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Combinations of closely situated cis-acting elements determine tissue-specific patterns and anterior extent of early *Hoxc8* expression

(mouse/neural tube/mesoderm/enhancer/transgenic/β-galactosidase reporter)

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ABSTRACT **We have used a transgene mutation approach to study how expression domains of *Hoxc8* are established during mouse embryogenesis. A cis-regulatory region located 3 kb upstream from the *Hoxc8* translational start site directs the early phase of expression. Four elements, termed A, B, C, and D, were previously shown to direct expression to the neural tube. Here we report that a fifth element, E, located immediately downstream of D directs expression to mesoderm in combination with the other four elements. These elements are interdependent and partially redundant. Different combinations of elements determine expression in different posterior regions of the embryo. Neural tube expression is determined minimally by ABC, ABD, or ACD; somite expression by ACDE; and lateral plate mesoderm expression by DE. Neural tube and lateral plate mesoderm enhancers can be separated, but independent somite expression has not been achieved. Furthermore, mutations within these elements result in posteriorization of the reporter gene expression. Thus, the anterior extent of expression is determined by the combined action of these elements. We propose that the early phase of *Hoxc8* expression is directed by two separate mechanisms: one that determines tissue specificity and another that determines anterior extent of expression.**

Homeobox genes specify positional information during embryonic development in diverse organisms. In mouse, there are four clusters of homeobox genes, termed *Hox* genes, which share a high degree of sequence, architectural and functional similarity with the *Drosophila* HOM-C genes (1). *Hox* genes specify positional identities along the anteroposterior embryonic axis. Each *Hox* gene is expressed in a characteristic pattern along this axis, with distinct anterior limits in both neurectoderm and mesoderm. *Hox* gene expression is regulated both spatially and temporally during embryonic development (1). We and others (2–9) have previously studied the region-specific expression and function of mouse *Hoxc8*. The expression of *Hoxc8* can be divided into an early or "establishment" phase and a late or "maintenance" phase. At 8.5 days postcoitum (d.p.c.), *Hoxc8* endogenous expression extends from the base of the allantois to the segmental plate mesoderm and to a more anterior region in the neurectoderm. The anterior boundary of *Hoxc8* at 9.5 d.p.c. is located in the neural tube at the level of the 9th somite, in the paraxial mesoderm at the 14th somite, and in the lateral plate mesoderm (LPM) at the 12th somite. Later in development, posterior expression of *Hoxc8* decreases, while intense expression is maintained within the brachial region of the neural tube.

To address the question of how *Hoxc8* expression is controlled, we have undertaken an extensive characterization of its cis-regulatory regions. Employing reporter gene analysis in transgenic mice, we have identified two distinct genomic regions that regulate the early and the late phase of *Hoxc8* expression (10–12). The early expression is regulated by elements present ≈3 kb 5′ from the translation start site of the gene, whereas the late expression is regulated by elements present 11–20 kb downstream of the start site (11, 12). At 9.5 d.p.c., the early *Hoxc8* transgene enhancer directs expression to posterior regions of the embryo with anterior boundaries in the neural tube at the 14th somite, in the paraxial mesoderm at the 18th somite, and in the LPM at the 20th somite. Our previous analysis of the early neural tube (ENT) enhancer revealed that four distinct elements, designated A, B, C, and D, located within a 135-bp region, determine posterior neural tube expression in an interdependent manner (11). We have now extended these studies to identify cis-acting sequences directing expression to posterior somites and the LPM. We demonstrate that different combinations of elements direct expression to the neural tube, somites, and the LPM.

