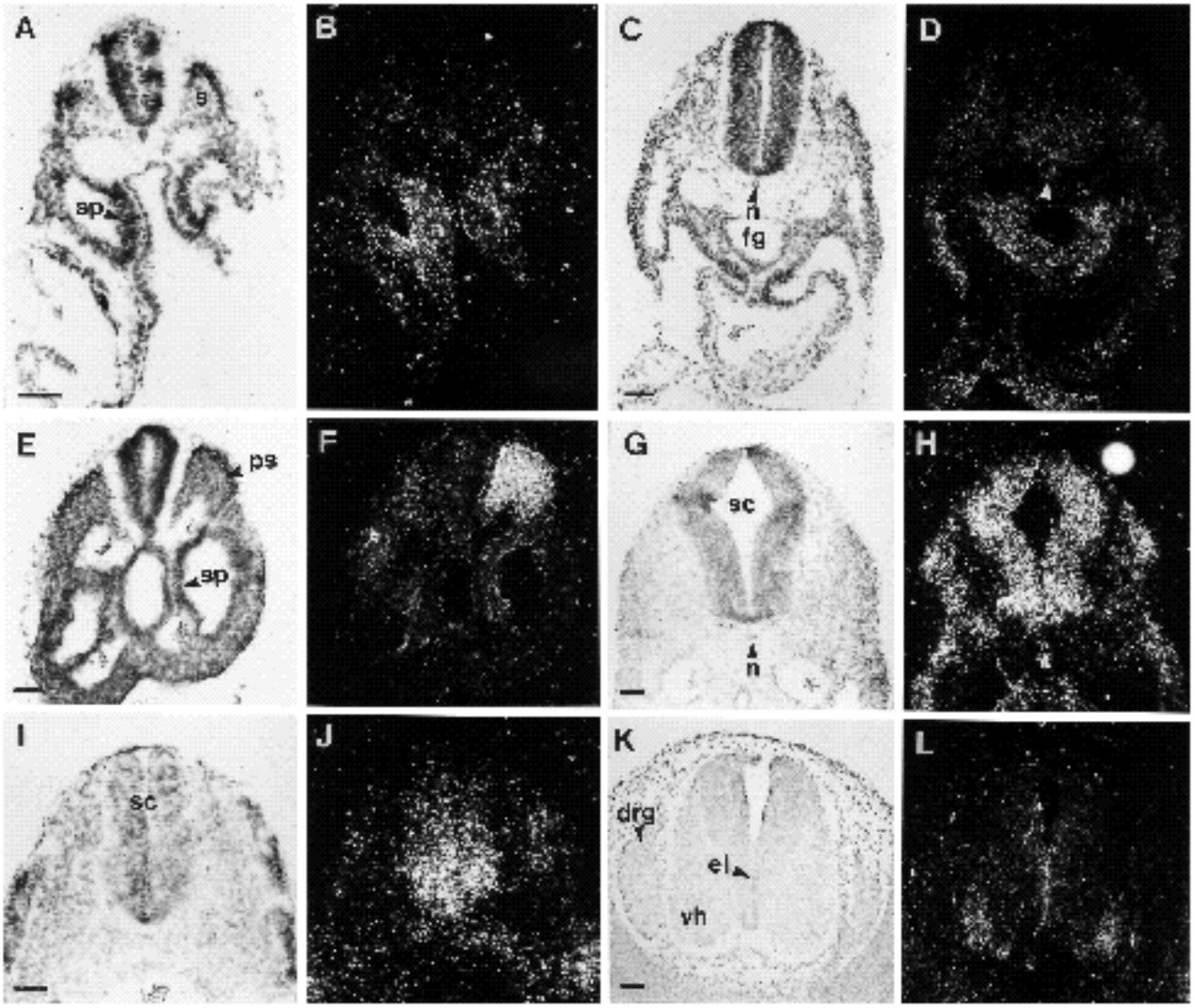

Fig. 6. *Sek* expression in the spinal cord. Transverse sections at different anterior-posterior levels through the spinal cord were hybridised with *Sek* probe. (A,B) anterior section at the 12-somite stage; (C,D) anterior section, and (E,F) posterior section, at the 25-somite stage (9.5 days); (G,H) anterior section and (I,J) posterior section, at 10.5 days; (K,L) anterior section at 12.5 days. In each pair of photographs, a bright-field image is to the left of the corresponding dark-field image. drg, dorsal root ganglion; el, ependymal layer; fg, foregut; n, notochord; ps, presomitic mesoderm; s, somite; sc, spinal cord; sp, splanchnic mesoderm; vh, ventral horn. The bar indicates 50 μm.

