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Comparative studies on mammalian *Hoxc8* early enhancer sequence reveal a baleen whale-specific deletion of a cis-acting element

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ABSTRACT **Variations in regulatory regions of developmental control genes have been implicated in the divergence of axial morphologies. To find potentially significant changes in cis-regulatory regions, we compared nucleotide sequences and activities of mammalian *Hoxc8* early enhancers. The nucleotide sequence of the early enhancer region is extremely conserved among mammalian clades, with five previously described cis-acting elements, A–E, being invariant. However, a 4-bp deletion within element C of the *Hoxc8* early enhancer sequence is observed in baleen whales. When assayed in transgenic mouse embryos, a baleen whale enhancer (unlike other mammalian enhancers) directs expression of the reporter gene to more posterior regions of the neural tube but fails to direct expression to posterior mesoderm. We suggest that regulation of *Hoxc8* in baleen whales differs from other mammalian species and may be associated with variation in axial morphology.**

The role of *Hox* genes in the evolution of axial morphology has long been a subject of speculation (1). Several mechanisms, including expansion in the number of genes, cluster duplications, and changes in expression patterns, have been implicated in the generation of diversity in the metazoan body plan (reviewed in refs. 2–5). Variations in expression patterns of *Hox* genes between different species may be brought about by changes in components of their transcriptional regulation. This may involve changes in cis-regulatory elements and trans-acting factors whose interactions determine embryonic expression patterns of *Hox* genes. Comparative analysis of vertebrate cis-regulatory regions in transgenic mouse embryos have, in general, demonstrated remarkable conservation of transcriptional regulation of *Hox* genes (reviewed in ref. 4). However, in a few instances, heterospecific *Hox* enhancer sequences have been shown to function differently from corresponding mouse *Hox* enhancers in transgenic mouse embryos (6–8). These variations may reflect changes in *Hox* gene expression patterns among different species and provide a genetic basis for divergence of axial morphologies (6, 7).

Transcriptional regulation of *Hoxc8* expression along the embryonic axis in the mouse is controlled by at least two separate cis-regulatory regions, the early enhancer located in the 5′ region and the late enhancer located in the 3′ region of the *Hoxc8* transcriptional unit (9–13). The early enhancer is required to initiate *Hoxc8* expression in the posterior regions of the day 8.5 mouse embryo and to establish spatial domains of *Hoxc8* expression in the neural tube and mesoderm. The late enhancer is required to maintain anterior *Hoxc8* expression and to down-regulate posterior expression after day 9.0 of mouse embryonic development. The *Hoxc8* early enhancer has been delimited to a 200-bp fragment by progressive deletions (12, 13). Contained within the 200 bp are at least five distinct cis-acting elements (A–E) that are partially redundant and interdependent as judged by reporter-gene analysis in transgenic mouse embryos. Different combinations of these elements govern both the anterior limits and the tissue-specific pattern of the reporter gene expression along the anteroposterior embryonic axis. Changes in the nucleotide sequence of any of these elements would be expected to result in alterations in *Hoxc8* expression and manifest in alterations of morphologic features. In support of this view, a comparison of mouse and chicken *Hoxc8* early enhancer sequences and activities revealed differences that correlated with the divergence of *Hoxc8* expression pattern (6). This divergence may, in turn, correspond to modifications in the organization of the body axis in these two taxa.

To find potentially significant variations in *Hoxc8* early enhancer sequences among closely related species, we performed a PCR-based survey on 29 species representing major mammalian clades. In this study, we found a remarkable degree of conservation of nucleotide sequences corresponding to the mouse *Hoxc8* early enhancer sequence. Strikingly, the baleen whale enhancer region shares a 4-bp deletion in element C. By using reporter-gene analysis in transgenic mice, we show that the baleen whale-specific deletion of this *Hoxc8* sequence greatly reduced posterior mesoderm expression and lessened the anterior extent of the neural tube expression. This change in enhancer activity supports the notion that modification in cis-regulatory elements of *Hox* genes is one mechanism that might have contributed to the evolution of body plan diversity during mammalian radiation.

