Development 130, 941-953
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00318



# Neural crest patterning: autoregulatory and crest-specific elements co-operate for *Krox20* transcriptional control

**Julien Ghislain, Carole Desmarquet-Trin-Dinh, Pascale Gilardi-Hebenstreit, Patrick Charnay* and Monique Frain**

Unité 368 de l'Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France

*Author for correspondence (e-mail: charnay@wotan.ens.fr)

*Accepted 29 November 2002*

## SUMMARY

**Neural crest patterning constitutes an important element in the control of the morphogenesis of craniofacial structures. *Krox20*, a transcription factor gene that plays a critical role in the development of the segmented hindbrain, is expressed in rhombomeres (r) 3 and 5 and in a stream of neural crest cells migrating from r5 toward the third branchial arch. We have investigated the basis of the specific neural crest expression of *Krox20* and identified a cis-acting enhancer element (NCE) located 26 kb upstream of the gene that is conserved between mouse, man and chick and can recapitulate the *Krox20* neural crest pattern in transgenic mice. Functional dissection of the enhancer revealed the presence of two conserved Krox20 binding sites mediating direct Krox20 autoregulation in the neural crest. In addition, the enhancer included another essential element containing conserved binding sites for high mobility group (HMG) box proteins and which responded to factors expressed throughout the neural crest. Consistent with this the NCE was strongly activated in vitro by Sox10, a crest-specific HMG box protein, in synergism with Krox20, and the inactivation of *Sox10* prevented the maintenance of *Krox20* expression in the migrating neural crest. These results suggest that the dependency of the enhancer on both crest- (Sox10) and r5- (Krox20) specific factors limits its activity to the r5-derived neural crest. This organisation also suggests a mechanism for the transfer and maintenance of rhombomere-specific gene expression from the hindbrain neuroepithelium to the emerging neural crest and may be of more general significance for neural crest patterning.**

Key words: *Krox20*/*Egr2*, High mobility group, *Sox10*, Hindbrain, Cranial neural crest, Transcriptional control, Pattern formation, Segmentation, Mouse, Chick

## INTRODUCTION

The patterning mechanisms controlling craniofacial morphogenesis constitute and important issue and an interesting paradigm for the study of vertebrate development. Numerous studies have demonstrated the crucial roles of the hindbrain and its associated neural crest in this process (Lumsden and Krumlauf, 1996; Le Douarin and Kalcheim, 1999). During early development, the hindbrain undergoes a transient segmentation which is visible at the morphological level by the appearance of 7 bulges known as rhombomeres (r) (Lumsden and Keynes, 1989; Lumsden, 1990). Each rhombomere consists of a polyclonal, cell lineage restricted, anteroposterior (AP) compartment that expresses a specific set of genes and behaves as a developmental unit (Fraser et al., 1990; Birgbauer and Fraser, 1994; Lumsden and Krumlauf, 1996). The cranial neural crest (NC) arises at the border between the neural plate and surface ectoderm. It comprises a pluripotent cell population that delaminates just prior to and following neural tube closure in the mouse and chick, respectively, and, after migration, differentiates into various tissues including ganglia, cartilage, bone and connective tissue (Le Douarin, 1982; Noden, 1988). In register with the rhombomeric organisation, the cranial NC cells migrate in three segmented streams adjacent to r2, r4 and r6 (Anderson and Meier, 1981; Lumsden et al., 1991; Serbedzija et al., 1992; Liem et al., 1995; Selleck and Bronner-Fraser, 1995) contributing to the V, VII/VIII and IX ganglia and colonising the 1st, 2nd and 3rd branchial arches, respectively (Le Lièvre and Le Douarin, 1975; Noden, 1983; Lumsden et al., 1991; Köntges and Lumsden, 1996). r3 and r5 produce fewer NC cells than even-numbered rhombomeres and these migrate rostrally and caudally to join the adjacent streams (Sechrist et al., 1993; Lumsden et al., 1991; Trainor et al., 2002a).

Previous studies, involving transplantations of neural tube or neural fold, led to the conclusion that the NC exerts an instructive role in the specification of the regional identity of the branchial arches (Le Douarin, 1982; Noden, 1983; Noden, 1988). Furthermore, the implication of Hox genes in the AP patterning of both rhombomeres and NC (Gendron-Maguire et al., 1993; Rijli et al., 1993; Zhang et al., 1994; Barrow and Capecchi, 1996; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1997; Manley and Capecchi, 1997), together with correlated Hox codes between NC cells and their

rhombomeric region of origin (Hunt et al., 1991a; Hunt and Krumlauf, 1991), led to the elaboration of a pre-patterning model for craniofacial development: NC cells acquire positional information (Hox gene pattern), according to their AP level of origin in the hindbrain, which is passively carried to peripheral tissues where it is used to instruct ectodermic, mesodermic and endodermic tissues in co-ordinated craniofacial development (Hunt et al., 1991b; Hunt et al., 1991c). This view has been recently tempered by several observations, including: (i) NC plasticity in Hox gene expression (Schilling et al., 2001; Trainor and Krumlauf, 2001; Couly et al., 2002; Trainor et al., 2002b); (ii) independent *Hoxa2* gene regulation in the hindbrain and in the NC (Prince and Lumsden, 1994; Maconochie et al., 1999); (iii) patterning roles of the isthmus, cranial mesoderm and foregut endoderm (Trainor and Krumlauf, 2000; Couly et al., 2002; Trainor et al., 2002b); (iv) acquisition of AP specification by the branchial arches in the absence of NC input (Veitch et al., 1999; Gavalas et al., 2001). Together these data suggest that the NC cells are able to respond to signals from the environment in which they migrate and that the regional specification of the branchial arches involves a complex integration of patterning information from different tissues.

In spite of this new evidence, the contribution of the NC to craniofacial development is likely to depend in part on pre-patterning events that define NC migration pathways and AP identity. We are investigating this question in the case of the *Krox20* gene. *Krox20* encodes a zinc finger transcription factor that is expressed in r3 and r5, and has been shown to play a key role in hindbrain segmentation: it controls and co-ordinates the formation of the r3 and r5 territories and their acquisition of odd-number characteristics (Schneider-Maunoury et al., 1993; Schneider-Maunoury et al., 1997; Giudicelli et al., 2001; Voiculescu et al., 2001). These roles are exerted through the transcriptional control of many genes, including Hox genes and members of the Eph receptor gene family (Sham et al., 1993; Vesque et al., 1993; Nonchev et al., 1996; Theil et al., 1998; Giudicelli et al., 2001; Manzanares et al., 2002). Together with their ephrin ligands, the Eph receptors prevent cell mingling between adjacent rhombomeres and are involved in controlling NC migration in separate streams (Bergemann et al., 1995; Gale et al., 1996; Smith et al., 1997). In the NC, *Krox20* expression is mainly restricted to cells migrating lateral to r6 towards the third branchial arch and presumably originating from r5 in the mouse (Schneider-Maunoury et al., 1993). *Krox20* is rarely expressed in the NC cells migrating rostral from r5 and has not been detected in those produced in r3 (Schneider-Maunoury et al., 1993). Indeed, fate-tracing analyses have not revealed any significant contribution of *Krox20*-expressing cells to the r3 NC (Voiculescu et al., 2001). In the r5-derived NC, Krox20 controls the transcription of several key regulatory genes, including *Hoxa2*, *Hoxb2*, *Hoxb3* and *EphA4* (Sham et al., 1993; Nonchev et al., 1996; Manzanares et al., 2002; Theil et al., 1998), and may therefore play an important patterning role.

In order to understand the regulatory network controlling *Krox20* in the NC, we have searched for cis-acting regulatory elements responsible for this aspect of its expression. In this paper we present the identification and characterisation of a neural crest-specific element (NCE) located 26 kb upstream of the mouse *Krox20* gene that drives specific expression in the r5 stream of the NC in transgenic mice. The NCE was shown to require both Krox20 and putative high mobility group (HMG) box binding sites for proper activity. The HMG box factor Sox10, expressed specifically in the NC, was found to co-operate with Krox20 to activate the NCE and to be required for the maintenance of *Krox20* expression in the r5-derived NC. This organisation suggests a mode of patterning of the r5-derived NC according to its rhombomeric origin: following the initial *Krox20* expression in the premigratory NC under the control of cis-regulatory elements active in r5, Krox20 and crest-specific Sox proteins combine to activate the NCE, maintaining its expression in the delaminating NC.

## MATERIALS AND METHODS

### Mouse lines and genotyping

The *Krox20*$^{Cre}$ (Voiculescu et al., 2000), *Krox20*$^{lacZ}$ (Schneider-Maunoury et al., 1993) and *Sox10*$^{Dom}$ (Herbarth et al., 1998; Southard-Smith et al., 1998) alleles and the –31/+7 *Krox20/lacZ* transgenic line (Ghislain et al., 2002) have been described previously. These lines were maintained in a mixed C57Bl6/DBA2 background. Genotyping was performed by PCR on extracted DNA using primers described previously (Schneider-Maunoury et al., 1993; Voiculescu et al., 2000; Ghislain et al., 2002) except for the *Sox10*$^{Dom}$ allele, which was genotyped as follows. PCR primers: 5′-ctccggatgcagcacaaaaaggacc-3′ (forward; T4 polynucleotide kinase labelled using [γ-$^{32}$P]ATP) and 5′-cctgaatagcagcagcccctccttg-3′ (reverse) were used to amplify sequences containing the extra nucleotide at position 929 of the *Sox10*$^{Dom}$ allele (Southard-Smith et al., 1998). The product of the wild-type and mutant alleles, 137 and 138 bp, respectively, were resolved by denaturing gel electrophoresis.

### DNA constructs and mutagenesis

The pEMBL2 cosmid clone 8/4 (Chavrier et al., 1989) was used as a source of murine *Krox20* extragenic sequences. Subfragments were cloned upstream of a minimal *β-globin* promoter/*lacZ* reporter in plasmid pBGZ40 (Yee and Rigby, 1993), as described previously (Ghislain et al., 2002). Site-directed mutagenesis of Krox20 binding sites was performed on fragment #11 cloned in pBS using the Transformer$^{TM}$ Site-Directed Mutagenesis kit (Clontech). The oligonucleotides used were: 5′-ggaggaaagcgtcggttgcaggcagg-3′ (binding site 1); 5′-gcctcgaagaagtcggcgggagcctc-3′ (binding site 2); 5′-ggaattcgatatctctagaatcgataccgtc-3′ (selection). Deletion of the putative HMG box binding sites was performed using the Exsite$^{TM}$ PCR-based site-directed mutagenesis kit (Stratagene) with the following oligonucleotides: 5′-ggctggggacgggca-3′ (forward); 5′-tgttctcctttccctgtctcagcag-3′ (reverse). All mutated fragments were verified by sequencing and cloned into pBGZ40. Multimerisation of sequences containing the putative HMG box binding sites (fragment #24) was performed on double-stranded oligonucleotides containing terminal *Spe*I sites by ligation into pBS. A 7× multimer was selected and cloned into pBGZ40.

