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Evidence for positive and negative regulation of the *Hox-3.1* gene

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ABSTRACT **The region-specific patterns of expression of mouse homeobox genes are considered important for establishing the embryonic body plan. A 5-kilobase (kb) DNA fragment from the *Hox-3.1* locus that is sufficient to confer region-specific expression to a β-galactosidase reporter gene in transgenic mouse embryos has been defined. The observed reporter gene expression pattern closely parallels endogenous *Hox-3.1* expression in 8- to 9.5-day postcoitum (p.c.) embryos. At 10.5 days p.c. and later, the pattern of β-galactosidase activity diverges from the *Hox-3.1* pattern, and an inappropriately high level of reporter gene expression is observed in posterior spinal ganglia. Inclusion of an additional 2 kb of upstream sequences is sufficient to suppress this aberrant expression in the developing spinal ganglia. Together, these results show that the control of early *Hox-3.1* expression is complex, involving at least one positively acting and one negatively acting element.**

A central problem in animal development is the regulation of gene expression underlying pattern formation and cellular differentiation. The discovery of homeobox genes has provided a molecular tool to begin to address this issue. Mammalian homeobox genes have been identified on the basis of their homology to certain *Drosophila* homeotic and segmentation genes that share the highly conserved 183-base-pair (bp) homeobox sequence. These developmental control genes encode transcription factors whose region-specific expression is instrumental in establishing the metameric body plan of *Drosophila* (1, 2). The precise expression patterns of *Drosophila* homeobox genes are determined by complex regulatory interactions among a network of many genes, and this network controls pattern formation (3). While the functions of homeobox genes in mammals remain to be elucidated, similarities between the organization and patterns of expression of vertebrate and *Drosophila* homeobox genes make it tempting to propose similar roles for these genes in disparate organisms.

The mouse *Hox* gene family constitutes a class of genes containing sequences homologous to the homeobox found in the *Antennapedia* locus in *Drosophila*. About 30 *Hox* genes have been identified in four large gene clusters, and several have been characterized in considerable detail (4, 5). Consistent with their hypothesized role in establishing the body plan, the *Hox* genes have been shown to be transcribed in distinct yet overlapping spatiotemporal domains along the anteroposterior axis during embryogenesis. To a great extent, *Hox* gene transcripts accumulate in region-specific rather than tissue-specific patterns during embryogenesis. Accordingly, the expression of a *Hox* gene often occurs in tissues derived from two or more germ layers but only within particular regions of the embryo. A remarkable feature of the *Hox* genes is that their linear order within a cluster is reflected in their anterior boundaries of expression: a gene 3′ in a cluster has a more anterior boundary of expression than a gene more 5′ within the same cluster (6–10). Furthermore, it appears that for at least some of the *Hox* genes, most notably *Hox-3.1* and *Hox-2.5*, the region-specific expression patterns are dynamic, showing two distinct phases: an "early" posterior pattern at 8.5 and 9.5 days postcoitum (p.c.) and a "late" more anterior pattern with altered dorsoventral localization within the neural tube at 10.5 days p.c. and later (11, 12). While the anterior shift could result from increased growth in the neural tube relative to the rest of the embryo, the dorsoventral localization for each gene represents a new cellular specificity.

Although the region-specific patterns of expression of mouse homeobox genes are considered important for embryonic development, little is known about how these expression patterns are generated. The expression of *Hox-3.1* has been well-characterized by *in situ* hybridization, making it an excellent candidate to begin to define the molecular mechanisms that establish and maintain its region-specific expression during embryogenesis (11, 13–16). As a first step in addressing this complex problem of gene regulation, we used transgenic mouse embryos to identify cis-acting DNA elements that can confer region-restricted expression on a heterologous gene.

Guided by the observation that several *Drosophila* homeobox-containing genes are controlled by elements located far upstream of their coding regions (17), we initially focused our attention on the entire genomic region between *Hox-3.1* and *Hox-3.2* (Fig. 1).