MATERIALS AND METHODS

Genomic DNA Samples. A plasmid containing human *HOXC8* genomic region and its partial sequences were kindly provided by E. Boncinelli (Department of Biological and Technological Research, San Raffaele Biomedical Park, Milan, Italy). We obtained armadillo and bat genomic DNAs from W. Bailey (Merck); giraffe genomic DNA from K. Weiss (Pennsylvania State University, State College, PA); camel, hippopotamus, and llama genomic DNAs from D. Irwin (University of Toronto, Canada); cow, ocelot, elephant, and beluga and humpback whale genomic DNAs from S. O'Brien (National Institutes of Health); and remaining whale genomic

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF099473–AF099500).

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DNAs from U. Arnason (University of Lund, Sweden). Systematic names of species analyzed follow in parentheses. Species studied include mouse (*Mus musculus*, strain C57BL/6J), armadillo (*Dasypus novemcinctus*), D'orbiguy's round-eared bat (*Tonatia sylvicola*), cow (*Bos taurus*), hippopotamus (*Hippopotamus amphibius*), ocelot (*Felis pardalis*), warabi wallaby (*Petrogale burbidoei*), elephant (*Loxodonta africana*), camel (*Camelus dromedarius*), llama (*Lama guanicoe*), giraffe (*Giraffa camelopardalis)*, Northern bottlenose whale (*Hyperoodon ampullatus*), common porpoise (*Phocoena phocoena*), narwhal (*Monodon monoceros*), beluga (*Delphinapterus leucas*), whitebeak dolphin (*Lagenorhynchus albirostris*), Indus river dolphin (*Platanista indi*), Antillean beaked whale (*Mesoplodon europaeus*), North Sea beaked whale (*Mesoplodon bidens*), La Plata dolphin (*Pontoporia blainvillei*), Baird's beaked dolphin (*Berardius bairdii*), great sperm whale (*Physeter macrocephalus*), pygmy sperm whale (*Kogia breviceps*), fin whale (*Balaenoptera physalus*), sei whale (*Balaenoptera borealis*), minke whale (*Balaenoptera acutorostrata*), Bowhead whale (*Balaena mysticetus*), and humpback whale (*Megaptera novaeanagliae*).

Isolation of Early *Hoxc8* Enhancer Orthologous Sequences from Different Mammalian Species. For most species, primers used for PCR amplification of the *Hoxc8* early enhancer region are: Jstabv (5′-CCCACCTCTCCTCTGCTCCTTTGCTGGAATCACAAAACCCTAAAG-3′) and PR6 (5′-CCTGCAGCTCCGTGGGCCATAG-3′). These primers amplified a 273-bp fragment. In some instances, a combination of Jstabv and Lcns2 (5′-GCCTCTAACATTGAGCAACAGCGCCACCTCGCGT-3′), which amplified an ≈800-bp DNA fragment were used. PCR amplification was performed with different buffer sets provided by Invitrogen and at different annealing temperatures on a trial-and-error basis. PCR amplification was done for 45 cycles in a thermocycler (Omnigene; Hybaid, Middlesex, U.K.). PCR products were cloned in TA cloning vector (Invitrogen) and sequenced by using a standard dideoxynucleotide method. Authenticity of the sequence was examined by analyzing multiple PCRs and different genomic DNA samples for each taxon. In general, diagnostic nucleotide substitutions for each species were identified to ensure lack of potential cross-contamination from genomic DNAs of the other species examined.

Reporter-Gene Analysis in Transgenic Mice. Production of transgenic embryos, preparation of DNA for microinjection, and staining for β-galactosidase activity have been described (12). The mouse reporter-gene construct has been described (13). The human reporter construct described in this study contains a 400-bp fragment corresponding to the mouse *Hoxc8* early enhancer. Sperm whale and fin whale reporter constructs containing a 273-bp fragment were generated by using primers Jstabv and PR6 (described above). A 4-bp deletion of element C in a mouse reporter construct was generated by using an overlapping-PCR strategy.

