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Evolution

# Modification of expression and *cis*-regulation of *Hoxc8* in the evolution of diverged axial morphology

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**ABSTRACT** **Differential *Hox* gene expression between vertebrate species has been implicated in the divergence of axial morphology. To examine this relationship, we have compared expression and transcriptional regulation of *Hoxc8* in chicken and mouse. In both species, expression of *Hoxc8* in the paraxial mesoderm and neural tube is associated with midthoracic and brachial identities, respectively. During embryogenesis, there is a temporal delay in the activation of *Hoxc8* in chicken compared with mouse. As a result, chicken *Hoxc8* expression in the paraxial mesoderm is at a posterior axial level, extending over a smaller domain compared with mouse *Hoxc8* expression. This finding is consistent with a shorter thoracic region in chicken compared with mouse. In addition, the chicken *Hoxc8* early enhancer, differing from its mouse counterpart in only a few specific nucleotides, directs a reporter gene expression to a more posterior domain in transgenic mouse embryos. These findings are consistent with the concept that the diversification of axial morphology has been achieved through changes in *cis*-regulation of developmental control genes.**

Genes that control axial patterning, such as *Hox* genes, are highly conserved across the animal kingdom (1). However, animals exhibit a high degree of diversity in the organization of the primary body axis. This phenomenon may be caused by conserved genetic programs having become variously modified in different organisms (2–4). Differences in *Hox* gene expression may contribute to an understanding of how modifications of developmental programs generate axial diversity between species (5–7).

Amniotes differ greatly in the number of segments contributing to individual anatomic regions along the vertebral column such as the cervical, thoracic, and lumbar regions. For example, the vertebral column of the mouse consists of seven cervical and 13 thoracic vertebrae, whereas the vertebral column of chicken displays 14 cervical and seven thoracic vertebrae. Comparisons between mouse and chicken show that differences in axial morphology are associated with differences in spatial domains of *Hox* gene expression (5, 6). For example, *Hoxc6* is expressed at the cervical/thoracic transition in both mouse and chick, but at different relative levels along the anteroposterior axis; likewise, *Hoxc8* is expressed in the thoracic region of both mouse and chicken (6).

Differences in expression patterns of *Hox* genes between different species may be brought about by changes in components of their transcriptional regulation, including changes in *cis*-regulatory elements and *trans*-acting factors whose interactions determine embryonic expression patterns of *Hox* genes. However, experimental exchanges of *Hox* genes and their *cis*-regulatory regions between different organisms have demonstrated a high degree of functional conservation (8–24). The expression of *trans*-acting factors of *Hox* genes probably are largely retained in amniotes, setting up a pre-pattern that provides positional coordinates along the body axis. We postulate that subtle changes in *cis*-regulatory elements leading to altered interactions with conserved *trans*-acting factors may contribute to diverged expression patterns of *Hox* genes among different species.

In previous studies, we identified *cis*-regulatory regions that control different phases of mouse *Hoxc8* expression (25–29). The *Hoxc8* early enhancer is involved in the establishment of the anteroposterior expression domain of *Hoxc8*, consistent with its role in the regionalization of the body axis (26, 29). The early enhancer has been delimited to a 200-bp DNA fragment by extensive deletion analyses in transgenic mice (26, 28). Five partially redundant elements within this region act in combination in determining early *Hoxc8* expression (26, 28). A survey among mammalian species reveals a remarkable conservation of the nucleotide sequence of the early enhancer (C.S.S., unpublished observations). Any difference in the nucleotide sequence and activity of this highly conserved and well-characterized enhancer may have strong implications on the divergence of *Hoxc8* expression between different species.

To test this hypothesis, we have studied *Hoxc8* expression during chicken and mouse embryogenesis and compared their early enhancer regions for nucleotide sequence similarities, and enhancer activities in transgenic mice. In this report we provide evidence suggesting that transposition of *Hoxc8* expression between the two species is achieved by differential activities of the *Hoxc8* early enhancer.