MATERIALS AND METHODS

Construction of Transgenes. *Hox-3.1/lacZ* transgenes were constructed from the *lacZ*-containing plasmid pCH110 (Pharmacia) and various subclones of the *Hox-3.1*-containing cosmid CosEa (18). To generate p3Z5.2ΔL, the *lacZ* gene was cloned downstream of a 5-kilobase (kb) *EcoRI* fragment from the upstream region of *Hox-3.1* (Fig. 1) carried in a pBluescript vector (Stratagene). The *lacZ* gene from pCH110, carried on a 3.7-kb *Hin*dIII–*Bam*HI fragment, was subcloned into a modified pGem (Promega) vector termed pSafyre (L. Bogorad and F.H.R., unpublished data) to create plasmid pLZRVA. To generate p3Z5.2, a 5.2-kb *Eco*RI–*Sac* II fragment from the *Hox-3.1* locus (Fig. 1) was cloned upstream of the *lacZ* gene in pLZRVA. To generate p3Z7.2, a 9-kb fragment carrying the *Hox-3.1/lacZ* transgene from p3Z5.2 was ligated to the upstream 2-kb *Eco*RI fragment containing the *Hox-3.2* homeobox carried in a pBluescript vector. In the resulting plasmid, the 7.2-kb of *Hox-3* complex sequences are identical to those found in the genome, with the exception of a 15-bp insertion at the *Eco*RI site located at position 1647 in the *Hox-3.1* sequence (18). In all three transgene constructs, the junction between the *lacZ* gene and the *Hox-3.1* sequences occurs within the mRNA leader

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Abbreviations: β-gal, β-galactosidase; p.c., postcoitum; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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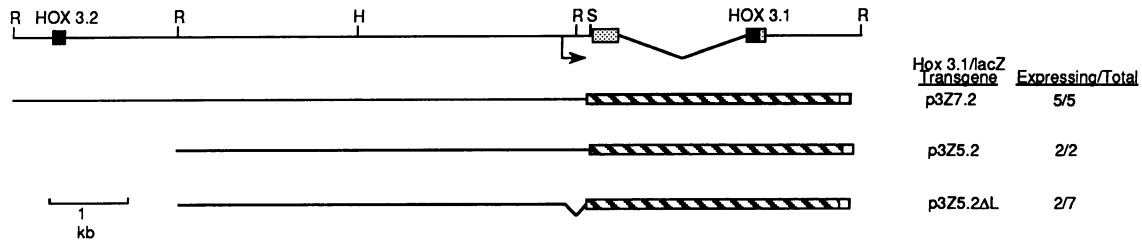

FIG. 1. Partial restriction map of the *Hox-3.1* locus and *Hox-3.1/lacZ* transgenes. Three different *Hox-3.1/lacZ* constructs (p3Z7.2, p3Z5.2, and p3Z5.2ΔL) were used to generate transgenic mice. The location of the *Hox-3.1* sequences in each construct is shown relative to a partial restriction map of the *Hox-3* locus, and the fraction of independent lines expressing the transgene is shown. The filled boxes represent the *Hox-3.2* and *Hox-3.1* homeoboxes. The stippled boxes represent the protein-coding region of *Hox-3.1*. The hatched boxes represent the *lacZ* sequences. The open boxes represent the simian virus 40 polyadenylylation signals. The arrow indicates the region of transcriptional initiation (18). H, *Hin*dIII; R, *Eco*RI; S, *Sac* II.

sequence of both genes. To prepare DNA for injection into single-cell embryos, p3Z5.2ΔL was digested with *Not* I and *Xho* I, and an 8.5-kb fragment was isolated; p3Z5.2 was digested with *Bam*HI and an 8.7-kb fragment was isolated; p3Z7.2 was digested with *Sac* II and *Xho* I and a 10.7-kb fragment was isolated. Following restriction enzyme digestion, fragments for injection were separated from vector sequences by sucrose gradient fractionation (19), dialyzed against 10 mM Tris/0.25 mM EDTA, pH 7.5, adjusted to a concentration of 1–3 μg/ml, and injected into (CD-1 × B6D2)F_1 single-cell embryos as described (20).

