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Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of *Hoxb4* expression

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SUMMARY

Hox genes are key determinants of anteroposterior patterning of animal embryos, and spatially restricted expression of these genes is crucial to this function. In this study, we demonstrate that expression of *Hoxb4* in the paraxial mesoderm of the mouse embryo is transcriptionally regulated in several distinct phases, and that multiple regulatory elements interact to maintain the complete expression domain throughout embryonic development. An enhancer located within the intron of the gene (region C) is sufficient for appropriate temporal activation of expression and the establishment of the correct anterior boundary in the paraxial mesoderm (somite 6/7). However, the *Hoxb4* promoter is required to maintain this expression beyond 8.5 dpc. In addition, sequences within the 3′ untranslated region (region B) are necessary specifically to maintain expression in somite 7 from 9.0 dpc onwards. Neither the promoter nor region B can direct somitic expression independently, indicating that the interaction of regulatory elements is crucial for the maintenance of the paraxial mesoderm domain of *Hoxb4* expression. We further report that the domain of *Hoxb4* expression is restricted by regulating transcript stability in the paraxial mesoderm and by selective translation and/or degradation of protein in the neural tube. Moreover, the absence of *Hoxb4* 3′-untranslated sequences from transgene transcripts leads to inappropriate expression of some *Hoxb4* transgenes in posterior somites, indicating that there are sequences within region B that are important for both transcriptional and post-transcriptional regulation.

Key words: *Hoxb4*, Paraxial mesoderm, Anterior boundary, Maintenance, Transcription, RNA stability, Translation, Mouse

INTRODUCTION

The Hox genes are a highly conserved gene family present in all animal phyla studied (Ferrier and Holland, 2001). They encode homeodomain-containing transcription factors that specify regional identities along the anteroposterior (AP) axis of the developing embryo. Crucial to this function is the spatially restricted expression of these genes, and particularly the formation of distinct anterior boundaries. Hox genes are organised in genomic clusters, although the number and structure of clusters have diverged significantly along different evolutionary lineages (Amores et al., 1998; de Rosa et al., 1999). Remarkably, the physical order of genes in a cluster corresponds both to the temporal order in which they are activated and to the anterior extents of their expression, a phenomenon known as colinearity. This feature is conserved even between the highly diverged Hox clusters of *Drosophila melanogaster* and the mouse, the two species in which the structure, function and regulation of Hox genes have been most intensively studied.

In flies, the establishment and maintenance of expression domains are mechanistically distinct events. These domains are initially defined by the regulatory cascade of gap, pair-rule and segmentation genes that also determines the segmental structure of the embryo (Jack and McGinnis, 1990). However, the segmentation genes are only transiently expressed during early embryonic development, and Hox gene domains are subsequently refined and maintained by auto- and cross-regulatory interactions between these genes (Miller et al., 2001). Moreover, genes of the Polycomb (Pc) and trithorax (trx) families are required for the maintenance of transcriptionally silent or active states of Hox genes, respectively (Kennison, 1995). The precise function of the products of Pc and trx genes has not yet been elucidated but growing evidence indicates that they are involved in modifying chromatin structure to maintain transcriptionally repressive or permissive environments (Petruk et al., 2001; Tie et al., 2001).

Studies of the regulation of murine anterior Hox genes using randomly-integrated transgenes have revealed distinct activation and maintenance phases similar to those in *Drosophila*. One of the best characterised examples is that of *Hoxb1*. This gene is initially expressed in the neural tube with

an anterior limit at the boundary between rhombomeres 3 and 4. Subsequently, expression regresses and is lost from the hindbrain, with the exception of rhombomere 4 (r4) in which high levels are maintained. Early neural expression of *Hoxb1* is controlled by retinoic acid through a response element located 3′ to the gene (Marshall et al., 1994; Studer et al., 1998), whereas maintenance of r4 expression is dependent on auto-regulation and cross-regulation by Hoxa1, in association with the cofactor Pbx1 (Pöpperl et al., 1995; Studer et al., 1998). Separate early and late phases of neural expression have been identified for several other Hox genes that have anterior boundaries in the hindbrain (Gould et al., 1997; Gould et al., 1998; Maconochie et al., 1997; Manzanares et al., 2001), but little is yet known about whether equivalent phases of expression occur in other tissues. Although many transgenic studies of more posterior Hox genes have been performed, there are currently few examples of separate enhancers that control early and late phases of expression. Such elements have been identified for *Hoxc8* (Bradshaw et al., 1996), although the molecular details of activation and maintenance have not yet been elucidated. Interestingly, Oosterveen et al. have recently identified a single retinoic acid response element that controls the late phase of expression of several genes (*Hoxb5*, *Hoxb6* and *Hoxb8*) in the posterior hindbrain (Oosterveen et al., 2003).

Many murine homologues of Pc and trx group genes have now been identified and shown to be involved in the regulation of Hox genes (Gould, 1997). For example, in mice lacking the trx group gene *Mll*, endogenous *Hoxa7* expression is established normally but is not maintained (Yu et al., 1998). Conversely, double mutation of the Pc group genes *Mel18* and *Bmi1* leads to de-repression of several Hox genes in anterior regions of the embryo (Akasaka et al., 2001). Interestingly, some murine Pc group genes may regulate Hox genes earlier in development. Early activation of *Hoxd11* transcription is observed in mice lacking the Pc group gene *M33*, although expression is apparently normal from 9.5 dpc onwards (Bel-Vialar et al., 2000). *Hoxd4* and *Hoxd10* are similarly affected when the global repression of the Hoxd cluster is disrupted by targeted genomic deletions (Kondo and Duboule, 1999). Thus, some Pc group genes may regulate the timing of Hox gene activation and contribute to the generation of co-linearity.

Sequences located within a 7.4 kb genomic fragment including *Hoxb4* are sufficient to recapitulate the full expression pattern of this gene in transgenic mice (Whiting et al., 1991). We have shown previously that the intronic enhancer (region C) is sufficient to establish transgene expression in the paraxial mesoderm with an anterior boundary equivalent to that of *Hoxb4* but that it cannot maintain this pattern (Gilthorpe et al., 2002). We have now characterised the loss of expression more fully and show that it proceeds by a gradual regression of the anterior boundary. We demonstrate that sequences within the *Hoxb4* promoter are necessary for continuation of the expression established by region C, and that regulatory elements in the 3′ untranslated region (UTR) of *Hoxb4* (region B) are required to maintain the correct anterior boundary in the paraxial mesoderm throughout embryonic development. We show that the domain of *Hoxb4* expression is restricted by regulating transcript stability in the paraxial mesoderm, and by selective translation and/or degradation of protein in the neural tube. Furthermore, we demonstrate that inappropriate expression of some *Hoxb4* transgenes in posterior somites is a result of escape from post-transcriptional regulation and that this is attributable to the absence of *Hoxb4* 3′-UTR sequences from transgene transcripts.

MATERIALS AND METHODS

Constructs and transgenic mice

Construct CHZ has been described previously (Gilthorpe et al., 2002). All other constructs were based on a *Hoxb4* promoter-*lacZ*-SV40 polyA reporter gene [construct 8 (Whiting et al., 1991)], referred to here as b4Z. The 1.4 kb *Sal*I-*Bgl*II region C fragment of *Hoxb4* was cloned upstream or downstream of b4Z to generate constructs Cb4Z and b4ZC, respectively. Similarly, the 3kb *Sal*I-*Hin*dIII fragment of *Hoxb4*, containing regions C and B, was cloned upstream or downstream of b4Z to generate constructs CBb4Z and b4ZCB, respectively. Specific details of all cloning steps are available on request. Transgenic mice carrying construct b4ZCBpA [construct 6 (Whiting et al., 1991)] were provided by R. Krumlauf (NIMR, Mill Hill). The production, PCR diagnosis and whole-mount staining of transgenic mice were performed as described previously (Gilthorpe and Rigby, 1999; Summerbell et al., 2000). In some cases, X-gal stained embryos were counter-stained in 0.1% aqueous acid fuchsin to facilitate precise identification of anterior somitic boundaries.