RESULTS

Comparison of Nucleotide Sequences of Mammalian Orthologs of Mouse *Hoxc8* Early Enhancer Region. A criterion for assessing the significance of the mouse *Hoxc8* early enhancer module with respect to patterning is its evolutionary conservation across species. A comparison of orthologous nucleotide sequences of mouse and human shows that they share ≈70% nucleotide sequence similarity over a 4.5-kb *Hoxc9–Hoxc8* intergenic region (Fig. 1). The highest level of similarity (90%) occurs in the region corresponding to the mouse *Hoxc8* early enhancer sequence with all five cis-acting elements (A–E) being invariant (Fig. 2). In several regions, nucleotide-sequence similarity >70% was found in the regions

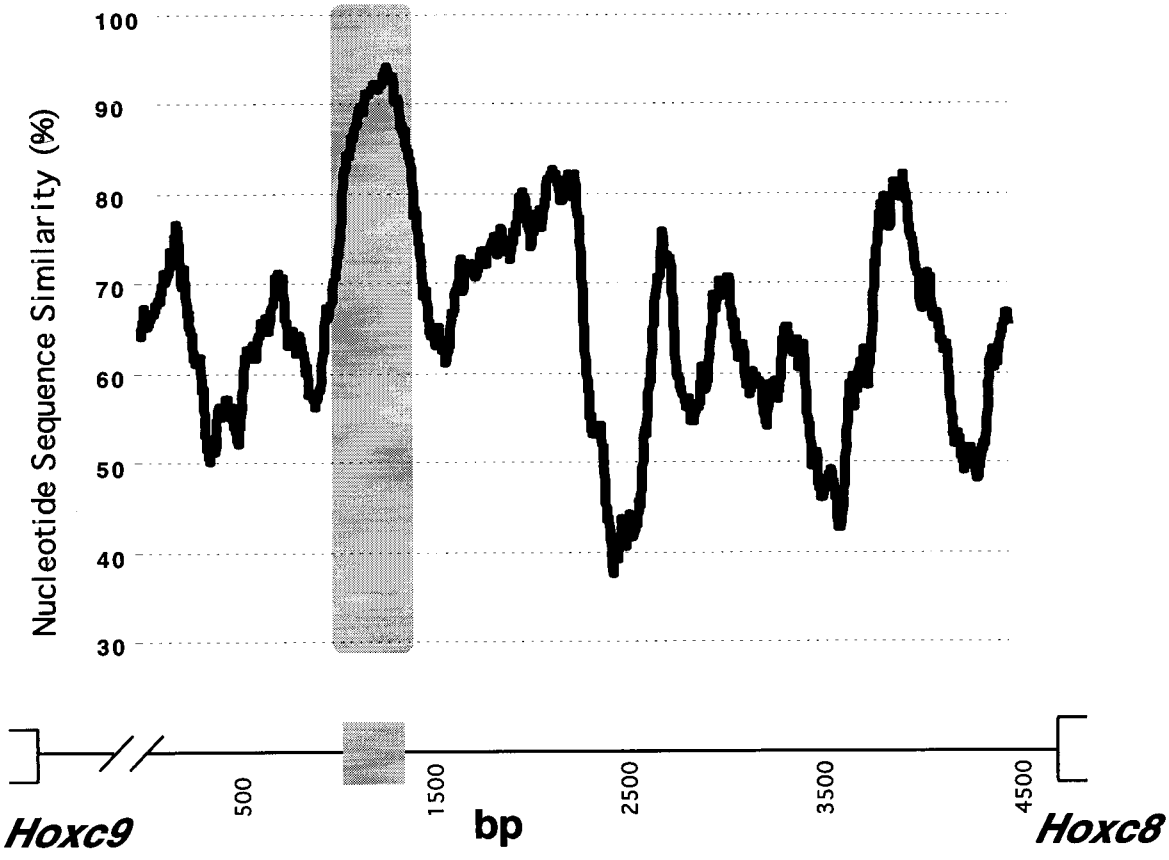

FIG. 1. Comparison of the nucleotide sequence of mouse and human *Hoxc8* genomic regions. A 4.5-kb *Hoxc9–Hoxc8* intergenic sequence flanking the early enhancer region (shaded region) was compared.