MATERIALS AND METHODS

Standard subcloning procedures were used for generating constructs for microinjection (13). Construct 1 has been described (11). A 0.6-kb *Bsp*EI–*Fsp*I fragment (see Fig. 1 for restriction map) was cloned by ligation at *Sal*I and *Sma*I sites present in the polylinker sequence of pHSF (11). Remaining constructs were generated by PCR, cloned, sequenced, and prepared for microinjection as described (11). The nucleotide sequence of the 399-bp *Hoxc8* early enhancer region is shown in Fig. 3 (14). The position of the nucleotides included in the constructs is shown in Fig. 1 (constructs 4–10). Alterations in the 399-bp reporter construct were introduced by an overlapping PCR strategy, using synthetic oligonucleotide primers containing appropriate changes in the nucleotide sequence (11). The changes introduced are indicated in the legend to Table 1. Production of transgenic embryos, Southern blot analysis, and detection of β-galactosidase activity have been described (11).

RESULTS

Regulatory Elements Specific for Mesoderm Expression Lie Adjacent to the ENT Enhancer. Our previous studies identified a 1.0-kb DNA fragment from the *Hoxc8* locus (Fig. 1, construct 1) that was capable of directing a pattern of reporter gene expression in the posterior neural tube and mesoderm (11). Two nonoverlapping fragments, a 335-bp *Bsp*EI–*Dra*I fragment and a 0.7-kb *Dra*I–*Hin*dIII fragment (Fig. 1, construct 2) were tested for enhancer activity. Whereas the 335-bp *Bsp*EI–

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Abbreviations: LPM, lateral plate mesoderm; d.p.c., days postcoitum; ENT, early neural tube.

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Table 1. Mutational analyses of the *Hoxc8* early enhancer region

Construct no.	Sites mutated	No. of transgenic embryos expressing *lacZ*	Expression		
			Neural tube	Somites	LPM
6	—	4	+++	+++	+++
11	E1	3	++	−	−
12	E2	3	++	−	−
13	A	3	+	−	+++
14	B	6	+++	+++	+++
15	C	3	+	+	+++
16	D	8	++	−	−
17	B, C	4	−*	−	++
18	B, D	2	−*	−	−
19	C, D	3	−*	−	−
20	A, D	2	−	−	−

β-Galactosidase activity in transgenic embryos carrying constructs 6 and 12–19 are shown in the Fig. 2. Mutations introduced at sites A and D: TTTATGG to TTGCGGG; B, CTGTTTGT to CTGGGGGT; C, TTAATT to TTCCTT; and E, CCTTTGA to CCGGGGA (E1) and to AATTTCA (E2). +++, Strong expression; ++, moderate expression; +, weak expression; and −, no expression.
*Restricted weak expression in the caudal neuropore.

*Dra*I fragment (ENT enhancer) directed expression to the posterior neural tube, neither construct directed expression to the posterior mesoderm (11). These initial studies suggested that mesoderm-specific expression relied on an interaction between cis-acting sequences present in the two fragments. Therefore, we examined this possibility by generating a series of deletions in the 1.0-kb *Bsp*EI–*Hin*dIII fragment. Constructs 2–8, carrying progressive deletions from the 3′ end, directed posterior neural tube and mesoderm expression (Figs. 1 and 2). Reporter gene expression in the transgenic embryos carrying a 399-bp fragment (Fig. 1, construct 6; and Fig. 2*A*) was similar to that observed with larger constructs (11). An intense staining for β-galactosidase activity was observed in caudal regions of the 9.5-d.p.c. embryos. Anterior boundaries of expression in the neural tube and mesoderm were at somite levels 14 and 18, respectively. This construct was employed as a minimal *Hoxc8* early enhancer for introducing mutations at specific sites described below.

Further deletions led to identification of mesoderm-specific elements. A shorter construct that contained a 365-bp fragment still directed expression to the posterior neural tube and mesoderm (Fig. 1, construct 8; and Fig. 2*B*). However, construct 9, which contained a 350-bp fragment (Fig. 1), failed to direct somite expression (Fig. 2*C*). The neural tube expression remained unaffected. Thus, an essential component of mesoderm expression lies within the overlapping regions of nucleotide positions 350–365 (Fig. 3). An examination of this nucleotide sequence revealed potential binding sites for high mobility group proteins (15, 16). This sequence, CCTTTG, designated as element E, when mutated to CCGGGG or to AATTTC (Fig. 1, constructs 11 and 12) resulted in loss of mesoderm expression in transgenic embryos (Fig. 2*D*, construct 12; construct 11 not shown). The reporter gene expression was confined to the neural tube with its anterior boundary relatively posterior to that observed with construct 6. These results delineate a fifth site that is important for early *Hoxc8* expression.

