Int. J. Dev. Biol. 46: 325-328 (2002)

Short Communication

Retinoic acid response element in *HOXA-7* regulatory region affects the rate, not the formation of anterior boundary expression

MYOUNG H. KIM*, JAE S. SHIN, SUNGDO PARK, MAN-WOOK HUR[1], MI-OK LEE[#,2], HYOUNGWOO PARK and CHUL-SANG LEE[3]

Dept. of Anatomy, Biochemistry and Molecular Biology[1], and Microbiology[2], Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul, Korea and Korea Research Institute of Bioscience and Biotechnology[3], Taejon Korea

ABSTRACT Since it is known that a 307 bp fragment of the position specific regulatory element of human *HOXA-7* contains two (DR3 and DR5) retinoic acid response elements (*RAREs*) at its 3' end, we constructed several deletion constructs containing different numbers of *RAREs* and examined their effects *in vitro* and *in vivo*. The 5' deletion constructs, BM112 and OM213, retaining both *RARE*s, were highly responsible (about 8 fold induction) for RA in F9 teratocarcinoma cells, versus NM307 (4-5 fold). The construct NS218, with both *RAREs* deleted but retaining the 5' sequences lost RA responsibility completely, whereas NR271, with one *RARE* (DR5) deleted retained a 50% inducibility (2.5 fold). *In vivo* transgenic analysis revealed that the constructs NM307 and NR271, but not OM213 nor BM112, directed the position specific expression pattern. Sequence analysis revealed that *HOXA-7* enhancer sequences, including the *RARE* repeat sequences, were well conserved in human, mouse and chick. Part of the *RAREs* overlaps with the CDX1 binding site, and sequences of the DR3 *RAREs* were identical in this species. Two GAGA binding sites were also found to be strictly conserved. Because OM213, which had one GAGA site disrupted but retaining both *RAREs*, did not direct anterior boundary formation in transgenic mice, these results suggest the importance of the 5' 94 bp region, including the GAGA binding site, in anterior boundary formation and the involvement of the *RARE* in the rate of expression not in anterior boundary formation.

KEY WORDS: *human HOXA-7, position specific regulatory element, retinoic acid response elements, GAGA*

Hox genes, which encode transcription factors, are known to be responsible for conferring regional identity in embryonic tissues, such as the limb buds, the neural tube, and the presomitic mesoderm (Min *et al.*, 1998; Haack and Gruss, 1993). Thus far 39 *Hox* genes, related to those of the *Drosophila* homeotic gene complex (*HOM-C*) have been identified in vertebrates. These genes are arranged in four clusters of approximately 100 kb that map to different chromosomes, and which are expressed in a sequential manner, i.e. colinear with the position on the cluster and the expression domain along the anteroposterior axis of the developing embryo and with embryonic development time.

Retinoic acid (RA), a metabolite of vitamin A (retinol), is known to be required for the normal development of the vertebrates (Hofmann and Eichele, 1994). A deficiency or excess of RA during embryogenesis leads to developmental abnormalities in several vertebrates, including the zebrafish, Xenopus, chickens, mice, and humans (Langston *et al.*, 1997). Moreover, evidence exists that RA regulates *Hox* gene expression (Huang *et al.*, 1998). When teratocarcinoma cells, such as F9 are treated with RA, most *Hox* genes are expressed sequentially, in accord with their positions in the clusters (Simeone *et al.*, 1990). During early development RA treatment causes an anterior shift of the expression boundary of several *Hox* genes, such as *Hoxa-1* and *Hoxb-1*. These are normally expressed in the neural tube with distinct anterior boundaries in the hindbrain and this shift is associated with the homeotic transformation of segments in the hindbrain (Marshall *et al.*, 1992; Kessel and Gruss, 1991). Furthermore, RA has been detected in the dorsal lip of Xenopus, in Hensen's node of the chick and in the primitive streak of the mouse, which are the areas responsible for organizing the embryo and the likely sites of *Hox* induction (Chen *et al.*, 1992; Wagner *et al.*, 1992). A graded anteroposterior

Abbreviations used in this paper: DR, direct repeat; *PSRE*, position specific regulatory element; RA, retinoic acid; *RARE*, retinoic acid response element.