## MATERIALS AND METHODS

FVB mice (Taconics) were used for obtaining staged embryos and for transgenic analysis. For mating, pairs were caged together at noon, and the females were examined for the presence of a vaginal plug the next morning, which was defined as day 0.5. Fertilized eggs from white leghorn hens (SPAFAS) were incubated at 37°C in an egg incubator. The chicken embryos were staged as described (30).

Immunohistochemistry was performed with a mAb, C592/7E, against the mouse *Hoxc8* protein (29) as described (31) with minor modifications.

For retrograde labeling experiments, mouse (10.5 day postcoital) and chicken [Hamburger and Hamilton (H&H) 24] embryos were fixed in 4% paraformaldehyde in PBS. Brachial motor neurons were retrograde-labeled by placing finely ground 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) crystals into one severed forelimb bud and a 1- to

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; H&H, Hamburger and Hamilton.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ223359).

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2-week incubation in PBS, 10 mM EDTA. Specimens were cut on a vibratome at 80–100 $\mu$m, and the image was captured with a black-and-white charge-coupled device camera. The same sections then were processed with *Hoxc8* mAbs, and the expression of *Hoxc8* was compared with the DiI label, side by side or by superimposing the captured images in pseudocolor.

Production of transgenic embryos, preparation of DNA for microinjection, and staining for $\beta$-galactosidase have been described (26). The reporter gene construct (construct A) carrying 399-bp *Hoxc8* early enhancer was described earlier (28). A 151-bp chicken enhancer was isolated by PCR using chicken genomic DNA as a template and synthetic oligonucleotide primers designed from the mouse enhancer sequence flanking the fragment. A 399-bp enhancer fragment in which 151 bp of the mouse sequence was replaced with the corresponding chicken DNA fragment was generated by overlapping PCR. The resulting fragment was cloned by ligation at appropriate restriction sites in the polylinker sequence of pHSF (26) to create a mouse/chicken hybrid construct (construct B).

## RESULTS

**Heterochronic Activation of *Hoxc8*.** Previous studies by Burke *et al.* (6), using RNA *in situ* procedures, showed that axial levels of *Hoxc8* expression differed between mouse and chicken midgestation embryos. To determine the temporal sequence of *Hoxc8* expression during mouse and chicken embryogenesis, we performed a comparative immunohistochemistry, using a mAb raised against mouse *Hoxc8* (29). The mouse embryonic expression pattern is described in detail elsewhere (29). Briefly, in the mouse, *Hoxc8* first is detected at the base of allantois in day 8 embryos having 6–7 somites (Fig. 1*A*). At this stage, the neural tube is still open, and the heart primordia have just formed (32). The earliest chicken embryos examined, stages 10 and 11, are similar in their developmental progression to day 8.0–8.5 mouse embryos. At these stages, the major events of gastrulation have occurred, and organogenesis is proceeding in the anterior portion of the embryos. Embryos of both species possess a similar number (8–12) of somites, and the degrees of the development of the heart and nervous system are comparable at these stages. However, in the chick, *Hoxc8* protein was not detected in stages 10 or 12 (data not shown), but first was detected in the posterior regions in stage 13 embryos having 18–19 somites (Fig. 1*E*). At this stage, the neural tube, with the exception of the caudal neuropore, is entirely closed, and the rostral portions, including fore-, mid- and hindbrain, the optic cups, and otic vesicles are formed. As in the mouse, chicken *Hoxc8* expression at this stage was diffuse in all embryonic tissues posterior to somite condensation. Thus, the onset of *Hoxc8* expression in the chicken is developmentally delayed compared with mouse.