Whole-mount in situ hybridisation and immunostaining

The *Hoxb4* probe was provided by R. Krumlauf (NIMR, Mill Hill). The *lacZ* probe has been described elsewhere (Teboul et al., 2002). In situ hybridisation was performed using an InsituPro robot (Intavis, Bergisch-Gladbach, Germany) essentially as previously described (Summerbell et al., 2000), but substituting Red-Phos (Research Organics, Cleveland, Ohio) for BCIP in the staining solution. The anti-Hoxb4 antibody was provided by A. Gould (NIMR, Mill Hill), and immunostaining was performed as previously described (Gould et al., 1997). For sectioning, embryos were embedded in 2% (w/v) agarose. 70 μm sections were cut using a vibrotome.

RESULTS

Failure of construct CHZ to maintain expression is manifest as a gradual regression of anterior boundaries

We have previously analysed the intronic enhancer (region C) of the mouse *Hoxb4* gene using a construct in which region C is positioned upstream of the *hsp68* promoter-*lacZ* reporter gene (Gilthorpe et al., 2002). We showed that this construct (CHZ; Fig.1B) is able to establish expression in the paraxial mesoderm with an anterior limit identical to that of *Hoxb4*, but that it is unable to maintain this boundary (Gilthorpe et al., 2002). To further characterise this change in expression, we analysed embryos from a transgenic line carrying CHZ at various times between 8.5 and 9.5 dpc. At 8.5 dpc, CHZ was expressed in the paraxial mesoderm with an anterior limit at the level of somite (so) 6/7 and in the neural tube up to the spinal cord/hindbrain boundary (Fig. 2A) (Gilthorpe et al., 2002). At 8.75 dpc, the rostral limits of CHZ expression had not changed but it was obvious that the anteriormost regions of both neural and mesodermal expression were considerably weaker (Fig. 2B). By 9.0 dpc the anterior limit of CHZ expression in the paraxial mesoderm was clearly posteriorised, lying at the level of so8/9, and by 9.25 dpc it had receded further to so10/11 (Fig. 2C,D). During this period, the neural

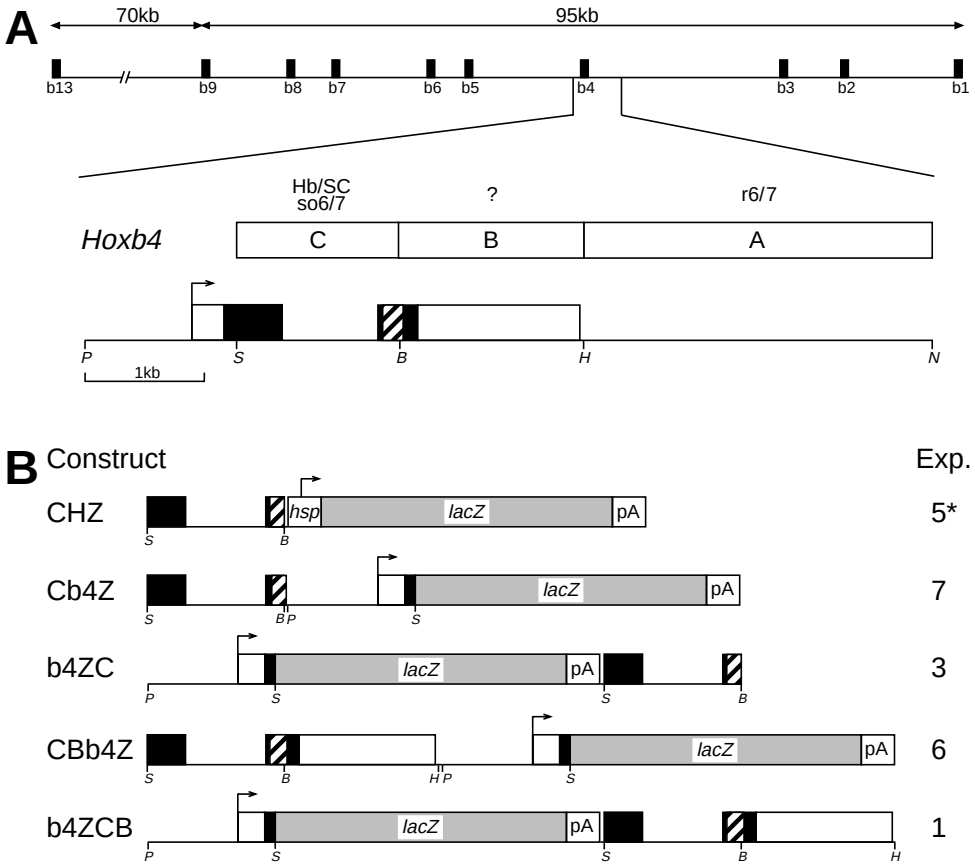

Fig. 1. (A) Details of the *Hoxb4* locus are shown along with an indication of its position within the Hoxb cluster. The first transcription start site (P1) (Gutman et al., 1994) is indicated with an arrow. Black and white boxes represent coding and untranslated sequences, respectively. The hatched region within exon 2 indicates the position of the homeobox. The positions and specificities of known regulatory regions (A, B, C) are indicated above the locus (Whiting et al., 1991). The ambiguous function of region B is indicated by a question mark. *B*, *Bgl*II; Hb, hindbrain; *H*; *Hin*dIII; *N*, *Nco*I; *P*, *Pst*I; r, rhombomere; *S*, *Sal*I; SC, spinal cord; so, somite. (B) Constructs used in this study. Fragments of the *Hoxb4* locus are represented as in A. *hsp* and pA indicate the *hsp68* promoter and SV40 polyadenylation signal, respectively. Exp denotes the total number of transgenic F_0 embryos and lines showing a consistent pattern of X-gal staining for each construct. Asterisk indicates that analysis of construct CHZ has been reported previously (Gilthorpe et al., 2002) and the data are given here for comparison.

boundary also regressed and lay alongside so5/6 at 9.0 dpc, and so6/7 at 9.25 dpc (Fig. 2C,D). Between 9.25 and 9.5 dpc, the anterior boundary of CHZ expression remained constant in the neural tube but receded still further in the paraxial mesoderm to so13/14 (Fig. 2E). These rostral limits were maintained until 12 dpc (Gilthorpe et al., 2002). These results demonstrate that the failure of CHZ to maintain rostral limits of expression from 8.5 dpc onwards is manifest as a gradual regression of anterior boundaries in both the somitic mesoderm and the neural tube.

Hoxb4 promoter-region C interaction maintains early expression but not the anterior somitic boundary

Previous studies on the regulation of *Hoxb4* identified region C as the only enhancer responsible for setting the proper limit of *Hoxb4* expression in the paraxial mesoderm (Whiting et al., 1991). Therefore, we reasoned that the failure of CHZ to maintain the anterior boundary of somitic expression was the result of a requirement for interaction between region C and the *Hoxb4* promoter. The promoter itself does not contain any relevant spatially-specific regulatory elements, as an *Hoxb4*

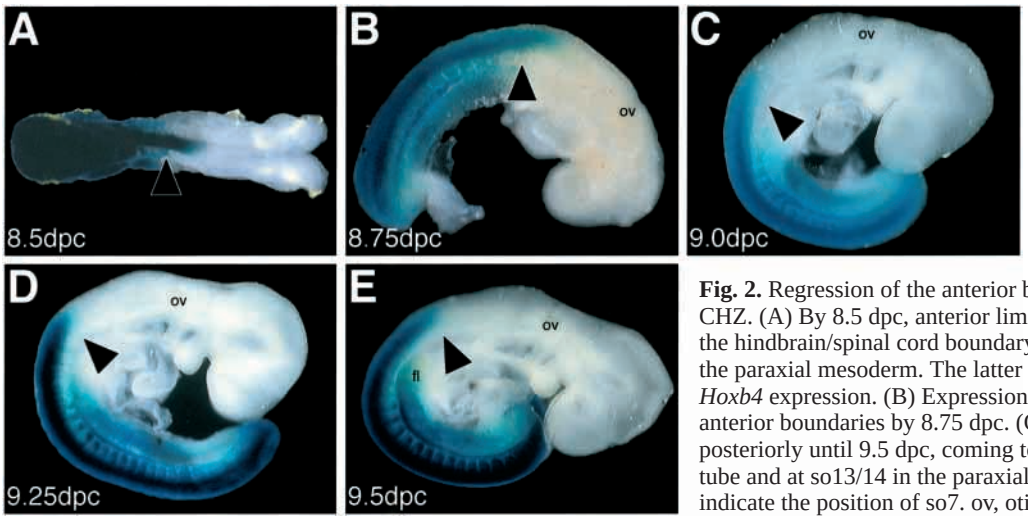

Fig. 2. Regression of the anterior boundaries of expression of construct CHZ. (A) By 8.5 dpc, anterior limits of expression were established at the hindbrain/spinal cord boundary in the neural tube, and at so6/7 in the paraxial mesoderm. The latter corresponds to the boundary of *Hoxb4* expression. (B) Expression was noticeably weaker at the anterior boundaries by 8.75 dpc. (C-E) Boundaries continued to shift posteriorly until 9.5 dpc, coming to rest alongside so6/7 in the neural tube and at so13/14 in the paraxial mesoderm. Black arrowheads indicate the position of so7. ov, otic vesicle; fl, forelimb bud.

promoter-*lacZ* reporter gene (b4Z) does not recapitulate any aspect of the normal *Hoxb4* expression pattern, although it consistently directs ectopic expression in the dorsal midbrain (Whiting et al., 1991).