Early Mesoderm Expression Requires Elements of the ENT Enhancer. Mesoderm expression of the transgene was not achieved independent of the ENT enhancer, indicating that some of the components of the ENT enhancer contribute to mesoderm expression. To delineate the role of elements A, B, C, and D of the ENT enhancer in directing the mesoderm expression, we introduced mutations at these sites both individually and in combination. The results of these mutations are summarized in Table 1. These mutations described below had varying effects on mesoderm expression.

Sites A and D contain a similar core sequence, TTTATGG, which is a potential binding site for caudal-related proteins (17, 18). To determine the importance of these sequences for mesoderm expression, three nucleotide substitutions were introduced in sites A and D (TTTATGG to TTGCGGG) individually and in combination in the 399-bp reporter (construct 6). Nucleotide substitution at site A (Table 1, construct 13) resulted in greatly reduced expression in the neural tube and somites (Fig. 2*E*). Neural tube expression was found in extreme caudal regions, with an anterior boundary at 22–23 somite levels in 25-somite stage embryos. In these embryos, somite expression was not observed. However, expression in the LPM was unaffected. In contrast, similar mutations at site D (Table 1, construct 16) resulted in complete abolition of expression in somites and the LPM (Fig. 2*H*). Neural tube expression was observed at somite levels 18–19, ≈4–5 somite levels posterior to that observed with the 399-bp reporter. Therefore, although sites A and D are identical in their core sequences, they are functionally distinct. When nucleotide substitutions were introduced at both sites A and D (Table 1, construct 20), no reporter gene expression was observed in the posterior neural tube and mesoderm (data not shown).

Site B contains a potential binding site, CTGTTTGT, for fork head-related proteins (19–21). Three nucleotide substitutions were introduced in the core sequence (CTGTTTGT to CTGGGGGT) in the 399-bp reporter (Table 1, construct 14). Individual mutations at site B had no discernible effect on the reporter gene expression (Fig. 2*F*). The reporter gene activity in these transgenic embryos was comparable to that of the unaltered 399-bp reporter gene (Fig. 2*A*, construct 6). However, mutations introduced simultaneously at sites B and C, or B and D, did result in pronounced changes in the reporter gene expression, as discussed below.

Site C contains a typical homeodomain binding site, TTAATT (22). When this sequence was altered to TTCCTT in the 399-bp reporter (Table 1, construct 15), posteriorization of neural tube and somite expression was observed (Fig. 2*G*). The neural tube expression in a 25-somite stage embryo was at somite levels 19–20. Unsegmented mesoderm in the caudal regions and the posterior-most one or two condensed somites stained for β-galactosidase activity. Expression in the LPM was relatively unaffected. The level of expression in these tissues varied somewhat among different founder generation embryos.

Pairwise mutations in sites B, C, and D resulted in dramatic reduction in neural tube and mesoderm expression. When site B and site C were mutated in the same construct (Table 1, construct 17), both somite and neural tube expression were greatly reduced, with the LPM expression less affected (Fig. 2*I*). Expression in the neural tube was limited to a few cells near the caudal neuropore. When sites B and D were mutated in the same construct (Table 1, construct 18), no expression was observed in somites and the LPM, whereas reduced neural tube expression was observed in the extreme posterior region (Fig. 2*J*). Similar results were obtained when sites C and D were mutated in the same construct (Table 1, construct 19; and Fig. 2*K*).

It can be concluded that mesoderm expression is directed by five distinct elements A to E. Mutations at these sites either singly or in combination affect expression in somite and the LPM to different degrees.

