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A long-range regulatory element of *Hoxc8* identified by using the pClasper vector

(yeast gap repair/homologous recombination/transgenic analysis/homeobox genes/cluster regulation)

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ABSTRACT **Hox genes are located in highly conserved clusters. The significance of this organization is unclear, but one possibility is that regulatory regions for individual genes are dispersed throughout the cluster and shared with other Hox genes. This hypothesis is supported by studies on several Hox genes in which even large genomic regions immediately surrounding the gene fail to direct the complete expression pattern in transgenic mice. In particular, previous studies have identified proximal regulatory regions that are primarily responsible for early phases of mouse *Hoxc8* expression. To locate additional regulatory regions governing expression during the later periods of development, a yeast homologous recombination-based strategy utilizing the pClasper vector was employed. Using homologous recombination into pClasper, we cloned a 27-kb region around the *Hoxc8* gene from a yeast artificial chromosome. A reporter gene was introduced into the coding region of the isolated gene by homologous recombination in yeast. This large fragment recapitulates critical aspects of *Hoxc8* expression in transgenic mice. We show that the regulatory elements that maintain the anterior boundaries of expression in the neural tube and paraxial mesoderm are located between 11 and 19 kb downstream of the gene.**

Hox genes are arranged in highly conserved clusters (1). The cluster organization has several striking features (2, 3). The position of genes within the cluster is colinear with the position of gene expression along the anteroposterior axis (spatial colinearity), such that the 3′ most genes have a more anterior limit of expression. Similarly, the 3′ genes are activated earliest in development (temporal colinearity) and are more sensitive to the effects of retinoic acid. This organization and order of genes along the cluster has been maintained over long periods of evolutionary time. The significance of cluster organization is unclear, but one possibility is that regulatory regions are widely dispersed, therefore requiring the cluster to be intact to establish the correct spatial and temporal expression pattern of individual Hox genes. Regulatory elements controlling the endogenous expression pattern have been described for a few Hox genes (4–6). However, for many Hox genes, including *Hoxc8, Hoxb6, Hoxb7*, and *Hoxb8* (7–10), extensive analysis of genomic regions surrounding the gene have failed to identify all of the elements required for endogenous gene expression. For instance, in the case of *Hoxb7*, >27 kb of the genomic region surrounding the gene has been assayed by transgenic mouse analysis but has failed to recapitulate the entire endogenous expression pattern, suggesting that larger regions of the cluster are required (10).

Similarly, only part of the endogenous pattern of mouse *Hoxc8* expression has been recapitulated by transgenic analysis (8, 11). *Hoxc8* expression can be divided into two phases: an early phase between 8.0 and 10.0 days postcoitum (d.p.c.) and a late phase after 10.5 d.p.c. (12–14). The early phase is characterized by the establishment (establishment phase) of anterior boundaries of expression in the neural tube and paraxial mesoderm. These boundaries are maintained (maintenance phase) during later periods of development. In the late phase, the anterior boundaries of expression remain constant, while expression becomes restricted to specific cell types. In previous studies, we have characterized elements that control the establishment phase of expression but do not regulate later expression patterns (8, 11).

We have used a different approach to identify additional regulatory elements for *Hoxc8* expression (15). This approach employs homologous recombination into the yeast/bacteria shuttle vector pClasper to create large reporter constructs for transgenic analysis. Using this strategy, we identified a regulatory region responsible for the maintenance of *Hoxc8* expression boundaries in the neural tube and paraxial mesoderm. We show here that regulatory regions responsible for maintaining the anterior boundaries of *Hoxc8* expression are located 11–19 kb downstream of the *Hoxc8* start site. This strategy allows us to efficiently assay large regions of the Hoxc cluster for enhancer activity, thus increasing the possibility of identifying regulatory regions acting over a long distance. We believe this strategy will be useful in the analysis of complex gene clusters.