### Generation of transgenic mice and in ovo electroporation

Transgenesis and identification of transgenic embryos by PCR was performed as described previously (Sham et al., 1993; Ghislain et al., 2002). In ovo electroporation in the chick neural tube was performed as described previously (Giudicelli et al., 2001). Krox20 transactivation studies were performed by coelectroporating the *lacZ* reporter constructs with a mouse *Krox20* expression plasmid, pAdRSV*Krox20* (Giudicelli et al., 2001).

### In situ hybridisation, X-gal staining and sections

Whole-mount in situ hybridisation was performed as described

previously (Wilkinson et al., 1992). Alkaline phosphatase activity was revealed using the NBT/BCIP substrate (Roche). Antisense RNA probes were prepared from a *Krox20* cDNA (Wilkinson et al., 1989) and from *Sox9* and *Sox10* est clones obtained from the MRC geneservice (IMAGE ID 4165469 and 3675437, respectively). Mouse and chick embryos were stained for β-galactosidase activity in toto following fixation in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 minutes at 4°C. Staining was performed in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 0.1% Tween-20 in PBS at 30°C for 15 hours. For staining prior to in situ hybridization embryos were incubated for a maximum of 4 hours in the staining solution containing 0.02% PFA. All tissues were postfixed in 4% PFA in PBS. Embyos for sectioning were dehydrated and embedded in paraffin wax and 10 μm sections were prepared. Sections were stained with Nuclear Fast Red at 1 mg/ml for 10 seconds, dehydrated and mounted in Eukitt (Merck).

### Isolation of the chick NCE

A pBS-*Hin*dIII sub-library of a chicken genomic BAC clone carrying the *Krox20* gene and flanking sequences (Giudicelli et al., 2001) was transferred to Hybond-N+ membranes and hybridised at low stringency with the mouse NCE fragment #17, labelled with [α-$^{32}$P]dATP by random priming, according to the manufacturers instructions (Amersham Pharmacia Biotech). A positive clone, containing a 6.4 kb insert, was digested with a series of restriction enzymes. Following gel electrophoresis, the DNA was transferred under alkaline conditions to Highland-N+ membrane and hybridised at low stringency with fragment #17. This identified a 1.7 kb *Apa*LI/*Stu*I subfragment that was cloned into pBS for sequencing and pBGZ40 for electroporation in the chick neural tube.

### DNase I footprinting and band shift assays

The mouse Krox20 protein was expressed in bacteria using the pET3a system (Novagen). Extracts were prepared from Krox20-expressing and control bacteria as described previously (Nardelli et al., 1992). For footprinting and band shift experiments, fragment #17, cloned in the *Spe*I site of pBS, was digested with either *Bam*HI or *Xba*I, dephosphorylated, T4 polynucleotide kinase labelled using [γ-$^{32}$P]ATP and digested with *Not*I or *Sma*I, respectively. Labelled fragments were purified by polyacrylamide gel electrophoresis. DNase I footprinting experiments were performed as described previously (Galas and Schmitz, 1978) and reaction products resolved on a 6% denaturing polyacrylamide gel. Band shift experiments have been described previously (Nardelli et al., 1992).

### Cell culture, transfection and β-galactosidase assay

HeLa cells were cultured in DMEM supplemented with 10% FCS. Cells (150,000/35 mm well) were transfected with 1 μg of DNA in duplicate using the Fugene 6 transfection reagent (Roche). Expression plasmids encoding the mouse Krox20 protein, pAdRSV*Krox20* (Giudicelli et al., 2001) and human Sox10 protein, pECE*SOX10* (Bondurand et al., 2000) were used. Reporter constructs were the same as those used in in vivo experiments. The total quantity of DNA was kept constant using empty pBS vector. Following transfection, cells were incubated for 48 hours and extracts were assayed for β-galactosidase activity using the chemiluminescent β-gal reporter gene assay (Roche). Transfections were normalised by cotransfecting a chloramphenicol acetyltransferase (CAT) expression plasmid (pSV2*CAT*) and quantitating protein using the CAT ELISA assay (Roche).

### Sequence alignment

The GenBank accession numbers for human and mouse genomic contigs containing the *Krox20* gene and flanking sequence are AL133417 and AC068424, respectively. The 1.7 kb chick sequences containing homology to the mouse NCE have been submitted to GenBank (accession number AY117679). Sequence alignments were performed using Dialign (Morgenstern et al., 1996) and identification of putative Krox20 binding sites using the rVista program (G. G. Loots, I. Ovcharenko, L. Pachter, I. Dubchak and E. Rubin, unpublished).

## RESULTS

### Autoregulation contributes to *Krox20* expression in the neural crest

Recent studies have suggested that autoregulatory mechanisms contribute to *Krox20* expression in the developing hindbrain (Giudicelli et al., 2001; Voiculescu et al., 2001). However, previous analysis of *Krox20* mutants homozygous for a *Krox20$^{lacZ}$* allele did not reveal any obvious difference in the level of β-galactosidase activity in the NC when compared to heterozygotes (Schneider-Maunoury et al., 1993). We reasoned that an autoregulatory role of *Krox20* in the NC may have been masked in these experiments by the presence of two copies of the *lacZ* gene in the homozygotes, leading to a higher initial expression relative to heterozygotes. To circumvent this problem we made use of another *Krox20* null allele, *Krox20$^{Cre}$*, and analysed compound mutant embryos, *Krox20$^{Cre/lacZ}$*, which carry only one copy of the *lacZ* gene. The effect of the *Krox20* mutation in r3 and r5 has been described previously (Schneider-Maunoury et al., 1993; Schneider-Maunoury et al., 1997; Voiculescu et al., 2001). Consistent with these studies r3 and r5 were dramatically affected in the compound mutants at both 9.0 and 9.5 dpc (compare Fig. 1A with B and C with D). In the NC, the β-galactosidase activity in heterozygotes (*Krox20$^{+/lacZ}$*) was similar to the compound mutants at 9.0 dpc (compare Fig. 1A with B), however, β-galactosidase-positive NC cells were seen to migrate both rostrally and caudally

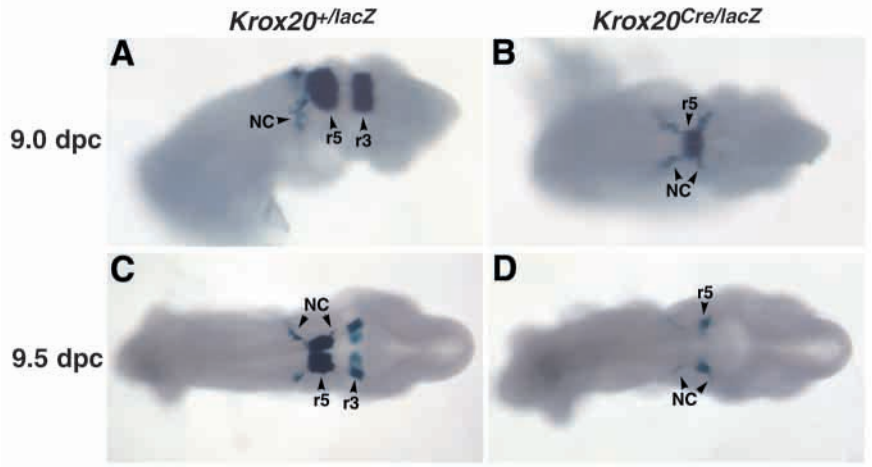



**Fig. 1.** Autoregulation contributes to *Krox20* expression in the neural crest. Heterozygous (A,C, *Krox20$^{+/lacZ}$*) and compound mutant (B,D, *Krox20$^{Cre/lacZ}$*) embryos were analysed for β-galactosidase activity in toto at 9.0 (A,B) and 9.5 (C,D) dpc. Expression in the neural crest and rhombomeres 3 and 5 are indicated (arrowheads). Embryos are oriented with anterior to the right. NC, migrating neural crest.

around the otic vesicle in the compound mutants, a profile described previously for the *Krox20* mutation (Schneider-Maunoury et al., 1993). At 9.5 dpc a significant down-regulation was observed in the compound mutants (compare Fig. 1C to D). Since the production and survival of the crest population which should express *Krox20* is not noticeably affected in *Krox20* null mutants (Schneider-Maunoury et al., 1993) (O. Voiculescu, personal communication), these data suggest a decrease in *Krox20/lacZ* expression and support an autoregulatory mechanism contributing to *Krox20* expression in the NC.

### Identification of an autoregulatory element controlling *Krox20* expression in the neural crest

In order to identify cis-acting regulatory elements controlling *Krox20* expression, we generated transgenic lines with several constructs containing large genomic regions surrounding a *Krox20* allele with a reporter *lacZ* gene inserted in-frame in exon 2 (*Krox20/lacZ*) (Ghislain et al., 2002). This insertion is identical to the knock-in allele ($Krox20^{lacZ}$) generated previously in our lab, which was shown to reproduce the expression profile of the endogenous gene (Schneider-Maunoury et al., 1993). One of these constructs, carrying sequences between –31 kb and +7 kb relative to the transcription start site of *Krox20* (–31/+7 *Krox20/lacZ*), was active at the dorsal edge of the neural tube at the level of r5 and in a stream of NC cells migrating caudally from r5, as early as 8.5 dpc (8-10 somites) in 3/3 transgenic lines (Fig. 2A, Fig. 3, construct #1). In the neuroepithelium, the β-galactosidase-expressing cells were largely restricted to the dorsal edge consistent with the premigratory NC (Fig. 2B). The presence of more ventrally located β-galactosidase-positive cells (Fig. 2B), which are probably of neuroepithelial identity, may be explained by a lack of commitment of some dorsal cells to a NC fate. The expression in migratory NC cells was maintained at later stages, the majority migrating caudal to the otic vesicle (Fig. 2C,D). By 9.5 dpc expression was reduced, particularly in those cells distal to their site of origin in the third branchial arch (Fig. 2D). A small number of *lacZ*-positive crest cells were also found to migrate rostal to the otic vesicle (Fig. 2C,D). This pattern of expression of the transgene in the r5-derived NC is identical to those described for knock-in *Krox20* alleles as well as for the endogenous gene (Fig. 1C) (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993; Nieto et al., 1995; Voiculescu et al., 2001).