Mesoderm-Specific Elements Influence Neural Tube Expression. The neural tube expression observed with the 399-bp early enhancer (Fig. 2*A*, construct 6) differed from that of the 335-bp ENT enhancer described earlier (11). With the larger 399-bp reporter, the anterior boundary of expression was found at the 14th somite, whereas with the smaller 335-bp reporter, the neural tube expression was at somite 17 (Table 2). Thus the larger construct directed expression to more anterior

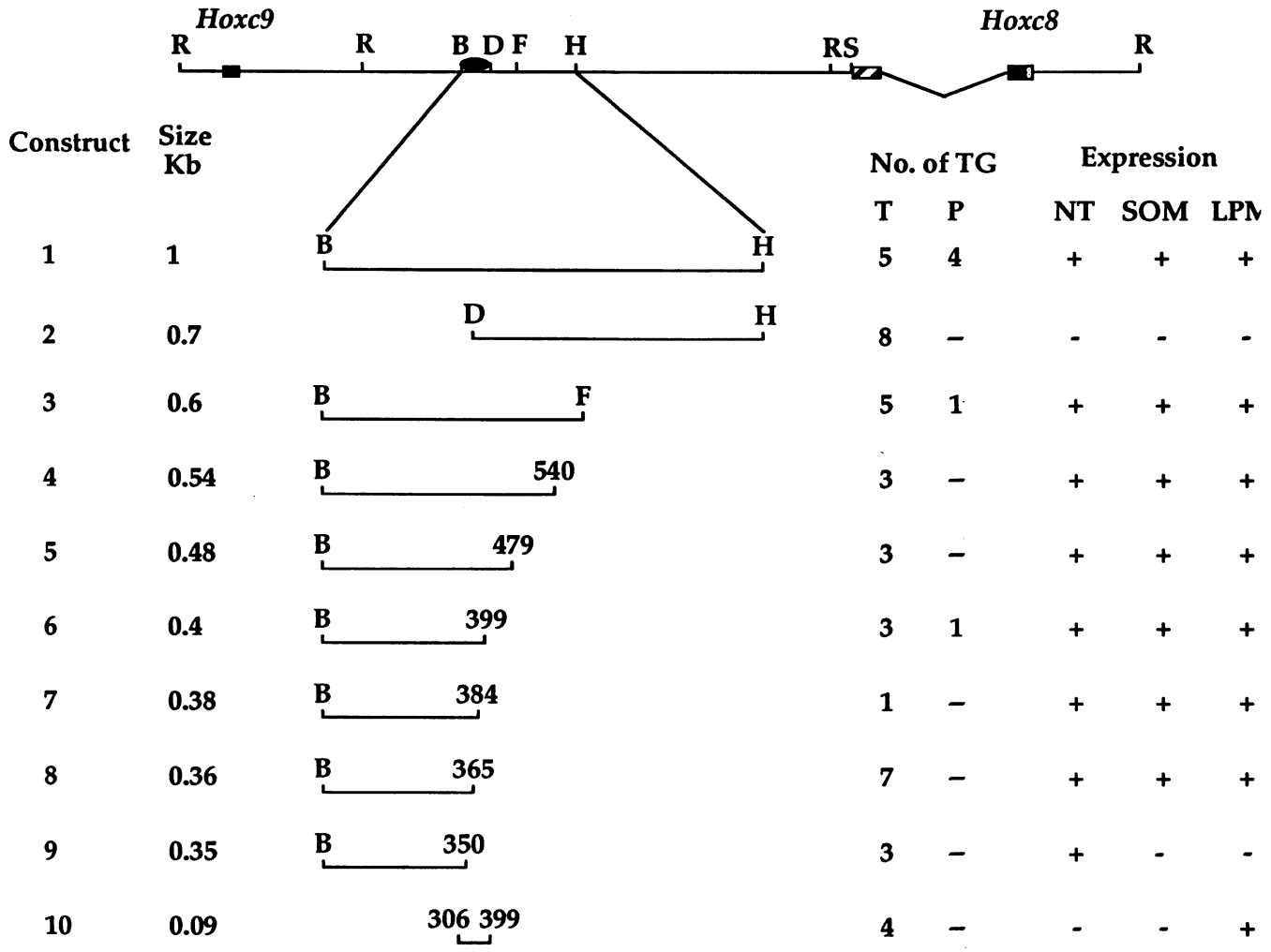

FIG. 1. Characterization of regulatory regions of the *Hoxc8* gene in transgenic mouse embryos. Schematic of reporter gene constructs and sites of expression. Partial restriction map of *Hoxc9–Hoxc8* intergenic region is represented above. Black boxes indicate homeoboxes of *Hoxc9* and *Hoxc8*; black sphere indicates the position of the ENT enhancer. Shaded box represents the first exon of the *Hoxc8*. Schematic of various regions of the *Hoxc8* genomic locus tested for enhancer activity in transgenic mice are shown below. The nucleotide positions correspond to the sequence in Fig. 3. Results of transgenic expression analysis are indicated. The first column represents the number of transgenic embryos (TG) that express the β-galactosidase gene: T, founder generation embryos; P, established transgenic lines; presence of expression (+); and absence of expression (−) is indicated in neural tube (NT), somites (SOM), and lateral plate mesoderm (LPM). R, *Eco*RI; B, *Bsp*EI; D, *Dra*I; F, *Fsp*I; H, *Hin*dIII; and S, *Sac*I.

levels. A comparison of anterior boundaries of neural tube expression of the constructs that contain mutations at various sites in the context of the 399-bp and the 335-bp reporter constructs is presented in Table 2. While mutation in site A in the 335-bp reporter completely abolished neural tube expression, the same mutation in the 399-bp reporter resulted in only a reduction in neural tube expression. These results suggest that elements present in the 399-bp reporter made additional