MATERIALS AND METHODS

Construction of the *Hoxc8* Transgene. pClasper is described elsewhere (15) (GenBank data base accession no. U38900). The targeting vector and cloning of the 27 kb *Hoxc8* insert to create recombinant pC827 is also described in detail elsewhere (15). The *lacZ/URA3* vector, pLZURA, was constructed in the following manner. The promoter- and start-site-deficient *lacZ* gene was isolated from pMC1871 (Pharmacia Biotech) by digestion with *Pst* I and *Sac* I. The 3′ end of the gene was replaced by the 3′ end of *lacZ*/SV40 poly(A) addition signal on a *Sac* I restriction fragment from the vector pLZRVA (8). Both fragments were inserted into pSafyre (8), previously digested with *Sac* I and *Sal* I, to create pLZFSV. The *URA3* gene was isolated on a 1.6-kb *Ssp* I fragment from pRS406 (Stratagene) and ligated 3′ to the *lacZ* gene in pLZFSV previously digested with *Xba* I and blunt-ended with the Klenow fragment of DNA polymerase I, to create pLZURA.

Homologous regions from *Hoxc8* for the *lacZ* insertion vector were made by PCR. Primers designed to hybridize with the second exon of *Hoxc8* amplified 257 bp from position 8652

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Abbreviations: YAC, yeast artificial chromosome; d.p.c., days postcoitum.

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to 8909 (GenBank data base accession no. M35603) and contained *Xho* I and *Eco*RV recognition sites for cloning. Primers designed to hybridize with the 3′ untranslated region of *Hoxc8* amplified 438 bp from position 9557 to 9995 and contained *Not* I and *Sal* I restriction sites. The exon 2 PCR product was fused in-frame to the *lacZ* gene at the *Sma* I restriction site of vector pLZURA. The 3′ PCR product was inserted in the Not I site downstream of the *URA3* gene in the same vector. The resulting insertion vector was digested with *Sal* I to release the *lacZ/URA3* cassette flanked by the recombinogenic ends, which was used to transform a *Saccharomyces cerevisiae* strain carrying pC827 (15). Transformants were selected on drop-out medium lacking leucine and uracil. Leu^+, Ura^+ colonies containing the homologous recombination event were identified by whole-cell PCR by using primers to the *lacZ* gene and verified by Southern blot analysis. The resulting construct is designated pC8-LZ.

Transgenic Analysis. pC8-LZ was digested to completion with an intron-encoded endonuclease, I-*Ppo* I (New England Biolabs), to release the vector from the insert. The 32-kb insert (construct C8-LZ) was separated from the vector by centrifugation through a sucrose gradient (16) for 9 h, then dialyzed against microinjection buffer (10 mM Tris·HCl, pH 7.5/0.25 mM EDTA) for 48 h. A total of 3.1 μg of DNA per ml was used for microinjection. For construct C8-SfiLZ, pC8LZ was double digested with *Sfi* I and I-*Ppo* I before being loaded on the sucrose gradient. Transgenic mice were produced and stained for β-galactosidase activity as described (8, 11). Staining of whole mount embryos with Hoxc8 antibody is described elsewhere (11).

RESULTS

Recombinational Cloning of a 27-kb *Hoxc8* Reporter. Our aim in this study was to reconstitute the complete mouse *Hoxc8* expression pattern in transgenic mice. To do this, we required large genomic regions of the cluster surrounding *Hoxc8*. Rather than attempt to insert multiple large DNA fragments into a reporter construct, we developed a method to clone specific, large genomic regions from yeast artificial chromosomes (YACs) into which a reporter gene can then be introduced. By using this method, large genomic regions can then be assayed in transgenic mice with minimal manipulation of the gene.

To clone large well-defined fragments from YACs, we designed a yeast/bacteria shuttle vector, pClasper, based on yeast gap-repair (15, 17). In this method, free ends of a replicating plasmid recombine with homologous regions in a YAC resulting in a circular product containing a copy of the region between the recombinogenic ends (Fig. 1). The resulting circular product can then be modified by homologous recombination in yeast and shuttled to bacteria for preparation of large quantities of plasmid DNA. The salient feature of pClasper (15) is the presence of the mini-F factor replicon for stable maintenance of very large inserts (>300 kb) in bacteria (19).