***Address correspondence to:** Dr. Myoung Hee Kim. Dept. of Anatomy, Yonsei University College of Medicine, C. P. O. Box 8044, Seoul 120-752, Korea. Tel: +82-2-361-5173. Fax: +82-2-365-0700. e-mail, mhkim1@yumc.yonsei.ac.kr

 # Present address: Dept. of Bioscience and Biotechnology. Sejong University, Seoul, Korea.

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

distribution of RA across the wing buds, and the respecification of positional identity by locally applied RA during the development or the regeneration of the vertebrate limb (Maden, 1985; Tabin 1992), strongly implies that RA is a natural morphogen in vertebrates, which can specify positional identity. Regulatory regions of several *Hox* genes, such as *Hoxa-1*, *Hoxb-1* (Langston *et al.,* 1997), *Hoxa-4* (Doerksen *et al.,* 1996) and *Hoxd-4* (Popperl and Featherstone, 1993) have been found to contain retinoic acid response elements (*RARE*s), which suggests that transcriptional regulation by RA is mediated by a direct mechanism involving the retinoic acid receptors (RARs) (Gudas, 1994). Previously we identified a 307 bp position specific regulatory element of human *HOXA-7* (Min *et al.,* 1998), which was sufficient to direct downstream reporter gene expression in a position specific manner with a distinct anterior boundary in transgenic embryos. Since the 307 bp of *HOXA-7* contained two *RARE* direct repeat sequences, we analyzed the function of these sequences *in vitro* and *in vivo* in transgenic mice.