We cloned region C upstream of b4Z to produce construct Cb4Z (Fig. 1B), and analysed expression in transgenic mice. At 10.5 dpc, Cb4Z was expressed in more anterior somites than CHZ, as expected (Fig. 3C-F). However, comparison with the distribution of the Hoxb4 protein (Fig. 3A,B) indicated that Cb4Z did not specify the correct boundary of expression in the somites at this stage of development. Moreover, a useful internal control provided by staining of the dorsal root ganglia (drg) confirmed this. The second drg (drg2) is identifiable at 10.5 dpc by its characteristic bipartite structure and by the fact that it is the most anterior drg visible, as drg1 degenerates to form a bar-like structure (Spörle and Schughart, 1997). The spinal nerve originating from drg2 passes through the rostral half of the sclerotome of so7 (Spörle and Schughart, 1997). It was clear that the spinal nerve emanating from drg2 did not pass through a somite in which Cb4Z was expressed (Fig. 3E,F**)**. However, the next somite caudally was expressing *lacZ*, although weakly, demonstrating that Cb4Z specifies a boundary of expression in the paraxial mesoderm at so7/8 at this stage. This pattern was seen in all F_0 embryos in which the transgene was expressed, although expression was often weak, not only in so8 but throughout the cervical region. Expression in the neural tube was also more anterior with Cb4Z than CHZ (Fig. 3C-F). The position of the neural boundary relative to drg2 indicates that Cb4Z was expressed up to the boundary of the spinal cord and hindbrain. Therefore, Cb4Z had maintained the neural boundary of expression that is established by region C at 8.5 dpc.

The inability of Cb4Z to specify the correct somitic boundary could have been caused by the incorrect position of region C and the promoter relative to each other, in comparison with their normal genomic arrangement. However, we observed an identical expression pattern in three F_0-transgenic embryos carrying a construct in which region C was cloned 3′ of the *Hoxb4* promoter-*lacZ* reporter gene (construct b4ZC; Fig.1B), which suggests that this is the genuine limit of region C activity in combination with the *Hoxb4* promoter (Fig. 3G,H).

Region B is required for maintenance of the somitic boundary

From these experiments it is clear that the Cb4Z construct lacks regulatory elements required to fully recapitulate the somitic expression of *Hoxb4*. All the enhancer elements necessary to recapitulate the full expression pattern of *Hoxb4* lie 3′ of its transcription start sites (Fig. 1A) (Whiting et al., 1991). Region A controls the proper boundary of expression in the neural tube and does not specify any mesodermal expression. Although only lung-specific enhancer activity has previously been ascribed to region B, constructs containing regions C and B are able to specify the so6/7 boundary (Whiting et al., 1991). Therefore, we cloned a DNA fragment consisting of regions C and B upstream of the b4Z reporter gene to test whether region B could alter the observed expression pattern (construct CBb4Z; Fig. 1B). At 10.5 dpc, expression of CBb4Z clearly extended one somite more rostrally than that of Cb4Z or b4ZC (Fig. 3I,J). Moreover, drg2 is again easily identifiable, and the nerve from drg2 was clearly passing through the most rostral-stained somite, identifying it as so7. This pattern was observed

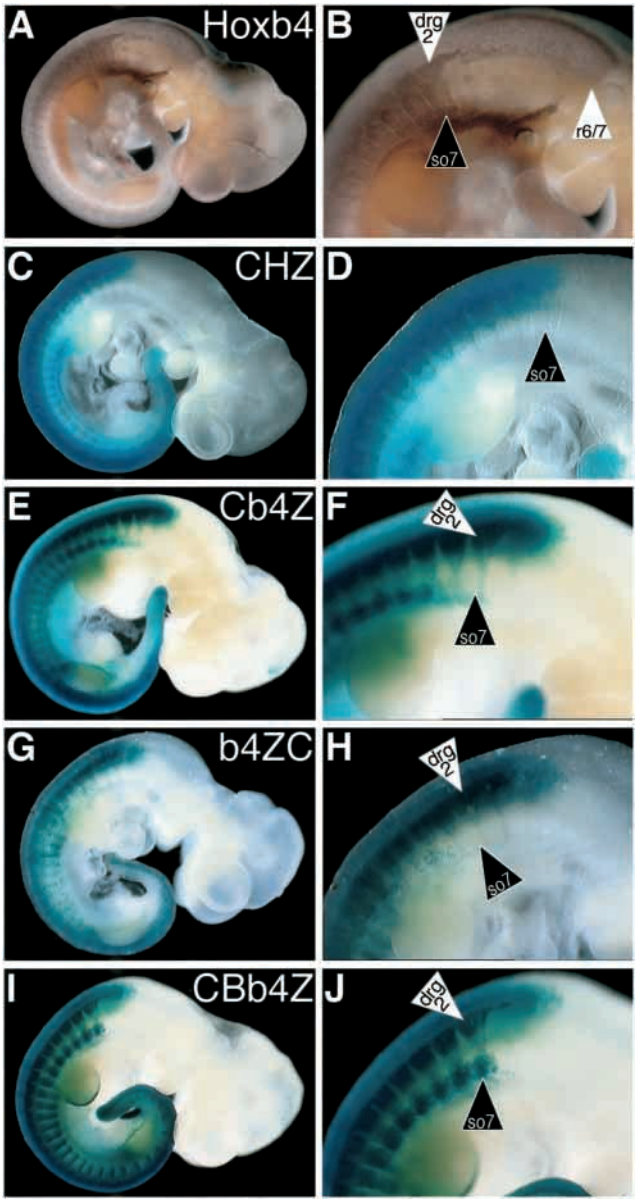

Fig. 3. Multiple regulatory elements are required to determine the anterior boundary of expression in the paraxial mesoderm. (A,B) Whole-mount immunostaining of a 10.5 dpc embryo, using an anti-Hoxb4 antibody. Hoxb4 protein was expressed up to the r6/7 boundary in the neural tube and up to so7 in the paraxial mesoderm. So7 is easily identified at this stage as it lies alongside the second dorsal root ganglion (drg2). Drg2 is the most anterior drg visible at this stage as drg1 has degenerated. (C-J) X-gal staining of 10.5 dpc transgenic embryos. (C,D) Construct CHZ was expressed only up to so14 at this stage. (E,F) Construct Cb4Z had an anterior boundary at so8, indicating that the *Hoxb4* promoter can maintain much of the somitic expression established by region C but not the correct anterior boundary. (G,H) Construct b4ZC had identical boundaries of expression to Cb4Z, demonstrating that the position of region C relative to the promoter does not affect the expression pattern. (I,J) Construct CBb4Z did recapitulate the somitic boundary of *Hoxb4* expression, identifying a requirement for region B in the regulation of *Hoxb4* in the paraxial mesoderm.

in all embryos expressing the CBb4Z construct, and also in a single embryo carrying a construct in which regions C and B were cloned 3′ of the *Hoxb4* promoter-*lacZ* reporter (Fig. 1B; data not shown). Interestingly, the anterior limit of neural tube expression was identical with Cb4Z and CBb4Z (Fig. 3E,F,I,J), which indicates that region B is involved in regulating mesodermal but not neural expression. In addition, both constructs gave staining in the dorsal midbrain that is attributable to ectopic activity of the *Hoxb4* promoter (Whiting et al., 1991).