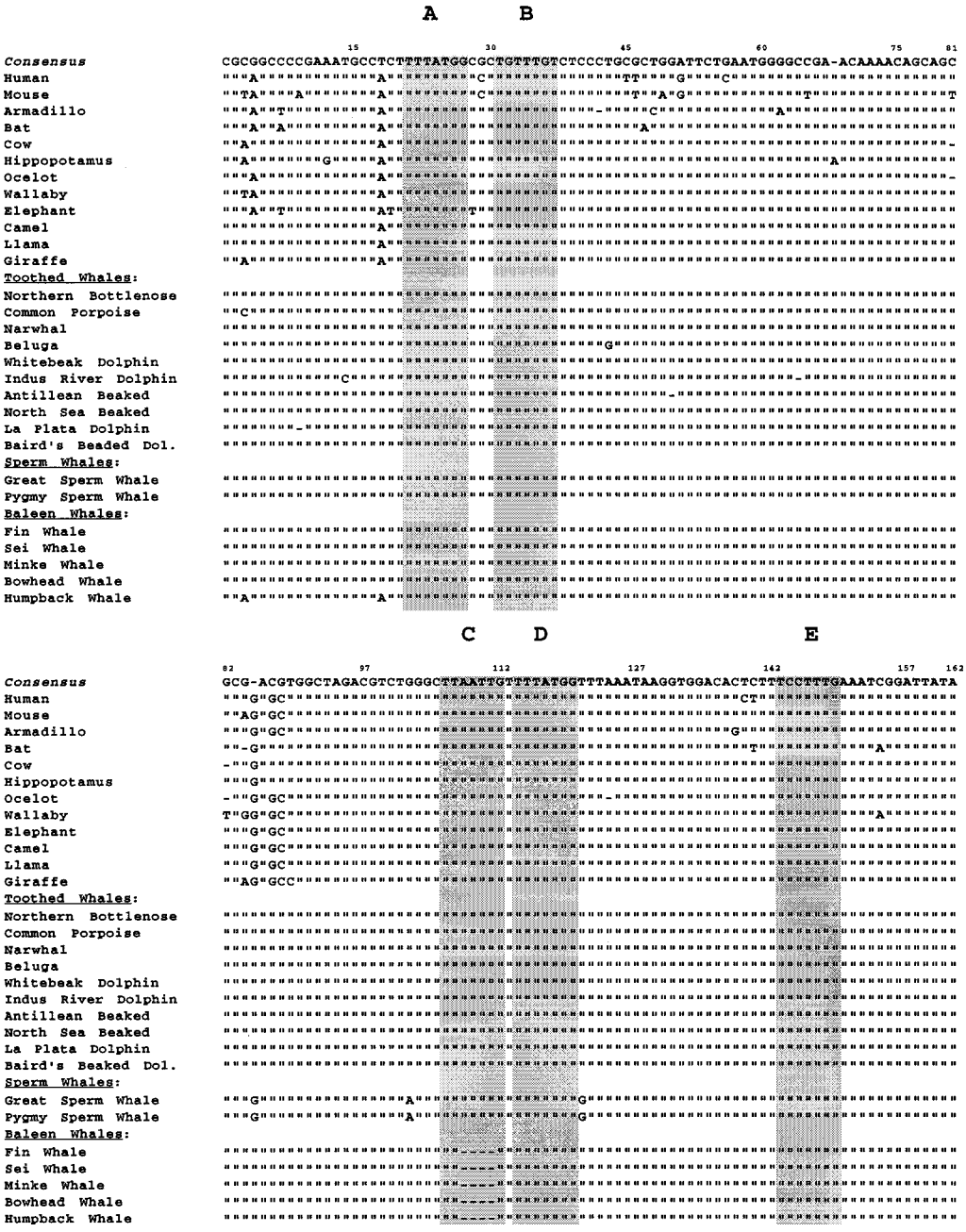

FIG. 2. Nucleotide sequences of mammalian *Hoxc8* early enhancers. Nucleotide sequences were compared against a derived consensus sequence. Similarity with the consensus sequence is denoted by quotation marks, a gap denotes a missing base, and variant bases are indicated. Shaded areas represent critical cis-acting elements (A–E) identified by transgene-mutation analysis in mouse. Scientific names of species studied are given in *Materials and Methods*.

flanking the early enhancer, although their functional significance has not yet been established.

