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Hoxb-2 transcriptional activation in rhombomeres 3 and 5 requires an evolutionarily conserved *cis*-acting element in addition to the Krox-20 binding site

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Segmentation is a key feature of the development of the vertebrate hindbrain where it involves the generation of repetitive morphological units termed rhombomeres (r). *Hox* genes are likely to play an essential role in the specification of segmental identity and we have been investigating their regulation. We show here that the mouse and chicken *Hoxb-2* genes are dependent for their expression in r3 and r5 on homologous enhancer elements and on binding to this enhancer of the r3/r5-specific transcriptional activator Krox-20. Among the three Krox-20 binding sites of the mouse *Hoxb-2* enhancer, only the high-affinity site is absolutely necessary for activity. In contrast, we have identified an additional *cis*-acting element, Box1, essential for r3/r5 enhancer activity. It is conserved both in sequence and in position respective to the high-affinity Krox-20 binding site within the mouse and chicken enhancers. Furthermore, a short 44 bp sequence spanning the Box1 and Krox-20 sites can act as an r3/r5 enhancer when oligomerized. Box1 may therefore constitute a recognition sequence for another factor cooperating with Krox-20. Taken together, these data demonstrate the conservation of *Hox* gene regulation and of Krox-20 function during vertebrate evolution.

Keywords: evolutionary conservation/hindbrain segmentation/*Hoxb-2*/*Krox-20*/transcriptional control

Introduction

The hindbrain region of the developing vertebrate central nervous system is subject to a transient segmentation process (Keynes and Lumsden, 1990). A series of seven to eight successive bulges, termed rhombomeres (r), are observed along the anteroposterior axis and have been shown to be associated with the reiterated organization of reticular neurons, branchiomotor nerves, sensory ganglia and to be correlated with the generation and migration of the neural crest in this region (Lumsden and Keynes, 1989; Lumsden *et al.*, 1991; Serbedzija *et al.*, 1992; Clarke and Lumsden, 1993; Sechrist *et al.*, 1993; Birgbauer *et al.*, 1995). In addition, the rhombomeres have been shown to constitute units of cell lineage restriction (Fraser *et al.*, 1990; Birgbauer and Fraser, 1994) as well as units of specific gene expression (reviewed in Wilkinson, 1993), which are properties they share with insect compartments. Taken together, these data suggest that rhombomeric organization in diverse vertebrates participates in a fundamental way in the establishment of the developmental pattern governing hindbrain and craniofacial morphogenesis.

A number of putative regulatory genes, encoding transcription factors, growth factors, tyrosine kinase receptors and retinoic acid receptor or binding proteins have been shown to be expressed in a rhombomere-restricted manner (Murphy *et al.*, 1989; Wilkinson *et al.*, 1989a,b; Sundin and Eichele, 1990; Wilkinson, 1990; Maden *et al.*, 1991, 1992; Morriss-Kay, 1991; Ruberte *et al.*, 1991, 1992; Gilardi-Hebenstreit *et al.* 1992; Zimmer and Zimmer, 1992; Becker *et al.*, 1994; Cordes and Barsh, 1994) and could play a role in the segmentation process or in the specification of regional identity. Indeed, in the mouse several of them have been shown to be required for normal segmentation (Carpenter *et al.*, 1993; Dolle *et al.*, 1993; Mark *et al.*, 1993; Schneider-Maunoury *et al.*, 1993; Swiatek and Gridley, 1993; Cordes and Barsh, 1994). Beside the precise definition of the functions of these genes, a major challenge for the future will be the analysis of the molecular basis of their regulation and more generally the elucidation of the genetic network governing hindbrain segmentation. Furthermore, while most of the expression, genetic and functional analysis has been done in mouse embryos an important goal will be to determine the degree to which such a hindbrain cascade is evolutionarily conserved in other vertebrates.

Among the regulatory genes associated with hindbrain segmentation, *Hox* genes are thought to play a particularly important role in the process of specifying positional identity (reviewed in Krumlauf, 1994). However, little is known about the genes or factors which function upstream in the cascade to establish the rhombomere-restricted *Hox* expression. In this regard, analysis of *Hox* regulation using *lacZ* reporter genes in transgenic mice has begun to identify *cis*-elements and *trans*-acting components of *Hox* genes in the hindbrain (Whiting *et al.*, 1991; Sham *et al.*, 1992; Marshall *et al.*, 1994; Studer *et al.*, 1994; Frasch *et al.*, 1995; Popperl *et al.*, 1995). Using transgenic mice to screen functionally for potentially homologous *Hox* regulatory regions from other species in combination with sequence comparisons, has facilitated the identification of conserved motifs critical for segmental regulation (Awgulewitsch and Jacobs, 1992; Malicki *et al.*, 1992; Marshall *et al.*, 1994; Studer *et al.*, 1994; Aparicio *et al.*,

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1995; Morrison et al., 1995; Popperl et al., 1995). This also suggests that the underlying regulatory mechanisms in hindbrain segmentation are likely to be conserved in vertebrates.

We have shown previously that the zinc finger transcription factor Krox-20 (Chavrier et al., 1988, 1990), expressed specifically in r3 and r5 in the hindbrain and essential for their morphogenesis (Wilkinson et al., 1989a; Schneider-Maunoury et al., 1993), constitutes a direct transcriptional activator of the two mouse paralogous genes *Hoxa-2* and *Hoxb-2* in these rhombomeres (Schneider-Maunoury et al., 1993; Sham et al., 1993; Nonchev et al., 1996a). This r3/r5 regulation is achieved via an enhancer located upstream of each gene and these enhancers contain several Krox-20 binding sites required for *in vivo* activity. We wanted to know if *Krox-20* played this role in other vertebrates and whether these multiple Krox-20 binding sites implied synergistic interactions necessary and sufficient for rhombomere-restricted expression. In the present work, we have used functional analysis of the *Hoxb-2* r3/r5 enhancer to evaluate the contribution of each Krox-20 binding site to r3/r5 activity and found that only one specific site was required. We also cloned and identified an r3/r5 enhancer upstream of the chicken *Hoxb-2* gene, which contained a single conserved Krox-20 binding site essential for its regulatory activity. This demonstrates the conservation of both Krox-20 function and the molecular basis of *Hoxb-2* regulation since avian/mammalian separation. The Krox-20 sites alone are not sufficient for r3/r5 regulation. Therefore, we have used sequence comparisons between the mouse and chick enhancers to identify a conserved motif 17 bp 5′ of the critical Krox-20 binding site which is required for enhancer activity and may constitute a target for a factor cooperating with Krox-20 in segmental regulation.

Results

The high-affinity Krox-20 binding site 1 is essential for the activity of the mouse Hoxb-2 r3/r5 enhancer

We have previously shown that the mouse *Hoxb-2* r3/r5 transcriptional enhancer contains three Krox-20 binding sites and that the introduction of point mutations within all three sites abolishes both *in vitro* binding of Krox-20 to the enhancer fragment and the r3/r5-specific activity of the enhancer in transgenic mice (Sham et al., 1993). More recently we have demonstrated that the activation of the mouse *Hoxa-2* gene in r3 and r5 also involves an upstream r3/r5 enhancer, that this enhancer contains two Krox-20 binding sites, and that mutation of both sites inactivates the enhancer (Nonchev et al., 1996a). The presence of multiple Krox-20 binding sites within these two enhancers raised the question of their possible redundancy and of their relative contributions to enhancer activity. In the case of the *Hoxb-2* enhancer, site 1 is a high-affinity binding site, while the two other have lower affinity for the protein *in vitro*, as determined by gel retardation assay (Sham et al., 1993). To evaluate the relative contribution of the three sites to r3/r5 expression, we have generated four *lacZ* reporter constructs in the context of a 2.1 kb *Bam*HI–*Eco*RI *Hoxb-2* enhancer fragment and analysed their activity in transgenic embryos between 8.5–9.5 days of development (Figure 1). The constructs were: (wild-type), all Krox-20 binding sites intact; (1*+ 2*+ 3*), all sites inactivated by specific point mutations as described previously (Nardelli et al., 1991; Sham et al., 1993); (1*), only the high-affinity binding site 1 inactivated; and (2*+ 3*), the low-affinity sites 2 and 3 both inactivated.

As previously reported, the wild-type construct gave rise to specific high levels of *lacZ* expression in r3 and r5, in addition to a weaker staining in r4 and in the crest derived from this rhombomere due to the presence of an r4-specific enhancer on the same fragment (Figure 1A; $n = 12$ embryos; also Sham et al., 1992). When all Krox-20 binding sites were mutated, high-level r3/r5-specific expression was abolished (Figure 1B; $n = 10$ embryos). Similar patterns where r3/r5 expression was specifically lost were obtained when only the high-affinity binding site 1 was mutated (Figure 1C; $n = 14$ embryos). In contrast, mutation of the two low-affinity binding sites (2 and 3) did not dramatically affect r3/r5-specific expression (Figure 1D; $n = 8$ embryos), although there was more variability in the levels of r3/r5 expression compared with the wild-type construct. In conclusion, these data indicate that the two low-affinity Krox-20 binding sites are not necessary for the r3/r5 activity of the mouse *Hoxb-2* enhancer, while the high-affinity binding site 1 is absolutely required. This high-affinity site is identical to one in the mouse *Hoxa-2* enhancer (Nonchev et al., 1996a). This suggests that cooperativity between several Krox-20 molecules bound at the enhancer does not play an essential role in transcriptional activation in r3 and r5 in the case of the mouse *Hoxb-2* enhancer, although the additional sites might influence levels of expression.

Since only site 1 appeared to be required for r3/r5 activity, we wanted to determine if it was sufficient. A construct containing three copies of this motif shared by *Hoxa-2* and *Hoxb-2* was linked to the *lacZ* reporter vector and tested in transgenics. We never detected expression in r3 and r5 in any of the eight transgenic embryos, although there were several embryos with ectopic expression in other sites, indicating that the reporter was capable of working (data not shown). We have also tested multimerized copies (3×, 6× and 9×) of an optimized version of a Krox-20 binding site originally found adjacent to the *Hoxa-4* gene (Chavrier et al., 1990) and never observed rhombomere-restricted expression (data not shown). These results suggest that additional *cis*-acting sequences in the r3/r5 enhancer and other factors potentially interacting with Krox-20 may be required for activity.