Given that autoregulation contributes to *Krox20* crest expression (Fig. 1), we asked whether such a mechanism may be involved in the control of the activity of the –31/+7 *Krox20/lacZ* transgene in the NC. When this transgene was analysed in a *Krox20* null background ($Krox20^{Cre/Cre}$), no β-galactosidase activity was detected in embryos at either 9.0 or 9.5 dpc (Fig. 2E,F). As the absence of β-galactosidase activity in the NC is not a consequence of the loss of these cells (Schneider-Maunoury et al., 1993), we conclude that *Krox20* is required for the expression of this transgene. Taken together, these results identify a neural crest-specific element (NCE) that recapitulates the wild-type *Krox20* expression in the NC and whose activity is dependent on an autoregulatory mechanism.

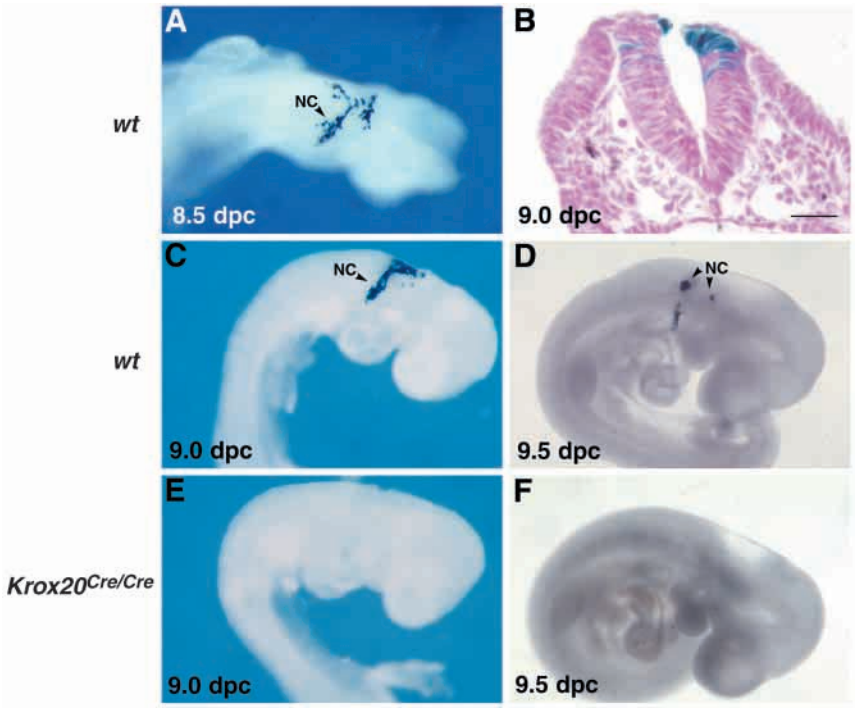


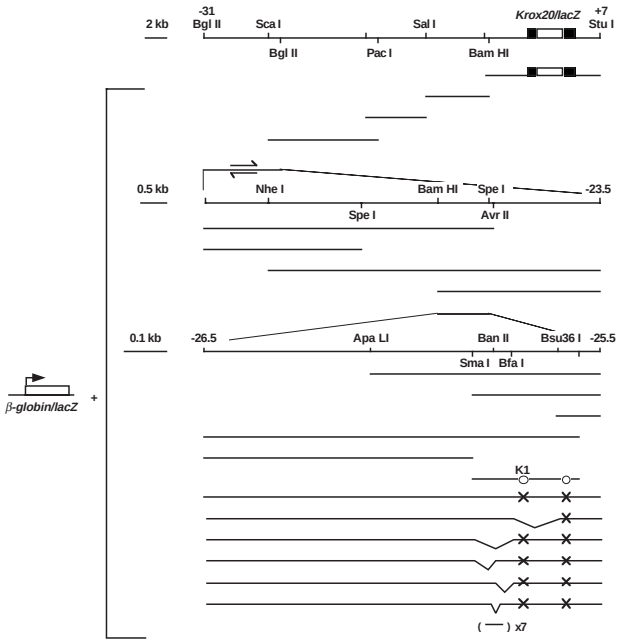

**Fig. 2.** Identification of a neural crest-specific, autoregulatory element controlling *Krox20* expression. (A-D) The transgenic line –31/+7 *Krox20/lacZ* (see Fig. 3, construct #1) was analysed for β-galactosidase activity in toto at 8.5 dpc (A), 9.0 dpc (C) and 9.5 dpc (D), or by transverse section through rhombomere 5 at 9.0 dpc (B, dorsal to the top). (E,F) The –31/+7 *Krox20/lacZ* transgene was analysed in a *Krox20* mutant background ($Krox20^{Cre/Cre}$) at 9.0 dpc (E) and 9.5 dpc (F). Embryos are oriented with anterior to the right (A, C-F). NC, migrating neural crest. *wt*, wild type. The scale bar in B represents 40 μm.

### Delimitation of the NCE

Although the genetic studies described above clearly indicate the involvement of autoregulation in the control of the activity of the NCE, the identification of the cis- and associated trans-acting factors is required to determine whether the role of Krox20 is direct or indirect. As a first step, the NCE was delimited from the original –31/+7 *Krox20/lacZ* transgene. Subfragments of the –31 kb to +7 kb region containing either the *Krox20/lacZ* chimera (Fig. 3, construct #2) or linked to a *β-globin* minimal promoter/*lacZ* reporter (Fig. 3, constructs #3-6) were tested by 'transient' transgenesis at 9.5 dpc. Of the five constructs spanning this region, only one was active in the NC, the –31 kb to –23.5 kb fragment, which reproduced the profile in 5/9 transgenic embryos (Fig. 3, Fig. 4A, construct #6S). Interestingly, one of the embryos, which was positive in the NC, also showed widespread expression in r5 (Fig. 3 and data not shown). Fragment #6 was also tested by transgenesis in antisense orientation relative to the promoter/*lacZ* reporter (Fig. 3, construct #6AS). This construct was active in 2/6 transgenic embryos in the NC, suggesting

that the NCE acts as a canonical transcriptional enhancer (data not shown).

In ovo electroporation in the chick neural tube offers a quick and easy procedure to test the activity of cis-regulatory elements in the neuroepithelium (Itasaki et al., 1999). To determine whether this approach could facilitate the delimitation of the NCE, constructs #2-6 were electroporated into chick embryo hindbrains at Hamburger and Hamilton (HH) stage 9 and analysed for β-galactosidase activity at HH stage 13-14. Similar to the results for these constructs in mouse transgenesis, constructs #2-5 were negative whereas construct #6 was active in the NC (Fig. 3, Fig. 4B). Interestingly, in chick electroporation this construct was also active in r3 and r5 (Fig. 4B; see discussion). These results support the applicability of in ovo electroporation to delimit the NCE.




| # | Mouse NC | Chick r3/r5+NC |
|---|---|---|
| 1 | 3/3 | ND |
| 2 | 0/6 | - |
| 3 | 0/6 | - |
| 4 | 0/2 | - |
| 5 | 0/10 | - |
| 6S | 5/9,1/9(r5) | + |
| 6AS | 2/6 | + |
| 7 | ND | + |
| 8 | ND | - |
| 9 | ND | + |
| 10 | ND | + |
| 11 | 8/15,1/15(r5) | + |
| 12 | ND | + |
| 13 | ND | + |
| 14 | ND | - |
| 15 | ND | + |
| 16 | ND | - |
| 17 | 3/9,2/9(r5) | + |
| 18 | 4/10(weak) | +(NC,weak) |
| 19 | ND | +(NC,weak) |
| 20 | ND | - |
| 21 | ND | - |
| 22 | ND | - |
| 23 | ND | - |
| 24 | 3/4(CNC) | +(CNC) |

**Fig. 3.** Genomic constructs analysed for NCE activity by transgenesis in the mouse and electroporation in the chick neural tube. The –31/+7 *Krox20/lacZ* transgenic construct containing a *lacZ* in-frame insertion in *Krox20* is indicated at the top. Restriction enzymes used to clone subfragments are shown. Distances are in kb and indicate the position relative to the start site of transcription of *Krox20*. Scale bars relevant to each subregion are indicated on the left. With the exception of constructs #1 and #2 which contain the *Krox20/lacZ* reporter, all subfragments were cloned into the *β-globin/lacZ* promoter/reporter vector. Fragment #6 was cloned in both the sense (S) and antisense orientations (AS). K1/K2, Krox20 binding sites (open circle, wild type; X, mutated). For details of the Krox20 binding sites, their mutation, the regions deleted in constructs #19-23 and the sequences multimerised in construct #24 see Fig. 5. In mouse transgenic experiments, embryos were analysed at 9.5 dpc for β-galactosidase activity. Those carrying construct #1 were analysed at $F_1$ and all others were analysed in 'transient' transgenesis. Ratios correspond to the number of β-galactosidase positive/total number of transgenic embryos. Unless indicated in brackets, mouse embryos were scored for expression in the r5 neural crest stream (NC). Embryos with expression in r5 were among those that showed expression in the neural crest. Chick embryos were scored at Hamburger and Hamilton stages 13-14 for β-galactosidase activity in r3, r5 and the r5 neural crest stream (r3/r5+NC), unless indicated in brackets. NC, expression restricted to the r5 neural crest stream; CNC, expression throughout the cranial neural crest; +, constructs for which at least 60% of the electroporated embryos gave the indicated profile; –, those that were essentially negative in these tissues in at least two sets of five electroporated embryos; weak, when the levels of β-galactosidase activity detected were considerably lower than for the controls; ND, not determined.

Subsequently, a series of deletions of fragment #6 were analysed for activity in the chick neural tube linked to the *β-globin/lacZ* promoter/reporter (Fig. 3, constructs #7-11). These studies led to the identification of an approximately 1 kb fragment extending from –26.5 kb to –25.5 kb which, much like construct #6, was active in the NC and r3 and r5 (Fig. 3, Fig. 4C, construct #11). When tested by transgenesis in the mouse, construct #11 produced a high frequency of expression in the NC and a low frequency in r5, similar to construct #6 (Fig. 3, Fig. 4D and data not shown). The NCE was further delimited by testing external 5′ and 3′ deletions of the 1 kb fragment (Fig. 3, constructs #12-16). Using chick electroporation, we identified a 247 bp sequence with activity similar to the original fragment (Fig. 3, Fig. 4E, construct #17). When this fragment was tested in mouse transgenesis, it gave results similar to those of fragment # 11, although the level of expression in the NC was reduced and the frequency of expression in r5 was increased (Fig. 3, Fig. 4F, construct #17). This raises the possibility that some cis-regulatory elements that contribute to the activity in the NC are located outside of fragment #17. However, as these sequences control only the level of activity in the NC and not the specificity, the characterisation of the 247 bp fragment was pursued.

