Characterization and Retinoic Acid Responsiveness of the Murine Hoxd4 Transcription Unit*

(Received for publication, May 16, 1997, and in revised form, July 18, 1997)

Adriana Folberg‡§, Erszebet Nagy Kovács‡, and Mark S. Featherstone‡§

From the ‡McGill Cancer Centre and the Departments of §Medicine (Division of Experimental Medicine) and ¶Oncology, McGill University, 3655 Drummond St., Montréal, Quebec H3G 1Y6, Canada

We have characterized the transcription unit of a murine Hox gene in the fourth paralogous group, Hoxd4. We have identified two Hoxd4 transcription start sites by S1 analysis. The upstream promoter (P2) is 5.2 kilobase pairs upstream from the coding region, while the downstream promoter (P1) is 1.1 kilobase pairs distant. Both promoters bear a cluster of start sites. Multiple transcripts were identified by Northern blot, originating from both promoters and multiple polyadenylation signals. Expression of P1 transcripts in the neural tube shows an anterior border at the rhombomere 6/7 boundary, corresponding to previous reports (Gaunt, S. J., Krumlauf, R., and Duboule, D. (1989) Development 107, 131–141; Morrison, A., Moroni, M. C., Ariza-McNaughton, L., Krumlauf, R., and Mavilio, F. (1996) Development 122, 1895–1907). A more posterior boundary in the central nervous system was observed for P2 transcripts. We observed strong expression up to somite 6 and weak expression in somite 5, correlating with the phenotype of Hoxd4 null mutant mice (Horan, G. S. B., Nagy Kovács, E., Behringer, R. R., and Featherstone, M. S. (1995) Dev. Biol. 169, 359–372). In response to retinoic acid, expression from P1 in the hindbrain was anteriorized after 4 or 24 h of treatment. P2 transcripts seemed to be less responsive and/or to have an indirect response to retinoic acid. The long 5'-untranslated region found in all Hoxd4 transcripts suggests that translation does not occur by a classical ribosome scanning mechanism.

Hox genes encode homeodomain-containing transcription factors that specify positional identity along the anteroposterior and appendicular axes of the developing embryo (4). Hox homologs can be found from Hydra to humans in the animal kingdom. This high degree of conservation throughout evolution suggests that these genes are indispensable participants in embryonic development.

Mammals have 39 Hox genes, organized in four clusters, namely Hox A, B, C, and D (4, 5). These multiple clusters are the result of duplication events from the original cluster. Therefore, corresponding genes are distributed in the same order along each cluster and are called paralogs. During embryogenesis, Hox genes are sequentially expressed both in time and space, according to their position in the cluster. The genes at the 3'-end are the first to be expressed and have the most anterior borders of expression. The genes more to the 5'-end are expressed later in time and have more posterior domains of expression (4).

To safeguard their correct expression and function, Hox genes must be under precise regulation. Misexpression caused by either loss or gain of function has shown that the mutant mice present transformations and malformations that can affect central nervous system organization, somite derivatives, limbs, and other structures (4, 6).

Murine Hoxd4 is expressed in the embryo from day 8.5 onward, declining by day 12.5 (1). Its expression is detected in the spinal cord and prevertebra (1, 7). Hoxd4 knockout results suggest that this transcription factor functions in the specification of vertebral identity along the cervical region (3).

The Hoxd4 gene bears an autoregulatory element and a retinoic acid response element in its 5'-flanking region, and both elements are functional in vitro in transfected P19 cells (8, 9). The retinoic acid response element is highly conserved in the human homolog (10), and it is required to partially recapitulate the endogenous expression of Hoxd4 in the central nervous system (CNS)1 of transgenic mice (2).

Although two regulatory elements have been mapped, a more basic feature of the murine Hoxd4 transcription unit remains unknown, the transcription start site(s). To better understand the regulation of murine Hoxd4, we have characterized its transcription unit. We have mapped two promoters, each one composed of a cluster of start sites. A complex splicing pattern gives rise to transcripts originating from the upstream start sites. We demonstrate that both promoters are active in the embryo and that a number of transcripts are expressed as a result of alternative promoter and/or polyadenylation signal usage. The anterior expression boundary of the upstream promoter is posterior to that of the downstream promoter and thus follows the colinearity rule. In addition, the two promoters respond differentially to RA induction.