Identification of an r3/r5 enhancer upstream of the chicken Hoxb-2 gene

We were interested in determining if the mechanism of regulating rhombomeric expression of *Hoxb-2* was conserved in other vertebrates and, if so, whether sequence comparisons of homologous enhancers between species could help to identify the additional *cis*-acting elements required for r3/r5 up-regulation by Krox-20. Birds, whose ancestors have diverged from those of mammals for 220 million years, might provide a useful comparison in this respect. Figure 2 presents a comparison of the expression profiles of *Hoxb-2* in the mouse and chick embryos at corresponding stages of development as assayed by *in situ*

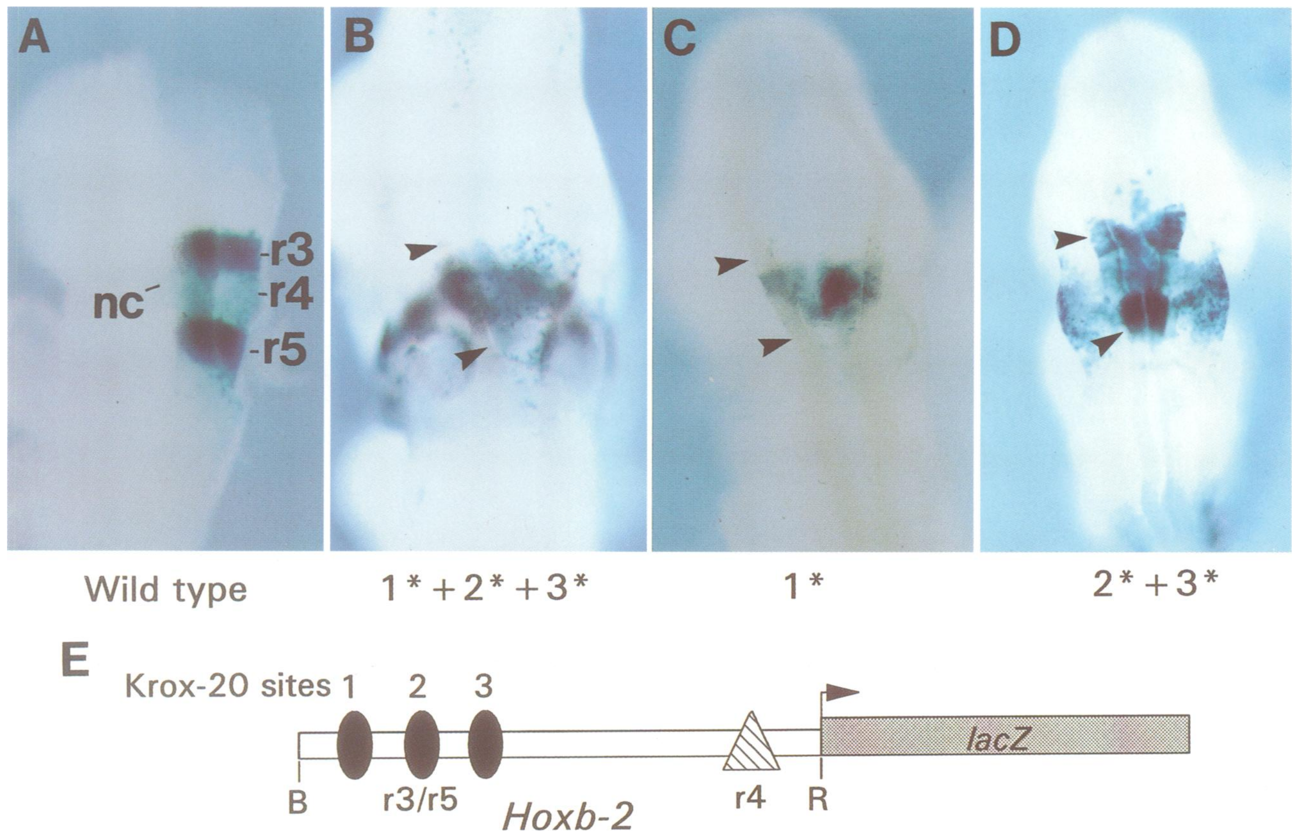


Fig. 1. Relative contributions of the three Krox-20 binding sites to the activity of the mouse *Hoxb-2* r3/r5 enhancer. (**A**) Dorsolateral view of an 8.5 dpc X-gal stained embryo generated with the 2.1 kb *Bam*HI–*Eco*RI fragment including both the r4 enhancer and the r3/r5 enhancer which contains three Krox-20 binding sites. Note the high levels of *lacZ* reporter expression in r3 and r5 and the weaker staining in r4 and in the associated neural crest. (**B**) Dorsal view of a 15-somite transgenic embryo obtained with the same construct but in which the three Krox-20 binding sites have been mutated. The high level expression in r3 and r5 is eliminated, but the staining is maintained in r4 and its derived neural crest. (**C**) Dorsal view of a 15-somite transgenic embryo in which only the high-affinity Krox-20 binding site 1 has been mutated. r3/r5 specific high-level expression is abolished as in (B). The apparently higher expression level in r4 in (B) and (C) is due to variations presumably linked to the site of insertion of the transgene. (**D**) Dorsal view of an 11-somite transgenic embryo in which the two low-affinity Krox-20 binding sites (2 and 3) have been mutated. r3/r5 specific up-regulation is maintained. (**E**) Schematic representation of the reporter construct containing the mouse *Hoxb-2* *Bam*HI–*Eco*RI fragment. The r4 enhancer, as well as the Krox-20 binding sites of the r3/r5 enhancer, are indicated. Rhombomeres (r) are shown in (A) and r3 and r5 are indicated by arrowheads elsewhere; nc, neural crest.

hybridization. The patterns are similar since the genes are expressed in the neural tube up to a common anterior boundary corresponding to the r2/r3 border. However, the relative level of expression in r3 and r5 is slightly lower in the chick and the labelling is restricted to rhombomere boundaries in this species. Nevertheless, these data are consistent with the idea that aspects of the regulation of the two genes have been conserved, and that activation of the chicken *Hoxb-2* gene in r3 and r5 might also rely on an r3/r5-specific enhancer.

We set out to search for such a regulatory region in the chicken *Hoxb-2* locus, postulating that activation in r3 and r5 might also be under the control of Krox-20. To identify Krox-20 binding sites in the vicinity of the chicken *Hoxb-2* gene, a 6 kb genomic fragment encompassing the gene and 2.5 kb of sequence 5′ to the initiator codon was digested with *Sau*3AI. The resulting fragments were then mixed with a bacterial extract containing the Krox-20 protein and subjected to a co-immunoprecipitation assay with an antibody directed against Krox-20. Four *Sau*3AI fragments were specifically retained in this assay (Figure 3). The presence of Krox-20 binding sites on these fragments was confirmed by gel retardation experiments (data not shown). Figure 4A indicates the positions of the two *Sau*3AI co-precipitated fragments (A and B) located 5′ to the initiator ATG. Since the mouse *Hoxb-2* enhancer is located upstream of the gene, we decided to first investigate the possibility that the putative r3/r5 chicken *Hoxb-2* enhancer might overlap with fragments A or B.

The activity of the chicken enhancer was tested in the mouse system, again assuming sufficient evolutionary conservation to allow inter-species recognition. A 2.5 kb *Hin*dIII–*Bam*HI genomic fragment encompassing both 5′ fragments A and B was cloned into an enhancer trap-type *lacZ* reporter vector (construct 8 of Whiting *et al.*, 1991) resulting in construct #1 (Figure 4A). Generation and analysis of transgenic embryos containing this construct demonstrated that the cloned fragment was able to drive expression of the *lacZ* reporter in r3 and r5 (Figure 4B). However, specific expression was also observed in the ventral part of r6 (Figure 4B). The expression in r3 was not uniform, being weaker in the central part of the rhombomere. These data were consistent with the idea that the *Hin*dIII–*Bam*HI fragment included an r3/r5 enhancer. Further constructs were used to localize the enhancer activity more precisely (Figure 4A). Constructs #3 and #4 were also found to lead to specific expression in r3 and r5 (Figure 4D and E). Construct #4 contains only the

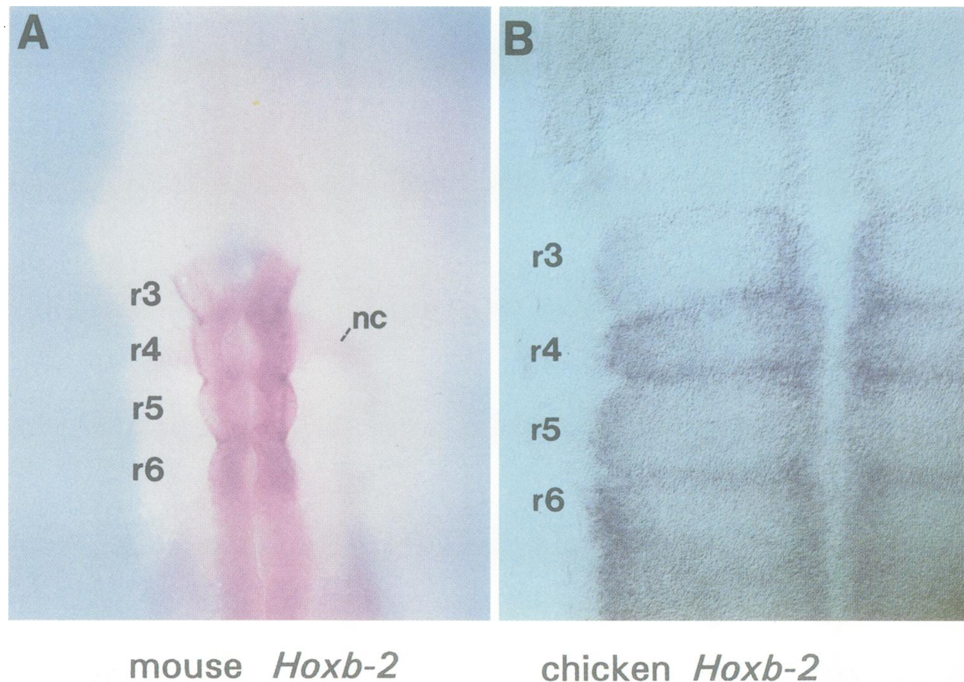


Fig. 2. Comparison of the hindbrain expression of the *Hoxb-2* gene in the mouse and chick embryos. (**A**) Whole-mount *in situ* hybridization of a 9 dpc mouse embryo with the *Hoxb-2* probe (dorsal view). Note the high level of expression in r3 to r5 and the decreasing level from r6 toward the posterior part of the neural tube. (**B**) Flat-mount of the hindbrain of a 15-somite chick embryo hybridized with the chicken *Hoxb-2* probe. Note the lower relative level of expression in r3 and r5 as compared with the mouse and the stronger signal along rhombomere boundaries. Rhombomeres (r) are indicated; nc, neural crest.