We used this recombination-based strategy to clone a 27-kb region surrounding *Hoxc8* from a 440-kb Hoxc YAC (18). To do this, PCR-generated recombinogenic ends were designed for pClasper to be homologous to the untranslated regions of the flanking genes, *Hoxc9* and *Hoxc6* (Fig. 1). The resulting targeting vector, pClC9C6, was transformed into the yeast strain YLC44-5 carrying the Hoxc YAC. The resulting circular homologous recombinant, pC827, contains a 27-kb *Hoxc8* insert (15). To construct a reporter for transgenic analysis, we introduced the *lacZ* gene into this recombinant by homologous recombination in yeast (Fig. 2). First, an insertion vector, pLZURA, containing a *lacZ/URA3* cassette in which the first eight amino acids of *lacZ* were deleted was made. Next, 257 bp of the second exon of *Hoxc8* was fused in-frame to the *lacZ* gene. Then, 438 bp of the 3′ untranslated region of *Hoxc8* was inserted downstream of the *URA3* gene. After transformation of yeast carrying pC827 with this insertion construct, colonies were selected for the acquisition of the URA3 phenotype and correct recombinants were identified by PCR and Southern analysis (data not shown). The recombinant with *lacZ* inserted in-frame in *Hoxc8*, was transferred to bacteria for the production of plasmid DNA for microinjection. Restriction enzyme digestion with I-*Ppo* I was used to remove all vector sequences, and the resulting 32-kb construct, C8-LZ (Fig. 2), was used to generate transgenic mice by pronuclear microinjection.

C8-LZ Recapitulates the Maintenance Phase of *Hoxc8* Expression. We analyzed the expression of C8-LZ first in founder generation embryos at 10.5 d.p.c. (data not shown). β-galactosidase expression was observed in both the neural tube and mesoderm in the posterior region of the embryo. The anterior boundary of expression in these tissues was comparable to that of endogenous Hoxc8. For detailed analysis we generated permanent transgenic lines.

We examined the expression of the C8-LZ reporter gene in the permanent lines at three developmental stages from 8.5 to

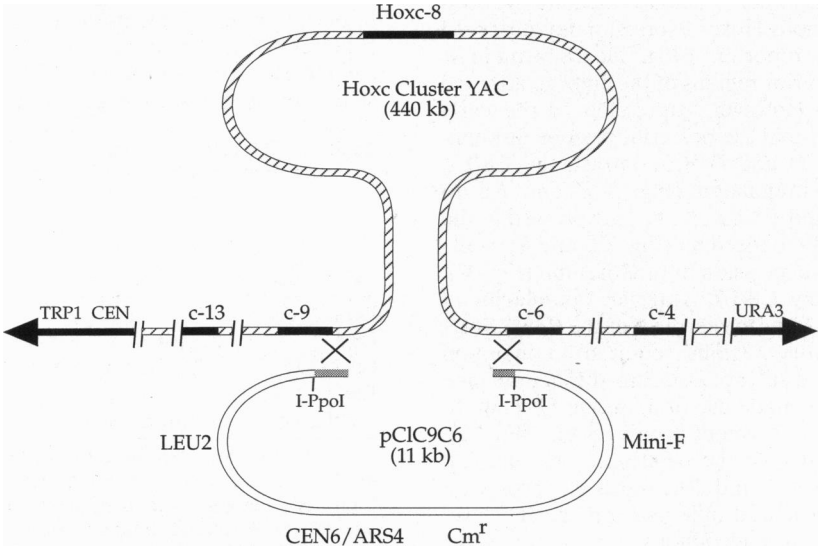

FIG. 1. Schematic of homologous recombination between the targeting vector and a Hoxc YAC. Top hatched line represents part of a 440-kb Hoxc YAC (yH3-44) (18). Black boxes represent Hox genes. Arrows represent YAC arms. Unfilled lines on bottom represent pClasper with recombinogenic ends (gray boxes) homologous to *Hoxc9* and *Hoxc6* creating targeting vector pClC9C6.