EXPERIMENTAL PROCEDURES

5'-Rapid Amplification of cDNA Ends (RACE)—First strand synthesis and tailing were performed under the conditions recommended by Life Technologies, Inc. 5'-RACE kit, except for the reverse transcriptase reaction, which was incubated at 42 and 37 °C for 30 min each (11). 1 μg of kidney or 11.5-day-old embryo total RNA was used for first strand cDNA synthesis. The RNA was annealed to antisense primer a (5'-CTCGCCTAGGTAAGCCACCCC-3') or e (5'-GAGATGGCGGCTTAATT-GCC-3') (Figs. 1 and 2). The cDNA was amplified using an antisense oligonucleotide from Hoxd4, primer b (5'-GCAAATATTCCTCG-CACCCA-3') or primer f (5'-TCTAGAAATCAGCAATTTACCT-3'), and an anchor primer complementary to the oligo(dC) tail (5'-UCUAUCUAUGCCACCGTGCAATGACCGGGGIIIIIGIIGIIGGIGG-3') supplied by the manufacturer. PCR conditions were 0.4 μm primer, 0.2 mM dNTP mix, 1.25 units of Taq polymerase (Boehringer Mannheim),

1 The abbreviations used are: CNS, central nervous system; RA, retinoic acid; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); UTR, untranslated region.
and PCR reaction buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.01% Nonidet P-40, and 0.01% Tween 20). Cycling parameters were one long cycle (95 °C for 5 min, 60 °C for 2 min, and 72 °C for 40 min) followed by 35 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 3 min (11). For the second round of amplification, 5 µl (10%) of the first PCR reaction was used as template, and the long cycle was omitted. Nested antisense oligonucleotide c (5'-TCCTTAATCTCA-CATGCCGCT-3') or g (5'-TTCCGGATCCCGTGCTGCTTTGGT-GCT-3') and a shorter version of the anchor primer (5'-CUACUAUC-AUCUCAGCCGGCGACTAGTAC-3') were used in the second PCR. For both rounds of PCR, the enzyme was added to the reaction after the denaturation step.

Specific PCR products (as assessed by Southern blot; data not shown) were gel-purified and cloned into EcoRV-linearized pBluescript KS (Stratagene) or T-tailed pBluescript KS. T-tailed vector was made by linearizing pBluescript KS with EcoRV and incubating with Top polymerase in the presence of 2 mM dTTP for 2 h at 70 °C. The T-tailed vector was purified by phenol extraction and ethanol precipitation. RACE clones were sequenced by the standard Sanger method (Pharmacia T7 sequencing kit).

**S1 Mapping**—S1 analysis was performed exactly as described (12). For single-stranded DNA probes, antisense oligonucleotide h (5'-TGG-TAGAGAAAGTACAGG-3') or d (5'-GGTCTGATACATTTACGC-3') was kidney-mediated, extended with Klenow using general murine Hoxd4 as template, and ligated to HindIII or KpnI, respectively. 5 x 10^6 cpm of probe was hybridized to 50 µg of 11.5-day-old embryo total RNA. The BamHI-AccI probe was a 2.1-kb fragment double-stranded, labeled at the AccI end.

**RNase Protection**—An RNase protection assay was performed to verify if the 5'-end of the RACE clone (shown in Fig. 1b) corresponded to a transcription start site (data not shown). Probe synthesis and hybridization were carried out as described (13). RNase T2 (Life Technologies, Inc.) was used for digestion (14), and protected fragments were resolved in a 6% denaturing acrylamide gel. The probe was made by subcloning a 493-bp EcoRI-KpnI fragment from the Hoxd4 5' region into pBluescribe (Stratagene), which was called EK (Fig. 4a). The template was linearized with EcoRI, and the antisense probe was synthesized using T3 RNA polymerase (Boehringer Mannheim). 5 x 10^6 cpm of probe was hybridized to 30 µg of 11.5-day-old embryo total RNA. A control reaction using tRNA was run in parallel.

**Northern Blot**—Northern blot was performed essentially as described by Chow et al. (15). 5 µg of poly(A)+ RNA (isolated using the Dynabeads system, Dynal) from 11.5-day-old mouse embryo was used. The 32P-labeled probe was added at 1 x 10^6 cpm/ml. Probe H-X was hybridized to the 3'-untranslated region (UTR), and it is a Hoxd9-XhoI fragment about 400 bp long. Probe A is a 2323-bp-long PCR fragment, from 1811 to 1489 from the coding region. Probe B is a PsI-EcoRI fragment within the intronic region just upstream of exon 5. Probe C is a 282-bp-long PCR fragment, from 402 to 121 from the coding region. To avoid cross-hybridization across parallel group 4, we designed probe C outside a region of high homology among the members of the Hoxd4 family (from 120 up to the coding region). The blot hybridized with probe C was run alongside commercial size markers (Promega).