The marked level of sequence conservation in regions flanking critical cis-acting elements of the *Hoxc8* early enhancer between mouse and human facilitated the design of PCR primers used to isolate orthologous early enhancer sequences from other mammalian taxa. PCR products isolated from 29 species belonging to 9 different mammalian orders were sequenced (Fig. 2). With the exception of the baleen whales discussed below, all other mammalian sequences examined were remarkably conserved, with all five cis-acting elements being invariant. Furthermore, 122 of 162 nucleotides (75%) were invariant, and of the 40 variant nucleotides, 20 were single substitutions or deletions (Fig. 2).

An exception to the conservation of the mammalian *Hoxc8* sequence was found in baleen whales. All five baleen whale species surveyed showed a 4-bp deletion within element C, earlier defined as a potential homeodomain binding site of the mouse *Hoxc8* early enhancer (12, 13). The presence of this deletion within a specific mammalian lineage is striking considering the high degree of sequence conservation of the *Hoxc8* region in a wide range of mammalian species surveyed in this study.

Reporter-Gene Analysis of Mammalian *Hoxc8* Enhancer Activities in Transgenic Mouse Embryos. To further investigate the putative functional significance of the baleen whale-specific deletion in element C, we compared the activities of mammalian Hoxc8 sequences as enhancers by using transgenic mouse assays (Figs. 3 and 4). The mouse *Hoxc8* early enhancer, which contains all five cis-acting elements, directs expression of a reporter gene to the neural tube, somites, and lateral-plate