447 bp *Sau*3AI fragment B (Figure 4A). Expression was much weaker in r3, the only positive cells being close to the boundaries of the rhombomere. This pattern was reminiscent of the expression profile of *Hoxb-2* in the chick rhombomeres (Figure 2). In contrast, expression in r5 appeared to be strong and uniform. In conclusion, these data indicate that fragment B contains a transcriptional enhancer with specificity for r3 and r5.

Spatiotemporal activity of the chicken r3/r5 enhancer

The expression patterns observed with constructs #1 and #4 were slightly dissimilar and also differed from the pattern obtained with the mouse *Hoxb-2* enhancer. To eliminate the possibility that these differences could be due to variations in the stages of development of the embryos, we generated transgenic lines with these two constructs to perform a detailed spatial and temporal analysis of the *lacZ* expression patterns during development. This study is presented in Figure 5 and shows that transgene expression was uniform in r5 with both constructs and reflected the timing and distribution of the endogenous Krox-20 in r5. However, in r3 expression was delayed and not uniform, as the staining was stronger along the boundaries of the rhombomere at early and late stages (Figure 5B, C, F and G). In addition, in contrast to both the mouse *Hoxb-2* enhancer and construct #4, construct #1 drove *lacZ* expression in the ventral part of r6 in addition to r3 and r5 (Figure 5C and G; Sham *et al.*, 1993). Therefore the three DNA fragments, while all being able to drive expression in r3 and r5, appear to present slight differences in the specificity of their respective enhancer activities. The differences between the two chicken constructs are likely to be due to the presence of additional regulatory sequences within construct #1.

The chicken Hoxb-2 enhancer contains a high-affinity Krox-20 binding site essential for its activity

The sequence of fragment B is presented in Figure 6A. Analysis of this sequence revealed only one motif (5′-CACCCACAC-3′, Site 1) which fits the consensus for a high-affinity Krox-20 binding site (Chavrier *et al.*, 1990; Nardelli *et al.*, 1991, 1992; Swirnoff and Milbrandt, 1995). This motif is also almost identical to the high-affinity Krox-20 binding site of the mouse *Hoxb-2* r3/r5 enhancer (5′-CACCCACGC-3′), differing at only one position which has been shown not to be involved in the specific contact made with the Krox-20 protein (Pavletich and Pabo, 1991). Because of degeneracy within the Krox-20 binding motif, low-affinity sites are difficult to identify simply by analysis of the nucleotide sequence. Therefore we performed a gel retardation analysis both to establish the binding of Krox-20 to Site 1 and to reveal the presence of putative additional binding sites. Fragment B was digested with *Eco*RI which cuts it approximately in the middle (Figure 6A). The two subfragments were subjected to gel retardation analysis in the presence of bacterial Krox-20 protein. Only the 5′ 245 bp *Sau*3AI–*Eco*RI fragment led to formation of retarded complexes in the presence of Krox-20 (Figure 6B and data not shown), indicating that the other fragment does not contain any Krox-20 binding site. In the case of the 245 bp fragment, two retarded complexes were observed and an increase in the amount of Krox-20 protein led to a parallel increase in the relative incorporation of the probe in the slower-

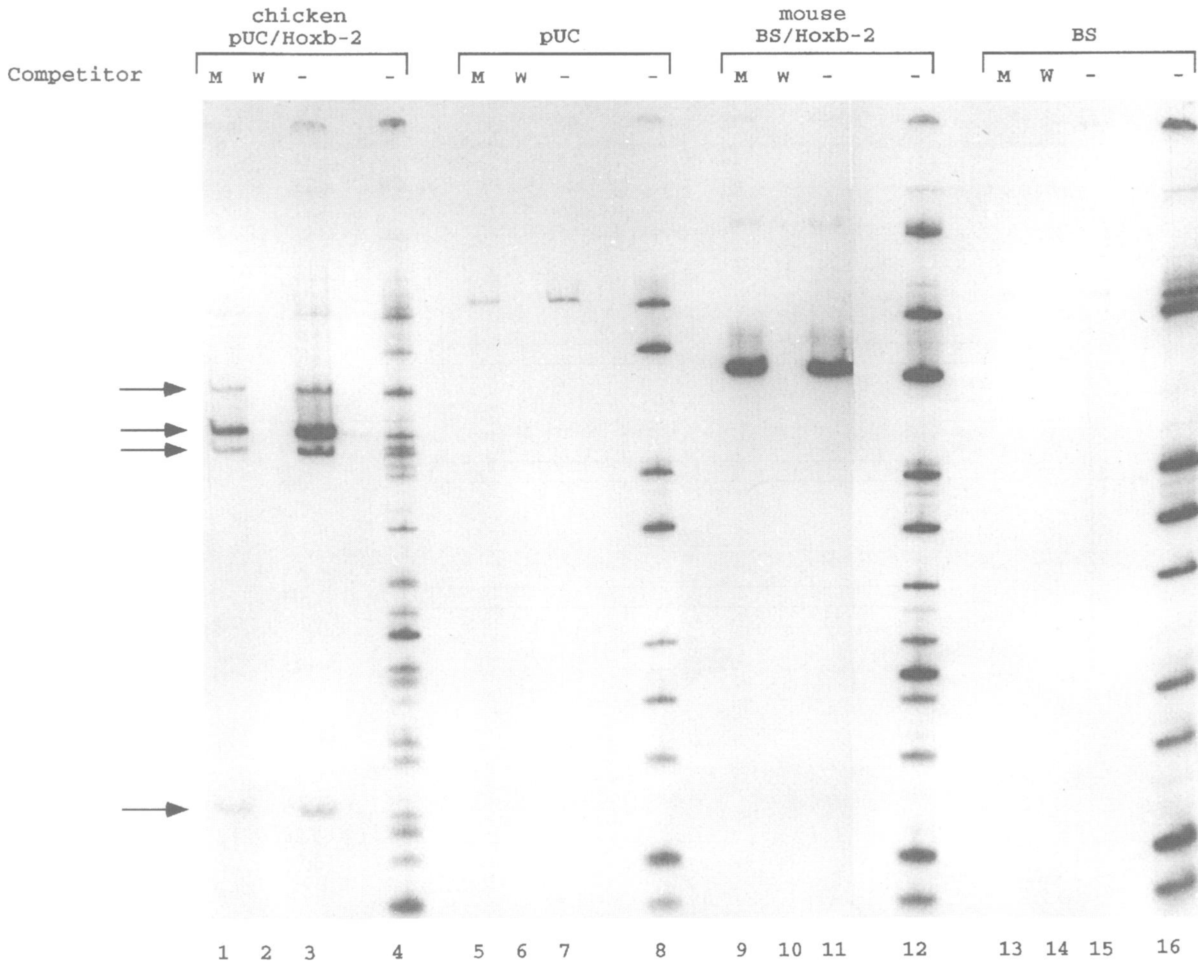


Fig. 3. Multiple Krox-20 binding sites are found in the vicinity and within the chicken *Hoxb-2* gene. A plasmid containing a 6 kb *Hin*dIII restriction fragment encompassing part of the chicken *Hoxb-2* gene and 2.5 kb of its 5′ flanking sequences (pUC/Hoxb-2) was digested with *Sau*3AI, subjected to co-immunoprecipitation in presence of Krox-20 protein and of an antibody directed against the protein and analysed by electrophoresis on polyacrylamide gel. The controls consist of similar analyses performed on the cloning vector (pUC), a plasmid containing the mouse *Hoxb-2* enhancer which carries three Krox-20 binding sites on a unique *Sau*3AI fragment (BS/Hoxb-2) and the corresponding cloning vector (BS). Binding was performed either in absence of specific competitor (lanes 3, 7, 11 and 15), or in presence of an oligonucleotide containing a high-affinity Krox-20 binding site (W) (lanes 2, 6, 10 and 14), or of a related oligonucleotide (M) carrying a mutation abolishing Krox-20 binding (lanes 1, 5, 9 and 13). Lanes 4, 8, 12 and 16: input DNA (one-tenth). The four chicken *Hoxb-2* fragments specifically co-immunoprecipitated are indicated by arrows.

migrating complex (C2). This suggested that the fragment contained two Krox-20 binding sites, Site 1 and a lower-affinity site whose location within the fragment has not been determined. Competitions for Krox-20 binding were performed with several oligonucleotides: CONS, which carried a well-characterized high-affinity consensus-like Krox-20 binding site (5′-GCGGGGGCG-3′), an oligonucleotide carrying the Site 1 motif and a related oligonucleotide carrying a mutated version of Site 1 (Site 1*). This mutation consisted of two nucleotide changes designed to abolish Krox-20 binding (Figure 6A; Nardelli *et al.*, 1991; Pavletich and Pabo, 1991; Swirnoff and Milbrandt, 1995). As expected, the CONS oligonucleotide was efficient in preventing Krox-20 binding to both sites of the 245 bp fragment (Figure 6B). The Site 1 oligonucleotide was almost as efficient, while the mutated Site 1* did not compete at all. Taken together, these data demonstrate that the Site 1 motif constitutes a high-affinity Krox-20 binding site and that, in addition to Site 1, the 245 bp *Sau*3AI–*Eco*RI fragment contains another Krox-20 binding site, presumably of lower affinity.