**Axial Levels of *Hoxc8* Expression.** A clear and differential anterior boundary of *Hoxc8* expression in the neural tube and paraxial mesoderm is established at subsequent stages in the mouse (day 8.5, Fig. 1*B*) and in the chicken embryos (stage 14, Fig. 1*F*). In the mouse, at days 9.5 and 10.5, anterior bound-

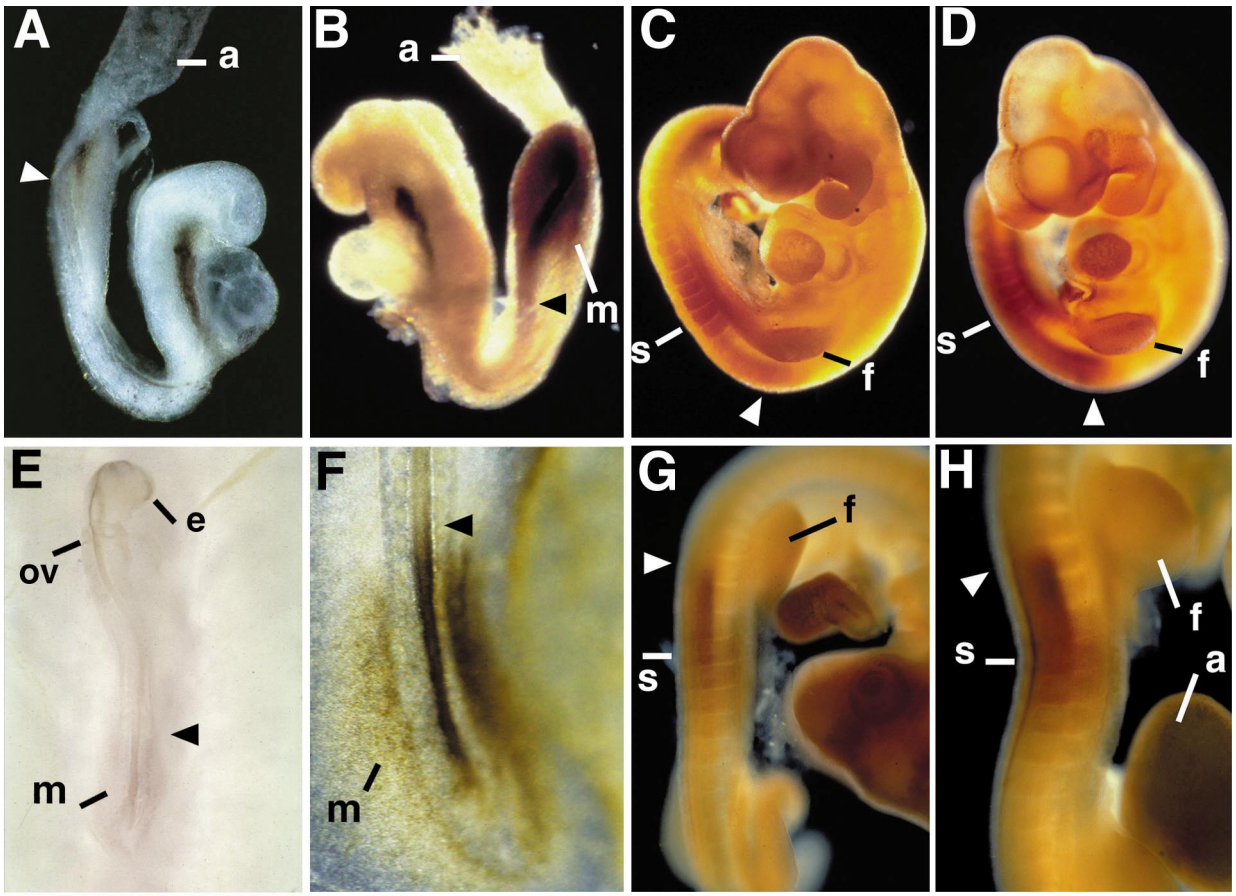


FIG. 1. Expression of *Hoxc8* during early mouse (*A–D*) and chicken (*E* and *F*) embryogenesis. The anterior boundary of expression in the neural tube is indicated by arrowheads. (*A*) Embryo with 7–8 somites. (*B*) Embryo with 10–12 somites. The staining of the foregut in *A* and *B* is caused by antibody trapping. (*C*) Embryo with 28–30 somites (9.5 day postcoital). (*D*) Embryo at 10.5 day p.c. Somite 17 is indicated in *C* and *D*. (*E*) Chicken embryo at H&H stage 13 (18–19 somites). (*F*) Embryo at H&H stage 14 (20 somites). (*G*) Embryo at H&H stage 21. (*H*) At H&H stage 23, somite 22 is indicated in *G* and *H*. The staining of the brain and the allantois is caused by antibody trapping. a, allantois; e, eye; f, forelimb bud, m, mesoderm; ov, otic vesicle; s, somite.