Southern Blot Analyses. Founder and F_1 generation transgenic mice were identified by Southern blot hybridization of *Sac* I-digested tail DNA with a single-stranded probe, M13-262, containing a 300-bp *Sac* I–*Bgl* II fragment from the *Hox-3.1* upstream region or by hybridization to *Eco*RI-digested tail DNA probed with a random hexamer-labeled 3.7-kb *Hin*dIII–*Bam*HI fragment from pCH110 (21). All founders appeared to have intact copies of their respective transgenes.

Detection of β-Galactosidase (β-gal) Activity. To determine the pattern of β-gal activity, embryos were fixed in 0.25% gluteraldehyde in phosphate-buffered saline for 30 min, then incubated at 37°C for 16 hr in a solution containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) at 0.5 mg/ml, 25 mM potassium ferrocyanide, 25 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mM spermidine, 0.02% Nonidet P-40 (Sigma), and 0.01% sodium deoxycholate. To prepare tissue sections, embryos were postfixed in 4% paraformaldehyde, embedded in paraffin, and sectioned as described (12).

***In Situ* Hybridization.** Hybridization was performed as described (12). The *Hox-3.1* probe was prepared from a ≈700-bp *Eco*RI/*Sal* I cDNA restriction fragment cloned into a pBluescript vector (18). This plasmid was also used to prepare a negative control probe of the opposite orientation for all experiments. The *lacZ* probe was prepared from pLZRVA. No specific labeling was observed using the negative control probe (data not shown).

RESULTS AND DISCUSSION

Five independent transgenic strains (designated J22-2, J22-3, J22-10, J22-14, and J22-17) were generated carrying a 7.2-kb fragment from the *Hox-3.1* upstream region driving expression of the *Escherichia coli lacZ* gene, which encodes the enzyme β-gal (Fig. 1). This fragment contains several closely spaced *Hox-3.1* transcriptional start-sites (Fig. 1; ref. 18) and encodes all but 25 bp of the 5′ untranslated mRNA sequence. The 5′ end of this fragment contains a portion of the presumptive protein coding region of the *Hox-3.2* gene and therefore includes the entire intergenic region between *Hox-3.1* and *Hox-3.2* (Fig. 1; ref. 15). Transgenic embryos from all five strains showed comparable levels of reporter gene expression in an identical region-restricted pattern 10.5 days p.c. Two of these lines, J22-3 and J22-17, were studied in detail to determine the precise temporal and spatial patterns of reporter gene activity. To test for expression of the *Hox-3.1/lacZ* transgenes, male transgenic animals were mated to normal CD-1 females, and embryos were dissected and assayed for β-gal activity by incubation in the presence of X-Gal. From 7.5 to 14.5 days p.c., both lines displayed indistinguishable patterns of β-gal expression.

Reporter gene expression was first detected in the posterior region of early somite-stage embryos approximately 8.0 days p.c. (Fig. 2*a*). Intense staining was observed in the base of the allantois, the primitive streak, and the posterior ectoderm. All of the β-gal activity was observed posterior to the region of somite formation. The pattern of expression of the reporter gene at this stage correlated very well with the localization of the authentic Hox-3.1 mRNA as assayed by *in situ* hybridization (14). Although Gaunt reported detection of Hox-3.1 transcripts in the allantois of 7.5-day embryos (14), we did not observe β-gal activity in any *Hox-3.1/lacZ* transgenic embryos at that stage. This discrepancy can probably be explained by differences in staging of embryos and/or the use of different mouse strains, rather than a true divergence between reporter and endogenous gene expression.