Since in the case of the mouse *Hoxb-2* r3/r5 enhancer, mutation of the unique high-affinity Krox-20 binding site is sufficient to inactivate the enhancer in r3 and r5, we tested whether Site 1 is required for the activity of the chicken *Hoxb-2* enhancer. Site 1 was mutated in the context of the 2 kb *Bam*HI fragment (Figure 4A, construct #3) into Site 1*, by the introduction of the same mutations which were shown to eliminate Krox-20 binding *in vitro*. The mutant fragment was then introduced into the *lacZ* reporter vector to generate construct #5 (Figure 4A). None of the 24 transgenic embryos obtained with construct #5 expressed *lacZ* in r3 or r5 (Figures 4A and 7B). In addition to the r3/r5 pattern, a consistent feature of construct #3 is expression in the atrium of the heart primordium (Figure 7A). This expression was reproducibly maintained in two of the transgenic embryos obtained with mutated construct #5 (Figure 7B), indicating that the promoter and *lacZ*

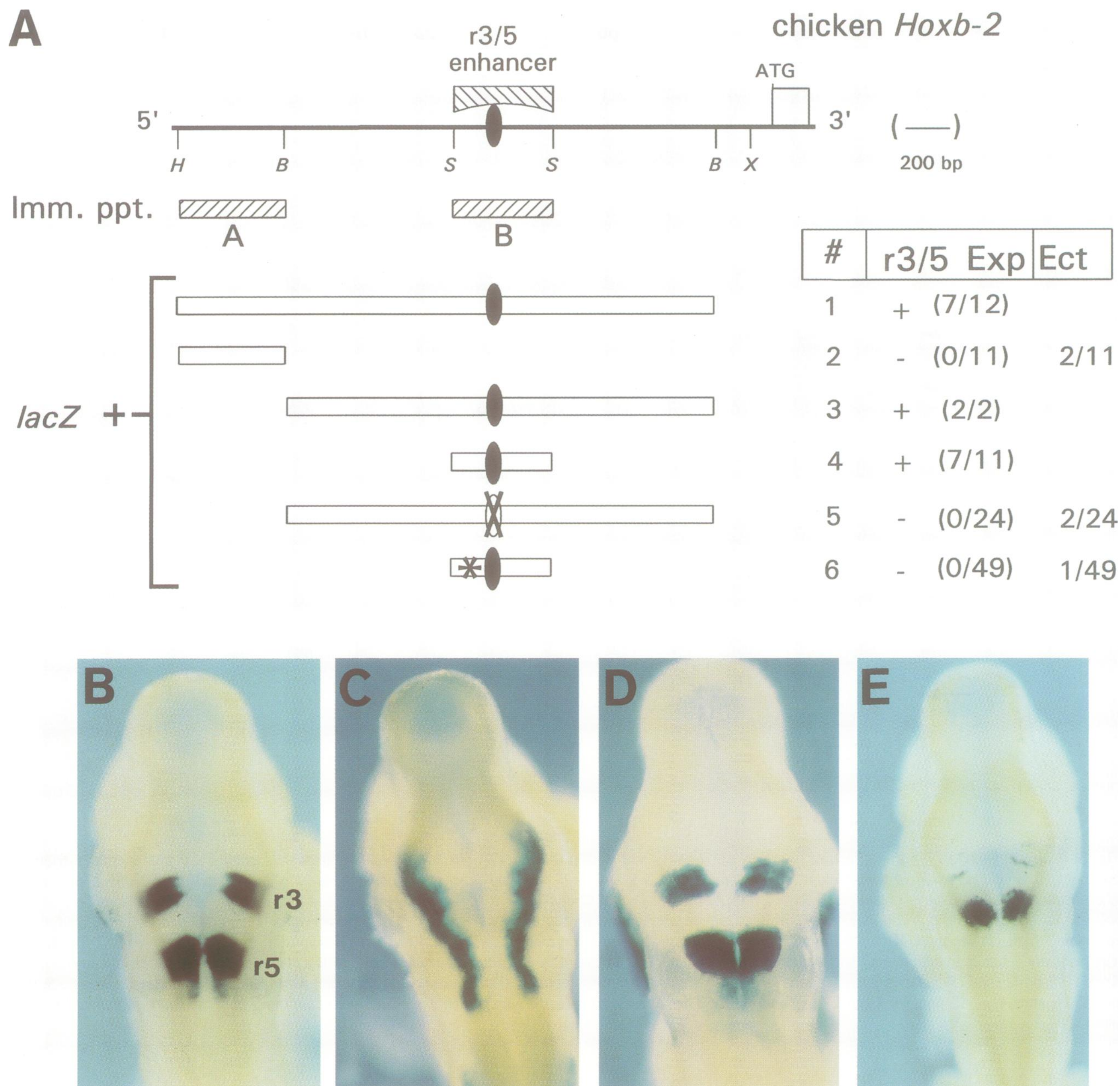


Fig. 4. Localization and characterization of a r3/r5 enhancer upstream of the chicken *Hoxb-2* gene by deletion and mutational analysis. (**A**) The indicated restriction fragments have been tested for r3/r5 enhancer activity after insertion into the vector p1084 and generation of transgenic animals. The striped boxes shows the localization of the *Sau*3AI fragments A and B which are co-immunoprecipitated in Figure 3. The closed ellipse indicates the position of the high-affinity Krox-20 binding site. Presence of a cross in construct #5 indicates that the site has been mutated. The star in construct #6 refers to the mutation introduced in Box1. The number of embryos showing r3/r5-specific or ectopic (ect) expression among total transgenic embryos are indicated. H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; S, *Sau*3AI. (B–E) show *LacZ* expression patterns in transgenic embryos generated with different chicken *Hoxb-2* constructs. Dorsal views of 9.5 dpc transgenic embryos are shown in all cases. (**B**) Embryo generated with construct #1. The X-gal staining is restricted to r3 and r5, with columns of positive cells in the ventral part of r6. Note the weaker staining in the central part of r3. (**C**) Embryo generated with construct #2. Expression of the reporter is localized over the ridges of the closing neural folds in the r2–r7 domain. (**D**) Embryo generated with construct #3 and displaying a pattern of X-gal staining identical to (B). (**E**) Embryo generated with construct #4. The transgene is expressed in the totality of r5, but in r3 staining is restricted to narrow bands one to two cells thick localized at rhombomere boundaries.

coding sequences were functional in this construct and that the mutation specifically abolished r3/r5 expression. In conclusion, the high-affinity Krox-20 binding Site 1 appears to be essential for the r3/r5 activity of the chicken *Hoxb-2* enhancer.

To further implicate a role for Krox-20 in the activity of the chicken *Hoxb-2* enhancer in r3 and r5, we introduced a construct leading to ectopic expression of *Krox-20* in r4 (Nonchev *et al.*, 1996a) in transgenic embryos carrying constructs #1 or #4. As shown above (Figure 5), lines carrying the *lacZ* reporter under the control of the r3/r5 enhancer in constructs #1 and #4 do not express the transgene in r4. However, when the r4/Krox-20 ectopic expression construct was introduced into this transgenic background, patchy but specific *lacZ* expression was induced in r4 (Figure 8A and B). This demonstrates that

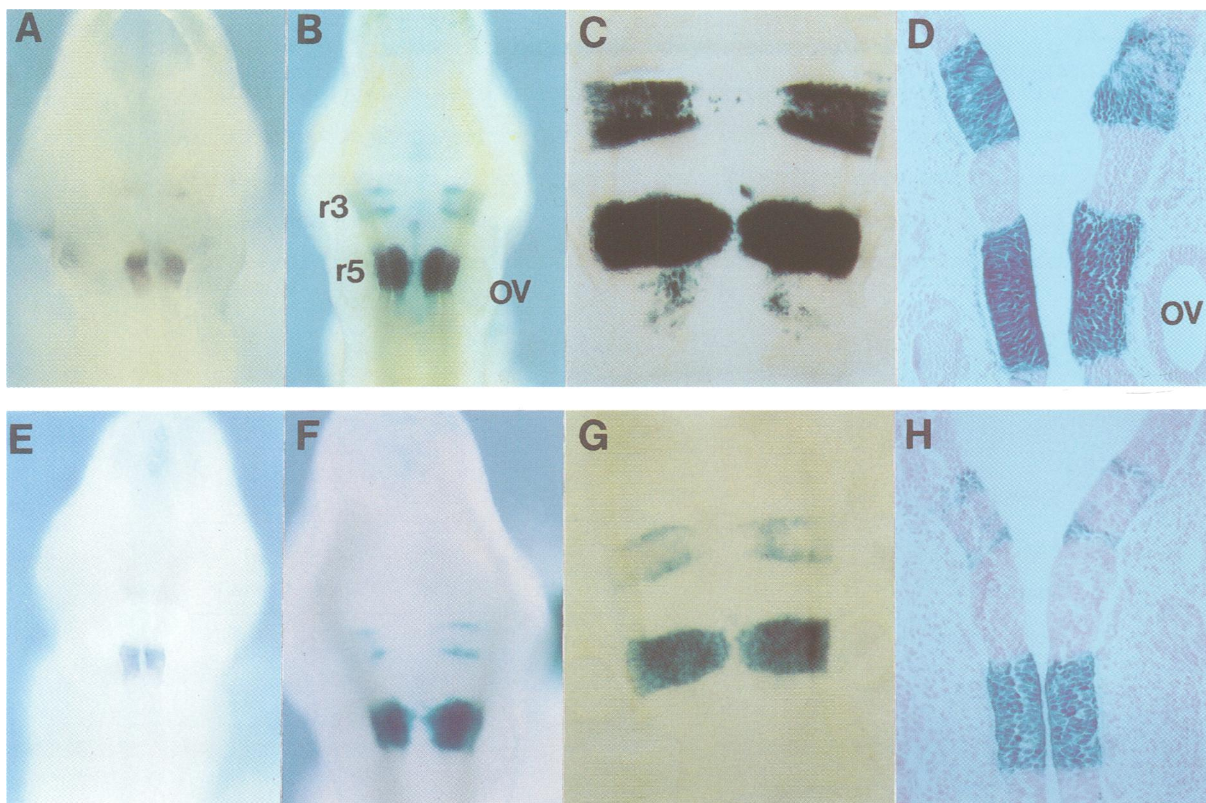


Fig. 5. Temporal and spatial patterns of activity of the chicken *Hoxb-2* r3/r5 enhancer. (A–D) and (E–H) correspond to embryos obtained from mouse transgenic lines generated with construct #1 and #4 respectively. (**A** and **E**) Dorsal views of X-gal stained 8.5 dpc embryos showing that both lines display similar patterns with high activity in r5 and a few positive cells in r3. (**B** and **F**) Dorsal views of 9.5 dpc embryos showing similar high-level expression in r5 between the two lines. In r3 however, differences in the intensity and the distribution of the staining are appearing. (**C** and **G**) Flat-mounts of the hindbrain region of 10.5 dpc embryos from both lines. The line generated with construct #1 shows high-level expression in r3 and r5, with nevertheless a band of weaker intensity in the middle of r3. Expression also extends to the ventral part of r6. In contrast, with the line generated with construct #4, no r6 expression is observed and staining in r3 is mainly restricted to the boundaries. (**D** and **H**) Coronal sections through the hindbrain of 11.5 dpc embryos. The differences observed between the two lines at previous stages are maintained. ov, otic vesicle.

the Krox-20 protein is able to transactivate through the chicken enhancer in transgenic mice.