To determine whether region B can function as an independent enhancer, a construct containing only region B cloned 5′ to the b4Z reporter gene was analysed in transgenic mice. Embryos were examined between 9.5 and 11.5 dpc but only random expression was observed (n=3; data not shown). This is consistent with the results of previous attempts to identify a specific enhancer function of region B in isolation (R. Krumlauf, personal communication). Therefore, we conclude that sequences within region B represent a component of the paraxial mesoderm enhancer of *Hoxb4* that is functionally dependent on elements located within region C. This interaction is necessary for expression in so7 at 10.5 dpc.

Region B is required from 9.0 dpc onwards

As construct CHZ is able to establish the so6/7 boundary at 8.5 dpc, we reasoned that Cb4Z and CBb4Z would also recapitulate this pattern and that the single somite difference seen at 10.5 dpc must arise between these two time points. As expected, both Cb4Z and CBb4Z were expressed with rostral limits at so6/7 in the paraxial mesoderm, and at the hindbrain/spinal cord boundary in the neural tube at 8.5 dpc (Fig. 4A,B). These boundaries were maintained with both constructs until 9.0 dpc (Fig. 4C,D). However, by 9.5 dpc the boundary in the paraxial mesoderm had shifted one somite caudally with Cb4Z, compared with CBb4Z, although the anterior limit of neural expression of both constructs remained identical (Fig. 4E,F). To confirm this subtle shift in the expression boundary, transgenic embryos previously stained for β-galactosidase activity were treated with the cytoplasmic stain acid fuchsin in order to visualise somites in which reporter genes were not expressed. At 9.0 dpc there was a two-somite gap between the limits of reporter gene expression in the paraxial mesoderm and neural tube for both Cb4Z and CBb4Z (Fig. 4G,H). However, at 9.5 dpc there was a clear three-somite difference between the mesodermal and neural boundaries of Cb4Z expression (Fig. 4I), whereas the gap remained two somites for CBb4Z (Fig. 4J).

These results demonstrate that elements within region B have a very specific role in maintaining expression in the anteriormost somite (so7) of the *Hoxb4* domain from 9.0 d*pc* onwards. Moreover, the ability of construct Cb4Z to maintain the somitic boundary between 8.5 and 9.0 dpc further underlines the importance of the *Hoxb4* promoter in preserving the expression established by region C. Thus, we have defined three phases in the early expression of *Hoxb4* in the paraxial mesoderm that are controlled by distinct regulatory elements: (1) establishment, dependent on region C; (2) general maintenance, dependent on the promoter; and (3) specific maintenance in so7, dependent on region B.

Differential downregulation of reporter genes during later embryonic development

We examined transgenic mice carrying reporter constructs CHZ, Cb4Z and CBb4Z during later stages of development,

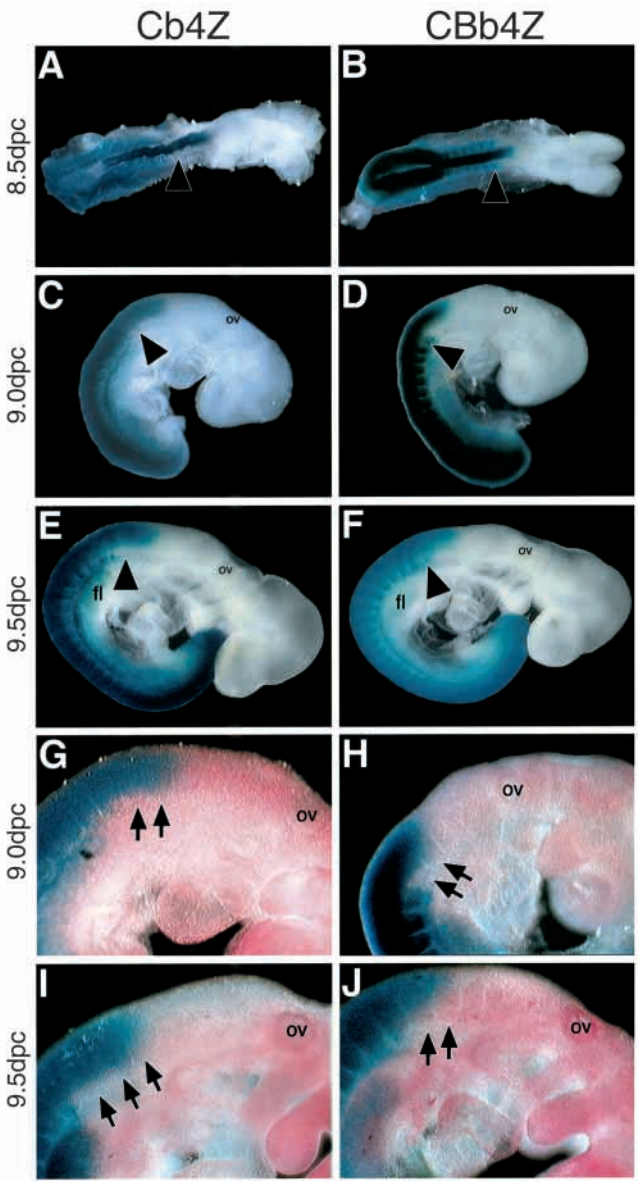

Fig. 4. Region B is required to maintain the anterior somitic boundary after 9.0 dpc. (A-F) X-gal staining of transgenic embryos. (A,B) Expression patterns for construct Cb4Z (A) and CBb4Z (B) were identical at 8.5 dpc. Both had an anterior boundary at so6/7 in the paraxial mesoderm. (C,D) Anterior boundaries of expression remained identical at 9.0 dpc. (E,F) By 9.5 dpc, the somitic boundary of Cb4Z expression shifted one segment posteriorly (E), whereas that of construct CBb4Z remains at so6/7 (F). Black arrowheads indicate the position of so7. (G-J) X-gal stained transgenic embryos counterstained with acid fuchsin. (G,H) At 9.0 dpc, two somites could be detected between the anterior boundaries of X-gal staining in the paraxial mesoderm and neural tube (black arrows) for constructs Cb4Z and CBb4Z. (I,J) By 9.5 dpc, the gap had extended to three somites with construct Cb4Z (I) but remained at two with CBb4Z (J), confirming the timing of the posterior shift of the expression boundary.

and observed further changes in expression patterns, notably two distinct events of downregulation. First, expression of all three constructs in the posterior mesoderm was downregulated between 11.0 and 12.5 dpc (Fig. 5A-I). For Cb4Z and CBb4Z this resulted in restriction of expression to the cervical region of the embryo, although the posterior boundary was not sharply defined for either construct (Fig. 5H,I). By contrast, expression of CHZ was lost from the cervical somites between 8.5 and 9.5 dpc (Fig. 2) and, therefore, downregulation in the posterior region at this stage completely eliminated mesodermal expression of this construct (Fig. 5G).

Second, the remaining neural expression of CHZ is downregulated after 12.5 dpc such that only weak patches of β-galactosidase activity were detected at 13.5 dpc (Fig. 5J). By contrast, expression of both Cb4Z and CBb4Z remained strong with distinct anterior boundaries (Fig. 5K,L). Moreover, the difference in the anterior limits of mesodermal expression, first observed at 9.5 dpc, was maintained throughout the developmental period analysed. The significance of the downregulation of transgene expression in the posterior mesoderm is unclear given that this domain does not reflect expression of the endogenous *Hoxb4* gene (see below). However, the differential downregulation of CHZ between 12.5 and 13.5 dpc, compared with Cb4Z and CBb4Z, suggests that the *Hoxb4* promoter is required to maintain late phases of expression, as well as to maintain the early expression established by region C.

CBb4Z was also expressed in other tissues in which both Cb4Z and CHZ were not. By 12.5 dpc, expression was visible in the follicles of the vibrissae in the snout and in the primordia of the mammary glands (Fig. 5I). 24 hours later, additional expression was detected in the follicles of the tactile hairs of the face (Fig. 5L). Moreover, diffuse staining was seen in the skin throughout the trunk region (Fig. 5L). This preceded the expression in the dermal placodes of the pelage hair follicles (data not shown), which has been reported previously for *Hoxb4* transgenes (Whiting et al., 1991).