**Whole Mount in Situ Hybridization**—Whole mount in situ hybridization was performed essentially as described (16), incorporating one extra step (17). Just before prehybridization, embryos were treated with 0.1% borohydride in PB (phosphate-buffered saline with 0.1% Tween 20) for 20 min at room temperature followed by three PBT washes. The alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was preadsorbed with embryo powder prepared as described (16) and used at a 1:5000 dilution. Embryos were incubated with antibody overnight at 4 °C; washed the next day, with five changes of the washing solution; and left overnight in TBST (25 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 2.7 mM CaCl2, 0.1% Tween 20, 2 mM levamisole). Color reaction was performed as described (16).

**Synthesis of Hybridization Probes**—Single-stranded RNA probes containing digoxigenin were synthesized from linearized template DNA exactly as directed by the manufacturer (DIG RNA labeling kit, Boehringer Mannheim). Probe BgH is a 950-bp-long BglII-HindIII fragment of murine Hoxd4 located in the homeobox, cloned into pGem1 (Promega) (1). The template was linearized with EcoRI, and the antisense strand was synthesized with SP6 RNA polymerase (Boehringer Mannheim). Probe Sma is a 470-bp Sma fragment starting 370 bp upstream of the Hoxd4 coding region, cloned into pBluescribe. Probe PEm is a 493-bp EcoRI-KpnI fragment of the downstream start site, cloned into pBluescribe. Both templates were linearized with EcoRI, and the probes were synthesized using T3 RNA polymerase (Boehringer Mannheim). The Kox20 probe was generously provided by Dr. D. Wilkinson.

**Administration of RA**—Pregnant CD-1 female mice were administered RA essentially as described by Conlon and Rossant (17). A stock solution of 25 mg/ml all-trans-retinoic acid (Sigma) in Me2SO was diluted 10 times in corn oil just before use, and 0.2 ml was delivered by gavage for a final dose of approximately 20 mg/kg of maternal body weight. Control mice were administered the same mixture without RA. For treatments of 4 h (short treatment), mothers were treated at 8 a.m. on embryonic day 8.5, and the embryos were recovered at noon of the same day. For treatments of 24 h (long treatment), mothers were treated at noon on embryonic day 8.5, and the embryos were recovered at noon on the following day. The day of appearance of the vaginal plug was taken as 0.5 days postcoitus. For all treatments, we analyzed between 10 and 25 embryos.

**RESULTS**

To map the transcriptional start site of Hoxd4, we performed an initial S1 analysis with mouse kidney RNA. Using a BamHI-AccI fragment as a probe, we observed two end points of protection (indicated as x and y in Fig. 1a). This suggested that murine Hoxd4 may have two promoters, as does its human homolog (18).

**Mapping the 5'-UTR Intronic Exon Boundaries**—We designed a 5'-RACE strategy based on the S1 end point of protection y (Fig. 1b). The first RACE product was obtained using oligonucleotide a as a primer for the reverse transcription reaction and using primers b and c for each round of PCR, respectively (Fig. 1b). Hoxd4-specific fragments (evaluated by Southern blot; data not shown) were cloned into pBluescript and sequenced. One clone, 140 bp long, showed homology to mouse Hoxd4 sequence; however, the homology was not contiguous. The 5'-
end of the clone started at position −1372 from the coding region, and at position −1300 the homology was interrupted. The sequence continued from position −402, and the 3′-end of the clone corresponded to primer e (Fig. 1b). Analysis of the genomic sequence flanking the point of discontinuity showed homology to the conserved consensus for 5′-donor and 3′-acceptor splice sites. An intron between positions −1300 and −402 within the 5′-UTR was thus delimited. Interestingly, neither splice site is conserved in the human HOXD4 sequence, which demonstrates a divergence between the two species (Fig. 2b).

RNase protection analysis showed a protection of the probe by sequences upstream of the 5′-end of the RACE product. This demonstrated that the RACE clone 5′-end did not correspond to a transcription start site (data not shown). S1 analysis was performed on sequences further upstream in an attempt to map the start site. An S1 analysis product was finely mapped to a cytidine at position −1817 from the coding region, which is preceded by CAAG, the known consensus for a 3′-splice acceptor site (see Fig. 2a). To test the possibility of another splice event, we designed a second 5′-RACE strategy (Fig. 2a). For the second RACE product, primer e was used for the reverse transcriptase reaction, and nested primers f and g were used for the two rounds of PCR, respectively. Specific fragments (evaluated by Southern blot; data not shown) were cloned into a T-tailed vector and sequenced. Eleven clones contained a sequence whose 3′-end corresponded to primer g but became discontinuous at position −1817, at the S1 end point of protection. The diverged 5′-end of these products was composed of three juxtaposed sequences of 85, 79, and 36 bp with homology to HOXD4 genomic sequences in a region between 4 and 5 kb upstream of the open reading frame. We compared the genomic flanking residues of these three segments and observed a very good homology with splice site consensus sequences (Fig. 2b). Therefore, this cDNA clone was composed of four exons, which we named 1, 2, 3, and 4. Overall, the two cDNA clones demonstrated that the transcript starting from an upstream promoter undergoes several splice events (Fig. 4a) and therefore is composed of multiple exons.