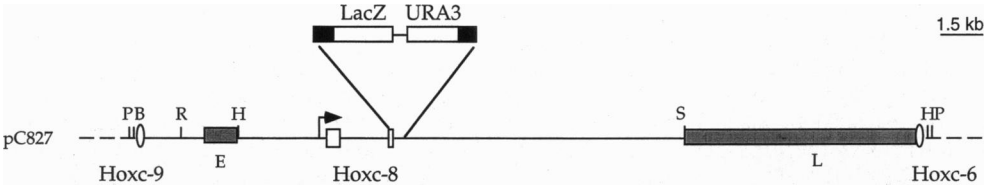

FIG. 2. Schematic of 27-kb genomic region isolated by homologous recombination. Regions of *Hoxc9* and *Hoxc6* used as recombinogenic ends are shown (ovals). *Hoxc8* exons (boxes) and start site (arrow) are shown. Gray boxes indicate the early (E) and late (L) regulatory regions. Dotted lines are vector sequences. The unique restriction sites are P, I-*Ppo* I and S, *Sfi* I. Restriction sites used for cloning are as follows: B, *Bam*HI; H, *Hin*dIII; and R, *Eco*RI. The insertion site of the *lacZ/URA3* cassette is shown. Black boxes represent homologous regions to the first exon and 3′ untranslated region of *Hoxc8*.

10.5 d.p.c. (Fig. 3 *D–F*). The expression of the reporter construct was compared with that of Hoxc8 protein as detected by monoclonal antibody staining (Fig. 3 *A–C*) and to the previously reported early enhancer reporter (8, 11) (Fig. 3 *G–I*). Hoxc8 protein is first detected at 8.0 d.p.c. in the posterior region of the embryo. At 8.5 d.p.c., both the C8-LZ and early enhancer reporters showed identical expression patterns to that of Hoxc8 protein, reflecting the establishment phase of *Hoxc8* expression (Fig. 3 *A, D,* and *G*). At 9.5 d.p.c., the anterior boundary of Hoxc8 protein is detected in the neural tube at the level of the 9th somite and in the somitic mesoderm at the level of the 13th somite (Fig. 3*B* and Table 1). In contrast, the anterior boundary of expression of the early enhancer reporter is five somites posterior to the Hoxc8 protein in both the neural tube and somites (Fig. 3*H* and Table 1), indicating that this reporter lacks information required for the maintenance of the anterior boundaries. The C8-LZ reporter is expressed more anterior to the early enhancer reporter at the level of the 10th and 14th somites in the neural tube and somites, respectively (Fig. 3*E* and Table 1). Thus, the C8-LZ reporter contains additional regulatory regions for the maintenance of the anterior boundaries. The difference in the anterior boundaries between the two reporters becomes more pronounced at 10.5 d.p.c. (Fig. 3 *C, F,* and *I*). C8-LZ and Hoxc8 protein maintain similar anterior boundaries in the spinal cord and somites, while the early enhancer reporter begins to lose expression at its anterior limits and regresses to a more posterior position. Taken together, these results indicate that while both reporter constructs contain the information to establish *Hoxc8* expression, C8-LZ contains additional information that controls the maintenance of expression at the anterior boundaries in the neural tube and somites.

The larger C8-LZ reporter construct also recapitulates two further aspects of endogenous Hoxc8 expression not observed with the early enhancer reporter. First, Hoxc8 protein is downregulated in the posterior regions of the embryo after 9.0 d.p.c. (Fig. 3 *B* and *C*). However, expression of the early enhancer reporter is intense in the posterior regions throughout development (Fig. 3 *H* and *I*). In contrast, the C8-LZ reporter is partially downregulated (Fig. 3 *E* and *F*). In addition, Hoxc8 protein and C8-LZ are both expressed in the apical ectodermal ridge of the forelimb (Fig. 3 *C* and *F*), while the early enhancer has no expression in the forelimb (Fig. 3*I*).