In 8.5- to 8.75-day p.c. transgenic embryos, β-gal activity persisted in the rapidly growing posterior region (Fig. 2 *b* and *c*), but the anterior limit of expression remained posterior to the region of somite formation (Fig. 2*g*). High levels of β-gal activity were also observed in the posterior region of 9.0- and 9.5-day embryos (Fig. 2 *d*, *e*, and *h–j*). The pattern of β-gal activity we observed correlated well with previously reported results of *in situ* hybridization studies with *Hox-3.1*-specific probes (11, 15). In the most posterior region of 9.5-day embryos, near the tip of the tail bud, virtually all cells showed β-gal activity (Fig. 2*i*). In a more anterior region, at approximately the level of the 17th somite, the neural tube, lateral mesoderm, and hindgut were more strongly labeled than the somites (Fig. 2*j*).

Surprisingly, in 10.5-day p.c. embryos, when *Hox-3.1* expression in the neural tube shifts anteriorly (11), the pattern of β-gal activity remained much the same as that seen at 9.5 days (Fig. 2*f*). The shift in expression that occurs for the endogenous *Hox-3.1* gene is not carried out by the *Hox-3.1/lacZ* transgenes along either the anteroposterior or dorsoventral axes. Furthermore, in contrast to the *Hox-3.1* gene, the *Hox-3.1/lacZ* transgenes continued to be expressed at high levels in the posterior region of the embryo. In older embryos of 11.5–14.5 days of gestation, β-gal activity persisted in posterior structures but did not appear in the more anterior region of the spinal cord characteristic of *Hox-3.1* (data not shown; refs. 11, 13–16). The persistence of the β-gal activity in posterior tissues may indicate that a negative element required for efficient down-regulation of *Hox-3.1* in this region is not present in the transgenes. Similarly, Puschel *et al.* (24) recently reported that a *Hox-1.1/lacZ* transgene

