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Nab proteins mediate a negative feedback loop controlling Krox-20 activity in the developing hindbrain

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SUMMARY

The developing vertebrate hindbrain is transiently subdivided along the anterior-posterior axis into metameric units, called rhombomeres (r). These segments constitute units of lineage restriction and display specific gene expression patterns. The transcription factor gene *Krox-20* is restricted to r3 and r5, and is required for the development of these rhombomeres. We present evidence that Krox-20 transcriptional activity is under the control of a negative feedback mechanism in the hindbrain. This regulatory loop involves two closely related proteins, Nab1 and Nab2, previously identified as antagonists of Krox-20 transcriptional activity in cultured cells. Here we show that in the mouse hindbrain, *Nab1* and *Nab2* recapitulate the *Krox-20* expression pattern and that their expression is dependent on Krox-20 function. Furthermore, misexpression of *Nab1* or *Nab2* in zebrafish embryos leads to alterations in the expression patterns of several hindbrain markers, consistent with an inhibition of Krox-20 activity. Taken together, these data indicate that Krox-20 positively regulates the expression of its own antagonists and raise the possibility that this negative feedback regulatory loop may play a role in the control of hindbrain development.

Key words: Krox-20, Nab1, Nab2, Transcriptional repression, Hindbrain development, Rhombomere identity, Cell segregation

INTRODUCTION

The development of the vertebrate hindbrain involves a transient segmentation process along the anterior-posterior (AP) axis, which leads to the formation of 7-8 morphological units termed rhombomeres (r) (Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996; Wingate and Lumsden, 1996; Schneider-Maunoury et al., 1998). The rhombomeres behave as units of lineage restriction (Fraser et al., 1990; Birgbauer and Fraser, 1994) and play an essential role in establishing the pattern of both hindbrain and craniofacial morphogenesis. In particular, this subdivision presages the periodic organisation of neurons (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993) and correlates with the pathways of neural crest migration into the branchial arches (Lumsden and Guthrie, 1991; Lumsden et al., 1991; Serbedzija et al., 1992; Birgbauer et al., 1995; Kontges and Lumsden, 1996).

A number of putative regulatory genes have been shown to present spatially restricted patterns of expression along the AP axis in the hindbrain, with limits corresponding to prospective or established rhombomere boundaries (reviewed in Lumsden and Krumlauf, 1996). Mutational analyses indicate that several of these genes do indeed play important roles in the control of hindbrain development (reviewed in Schneider-Maunoury et al., 1998). Among them, *Krox-20*, which encodes a zinc finger transcription factor (Chavrier et al., 1988, 1990), appears to be essential for the formation and specification of r3 and r5. *Krox-20* is successively activated in two transverse stripes, which prefigure and subsequently coincide with r3 and r5 (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993). It is also expressed in neural crest cells emigrating from r5 toward the third branchial arch (Schneider-Maunoury et al., 1993; Nieto et al., 1995). Targeted inactivation of *Krox-20* leads to progressive disappearance of the r3 and r5 territories (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993) indicating that this gene participates in the control of the segmentation process. Furthermore, Krox-20 has been shown to regulate the transcription of several *Hox* genes in r3 and/or in r5, directly or indirectly (Sham et al., 1993; Nonchev et al., 1996a,b; Vesque et al., 1996; Seitanidou et al., 1997). Since the combinatorial expression of *Hox* genes is believed to determine the AP positional identity of the rhombomeres, this indicates that Krox-20 also plays an important role in the specification of positional information. Finally, Krox-20 has been shown to directly control the transcription of *EphA4*, which encodes a transmembrane tyrosine kinase receptor (Theil et al., 1998). Since Eph family members and their ligands have been implicated in the segregation of cells between odd and even rhombomeres (Xu et al., 1995, 1999; Mellitzer et al., 1999), this observation suggests that Krox-20 could participate in the

establishment of lineage restrictions within the hindbrain. Taken together these various data indicate that Krox-20 is a key regulator of gene expression in r3 and r5 and co-ordinates various aspects of the segmentation and specification processes.

While several of the molecular targets of Krox-20 have been identified, very little is known about the regulation of *Krox-20* expression and activity. Two potential modulators of Krox-20 transcriptional activity, Nab1 and Nab2, have been discovered (Russo et al., 1993, 1995; Svaren et al., 1996). These proteins were identified on the basis of their capacity to directly interact with Krox-24 (also known as NGFI-A, Egr1 or Zif268), a zinc finger transcription factor closely related to Krox-20 (Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988). It was shown that they can also bind to Krox-20 and that they inhibit transcriptional activation by both zinc finger proteins in cultured cells (Russo et al., 1995). This inhibition presumably involves the formation of a ternary complex between the DNA target sequence, Krox-20 or Krox-24, and the Nab protein, the latter carrying a transcription repression domain (Svaren et al., 1998; Swirnoff et al., 1998). The potential involvement of *Nab1* and *Nab2* in the modulation of Krox-20 function led us to investigate their expression in the developing hindbrain. We found that their expression patterns closely overlap with that of *Krox-20* in r3 and r5. Furthermore, in *Krox-20*$^{-/-}$ embryos, *Nab1* and *Nab2* are not expressed in the hindbrain, indicating that these genes are downstream of *Krox-20*. Finally, misexpression experiments performed in zebrafish embryos suggest that both Nab1 and Nab2 can antagonise Krox-20 action. In conclusion, these data indicate that Krox-20 controls the expression of its own antagonists and suggest that this feedback regulatory loop plays an important role in the development of the hindbrain.

MATERIALS AND METHODS

Isolation of mouse *Nab1* and *Nab2* cDNAs and RNA preparation

Mouse cDNAs corresponding to the complete coding sequence of *Nab2* (1575 nucleotides encoding the 525-amino-acid protein) and the near-complete coding sequence of *Nab1* (1383 nucleotides encoding the 461 N-terminal amino acids of the 489-amino-acid protein) were isolated by reverse transcription-PCR amplification. Total RNA was prepared as described (Chomczynski and Sacchi, 1987), from newborn mouse brain (*Nab1*) or Ras-transformed NIH3T3 fibroblasts (*Nab2*). cDNA was prepared with MMLV Reverse Transcriptase using degenerated hexamers (pDN6, Pharmacia, Uppsala, Sweden), following the conditions of the manufacturer (Life Technologies, Bethesda, MD). The product of the reaction was diluted 20-fold for PCR amplification in the buffer recommended by the manufacturer of the Taq DNA Polymerase (Eurobio). The sequences of the primers were deduced from the published *Nab1* and *Nab2* nucleotide sequences (Russo et al., 1995; Svaren et al., 1996): *Nab1*, 5′-ATGGCCACAGCCTTACCTAGGAC-3′ and 5′-CCCAAGGCTC-TCTGAGGAGTGGG-3′; *Nab2*, 5′-ATGCACAGAGCTCCCTCTCC-CACAG-3′ and 5′-TCACTGCCGGCTGGCTTCTGCCTC-3′. 25 cycles of amplification were carried out at 94°C for 1 minute, 55°C for 1 minute, 30 seconds and 72°C for 1 minute, 30 seconds. The expected products of amplification were purified by electrophoresis on 1% agarose gel (Quiaex kit, Quiagen) and inserted into the PCRIITm plasmid by TA cloning (TA cloning kit, Invitrogen, La Jolla, CA). The fragments were subsequently recloned into Bluescript KS+, sequenced and shown to encode bona fide Nab1 and Nab2 proteins. For the preparation of sense RNA for injection in zebrafish embryos, the cDNAs were recloned into the pCS2+ vector (Turner and Weintraub, 1994). The concentrations of RNA preparations were estimated by gel electrophoresis analysis.