Sek gene expression is highly dynamic, since somites form at 2-3 hour intervals in the mouse.

Expression of Sek *in mesodermal derivatives*

In addition to the domains of high relative *Sek* expression in presumptive somites, expression is detected at lower levels in other mesoderm derivatives. In 12-somite (Fig. 6A,B) and 25-somite (Fig. 6C-F) embryos expression is detected in splanchnic mesoderm, a derivative of lateral mesoderm that forms the medial wall of the developing coelum and lies adjacent to the gut. Moreover, although expression does not occur in the forming notochord in caudal regions of the embryo (adjacent to the anterior segmental plate; Fig. 6E,F), expression is consistently detected at low levels in more mature notochord (for example, Figs 3A,B,D,E, 6C,D,G,H). Finally, *Sek* expression is detected in paraxial mesenchyme adjacent to the hindbrain in the 9.5-day embryo (Fig. 4A,B,D,E). This expression is in head mesoderm, rather than neural crest, since hybridisation of an adjacent section with AP-2, a marker for neural crest and epidermis (Mitchell et al., 1991) does not detect this population of *Sek*-expressing cells (compare Fig. 4B with C). There is no obvious periodicity in this expression that would suggest a correlation with head somitomeres.

Sek *expression in neural crest*

The analysis of serial transverse sections revealed *Sek* expression in neural crest adjacent to r6 (Fig. 8A,B; compare with AP-2, a marker of neural crest, shown in Fig. 8D,E), but not in more rostral regions of the 9.5-day embryo. This expression pattern is reminiscent of that of *Krox-20*, which is transiently expressed in crest adjacent to