contributions toward neural tube expression. There was no significant change in the anterior limits of neural tube expression when site B was mutated. A comparison of mutations at sites C and D revealed no significant difference in the anterior limits of expression in the context of the 335-bp construct. Individual mutations at C and D resulted in posteriorization of neural tube expression by four to six somite levels when compared with the anterior boundary of the the 399-bp reporter (Table 2). Mutations at site E in the context of the larger construct also resulted in the posteriorization of neural tube expression; reporter gene expression was observed at somite levels 18-19, 4-5 somite levels posterior to that observed with the unaltered 399-bp reporter. These results taken together indicate that each of the elements, A, C, D, and E, contribute to the anterior boundary of neural tube expression.

Early LPM Expression Can Be Achieved Independent of Neural Tube and Somite Expression. Mutational analyses described above indicate that somite expression was inseparable from neural tube and LPM expression because of interdependency among all but element B. In contrast, lateral plate expression was affected only when sites D and E were mutated. To test whether expression in the LPM can be achieved independent of neural tube and somite expression, a reporter gene construct containing sequences spanning nucleotide positions 306–399 was generated (Fig. 1, construct 10). This construct contains elements C, D, and E. As shown in Fig. 2*L*, construct 10 can direct expression in the posterior LPM. The expression of the transgene was not detected in neural tube and somites. Thus a regulatory component of the mesoderm expression can be isolated independent of neural tube expression.

DISCUSSION

The mechanisms regulating *Hox* gene expression provides information concerning the establishment of the vertebrate body plan. Many studies have identified DNA fragments that direct *Hox* gene expression (1), but only a few of these have