X-gal staining and whole-mount in situ hybridisation on mouse embryos

Postimplantation mouse embryos were recovered at the appropriate stage (8-10 days post coitum: dpc) and fixed at 4°C in PBS containing 4% paraformaldehyde for 30 minutes for X-gal staining or for 8 hours for direct in situ hybridisation. X-gal staining was performed as described (Sham et al., 1993). Embryos were refixed for 8 hours after X-gal staining, dehydrated in methanol and processed for whole-mount in situ hybridisation as described (Seitanidou et al., 1997). Digoxigenin antisense RNA probes were generated using a Stratagene RNA transcription kit.

Zebrafish embryo injection, X-gal staining and whole-mount in situ hybridisation

Zebrafish eggs were obtained by natural spawning, collected and maintained at 28.5°C in E3 embryo medium supplemented by Methylene Blue (4 μg/ml) to inhibit fungal growth (Haffter, 1996). Embryos were staged according to Kimmel et al. (1995). They were injected at the 2-4-cell stage with 200 pl of a solution containing the RNA and 0.2% Phenol Red as described (Wittbrodt and Rosa, 1994). They were then allowed to develop until the 5- to 10-somite stage and were fixed for 30 minutes at room temperature in 4% paraformaldehyde in PBS containing 0.1% Tween 20 (PBT). After three washes in PBT, they were dissected from the vitellus and stained with X-gal for 15 minutes as described (Haddon et al., 1998). The embryos were then refixed overnight at 4°C, dehydrated in methanol and processed for in situ hybridisation (Oxtoby and Jowett, 1993). Single in situ hybridisations were performed with digoxygenin-labelled probes. Double in situ hybridisations also included a fluorescein-labelled *Krox-20* probe and were performed as described (Hauptmann and Gerster, 1994; Prince et al., 1998), except that we used BM purple (Boehringer) as a substrate for the detection of digoxygenin instead of NBT and BCIP. After hybridisation, the embryos were stored in PBT at 4°C, flattened under a coverslip and photographed in 100% glycerol. Several concentrations of *Nab1* or *Nab2* RNAs were initially tested (5, 10, 20, 50 and 100 ng/μl) in these injection experiments. The highest concentrations (50 and 100 ng/μl) led to a high proportion of embryos with gastrulation defects and the lowest (5 ng/μl) did not induce any phenotype. We therefore selected the concentration of 20 ng/μl for further experiments and coinjected *lacZ* RNA with *Nab* RNA to reach a constant total RNA concentration of 100 ng/μl.

RESULTS

Overlapping expression patterns of *Krox-20*, *Nab1* and *Nab2* in the hindbrain

To investigate a role for Nab1 or Nab2 in the modulation of Krox-20 transcriptional activity in the hindbrain, we examined their expression patterns by whole-mount in situ hybridisation during the period of *Krox-20* expression in r3 and r5, i.e. between 8 and 10 dpc. During this period, we found that, like *Krox-20*, the expression of both genes was restricted to r3 and r5 (Fig. 1). The localisation of the sites of expression was first estimated according to morphological landmarks (pre- and post-otic sulci, otic placode). *Nab1* is activated in the hindbrain at around the 6- to 8-somite stage approximately simultaneously in prospective rhombomeres (pr) 3 and 5

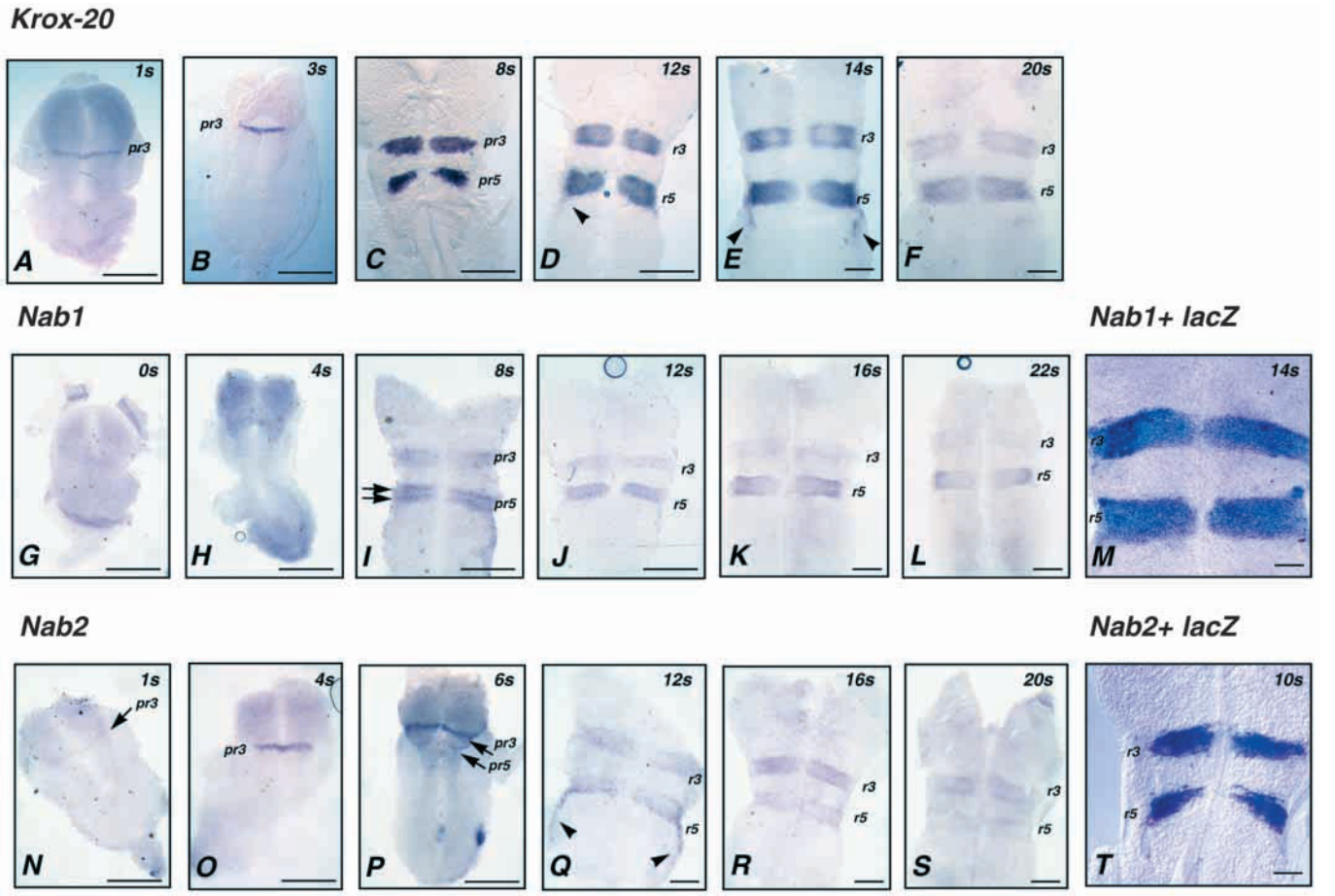
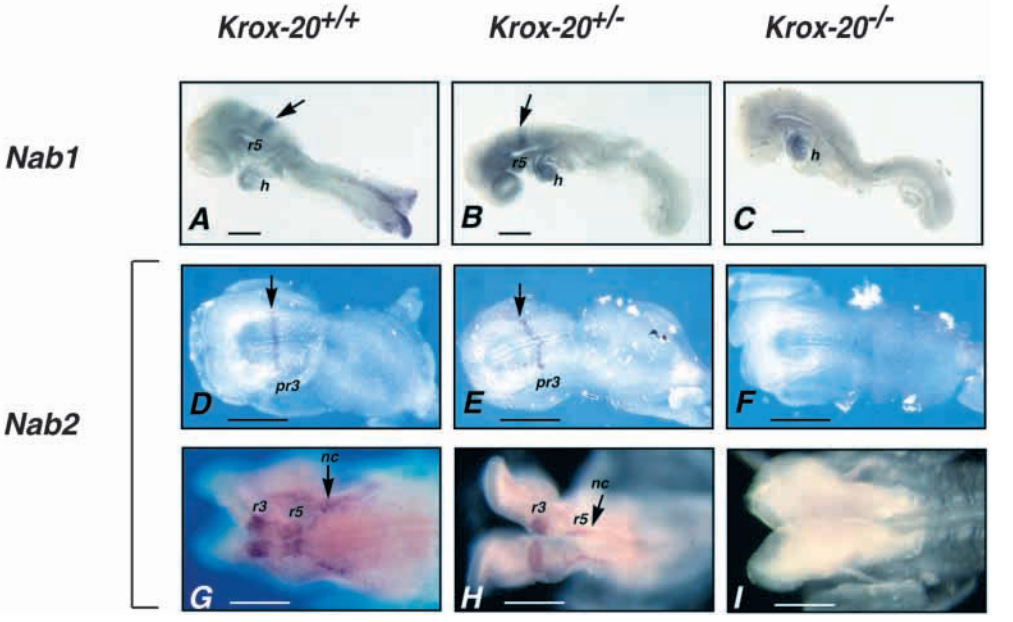