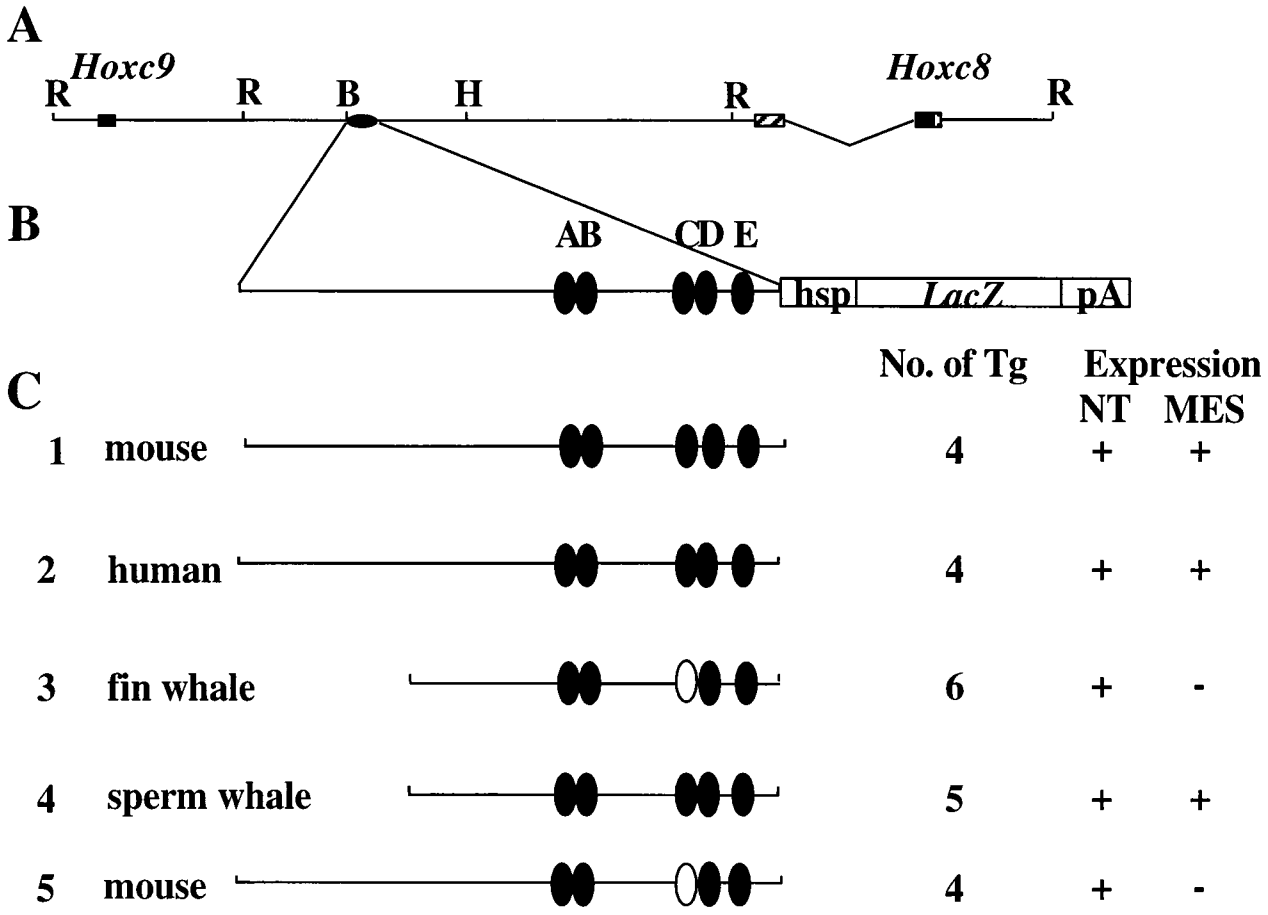

FIG. 3. Schematics of the early enhancer region and reporter gene constructs. (*A*) Partial restriction map of the *Hoxc9–Hoxc8* intergenic region. Black boxes indicate *Hoxc9* and *Hoxc8* homeoboxes. The hatched box indicates the first exon of *Hoxc8*. The black oval is the *Hoxc8* early enhancer. The design of reporter constructs is shown in *B*. Ellipses represent five cis-acting elements (A–E). The structure of reporter-gene constructs and results of transgenic analysis are shown in *C*. The first column represents number of transgenic embryos (Tg) that express the β-galactosidase reporter gene. Presence of expression (+) and absence of expression (−) is indicated in the neural tube (NT) and mesoderm (MES). R, *Eco*RI; B, *Bsp*EI; H, *Hin*dIII; hsp, mouse heat shock protein 68 promoter; pA, simian virus (SV) 40 polyadenylation signal.

mesoderm (Figs. 3 and 4*A*; ref. 13). The anterior boundary of expression in the neural tube and somites in a day 9.5 transgenic embryo is at the 14th and 18th somite levels, respectively. Human and sperm whale *Hoxc8* early enhancers, each containing an intact element C, also direct expression to the posterior neural tube and mesoderm (Fig. 4 *B* and *C*). Thus, the reporter-gene activities by mouse, human, and sperm whale enhancer sequences are comparable to one another. These findings also indicate that few changes in sequences outside of the conserved cis-acting elements (A–E) do not significantly affect expression patterns of the reporter gene in transgenic embryos. In contrast, when the construct containing the fin whale enhancer with a 4-bp deletion in element C was analyzed, the anterior boundary of expression in the neural tube was 4–5 somite levels posterior as compared with the pattern directed by mouse, human, or sperm whale *Hoxc8* enhancers. Furthermore, there was no expression of the reporter gene in the posterior mesoderm (Fig. 4*D*). To test whether the 4-bp deletion is responsible for the lack of activity in the posterior mesoderm, we introduced the 4-bp deletion into the mouse enhancer by site-specific mutagenesis. The mutated mouse enhancer showed a pattern of activity similar to that of the baleen whale enhancer (Fig. 4*E*). These results show that this 4-bp deletion effects a significant change in reporter-gene expression in the posterior mesoderm of transgenic mouse embryos. Thus, regulation of *Hoxc8* in baleen whales differs from that of other mammalian species.