The Region Maintaining C8-LZ Anterior Boundaries of Expression Is Located >11 kb Downstream of the *Hoxc8* Start Site. To localize regulatory regions required to maintain C8-LZ anterior boundaries of expression in the neural tube and somitic mesoderm, we made use of a unique *Sfi* I site to delete the 3′-most 8.5-kb fragment from C8-LZ (Fig. 2). Transgenic mice produced with the deletion (construct C8-SfiLZ) expressed *lacZ* in 9.5- and 10.5-d.p.c. embryos with anterior boundaries in the neural tube and somites at a level five somites posterior to the endogenous gene at this stage (Fig. 3 *K* and *L*; Table 1). The expression pattern of the deletion is identical to that seen for the early-enhancer reporter construct. In addition, there is no downregulation of expression in the posterior regions of the embryo, nor is there any expression in the forelimb apical ectodermal ridge. These results indicate that the regulatory regions required to maintain the correct anterior boundaries of late *Hoxc8* expression in the neural tube, somites, and forelimb are contained in the deleted 8.5-kb 3′ fragment. Thus, we have located a late regulatory element 11–19 kb downstream of the transcriptional start site of *Hoxc8*.

DISCUSSION

We have used a 27-kb genomic fragment cloned by homologous recombination from a YAC to recapitulate significant aspects of *Hoxc8* expression. Fig. 3*J* summarizes the expression pattern of the Hoxc8 protein and the three reporter constructs in 9.5-d.p.c. embryos. Previous studies have identified an early enhancer located 3 kb upstream of the start site that contains regulatory regions for the establishment of *Hoxc8* gene expression but does not recapitulate the complete endogenous pattern (11). Here, we have shown that a 27-kb C8-LZ genomic fragment is more faithful to the endogenous pattern. In particular, this large reporter recapitulates the maintenance phase of Hoxc8 expression. In contrast to the early-enhancer reporter construct, the C8-LZ reporter construct directs *lacZ* expression at more anterior boundaries in the neural tube, somites, and mesoderm after day 9.5 d.p.c. The anterior limit of expression is about one somite posterior to that of the Hoxc8 protein. Studies on other Hox genes have shown *lacZ* constructs with anterior boundaries of expression that are also slightly posterior to that of the endogenous gene (7, 10). Downregulation of expression in the posterior region does occur with the large C8-LZ reporter, but it is slower and less complete than the downregulation of Hoxc8. This may be due, in part, to a difference in the stability of the Hoxc8 and lacZ proteins. Several studies on Hox gene regulation have reported similar deficiencies in down-regulation of *lacZ* reporter constructs in the posterior region of the embryo (7, 20, 21). We

Table 1. Reporter constructs used to generate transgenic mice

Construct	R. E.	Size, kb	T. G., *n* T	T. G., *n* P	*lacZ* expression NT	*lacZ* expression Som/Mes
Hoxc8*					9	13
C8-LZ	I-*Ppo* I	32	1	2	10	14
C8-SfiLZ	I-*Ppo* I, *Sfi* I	23.5	15	0	14	18
Early enhancer†	*Bsp*EI, *Hin*dIII	1	5	4	14	18

Name of construct is indicated with restriction enzyme(s) (R. E.) used to release it from pC8-LZ. T. G. is number of transient (T) transgenic embryos and permanent (P) β-galactosidase-expressing lines analyzed. Anterior boundary of *lacZ* expression at 9.5 d.p.c. in the neural tube (NT) and somites and mesoderm (Som/Mes) is indicated by somite number.

*Hoxc8 protein detected by monoclonal antibody.

†Early-enhancer fragment is fused to *hsp68/lacZ* cassette.