Fig. 1. Comparison of *Krox-20*, *Nab1* and *Nab2* expression patterns in the developing mouse hindbrain. Whole-mount in situ hybridisations were performed with *Krox-20* (A-F), *Nab1* (G-L) or *Nab2* (N-S) probes on wild-type embryos at the indicated somite stage. Whole embryos are shown in (A,B,G,H,N-P) and flat mounts of the hindbrain opened on the dorsal edge in (C-F,I-M,Q-T). Rostral is to the top. Note in (I) the higher level of *Nab1* expression at the boundaries of r5 (arrows) and in (Q) the expression of *Nab2* in neural crest cells presumably emigrating from r5 (arrowheads). (M) and (T) show double labelling by in situ hybridisations with the *Nab1* (M) and *Nab2* (T) probes and X-gal staining to mark r3 and r5 on an embryo heterozygous for the *Krox-20/lacZ* knock-in. Note the overlap between the two types of signal, indicating that *Nab* expression is restricted to r3 and r5. pr, prospective rhombomere; r, rhombomere. Bars, 60 μm (A-L,N-S), 30 μm (M,T).

Fig. 2. Krox-20 is required for *Nab1* and *Nab2* expression in the hindbrain. Wild-type (A,D,G), heterozygous (B,E,H) and homozygous *Krox-20* mutant embryos (C,F,I) were analysed by whole-mount in situ hybridisation using *Nab1* (A-C) or *Nab2* (D-I) probes. The stages of the embryos, 8-10 somites for *Nab1* and 4-6 and 10-12 somites for *Nab2*, were chosen to maximise the detection of the gene while r5 (C,I) or r3 (F) were still present in the homozygous mutants, or to analyse the expression in the neural crest (I). Embryos are oriented rostral to the left. Note the absence of *Nab1* mRNA in the homozygous *Krox-20* mutant (C) in r5 (r3 has already almost disappeared at this stage), while the expression is maintained in the heart (h). Similarly, note the absence of *Nab2* mRNA in r3 (F) and in r5 and r5-derived neural crest (nc) (I). Bars, 100 μm.



(Fig. 1I). At this stage, the level of expression appears stronger in pr5 than in pr3 and close to the interface between these prospective rhombomeres and the adjacent ones. Subsequently, the expression becomes progressively more uniform within r3 and r5, while remaining higher in the latter rhombomere (Fig. 1J). Finally *Nab1* expression fades away in r3 and in r5, starting from their ventral part (Fig. 1K,L), persisting in dorsal r5 until the 30-somite stage (data not shown). The pattern of *Nab1* extinction in r3 and r5 is very similar to that of *Krox-20* (Fig. 1D-F; Irving et al., 1996).

Nab2 activation in the hindbrain occurs earlier than *Nab1*, at around the 1-somite stage, and is first restricted to a narrow stripe likely corresponding to pr3 (Fig. 1N). Later on, this expression becomes more intense (Fig. 1O) and at around the 6-somite stage a second, more caudal stripe of expression is observed coinciding with pr5 (Fig. 1P). This sequential activation of *Nab2* in pr3 and pr5 faithfully reflects the pattern of activation of *Krox-20* in these prospective rhombomeres (Fig. 1A-C; Irving et al., 1996). At later stages *Nab2* is expressed at similar levels in r3 and r5, with a slight reinforcement at the boundaries of these rhombomeres (Fig. 1Q-S). At around the 12-somite stage, *Nab2* expression was also detected in the r5-derived neural crest migrating toward the third branchial arch (Fig. 1Q, arrowheads). From the 16-somite stage, *Nab2* became progressively downregulated in r3 and r5 and the RNA was not detected beyond the 25-somite stage (data not shown).

To confirm the coexpression of *Nab1* and *Nab2* with *Krox-20* in r3 and r5, we performed double labelling experiments. For this purpose, we made use of a mouse line carrying a 'knock-in' of *lacZ* into the *Krox-20* locus (Schneider-Maunoury et al., 1993). Embryos heterozygous for this mutation develop normally and the *Krox-20/lacZ* gene recapitulates the normal expression of *Krox-20*. *Nab1* and *Nab2* mRNAs were detected by in situ hybridisation and *Krox-20* expression by X-gal staining. The *Nab1* and *Nab2* expression domains were completely included within the X-gal positive regions (Fig. 1M,T). The *Nab1* signal was only slightly visible (i.e. not completely masked by the X-gal) close to the transversal limits of r5 (Fig. 1M), confirming that the stripes of reinforced expression abut these boundaries.