DISCUSSION

Hox genes exhibit remarkable conservation among metazoans with respect to their sequence, clustered genomic organization, and collinear expression along the body axis. Yet, animals exhibit a high degree of diversity in the organization of the primary body axis. This phenomenon may be caused by modification of Hox gene expression associated with changes in their transcriptional regulatory controls. We have examined to what extent conserved cis-regulatory sequences determine early phases of *Hoxc8* expression during embryogenesis. In previous studies, noncoding regions of Hox genes of diverse organisms such as fish, chicken, and mouse were compared and highly conserved regions for potential cis-regulatory sequences were identified (7, 14–21). In contrast, we chose as a model a well-defined cis-regulatory sequence, the *Hoxc8* early enhancer, to compare closely related species belonging to a single class, Mammalia, and to look for potentially significant sequence variations. The effects of such sequence variations in cis-acting sequences were further tested in transgenic mice. For our approach, the *Hoxc8* early enhancer was ideal, as its sequence has been delimited to a small region (200 bp), and several elements (A–E) critical for expression of the reporter gene in posterior neural tube and mesoderm have been identified by mutational analyses (12, 13).

The *Hoxc8* early enhancer region can be independently identified as the region of highest level of nucleotide-sequence similarity by a comparison of human and mouse orthologous

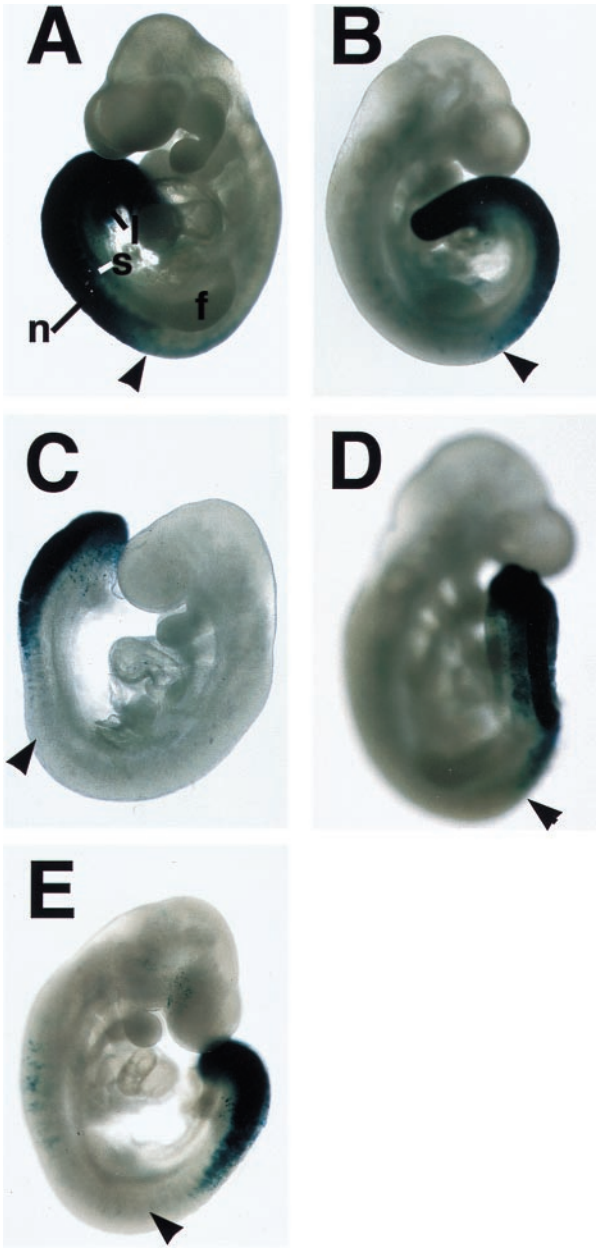

FIG. 4. Expression patterns of reporter genes in transgenic embryos. Expression of the β-galactosidase gene is detected in the posterior regions of transgenic embryos. The transgenic embryos are staged between days 9.0 and 9.5 of embryonic development. Arrows indicate position of the 14th somite. Enhancer activity of 400-bp mouse (*A*) and human (*B*) constructs are shown in the first row. Enhancer activity of 273-bp fin whale (*C*) and sperm whale (*D*) constructs are shown in the second row. Enhancer activity of a 400-bp mouse (*E*) construct carrying the 4-bp deletion of element C is shown in the third row. The structure of mammalian enhancers is described in Fig. 3. f, forelimb; n, neural tube; s, somites.