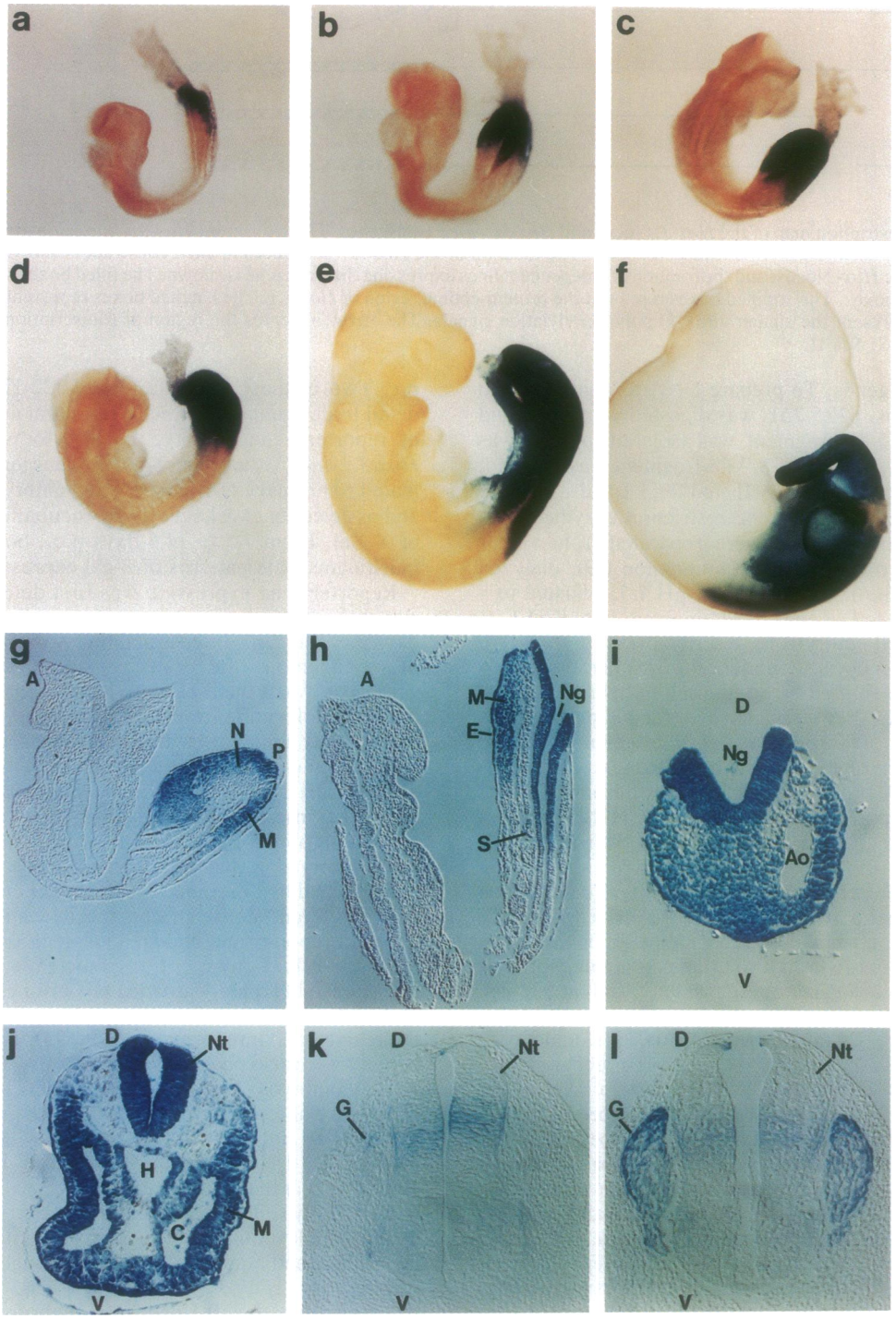

FIG. 2. β-gal activity in 8.0- to 10.5-day p.c. embryos, Theiler stages 12–17 (23). In whole mounted embryos (*a–f*), β-gal activity is localized posteriorly. (*a*) Four-somite, stage 12 embryo. (*b*) Eight-somite, stage 13 embryo. (*c*) Twelve-somite, stage 13 embryo. (*d*) Fourteen-somite, stage 14 embryo. (*e*) Twenty-five-somite, stage 15 embryo. (*f*) Approximately 39-somite, stage 17 embryo. (*g*) Parasagittal section through a stage 13 embryo. β-gal activity is observed in the posterior neural groove, presomitic and lateral plate mesoderm, and ectoderm. (*h*) Parasagittal section through a stage 14 embryo. β-gal activity is observed posterior to the 13th somite in the neural tube, presomitic and lateral plate mesoderm, and ectoderm. (*i*) Cross section through a stage 15 embryo at the level of the tailbud. (*j*) Cross section through a stage 15 embryo at about the level of the 17th somite. (*k*) Cross section through a stage 17 embryo carrying p3Z7.2 (Fig. 1) just posterior to the forelimb bud. β-gal activity is present in two regions of the neural tube: a centrally located band of cells and a small cluster of cells in the roof plate. A group of cells in the dorsolateral region of the spinal ganglia also show β-gal activity. (*l*) Cross section through a stage 17 embryo carrying p3Z5.2 (Fig. 1) just posterior to the forelimb bud. The pattern of β-gal activity looks similar to that shown in *k*, except that virtually all cells in the spinal ganglia show strong β-gal expression. A, anterior; Ao, aorta; C, coelom; D, dorsal; E, ectoderm; G, spinal ganglion; H, hindgut; M, lateral mesoderm; N, neural ectoderm; Ng, neural groove; Nt, neural tube; P, posterior; S, somite; V, ventral. Embryos in *a–f* and *k* were sired by F_1 transgenic male offspring of founder mouse J22-3, which carries transgene p3Z7.2 (Fig. 1). Embryos in *g–j* and *l* were sired by transgenic male F_1 offspring of founder pG+1, which carries transgene p3Z5.2 (Fig. 1). Whole-mount photographs were taken on a Leitz M400 microscope. (*a–d*, ×15; *e*, ×15; *f* ×10; *g*, ×45; *h*, ×40; *i*, ×70; *j* and *k* ×100.)

that is correctly expressed 7.5–8.5 days p.c. fails to be properly down-regulated in posterior tissues after day 8.5 p.c.