aries of expression of *Hoxc8* were observed in the neural tube at the level of 10th somite and in the paraxial mesoderm at the level of 14th somite (Fig. 1 *C* and *D*). In the chick, however, at stages 21 and 23, anterior boundaries of expression of *Hoxc8* were observed in the neural tube at the level of the 18th somite and in the paraxial mesoderm at the level of 20th somite. Thus, the axial level of expression of *Hoxc8* in chicken embryos is 6–8 somites more posterior than that observed in the mouse embryos.

At these stages, in both mouse and chicken embryos, *Hoxc8* expression declines in the tailbud region, thus defining posterior boundaries of expression. However, these boundaries are unlike the anterior boundaries of expression, having indistinct margins. In the mouse, *Hoxc8* expression in the neural tube spans about six somite levels (from somites 10–15) and in the paraxial mesoderm about 7–8 somite levels (somites 13–21, with weaker levels of expression at somites 13 and 21). In the chick, *Hoxc8* expression in the neural tube spans about six somite levels (somites 18–23; strong expression in somites 19–20) and in paraxial mesoderm about five somite levels (somites 21–25). Thus *Hoxc8* expression in paraxial mesoderm is 2–3 somite levels shorter in the chicken compared with mouse. Although the expression domain of *Hoxc8* in the paraxial mesoderm is smaller and more posterior compared with mouse, the expression pattern is coincident with the smaller thoracic region in chick. Thus, *Hoxc8* expression in the paraxial mesoderm is different not only with respect to absolute axial levels but also in the number of expressing somites.

**Early and Late Phases of Neural Tube Expression of *Hoxc8*.** In the mouse, two phases of *Hoxc8* expression can be distinguished in the neural tube, an early and a late phase (29). In the early phase (day 8–9.5), *Hoxc8* expression is found in most, if not all, cells along the dorsoventral extent of the neural tube (data not shown). In the late phase (after day 10.5), *Hoxc8* expression is restricted to differentiating neurons, predominantly in motor neurons in the ventrolateral region of the neural tube (Fig. 1*D*). In the chick, similar phases of *Hoxc8* expression in the neural tube are observed. In the early phase (stage 13–15) *Hoxc8* is distributed uniformly in the neural tube, whereas in the late phase *Hoxc8* is distributed predominantly in the motor neurons in the ventrolateral region (Fig. 1*H*). The distribution of *Hoxc8* within the subregions of the spinal cord at subsequent stages is very similar in both species.

**Association of *Hoxc8* Expression with Motor Neurons.** To determine whether the axial shift of *Hoxc8* expression in the neural tube of mouse and chicken corresponds to a transposition of regional identity of spinal nerves, brachial motor neurons were identified and tested for colocalization with *Hoxc8* protein. Brachial motor neurons of day 10.5 mouse and stage 24 chicken embryos were retrograde-labeled with DiI as described in *Materials and Methods*. The embryos were sectioned serially, and the DiI signal was compared with the distribution of *Hoxc8* protein on the same sections. In both mouse and chick, sections through the anterior brachial neural tube at somite levels 9 and 17, respectively, showed no *Hoxc8* expression (Fig. 2 *A* and *D*). At more posterior levels, (at somite levels 11 in mouse and 19 in chick), however, DiI label coincided with the domain of *Hoxc8* expression (Fig. 2 *B* and *D*). These findings were confirmed on horizontal sections (data not shown). Thus, *Hoxc8* expression in the central nervous system is transposed according to the functional identity of expressing motor neurons.