Comparison of the mouse and chicken enhancers identifies another essential cis-acting element

In order to search for possible evolutionary conservation of important sequences between the mouse and chicken *Hoxb-2* enhancers, we have performed an alignment comparison of the nucleotide sequences of the mouse 691 bp (Sham *et al.*, 1993) and chicken 447 bp *Sau*3AI fragments carrying the respective enhancers. No long stretch of homology was detected, except for a 54/52 bp region (mouse/chick) surrounding the high-affinity Krox-20 binding sites where 75% identity is observed (Figure 7E). In this region, we were particularly intrigued by the occurrence of a fully conserved 8 bp partially palindromic sequence, 5′-GAAACTTT-3′, at an identical position 17 bp upstream to the Krox-20 binding site in both enhancers. In order to investigate its possible functional significance, this motif (called Box1) was mutated in the context of construct #4 by replacement with an unrelated sequence, 5′-TCGGATCC-3′ (Figure 7E). The resulting plasmid, construct #6, was used to generate a series of transgenic embryos (Figure 4A). While expression in r3 and r5 was observed at a frequency higher than 50% with construct #4, no X-gal staining was detected in the hindbrains of the 49 transgenic embryos generated with construct #6, with one case of ectopic expression (Figures 4A and 7C and D). These data therefore demonstrate that the chicken *Hoxb-2* r3/r5 enhancer contains a sequence motif which is shared with the mouse enhancer and is absolutely required in addition to the high-affinity Krox-20 binding site for enhancer activity.

To investigate whether the association of the Box1 and Krox-20 sites might constitute a minimal r3/r5 enhancer, we synthesized a 44 bp oligonucleotide spanning the two sites in the chicken context. Three copies of this sequence were linked to the *lacZ* reporter vector and this construct was tested in transgenic mice. In contrast to the multimerization of the Krox-20 binding site alone, this combination led to r3/r5-specific expression (Figure 8C and D) in all embryos showing reporter expression ($n = 3$). The staining was strong and rather uniform in r5, but patchy and more restricted to rhombomeric boundaries in r3. In conclusion, a short oligomerized sequence spanning both the Box1 and Krox-20 sites is able to mimic the activity of the chicken r3/r5 enhancer in the mouse, raising the possibility that the Box1 and Krox-20 sites are both necessary and sufficient for activity.

Discussion

In the present study, we have investigated the role of the transcription factor Krox-20 in the control of the expression

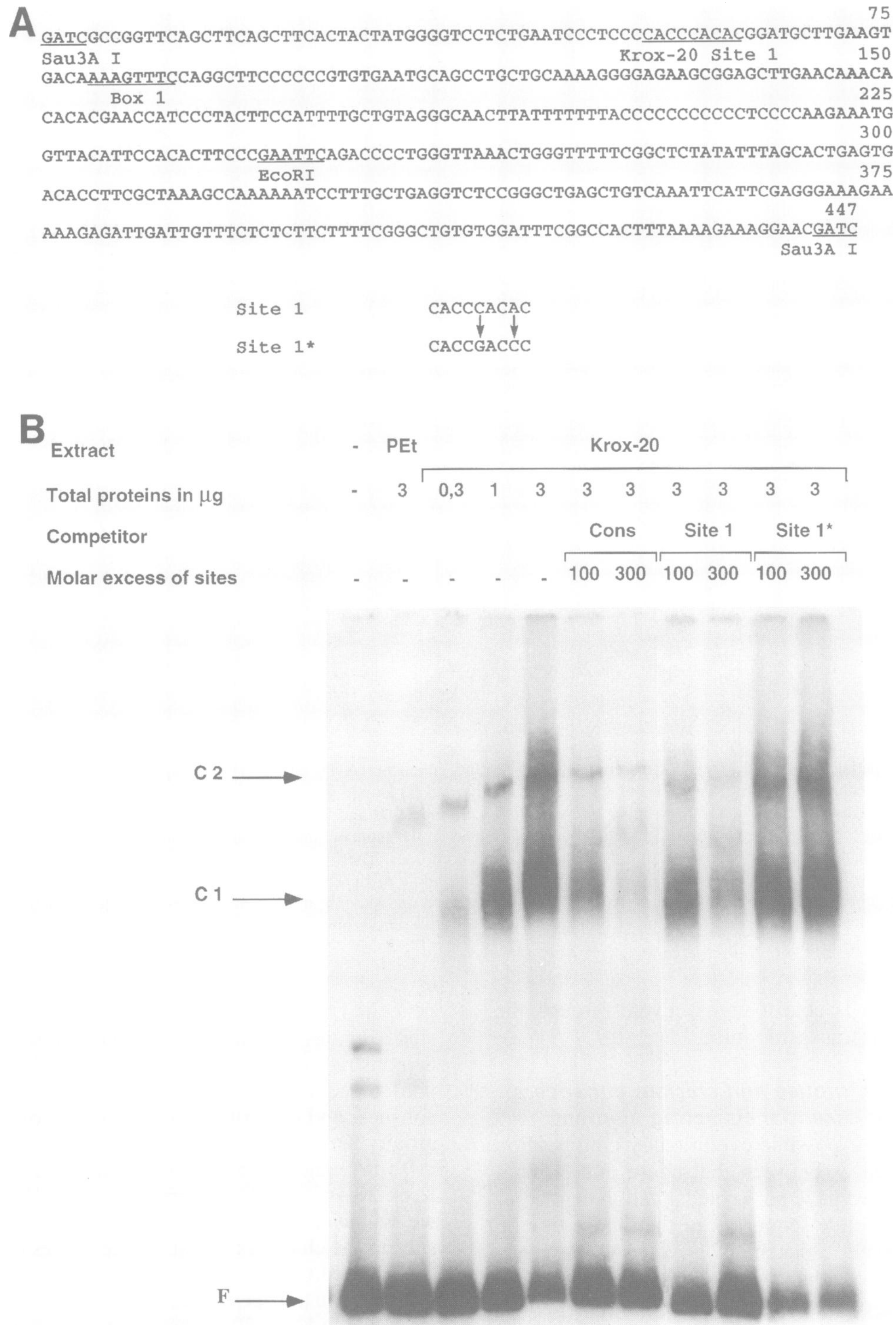
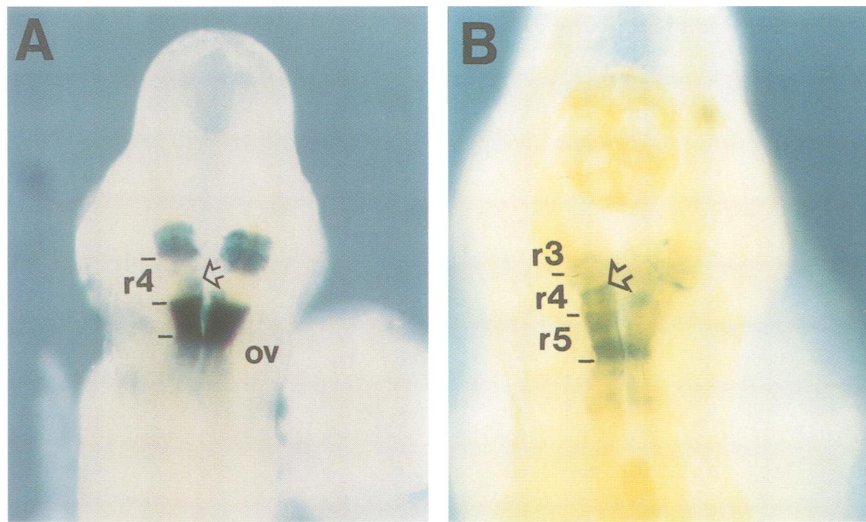
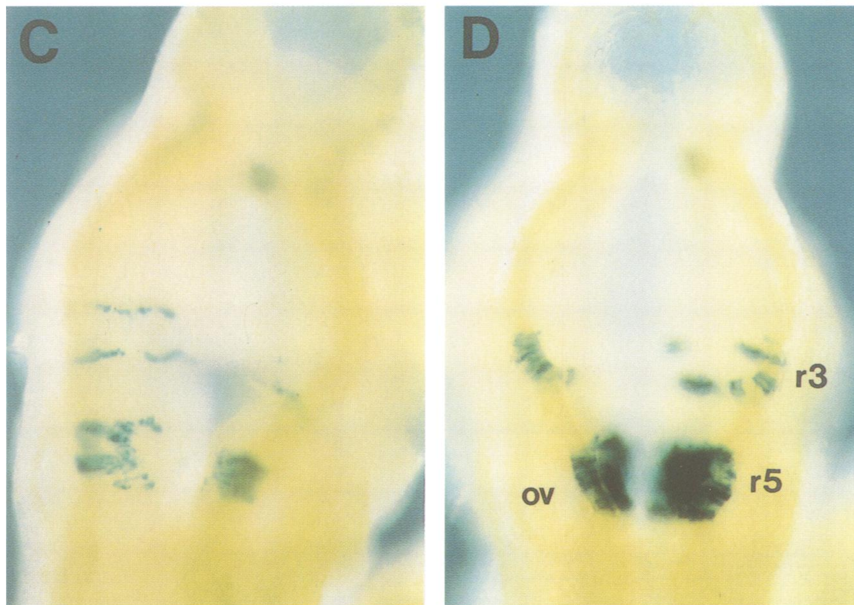


Fig. 6. The chicken *Hoxb-2* r3/r5 enhancer contains a high-affinity Krox-20 binding site. (**A**) Nucleotide sequence of the 447 bp chicken *Hoxb-2 Sau*3AI enhancer fragment. The sequence of the high-affinity Krox-20 binding site (site 1) is underlined, as well as the Box1 sequence and the *Eco*RI and *Sau*3AI restriction sites. The two nucleotide changes introduced to inactivate the Krox-20 binding site 1 (resulting in site 1*) are indicated underneath. (**B**) Gel retardation analysis demonstrating specific Krox-20 binding to the 245 bp *Sau*3AI–*Eco*RI fragment of the chicken enhancer. The probe was incubated either with a control bacterial extract (PEt) or with increasing amounts of an extract containing the Krox-20 protein. Competitions were performed at the indicated molar excess of sites with the following oligonucleotides: Cons carries a previously characterized, high-affinity Krox-20 binding site; Site 1 carries the Krox-20 site 1 of the chicken *Hoxb-2* enhancer; Site 1* carries the mutated version of this Krox-20 binding site. F, free probe; C1 and C2, the two retarded complexes.