In conclusion, both *Nab1* and *Nab2* present patterns of expression within the developing hindbrain which strikingly overlap with that of *Krox-20.* More specifically, the activation pattern of *Nab2* and the downregulation profile of *Nab1* very closely resemble those of *Krox-20*. An interesting feature of the *Nab1* pattern is the reinforcement at the boundaries of r3 and r5 at around the 8-somite stage, which does not reflect the rather uniform expression of *Krox-20* in these rhombomeres, as seen by in situ hybridisation, reporter tracing and protein immunochemistry (Fig. 1C and data not shown; Schneider-Maunoury et al., 1993; Irving et al., 1996). Together these data raise the possibility that the Nab proteins could indeed modulate the transcriptional activity of Krox-20 in the hindbrain.

Krox-20 regulates *Nab1* and *Nab2* in the hindbrain

The overlapping patterns of expression of the three genes in the developing hindbrain raise the possibility that *Nab1* and *Nab2* may lie downstream of *Krox-20* in a regulatory pathway. To test this possibility, we examined *Nab1* and *Nab2* expression in *Krox-20*$^{-/-}$ embryos. Although *Krox-20* inactivation leads to the disappearance of r3 and r5, this phenomenon is progressive and the r3 and r5 territories can be observed until the 6-8 and 12- to 14-somite stages, respectively (Schneider-Maunoury et al., 1993, 1997; Seitanidou et al., 1997). In the case of *Nab1*, the late and weak activation in r3, at around the 8-somite stage, precludes the investigation of its possible regulation by Krox-20 in this rhombomere. In contrast, we could show that the stripe of *Nab1* mRNA corresponding to r5 is not observed in *Krox-20* homozygous mutant embryos, while it is present in both wild type and heterozygous embryos at the 8- to 10-somite stage (Fig. 2A-C). The persistence of the *Nab1* signal in the heart region in *Krox-20*$^{-/-}$ embryos attests that hybridisation occurred normally (Fig. 2C). In the case of *Nab2*, the highest level of expression is observed in wild-type embryos in r3 at around the 4- to 6-somite stage (Fig. 1N-S). We therefore first performed an analysis at this stage and observed that in *Krox-20*$^{-/-}$ embryos no activation of *Nab2* occurs in the prospective r3 territory (Fig. 2D-F). Examination of older embryos (10- to 12-somite) demonstrated that in *Krox-20*$^{-/-}$ embryos *Nab2* expression is also lost in r5 and in the r5-derived neural crest cells (Fig. 2G-I). These latter cells, which express *Krox-20* in the wild-type situation, have been shown previously not to be affected at this stage in their survival by the mutation (Schneider-Maunoury et al., 1993; Seitanidou et al., 1997). In contrast *Nab2* expression is maintained in the developing heart (data not shown).

In conclusion, these data indicate that Krox-20 function is required for expression of *Nab1* at least in r5 and of *Nab2* in r3, r5 and the r5-derived neural crest. Therefore, in these territories, the transcription factor Krox-20 appears to positively regulate the transcription of genes encoding putative antagonists of its own transcriptional activity.

Nab1 or *Nab2* misexpression results in alterations in Krox-20 target gene pattern

The analysis of *Nab1* and *Nab2* mRNA patterns indicated that these genes are essentially coexpressed with *Krox-20* during hindbrain development, raising the possibility that they may antagonise its activity. Therefore we decided to investigate whether overexpression or premature activation of these genes might affect Krox-20 transcriptional activity. Ectopic expression can be very efficient in the zebrafish (*Brachydanio rerio*), following injection of RNA into early embryos. On the basis of the strong conservation of the *Krox-20* expression pattern and of the amino acid sequence between mouse and fish (72% identity; Oxtoby and Jowett, 1993), it is likely that the protein plays very similar or an identical function during hindbrain development in the two species. Furthermore, since the region of the Krox-20 protein known to interact with the Nab proteins (Russo et al., 1995) shows an even higher degree of similarity between the two species (94% between residues 244-277 and 226-259 in the mouse and zebrafish sequences, respectively), it is also likely that mouse Nab1 and Nab2 can bind to the fish and mouse Krox-20 proteins with similar affinities.

Therefore we decided to investigate the capacity of Nab protein to antagonise Krox-20 function during hindbrain development by injecting mouse *Nab1* or *Nab2* RNA into zebrafish embryos at the 2- to 4 cell stages. To evaluate the effect of this ectopic expression of *Nab* genes, we studied its consequences on the expression of a Krox-20 target gene,

EphA4 (previously known as *Sek1* in the mouse and *rtk1* in zebrafish), by whole-mount in situ hybridisation at the 6- to 8-somite stage. In the zebrafish hindbrain, *EphA4* was shown to be expressed in r3 and r5, a pattern very similar, although not identical, to that of the mouse (MacDonald et al., 1994; Xu et al., 1994, 1995). In the mouse, *EphA4* was demonstrated to be under the direct transcriptional control of Krox-20 in r3 and r5 (Seitanidou et al., 1997; Theil et al., 1998).

The optimal amount of *Nab* RNA to inject was determined in a first series of experiments (see Materials and Methods). In the following experiments, the *E. coli lacZ* RNA was coinjected as a lineage tracer and β-galactosidase activity was detected by X-gal histochemistry. Embryos injected with *lacZ* RNA only or a *Nab2* RNA carrying a frame-shift mutation immediately 3′ to the initiation codon (*Nab2mut*) were used as controls. In uninjected and control embryos, at the 6- to 8-somite stage, *EphA4* transcripts were detected in the forebrain, the otic and olfactory placodes, the notochord, r3 and r5 (Fig. 3A,B and data not shown). In the hindbrain, the r3 and r5 territories of *EphA4* expression appear as even transversal stripes, with straight interfaces orthogonal to the AP axis. In contrast, more than 40% of embryos injected with *Nab1* (Fig. 3C-H) or *Nab2* (Fig. 3I-N) present obvious alterations in the segmental *EphA4* expression pattern (Table 1A), while the general organisation of the embryos did not seem to be affected except for a slight, general widening of the neural tube. The r3/r5 *EphA4* stripes were irregular in shape and in some cases their extension was reduced as if parts of the expression domains had been eliminated. We also observed situations in which the level of *EphA4* mRNA was reduced in a few cells, possibly directly reflecting a downregulation of *EphA4* expression (Fig. 3H). The missing parts of r3 or r5 did not always coincide with areas of *lacZ* expression. Alterations of *EphA4* expression were observed at the levels of r3 or r5 with comparable frequencies (Table 1A). Alterations affecting both r3 and r5 were less frequent (Table 1A) and were generally associated with a higher number and a more widespread distribution of X-gal-positive cells.