To further delineate the cis-acting sequences required to confer the region-restricted pattern of expression, a fragment containing only 5-kb of *Hox-3.1* upstream sequences was used to direct *lacZ* expression (Fig. 1; p3Z5.2). Two independent founder animals were generated carrying this construct: one, designated pG+0, was assayed at 10.5 days p.c. while the other, designated pG+1, was allowed to develop to term and its offspring were assayed in a developmental time course for β-gal activity. The pattern of reporter gene activity observed in 8.0- to 9.5-day p.c. transgenic embryos was indistinguishable from that seen in embryos carrying the larger 7.2-kb upstream region in p3Z7.2. However, at 10.5 days p.c., a striking difference was observed in the pattern of β-gal activity. Embryos from the two independent strains carrying only 5 kb of *Hox-3.1* upstream sequences driving the *lacZ* gene displayed very strong β-gal activity in the posterior spinal ganglia (Fig. 2*l*). In contrast, weak β-gal activity was observed in the corresponding ganglia of transgenic embryos carrying p3Z7.2 (Fig. 2*k*) in good agreement with the low level of *Hox-3.1* expression detected in developing spinal ganglia in 10.5-day p.c. embryos (25). These data suggest that an element that may down-regulate the reporter gene in spinal ganglia is present in the upstream 2.0-kb *Eco*RI fragment (Fig. 1). In the absence of this negatively acting region, inappropriately high levels of reporter gene activity in spinal ganglia were observed.

This apparent overexpression in spinal ganglia was also observed in embryos from two independent strains, designated Z3-1 and Z3-7, carrying p3Z5.2ΔL (Fig. 1). This construct differs from p3Z5.2 in that 235 bp of the 5′ untranslated region of *Hox-3.1* has been deleted. The pattern of β-gal activity seen in embryos carrying this construct is indistinguishable from that seen in embryos carrying p3Z5.2, indicating that the deleted region is not required to specify the early pattern of expression. However, consistently high levels of β-gal activity were seen in embryos from only one transgenic strain carrying p3Z5.2ΔL. Transgenic embryos from a second strain carrying this construct showed variable levels of β-gal activity ranging from no expression to strong expression. In five other strains carrying p3Z5.2ΔL, we were unable to demonstrate β-gal activity in transgenic embryos. These observations suggest that while the deleted portion of the 5′ untranslated leader appears not to contribute to the region-specificity of expression, its presence may lead to increased reporter gene expression in a broader array of integration sites, perhaps by transcript stabilization.

As a direct comparison of the expression of the endogenous *Hox-3.1* gene with the expression of the reporter, *in situ* hybridization to mRNA was performed by using probes specific for either the *Hox-3.1*/*lacZ* gene or the endogenous *Hox-3.1* gene on serial sections of 8.5-day transgenic embryos (Fig. 3 *a*/*a*′ and *b*/*b*′). Transcripts from both genes were localized posterior to the region of somite formation with nearly identical anterior boundaries of expression in both ectoderm and mesoderm, but Hox-3.1 transcript accumulation extended slightly more anterior. Within the limits of quantitation afforded by *in situ* hybridization, the levels of transcript accumulation appeared to be similar. These results provided a direct confirmation that the transgene expression closely paralleled *Hox-3.1* expression at 8.5 days of gestation and clearly demonstrated the regional specificity of transgene expression at the level of mRNA.