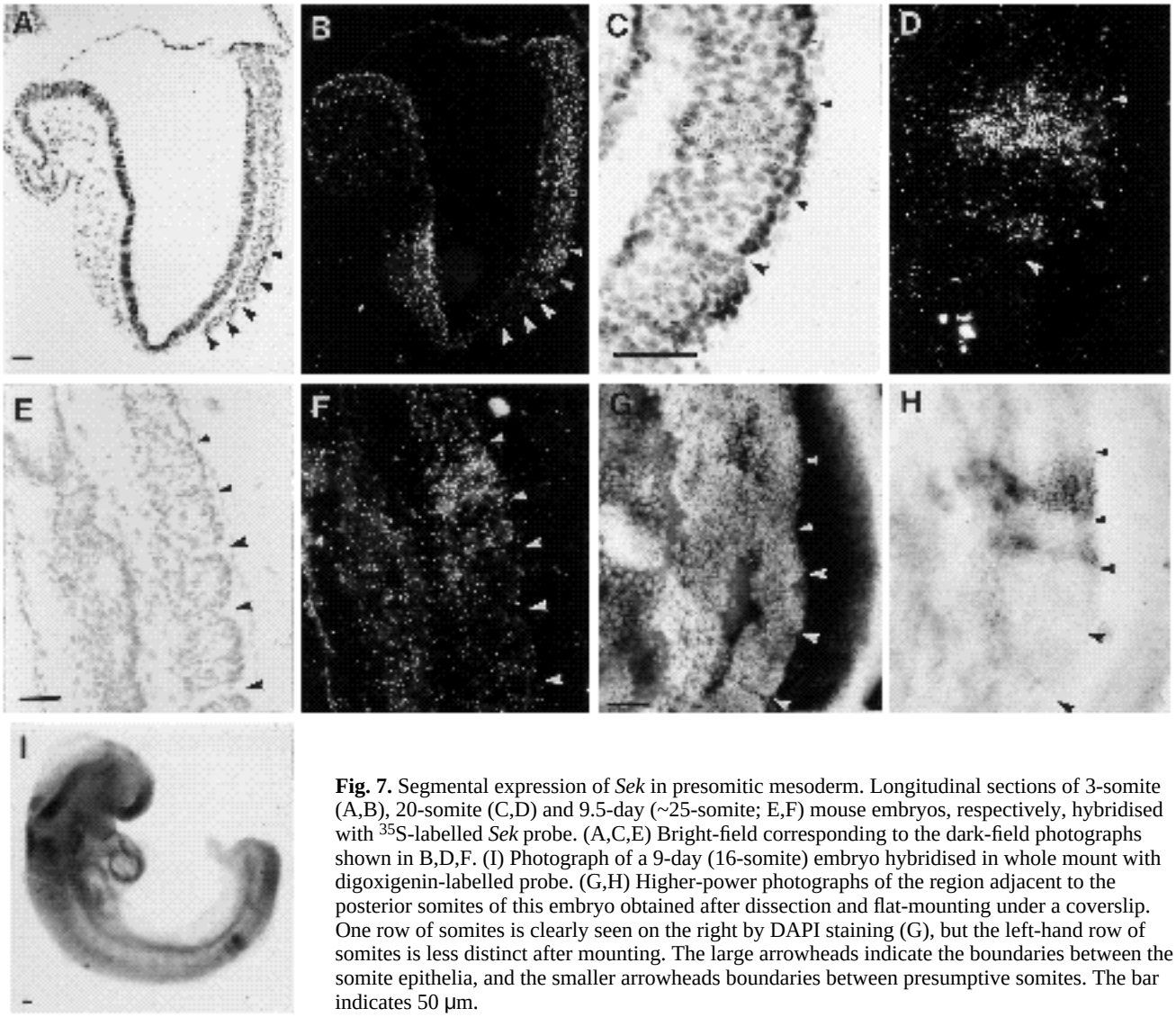

Fig. 7. Segmental expression of *Sek* in presomitic mesoderm. Longitudinal sections of 3-somite (A,B), 20-somite (C,D) and 9.5-day (~25-somite; E,F) mouse embryos, respectively, hybridised with ^{35}S-labelled *Sek* probe. (A,C,E) Bright-field corresponding to the dark-field photographs shown in B,D,F. (I) Photograph of a 9-day (16-somite) embryo hybridised in whole mount with digoxigenin-labelled probe. (G,H) Higher-power photographs of the region adjacent to the posterior somites of this embryo obtained after dissection and flat-mounting under a coverslip. One row of somites is clearly seen on the right by DAPI staining (G), but the left-hand row of somites is less distinct after mounting. The large arrowheads indicate the boundaries between the somite epithelia, and the smaller arrowheads boundaries between presumptive somites. The bar indicates 50 μm.

r6 (Wilkinson et al., 1989a; R. DasGupta and D.G.W., unpublished data). We therefore compared the expression of *Sek* (Fig. 8B) and *Krox-20* (Fig. 8C) on adjacent sections. The data suggest that *Sek* expression occurs in the cells that express *Krox-20*, but the former gene is also expressed in more ventral neural crest cells. The simplest explanation of these data is that these genes are co-expressed in early crest, but whereas *Krox-20* expression is brief, *Sek* expression persists as these cells migrate into ventral regions of the head.

Other sites of Sek *expression*

Finally, our analysis of *Sek* transcripts has revealed two further sites of expression: in the early thyroid (Fig. 8A,B), and in the otic placode (Fig. 3D,E) and subsequently in the otic vesicle (Figs 1, 2N).

Discussion

We identified *Sek* through a screen for kinases segmentally expressed in the developing hindbrain, and it is likely that the transmembrane receptor tyrosine kinase encoded by this gene (Gilardi-Hebenstreit et al., 1992) is involved in the transduction of extracellular signals in specific hindbrain segments. Our finding that *Sek* is also expressed in a restricted pattern in a number of other tissues, both segmented and non-segmented, is not surprising, since the involvement of receptor kinases in diverse developmental processes has many precedents, for example the role of the EGF receptor in *Drosophila* in the early embryo and the eye (reviewed by Shilo and Raz, 1991), and the involvement of *c-kit*/*W* in the development of germ cells, melanoblasts and haemopoietic cells in the mouse (Nocka et al., 1989). Before discussing the relationship between *Sek* expression and segmentation, we consider what other roles *Sek* may have in mouse development.