of a vertebrate *Hox* gene transcribed in r3 and r5. We have identified an r3/r5-specific enhancer upstream of the chicken *Hoxb-2* gene. This enhancer is functional in the mouse and shares with the homologous mouse enhancer several properties, in particular the requirement for one high-affinity Krox-20 binding site as well as an additional





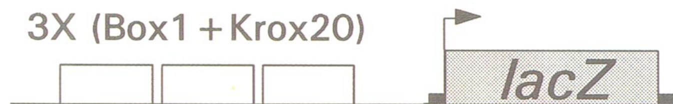


Fig. 7. The chicken *Hoxb-2* r3/r5 enhancer requires the integrity of both the Krox-20 Site 1 and the Box1 sequence. (A and B) Effect of the mutation of the high-affinity binding site on *lacZ* reporter expression in 9.5 dpc transgenic embryos. (**A**) Embryo generated with construct #3. (**B**) Embryo generated with the related construct #5 in which the Krox-20 site 1 has been mutated. r3 and r5 expression is specifically abolished by the mutation, while staining in the atrium anlage is maintained. (C and D) Effect of the mutation in the Box1 sequence. (**C**) Dorsal view of a 9.5 dpc transgenic embryo generated with the minimal chicken *Hoxb-2* enhancer fragment retaining r3/r5 activity (construct #4). (**D**) Dorsal view of the only transgenic embryo (8.5 dpc) expressing the *lacZ* reporter among those generated with the related construct #6 in which the Box1 motif is mutated. This expression is ectopic and no staining is observed in r3 or r5. (**E**) Alignment of the nucleotide sequences of the mouse and chicken *Hoxb-2* r3/r5 enhancers in the regions of the high-affinity Krox-20 binding sites. The Krox-20 Site 1 and Box1 sequences are indicated and the mutations introduced into these sequences in constructs #5 and #6 respectively are presented underneath. Above are shown putative Box1 and Krox-20 sites observed within the Fugu *Hoxb-2* r3/r5 enhancer (Nonchev *et al.*, 1996b) and upstream of the human *Hoxb-2* gene (Vieille-Grosjean and Huber, 1995; this latter Krox-20 site is likely to be of lower affinity). Note that in these cases the spacing between the two sites is not conserved.

cis-acting element. This latter motif might constitute a binding site for a factor cooperating with Krox-20. These data illustrate the conservation of *Hox* gene regulation and of Krox-20 function during vertebrate evolution.

The chicken and mouse Hoxb-2 r3/r5 enhancers constitute homologous regulatory elements

The element identified upstream of the chicken *Hoxb-2* gene is able to drive specific expression of a reporter gene in r3

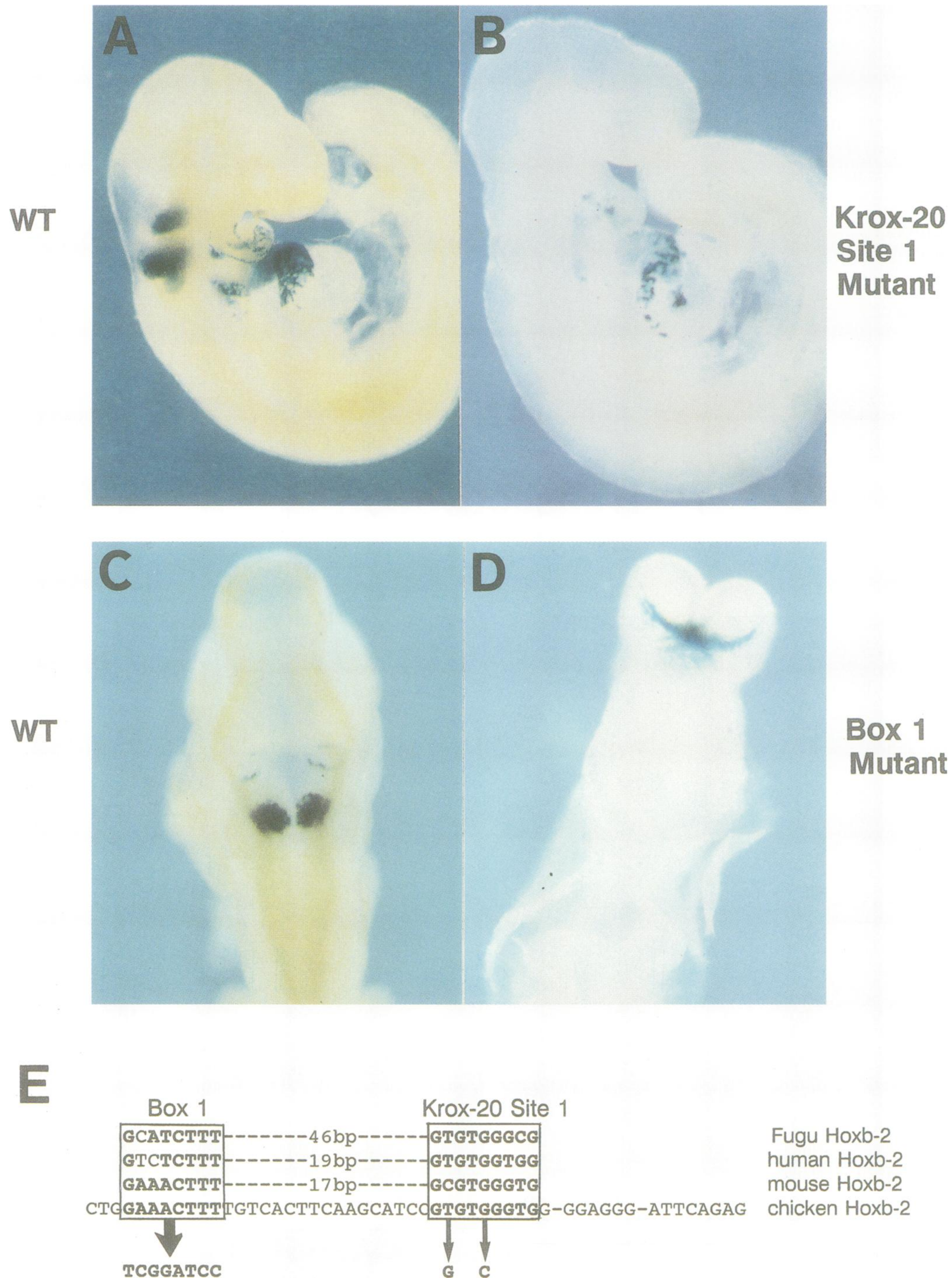


Fig. 8. Transactivation by ectopic Krox-20 and sufficiency of the sequence spanning the Box1 and Krox-20 sites to direct r3/r5 expression. (**A** and **B**) Dorsal views of double transgenic embryos carrying either construct #1 (A) or #4 (B), in which a construct expressing *Krox-20* in r4 induces *lacZ* expression specifically in this rhombomere, in addition to the r3/r5 expression due to the endogenous Krox-20. Open arrows indicate X-gal staining in r4. (**C** and **D**) Dorsal views of two transgenic embryos generated with the construct containing the basal *lacZ* vector linked to three copies of a 44 bp oligonucleotide spanning the chicken Box1 and Krox-20 sites (schematic representation below). This sequence is sufficient to mediate expression in r5 and part of r3. ov, otic vesicle.

and r5. Since it is active irrespective of the orientation of the fragment, it is likely to constitute a transcriptional enhancer. This element shares several properties with the r3/r5 enhancer identified in the vicinity of the mouse *Hoxb-2* gene (Sham *et al.*, 1993): (i) the two elements have analogous locations respective to the *Hoxb-2* gene; (ii) their specificity appears very similar, although not identical (see below); (iii) their activity in r3 and r5 requires the presence of a high-affinity Krox-20 binding site; and (iv) another *cis*-acting sequence necessary for r3/r5 activity is present at the same location respective to the Krox-20 high-affinity binding site. For these reasons we think that these enhancers constitute homologous regulatory elements which control *Hoxb-2* gene expression.

Two important differences have been observed in the specificities of the mouse and chicken enhancers: (i) the chicken element is active in the ventral region of r6, while the mouse enhancer is not; (ii) the mouse enhancer drives *lacZ* expression within the entire body of rhombomere 3, while the activity of the chicken enhancer in r3 is restricted to the regions of the boundaries with adjacent rhombomeres. Furthermore, in contrast to the mouse element, the chicken enhancer activity appears weaker and delayed in r3 as compared with r5. Such differences between the two enhancers might be due to the fact that the chicken enhancer is tested in an heterologous system. However, we think that the boundary restriction accurately reflects the normal expression pattern of the chicken gene, which, in contrast to the mouse gene in early stages, is expressed at higher levels in the vicinity of rhombomere boundaries (Figure 2). However, it is important to note that in the mouse during the late phase (11.5 dpc) when Krox-20 is being down-regulated in r3 and r5, expression persists longer at the rhombomere boundaries. This suggests that either Krox-20 is independently regulated at the boundaries versus the middle of the rhombomeres or that the level of expression is actually higher in this region. This is also consistent with the observations that a number of other genes are specifically expressed in cells bordering the junction between rhombomeres rather than throughout an entire rhombomere (Cook *et al.*, 1995; Heyman *et al.*, 1995; Mahmood *et al.*, 1995).

The control of Hox gene expression by Krox-20 has been maintained during vertebrate evolution

We have previously shown that the mouse *Hoxb-2* gene and its unique paralogous gene, *Hoxa-2*, are under the direct transcriptional control of Krox-20 in r3 and r5 (Schneider-Maunoury *et al.*, 1993; Sham *et al.*, 1993; Nonchev *et al.*, 1996a). In the present report, we show that chicken *Hoxb-2* gene expression in r3 and r5 is dependent on Krox-20 binding to an homologous enhancer element. The chicken and mouse *Hoxb-2* genes have been diverging since the avian/mammalian separation which occurred about 220 million years ago. The four vertebrate *Hox* gene clusters are thought to have been generated from a unique cluster present in primitive chordates by successive duplications which occurred during early vertebrate radiation, about 500 millions years ago (Garcia-Fernandez and Holland, 1994). Therefore, essential aspects of the regulation of the expression of two *Hox* genes and of the function of Krox-20 have been conserved during most of vertebrate evolution. If Krox-20 played a fundamental role in the regulation of one ancestral *Hox* gene, it is legitimate to question whether it could not also be involved in the control of other vertebrate *Hox* genes expressed in these rhombomeres. For example, members of paralogous group three are transcribed at high levels in r5. Recently we have investigated the expression of *Hoxb-3* in *Krox-20* homozygous mutants and found that the mutation prevents the activation of the gene in r5 (T.Seitanidou, S.Schneider-Maunoury and P.Charnay, in preparation). Therefore, although we do not know yet whether Krox-20 is actually a direct activator in this latter case, it appears as a key regulator of *Hox* gene expression in the developing hindbrain, a function that it is likely to have acquired early during chordate evolution.