To determine whether the failure of the reporter genes to be expressed in the typical *Hox-3.1* late pattern and whether the apparent overexpression in spinal ganglia were due to regulation at the transcriptional or translational level, we performed *in situ* hybridization to mRNA in serial sections of 10.5-day p.c. transgenic embryos carrying p3Z5.2ΔL.

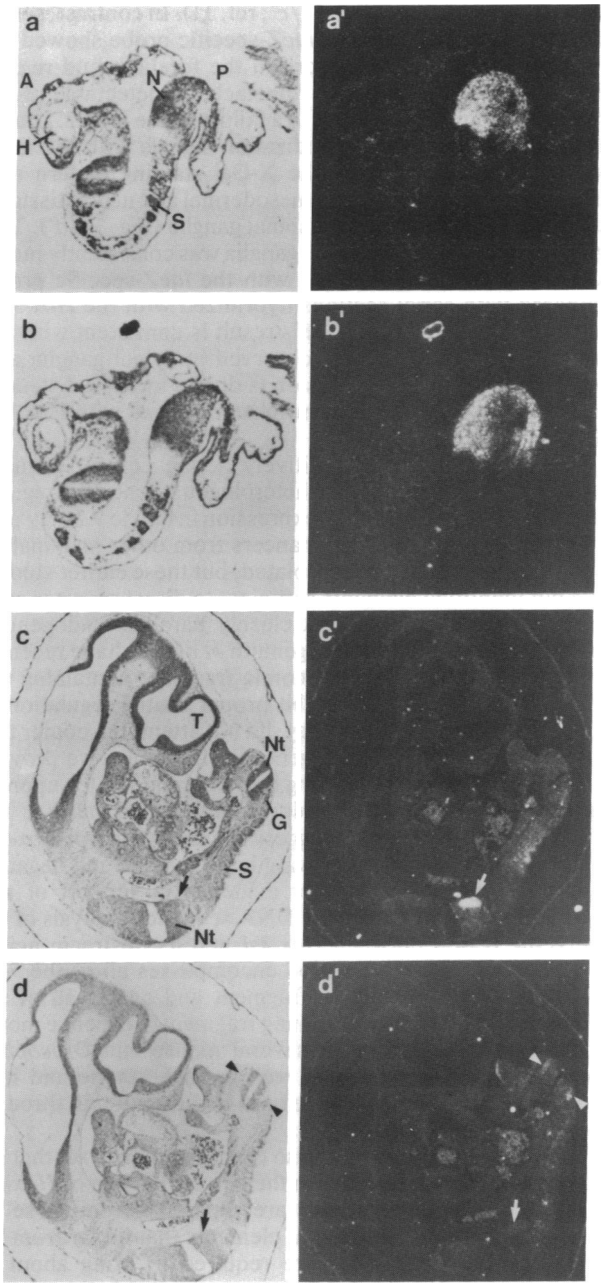

FIG. 3. *In situ* hybridization to mRNA of transgenic embryos. (*a*/*a*′) Bright- and dark-field views of a parasagittal section of an 8.5-day p.c. embryo hybridized with the *Hox-3.1*-specific probe. (*b*/*b*′) Bright- and dark-field views of a section adjacent to *a*/*a*′ that was hybridized with the *lacZ*-specific probe. (*c*/*c*′) Bright- and dark-field views of a parasagittal section of a 10.5-day p.c. embryo hybridized with the *Hox-3.1*-specific probe. The major site of hybridization is the ventral cervical spinal cord (arrows), with weaker labeling apparent over thoracic somites. (*d*/*d*′) Bright- and dark-field views of a section adjacent to *c*/*c*′ that was hybridized with the *lacZ*-specific probe. No hybridization is observed in the cervical spinal cord. The most intense hybridization is observed over posterior spinal ganglia (arrowheads). The 8.5-day p.c. embryos were sired by transgenic F_1 male offspring of founder pG+1, and 10.5-day p.c. embryos were sired by transgenic F_1 male offspring of founder Z3-1. A, anterior; G, spinal ganglia; H, heart; N, neural groove; Nt, neural tube; P, posterior; S, somites; T, telencephalon. (*a*/*a*′ and *b*/*b*′, ×20; *c*/*c*′ and *d*/*d*′, ×10.)