Expression in the forebrain and spinal cord

We find that expression of *Sek* is initiated at the 2 somite stage throughout the neural epithelium corresponding to the prospective telencephalon and diencephalon. During the

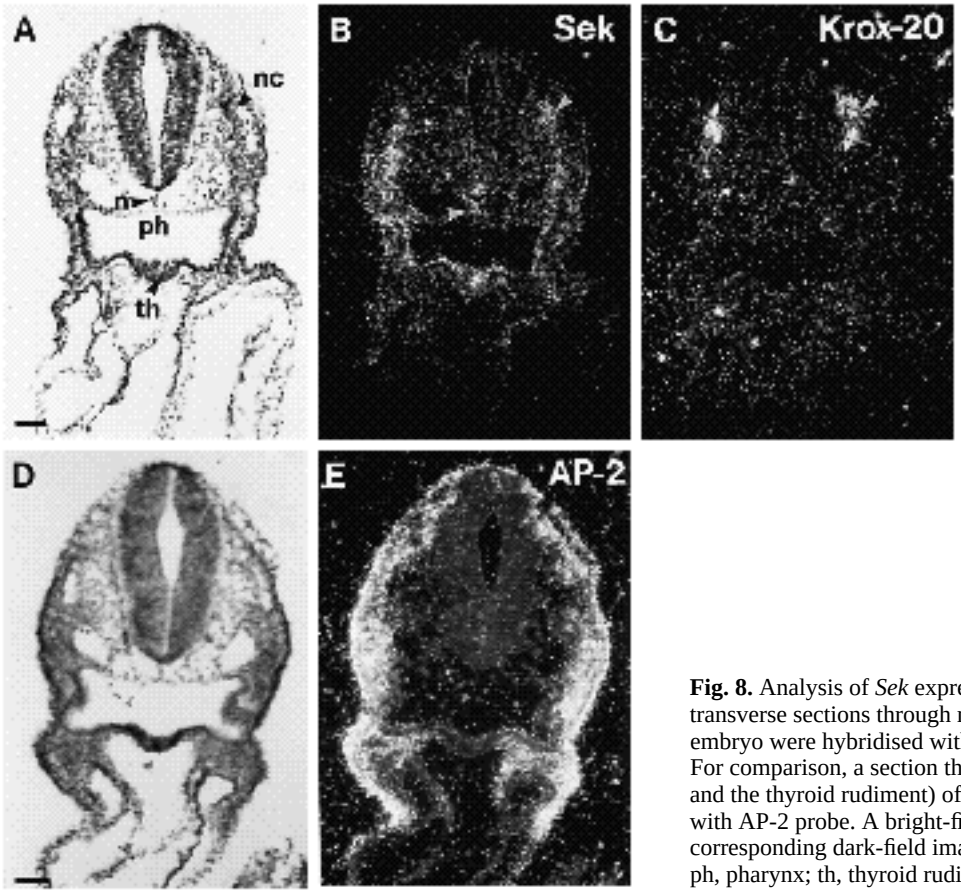

Fig. 8. Analysis of *Sek* expression in neural crest. (A-C) Adjacent transverse sections through rhombomere 6 of the 25-somite embryo were hybridised with *Sek* (B) or *Krox-20* (C) probe. (D,E) For comparison, a section through the same level (rhombomere 6 and the thyroid rudiment) of a different embryo was hybridised with AP-2 probe. A bright-field image is shown to the left of the corresponding dark-field image(s). n, notochord; nc, neural crest; ph, pharynx; th, thyroid rudiment.