Mapping of the Upstream Promoter (P2) —We had yet to confirm whether the 5′-end of the second RACE clone corresponded to a transcription start site. For that purpose, we did an S1 analysis using a probe made by extension from primer h, located within exon 1, and extending up to a HindII site (Fig. 3, a and c). We obtained 10 end points of protection covering 70 nucleotides. These were further confirmed on a second S1 analysis using a longer probe linearized at the PstI site and gave one more protection (data not shown). This gave a total of 11 start sites spanning a region of 120 nucleotides. In this manner, we mapped a distal promoter (P2) 5.2 kb upstream from the coding region, whose transcripts are processed by the splicing machin-
The 2.6-kb band corresponds to the predicted size of transcripts originating from either P1 or P2 (assuming that all introns have been spliced out of the P2 transcript). The two known poly(A) signals are 270 and 572 nucleotides downstream of the stop codon. We believe that the broad 2.6-kb band comprises transcripts terminating at both of these sites.

In the human HOXD4 homolog, there are poly(A) signals 3 kb downstream of the stop codon (18). This suggests the possibility of additional poly(A) signals further downstream in the mouse Hoxd4 homolog as well. Using a HindIII-Xba fragment as a probe (Fig. 4a), we detected two bands corresponding to the larger size range, at 4.2 and 5.6 kb (data not shown). Based on this result, we propose two additional polyadenylation signals further downstream. Alternatively, there could be only one additional poly(A) signal, and the transcripts would be subject to additional post-transcriptional processing at the 3'-end. Either option would result in two more variations in the 3'-UTR.

Therefore, we conclude that both promoters are used in the mouse embryo and that the variant of molecular weights detected in the Northern blots is due to the presence and usage of various poly(A) signals.

**Differential Expression of Alternative Hoxd4 Transcripts in the Mouse Embryo**—Having demonstrated that at least two Hoxd4 promoters are used in the mouse, we wanted to examine how these promoters are regulated in the mouse embryo, for which we used whole mount in situ hybridization. We first hybridized 9.5-day embryos with probe BgH (Fig. 4a). Expression in the CNS confirmed the anterior boundary at the border of rhombomeres 6 and 7 (Fig. 5, a and b), as earlier described (1). In the somitic mesoderm, expression was strong in somite 6 and posterior and was weak in somite 5 (Fig. 5b). There was strong expression in the fore limb bud, as previously shown (19) and along the tail bud. In addition, we detected expression in the fourth branchial arch (Fig. 5a).

We used different probes to compare the expression of the P1 and P2 promoters. Probe EK detects only transcripts originating at P2 (Fig. 4a). Probe Sma detects transcripts originating at both promoters (Fig. 4a). Thus, expression domains uniquely detected by the P1-P2 Sma probe reflect the specific activity of the P1 promoter.

In both 8.5- and 9.5-day-old embryos, the staining was different for the two probes, especially in the CNS, thus demonstrating differential activity of the two promoters (Fig. 5, c and d). The staining with the P1-P2 Sma probe reproduced the pattern observed with probe BgH, which is consistent with the predicted ability of both probes to detect all transcripts from the Hoxd4 locus (Fig. 5c, embryo on bottom; d, embryo on left). However, transcripts detected with the P2-specific EK probe have an anterior border in the CNS that is more posterior (Fig. 5c, embryo on top; d, embryo on right). This result shows that the more anterior expression domain in the CNS is due to P1 activation but not P2. In the other domains, including the entire somitic column, the activity of both promoters overlaps (Fig. 5, c and d).

**Retinoic Acid Differentially Affects Each Hoxd4 Promoter**—Both mouse and human Hoxd4 transcripts are induced in cell culture upon treatment with RA. A retinoic acid response element is conserved in the genes of both species (2, 9, 10), located between the P1 and P2 promoters as mapped here (Fig. 4a). We wished to examine how each promoter would respond to retinoic acid in vivo.

After a 4-h RA treatment on day 8.5, embryos hybridized with the P1-P2 Sma probe showed an anterior shift of Hoxd4 expression in the hindbrain. This anteriorization is especially

---

2 M. S. Featherstone, unpublished results.
evident in embryos labeled simultaneously for Hoxd4 and Krox20 (Fig. 6a). In the RA-treated embryos, the Hoxd4 boundary is pushed anteriorly toward r5 (Fig. 6a, embryo on the right). Embryos stained with P2-specific EK probe showed no difference in expression after 4 h of RA treatment (data not shown).