EphA4 expression in the forebrain or notochord was never affected by *Nab1* or *Nab2* injection (Fig. 3C-E, I-K and data not shown). Furthermore, cohybridisation with a *Paxb* probe, which labels a transversal territory at the level of the mid/hindbrain junction (Krauss et al., 1991), indicated that the *Paxb*-positive stripe was not affected like the r3/r5 *EphA4* positive domains by the injection (Fig. 3E,H,K,N). Therefore, effects of *Nab* misexpression on *EphA4* expression appeared to be specific and were only observed in territories where Krox-20 is known to be involved in the activation of *EphA4* transcription, suggesting that the Nab proteins are indeed acting by interfering with the normal Krox-20 function and that this might include repression of transcriptional activation. This latter possibility is reinforced by the observation of cells where the level of expression of *EphA4* is simply reduced (Fig. 3H, arrow).

To investigate directly whether the Nab proteins were acting solely by antagonising Krox-20, we attempted to rescue the *Nab*-mediated phenotype by coinjection of *Nab1* or *Nab2* with *Krox-20* RNA. Preliminary experiments indicated that injection of *Krox-20* mRNA at concentrations higher than 0.2 ng/μl resulted in a very high incidence of major developmental defects, with more than 70% of dead or grossly abnormal embryos at the stage of examination (data not shown). In addition, coinjection of 0.2 ng/μl of *Krox-20* mRNA with *Nab* mRNAs was not effective in rescuing the *EphA4* hindbrain phenotype (data not shown). Therefore, the induction of early defects during gastrulation by *Krox-20* misexpression precluded our analysis of its capacity to rescue the *Nab*-mediated phenotype at the 6- to 8-somite stage.

Nab1 or *Nab2* misexpression results in alterations of *Krox-20*-positive territories

The observation that loss of *EphA4* expression is not always associated with *lacZ* expression in the *Nab* RNA injection experiments suggests that *Nab* misexpression might modify the fate of the *Nab*-expressing cells or even of adjacent *Nab*-negative cells. To investigate this possibility, we repeated the *Nab* RNA injection experiments and analysed the effects on the *Krox-20*-positive territories by whole-mount in situ hybridisation. Uninjected and control embryos (Fig. 4A,B and data not shown) presented a typical *Krox-20* (*Krx-20*) expression pattern with two even transversal stripes corresponding to r3 and r5 and the r5-derived neural crest. In contrast, in embryos injected with *Nab1* (Fig. 4C-E) or *Nab2* (Fig. 4F-H), the stripes were distorted, with regions severely narrowed or even eliminated. The phenotype appeared to affect more frequently and, in a more dramatic manner, r5 than r3 (Table 1B, Fig. 4C,F), although r3 was also grossly abnormal in some cases (Fig. 4H). The affected regions were often X-gal-negative. The phenotypes and frequencies appeared similar in the cases of injection of *Nab1* or *Nab2* (Table 1B). In addition, the percentage of affected embryos was about twofold lower than in the case of the analysis of the consequences on *EphA4* expression (Table 1B). As indicated above, the *Paxb*-positive stripe did not appear to be affected by the misexpression of the *Nab* genes (Fig. 4E,H), suggesting that the phenotype is restricted to the r3-r5 region. Finally, we also noted occasional alterations in the pattern of migration of r5-derived *Krox-20*-positive neural crest cells (data not shown).

In conclusion, *Nab1* or *Nab2* injection appears to affect not only the expression of a Krox-20 target gene, but also the shape and extension of the domains of expression of *Krox-20* itself, suggesting a role for the Nab proteins either in the control of *Krox-20* transcription or on the fate of *Krox-20*-positive cells.

Nab1 or *Nab2* misexpression also affects the r4 territory

Since *Nab* misexpression often led to modifications of the domains of *Krox-20* expression, we wondered whether this was accompanied by an alteration of the r4 territory. The *Hoxb1* gene is specifically expressed in this rhombomere at the 6- to 8-somite stage (Prince et al., 1998). As anticipated, whole-mount *Hoxb1* in situ hybridisation on uninjected or control embryos revealed a single transversal stripe of expression corresponding to r4 (Fig. 5A,B and data not shown). In contrast, about 20% of the embryos injected with *Nab1* or *Nab2* RNA presented visible alterations of the *Hoxb1*-positive territory (Table 1C). Frequently, these alterations concerned the posterior part of the territory, with caudal extensions; however, these did not cover the entire medio-lateral width of the rhombomere, the rest of it appearing sometimes reduced in width (Fig. 5D,E,G,H). Finally, by performing double in situ hybridisations, we examined whether the variations in shapes

and extent of the *Krox-20*- and *Hoxb1*-positive territories maintained the complementarity between the respective expression domains. In embryos injected with *Nab1* or *Nab2* RNA, *Krox-20*- and *Hoxb1*-positive domains were found perfectly adjacent, with no cell in between expressing either marker (Fig. 5F,I).