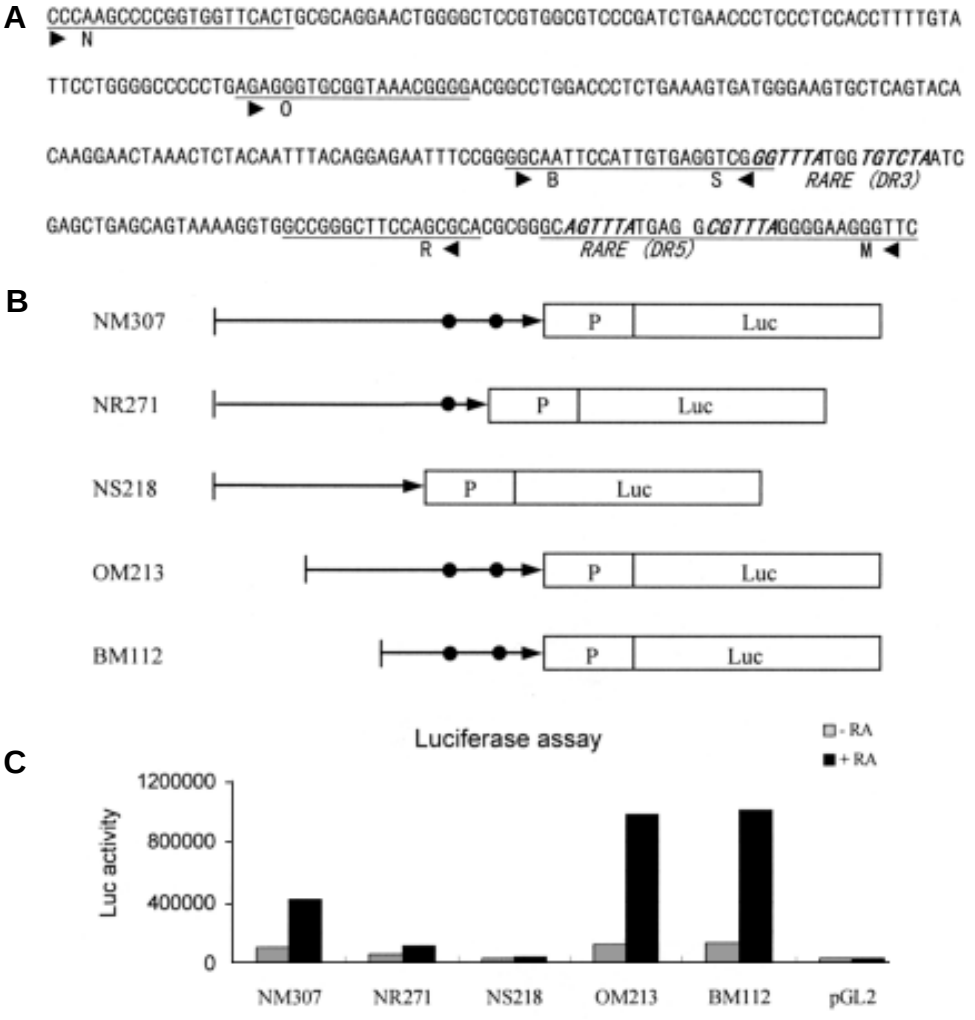

Fig. 1. *In vitro* analysis of deletion constructs containing different numbers of *RAREs*. (A) *The nucleotide sequences of the 307 bp (NM307) position specific regulatory element of HOXA-7. The primer sequences (N, O, B, S, R, M) used for PCR are underlined with arrowheads indicating the 3' direction. The RARE repeat sequences DR3 and DR5 are written in bold italics.* **(B)** *The reporter deletion constructs of the HOXA-7 control element. The RARE repeat sequences are marked as* ●. **(C)** *Luciferase activity of each construct in the presence or in the absence of RA.*

In Vitro *Effect of RAREs on Gene Expression*

Previous transgenic analysis has shown that the 307 bp fragment (NM307) in the *HOXA-7* upstream sequence (Fig. 1A) is sufficient to set the anterior boundary of expression (Min *et al.*, 1998). The finding of two putative direct repeat (DR) sequences of the retinoic acid response element (*RARE*) located in the 3' region of the fragment (Fig. 1A); i.e., nt 217-231 (DR3) and nt 279-295 (DR5) was of particular interest. In order to characterize the *RARE* function of *HOX* gene expression, several fragments containing different number of *RAREs* (NM307, NR271, NS218, OM213, and BM112) were generated as described in 'Experimental Protocols' and cloned into the *Sma*I site of the pGL2-promoter vector harboring a luciferase gene as a reporter (Fig. 1B). After transient transfection into the F9 teratocarcinoma cells, luciferase activity was analyzed in the presence or absence of RA.

As shown in Fig. 1C, all pGL2-px reporter constructs showed basal level activity in the absence of RA (Fig. 1C). However, RA treatment led to a 4-fold induction of luciferase activity in cells carrying the NM307 construct. The constructs deleted one (NR271) putative *RARE* reduced the luciferase induction by 50% (2.5-fold induction) whereas the cells carrying the NS218 in which both *RAREs* were absent were not induced at all in the presence of RA. The construct having a 94-bp deletion at the 5' end (OM213), however, showed a 10-fold induction, which is almost as same as that of BM112 having a further deletion at the 5' end but retaining both *RARE*s (Fig. 1B, C).

These results altogether indicate the existence of a plausible negative regulatory element located in the 5' 94 bp region deleted in OM213, and an enhancer element responsible to RA in the 112 bp (BM112) region. Since a transgenic mice carrying the OM213 construct expressed the reporter gene along the whole neural tube (NT) without restriction (Min *et al.,* 1998), a negative regulatory element located at the 5' 94 bp region was speculated to be involved in anterior boundary formation *in vivo.*

In Vivo *Effect of RAREs on Anterior Boundary Formation*

Since transfection analysis revealed that both *RAREs* were required for full induction of transgene, we examined whether both *RAREs* are also required to set the anterior boundary of expression. To do this, two kinds of transgenic mice were generated; NM307 (307 bp/*LacZ*) and NR271 (271 bp/*Lac*Z) harboring two and one *RARE* binding site, respectively. In the case of NM307, four (1 female and 3 male) founder (F0) mice were obtained and three were found to express *LacZ*