Stabilisation of transcripts underlies inappropriate transgene expression in posterior somites

Strong reporter gene expression in posterior somites was characteristic of all the constructs used in this study (Fig. 3). However, this is not a domain of expression of either *Hoxb4* mRNA or protein (Fig. 6). Whole-mount in situ hybridisation detected *Hoxb4* transcripts in so7 to so13 of 10.5 dpc embryos but not in more posterior somites (Fig. 6A), and an identical distribution of Hoxb4 protein was revealed by whole-mount immunostaining (Fig. 6E). These observations were confirmed by cutting sections of embryos (Fig. 6I-L). In transverse sections at the level of the forelimb bud, *Hoxb4* transcripts were detected in all tissue surrounding the neural tube with

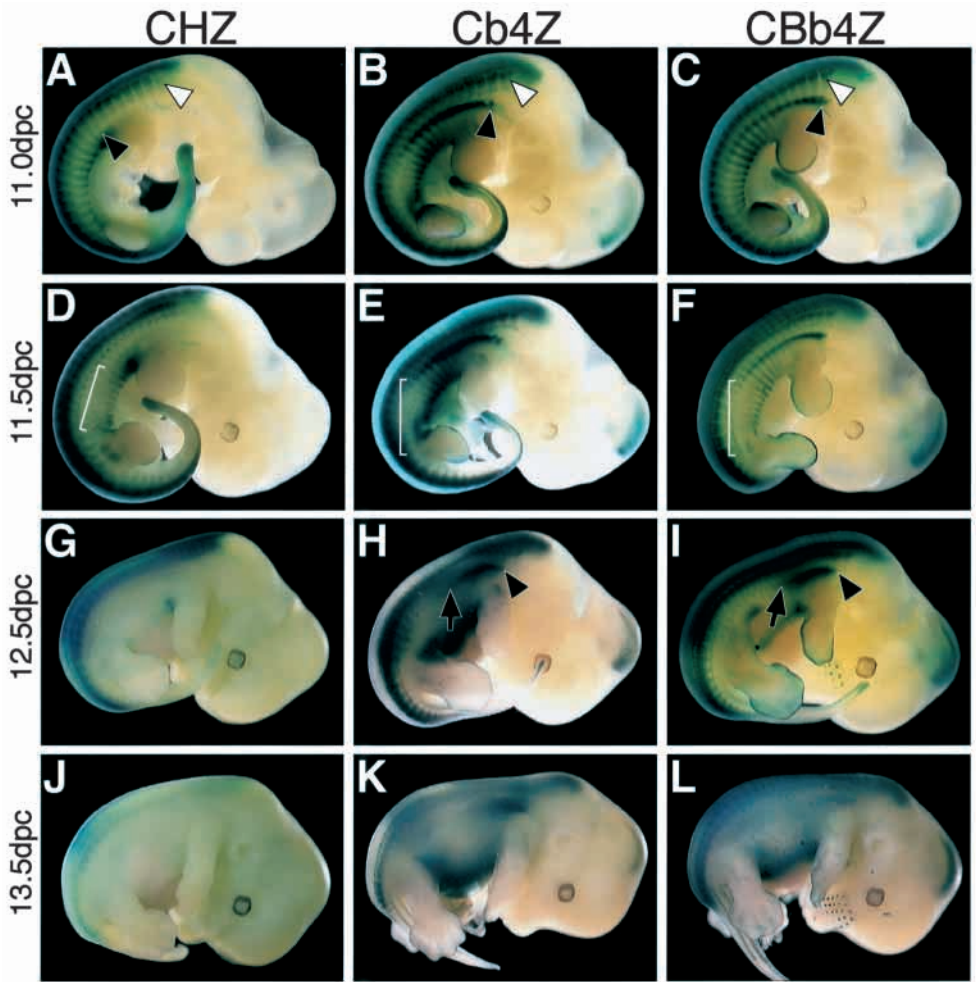

Fig. 5. Downregulation of transgene expression in later development. (A-C) At 11.0 dpc, the anterior boundaries and overall expression patterns of constructs CHZ (A), Cb4Z (B) and CBb4Z (C) were identical to those seen at earlier stages of development. (D-F) By 11.5 dpc, downregulation of expression had commenced in the posterior mesoderm with all three constructs (white brackets). (G-I) Downregulation was complete by 12.5 dpc. Mesodermal expression was absent from embryos carrying construct CHZ (G) and was restricted to cervical regions for constructs Cb4Z (H) and CBb4Z (I). (J-L) At 13.5 dpc, neural expression of CHZ was also downregulated (J), whereas neural and mesodermal expression was maintained by constructs Cb4Z (K) and CBb4Z (L). White arrowheads indicate drg2. Black arrowheads and arrows indicate the anterior and posterior boundaries of mesodermal expression, respectively.