sequences. A PCR survey among mammals revealed that this conservation extends to many mammals. In light of this sequence conservation, the 4-bp deletion within element C, which is shared among all of the baleen whales included in this study, stands out as a distinctive feature. The potential functional significance of this deletion was assessed with reporter-gene assays in transgenic mouse embryos. Unlike other mammalian *Hoxc8* early enhancers, the baleen whale enhancer and the mouse enhancer into which the 4-bp deletion was introduced failed to direct expression of the reporter gene to mesoderm. Additionally, the expression in the neural tube posteriorized by several somites. We note further that the baleen whale-specific deletion overlaps with the C element (5′-TTAATTG-3′) of the early enhancer. In previous studies, we showed that replacement of the 5′-TT*AA*TTG-3′ sequence with 5′-TT*CC*TTG-3′ resulted in posteriorization of reporter-gene expression in the neural tube and reduction of expression in the mesoderm (13). Compared with this, the 4-bp deletion, 5′-TT----G-3′ shown in this report resulted in complete abolition of mesoderm activity. The difference in these results may represent differences in the type of modification (substitution vs. deletion).

The nucleotide sequence of element C is a potential binding site for interactions with homeodomain proteins (22). *Hox* genes have been implicated in cross- and autoregulatory interactions (4, 23). Hence, the deletion of a potential homeodomain binding site in baleen whales may represent a modification of the Hox transcriptional regulatory network that controls patterning in the neural tube and mesoderm.

Mice that do not have a functional *Hoxc8* gene are often viable, although homeotic transformation of the first lumbar vertebra to a thoracic vertebra and defects in brachial spinal nerves are present (24, 25). Considering the multicomponent and redundant nature of the *Hoxc8* early enhancer, a deletion of any of its cis elements critical for functioning may selectively modify *Hoxc8* expression, resulting in subtle alterations in the axial patterning. Differences in the axial organization between mouse and chicken correlate well with variations in spatial domains of *Hoxc8* expression between the two species (6, 26). The differences in mouse and chicken *Hoxc8* expression patterns are correlated with changes in their early enhancers (6). Because the baleen whale enhancer fails to direct expression in mesoderm, it is possible that the 4-bp deletion in the *Hoxc8* early enhancer could be correlated with aspects of vertebral identity in baleen whales. Whales not only show great variation in the number of thoracic vertebrae but also show modification of axial structures and appendages as a consequence of their secondary adaptation to aquatic life. However, we are unable to directly correlate the difference in baleen whale *Hoxc8* enhancer sequences with any specific morphological trait. This correlation requires an understanding of the effect of the deletion of the cis-acting sequence on *Hoxc8* expression and subsequent effect on morphological features in experimental organisms such as the mouse.

Within the order Cetacea, the relationship among baleen, toothed, and sperm whale lineages are controversial. The traditional view, supported by recent molecular data, divides extant cetaceans into two suborders, Odontoceti (toothed whales including sperm whales) and Mysticeti (baleen whales; refs. 27–29). In contrast, other molecular studies suggest that sperm whales are more closely related to baleen whales (30, 31). The characteristic deletion in the *Hoxc8* early enhancer occurring only among baleen whales does not necessarily support the inclusion of sperm whales in this group. The origin of the order Cetacea has been the subject of many investigations. Recent molecular evidences suggest that Cetaceans are monophyletic with Artiodactyla (32–37). Members of the order Artiodactyla surveyed in this study (cow, camel, llama, hippopotamus, and giraffe) do not show any significant variations in their *Hoxc8* early enhancer sequences.

The high degree of nucleotide sequence conservation of the *Hoxc8* enhancer region provides few informative positions for a phylogenetic analysis. Nucleotide sequences of regions that immediately surround the early enhancer region may have utility in addressing phylogenetic questions within Mammalia. In a number of studies, mitochondrial and nuclear genes containing a large number of informative positions have proven useful for resolving phylogenetic branching arrangements (for examples, see refs. 37–43). We, however, recognize the potential of enhancer sequences as useful phyletic molecular markers, as even minor variations in enhancer regions are

able to modify the expression of the regulated gene. Such variations in *Hox* gene enhancers have the potential to modify developmental programs and thus contribute significantly toward knowledge of the evolution of mammalian species.

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