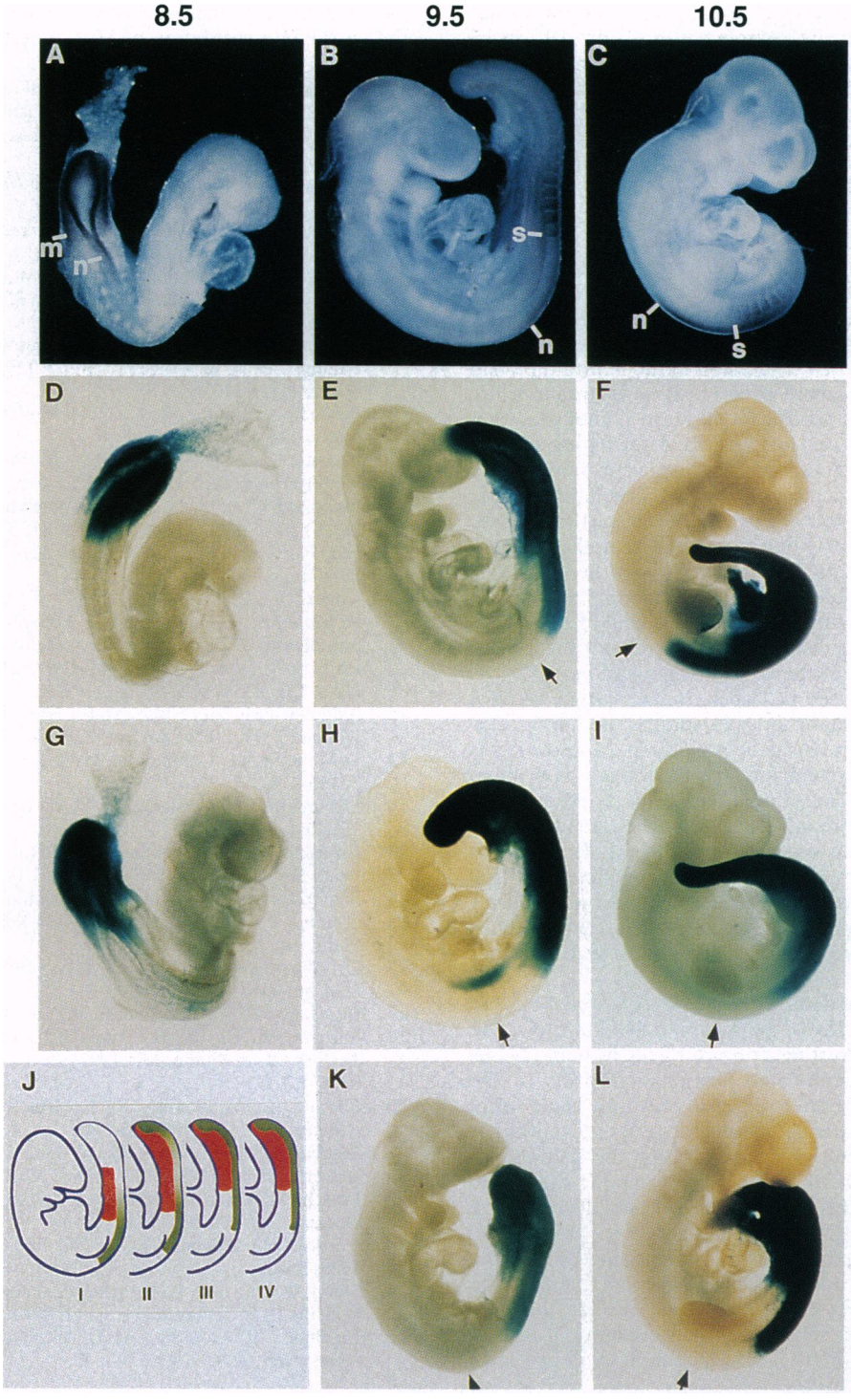

FIG. 3. *Hoxc8* and transgene expression in whole-mount embryos. Developmental stage of embryos is indicated (in d.p.c.) above each column. (*A–C*) Hoxc8 protein detected by monoclonal antibody staining. The transgenes are as follows: (*D–F*) full-length 32-kb construct C8-LZ detected in a permanent line; (*G–I*) 1-kb early enhancer/*hsp68*/*lacZ* reporter construct detected in a permanent line (8); (*K* and *L*) deletion of downstream 8.5-kb *Sfi* I fragment of C8-LZ to create construct C8-SfiLz detected in transient embryos. Anterior boundary of expression is indicated in the neural tube (n), mesoderm (m), and somites (s) in the antibody stained embryos (*A–C*). The same somite level indicated for the neural tube anterior boundary in the antibody stained embryos (somite 10) is indicated by an arrow in the *lacZ*-stained embryos as a reference point. The forelimb β-galactosidase staining in *H* is ectopic and transgenic-line dependent. Staining with Hoxc8 monoclonal antibody and detection of β-galactosidase activity was as described (8, 11). (*J*) Schematic representation of *Hoxc8* and reporter construct expression in 9.5-d.p.c. embryos. I, Hoxc8 protein; II, C8-LZ reporter construct; III, early-enhancer reporter construct; and IV, *Sfi* I-fragment deletion of C8-LZ (C8-SfiLZ) reporter construct. Somite expression is indicated in red. Neural tube expression is indicated in green. The relative quantity of expression is indicated by the relative amounts of green shown.

conclude that this 27-kb fragment is missing additional regulatory regions required for the expression of the complete endogenous pattern.

In a few instances, the endogenous Hox expression pattern appears to be to be completely reconstituted in transgenic mice when using relatively small genomic fragments (4, 6). For the

majority of Hox genes analyzed to date, the authors do not claim to have identified the complete expression pattern by transgenic mouse analysis even when large genomic regions have been surveyed for regulatory elements in transgenic mice (5, 22–24). For example, 15 kb and 27 kb of genomic region was analyzed for *Hoxb6* and *Hoxb7*, respectively (9, 10). In both instances, the complete endogenous expression patterns were not reconstituted. In addition, there is already good evidence that enhancers may be shared between several Hox genes (4, 24). This lends support to the hypothesis that the conserved cluster organization is involved in gene regulation. The techniques using the pClasper vector described here are useful for addressing the problem of cluster regulation.