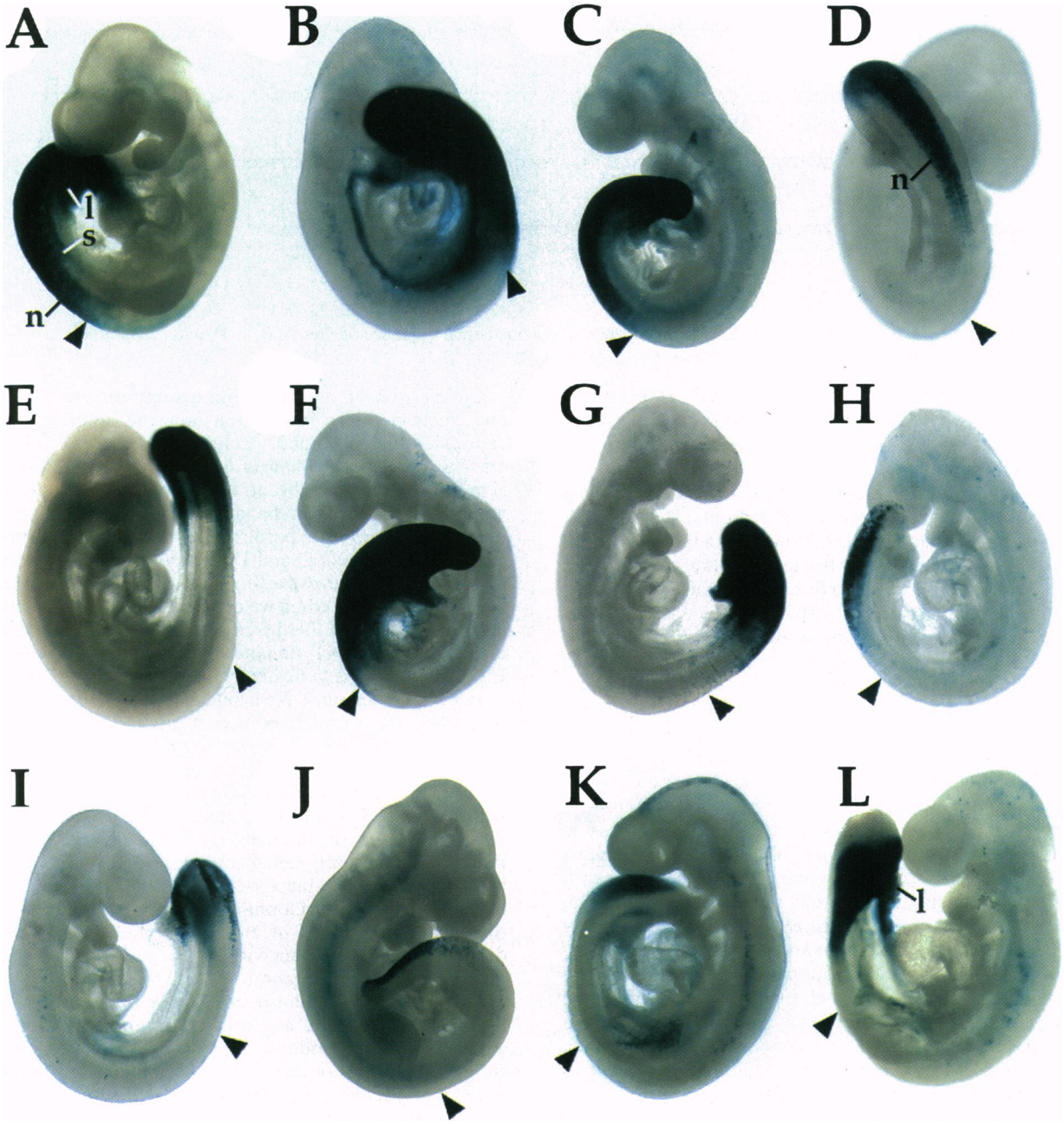

FIG. 2. Expression patterns of reporter genes in transgenic embryos. Expression of the β-galactosidase gene is detected in the posterior regions of transgenic embryos. The transgenic embryos are staged between 9.0 and 9.5 d.p.c. Constructs are shown in Fig. 1 and Table 1. Arrowheads indicate position of the 14th somite. Enhancer activity of deletion constructs 6 (*A*), 8 (*B*), and 9 (C) are shown (Fig. 1). (*D–K*) Reporter gene activity of constructs 12–19 (Table 1). (*L*) Reporter gene activity of construct 10 (Fig. 1). Additional sites of expression unrelated to *Hoxc8* early enhancer activity are due to integration site effect and are unique to each transgenic embryo, except in the case of *B*. Three independent transgenic embryos show ventral staining along the axis. This ectopic expression could be due to elimination of negative elements from the larger constructs. n, Neural tube; s, somites; and l, lateral plate mesoderm.