As *Krox20* was shown to act upstream of the NCE in the mouse (Fig. 2C-F), we tested the effect of *Krox20* ectopic expression on the activity of this element by co-electroporation of construct #17 with a *Krox20* expression vector (Giudicelli et al., 2001) in the chick hindbrain. In contrast to construct #17 alone, whose activity was limited to the endogenous *Krox20* expression domain, co-electroporation led to activation of the reporter throughout the electroporated region (Fig. 3, construct #17+; compare Fig. 4E with G). This unrestricted response to Krox20 provides additional evidence in favour of an autoregulatory mechanism controlling the activity of the NCE.

In summary, deletion studies identified a fragment (#11) located between –26.5 kb and –25.5 kb, which reproduced the profile of the –31/+7 *Krox20/lacZ* transgene in the NC. Further deletions of this element resulted in the isolation of a minimal, 247 bp, Krox20-responsive NCE, which contains all of the sequences necessary for specific activity in the NC.

## Isolation of a conserved chick NCE

As the mouse NCE performed comparably in the NC of both

mouse and chick, we searched for orthologous regulatory elements in the chicken genome to aid the identification of cis- and trans-acting factors regulating *Krox20* in the NC. The putative chick NCE was isolated from a bacterial artificial chromosome (BAC) clone containing the chicken *Krox20* genomic sequence and flanking regions (Giudicelli et al., 2001) by hybridisation at low stringency, using fragment #17 as a probe. A 1.7 kb, cross-hybridising subfragment was tested in chick electroporation after linking to the *β-globin/lacZ* promoter/reporter. This construct was active in the r5 NC stream and in r3 and r5 similar to the mouse NCE, although occasional ectopic expression in the rostral neuroepithelium was also observed (Fig. 4H). The nucleotide sequences of mouse fragment #11 and of the 1.7 kb chick fragment were then established and compared together with the human sequences that were available in GenBank. These analyses revealed the existence of blocks of homology corresponding approximately to mouse fragment #17 (247 bp) and consisting of 211 bp in the chick and 243 bp in man. They showed 65% and 83% sequence identity to mouse fragment #17, respectively (Fig. 5). Interestingly, despite the indication that sequences flanking fragment #17 participate in NC activity, no significant homology was detected in this region in mouse/chick comparisons, supporting the conclusion that the essential cis-regulatory sequences are located within fragment #17 (data not shown).

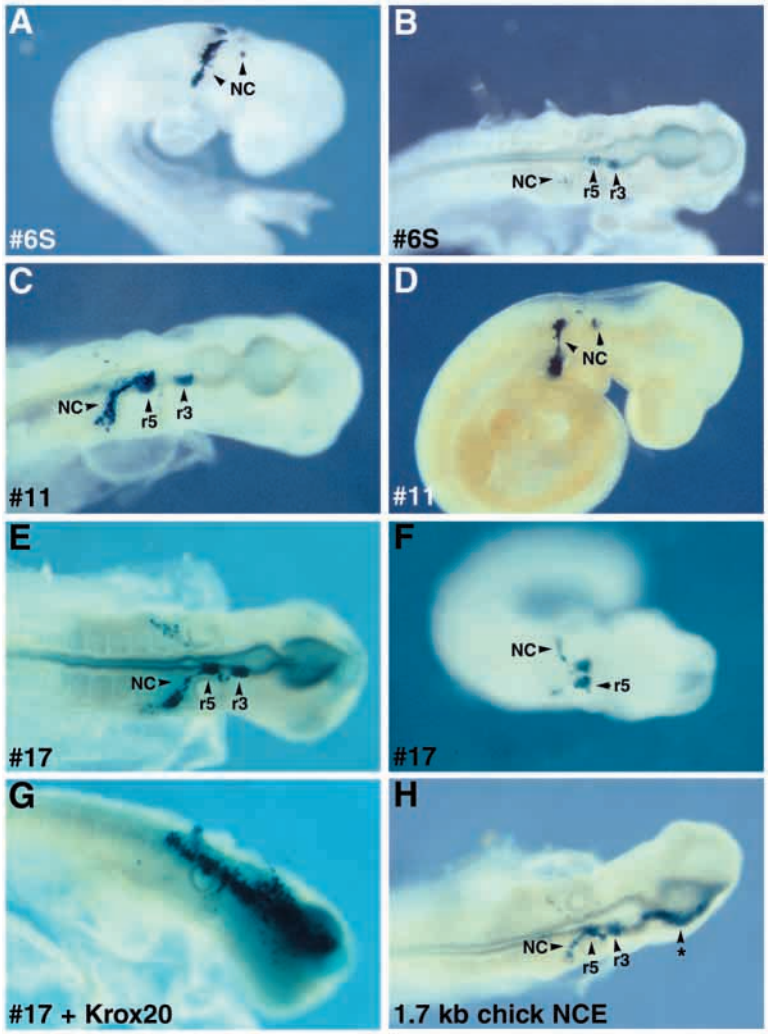



**Fig. 4.** Localisation of the mouse and chick NCE. Genomic fragments were fused to the *β-globin/lacZ* promoter/reporter and analysed for β-galactosidase activity by electroporation in the chick neural tube (B,C,E,G,H) or by transgenesis in the mouse at 9.5 dpc (A,D,F). (A,B) Mouse sequences between –31 kb and –23.5 kb relative to *Krox20* (Fig. 3, construct #6S). (C,D) Sequences between –26.5 kb and –25.5 kb (Fig. 3, construct #11). (E-G) A 247 bp subfragment of this region either alone (E,F; Fig. 3, construct #17) or co-electroporated with a *Krox20* expression construct (G; Fig. 3, construct #17+). (H) A 1.7 kb chick sequence with homology to mouse fragment #17. All embryos are whole mounts with anterior to the right. r3, r5 and neural crest (NC) are indicated by arrowheads. *, occasional ectopic expression.

### Direct regulation of the NCE by *Krox20*

The availability of NCE sequences in different species was then used to investigate whether the activity of fragment #17 was mediated by direct binding of Krox20 to sequences within this fragment. A matrix for the Krox20 binding site consensus sequence (Swirnoff and Milbrandt, 1995; Wingender et al., 2000) was used to search for putative Krox20 sites within fragment #17 and the human and chick orthologues. This led to the identification of three conserved candidate binding sites (data not shown). The capacity of these sites to bind Krox20 was assayed by footprinting with bacterially expressed Krox20 protein (Fig. 6). These analyses revealed the existence of two protected regions of about 20 bp, each centred over two of the three conserved sequences (Fig. 5, Fig. 6A). No other binding site was detected. In order to analyse the role of these two Krox20 binding sites, they were mutagenised by introducing a single G to C substitution, a mutation that has previously been shown to eliminate Krox20 binding activity both in vitro and in vivo (Nardelli et al., 1992; Sham et al., 1993). Indeed, bandshift experiments performed with wild-type fragment #17 and bacterially expressed Krox20 revealed specific complexes that were essentially eliminated when both Krox20 sites were mutated (Fig. 6B). These mutations were then introduced into fragment #11 and their effect was tested in both transgenesis in the mouse and electroporation in the chick (Fig. 3, construct #18). Inactivation of both Krox20 binding sites led to a dramatic decrease in the activity of the element in the NC in both the chick and the mouse (compare Fig. 4C,D with Fig. 7A,B). In addition, in the chick, where the NCE is normally active in r3 and r5, fragment #18 was completely inactive in these rhombomeres (Fig. 7A). These results indicate that the activity of the NCE is largely dependent on its Krox20 binding sites and strongly suggest the implication of a direct autoregulatory mechanism.

In conclusion, together with the genetic data obtained with the *Krox20* null allele, these results demonstrate the existence of a direct *Krox20* autoregulation in the NC that is required for the majority of the NCE activity. The limited crest activity that remains following the elimination of the two Krox20 binding sites may be due to either residual in vivo Krox20 binding activity and/or the action of other factors.

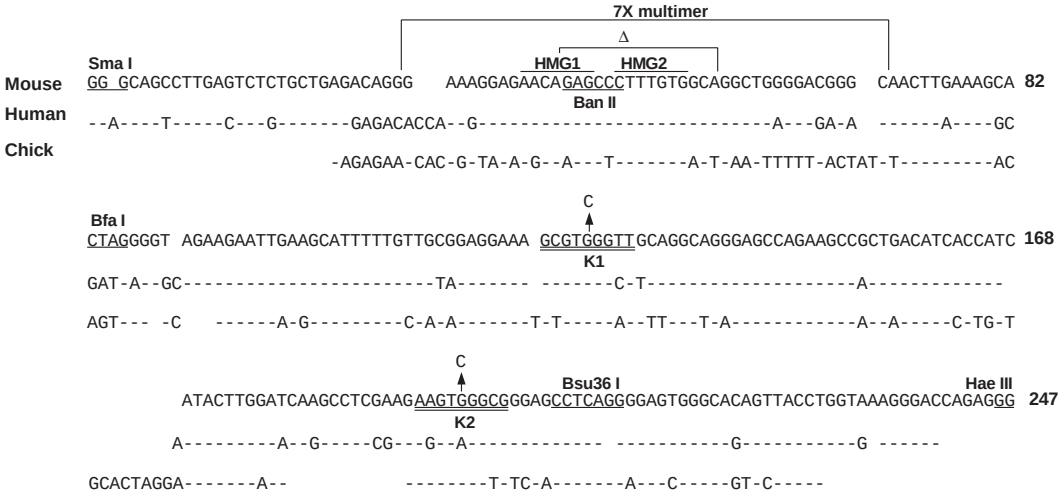



**Fig. 5.** Mouse, human and chick interspecies comparisons reveal sequence conservation within the NCE. The nucleotide sequence of mouse fragment #17 (Fig. 3) is shown. Restriction sites used to generate internal deletion fragments #19 (*Bfa*I and *Bsu*36I), #20 (*Sma*I and *Bfa*I), #21 (*Sma*I and *Ban*II) and #22 (*Ban*II and *Bfa*I) are underlined. Δ, indicates the region deleted in construct #23. Sequences multimerised in fragment #24 are shown. The putative, head-to-head HMG box binding sites are indicated (HMG1/HMG2). Conserved sequences identified in the human genome and in the chick 1.7 kb NCE (Fig. 4) are aligned to the mouse sequence. Conserved residues are indicated as a dash in the human and chick sequences. Sequences satisfying the known Krox20 binding site matrix in all three species and footprinted by the protein in vitro (Fig. 6) are double underlined (K1 and K2). The G to C substitutions used to inactivate the Krox20 binding sites in constructs #18-23 are shown. Nucleotide numbering corresponds to the mouse sequence.