The 24-h RA treatment also led to anteriorization in the CNS of transcripts detected with the P1/P2 Sma probe. The anterior boundary is now halfway overlapping with the otic vesicle at the rhombomere 5 and 6 boundary, which is one rhombomere more anterior than the normal border (Fig. 6b, embryo on the right). Embryos stained with the P2-specific EK probe showed anteriorization in one limited domain, ventral to the neural tube (Fig. 6c, embryo on the right).

Overall, these results suggest that embryonic Hoxd4 expression is responsive to RA treatment. This response is primarily from P1, which is affected after 4 h of treatment. Thus, P1 is more sensitive to RA and probably subject to a direct effect of RA. P2 showed a delayed and limited response; therefore, we concluded that it is less sensitive to RA and responds indirectly to its action.

FIG. 5. Expression of Hoxd4 in the mouse embryo and comparison of the P1 and P2 promoters. Panel a, in situ hybridization with probe BgH (Fig. 4a) in 9.5-day-old embryo. All Hoxd4 transcripts should be detected. In the CNS, expression starts just posterior to the otic vesicle and extends caudally. There is staining throughout the limb bud and in the fourth branchial arch. Panel b, dorsal view of the embryo shown in panel a, where strong somitic expression up to somite 6 and weak expression in somite 5 (s5) can be visualized. Panels c and d, 8.5- and 9.5-day-old embryos were labeled with either the P1/P2 Sma probe or with the P2-specific EK probe. P2 transcripts have an anterior border of expression in the CNS that is posterior to P1 transcripts at both ages. The arrowhead shows the P1 border of expression. In panel c, the top embryo is labeled with probe EK, and the bottom one is labeled with probe Sma. In panel d, the embryo on the left is labeled with probe Sma, and the one on the right is labeled with probe EK. OV, otic vesicle; IV, fourth branchial arch; L, forelimb bud. Magnification in panels a, b, and c is ×250, and in panel d it is ×120.

FIG. 6. Expression of Hoxd4 is affected by RA treatment. Panel a, dorsal view of 8.5-day-old embryos treated for 4 h with RA and hybridized with the P1/P2 Sma probe. P1 transcripts are anteriorized, since the P2-specific probe showed no change in expression pattern. Krox20 labeling was used to identify rhombomeres 3 and 5. Note the distance between the Hoxd4 rostral border (arrow) and rhombomere 5 in untreated and RA-treated embryos. This anteriorization was persistent after a 24-h treatment as shown in panel b. Panel b, in RA-treated 9.5-day-old embryos, the anterior border of P1 transcripts is at the boundary of rhombomeres 5 and 6 (open arrow), as revealed by Hoxd4 expression adjacent to rhombomere 5 (showing Krox20 expression). Panel c, P2 transcripts in the CNS were anteriorized after a 24-h RA treatment. The arrowheads point to anteriorized P2 transcripts in a domain ventral to the neural tube. Control embryos are on the left; RA-treated embryos are on the right. r3, rhombomere 3; r5, rhombomere 5; OV, otic vesicle.

DISCUSSION

We have analyzed various aspects of the murine Hoxd4 transcription unit. First, we mapped two transcription start sites that drive Hoxd4 expression and dissected the ensemble of
transcripts present in 11.5-day-old embryos. Second, we characterized the differential usage of these promoters in the mouse at 8.5 and 9.5 days postcoitus and their response to exogenous retinoic acid.

Murine Hox4 Has Two TATA-less Promoters—Both the upstream and the downstream promoters of murine Hoxd4 are composed of a cluster of start sites and do not bear a recognizable TATA box. TATA-less promoters were originally found in so-called “house-keeping” genes but later became a common feature especially among genes that are differentially expressed during development, such as oncogenes, growth factors, growth factor receptors, and transcription factors (20). In the absence of a TATA box, another class of binding sites might be present, the initiator motif (21). There are different classes of initiator elements, and they can bear binding sites for specific DNA-binding proteins (22). We were unable to detect any such consensus sequence elements in the vicinity of the Hoxd4 start sites. Nonetheless, since this is a more degenerate class, it would not be surprising if Hoxd4 would have an element with no homology with the ones described so far.

Alternative Promoters in the Hoxd4 Transcription Unit—Alternative promoter usage has been observed for many genes (23). It has been suggested that one single promoter region may not always be sufficient to accommodate all of the required information for a gene to be correctly expressed in time and space (23). This is particularly true and necessary for genes that are expressed in more than one tissue and/or developmental stage and which therefore may respond to different stimulatory signals. Hox genes may well form such a class.