Functional organization of the r3/r5 enhancer and involvement of multiple trans-acting factors

We have initiated an analysis of the functional organization of the mouse and chicken r3/r5 *Hoxb-2* enhancers. Evaluating the relative contribution of the three Krox-20 binding sites to the activity of the mouse enhancer, we have found that only the unique high-affinity binding site is required, suggesting that cooperative interactions between different Krox-20 molecules are not essential. Nevertheless, we cannot exclude the possibility that *in vivo* the two other sites might play some role in fine-tuning the level of *Hoxb-2* transcription. Targeted mutations of these sites will be required to address this issue. Similarly, the chicken enhancer displays analogous properties since it contains two Krox-20 binding sites and the mutation of the high-affinity site is sufficient to abolish activity.

The present work also provides two arguments in favour of the involvement of additional *trans*-acting factors in the activity of the *Hoxb-2* r3/r5 enhancers: (i) The identification of the Box1 sequence which is conserved between the mouse and chicken enhancers and is required for the activity of the chicken enhancer. To extend this evolutionary analysis, we have also sequenced the analogous region of the puffer-fish (*Fugu rubripes*) *Hoxb-2* gene, and used available sequence of the human gene (Vieille-Grosjean and Huber, 1995) to search for Box1 homologies. We found Box1-like sequences situated in close proximity to a putative Krox-20 binding site. The relative spacing of this motif from the Krox-20 site is 17 bp, 17 bp, 19 bp and 40 bp in the chicken, mouse, human and *Fugu Hoxb-2* genes respectively (Figure 7E). Furthermore, while the Box1 and Krox-20 motifs are conserved there is little sequence similarity in the spacer region between them in these species. (ii) The observation of a restricted expression of the *lacZ* transgene to the boundary regions in r3, while Krox-20 itself is present and active in the entire rhombomere. This indicates that either Krox-20 needs to cooperate with another factor(s) restricted to or at higher levels in the boundary regions or its activity is inhibited by factor(s) present in the complementary region of r3. Furthermore, the homogeneous activity of the chicken enhancer in r5 suggests that the distribution of these cofactors is different between r3 and r5. We also note that the 44 bp element works more efficiently in r5 than in r3.

Mode of action of the GAAACTTT motif

Different possibilities can be envisaged for the mode of action of the Box1 sequence, 5′-GAAACTTT-3′. One striking observation is that this sequence is found at exactly the same position relative to the Krox-20 high-affinity binding sites in both the mouse and chicken enhancers (17 bp) and is close in the *Fugu* and human cases (Figure 7E). This could mean that this sequence is also recognized by Krox-20 or that it participates to its binding to DNA. Indeed, another zinc finger transcription factor, MIG1, which possesses two zinc fingers similar to those of Krox-20, has been shown to bind to its target sequence only in the close context of an AT-rich sequence (Lundin *et al.*, 1994). However, we have compared the binding of Krox-20 to the 245 bp *Sau*3AI–*Eco*RI *Hoxb-2* enhancer fragment by gel retardation analysis with and without the mutation in Box1 and have found no difference

(data not shown). Therefore, Box1 sequence is not required for *in vitro* binding of Krox-20.

In these conditions, a more likely possibility is that the 5′-GAAACTTT-3′ sequence constitutes a binding site (or part of such a site) for another factor which cooperates with Krox-20. However, screening of the TF sites databases did not reveal any published transcription factor with a recognition sequence close to this motif. The conservation of the spacing between Box1 and the Krox-20 binding site could mean that if a factor binds to Box1, it might interact directly with Krox-20. The putative cooperating factor would have to be present in the neural tube at least in r3 and r5. Since we have shown that, in the presence of ectopic Krox-20, the mouse and chicken *Hoxb-2* enhancers are active in r4 or in the neural tube posterior to r5 (this work; Sham *et al.*, 1993 and data not shown), the cooperating factor could actually be much more widely distributed than Krox-20. So far, only one protein, NAB1, has been demonstrated to be able to interact with Krox-20 (Russo *et al.*, 1995); however, this appears to repress Krox-20 transcriptional activity in cells in culture and has not been shown to bind DNA.

Finally, the reconstitution of an r3/r5 enhancer, although partially active in r3, by oligomerization of the 44 bp oligonucleotide raises the possibility that only the Box1 and Krox-20 sites are required for this activity. Further experiments will be necessary to determine whether other sequences included within the oligonucleotide play a role in enhancer activity, to define precisely the active sequence within Box1, and eventually to characterize the putative binding factor.

Materials and methods

Plasmid constructions and mutagenesis

The chicken *Hoxb-2* reporter constructs were derived from the vector 1084 (construct #8 of Whiting *et al.*, 1991) by insertion of a series fragments into the *Bam*HI site or into a polylinker introduced into this site. The different fragments were derived from the genomic region 5′ to the ATG of the chicken *Hoxb-2* gene (see Figure 4A). Construct #1 contains a 2.5 kb *Hin*dIII–*Bam*HI fragment, inserted into the vector in the 5′ to 3′ orientation. Construct #2 contains the co-immunoprecipitated *Sau*3AI fragment A (Figure 4A), inserted into the vector in the 3′ to 5′ orientation. Construct #3 contains a 2 kb *Bam*HI fragment inserted into the vector in the 3′ to 5′ orientation. Construct #4 contains the co-immunoprecipitated *Sau*3AI fragment B inserted into the vector in the 3′ to 5′ orientation. Construct #5 is identical to plasmid #3, except that the fragment contains two single points mutations within the Krox-20 binding Site 1 (Figure 6A). Construct #6 is identical to plasmid #4, except that the Box1 motif 5′-GAAACTTT-3′ has been replaced by the sequence 5′-TCGGATCC-3′, therefore creating a new *Bam*HI site.

In the case of construct #5, the mutagenesis was performed on the 2 kb *Bam*HI fragment cloned in Bluescript using the mutagenic oligonucleotide 5′-CCCTCCCCACCGACCCGGGGA-3′ and according to the procedure of Kunkel *et al.* (1987). In the case of construct #6, the mutation was introduced by ligating two PCR products through their new *Bam*HI site. Two distinct sets of oligonucleotides were used, two of these being mutagenic: 5′-CAGGATCCGACAGGCTTCCCCCCG-TGT-3′ and 5′-TCGGATCCTGTCACTTCAAGCATCC-3′. The amplified fragments were digested with *Xba*I and *Bam*HI and *Bam*HI and *Hin*dIII respectively and subsequently ligated with the 1084 vector in the 3′ to 5′ orientation. The nucleotide sequences of the different mutagenized fragments were verified using the dideoxy-sequencing procedure with a Pharmacia kit.

In the case of the mouse *Hoxb-2* enhancer, the individual Krox-20 binding sites were mutated in the context of a 2.1 kb *Bam*HI–*Eco*RI fragment (Sham *et al.*, 1992). Oligonucleotides 5′-GTAACGTCCGC-GTCGGTGGTGGTGGAG-3′ and 5′-CCGGAGAGCTCCGACGGCT-CTGGCCTCCCATACGCATACTCGCTCTCTGTCG-3′ were used to introduce a point mutation within site 1 and points mutations within sites 2 and 3 respectively. To mutate all three sites, both oligonucleotides were used in the same mutagenesis reaction. The mutagenesis was performed using the Sculptor^{TM} *in vitro* mutagenesis system (Amersham) following the manufacturer's instructions. After sequencing on both strands, the mutated fragments were cloned into the end-filled *Spe*I site of construct BGZ40, upstream of the human β-globin promoter (Yee and Rigby, 1993).

To generate the 44 bp enhancer element, two oligonucleotides (5′-CTAGCCTG**GAAACTTT**TGTCACTTCAAGCATCC**GTGTGGGTG**-GGGAG-3′ and 5′-CTAGCTCCC**CACCCACAC**GGATGCTTGAAG-TGCAC**AAAGTTTC**CAGG-3′ corresponding to the chicken *Hoxb-2* sequence spanning the Box1 and Krox-20 sites (bold characters) were phosphorylated, annealed and ligated according to standard procedures. Trimers were purified by electrophoresis on polyacrylamide gel and inserted into the *Xba*I site of the 1084 vector.

Transgenic embryo generation and analysis

Transgenic embryos and mice were generated by microinjection of fertilized eggs from crosses between F1 hybrids (CBA×C57). PCR genotyping of embryos and mice and X-gal staining for revelation of β-galactosidase activity were performed as described previously (Whiting *et al.*, 1991). Transgenic lines (four for construct #1 and three for construct #4) with reproducible and consistent patterns of reporter gene expression were used to perform the time-course analysis of the activity of the respective regulatory elements.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed on mouse and chick embryos as described (Wilkinson and Green, 1990). The mouse *Hoxb-2* probe was obtained by transcription with T7 polymerase of a derivative of pKS containing a 2.2 kb *Eco*RI–*Not*I *Hoxb-2* genomic fragment and linearized with *Eco*RI. The chicken *Hoxb-2* probe was obtained by transcription with T7 polymerase of a pGEM4 derivative containing a 700 bp *Eco*RI–*Hin*dIII *Hoxb-2* genomic fragment and linearized with *Eco*RI.

In vitro DNA binding assays

The co-immunoprecipitation assay was performed as described previously (Sham *et al.*, 1993) using 20 μg of total protein from bacterial extracts (Chavrier *et al.*, 1990) and a 40-fold dilution of a rabbit polyclonal antiserum directed against Krox-20 (antiserum #539A, Nardelli *et al.*, 1991). The DNA fragments were generated by *Sau*3AI digestion of a 6 kb *Hin*dIII genomic fragment encompassing the chicken *Hoxb-2* gene and ^{32}P-labelled at their extremities with the Klenow enzyme. Competitions were performed with 200 ng of the double-stranded oligonucleotides W or M, whose sequences are shown below (the Krox-20 binding site and its mutated version are indicated in bold characters). W: 5′-CCGAGCTCTGTAC**GCGGGGGCG**GTTGAGGA-3′; M: 5′-CCGAGGGGAGCGC**GCGGCGGCG**GCGGAG-3′.