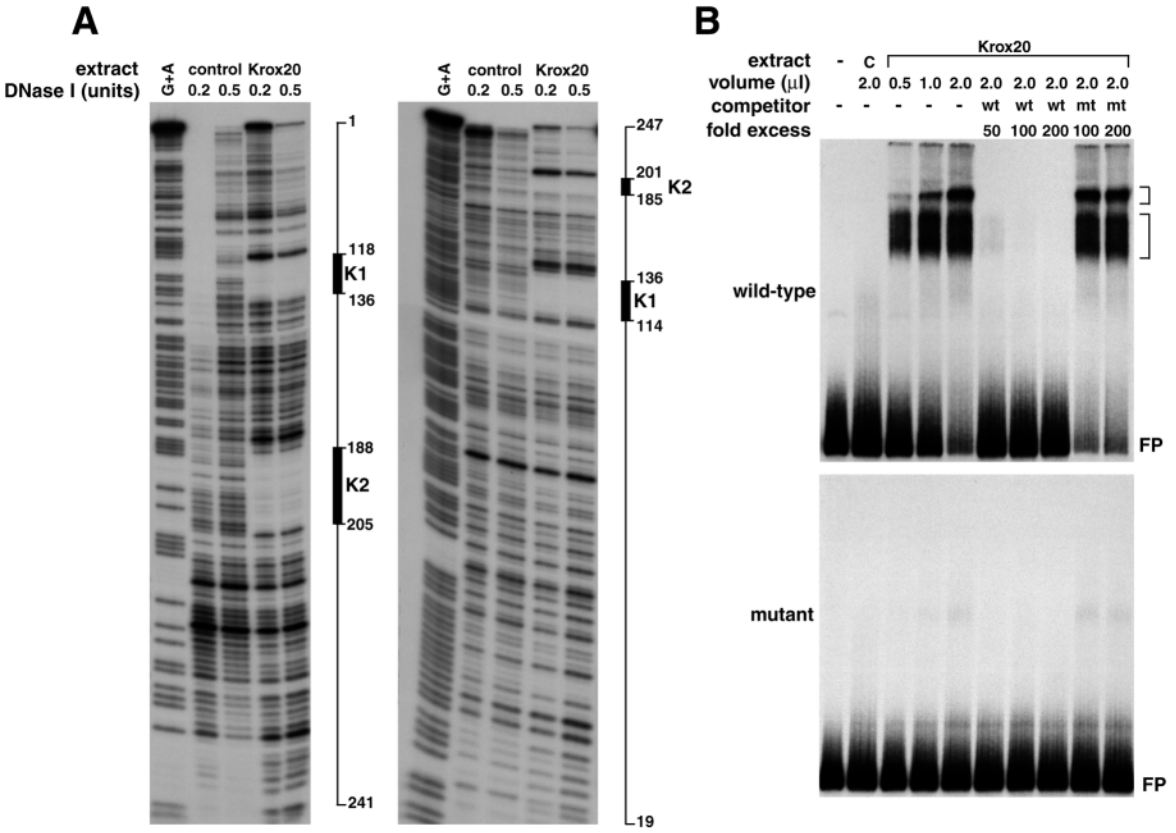



**Fig. 6.** In vitro analyses of the NCE identify two Krox20 binding sites. (A) Both the upper (left) and the lower (right) strand of the 247 bp fragment #17 (Fig. 3) were analysed for Krox20 binding sites in DNase I footprinting assays using extracts from control or Krox20-expressing bacteria. Two doses of DNase I were used (0.2 and 0.5 units). The G+A reaction is included for both strands. The position of the two Krox20 footprints and the range of sequence analysed are indicated for both strands. (B) Control and Krox20-expressing bacterial extracts were analysed in bandshift assays using either the wild-type (upper) or Krox20 binding site mutant (lower) fragment #17 as a probe. The volume of bacterial extract was varied between 0.5-2.0 μl. To identify specific complexes, unlabelled competitor oligonucleotides corresponding to a high affinity Krox20 binding site (wt) or a mutant version (mt) were included in the binding reaction at 50-200-fold molar excess (Sham et al., 1993). Specific complexes are indicated with brackets. The different mobility protein-DNA complexes are likely a result of the presence of two Krox20 binding sites, one of which is asymetrically localised (Fig. 5). FP, free probe.

### An NCE subregion containing HMG box binding sites is involved in crest-specific expression

In subsequent experiments, we searched for cis-acting sequences involved in the weak crest activity observed with mutant fragment #18. We introduced a series of internal deletions into fragment #18 and tested their effects in the chick electroporation assay. We focused our analysis on the region of interspecies sequence similarity since it is likely to contain the essential regulatory elements (Fig. 5). Two deletions, together covering the majority of this region, were first generated (Fig. 3, constructs #19 and #20). Whereas construct #19 behaved in a manner similar to the original construct #18, construct #20 was completely inactive (data not shown). The region deleted in construct #20 was further subdivided into two deletions (Fig. 3, constructs #21 and #22). Interestingly, both deletions were inactive in the NC (data not shown), raising the possibility that important regulatory sequences may lie at the junction between the two regions.

The sequences deleted in construct #20 were analysed for the presence of putative transcription factor binding sites that were conserved between the mouse, human and chick sequences. We identified two adjacent, conserved sequences in a head-to-head orientation, similar to HMG box protein binding sites (A/TA/TCAAAG, Fig. 5) (Travis et al., 1991; van de Wetering et al., 1991; Harley et al., 1994). These sites are located precisely at the junction between the regions deleted in fragments #21 and #22. A mutant fragment consisting of a 15 nt deletion was generated to eliminate the two putative HMG box binding sites (Fig. 5). When tested in chick electroporation this construct was inactive, suggesting that these sites contribute to the activity of the NCE (Fig. 3, Fig. 7C, construct #23). A 41 bp sequence encompassing these sites (Fig. 5) was then multimerised and tested for enhancer activity in both chick electroporation and mouse transgenesis (Fig. 3, construct #24). In the chick, this construct was active throughout the NC along the electroporated region (Fig. 7D). A similar profile was observed in mouse transgenesis: although weak, NC cells migrating from r2, r4 and r5/6 were clearly labelled as well as some trunk crest cells (Fig. 7E).

In conclusion, the search for additional cis-acting sequences in the NCE identified an essential subregion containing conserved sequences similar to HMG box binding sites which, when multimerised, appears to exhibit enhancer activity throughout the NC, implicating the action of crest-specific HMG box factors.

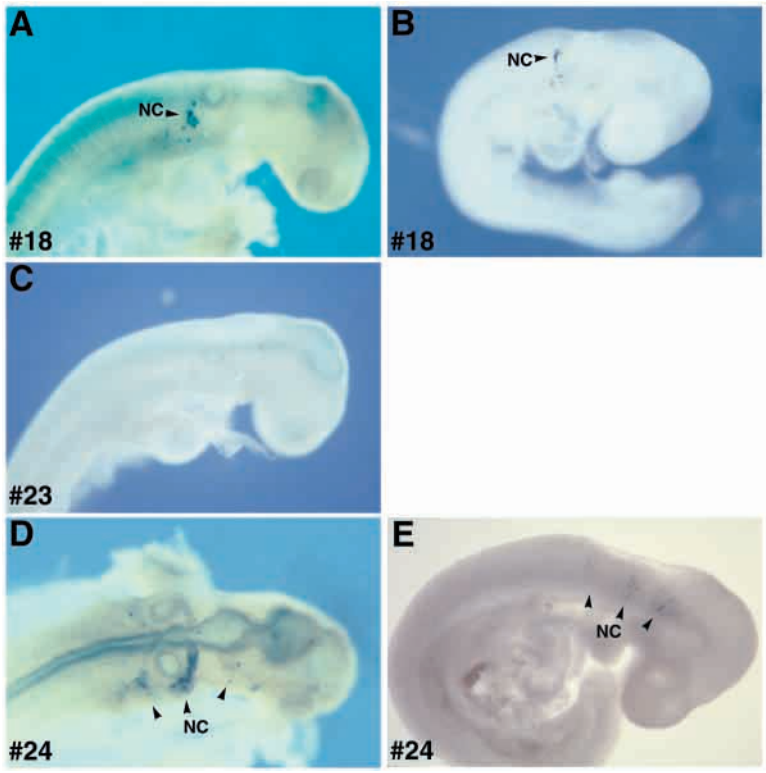



**Fig. 7.** In vivo analysis of essential NCE elements. (A-C) The activity of the NCE carrying mutations in the Krox20 binding sites alone (A,B; Fig. 3, construct #18) or both the Krox20 binding site mutations and a 15 nt deletion to inactivate the putative HMG box binding sites (C; Fig. 3, construct #23), were analysed by chick electroporation (A,C) or by mouse transgenesis (B). β-galactosidase activity was dramatically reduced (A,B) or eliminated (C) as compared to the wild-type construct (#11, see Fig. 4B-D). (D,E) A 41 bp sequence encompassing the putative HMG box binding sites (Fig. 5) was multimerised, linked to the reporter (Fig. 3, construct #24) and analysed by chick electroporation (D) and mouse transgenesis (E). Weak β-galactosidase activity is observed throughout the cranial neural crest. Embryos are whole mounts with anterior to the right. NC, neural crest.

### Synergistic activation of the NCE by Krox20 and Sox10

Several HMG box, SRY-related *Sox* genes are expressed in the NC and may therefore participate in *Krox20* expression. These include two group E *Sox* genes, *Sox9* and *Sox10* (Ng et al., 1997; Zhao et al., 1997; Kuhlbrodt et al., 1998; Southard-Smith et al., 1998). Recently, Sox9 was shown to be important in the specification of NC cells in *Xenopus* (Spokony et al., 2002). Sox10, although not required for crest formation and migration, participates in subsequent differentiation steps (Herbarth et al., 1998; Southard-Smith et al., 1998; Kelsh and Eisen, 2000; Britsch et al., 2001; Dutton et al., 2001). The possibility that *Sox10* contributes to *Krox20* expression in the NC was investigated. Firstly, we analysed its expression profile by in situ hybridisation. *Sox10* expression was detected as early as 8.5 dpc in the pre/post migratory NC, with the highest levels in r2 and r4 (Fig. 8A). However, significant levels were also detected in dorsal r5 (Fig. 8A). Double labelling of *Krox20*, by detection of β-galactosidase activity in *Krox20*$^{+/lacZ}$ embryos, and *Sox10* revealed a co-localisation in migrating NC, a profile that continued throughout the stages of crest migration (Fig. 8B and data not shown).