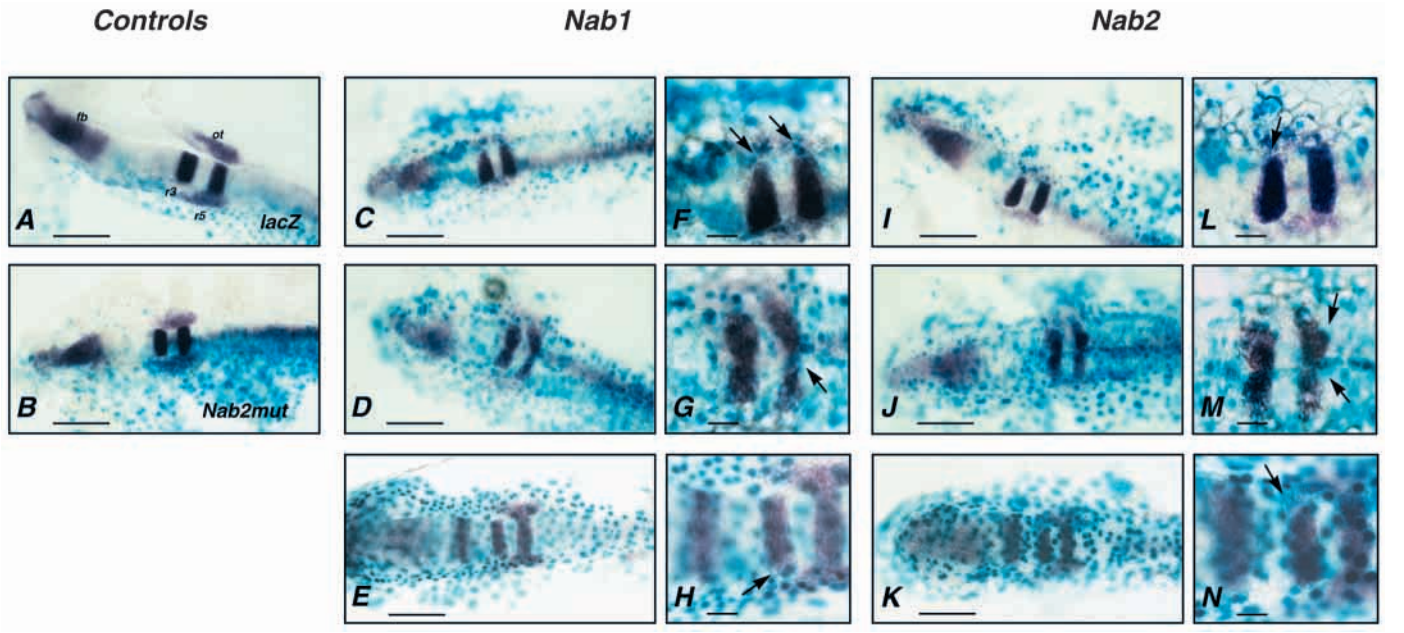


Fig. 3. Alterations of *EphA4* hindbrain expression pattern following *Nab* mRNA injection. mRNAs encoding *Nab1* or *Nab2* were microinjected together with *lacZ* mRNA into zebrafish eggs at the 2- to 4 cell stage. The embryos were allowed to develop until the 6- to 8-somite stage, stained for β-galactosidase activity and analysed by whole-mount in situ hybridisation with an *EphA4* (*rtk1*) probe or with both *EphA4* and *Paxb* probes. Embryos were mounted under a coverslip and photographed from a dorsal view. Rostral is to the left. In situ hybridisation labelling appears as a brown-purple precipitate, while X-gal staining is blue. (A) Embryo injected with *lacZ* mRNA only. (B) Embryo injected with a mutant *Nab2* mRNA encoding a non-functional protein. (C-E) Low magnifications of embryos injected with *Nab1* and revealed with *EphA4* (C,D) or *EphA4* and *Paxb* (E) probes. (F-H) High magnification of the same embryos. (I-K) Low magnification of embryos injected with *Nab2* and revealed with *EphA4* (I,J) or *EphA4* and *Paxb* (K) probes. (L-N) High magnification of the same embryos. The arrows identify regions of r3 and r5 where the expression of the *EphA4* gene appears the most dramatically affected. r, rhombomere; fb, forebrain; ot, otic placode. Bars, 200 μm (A-D,G,H), 40 μm (E,F,I,J).

Table 1. *Nab1* or *Nab2* mRNA injection in zebrafish embryos

	mRNA injected			
Experiment	*lacZ*	*lacZ+Nab2mut*	*lacZ+Nab1*	*lacZ+Nab2*
A				
EphA4 probe				
Total number of β-galactosidase-positive embryos	39	33	92	123
Embryos with alteration of *EphA4* expression in r3	1 (3%)	0 (0%)	18 (19.6%)	20 (16.4%)
Embryos with alteration of *EphA4* expression in r5	0 (0%)	0 (0%)	14 (15.2%)	23 (18.6%)
Embryos with alteration of *EphA4* expression in r3+r5	0 (0%)	0 (0%)	9 (9.8%)	11 (8.9%)
Embryos with no visible phenotype	38 (98%)	33 (100%)	51 (55.4%)	69 (56.1%)
B				
Krx-20 probe				
Total number of β-galactosidase-positive embryos	30	18	54	77
Embryos with alteration of *Krx-20* expression in r3	0 (0%)	0 (0%)	3 (5.7%)	4 (5.2%)
Embryos with alteration of *Krx-20* expression in r5	0 (0%)	0 (0%)	7 (12.9%)	11 (14.3%)
Embryos with alteration of *Krx-20* expression in r3+r5	0 (0%)	0 (0%)	3 (5.5%)	6 (7.8%)
Embryos with no visible phenotype	30 (100%)	18 (100%)	41 (75%)	56 (72%)
C				
Hoxb1 probe				
Total number of β-galactosidase-positive embryos	17	14	22	31
Embryos with alteration of *Hoxb1* expression	0 (0%)	0 (0%)	5 (23%)	6 (19%)
Embryos with no visible phenotype	17 (100%)	14 (100%)	17 (67%)	25 (81%)

Consequences of *Nab1* or *Nab2* ectopic expression have been followed by whole-mount in situ hybridisation with the *EphA4* (A), *Krx-20* (B) and *Hoxb1* (C) probes. *lacZ* mRNA was added to *Nab1* or *Nab2* mRNA (20 ng/μl) to reach a total RNA concentration of 100 ng/μl. The numbers of X-gal-positive embryos presenting alterations in the pattern of expression of each gene are indicated as well as their percentage among β-galactosidase-positive embryos (in parentheses). Embryos showing serious gastrulation defects were not scored (these represented fewer than 5-10% of the total number of injected embryos).

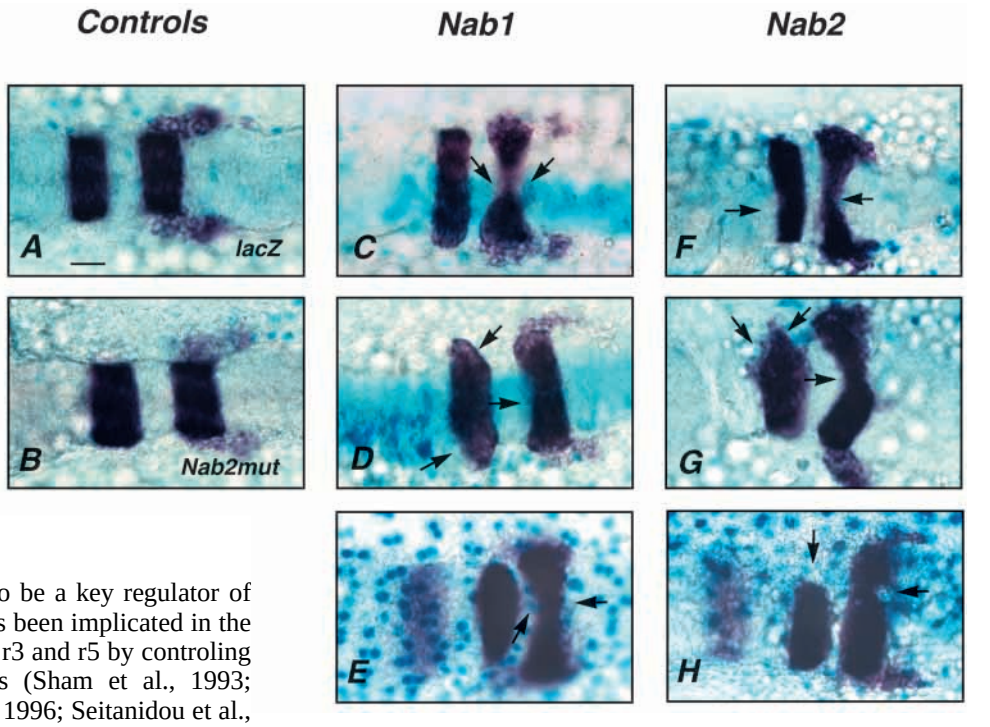


Fig. 4. *Nab* ectopic expression affects the *Krox-20* mRNA pattern in the hindbrain. Embryos were treated as described in Fig. 3 and analysed by whole-mount in situ hybridisation with a *Krox-20* probe or with both *Krox-20* and *Paxb* probes. (A) Embryo injected with *lacZ* mRNA only. (B) Embryo injected with a mutant *Nab2* mRNA encoding a non-functional protein. (C-E) Embryos injected with *Nab1* mRNA and revealed with *Krox-20* (C,D) or *Krox-20* and *Paxb* (E). (F-H) Embryos injected with *Nab2* mRNA and revealed with *Krox-20* (F,G) or *Krox-20* and *Paxb* (H). The arrows point to areas where the expression of *Krox-20* appears to be lost. Bar, 40 μm.