The use of multiple promoters is not without precedent for members of the Hox gene cluster. In paralog group 4, for example, both mouse Hoxa4 and Hoxd4 have two promoters each (24, 25). At least in one instance, for the mouse Hoxb3 gene, multiple promoters are differentially expressed in the embryo, providing elasticity in gene expression (26).

The multiple human HOXD4 transcripts are also generated from two alternative promoters and various polyadenylation signals (18). However, while the upstream promoter is located at position -1142 from the ATG, the downstream promoter is only 20 bp 5’ of the first codon (18). The mouse downstream promoter appears identical to the human upstream one, suggesting conservation between the two genes. However, we have been unable to detect anything comparable with the far downstream human promoter. This suggests that the two species have both conserved and diverged regulatory pathways for the control of Hoxd4 expression.

Domains of Expression of Hoxd4 in the Mouse Embryo—In the CNS, the transcripts detected with both P1-P2 and BgH probes have an anterior border in the hindbrain between rhombomeres 6 and 7, characteristic of Hoxd4 expression (1, 2). For the transcripts detected with the P2-specific probe that detects transcripts from the upstream promoter, the anterior border is more caudal and diffuse. This shows (a) that only the downstream promoter, P1, is active in the anterior-most domains of Hoxd4 expression in the hindbrain, and consequently (b) that there is a differential regulation of the two promoters. The possible origin of the upstream promoter might explain its more posterior expression in the hindbrain. The analysis of cDNA clones from the human HoxC cluster has revealed transcripts from the human HOXC4 gene originating in a promoter more than 35 kb upstream, in the HOXC6 gene (27). There is one long multigenic transcript in which the 5’ exon is shared between three genes, HOXC6, -C5, and -C4 and is brought into proximity with sequences from each gene by alternative splicing (27). At the same time, in the HoxD cluster, the members of paralog groups 5–7 are missing. It is possible that these genes were present in the ancestral vertebrate cluster and then were lost in the course of evolution (28). Potentially, regulatory regions left from the missing genes could now be acting on the next proximal gene, which is Hoxd4. The CNS expression of the Hoxd4 upstream promoter could indicate regulation by elements from ancestral 5’-genes (hence its more posterior expression domain). Hoxb5 expression in 12.5-day-old embryos is most intense directly posterior to the hindbrain, but it does extend into the hindbrain region (29). The hindbrain-spinal cord junction corresponds roughly to the midpoint between the otic vesicle and the beginning of the fore limb bud. The P2 anterior border is diffuse but extends anteriorly beyond this midpoint. Therefore, the P2 anterior border could reflect the paralog group 5 border. Interestingly, Hox8 expression in the CNS extends up to the hindbrain-spinal cord junction, far more anterior than its expression in mesoderm derivatives (30). This finding suggests that Hoxd8 and Hoxd4 P2 might share an enhancer element that drives their expression in the CNS.

With respect to the somitic expression, the initial description defined the border of Hoxd4 and Hoxd8 at prevertebrae 1 and that of Hoxd4 at prevertebrae 2 (1). We detected strong expression up to and including somite 6 and weak expression in somite 5. Despite being weak, this somite 5 expression might play a role in the patterning of the structures derived from it. Of all the paralog group 4 null mutants (3, 31–33), Hoxd4-/-mice show the most anterior transformations in somite-derived structures. These are transformations in the basicipital bone (3), which is partially derived from somite 5 (34). This phenotype can now be better understood by the endogenous expression of Hoxd4 in this somite.

All of our probes detected strong expression of Hoxd4 in the limb bud and in the tail bud region. Interestingly, a Hoxd4-lacZ transgene, containing about 6 kb of sequence upstream and 5 kb downstream of the coding region, was not expressed in these regions.3 This suggests that these domains are under the control of elements located far from Hoxd4, which is consistent with recent evidence for a limb bud enhancer in the HoxD cluster (35).

Hoxd4 Promoters Respond Differently to Retinoic Acid—Hox genes from the anterior (groups 1–3) and medial (groups 4–8) paralog groups can be anteriorized upon exogenous RA treatment (17, 36). This response depends on the gestation time when the treatment is administered. Each gene responds within a time window, after which it becomes refractory to the treatment. The RA-sensitive window functions under the collinearity rule; the genes more 3’ to the cluster are most sensitive in the early stages, and as time goes by the window moves along the cluster. Hoxb4 is anteriorized when embryos are treated at 8 a.m. of day 8 (17). Hoxd4 transcripts from the downstream promoter are also anteriorized upon this RA regime. This indicates that these two group 4 paralog genes are responsive within the same RA-sensitive window, as would be expected.