In a second series of experiments we investigated the possibility that Krox20 and Sox10 co-operate in the activation of the NCE. This was performed in a transactivation assay in cultured HeLa cells, which provides a quantitative evaluation of the contribution of each factor (Fig. 9). Using the wild-type NCE construct #11 as reporter, transfection of either a Krox20 or Sox10 expression construct led to a moderate level of induction. In contrast, the combination led to a synergistic induction. In order to investigate whether the synergism between these

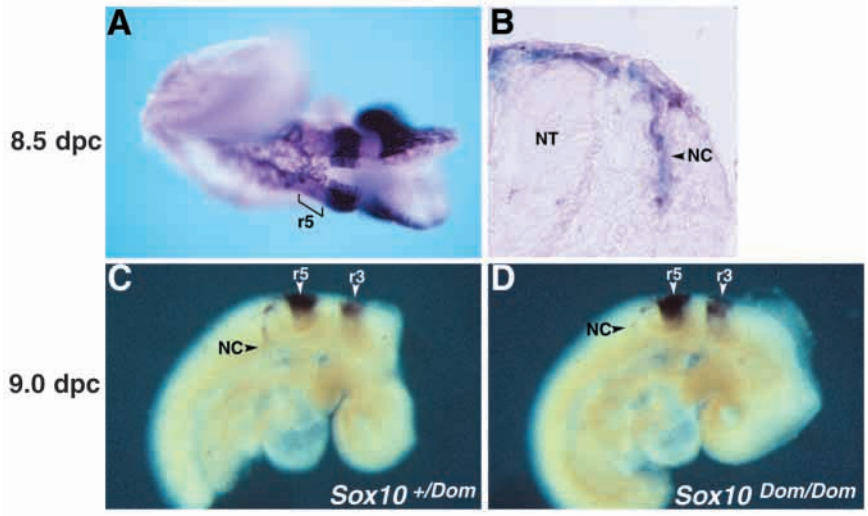



**Fig. 8.** Maintained *Krox20* expression in the migrating neural crest requires *Sox10*. (A,B) The expression of *Sox10* was analysed by in situ hybridisation in mouse embryos at 8.5 dpc. (A) Whole mount of a wild-type embryo. The territory corresponding to r5 is indicated. (B) Transverse section caudal to r5 of a *Krox20*$^{+/lacZ}$ embryo stained for both β-galactosidase activity (light blue) and *Sox10* (purple) revealing their co-expression in the migrating neural crest. (C,D) *Krox20* expression was analysed by whole-mount in situ hybridisation in *Sox10* heterozygous (*Sox10*$^{+/Dom}$) and homozygous (*Sox10*$^{Dom/Dom}$) mutant embryos at 9.0 dpc (20 somites). Embryos are presented with anterior to the right. NC, neural crest; NT, neural tube.

factors requires the elements important in NCE activity, a series of NCE mutants were tested. Construct #18, mutated in the two Krox20 binding sites, was weakly responsive to Krox20, confirming that the majority of the Krox20 binding activity had been eliminated. Although the Sox10 response was not affected, the combination of Krox20 and Sox10 resulted in a considerably reduced synergistic induction when compared to the wild-type construct. This suggests that the two Krox20 binding sites are required but that additional, weaker sites, are active. These results are also consistent with our in vivo data showing that construct #18 is weakly active in the NC (Fig. 7A,B). A new mutant derivative of construct #11, carrying uniquely the 15 nt deletion of the putative HMG box binding sites (Fig. 5), was tested. While the response to Krox20 was similar to the wild-type construct, this construct responded poorly to Sox10, consistent with an interaction of this factor with these sites. The combination of Krox20 and Sox10 together resulted in a considerably weaker synergistic response than with the wild-type construct. Although the reduced activity implicates additional sites, these results suggest an important contribution of the putative HMG box binding sites. Indeed, a construct containing a 7X multimer of these sites (#24) was dramatically induced by Sox10. Finally, a construct deficient in both the Krox20 and HMG box binding sites (#23) showed only an additive response to the combination of Krox20 and Sox10, definitively establishing the importance of these sites in the synergistic response. This latter result is consistent with our in vivo data indicating that construct #23 is defective in NC activity in chick electroporations (Fig. 7C).

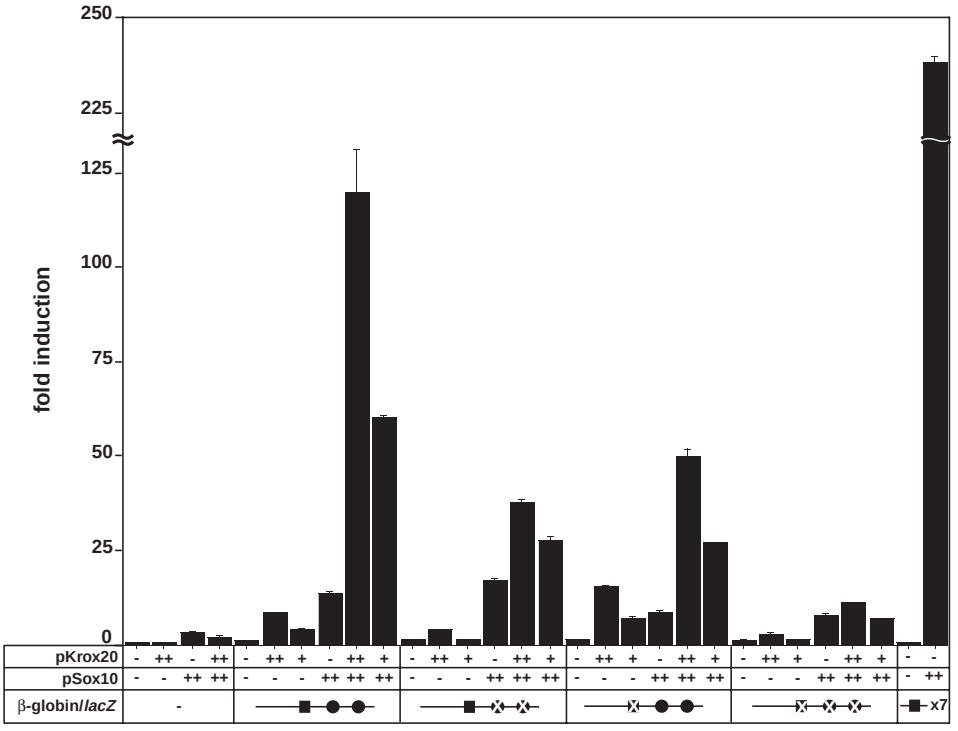



**Fig. 9.** Synergistic activation of the NCE by Krox20 and Sox10. Expression constructs encoding Krox20 (pKrox20) and Sox10 (pSox10) or the empty plasmid were transfected into cultured HeLa cells along with either the empty *β-globin/lacZ* promoter/reporter plasmid or the wild-type NCE (Fig. 3, fragment #11) or its derivatives fused to the promoter/reporter as indicated. The putative HMG box and Krox20 binding sites are indicated with squares and circles, respectively. X, represents the mutation of these sites. A 7X multimer of a 41 nucleotide sequence spanning the putative HMG box binding sites (Fig. 5) is indicated (right). Expression plasmids were transfected at either 100 ng/plate (++) or 20 ng/plate (+). The data show the β-galactosidase activities of one experiment performed in duplicate and is representative of two independent experiments. Values from transfections with the empty promoter/reporter and expression plasmids were arbitrarily set to one. Data from all other transfections are presented as the fold induction over this level. Error bars represent the standard error.

### *Sox10* is required for the maintenance of *Krox20* expression in the migratory neural crest

To establish the involvement of *Sox10* in *Krox20* regulation in vivo we analysed its expression in the *Sox10* mutant line, *Dom* (Herbarth et al., 1998; Southard-Smith et al., 1998). Comparison of wild-type (data not shown), heterozygous (Fig. 8C) and homozygous mutant embryos (Fig. 8D) at 9.0 dpc revealed a strong decrease in *Krox20* levels in the migrating neural crest of the homozygous mutant, whereas the heterozygote was not affected. This phenotype was observed throughout the stages of NC migration (data not shown). As *Sox10* mutation does not affect neural crest production and migration (Southard-Smith et al., 1998; Britsch et al., 2001), this effect is likely due to a decrease in gene transcription.

In conclusion our experiments indicate that Krox20 and Sox10 are co-expressed in the migrating NC, they can co-operate in the autoactivation of the NCE in vitro and Sox10 is involved in the control of *Krox20* expression in vivo. This suggests that Sox10 is an essential coactivator of *Krox20* autoregulation in the NC.

## DISCUSSION

This paper presents the identification and characterisation of a transcriptional enhancer element, the NCE, responsible for direct, positive autoregulation of the *Krox20* patterning gene in the r5-derived NC. In addition to the Krox20 binding sites, the NCE contains essential recognition sequences for a putative, crest-specific HMG box factor. A candidate for such a factor, Sox10, was shown to be involved in *Krox20* crest expression and to synergise with Krox20 to promote NCE activity. These data provide insight into the molecular mechanisms controlling the identity of postmigratory NC cells and how this identity is maintained.