subsequent morphological differentiation of the forebrain, expression becomes progressively more restricted: by 10.5 days to the telencephalon and dorsal diencephalon, and by 12.5 days to the prospective cerebral cortex and basal telencephalon. Little is known of cellular or molecular mechanisms that underlie the development of this region of the CNS, but recently a number of genes have been described with spatially restricted expression suggestive of roles in the regionalisation of the telencephalon and/or diencephalon. These include a number of homeobox genes, *Dlx* (Price et al., 1991), *Dlx-2* (Robinson et al., 1991), TTF-1 (Lazzaro et al., 1991), *Nkx-2.2* (Price et al., 1992), and *Otx-1* and *Otx-2* (Simeone et al., 1992) and the *Wnt-3* and *Wnt-3a* genes that encode putative cell signalling proteins (Roelink and Nusse, 1991). The *Emx-1* and *Emx-2* homeobox genes are also expressed in regions of the diencephalon and telencephalon (Simeone et al., 1992), but unlike *Sek* these domains also extend into the mesencephalon. At early stages, *Sek* expression encompasses the domains of all of these genes in the diencephalon and telencephalon, and upon restriction of *Sek* expression to the telencephalon, overlaps occur with the *Dlx*, *Emx*, and *Otx* family members and with *Wnt-3a*. However, there is no one-to-one correlation between the spatial expression pattern of any of these genes and *Sek* that would be suggestive of a simple regulatory link.

The expression of *Sek* in the spinal cord is distinct from that in the forebrain and hindbrain, in that it is initiated much later, at the onset of neuronal differentiation, and occurs in a dynamic pattern in the dorsoventral axis. In anterior regions (and at later stages, in posterior regions) of the spinal cord, expression is ventrally restricted at 9.5 days, then becomes more widespread, with higher levels in dorsolateral and ventral regions, at 10.5 days, and then is ventrally restricted at 12.5 days. The initial phases of this expression correlate with the general ventral-to-dorsal gradient in the birth of major classes of neurons, with motor neurons first to form in the ventral spinal cord, followed by relay neurons in intermediate regions and then the dorsal interneurons (Altman and Bayer, 1984). The initial ventral expression of *Sek* matches the birth of motor neurons, while the relatively widespread expression at 10.5 days is consistent with the considerable overlap in the birth of these classes of neurons. Based on this we suggest that *Sek* may have a role in the initial steps of neuronal differentiation in the spinal cord, while the later restriction to ventral regions reflects a continued function in motor neurons. The dynamic pattern of *Sek* expression is in contrast to the expression of the *Pax* genes, whose transcripts become restricted to specific dorsal (*Pax-3*: Goulding et al., 1991; *Pax-7*: Jostes et al., 1991) or ventral (*Pax-2*: Nornes et al., 1990; *Pax-6*: Walther and Gruss, 1992) regions of the spinal cord, suggestive of roles in regional specification. However, dynamic dorsoventral restrictions have been observed to occur in *Hox-2* gene expression, and suggested to indicate a role in conferring A-P positional identity to neurons (Graham et al., 1991). Up to 10.5 days, members of the *Hox-2* gene complex are expressed uniformly across the neural tube

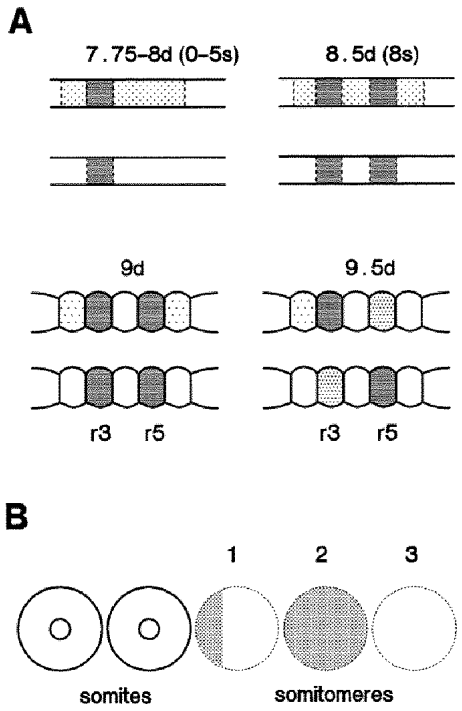

Fig. 9. Summary of segmental expression of *Sek*. (A) The time course of *Sek* expression in the hindbrain compared with *Krox-20* expression is depicted. For each stage, *Sek* expression is indicated by shading in the upper diagram and *Krox-20* expression in the lower diagram. We have not indicated the relationship between the timing of expression and the order of rhombomere boundary formation. s, somite stage; r, rhombomere. (B) Summary of the expression of *Sek* in mesoderm. Due to the progressive anterior-posterior segmentation of mesoderm, the diagram represents a time course, from right to left, of somite formation. The location of the stripes of expression suggests that the posterior domain correlates with somitomere 2, and the anterior domain with the anterior part of somitomere 1.

within their anterior-posterior domain of expression. Subsequently, expression is laterally restricted by 11.5 days, dorsally restricted at 12.5 days, then spreads into ventral regions by 14.5 days. This expression pattern does not correlate spatially or temporally with *Sek*, so it is not likely that there is a direct regulatory relationship between these genes.