***Hoxc8* Early Enhancer of Mouse and Chick.** The mouse *Hoxc8* early enhancer is involved in the establishment of spatial domains of *Hoxc8* expression (25, 26, 29). We isolated the chicken *Hoxc8* early enhancer to test whether the difference in the spatiotemporal pattern of chicken *Hoxc8* expression compared with mouse is caused by differences in the chicken enhancer. Primers were designed, based on most conserved regions of *Hoxc8* early enhancer, to amplify a 151-bp fragment

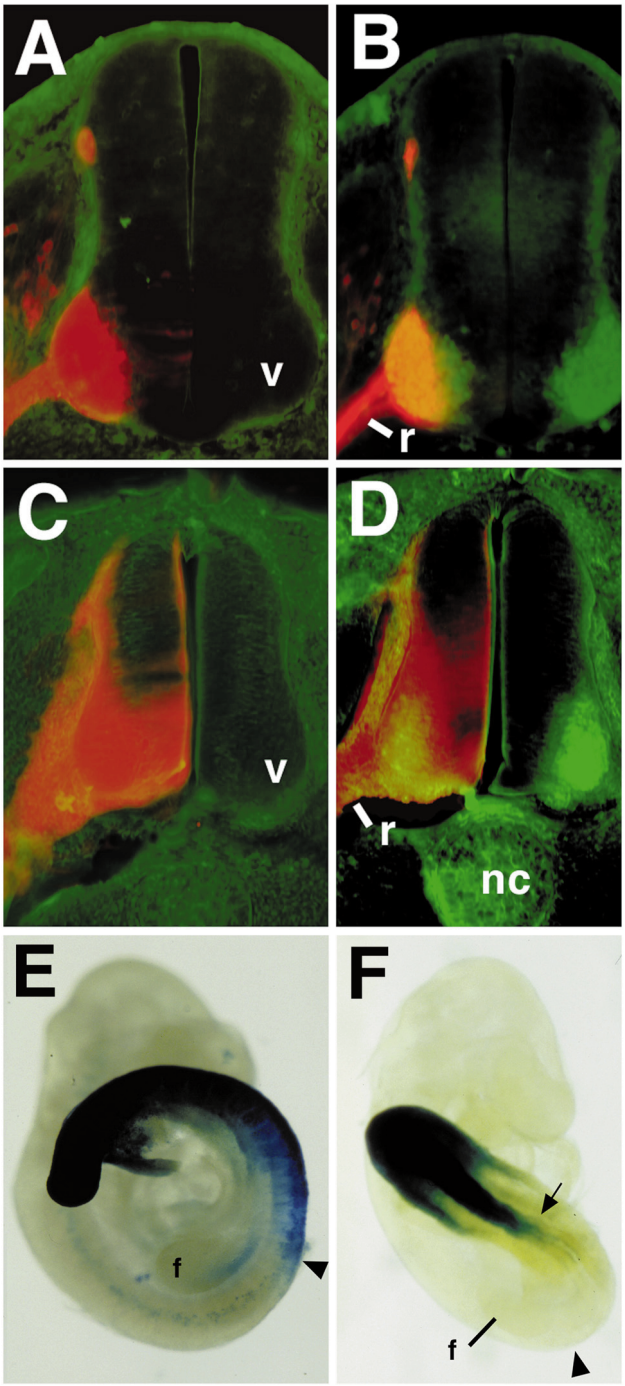


FIG. 2. (*A–D*) Expression of *Hoxc8* in the brachial region of the neural tube. Cross-section through the neural tube of a mouse embryo at the level of the ninth (*A*) and 11th (*B*) somite. (*C* and *D*) Cross-sections through the neural tube of a chicken embryo at the level of the 17th and 19th somites. *Hoxc8* expression is shown in green, DiI label in red and resulting overlap in yellow. (*E* and *F*) Expression of mouse and chicken reporter genes in transgenic mouse embryos. (*E*) Construct A (399-bp mouse sequence); B, construct B (399-bp mouse-chicken hybrid construct). The arrowheads indicates somite 14. The arrow indicates the anterior limit of reporter gene expression mediated by construct B (*F*). f, forelimb bud; nc, notochord; r, ventral root; v, ventral horn.