The Hoxd4 transcripts originating from the upstream promoter showed a limited and delayed response to RA, suggesting an indirect response. The human HOXD4 promoters are differentially responsive to RA as well (37). In vitro stimulation of NT2/D1 cells with RA has shown that the putative transcripts from the human downstream promoter are responsive to lower concentrations, while transcripts from the upstream promoter are responsive to higher concentrations of RA (37).

Regulation of Hoxd4 at the Post-transcriptional Level—Two important features of Hoxd4 transcripts suggest that post-

---

3 Zhang, F., Pöpperl, H., Morrison, A., Nagy Kovács, E., Prideaux, V., Schwarz, L., Krumlauf, R., Rossaut, J., Featherstone, M. S. (1997) Mech. Dev., in press.
transcriptional regulation takes place during Hoxd4 expression. At the 3'-end of the murine Hoxd4 messages, at least three polyadenylation signals are used (this paper, and see Footnote 2). The process of polyadenylation may be used to regulate gene expression. There are many proteins involved in polyadenylation, and the poly(A) tail plays a dual role in transcription initiation and RNA turnover (38). In oocytes of Xenopus and mice, differential polyadenylation and deadenylation regulates the translation of maternal mRNAs (39). The alternative usage of the different Hoxd4 poly(A) signals may therefore be another regulatory step in Hoxd4 expression.

Different Hox genes show an extensive nucleotide conservation at the 5’-noncoding region immediately preceding the initiator AUG (40). Hoxd4 messages starting from either promoter have a long 5’-UTR of 1.1 kb and share the last 400 nucleotides preceding the initiator AUG. According to the classical scanning model of translation, the long Hoxd4 5’-UTRs would be inefficiently translated (41). For one, the leaders bear multiple cryptic AUGs. In the last 400 nucleotides only, which are shared between both transcripts, there are 11 AUGs. Additionally, the leaders are likely to form an unfavorable secondary structure, and the long distance between the cap and the true AUG might impede the ribosomes through a “translation traffic jam” (42). This might not be a problem if another translation strategy is used. For transcripts from the Drosophila Hoxa gene Antennapedia, translation initiation takes place through internal ribosome binding (43). It is tempting to speculate that the shared sequences immediately preceding the Hoxd4 initiator AUG may likewise provide an internal ribosome entry site.

Conclusion—Alternative promoters may provide flexibility in response to different combinations of transcription factors. This is the situation for the Drosophila Adh gene. This gene has two promoters, which are differentially deployed during embryonic development (44). This happens due to differences in the core promoter elements, and the selective Adh promoter utilization is mediated by a specific TBP-TAF complex in combination with TFIID (44). By analogy, we could envisage a similar situation taking place in the murine Hoxd4 gene. The two promoters are differentially expressed, and we have shown that they respond differently to the same stimulus (RA). This may reflect differences in the basal transcriptional machinery bound to the core promoter elements and its interaction with enhancer factors like the retinoic acid receptors.

The colinearine rule states that Hox genes are expressed in a sequential order that respects their position in the cluster and that also correlates with their sensitivity to RA (4). Our characterization of the Hoxd4 transcription unit shows that this rule can also be applied within a single gene, whereby the promoter in a more 3’ position has a more anterior border of expression and is more responsive to RA, while the promoter more 5’ has a more posterior border of expression and is less responsive to RA.

Acknowledgments—We are grateful to R. Conlon and I. Dussault for advice on whole mount in situ hybridization; L. Chow for the protocol for Northern blot analysis; D. Wilkinson for the krox20 probe; and E. Daniels, F. Zhang, and members of the laboratory for helpful discussions.