Expression during mesoderm development

Analysis of early gastrulation-stage embryos reveals *Sek* transcripts in mesoderm adjacent to the primitive streak (M.A.N., unpublished observations), and this expression in early mesoderm occurs throughout gastrulation. Later in mesoderm development, expression is sharply up-regulated in presumptive somites (to be discussed below) and is found at lower levels in splanchnic mesoderm, the notochord, and head mesoderm. The significance of *Sek* expression in these sites is at present unclear, but the expression in early mesoderm is intriguing in view of the implication of various secreted growth factors, members of the FGF and TGF-β families (reviewed by Whitman and Melton, 1989), in the induction of mesoderm formation. The receptor tyrosine kinase encoded by *Sek* is not related to the known FGF or TGF-β receptors, so it will be important to identify its ligand in order to test its possible function, both in early mesoderm and in later derivatives.

Expression in neural crest

The expression of *Sek* in neural crest cells adjacent to rhombomere 6 is intriguing since *Krox-20*, too, is expressed in crest cells in this region (Wilkinson et al., 1989a). A detailed analysis of *Krox-20* expression in the mouse shows that transcripts are found only in crest cells in dorsal regions, presumably because expression is downregulated as these cells migrate ventrally (R. DasGupta et al., unpublished data). Based on the comparison presented here, we suggest that *Sek* and *Krox-20* are initially co-expressed in neural crest cells and that, unlike *Krox-20*, *Sek* transcripts persist during the migration of these cells. Thus, as also found for the rhombomeres, although *Sek* is up-regulated at the same time and in the same cell population as *Krox-20*, these genes are down-regulated at different times.

Potential roles of Sek *in hindbrain segmentation*

In the hindbrain, the spatial restriction of *Sek* transcripts precedes the formation of rhombomeres and the *Sek* gene product may therefore be involved in events that lead to the subdivision of the neural epithelium. The significance of the segment-restricted quantitative differences in *Sek* expression is at present difficult to assess, but it is striking that the alternating domains of high-level expression correlate with other aspects of hindbrain segmentation. Grafting experiments indicate that a cellular property that alternates between rhombomeres underlies the formation of boundaries (Guthrie and Lumsden, 1991), and the expression of *Sek* in presumptive r3 and r5 suggests that its product might have a role in cell-cell interactions that establish these cellular differences.

The up-regulation of *Sek* in the sites of *Krox-20* expression, in prospective rhombomeres and in crest, raises the possibility that they may be linked in a regulatory cascade, but it is not possible to infer which gene lies upstream in such a cascade from the relative timing of their expression (Fig. 9A). The expression of *Sek* initially in a broader domain than *Krox-20* argues that the latter is not involved in activation of the *Sek* gene, but could have a later role in regulating the segment-restricted high level expression of *Sek*. On the other hand, our data are consistent with *Sek* acting in signal transduction during the up-regulation of *Krox-20*. This latter possibility is particularly intriguing since *Krox-20* was initially identified as a gene that is transcriptionally up-regulated as an immediate-early response when fibroblasts are exposed to extracellular growth factors (Chavrier et al., 1988). It is not known whether the rapid up-regulation of *Krox-20* expression in embryos is also regulated through signal transduction pathways, but it will be interesting to test this, and to examine whether Sek protein might be a component of such a pathway. However, it must be emphasised that, if there is a regulatory relationship between *Sek* and *Krox-20*, it is not likely to be simple since although both are up-regulated at the same time, their down-regulation is not coordinate in the hindbrain (Fig. 9A) or crest.