clearly identified cis-acting elements and upstream factors involved in *Hox* gene regulation (23–28). We have employed a mutational approach in defining cis-acting elements of the *Hoxc8* gene. In a previous study, we identified four elements, termed A, B, C, and D, involved in early neural tube expression (11). These elements are located within a 135-bp region upstream of the *Hoxc8* coding region. In the present study, we report on the identification of a fifth element, E, which is essential for early mesoderm expression. Two separate mutations in element E which abolish mesoderm expression define a core sequence 5′-CCTTTG-3′. This has been previously shown to be a potential site for interactions with a class of sequence-specific high mobility group proteins (15, 16). A large number of genes belonging to the high mobility group, including LEF1, SRY, and Sox genes, are expressed in developing embryos both in the central nervous system and mesoderm derivatives (29–37).

A salient feature of the *Hoxc8* early enhancer region is the involvement of multiple elements in directing region-specific expression. Studies on other Hox genes, employing significantly larger DNA fragments than the *Hoxc8* early enhancer, have indicated the requirements of widely separated cis-regulatory regions for directing expression (1). In a few instances, multiple copies of similar elements have been shown

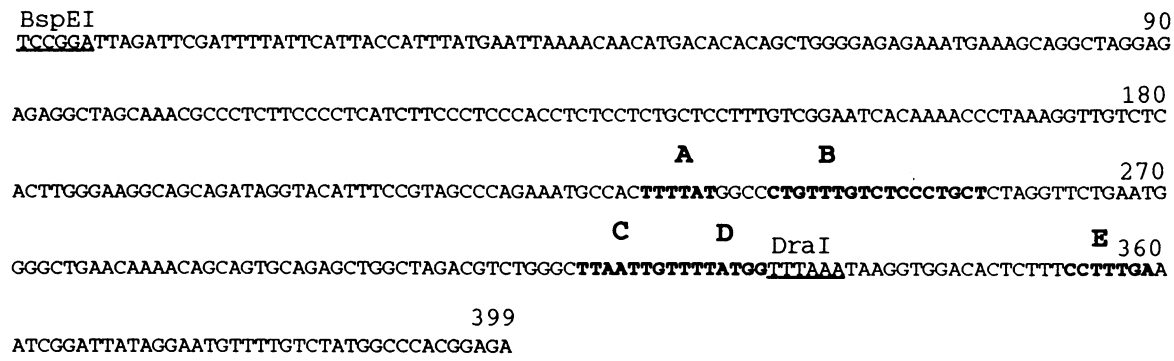

FIG. 3. Nucleotide sequence of the 399-bp *Hoxc8* early enhancer. The nucleotide sequence of sites A, B, C, D, and E are indicated in boldface. Underlined sequences represent recognition sequences for restriction enzymes.

to mediate expression (23, 27). In contrast, *Hoxc8* early enhancer comprises a compact region containing four classes of separate elements acting in concert in determination of posterior expression.

Our analysis of the ENT enhancer has revealed that while element A was essential, three other elements, B, C, and D, were functionally redundant (11). However, in the presence of additional 3′ sequences, each of these elements can be assigned distinctive roles. Of particular interest is the differential roles played by elements A and D, which share a similar core sequence, in directing expression to the neural tube, somites, and the LPM (Table 3). Both A and D are necessary for *Hoxc8* expression in somites. A is necessary for expression in the neural tube, while D is minimally involved. D is necessary for expression in the LPM, while A plays no detectable role. Thus, the neural tube, somites, and LPM elicit three different developmental responses from the *Hoxc8* early enhancer. This may be explained in terms of different isoforms of transcription factors, their concentrations and/or time of expression influencing early enhancer activity. With respect to elements A and D, it is possible that they interact preferentially with different Cdx proteins in different regions of the embryo. Alternatively, since none of these elements are sufficient by themselves in directing expression and require other elements, different protein–protein interactions may determine expression. These possibilities are not mutually exclusive. Similarly, roles of elements B and C, which were indistinguishable in the context of the ENT enhancer, can now be distinguished. Whereas individual mutations at site B are not sufficient to alter the reporter gene expression pattern, concomitant mutations at either C or D can affect expression dramatically. In contrast, the effect of mutations at element C can readily be recognized in terms of a general posteriorization of expression in the neural tube and mesoderm.