We have localized regulatory regions that maintain the anterior boundary of *Hoxc8* expression in the neural tube and somites in an 8.5-kb fragment over 11 kb downstream of the start site. This result is significant because it indicates that long-range interactions are involved in determining correct expression boundaries. These interactions occur between the proximal cis-acting elements and the late regulatory region shown here to be 11–19 kb downstream of the start site. These results also raise the intriguing possibility that the late enhancer may be shared with *Hoxc6* since it is in the vicinity of its promoter region. *Hoxc6* has an overlapping expression domain with *Hoxc8* but with a more anterior limit of expression (25). As discussed above, dispersed and shared enhancers may be an important aspect of the cluster organization.

The recombination-based pClasper cloning system we have used here provides an opportunity to address the importance of cluster organization in Hox gene regulation. Moreover, it is a generally applicable system for functional analysis of large genes or gene clusters. This strategy relies on an extension of gap-repair, which is commonly used for allelic rescue of a few hundred base pairs from yeast chromosomes (17). We have improved the method to isolate much larger sequences (1–200 kb) from YACs. We have demonstrated that we can capture a 27-kb DNA fragment by using this strategy. These studies have been extended to capture regions of 60 and 130 kb from other YACs (15).

In this report, we have demonstrated the isolation of a 27-kb region surrounding the *Hoxc8* gene from a 440-kb YAC. The gene was modified by inserting a reporter gene by homologous recombination and recapitulated significant aspects of the endogenous expression pattern of *Hoxc8* in transgenic mice. These analyses can be extended to include progressively larger domains of the Hoxc cluster by substituting the recombinogenic ends in pClasper with homologous genomic sites more distant from *Hoxc8*. In this way, regulatory elements which are still missing from the 27-kb fragment can be assayed. In addition, shared and separate enhancers contained within the cluster can be dissected. Alternatively, shorter fragments of the 27-kb reporter construct can be made to localize the late-enhancer region without relying on the availability of restriction sites or long-range PCR. These techniques will be useful for the continued analysis of the role of cluster organization in the coordinate regulation of Hox genes.

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