The gel retardation was carried out as described previously (Chavrier *et al.*, 1990; Nardelli *et al.*, 1991). The probe was end-labelled with the Klenow enzyme and the sequences of the competitor oligonucleotides were as follows (the Krox-20 binding sites are indicated in bold): CONS: 5′-CCGAGCTCTGTAC**GCGGGGGCG**GTTGAGGA-3′; Site 1: 5′-CCCTCCC**CACCCACAC**GGAT-3′; Site 1*: 5′-CCCTCCC**CACCG-ACCC**GGAT-3′.

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References

Aparicio,S., Morrison,A., Gould,A., Gilthorpe,J., Chaudhuri,C., Rigby,P., Krumlauf,R. and Brenner,S. (1995) Detecting conserved regulatory elements with the model genome of the Japanese puffer fish, *Fugu rubripes*. *Proc. Natl Acad. Sci. USA*, **92**, 1684–1688.

Awgulewitsch,A. and Jacobs,D. (1992) Deformed autoregulatory element from *Drosophila* functions in a conserved manner in transgenic mice. *Nature*, **358**, 341–344.

Becker,N., Seitanidou,T., Murphy,P., Mattei,M.G., Topilko,P., Nieto, M.A., Wilkinson,D.G., Charnay,P. and Gilardi-Hebenstreit,P. (1994) Several receptor tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. *Mech. Dev.*, **47**, 3–17.

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

Birgbauer,E., Sechrist,J., Bronner-Fraser,M. and Fraser,S. (1995) Rhombomeric origin and rostrocaudal reassortment of neural crest cells revealed by intravital microscopy. *Development*, **121**, 935–945.

Carpenter,E.M., Goddard,J.M., Chisaka,O., Manley,N.R. and Capecchi, M.R. (1993) Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. *Development*, **118**, 1063–1075.

Chavrier,P., Zerial,M., Lemaire,P., Almendral,J., Bravo,R. and Charnay,P. (1988) A gene encoding a protein with zinc fingers is activated during G_0/G_1 transition in cultured cells. *EMBO J.*, **7**, 29–35.

Chavrier,P., Vesque,C., Galliot,B., Vigneron,M., Dolle,P., Duboule,D. and Charnay,P. (1990) The segment-specific gene Krox-20 encodes a transcription factor with binding sites in the promoter region of the Hox-1.4 gene. *EMBO J.*, **9**, 1209–1218.

Clarke,J.D. and Lumsden,A. (1993) Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development*, **118**, 151–162.

Cook,M. *et al.* (1995) Expression of the zinc-finger gene PLZF at rhombomere boundaries in the vertebrate hindbrain. *Proc. Natl Acad. Sci. USA*, **92**, 2249–2253.

Cordes,S.P. and Barsh,G.S. (1994) The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. *Cell*, **79**, 1025–1034.

Dolle,P., Lufkin,T., Krumlauf,R., Mark,M., Duboule,D. and Chambon,P. (1993) Local alterations of Krox-20 and Hox gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null Hoxa-1 (Hox-1.6) mutant embryos. *Proc. Natl Acad. Sci. USA*, **90**, 7666–7670.

Frasch,M., Chen,X. and Lufkin,T. (1995) Evolutionary-conserved enhancers direct region-specific expression of murine Hoxa-1 and Hoxa-2 loci in both mice and *Drosophila*. *Development*, **121**, 957–974.

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

Garcia-Fernandez,J. and Holland,P.W.H. (1994) Archetypal organization of the amphioxus Hox gene cluster. *Nature*, **370**, 563–566.

Gilardi-Hebenstreit,P., Nieto,M.A., Frain,M., Mattei,M.G., Chestier,A., Wilkinson,D.G. and Charnay,P. (1992) An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. *Oncogene*, **7**, 2499–2506.

Heyman,I., Faissner,A. and Lumsden,A. (1995) Cell and matrix specialization of rhombomere boundaries. *Developmental Dynamics*, **204**, 301–315.

Keynes,R. and Lumsden,A. (1990) Segmentation and the origin of regional diversity in the vertebrate central nervous system. *Neuron*, **4**, 1–9.

Krumlauf,R. (1994) Hox genes in vertebrate development. *Cell*, **78**, 191–201.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.

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

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.

Lundin,M., Olof Nehlin,J. and Ronne,H. (1994) Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. *Mol. Cell. Biol.*, **xx**, 1979–1985.

Maden,M., Hunt,P., Eriksson,U., Kuroiwa,A., Krumlauf,R. and Summerbell,D. (1991) Retinoic acid-binding protein, rhombomeres and the neural crest. *Development*, **111**, 35–43.

Maden,M., Horton,C., Graham,A., Leonard,L., Pizzey,J., Siegenthaler,G., Lumsden,A. and Eriksson,U. (1992) Domains of cellular retinoic acid-binding protein I (CRABP I) expression in the hindbrain and neural crest of the mouse embryo. *Mech. Dev.*, **37**, 13–23.

Mahmood,R., Keifer,P., Guthrie,S., Dickson,C. and Mason,I. (1995) Multiple roles for *Fgf-3* during cranial neural development in the chicken. *Development*, **121**, 1399–1410.

Malicki,J., Cianetti,L.C., Peschle,C.P. and McGinnis,W. (1992) A human HOX4B regulatory element provides head-specific expression in *Drosophila* embryos. *Nature*, **358**, 345–347.

Mark,M., Lufkin,T., Vonesch,J.L., Ruberte,E., Olivo,J.C., Dolle,P., Gorry,P., Lumsden,A. and Chambon,P. (1993) Two rhombomeres are altered in Hoxa-1 mutant mice. *Development*, **119**, 319–338.

Marshall,H., Studer,M., Popperl,H., Aparicio,S., Kuroiwa,A., Brenner,S. and Krumlauf,R. (1994) A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature*, **370**, 567–571.

Morrison,A., Chaudhuri,C., Ariza-McNaughton,L., Muchamore,I., Kuroiwa,A. and Krumlauf,R. (1995) Comparative analysis of chicken *Hoxb-4* regulation in transgenic mice. *Mech. Dev.*, **53**, 47–59.

Morriss-Kay,G. (1991) Retinoic acid, neural crest, and cranio-facial development. *Semin. Dev. Biol.*, **2**, 211–218.

Murphy,P., Davidson,D.R. and Hill,R.E. (1989) Segment-specific expression of a homoeobox-containing gene in the mouse hindbrain. *Nature*, **341**, 156–159.

Nardelli,J., Gibson,T.J., Vesque,C. and Charnay,P. (1991) Base sequence discrimination by zinc-finger DNA-binding domains. *Nature*, **349**, 175–178.

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.

Nonchev,S. *et al.* (1996a) Segmental expression of *Hoxa-2* in the hindbrain is directly regulated by Krox-20. *Development*, **122**, 543–554.

Nonchev,S. *et al.* (1996b) The conserved role of *Krox-20* in directing *Hox* gene expression during vertebrate hindbrain segmentation. *Proc. Natl Acad. Sci. USA*, in press.

Pavletich,N.P. and Pabo,C.O. (1991) Zinc finger-DNA recognition: crystal structure of a Zif268–DNA complex at 2.1 Å. *Science*, **252**, 809–817.

Popperl,H., Bienz,M., Studer,M., Chan,S.K., Aparicio,S., Brenner,S., Mann,R.S. and Krumlauf,R. (1995) Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. *Cell*, **81**, 1031–1042.

Ruberte,E., Dolle,P., Chambon,P. and Morriss-Kay,G. (1991) Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development*, **111**, 45–60.

Ruberte,E., Friederich,V., Morriss-Kay,G. and Chambon,P. (1992) Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. *Development*, **115**, 973–987.

Russo,M.W., Sevetson,B.R. and Milbrandt,J. (1995) Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc. Natl Acad. Sci. USA*, **92**, 6873–6877.

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.

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.

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

Sham,M.H., Hunt,P., Nonchev,S., Papalopulu,N., Graham,A., Boncinelli,E. and Krumlauf,R. (1992) Analysis of the murine Hox-2.7 gene: conserved alternative transcripts with differential distributions in the nervous system and the potential for shared regulatory regions. *EMBO J.*, **11**, 1825–1836.

Sham,M.H. *et al.* (1993) The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell*, **72**, 183–196.

Studer,M., Popperl,H., Marshall,H., Kuroiwa,A. and Krumlauf,R. (1994) Role of a conserved retinoic acid response element in rhombomere restriction of Hoxb-1. *Science*, **265**, 1728–1732.

Sundin,O.H. and Eichele,G. (1990) A homeo domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.*, **4**, 1267–1276.

Swiatek,P.J. and Gridley,T. (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. *Genes Dev.*, **7**, 2071–2084.

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.

Vieille-Grosjean,I. and Huber,P. (1995) Transcription factor GATA-1 regulates human HOXB2 gene expression in erythroid cells. *J. Biol. Chem.*, **270**, 4544–4550.

Whiting,J., Marshall,H., Cook,M., Krumlauf,R., Rigby,P.W., Stott,D. and Allemann,R.K. (1991) Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. *Genes Dev.*, **5**, 2048–2059.

Wilkinson,D.G. (1990) Segmental gene expression in the developing mouse hindbrain. *Semin. Dev. Biol.*, **1**, 127–134.

Wilkinson,D.G. (1993) Molecular mechanisms of segmental patterning in the vertebrate hindbrain. *Perspect. Dev. Neurobiol.*, **1**, 117–125.

Wilkinson,D.G. and Green,J. (1990) In situ hybridization and three-dimensional reconstruction of serial sections. In Copp,A.J. and Cockcroft,D.L. (eds), *Postimplantation Mouse Embryos: A Practical Approach*. IRL Press, Oxford, pp. 155–171.

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

Wilkinson,D.G., Bhatt,S., Cook,M., Boncinelli,E. and Krumlauf,R. (1989b) Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. *Nature*, **341**, 405–409.

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.

Zimmer,A. and Zimmer,A. (1992) Induction of a RAR beta 2-lacZ transgene by retinoic acid reflects the neuromeric organization of the central nervous system. *Development*, **116**, 977–983.

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