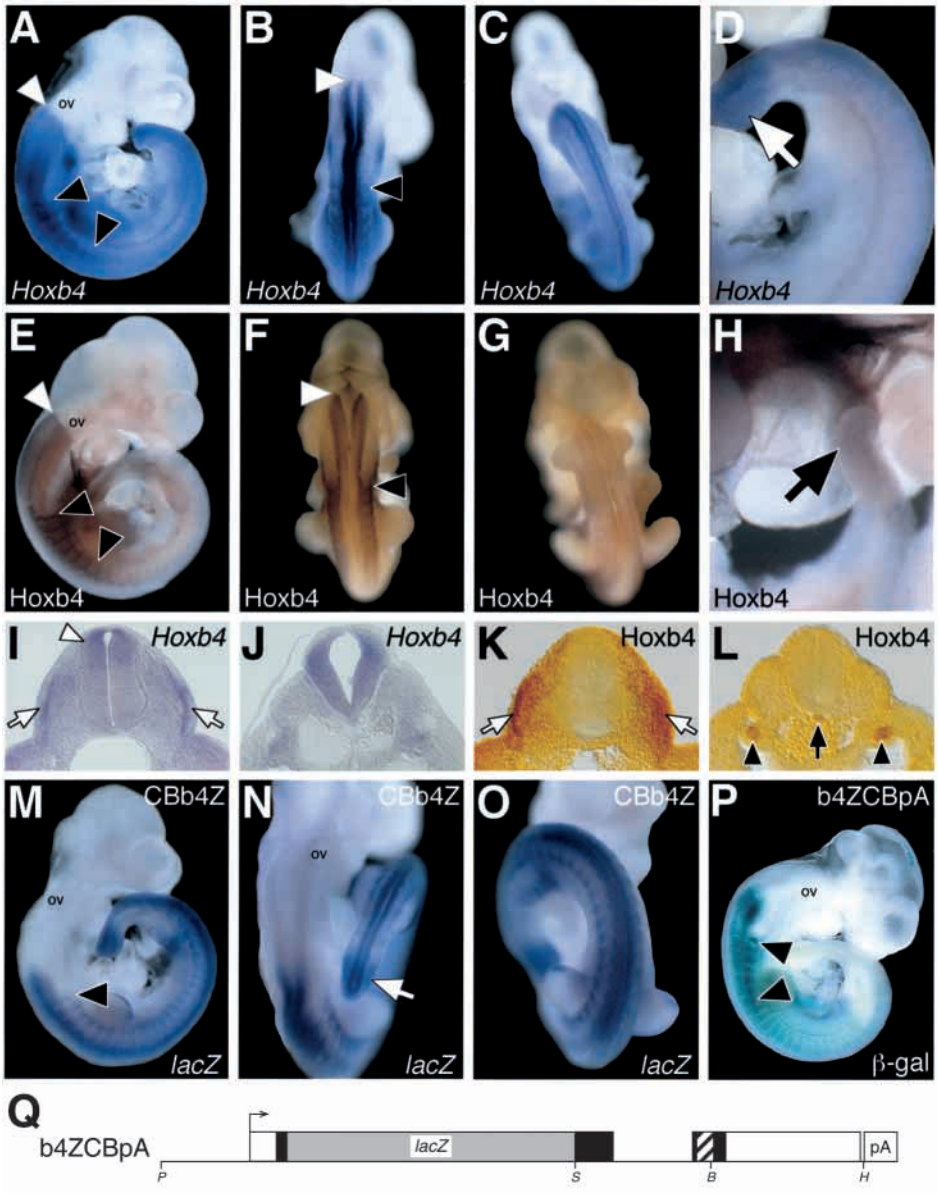

Fig. 6. Post-transcriptional regulation of *Hoxb4*. (A-D) In situ hybridisation for *Hoxb4*. Transcripts were present in so7-13 (black arrowheads) but not in more posterior somites. By contrast, *Hoxb4* was expressed throughout the neural tube posterior to the r6/7 boundary (white arrowhead). (D) Strong staining was seen in the tailbud (white arrow). (E-H) Whole-mount immunostaining for Hoxb4. Protein was detected in so7-13 and in the posterior hindbrain (E,F), but was absent from posterior neural tube and somites (G). (H) Protein was not expressed in the tailbud (black arrow). (I-J) Transverse section of embryos subjected to in situ hybridisation for *Hoxb4* (I,J) or immunostaining for Hoxb4 (K,L). (I) Transcripts were widely distributed at forelimb level with relatively high levels in the dorsal neural tube (white arrowhead) and dermomyotome (white arrows). (J) At posterior levels transcripts were expressed throughout the neural tube but not in the adjacent somites. (K) At forelimb level, Hoxb4 protein was not detected in the neural tube but was expressed in adjacent tissue with a high level in the dermomyotome (white arrows). (L) In the posterior embryo, protein was detectable in neither somites nor neural tube, but was seen in the notochord (black arrow) and the mesonephric ducts (black arrowheads). (M-O) In situ hybridisation of a *lacZ* probe to a transgenic embryo carrying construct CBb4Z. The pattern was identical to that seen with X-gal staining (Fig. 3I,J), with strong expression in all somites posterior to so7 (black arrowhead) and in the tailbud (white arrow). (P) X-gal staining of a 10.5 dpc transgenic embryo carrying construct b4ZCBpA. Strong staining was seen only in so7-13 (black arrowheads). (Q) Structure of construct b4ZCBpA [construct 6 (Whiting et al., 1991)]. This schematic follows the format used in Fig. 1.

noticeably higher expression in the dermomyotome (Fig. 6I). Hoxb4 protein was not detectable in the neural tube at this axial level but was distributed in other tissues in a similar pattern to transcripts, with relatively high dermomyotomal expression (Fig. 6K). At posterior levels, neither RNA nor protein were detected in somites (Fig. 6J,L).

To further investigate the misexpression of transgenes in posterior somites, we performed in situ hybridisation on transgenic mice using a probe for *lacZ* mRNA. We found that the distribution of *lacZ* mRNA was identical to that of the β-galactosidase protein, with transcripts detectable in all somites posterior to the forelimb bud, as well as in the anterior somitic and tailbud domains that are characteristic of the endogenous *Hoxb4* gene (Fig. 6M-O). This could represent ectopic transcription that is normally suppressed by sequences not present in these transgenes. However, comparison with a construct in which the genomic arrangement of the *Hoxb4* locus is maintained around the inserted *lacZ* gene makes this unlikely (Fig. 6P,Q) (Whiting et al., 1991). This construct contains identical *Hoxb4* sequences to constructs CBb4Z and b4ZCB, but more closely recapitulated the somitic expression of *Hoxb4*, with strong expression in so7-13 and little or no expression in somites posterior to the forelimb (Fig. 6P). Therefore, it is likely that the strong posterior expression resulted from the stabilisation of transcripts, which must reflect a feature that is common to the constructs we have employed here. All transgenes used in the present study contain a *lacZ*-SV40 polyA reporter gene (Fig. 1B). By contrast, the constructs used by Whiting et al. (Whiting et al., 1991) used a *lacZ* gene that was not coupled to a SV40 polyA signal; transcript termination was controlled by the polyA signal of *Hoxb4*, or by an SV40 polyA sequence cloned at the 3′ end of the construct (Fig. 6Q). Thus, these constructs retained the 3′ UTR of *Hoxb4*, and we therefore infer that the presence of this 3′ UTR is necessary to confer instability on the transcripts of transgenes and the endogenous gene in posterior somites. As region B contains the entire 3′ UTR, we conclude that this fragment contains sequences that are crucial for post-

transcriptional regulation of *Hoxb4* expression, in addition to the sequences involved in transcriptional regulation that we have identified in this study.

Translational regulation of *Hoxb4* expression in the neural tube

In contrast to the paraxial mesoderm, the distribution of transcripts and protein was not identical in the neural tube. *Hoxb4* transcripts were detected throughout the neural tube from the rhombomere 6/7 boundary to the posterior tip of the embryo, although staining was often noticeably weaker in the interlimb region (Fig. 6A-C). By contrast, Hoxb4 protein was only detectable in the posterior hindbrain and not in more posterior regions of the neural tube (Fig. 6E-G). At the level of the forelimb bud, *Hoxb4* transcripts were distributed throughout the neural tube, although expression was clearly stronger in the dorsal region (Fig. 6I). By contrast, Hoxb4 protein was not detectable in the neural tube at this axial level (Fig. 6K). At posterior levels, transcripts were detected in the neural tube but not in the adjacent somites (Fig. 6J), whereas protein was not present in either of these tissues (Fig. 6L). These differences in the distribution of *Hoxb4* transcripts and Hoxb4 protein were evident from the earliest stages we examined (8.5 dpc; data not shown) indicating that translational and/or post-translational regulation is a crucial mechanism in determining the domain of Hoxb4 function in the neural tube. In addition, strong staining was seen in the tailbud by in situ hybridisation but protein was not detectable, which indicates that Hoxb4 is also regulated at the level of translation in this region (Fig. 6D,H).

DISCUSSION

In this study we show that control of *Hoxb4* expression in the mouse embryo is complex, involving transcriptional, post-transcriptional and translational regulation. Moreover, the relative contribution of each mechanism is different for the same gene in different tissues. We demonstrate that specification of the anterior boundary of *Hoxb4* expression in the paraxial mesoderm is controlled by multiple transcriptional regulatory elements, each of which has a distinct role and acts at a particular time during development. We have shown previously that the intronic enhancer region C is sufficient to establish expression in the paraxial mesoderm with the correct anterior limit but that it cannot maintain this pattern (Gilthorpe et al., 2002). We now demonstrate that maintenance of the full expression pattern of *Hoxb4* in the somites is dependent on sequences in both the promoter and the 3′ UTR (region B). Moreover, these elements do not direct somitic expression independently. Therefore, interaction of regions C and B, and the *Hoxb4* promoter are required to specify paraxial mesoderm expression of *Hoxb4* throughout development, and the somitic enhancer is correctly defined as a fragment comprising regions C and B. The roles of each of these elements and the overall regulatory organisation of *Hoxb4* are summarised in Fig. 7A.

Region B and maintenance of the somitic boundary

Sequences within region B play a specific role in the regulation of *Hoxb4* expression in the paraxial mesoderm, i.e. in the maintenance of expression in so7 from 9.0 dpc onwards. In the absence of this fragment, the anterior boundary of reporter