of the chicken enhancer by PCR. This enhancer is highly conserved between the mouse and chicken with respect to structure and overall sequence similarity (Fig. 3*A*). The sequence similarity over 151 bp is 80%. The critical elements (Fig. 3*A*, A–E) required for the mouse enhancer activity in

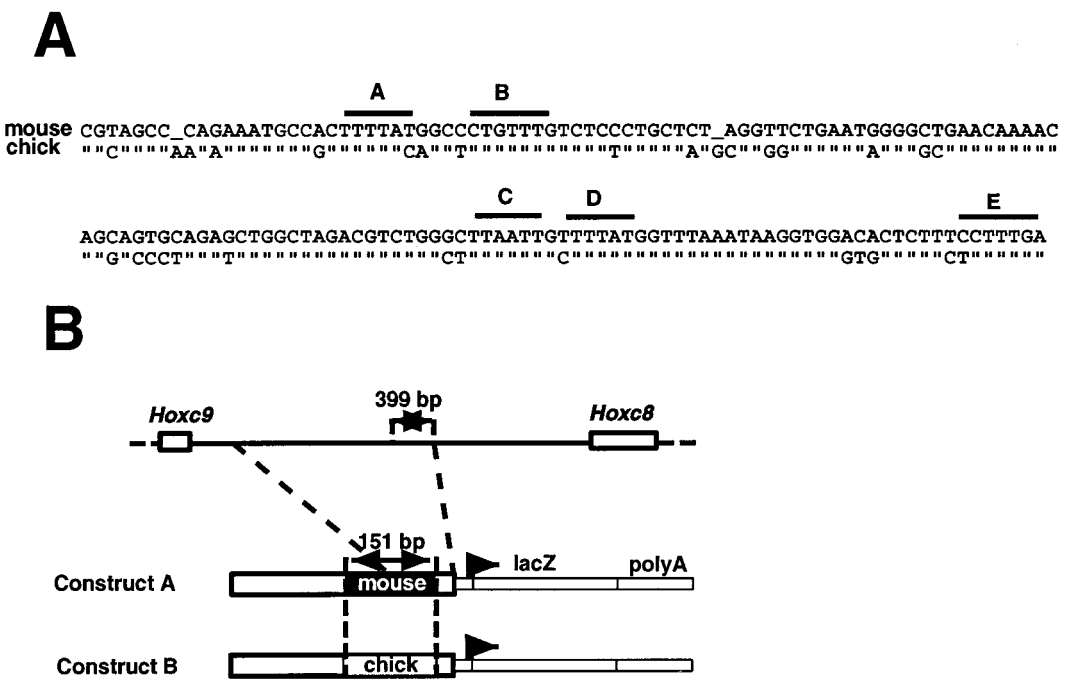


FIG. 3. The *Hoxc8* early enhancer in mouse and chick. (*A*) Nucleotide sequence comparison of the critical region of chicken and mouse *Hoxc8* early enhancers. One hundred fifty-one bp of the critical region of the early enhancer are shown. Five elements essential for the activity of the early enhancer are indicated (A–E). (*B*) Design of mouse and mouse-chicken hybrid reporter constructs. Construct A consists of a 399-bp early *Hoxc8* enhancer ligated to a mouse *hsp68/lacZ* reporter gene. Construct B was generated by replacing 151 bp of the critical enhancer region of the mouse with that of chick.

transgenic mice are arranged identically in the chicken enhancer. In addition to clusters of substitutions between the known sites, there are several differences within and in proximity to these elements.