Sek *and mesoderm segmentation*

The significance of the pattern of *Sek* expression for molecular mechanisms of mesoderm segmentation is difficult to assess since, thus far, no other genes have been implicated in this process, but the pattern does correlate with several cellular aspects of somitogenesis. Scanning electron microscope studies have revealed loose aggregations of cells, termed somitomeres, in paraxial mesoderm in the head, cervical and trunk regions (Meier, 1979; Tam et al., 1982). In the head, somites do not form, but in more posterior regions, somitomeres appear to be precursors to the epithelial somite (reviewed by Jacobson, 1988). However, somitomeres are not definitive segments since grafting experiments indicate cell mixing in the segmental plate (Tam, 1988) and clonal analysis reveals that cells are not allocated to specific somites until just prior to somite formation (Stern et al., 1988). Thus, it seems that critical events in segmentation occur in anterior presomitic mesoderm. Our observations indicate that *Sek* expression in paraxial mesoderm correlates with the formation of somites. In the head, we find only a low level of *Sek* expression in mesoderm, whereas in more posterior regions where somites are forming there is a sharp up-regulation of *Sek* expression in a presumptive somite, presumably the second (somitomere 2) relative to the most recently formed somite (Fig. 9B). These data suggest that Sek protein may have a role in cellular interactions that lead to the condensation of these cells into an epithelium. The potential significance of the subsequent anterior restriction of *Sek* expression in somitomere 1 is less clear. The anterior and posterior halves of each somite, and subsequently it's sclerotome derivative, differentially express a number of gene products, including cytotactin/tenascin (Tan et al., 1987, 1991; Mackie et al., 1988; Stern et al., 1989), cholinesterases (Layer et al., 1988), T-cadherin (Ranscht and Bronner-Fraser, 1991) and a number of proteins detected by two-dimensional gel analysis (Norris et al., 1989). Certain of these molecular differences may be involved in the subsequent migration of neural crest cells only through anterior half sclerotome (Ranscht and Bronner-Fraser, 1991; Tan et al., 1991; but see Stern et al., 1989). In addition, a peanut agglutinin-binding protein present only in posterior half sclerotome has been implicated in restricting the outgrowth of spinal motor axons to anterior sclerotome (Keynes and Stern, 1984; Stern et al., 1986; Davies et al., 1990). Might the anterior restriction of *Sek* expression be involved in generating differences between these anterior and posterior halves? Since we observe variation between embryos in the width of the anterior stripe (M. A. N., unpublished observations) it seems that there is a progressive posterior-to-anterior downregulation of expression in somitomere 1, rather than cessation throughout the posterior half and then the anterior half. Thus, we suggest that it is more likely that Sek protein is expressed in a gradient, rather than with a sharp boundary that would correlate with, and perhaps later define, the anterior and posterior halves of somites.

Significance of segmental expression in both mesoderm and the hindbrain

It is curious that *Sek* is expressed in prospective segmental units in both mesoderm and the hindbrain since, based on the overt morphological differences in segmentation, it is assumed that the underlying molecular mechanisms are distinct (reviewed by Stern, 1990); somites form sequentially by the condensation of mesenchymal cells, whereas rhombomeres form by the subdivision of a pre-existing epithelium and in a non-sequential manner. These gross differences are generally interpreted as evidence, albeit weak, for a separate evolutionary origin of segmentation in these tissues. If this view is correct, it follows that *Sek* has been independently recruited during evolution to the segmental patterning of mesoderm and the hindbrain. On the other hand, it is possible that the expression of *Sek* in prospective somites and rhombomeres is not fortuitous, but reflects a conserved mechanism involved in the formation of these segments. Regardless of whether *Sek* expression reflects a conservation or a convergence of function in segmentation, might it correlate with any similarities in the mechanisms by which somites and rhombomeres form? One potential link is that the aggregation of mesoderm cells and the alternation in cellular properties that restricts cells to specific rhombomeres (Guthrie and Lumsden, 1991) may both involve the regulated expression of cell adhesion molecules. Intriguingly, Sek protein, like other members of the *Eph*/*Elk* family (Pasquale, 1991), contains two fibronectin type III repeats in its extracellular domain, and thus it is possible that it directly mediates cell adhesion in presumptive somites and in alternating rhombomeres, r3 and r5. Recent studies of integrin receptors (reviewed by Hynes, 1992) provide precedents that such a role in adhesion could be coupled to signal transduction. Based on these ideas, it will be of interest to test for a function of *Sek* in cell adhesion and to analyse the effects of disrupting the normal expression or function of Sek protein in presumptive segments.

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