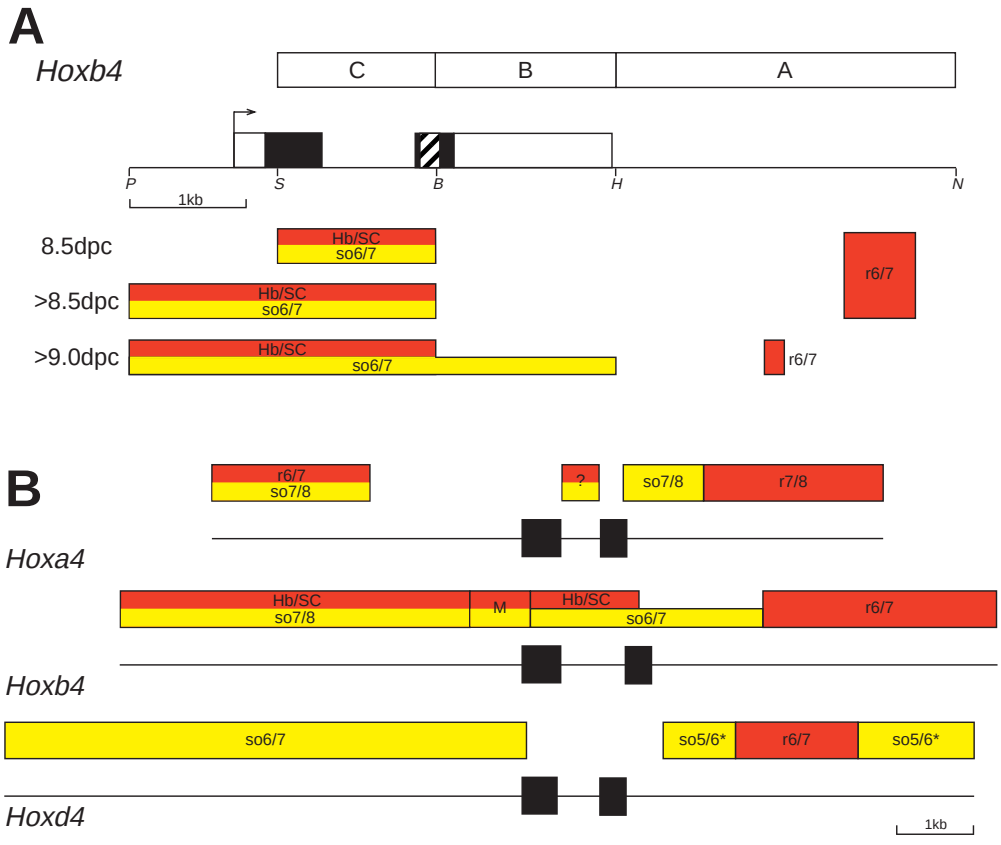

Fig. 7. Regulatory organisation of paralogous group 4 Hox genes in the mouse. (A) A revised version of Fig. 1A incorporating the data presented in this study. The spatial and temporal specificities of regulatory regions are indicated below the Hoxb4 locus. Red and yellow boxes indicate neural and paraxial mesodermal specificity, respectively. Early and late regulatory elements have been more precisely mapped within region A and are indicated accordingly (Gould et al., 1997; Gould et al., 1998). (B) Comparison of the regulatory organisation of *Hoxa4*, *Hoxb4* and *Hoxd4*. Black boxes represent the coding sequences of each gene. The question mark represents the ambiguous role of the *Hoxa4* intron (see Discussion). Asterisks indicate that enhancer activity may be located in either or both of the regions 3′ of *Hoxd4*. M, maintenance. This diagram incorporates data from Behringer et al. (Behringer et al., 1993), Keegan et al. (Keegan et al., 1997), Morrison et al. (Morrison et al., 1997), Sharpe et al. (Sharpe et al., 1998), Whiting et al. (Whiting et al., 1991) and Zhang et al. (Zhang et al., 1997).

gene expression shifts caudally, after 9.0 dpc, from so6/7 to so7/8. Interestingly, this bears a striking similarity to the regulation of *Hoxb4* in the neural tube. Proper neural expression of *Hoxb4* is controlled by the region A enhancer (Fig. 7A) (Whiting et al., 1991), and specific elements responsible for early and late phases of expression have been identified within this region (Gould et al., 1998). The early neural enhancer (ENE) is responsible for establishing the anterior boundary of expression between rhombomeres 6 and 7 by 8.5 dpc. However, between 9.0 and 9.5 dpc, expression of a reporter gene controlled by the ENE regresses to the r7/8 boundary, approximately. The late neural enhancer (LNE) also directs expression up to the r6/7 boundary, but it is only active from 9.0 dpc onwards. Therefore, it seems that 9.0 dpc represents the time at which regulation of *Hoxb4* switches from an early activation phase to a maintenance phase in both the neural tube and the paraxial mesoderm.

However, it is important to note that the molecular mechanisms of activation and maintenance are likely to be significantly different in the neural tube and paraxial mesoderm. The activation of neural expression through the ENE is directly controlled by retinoid signalling (Gould et al., 1998). Region C is sufficient to establish somitic expression of *Hoxb4*, and presumably contains all the cis-acting regulatory elements required to respond to the inductive signals that activate *Hoxb4* expression in this tissue. We have analysed region C in detail (Gilthorpe et al., 2002) and have found no evidence for direct regulation of this enhancer by retinoid signalling. The late phase of *Hoxb4* expression in the neural tube is controlled by autoregulation, and by crossregulation by other Hox proteins (Gould et al., 1997). As the LNE is a Hox-responsive element, it is active in isolation from the ENE. By contrast, region B does not function as an independent enhancer and apparently requires interaction with region C to drive expression in so7. Although this does not rule out the involvement of Hox proteins in maintaining somitic expression of *Hoxb4*, the mechanism is clearly more complex than a Hox-responsive element in region B that is equivalent to that in the LNE. In addition, the r6/7 boundary can be maintained by the neural regulatory elements on heterologous promoters (Gould et al., 1997; Gould et al., 1998; Whiting et al., 1991), whereas maintenance of the somitic boundary is dependent on the *Hoxb4* promoter (Gilthorpe et al., 2002).

Enhancer/promoter interactions in the regulation of *Hoxb4*

We have previously shown that in the absence of interaction with specific enhancers, the *Hoxb4* promoter cannot recapitulate any aspect of the proper expression pattern (Whiting et al., 1991). However, we have now demonstrated that the *Hoxb4* promoter is required to maintain the anterior boundaries of expression that are established by the region C enhancer. To our knowledge, this represents the first example of an active role for the promoter of a Hox gene in the maintenance of expression. The failure of construct CHZ to maintain expression that was initially established in the correct somitic domain is suggestive of the involvement of the Trithorax group proteins. It is interesting to note that in mice mutant for the *trithorax* gene *Mll*, *Hoxa7* expression is established normally during late gastrulation but is completely downregulated by 9.5 dpc (Yu et al., 1998). Consistent with this, we observe gradual regression of the anterior boundaries of CHZ expression between 8.5 and 9.5 dpc. However, we do not yet have any evidence for TrxG proteins interacting with the *Hoxb4* promoter. There may also be a requirement for the *Hoxb4* promoter in maintenance during late embryonic development, as expression of the CHZ reporter is completely downregulated between 12.5 and 13.5 dpc; constructs containing the *Hoxb4* promoter continue to express strongly during this period.

The detailed characteristics of core promoters are important in determining the specificity of enhancer/promoter interactions (Smale, 2001). This is likely to be especially pertinent to the tightly clustered Hox gene loci, where such interactions must be correctly established and maintained in a complicated regulatory environment; evidence from the *Hoxb* cluster supports this assertion. The LNE in region A controls the late expression of *Hoxb3*, as well as that of *Hoxb4* (Gould et al., 1997). Similarly, a mesodermal enhancer located upstream of *Hoxb4* (Fig. 7B) can activate expression through the *Hoxb4* or the *Hoxb5* promoters (Sharpe et al., 1998). By contrast, neural- and limb-specific enhancers in the same DNA fragment demonstrate a selective interaction with *Hoxb4*, and are seemingly unable to activate transcription through the *Hoxb5* promoter (Sharpe et al., 1998). Furthermore, a separate neural enhancer in the *Hoxb5-Hoxb4* intergenic region can drive expression through either promoter but, when placed between them, interacts exclusively with *Hoxb4*, indicating that the promoters of these two genes may compete for certain enhancers (Sharpe et al., 1998). The details of the interactions between enhancers and promoters that determine sharing, selectivity and competition in the Hoxb cluster have not yet been elucidated but it is interesting to note the results of recent studies in *Drosophila*. The presence or absence of certain components of the core promoter (the TATA box, initiator and downstream promoter element) can define the specificity of enhancer/promoter interactions (Ohtsuki et al., 1998; Butler and Kadonaga, 2001). Although the promoters of the majority of mouse Hox genes are poorly characterised, we have shown that the *Hoxb4* promoter has an unusual architecture (Gutman et al., 1994). It does not contain a TATA box but includes two initiators located approximately 80 bp apart that determine the start sites of alternative transcripts. It will be interesting to see how the specific characteristics of this and other promoters in the Hoxb cluster contribute to the proper spatiotemporal regulation of these genes

Regulatory organisation of PG-4 Hox genes

Murine Hox genes of paralogous group 4 (PG-4) have regulatory regions that are organised in a broadly similar manner (Morrison et al., 1997). Fig. 7B summarises all the known neural- and paraxial mesoderm-specific enhancers located close to the *Hoxa4*, *Hoxb4* and *Hoxd4* genes, and incorporates the results of this study. Enhancers located 3′ of *Hoxa4* and *Hoxd4* direct somitic expression with appropriate anterior boundaries for each gene. We have now demonstrated that sequences 3′ of *Hoxb4* are similarly required for proper expression of this gene in the paraxial mesoderm. However, region B has a restricted role in maintaining the anterior boundary and is dependent on sequences within region C to drive somitic expression (this study). By contrast, the 3′ enhancers of both *Hoxa4* and *Hoxd4* can function as regulatory

regions independently (Morrison et al., 1997). Interestingly, there may be some interaction between the intron and the 5′ region of *Hoxa4*. A transgene containing these sequences is expressed in the paraxial mesoderm with a boundary equivalent to that of *Hoxa4* and deletion of a 2 kb fragment from the 5′ region abolishes reporter gene expression (Behringer et al., 1993). Using the same parent construct, mutation of Hox binding sites in the intron eliminates expression in the paraxial mesoderm and the posterior neural tube (Keegan et al., 1997). Thus, both the upstream region and the intron would seem to be required. However, the ability of either to function as an independent enhancer has not yet been tested, and the relative contribution of these regions and the 3′ enhancer to the establishment and/or maintenance of somitic expression remains unclear.