DISCUSSION

Krox-20 has previously been shown to be a key regulator of gene expression in the hindbrain. It has been implicated in the determination of segmental identity in r3 and r5 by controling the expression of several *Hox* genes (Sham et al., 1993; Nonchev et al., 1996a,b; Vesque et al., 1996; Seitanidou et al., 1997) and in the segregation between even and odd numbered rhombomeres through regulation of a member of the *Eph* family, *EphA4* (Seitanidou et al., 1997; Theil et al., 1998). Furthermore, Krox-20 has been shown to be required for the maintenance of the r3 and r5 territories, which might involve a control on cell proliferation or survival (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993). Therefore, Krox-20 appears as a central co-ordinator of several essential cellular functions in these rhombomeres. The present work demonstrates that two additional genes, *Nab1* and *Nab2*, are downstream to Krox-20, and introduces additional complexity in the regulatory cascade. It suggests the existence of a negative feedback regulatory loop, since *Nab1* and *Nab2* encode proteins able to antagonise Krox-20 transcriptional activity in the hindbrain. Therefore, the *Nab* genes are likely to play important roles in the development of the hindbrain by controlling Krox-20 activity.

Krox-20 regulates the expression of its own antagonists in the hindbrain

We have shown that *Nab1* and *Nab2* present spatial-temporal patterns of expression within the hindbrain and the neural crest cells, that closely overlap with that of *Krox-20*. Furthermore, targeted inactivation of *Krox-20* prevents the expression of these two genes in these territories of overlap (i.e. r3, r5 and the r5-derived neural crest), but does not affect their expression

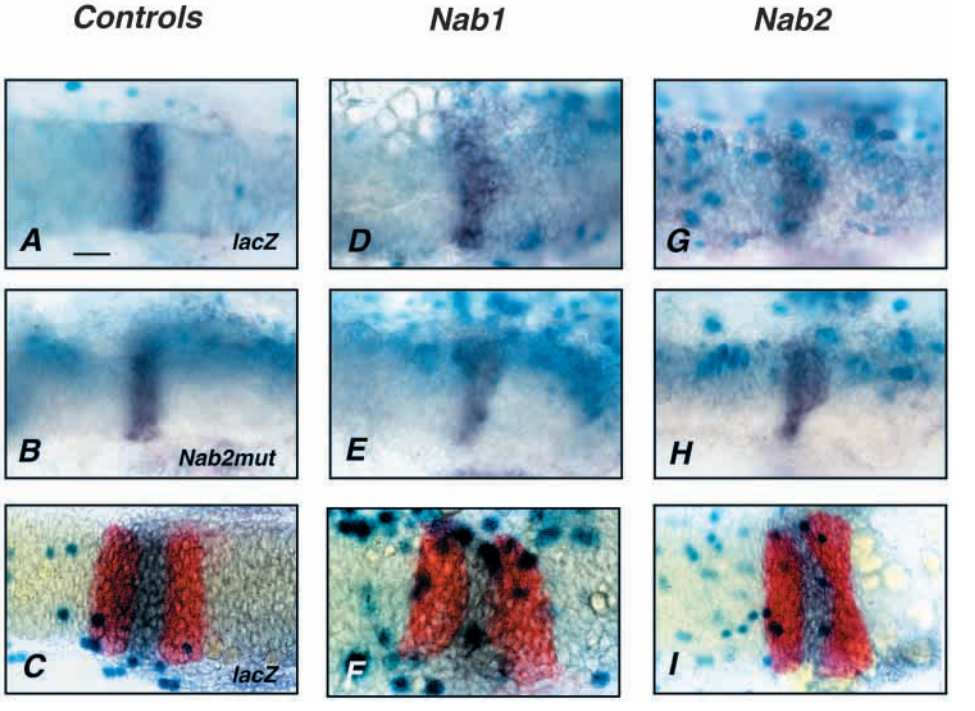


Fig. 5. *Nab* ectopic expression results in an extension of the *Hoxb1* expression domain. Embryos were treated as described in Fig. 3 and analysed by whole-mount in situ hybridisation with an *Hoxb1* probe (A,B,D,E,G,H) or by double in situ hybridisation with *Hoxb1* (blue) and *Krox-20* (red) probes (C,F,I). (A,C) Embryos injected with *lacZ* mRNA only. (B) Embryo injected with a mutant *Nab2* mRNA encoding a non-functional protein. (D-F) Embryos injected with *Nab1* mRNA. (G-I) Embryos injected with *Nab2* mRNA. Note the frequent caudal extension of the *Hoxb1* expression domain, which does not always coincide with *lacZ*-positive cells, and the perfect contiguity between *Hoxb1*- and *Krox-20*-positive cells. Bar, 40 μm.

in the developing heart where *Krox-20* is not expressed. These data indicate that the *Nab* genes are downstream of *Krox-20* in a regulatory pathway acting in r3, r5 and the r5-derived neural crest, and that they can also present other modes of regulation, independent of Krox-20. Since Krox-20 can act as a positive transcriptional activator in the hindbrain, these data raise the issue of whether its involvement in *Nab1* and *Nab2* transcription control is direct or indirect. Analysis of the *cis*-acting elements governing the expression of the *Nab* genes will be required to answer this question.

Although the patterns of expression of the *Nab* genes overlap with that of *Krox-20* in the hindbrain, they are not identical. Three features appear different: (1) *Nab1* is activated much later in r3 than *Krox-20*; (2) *Nab2* downregulation in r5 precedes that in r3, in contrast to *Krox-20*; (3) the levels of expression of the *Nab* genes are not uniform along the AP axis in r3 and r5, showing reinforcements close to the rhombomere boundaries. This character is more pronounced in the case of *Nab1* in r5 and contrasts the uniform pattern of expression of *Krox-20* at the same stages in r3 and r5. These differences between *Nab1*, *Nab2* and *Krox-20* expression patterns suggest either that other factors, in addition to Krox-20, participate in the transcriptional regulation of the *Nab* genes, or that differential post-transcriptional mechanisms are involved. In this respect, it is interesting to note that an r3/r5-specific, Krox-20-dependent transcriptional enhancer from the chicken *Hoxb2* gene also gives rise to reinforced expression close to the rhombomere boundaries in r3 (Vesque et al., 1996), supporting the view that in the case of the *Nab* genes this effect might also have its origin at the transcriptional level.