### *Krox20* autoregulation

We have identified the NCE as an enhancer located upstream of *Krox20*, which can drive specific expression in the r5-derived NC stream in transgenic mice, reflecting the normal *Krox20* expression pattern in the NC. The activity of this element is largely dependent on the integrity of two Krox20 binding sites and is abolished in a *Krox20* null background. These data establish that the NCE can mediate direct, positive autoregulation of Krox20 in the NC, raising the question as to what extent this element contributes to *Krox20* expression. Sequence similarity searches identified elements in both man and chick that are the orthologues of the mouse NCE (Fig. 5). The chick element exhibited an activity in the NC similar to that of the mouse element when tested by electroporation in the chick neural tube. These observations suggest that the NCE probably has an important function in these cells as it has been conserved throughout the evolution of birds and mammals. More directly, analyses performed on compound mutant embryos, $Krox20^{Cre/lacZ}$, revealed a role of *Krox20* in maintaining the level of β-galactosidase activity and therefore its own level in the NC (Fig. 1). Furthermore the contribution of autoregulation may be partially masked in these studies by the stability of the β-galactosidase protein which is likely to be higher than that of the Krox20 protein itself. We can conclude that autoregulation allows for prolonged *Krox20* expression in the NC following the migration of these cells from the neural tube. This may be critical for maintaining NC positional identity since several Hox genes (*Hoxa2*, *Hoxb2* and *Hoxb3*) are under *Krox20* regulation (Sham et al., 1993; Nonchev et al., 1996; Manzanares et al., 2002). In addition to Hox genes, *Krox20* controls the expression of the receptor tyrosine kinase gene, *EphA4*, in the NC (Seitanidou et al., 1997; Theil et al., 1998). By repulsion from cells expressing their Ephrin ligands, the Eph/Ephrin signalling system may guide NC cells along their pathway (reviewed by Holder and Klein, 1999). Indeed, blocking EphA4 function in *Xenopus* embryos causes r5-derived NC cells, normally destined for the third arch, to migrate into the second and fourth arches (Smith et al., 1997). This may partially explain why *Krox20*-expressing NC, which normally migrate caudally to the otic vesicle, migrate both rostrally and caudally in *Krox20* mutant mice (Schneider-Maunoury et al., 1993).

The involvement of autoregulation in the control of *Krox20* expression is not specific to the NC. We have also observed this mode of regulation in r3 and r5 (Giudicelli et al., 2001; Voiculescu et al., 2001) and in the bone-forming cells (M. F., unpublished). Autoregulation secures *Krox20* expression when the factors required for its initiation are no longer present, reflecting the importance and the duration of the role of this factor in developmental processes. Interestingly, distinct cis-acting elements mediate autoregulation in these tissues (this study; M. F., unpublished; D. Chomette, unpublished). The existence of multiple autoregulatory elements may derive from the necessity of tight coupling of autoregulation with the presence of other tissue-specific factors that have binding sites within each of these elements.

### Sox proteins as partners for *Krox20* autoregulation in the neural crest

Analysis of the NCE carrying mutations in the two Krox20 binding sites led to the identification of an essential subregion containing two adjacent, head-to-head, putative HMG box binding sites (Fig. 7C). In addition, when a multimerised oligonucleotide containing these sites was tested in vivo, enhancer activity was detected throughout the NC (Fig. 7D,E). Together these studies suggested the action of crest-specific HMG box proteins in *Krox20* autoregulation. We then provided evidence specifically implicating members the group E Sox subfamily of HMG box proteins: (i) two members of this group, *Sox9* and *Sox10*, are expressed in the pre/post migratory NC (Ng et al., 1997; Zhao et al., 1997; Kuhlbrodt et al., 1998; Southard-Smith et al., 1998) and *Sox10* is co-expressed with *Krox20* in r5-derived NC (Fig. 8); (ii) Sox10 can synergise with Krox20 in NCE transactivation studies in cell culture (Fig. 9); (iii) *Krox20* levels in migrating neural crest are not maintained in *Sox10* mutant embryos (Fig. 8). Together these data indicate that Sox10 is a crest-specific factor essential for *Krox20* autoregulation in the NC. This conclusion is consistent with the co-operation recently observed between Sox10 and Krox20 in the activation of the connexin 32 gene in the Schwann cell lineage (Bondurand et al., 2001), suggesting that such co-operation may be a more general phenomenon.

Although our studies highlight an important role of *Sox10*, it may not be the only HMG box factor co-operating with Krox20 in the r5-derived NC. *Sox9*, which is expressed in the newly formed r5-derived neural crest (data not shown) may

also be involved in an earlier step of *Krox20* autoregulation. Similarly, although the mutation of either the Krox20 or HMG sites resulted in a significant decrease in the synergistic response, the response was not completely eliminated, suggesting that additional Krox20 and HMG binding sites may be involved. This is supported by sequence analyses of the NCE indicating the presence of additional putative binding sites for both factors (data not shown).

In addition to the demonstration of synergism between Krox20 and crest-specific Sox proteins, our data suggest that these factors may also have distinct roles in the activation of the NCE. In the mouse, the NCE is generally active in r5-derived NC and less frequently in r5. In the electroporated chick neural tube, the NCE is active in all territories expressing *Krox20*: r3, r5 and r5-derived neural crest. Furthermore, in the chick, ectopic expression of *Krox20* leads to generalised activation of the NCE in both the neuroepithelium and the NC. A hypothesis to explain these data is that activation of the NCE by Krox20 may be very dependent on the chromatin state of the NCE: in normal chromosomal chromatin configuration, Krox20 absolutely requires co-operation with a crest-specific factor to activate the NCE; in contrast, in 'relaxed chromatin' configurations, reached at a low frequency in mouse transgenesis, where it may depend on the site of insertion of the transgene, and systematically after electroporation in the chick neural tube, where the reporter plasmid is likely to remain episomal, Krox20 can activate the NCE without the crest factor. According to this hypothesis, the crest-specific factor might modify the local conformation of the chromatin in order to allow activation by Krox20. The properties of Sox proteins are consistent with such a possibility (Wegner, 1999).

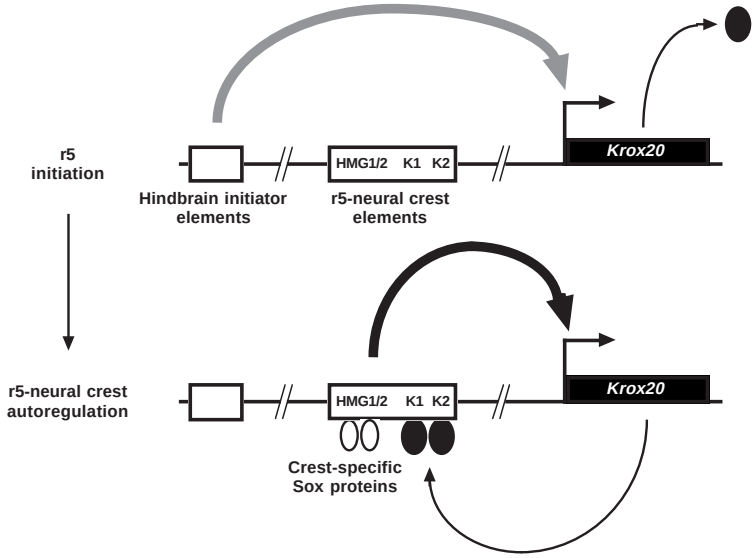



**Fig. 10.** A schematic model for the regulation of *Krox20* expression in r5-derived neural crest. We propose that the establishment of *Krox20* expression in r5-derived neural crest involves two steps. *Krox20* is first activated in the entire r5 territory under the control of an initiator cis-acting element distinct from the NCE (large grey arrow). In r5 premigratory NC cells, the Krox20 protein and crest-specific Sox proteins bind to sequences in the NCE and co-operate in the establishment of a positive, autoregulatory loop (large black arrow). This autoregulatory mechanism is then sufficient to maintain *Krox20* expression in migratory NC cells. K1 and K2, Krox20 binding sites; HMG1/2, putative Sox protein binding sites.

## A model for *Krox20* regulation in the neural crest

The present data allow us to propose a model on how *Krox20* expression is set up in r5-derived NC according to its level of origin in the hindbrain. This study has established an absolute requirement for Krox20 in NCE activity (Fig. 2). Consequently, the Krox20 protein must accumulate in the premigratory NC prior to the activation of the NCE by another mechanism. This may involve the action of regulatory elements that establish *Krox20* expression in the neuroepithelium, without discrimination between neural and NC precursors. We propose that establishment of *Krox20* expression in r5-derived NC involves the following steps (Fig. 10). (i) Initiation of *Krox20* expression in r5 by hindbrain cis-acting initiator element(s). We have recently identified an r3/r5-specific regulatory element, located upstream of the NCE, that constitutes a good candidate to perform this function (D. Chomette, unpublished). (ii) Activation of the NCE in premigratory NC cells in r5 by co-operation between Krox20 and crest-specific Sox proteins. (iii) Maintenance of *Krox20* expression under control of the NCE in postmigratory r5-derived NC once the initiators are downregulated. The model explains the establishment of a registry between *Krox20*-expressing NC and r5 on a cell lineage basis: sufficient amounts of Krox20 have to be inherited in delaminating NC cells for them to initiate or maintain the autoregulatory loop under the control of crest-specific Sox proteins. Such a mechanism ensures the acquisition of positional information in the NC according to its rhombomeric origin and may be involved in the control of the expression of other patterning genes. More generally, autoregulatory mechanisms, such as those controlling the expression of Hox genes in the hindbrain neural crest (Pöpperl et al., 1995; Maconochie et al., 1997), are likely to be important in maintaining the neural crest pre-pattern.

We thank Sylvie Schneider-Maunoury, Christine Vesque-Kishtoo and Marion Wassef for critical reading of the manuscript. We are greatful to Michel Goossens for providing us with the *Sox10$^{Dom}$* mouse line. J. G. was supported by postdoctoral fellowships from the European Union (TMR), the AFM and the Institut de France. This work was supported by grants from INSERM, MENRT, EC, ARC and AFM.

## REFERENCES

**Anderson, C. B. and Meier, S.** (1981). The influence of the metameric pattern in the mesoderm on migration of cranial neural crest cells in the chick embryo. *Dev. Biol.* **85**, 385-402.

**Barrow, J. R. and Capecchi, M. R.** (1996). Targeted disruption of the Hoxb-2 locus in mice interferes with expression of Hoxb-1 and Hoxb-4. *Development* **122**, 3817-3828.

**Bergemann, A. D., Cheng, H. J., Brambilla, R., Klein, R. and Flanagan, J. G.** (1995). ELF-2, a new member of the Eph ligand family, is segmentally expressed in mouse embryos in the region of the hindbrain and newly forming somites. *Mol. Cell. Biol.* **15**, 4921-4929.

**Birgbauer, E. and Fraser, S. E.** (1994). Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**, 1347-1356.

**Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Caignec, C. L., Wegner, M. and Goossens, M.** (2000). Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* **9**, 1907-1917.

**Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O. and Goossens, M.** (2001). Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Hum. Mol. Genet.* **10**, 2783-2795.

**Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M.** (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78.

**Chavrier, P., Janssen-Timmen, U., Mattei, M. G., Zerial, M., Bravo, R. and Charnay, P.** (1989). Structure, chromosome location, and expression of the mouse zinc finger gene Krox-20: multiple gene products and coregulation with the proto-oncogene c-fos. *Mol. Cell. Biol.* **9**, 787-797.