As noted above, the enhancer 5′ of *Hoxb4* can function independently and can interact with the promoter of either of the neighbouring genes (Sharpe et al., 1998). Although this region specifies an anterior somitic boundary characteristic of *Hoxb5*, this does not rule out involvement in the regulation of *Hoxb4*, a hypothesis supported by comparison to *Hoxd4*. Similar to *Hoxb4*, the 5′ mesodermal enhancer of *Hoxd4* directs expression with an anterior boundary caudal to that of the endogenous gene (Zhang et al., 1997). The nearest gene 5′ to *Hoxd4* is *Hoxd8*, the somitic expression of which has an anterior boundary in the lower thoracic region (Izpisùa-Belmonte et al., 1990), and it is thus unlikely to be regulated by the enhancer 5′ of *Hoxd4*. Therefore, the assumption is that this enhancer does regulate *Hoxd4* expression. Thus it seems likely that regulation of PG-4 Hox genes involves integration of inputs from upstream and downstream elements, and this arrangement presumably serves to determine appropriate levels of expression. Finally, no regulatory function has yet been ascribed to the intron of *Hoxd4*. Deletion of the intron from transgenes based on the mouse or human *Hoxd4* genes has no effect on the observed expression patterns, and the intron of the human gene does not function as an independent enhancer (Morrison et al., 1997; Zhang et al., 1997). We have previously identified a conserved block of sequence within the introns of PG-4 genes (Gilthorpe et al., 2002). Interestingly, the *Hoxd4* sequences showed the least identity in these alignments. Whether this is related to the apparent reduction in the regulatory function of the *Hoxd4* intron has not yet been determined.

It seems that the relative inputs of 5′, 3′ and intron sequences to PG-4 regulation have diverged over the course of vertebrate evolution but that the similar regulatory organisation of these genes reflects that of an ancestral Hox4 gene. Unfortunately, details of the regulation of Hox genes in species other than the mouse are extremely scarce. However, we note that the regulatory function of intronic sequences is apparently not equivalent for the *Hoxb4* genes of all vertebrate species. The intron of the chicken gene drives expression only in posterior neural and mesodermal tissue in transgenic mice, while the equivalent region of the pufferfish (*Fugu rubripes*) gene completely lacks enhancer activity in this assay (Morrison et al., 1995). This is in marked contrast to the 3′ neural enhancer (region A), as similar fragments of the chicken and pufferfish genes are able to recapitulate the r6/7 boundary in transgenic mice (Aparicio et al., 1995; Morrison et al., 1995). It is an intriguing possibility that the chicken and pufferfish genes have retained a more robust mesodermal enhancer function in 3′ regions that is characteristic of the ancestral condition, and that the mouse gene has evolved to rely more heavily on sequences within the intron. Further analysis of the regulatory regions of PG-4 genes from these and other vertebrate species should provide valuable insights into the evolution of regulatory organisation in the Hox clusters, and whether or not this can be correlated with changes in expression boundaries.

Regulation of transcript stability in the paraxial mesoderm

In the paraxial mesoderm, the distribution of *Hoxb4* transcripts is restricted to so7-13, and protein is produced wherever transcripts are present. By contrast, all of the constructs used in this study are expressed in somites posterior to so13, at both the transcript and the protein level. Comparison with other *Hoxb4* transgenes (Whiting et al., 1991), indicates that the 3′ UTR of *Hoxb4* is required to destabilise transcripts in the posterior somitic domain and thus restrict *Hoxb4* expression to so7-13. Therefore, although other possibilities exist, we feel it is likely that *Hoxb4* is transcribed in all somites posterior to the so6/7 boundary, and that post-transcriptional regulation determines the posterior boundary and thus the definitive domain of *Hoxb4* expression. Further complexity in the regulation of *Hoxb4* is revealed by the downregulation of constructs CHZ, Cb4Z and CBb4Z in the posterior domain after 11.5 dpc. However, we do not yet know whether this late phase of regulation involves changes in transcriptional or post-transcriptional regulation.

Interestingly, construct CHZ uncouples the two domains of somitic expression during early development, as it maintains expression only posterior to so14 and not in the anterior definitive domain of *Hoxb4* expression. We have shown that maintenance of expression in the latter domain is dependent on the *Hoxb4* promoter and have attributed this to a requirement for the promoter in the maintenance of transcription. However, it is possible that sequences within this region (presumably within the 5′ UTR) are necessary for the stabilisation of transcripts, and that CHZ transcripts are generated in so7-13 after 8.5 dpc but are rapidly degraded. Should this be true, it would identify contrasting roles for the 5′ and 3′ UTRs of *Hoxb4*, the former being required to stabilise transcripts and the latter to destabilise them, albeit in different domains of the paraxial mesoderm.

We have found interesting parallels between our observations on the regulation of *Hoxb4* expression and recent work on *Hoxd1* (Zákány et al., 2001). This is an unusual Hox gene in that it is not expressed in somites but is expressed in the anterior presomitic mesoderm. This expression occurs in pulses associated with the formation of each somite, and *Hoxd1* transcripts are rapidly excluded from somites once they have formed. However, when a *lacZ* reporter was inserted into the endogenous *Hoxd1* gene, stable transcripts accumulated and were retained in somites well after their formation. We note that in this experiment the *lacZ* gene was coupled to SV40 polyA sequences, thus removing *Hoxd1* 3′ UTR sequences from the transcripts generated from this locus. This correlates with our observations that *Hoxb4* transgenes containing *lacZ*-SV40 polyA cassettes generate transcripts that are stable in posterior somites, whereas those that contain *Hoxb4* 3′ UTR sequences are not. We note that many, although not all, Hox genes have restricted domains of expression in the paraxial

mesoderm, with both anterior and posterior boundaries (Burke et al., 1995), and propose the following general mechanism of *Hox* gene regulation in this tissue. Specification of the anterior boundary of a given gene is determined by the timing of transcriptional activation and is linked to the segmentation clock that regulates somitogenesis (Dubrelle et al., 2001; Zákány et al., 2001). Transcription then occurs in all somites posterior to this point and the definitive posterior boundary, if any, is determined by regulating the stability of transcripts in posterior regions through sequences in the 3′ UTR.

Translational regulation of *Hoxb4* in the neural tube

We have compared the distribution of *Hoxb4* transcripts and Hoxb4 protein and observed that different regulatory strategies are employed in the neural tube and paraxial mesoderm to achieve the same end: the spatial restriction of *Hoxb4* function in the embryo. In the neural tube, detectable levels of the protein accumulate only in an anterior subdomain of the region in which *Hoxb4* is transcribed. Although the mechanism by which this is achieved is not yet known, we envisage two likely scenarios. Either transcripts are selectively translated in the hindbrain and anterior spinal cord, or Hoxb4 protein is produced throughout the neural tube and actively and rapidly degraded in posterior regions. In *Drosophila*, the homeodomain protein Bicoid (Bcd) regulates the expression of another homeodomain protein Caudal (Cad) at the translational level. Bcd binds in a sequence-specific manner to the 3′ UTR of *cad* transcripts and represses translation by interacting with proteins bound to the 5′ cap (Niessing et al., 2000; Niessing et al., 2002). *bcd* has evolved from an ancestral PG-3 Hox gene (Stauber et al., 1999) and, although no Hox proteins have yet been shown to bind RNA, it is an intriguing possibility that translational repression of Hoxb4 is mediated by more posteriorly expressed Hox proteins in the same manner that Bcd regulates Cad.

Although data on the distribution of Hox proteins in the mouse embryo are scarce, both Hoxb5 and Hoxc8 are expressed in spatially restricted domains along the AP axis of the neural tube (Belting et al., 1998; Sharpe et al., 1998; Wall et al., 1992). However, for these genes the RNA is similarly localised and the regulation of expression appears to be primarily transcriptional (Awgulewitsch and Jacobs, 1990; Conlon and Rossant, 1992). Thus, the relative importance of transcriptional and translational control in the neural tube may differ for individual Hox genes.

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