The chicken enhancer was tested for its ability to direct the expression of a reporter gene (*hsp68-lacZ*) in transgenic mice to determine whether differences in its nucleotide sequence from that of mouse affects enhancer activity. A 399-bp DNA fragment containing the mouse early enhancer region (construct A, Fig. 3*B*) directs the reporter gene expression in day 9.5 embryos to the neural tube and paraxial mesoderm at the level of somite 14 and 19, respectively (ref. 28 and Fig. 2*E*). From this construct, the 151-bp fragment of the mouse enhancer containing the critical elements of the enhancer was replaced with the corresponding sequences from the chick. The resulting construct (construct B, Fig. 3*B*) directed the expression of the reporter gene in day 9.5 embryos to more posterior regions of the embryo in both neural tube and mesoderm (Fig. 2*F*). Five founder embryos that were generated displayed identical patterns. Expression in the neural tube was consistently found posterior to the 18th somite, whereas expression in the mesoderm was several somites posterior to that in the neural tube. Thus, chicken *Hoxc8* enhancer differing from the mouse counterpart in a few nucleotides directs the reporter gene expression to a different anterior boundary in the neural tube and mesoderm. This posteriorization of the reporter gene activity in the neural tube and mesoderm is consistent with the more posterior localized expression of *Hoxc8* in the chick.

## DISCUSSION

The investigation of the genetic basis of morphological diversity among animals now has become feasible because of the identification of highly conserved regulatory genes that control embryonic patterning and morphogenesis. To examine the correlation of *Hox* gene expression and axial variation, we have compared the spatiotemporal distribution of *Hoxc8* in mouse and chick. Our findings can be summarized as follows: *Hoxc8* expression is modified in concert with variation in axial morphology within the paraxial mesoderm and the neural tube (Fig. 4). Posteriorization of *Hoxc8* expression in chicken is achieved through a temporal delay of activation compared

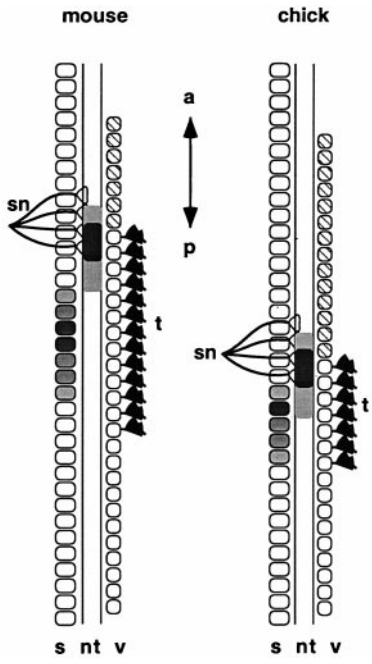


FIG. 4. Schematic comparison of *Hoxc8* expression in chicken and mouse in relationship to morphological landmarks. Cervical, thoracic, and lumbar regions of the vertebral column and the brachial region of the neural tube are indicated. Brachial spinal nerves C6, C7, C8, and T1 in mouse and C13, C14, C15, and T1 in chicken are shown. Shaded region in somites and neural tube represent *Hoxc8* expression. Regions of highest expression are indicated in dark shades. The double-headed arrow indicates the anteroposterior orientation of the body axis. a, anterior; p, posterior; nt, neural tube; t, thoracic vertebrae; s, somites; sn, spinal nerves; v, vertebrae.

with mouse. The comparison of chicken and mouse enhancer elements shows that only a few nucleotide changes within the critical region of the early *Hoxc8* enhancer suffice to transpose reporter gene expression to more posterior body regions.

The role of *Hox* genes in the regionalization of the nervous system has been examined most closely in the hindbrain (33). Within the hindbrain *Hox* genes are expressed in the same segments in mouse and chick, reflecting that the organization, in respect to the number of rhombomeres, has been conserved between these two species (34–36). In contrast, the brachial region of the spinal cord is transposed in these species and our results show that the shift of *Hoxc8* expression along the body axis corresponds to this anatomical modification.