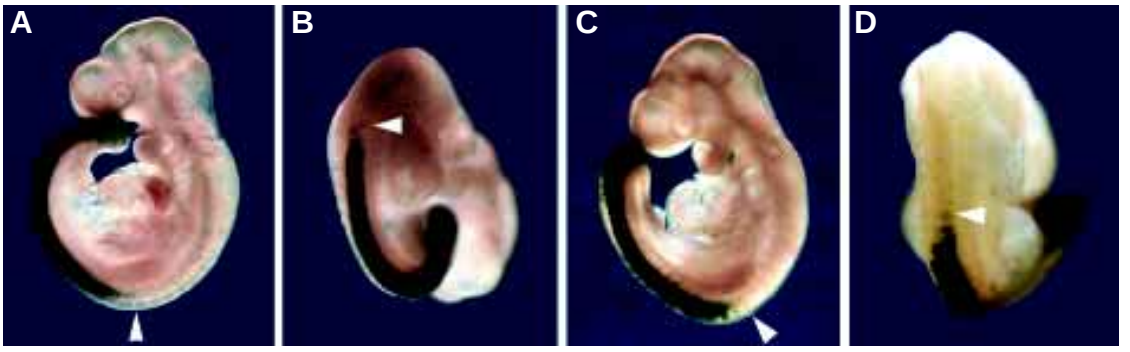

Fig. 2. Whole mount *LacZ* expression pattern in NM307 (A,B) and NR271 (C,D) transgenic embryos. *Lateral (A, C) and dorsal (B, D) views of transgenic embryos at day 9.5 p.c. The anterior limit of expression is marked with white arrowheads.*

with a sharp anterior boundary (Fig. 2 A,B). Whereas NR271, all three founder mice (1 female and 2 males) obtained here expressed the transgene; two exhibited the characteristic position-specific expression pattern with an anterior boundary (Fig. 2 C,D), while one showed an ectopic pattern (data not shown). The reporter *lacZ* was expressed in the ectoderm-derived neural tube, and very faintly in the spinal ganglia, but not in the mesoderm derived somite. This expression pattern is somewhat different from previous transient expression patterns associated with NM307 (Min *et al*., 1998), in which *lacZ* expression was rather strong in the spinal ganglia. The discrepancy could be partly due to the genetic background of the transgenic embryos. In the case of the line analyzed here, the founder mice of C57BL/6XDBA background were mated with ICR mice, so the F1 embryos analyzed here must have a 50% ICR background, whereas this was not the case in transient analysis, i.e. F0 has no ICR background. When F1 embryos did not produce a sufficiently strong LacZ expression, F2 embryos were analyzed after generating F1 mice by mating with ICR mice. In this case, the F2 transgenic embryos expressed transgene more strongly than the F1 embryos. During this analysis, we found that F2 transgenic embryos, having more ICR background, expressed reporter gene more strongly than F1 embryos, without altering the expression boundary. Although it is not clear why the rate/intensity of the expression is different among the generations, all the expressing embryos containing either NM307 or NR271 constructs exhibited a distinct anterior limit of expression in the neural tube, i.e., at the level of somite 10 (S10) in the embryo at day 9.5 post coitum (p.c.), indicating that one *RARE* binding site (DR3) is sufficient to set the anterior boundary of expression (Fig. 2).