REFERENCES

1. Gaunt, S. J., Krumlauf, R., and Duboule, D. (1989) Development 107, 131–141
2. Morrison, A., Moroni, M. C., Ariza-McNaughton, L., Krumlauf, R., and Duboule, D. (1989) Development 107, 131–141
3. Dorfman, S. F. B., Nagy, M., and Seidman, J. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5995–6000
4. Krumlauf, R. (1994) Cell 78, 191–201
5. Zeltser, L., Desplanc, C., and Heintz, N. (1996) Development 122, 2475–2484
6. St-Jacques, B., and McMahon, A. P. (1996)Curr. Opin. Genet. Dev. 6, 439–444
7. Krell, M., and Gruss, P. (1994) Cell 78, 191–201
8. Pooper, H., and Beilinson, E. (1996) Cell 84, 819–829
9. Kessel, M., and Gruss, P. (1991) Cell 64, 489–499
10. Visconti, A., Acampora, D., D’Esposito, M., and Boncinelli, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 330, 673–677
11. Izpisua-Belmonte, J.-C., Dolle, P., Zappavigna, V., Falkenstein, M., and Krumlauf, R. (1993) Development 116, 373–383
12. Roh, G. S. R., Nagy-Kovacs, E., Behringer, R. R., and Featherstone, M. S. (1995) Dev. Biol. 169, 359–372
13. Nolte, P., Drexler, G. S., Daniels, E., Turcotte, C., Chow, B., Basle, A. L., Holmes, K. V., and Beauchemin, N. (1994) J. Virol. 68, 4525–4537
14. Saucerman, C. F., Bordone, M., Chen, J. S., and Nordstrom, J. L. (1992) Trends Biochem. Sci. 17, 434–447
15. Chow, L. M. L., Ratcliffe, M. J. H., and Veillette, A. (1992) Mol. Cell. Biol. 12, 1226–1233
16. Wilkinson, D. G., and Nieto, M. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11643–11648
17. Conlon, R. A., and Rossant, J. (1992) Development 111, 357–368
18. Cianetti, L., Cristofaro, A. D., Zappavigna, V., Bottero, L., Bocell, G., Testa, U., Russo, G., Benincelli, E., and Peschle, C. (1990) Nucleic Acids Res. 18, 4361–4366
19. Roll, P., Izpisua-Belmonte, J.-C., Falkenstein, H., Renucci, D., and Duboule, D. (1990) Nature 342, 4146–4154
20. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Struhl, K. (1989) Current Protocols in Molecular Biology, Vol. 1, pp. 4.6.1–4.6.13, Wiley Interscience, New York
21. Smale, S. T., and Baltimore, D. (1989) Cell 57, 103–113
22. Zawel, L., and Reinberg, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4657–4660
23. Ayoubi, T. A. Y., and van de Ven, W. J. M. (1996) FASEB J. 10, 453–460
24. Galbiot, H., Dalle, P., Vigneron, M., and Featherstone, M. S., Baron, A., and Duboule, D. (1989) Development 107, 343–359
25. Gutman, A., Gilthorpe, J., and Rigby, P. W. J. (1994) Mol. Cell. Biol. 14, 4109–4114
26. Sham, M. H., Hunt, P., Nonner, S., Papalopulu, N., Graham, A., Benincelli, E., and Krumlauf, R. (1992) EMBO J. 11, 1825–1836
27. Simeone, A., Finamore, M., Acampora, D., D’Esposito, M., and Benincelli, E. (1988) Nucleic Acids Res. 16, 2379–2386
28. Ruddle, F. H., Bartels, J. L., Bentley, K. L., Kappan, C., Murtha, M. T., and Pardeton, J. W. (1994) Annu. Rev. Genet. 28, 423–442
29. Krell, M., and Gruss, P. (1994) Cell 78, 191–201
30. Izpisua-Belmonte, J.-C., Roll, P., Renucci, D., Zappavigna, V., Falkenstein, H., and Duboule, D. (1990) Development 110, 733–745
31. Ramirez-Solis, R., Zheng, H., Whiting, J., Krell, M., and Bradley, A. (1993) Cell 73, 279–294
32. Horan, G. S. B., Wu, K., Wolgemuth, D., and Behringer, R. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 91, 12644–12648
33. Boulet, A. M., and Capecchi, M. R. (1996) Dev. Biol. 177, 232–249
34. Couly, G. F., Colsey, P. M., and Le Douarin, N. M. (1993) Development 117, 409–429
35. van der Hoeven, F., Zaks, M., and Duboule, D. (1996) Cell 85, 1025–1035
36. Kesel, M., and Gross, P. (1991) Cell 67, 89–104
37. Simeone, A., Acampora, D., Arioni, L., Andrews, P. W., Benincelli, E., and Mavilio, F. (1990) Nature 346, 755–760
38. Proudfoot, N. (1996) Cell 87, 779–781
39. Wirth, M. (1993) Curr. Opin. Genet. Dev. 5, 950–954
40. Burglin, T. R., Wright, C. V. E., and DeRobertis, E. M. (1987) Nature 320, 361–367
41. Kessel, M., and Gruss, P. (1994) Cell 78, 191–201
42. Geballe, A. P., and Morris, D. R. (1994) Trends Biochem. Sci. 19, 159–164
43. Oh, S.-K., Scott, M. P., and Sarnow, P. (1992) Genes Dev. 6, 1643–1653
44. Hansen, S. K., and Tjian, R. (1995) Cell 82, 565–575