Table 2. Anterior limits of neural tube expression in the presence or absence of mesoderm specific enhancer elements

Sites mutated	Somite no.* Reporter,† 399 bp	Somite no.* Reporter,‡ 335 bp
None	14	17
A	22, 23	—§
B	14	18
C	19, 20	19, 20
D	18, 19	19, 20
E	18, 19	—

*Somite no. at which reporter gene expression is observed at its anterior limit.
†This study.
‡Ref. 11.
§Residual expression at the caudal neuropore.

Comparison of reporter gene activity directed by various constructs provides insights on how the anterior extent of expression is determined. Addition of mesoderm-specific elements to the ENT enhancer extends the neural tube expression more anteriorly by at least three somite levels. This anterior extension can be attributed to element E, since mutation at this site posteriorizes neural tube expression. Besides E, elements C and D also contribute toward anteriorizing neural tube expression. Roles of C and D elements become more clear when we compare mutations at these sites in the context of the 399-bp early enhancer than in the context of the shorter ENT enhancer. The anterior boundary of expression appears to be determined by the cumulative action of individual elements. We hypothesize that this is achieved by increasing the strength of the promoter. Extension of the anterior boundary by multimerizing *Hox* enhancers has been reported (38), suggesting that a positive relationship exists between element number and rostral extent of expression. Our study suggests that anterior extension can also be achieved by different classes of elements interacting together. Furthermore, since the early enhancer activity does not reflect the maintenance of the anterior expression of endogenous *Hoxc8* beyond day 8.5 of development (11, 12), it can be postulated that additional classes of elements found outside and at a distance (12) may interact with the early enhancer to maintain anterior levels of expression.

Two separate mechanisms can be distinguished in the determination of somite expression. Mutations in elements D and E abolish mesoderm expression completely and may reflect an inability of mesoderm-specific transcription factors to interact with these mutated elements. In contrast, mutations at elements A and C result in the posteriorization of expression. As a consequence of this posteriorization, expression in the mesoderm is largely confined to unsegmented regions.

The multicomponent, interdependent, and partially redundant nature of the *Hoxc8* early enhancer has several interesting implications. First, these findings suggests that *Hoxc8* expression is determined by combinatorial interactions among transcription factors acting upstream of the early enhancer. These factors may be expressed in a broader domain and the overlap among these factors may account for region-specific expression. Second, from an evolutionary perspective, the *Hoxc8* early enhancer may represent a prototype developmental module. Such a module can be easily modified to achieve subtle differences in anterior extent of expression in neural

Table 3. Minimal combinations of elements determining differential expression of the reporter gene

Tissue	Element combination
Neural tube	ABC, ABD, ACD
Somites	ACDE
LPM	DE

tube and mesoderm in different organisms with concomitant variation in body plan (H.-G. Belting and C.S.S., unpublished observations). Variations of this basic enhancer may be found in different Hox enhancers. Supporting this view, critical regions of the *Hoxb4* enhancer contain sequences strongly resembling many of the elements of the *Hoxc8* early enhancer (39). In conclusion, we have identified critical elements that direct early neural tube and mesoderm expression in a combinatorial fashion. We believe that the *Hoxc8* enhancer system will prove useful in further investigating mechanisms underlying *Hox* gene regulation in regard to both development and evolution.

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