Phenotypic manifestations of *Nab1* and *Nab2* misexpression reflect inhibition of Krox-20 function

Nab1 or *Nab2* RNA injection in zebrafish embryos results in specific modifications of the expression patterns of *EphA4*, an established transcriptional target of Krox-20 in r3 and r5 in the mouse, of *Krox-20* itself and of *Hoxb1*, a marker of r4. In the case of *EphA4*, these modifications appear as distortions and restrictions of the expression territories and affect r3 and r5 with similar frequencies (Table 1A). This effect is specific for this region of the central nervous system since the stripe of *Paxb*-positive cells at the mid/hindbrain junction is not altered. Since the Nab proteins are able to antagonise Krox-20 transcriptional activity in vitro, these observations could be interpreted as a simple inhibition of *EphA4* activation due to much too high levels of Nab proteins in the affected cells. This possibility is supported by the reduced level of *EphA4* expression observed in some cells (Fig. 3E,H). However, this interpretation does not directly account for the fact that the cells within the territories where *EphA4* expression appears lost are not always all positive for the coinjected *lacZ* marker. Furthermore, it does not explain why the *Krox-20*-positive territories are also affected. Finally, another interesting observation to take into account is the clear segregation between *EphA4*-positive and -negative cells in injected embryos: no 'salt and pepper' distribution was observed. These additional data lead us to propose two other interpretations, which are not exclusive with the first one.

(1) By antagonising Krox-20 activity and possibly other Nab targets, the overexpression of *Nab* genes may lead to a change of identity of odd-numbered rhombomere cells into even-numbered. This change of identity would be accompanied by a loss of *Krox-20* expression, an activation of even-numbered rhombomere markers (e.g. *Hoxb1*), and the acquisition of adhesion properties of even-numbered cells. This latter manifestation may be the sole consequence of the loss of *EphA4* expression, due to the co-ordinated cessation of expression of several Eph family receptors that are normally present in odd-numbered rhombomeres (Xu et al., 1994; the different Eph receptor genes might all be under the transcriptional control of Krox-20), or involve additional genes. Expression of dominant-negative forms of these receptors has previously been shown to affect segmental restriction in the hindbrain (Xu et al., 1995). This interpretation is obviously consistent with the role of Krox-20 in controlling the expression of several *Hox* genes, which have themselves been implicated in the specification of cell identity along the AP axis.

(2) A second interpretation, which we favour, is that inhibition of Krox-20 transcriptional activity by overexpression of *Nab* genes prevents not only *EphA4* expression, but also cell proliferation or survival, a possibility consistent with the phenotype associated with the *Krox-20* null mutation (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) and supported by our recent analysis of the fate of r3 and r5 cells in *Krox-20*$^{-/-}$ embryos (O. Voiculescu, P. Charnay and S. Schneider-Maunoury, unpublished results). This interpretation would explain why the territories of expression of *Krox-20* itself are also reduced in some injected embryos. The caudal expansion of the *Hoxb1* territory observed in some injected embryos may result from the invasion by *Hoxb1*-positive cells of the adjacent territory left over by the missing r3 or r5 cells, consistent with our double in situ analysis. We attempted to study modifications of the apoptotic pattern in injected embryos, but such an analysis was precluded by the high variability of the endogenous pattern of cell death at the stages analysed (data not shown).

In conclusion, our data are consistent with the idea that an abnormally high level of Nab proteins in r3 or r5 cells may lead to the inhibition of at least three manifestations of the Krox-20 activity: transcriptional activation of specific targets, like *EphA4*, segregation from cells with even-numbered identity and control of cell proliferation/survival. As indicated above, the different interpretations are not exclusive and the phenotype might result from a combination of different behaviours at the cellular level. In particular, the different targets of Krox-20 might not be sensitive to the inhibition by Nab proteins at the same level. This possibility is consistent with the higher incidence of alterations of the *Krox-20*-positive domain corresponding to r5 following *Nab* misexpression, whereas *EphA4*-positive territories are equally affected in r3 and r5 (compare Table 1A and B).

While the specific effect of *Nab* misexpression in r3 and r5 argues in favor of an interference with the Krox-20 function, we cannot exclude that these two proteins might have additional targets. Indeed, we noticed that injection of *Nab* RNA into zebrafish embryos, besides its specific effect in the hindbrain, also leads to a general widening of the neural tube. This might reflect an interference with the convergence-extension process occurring during gastrulation. In this respect, it is interesting to note that *Xegr-1*, the *Xenopus laevis* ortholog of *Krox-24/Egr-1* is expressed in the Spemann organiser region

and during gastrulation in the involuting mesoderm with a pattern very similar to that of *Xbra* (Panitz et al., 1998). Therefore, the observed phenotype in injected embryos might reflect an interference of *Nab* ectopic expression with an early function of Krox-24/Egr-1.

Physiological functions of Nab1 and Nab2

Our injection experiments suggest that the mouse Nab proteins can interfere with the zebrafish Krox-20 function and that, at least in this assay, Nab1 and Nab2 are functionally interchangeable since no significant differences were observed in the phenotypes (Table 1). In this respect, it is interesting to note that the Nab1 protein used in these experiments was deleted of its 38 C-terminal amino acids. Therefore, this part of the protein is not necessary for the tested activity. The functional similarity of Nab1 and Nab2 is not surprising considering the homology between the two proteins, which is particularly high in the regions involved in the interactions with members of the Krox-20 family and in transcriptional repression (90% and 72% amino acid identity, respectively). Further studies will be required to determine whether the activities of the two proteins differ in other aspects.

What are the physiological functions of Nab1 and Nab2? In the hindbrain, our data are consistent with the existence of a negative feedback loop regulating Krox-20 function. Such a loop might be involved in ensuring a precisely defined level of Krox-20 activity in r3 and r5, which itself might be required to obtain an equilibrated expression of its multiple target genes. These latter genes may display different sensitivities to Krox-20 concentration, and deviation from the normal Krox-20 level may affect this sensitive balance. Since Krox-20 controls multiple aspects of cell behaviour, any unbalance may favour one function of Krox-20 versus the others (e.g. proliferation versus specification) and result in dramatic alterations in cell fate. More specifically, the reinforced expression of *Nab1* close to the rhombomere boundaries could be responsible, in r3 and r5, for the reduced cell proliferation rates observed in these domains and involved in the narrowing of the neural tube width at this level (Guthrie et al., 1991). In this respect, the most evident effect of *Nab* misexpression in zebrafish embryos, distortion of the boundaries of the r3 and r5 territories, might reflect interference with this particular function.

Finally, Krox-20 has also been shown to be required for normal myelination by Schwann cells in the peripheral nervous system (Topilko et al., 1994). Recently, a mutation affecting a precise Krox-20 amino acid required for the Krox-24/Krox-20 interaction with the Nab proteins (Russo et al., 1995) has been observed in a recessive form of human peripheral myelanopathy (Warner et al., 1998). This suggest that the Nab proteins play an important role in the control of myelination in the peripheral nervous system and provides additional strong evidence for their involvement in regulating Krox-20 function. It will of course be of great interest to investigate the effects of this mutation on the development of r3 and r5. This should provide a direct assessment of the functions of the *Nab* genes in the developing hindbrain.

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