The different axial position and extent of *Hoxc8* expression in the segmental mesoderm reflects two major differences in the axial organization of the vertebral column of chicken and mouse. First, the relative expansion of the cervical region in the chicken is reflected by a posteriorization of *Hoxc8* expression. Second, the overall reduction of thoracic segments is reflected by a reduced number of somites expressing *Hoxc8* compared with the mouse. This association of *Hoxc8* expression with regional morphology of vertebrae suggests a role in the specification of midthoracic identity within the paraxial mesoderm. The axial fate of somites already is established in the presomitic mesoderm (37). Thus, the observation that *Hoxc8* is found at the correct place and at the right time in the segmental plate and before somite condensation is consistent with a role in the establishment of thoracic identity in the paraxial mesoderm. Further support for a causal relationship between *Hoxc8* expression and midthoracic identity stems from genetic analyses in mice. Ectopic expression of *Hoxc8* in somites of the lower thoracic and upper lumbar region leads to anterior transformations within this region, including the formation of lumbar ribs (38). Similarly, disruption of *Hoxc9* causes a posterior expansion of *Hoxc8* expression and the appearance of supernumerary ribs as well (39). These results also demonstrate that anatomical regions can be expanded by an extension of *Hox* gene expression along the primary body axis and agrees well with the finding that a larger expression domain of *Hoxc8* in the mouse, compared with the chick, is linked to a higher number of thoracic vertebrae. This finding suggests that axial variation among amniotes is not only generated by axial shifts in the anterior expression boundaries of *Hox* genes, but also by expansion or reduction of their overall expression domains.

The axial variation in *Hoxc8* expression may be caused by changes in the transcriptional regulation of *Hoxc8*. Differences in *Hox* gene expression could be caused by genetic changes in *cis* elements and/or *trans*-acting factors. Changes in *trans*-regulation are more difficult to study because of the multiplicity of potential (and lack of bona fide) regulatory proteins. Changes in *cis*-elements in this report were studied by comparing a minimal sequence of the *Hoxc8* early enhancer. The nucleotide sequence of the early enhancer region is highly conserved among mammals and the sequence of *cis*-acting elements (A–E) are invariant. Compared with mammalian *Hoxc8* early enhancer sequence, the chicken enhancer sequence showed more nucleotide sequence changes. Many of the nucleotide differences were observed in the vicinity of the genetically defined sites, A, D, and E. It is conceivable that these and other specific nucleotide differences contribute toward overall posteriorization of the reporter gene expression mediated through the chicken enhancer. In the case of the mouse enhancer, mutations at individual sites A, C, D, and E, lead to posteriorization of the reporter gene expression (28). The anterior extent of the reporter gene expression is determined by combinatorial interactions at these elements. In addition, nucleotide changes outside of the defined elements in the chicken enhancer may be affecting hitherto undefined *cis*-acting elements. A systematic exchange of nucleotide sequences between mouse and chicken enhancers will pinpoint critical nucleotides involved in the posteriorization of the reporter gene expression directed by the chicken enhancer.

Comparative analysis of vertebrate *cis*-regulatory regions, using reporter gene assays in transgenic mouse embryos, have shown remarkable conservation of transcriptional regulation of *Hox* genes (13–15, 17–19, 22). Many of these elements direct reporter gene expression to similar spatial domains in transgenic mouse embryos. On the other hand, a chicken *Hoxb4* enhancer, although capable of directing expression of the reporter gene to the correct anterior boundary in the neural tube, directed expression to a more posterior boundary in the mesoderm, suggesting a species-specific differences in the enhancer activity (18). Transcriptional heterochrony also has been suggested to be an important mechanism by which subtle changes in temporal colinearity of *Hox* genes may result in the evolution of body plans (40). A replacement of a conserved mouse *Hoxd11* regulatory region with its zebrafish counterpart lead to a slightly premature activation of *Hoxd11*, leading to rostral shift of its expression boundary and anterior transposition of the sacrum (22).

In conclusion, we have shown that the *Hoxc8* expression in mouse and chicken is similar with respect to anatomical features such as the brachial spinal nerves and the midthoracic region of the vertebral column. However, significant differences also exist in relational features of the body plan such as the ratio of cervical and thoracic domains. We also show in mouse transgenic experiments where we compare the early enhancers of mouse and chick that chicken enhancer constructs simulate a chicken relational pattern of expression. Additional experiments will be required to determine the specificity of nucleotide changes in the regulation of *Hoxc8* expression pattern and correlated modifications of the body plan.

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