**Couly, G., Creuzet, S., Bennaceur, S., Vincent, C. and le Douarin, N. M.** (2002). Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* **129**, 1061-1073.

**Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N.** (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.

**Fraser, S., Keynes, R. and Lumsden, A.** (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

**Galas, D. J. and Schmitz, A.** (1978). DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5**, 3157-3170.

**Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G. et al.** (1996). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **17**, 9-19.

**Gavalas, A., Davenne, M., Lumsden, A., Chambon, P. and Rijli, F. M.** (1997). Role of Hoxa-2 in axon pathfinding and rostral hindbrain patterning. *Development* **124**, 3693-3702.

**Gavalas, A., Trainor, P., Ariza-McNaughton, L. and Krumlauf, R.** (2001). Synergy between Hoxa1 and Hoxb1: the relationship between arch patterning and the generation of cranial neural crest. *Development* **128**, 3017-3027.

**Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T.** (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

**Ghislain, J., Desmarquet-Trin-Dinh, C., Jaegle, M., Meijer, D., Charnay, P. and Frain, M.** (2002). Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. *Development* **129**, 155-166.

**Giudicelli, F., Taillebourg, E., Charnay, P. and Gilardi-Hebenstreit, P.** (2001). Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev.* **15**, 567-580.

**Goddard, J. M., Rossel, M., Manley, N. R. and Capecchi, M. R.** (1996). Mice with targeted disruption of Hoxb-1 fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.

**Harley, V. R., Lovell-Badge, R. and Goodfellow, P. N.** (1994). Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res.* **22**, 1500-1501.

**Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M.** (1998). Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. *Proc. Natl. Acad. Sci. USA* **95**, 5161-5165.

**Holder, N. and Klein, R.** (1999). Eph receptors and ephrins: effectors of morphogenesis. *Development* **126**, 2033-2044.

**Hunt, P. and Krumlauf, R.** (1991). Deciphering the Hox code: clues to patterning branchial regions of the head. *Cell* **66**, 1075-1078.

**Hunt, P., Gulisano, M., Cook, M., Sham, M. H., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R.** (1991a). A distinct Hox code for the branchial region of the vertebrate head. *Nature* **353**, 861-864.

**Hunt, P., Wilkinson, D. and Krumlauf, R.** (1991b). Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. *Development* **112**, 43-50.

**Hunt, P., Whiting, J., Muchamore, I., Marshall, H. and Krumlauf, R.** (1991c). Homeobox genes and models for patterning the hindbrain and branchial arches. *Development* **Suppl. 1**, 187-196.

**Itasaki, N., Bel-Vialar, S. and Krumlauf, R.** (1999). 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.* **1**, 203-207.

**Kelsh, R. N. and Eisen, J. S.** (2000). The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. *Development* **127**, 515-525.

**Köntges, G. and Lumsden, A.** (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

**Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M.** (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-250.

**Le Douarin, N. M.** (1982). *The Neural Crest*, 2nd edn. Cambridge: Cambridge University Press.

**Le Douarin, N. M. and Kalcheim, C.** (1999). *The Neural Crest:* New York: Cambridge University Press.

**Le Lièvre, C. S. and le Douarin, N. M.** (1975). Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **34**, 125-154.

**Liem, K. F., Jr, Tremml, G., Roelink, H., Jessell, T. M., Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M. et al.** (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

**Lumsden, A.** (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **13**, 329-335.

**Lumsden, A. and Keynes, R.** (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

**Lumsden, A. and Krumlauf, R.** (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

**Lumsden, A., Sprawson, N. and Graham, A.** (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

**Maconochie, M. K., Nonchev, S., Studer, M., Chan, S. K., Popperl, H., Sham, M. H., Mann, R. S. and Krumlauf, R.** (1997). Cross-regulation in the mouse HoxB complex: the expression of Hoxb2 in rhombomere 4 is regulated by Hoxb1. *Genes Dev.* **11**, 1885-1895.

**Maconochie, M., Krishnamurthy, R., Nonchev, S., Meier, P., Manzanares, M., Mitchell, P. J. and Krumlauf, R.** (1999). Regulation of Hoxa2 in cranial neural crest cells involves members of the AP-2 family. *Development* **126**, 1483-1494.

**Manley, N. R. and Capecchi, M. R.** (1997). Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. *Dev. Biol.* **192**, 274-288.

**Manzanares, M., Nardelli, J., Gilardi-Hebenstreit, P., Marshall, H., Giudicelli, F., Martinez-Pastor, M. T., Krumlauf, R. and Charnay, P.** (2002). Krox20 and kreisler co-operate in the transcriptional control of segmental expression of Hoxb3 in the developing hindbrain. *EMBO J.* **21**, 365-376.

**Morgenstern, B., Dress, A. and Werner, T.** (1996). Multiple DNA and protein sequence alignment based on segment-to-segment comparison. *Proc. Natl. Acad. Sci. USA* **93**, 12098-12103.

**Nardelli, J., Gibson, T. and Charnay, P.** (1992). Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis. *Nucleic Acids Res.* **20**, 4137-4144.

**Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S. and Koopman, P.** (1997). SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183**, 108-121.

**Nieto, M. A., Sechrist, J., Wilkinson, D. G. and Bronner-Fraser, M.** (1995). Relationship between spatially restricted Krox-20 gene expression in branchial neural crest and segmentation in the chick embryo hindbrain. *EMBO J.* **14**, 1697-1710.

**Noden, D. M.** (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev. Biol.* **96**, 144-165.

**Noden, D. M., le Lievre, C. S. and le Douarin, N. M.** (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103**, 121-140.

**Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P.** (1996). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-554.

**Pöpperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S.,**

**Mann, R. S. and Krumlauf, R.** (1995). Segmental expression of Hoxb1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. *Cell* **81**, 1031-1042.

**Prince, V. and Lumsden, A.** (1994). Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.

**Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P.** (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-1349.

**Schilling, T. F., Prince, V. and Ingham, P. W.** (2001). Plasticity in zebrafish hox expression in the hindbrain and cranial neural crest. *Dev. Biol.* **231**, 201-216.

**Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P.** (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-1214.

**Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A.** (1997). Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. *Development* **124**, 1215-1226.

**Sechrist, J., Serbedzija, G. N., Scherson, T., Fraser, S. E. and Bronner-Fraser, M.** (1993). Segmental migration of the hindbrain neural crest does not arise from its segmental generation. *Development* **118**, 691-703.

**Seitanidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D. G. and Charnay, P.** (1997). *Krox20* is a key regulator of rhombomere-specific gene expression in the developing hindbrain. *Mech. Dev.* **65**, 31-42.

**Selleck, M. A. and Bronner-Fraser, M.** (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-538.

**Serbedzija, G. N., Bronner-Fraser, M. and Fraser, S. E.** (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.

**Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Gupta, R. D., Whiting, J., Wilkinson, D., Charnay, P. and Krumlauf, R.** (1993). The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell* **72**, 183-196.

**Smith, A., Robinson, V., Patel, K. and Wilkinson, D. G.** (1997). The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr. Biol.* **7**, 561-570.

**Southard-Smith, E. M., Kos, L. and Pavan, W. J.** (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat. Genet.* **18**, 60-64.

**Spokony, R. F., Aoki, Y., Saint-Germain, N., Magner-Fink, E. and Saint-Jeannet, J. P.** (2002). The transcription factor Sox9 is required for cranial neural crest development in Xenopus. *Development* **129**, 421-432.

**Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R.** (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. *Nature* **384**, 630-634.

**Swirnoff, A. H. and Milbrandt, J.** (1995). DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol. Cell. Biol.* **15**, 2275-2287.

**Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P. and Wilkinson, D. G.** (1998). Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development* **125**, 443-452.

**Trainor, P. and Krumlauf, R.** (2000). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat. Cell Biol.* **2**, 96-102.

**Trainor, P. A. and Krumlauf, R.** (2001). Hox genes, neural crest cells and branchial arch patterning. *Curr. Opin. Cell Biol.* **13**, 698-705.

**Trainor, P. A., Sobieszczuk, D., Wilkinson, D. and Krumlauf, R.** (2002a). Signalling between the hindbrain and paraxial tissues dictates neural crest migration pathways. *Development* **129**, 433-442.

**Trainor, P. A., Ariza-McNaughton, L. and Krumlauf, R.** (2002b). Role of the isthmus and FGFs in resolving the paradox of neural crest plasticity and prepatterning. *Science* **295**, 1288-1291.

**Travis, A., Amsterdam, A., Belanger, C. and Grosschedl, R.** (1991). LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. *Genes Dev.* **5**, 880-894.

**van de Wetering, M., Oosterwegel, M., Dooijes, D. and Clevers, H.** (1991). Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J.* **10**, 123-132.

**Veitch, E., Begbie, J., Schilling, T. F., Smith, M. M. and Graham, A.** (1999). Pharyngeal arch patterning in the absence of neural crest. *Curr. Biol.* **9**, 1481-1484.

**Vesque, C., Topilko, P., Becker, N. and Charnay, P.** (1993). Molecular analysis of the development of the rhombencephalon. *C. R. Seances Soc. Biol. Fil.* **187**, 364-367.

**Voiculescu, O., Charnay, P. and Schneider-Maunoury, S.** (2000). Expression pattern of a Krox-20/Cre knock-in allele in the developing hindbrain, bones, and peripheral nervous system. *Genesis* **26**, 123-126.

**Voiculescu, O., Taillebourg, E., Pujades, C., Kress, C., Buart, S., Charnay, P. and Schneider-Maunoury, S.** (2001). Hindbrain patterning: Krox20 couples segmentation and specification of regional identity. *Development* **128**, 4967-4978.

**Wegner, M.** (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.* **27**, 1409-1420.

**Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P.** (1989). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.

**Wilkinson, D. G.** (1992). Whole-mount in situ hybridisation of vertebrate embryos. In *In Situ Hybridisation: A Practical Approach* (ed. D.G. Wilkinson), pp. 75-83. Oxford: IRL Press.

**Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I. and Schacherer, F.** (2000). TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res.* **28**, 316-319.

**Yee, S. P. and Rigby, P. W.** (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.

**Zhang, M., Kim, H. J., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. and Grippo, J. F.** (1994). Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. *Development* **120**, 2431-2442.

**Zhao, Q., Eberspaecher, H., Lefebvre, V. and de Crombrugghe, B.** (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev. Dyn.* **209**, 377-386.