Parasagittal sections through embryos hybridized with the *Hox-3.1*-specific probe showed strong hybridization in the ventral portion of the spinal cord at the level of the forelimb bud and weaker hybridization over somites more posterior to

the spinal cord labeling (Fig. 3*c*/*c*'; ref. 11). In contrast, serial sections hybridized with the *lacZ*-specific probe showed no hybridization in the spinal cord in the forelimb bud region (Fig. 3*d*/*d*'), indicating that the lack of detectable β-gal activity in that region was due to a failure of the Hox-3.1/lacZ mRNA to accumulate. Hybridization signals with the *lacZ* probe correlated well with the X-Gal staining pattern and were observed over posterior mesodermal and neural tissues, including a strong signal over spinal ganglia (Fig. 3*d*/*d*'). The signal over the posterior spinal ganglia was consistently much higher on sections hybridized with the *lacZ*-specific probe compared with serial sections hybridized with the *Hox-3.1*-specific probe (Fig. 3*c*/*c*'). This result is consistent with the high levels of β-gal activity observed in spinal ganglia and suggests that the overexpression is due to increased steady-state levels of reporter gene mRNA compared with Hox-3.1 mRNA.

We have identified a positively acting control element sufficient to confer upon a heterologous gene the region-restricted pattern of *Hox-3.1* expression in 8.0- to 9.5-day p.c. embryos. Region-specific enhancers from other mammalian homeobox genes have been isolated, but these earlier studies have not identified elements sufficient to direct reporter gene expression in a manner that closely parallels endogenous gene expression (22, 26). Wolgemuth *et al.* (27) have recently demonstrated that a large genomic fragment containing the entire *Hox-1.4* gene can exhibit proper spatial regulation in 12.5-day p.c. embryos, but they did not attempt to confer this pattern onto a heterologous gene. Furthermore, we provide evidence for a negatively acting element required to suppress reporter gene expression in developing spinal ganglia. Together, these observations suggest that the control of *Hox-3.1* expression is complex, involving both positive and negative regulatory pathways. Further evidence in support of this suggestion has come from the DNA sequence analysis of the intergenic region between *Hox-3.1* and its upstream neighbor, *Hox-3.2* (18). This region encompasses all of the 5-kb positive-element-containing fragment and nearly all of the 2-kb negative-element-containing fragment. Sequence motifs similar to binding sites for several mouse and *Drosophila* homeobox gene products as well as to hormone and heat shock response elements have been found scattered throughout the intergenic region (18).

The results reported here also clearly demonstrate that the elements required to establish the correct pattern of *Hox-3.1* expression in early embryos are separable from those required later. It is possible that elements that lie far from the coding region of *Hox-3.1* are required to bring about an appropriate level or pattern of expression, as is the case for the dominant control region of the human β-globin locus (28). A similar regulatory element within the *Hox-3* locus could act alone or in concert with more proximal elements to influence expression of *Hox-3.1* and possibly other genes within the cluster. Such elements that influence the expression of several genes might provide an evolutionary force to maintain a clustered arrangement of homeobox genes.

The identification of a cis-acting DNA fragment able to confer the normal region-specific expression pattern of *Hox-3.1* to a heterologous gene in early embryos represents an important step in understanding the molecular mechanisms that control the complex patterns of mammalian homeobox gene expression. This control element can be used to force other homeobox genes to be expressed in the *Hox-3.1* early pattern, thereby perturbing their expression in a defined way. Clearly, such an approach is now feasible and may yield valuable insights into the regulation of expression as well as the function of homeobox genes in development.

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