Sequence Analysis

Since ontogeny is a recapitulation of phylogeny, *Hoxa-7* enhancer sequences were compared for vertebrates, such as the human, mouse and chick. As shown in Fig. 3, enhancer sequences, including the *RARE* repeat sequences were well conserved among the species. Interestingly, both *RARE* repeat sequences overlap with the CDX1 binding site (5'-TTTATG), which has been reported to be important for *Hox* gene expression (Subramanian *et al*., 1995). Deletion of one *RARE* (DR5)/CDX1 binding site located at the 3' end, which is less well conserved than the 5' CDX1 binding site in DR3, did not affect the position-specific expression pattern *in vivo*, whereas *in vitro* the induction rate was reduced about 50%. Since the *in vitro* analysis involved testing the function of *RAREs* in the presence or absence of RA, not the CDX1 function, we cannot rule out the possibility that the concomitant deletion of the CDX1 binding site could have contributed to the induction rate. Co-transfection analysis of Cdx1 and NR271/NM307 constructs could reveal the effect of CDX1 on the expression of the *Hox* gene in the future.

```
Human-A7    CCCAA-------GCCCCGGTGGTTCACTGCGCAGGAACTGGGGCTCCGTGGCGTCCC---
Mouse-a7    -CCAAAACCTGAGCCC------TTTACTAGGTAGGAGCTGGGGTTC------GTTCCAGC
Chick-a7    ------------------------------------------------------------

Human-A7    --GATCTGAACCCTCCCTCCACCTTTTGTA-TTCCTGGGGCCCCCTgagaGGGTGCGGTA
Mouse-a7    GGGATTTGAACA-TCCCTCTGTCTTTTGTGCTTCCAAGACCCCCCTgagaGGATGCGGTA
Chick-a7    ------------------------------------------------------------

Human-A7    AACGGGGACGG---------------CCTGGACCCTCTGAAAGTGATGGGAA-GTGCTCA
Mouse-a7    AACGGGGGTGGGGTAGGTGGAGGCAGCCTGGACCCACTGAAAGTGATAAGAAAGTGCTTT
Chick-a7    ----------------------------------------AAGTGGTGAGAAAGT-CTTT

Human-A7    GTACACAAGGAACTAAACTCTACAATTTACAGgagaATTTCCGGGGCAATTCCATTGTGA
Mouse-a7    GTACACCAGGAACCAAACTCTATAATTTAGAGgagaATTTCCGGGGCAATTCCATTGTGA
Chick-a7    GTACACGGGGCACCAAACT--ATAATTGAAAGgagaATTTCCGGGGCAATTCCATTGTGC

Human-A7    GG--TCGGGTTTATGGTGTCTAATCGAGCTGAGCAGTAAAAGGTGGCCGGGCTT--CCAG
Mouse-a7    AG--TCGGGTTTATGATGTGCAATCGAGCTGAGCAGTAAAAGGTCGCCAGTCTT--CCAG
Chick-a7    TCCTTCCAGTTTATGATGTGCAATACAGGCAGGCAGTAAAAGCT-----GTCTTTACCAG

Human-A7    C---GCACGC--GGG-----------CAGTTTATGAGGCGTTTAGGGGAAGGGTTC
Mouse-a7    CA-TG---GCCTGAG-----------CAGTTTATGAGGCGTTTAGGGGAAGGGTTC
Chick-a7    GACTCCCTGTCTGAGGGAAGGAAACTCATTTTACGATCCGTTTAAGGAAGGG---
```

Fig. 3. Nucleotide sequence alignment of *HOXA-7* enhancers from human (GenBank accession number, X81029), mouse (U15972), and chick (Y18147). *Gaps introduced for maximal similarity are indicated by a hyphen, identical nucleotides between sequences are shaded. GAGA, and CDX1 binding sites, and RAREs are written in small, italic, and bold letters, respectively.*

The GAGA binding sites also proved interesting, as they were strictly conserved among the species compared (Fig. 3). The GAGA factor is a trithorax-like (*trl*) protein, which is one of the trithorax group (*trxG*) gene products. Initially the *trxG* genes were reported to be required for the maintenance of the spatially localized expression patterns of the homeotic genes beyond mid-embryogenesis (Deschamps *et al.,* 1999). Later, they were found to participate in the assembly of PcG complexes at *PRE* (polycomb response element), which rather mediates the silencing of the homeotic genes (Horard *et al*., 2000). A previous transgenic experiment showed that OM213 having one GAGA binding site disrupted, did not give the correct anterior expression boundary (Min *et al*., 1998), suggesting the possible importance of GAGA in the position specific expression of

the *Hox* gene, but not the rate of induction. Since the position specific expression means the activation and repression of gene expression at different regions of the embryo, positive and/or negative regulatory elements are speculated to be exerted in these different areas.

Experimental Procedures

Reporter Constructs

Transgenic reporters, NM307 and NR271 were generated as described previously, except for the primer sets (see Min *et al.,* 1998).

Reporter constructs for transient transfection experiments were prepared as follows: NR271 (271 bp), NS218 (218 bp), OM213 (213 bp), and BM112 (112 bp) fragments were amplified by the PCR in the presence of template NM307 (307 bp) and the appropriate primers (N,R,S,O,M and B) and cloned into the *Sma*I site of the vector, pGL2-promoter (Promega), giving rise the reporter constructs, pGL2-pNM307, -pNR271, -pNS218, -pOM213, and -pBM112. All constructs were sequenced to confirm the integrity and orientation of the inserted fragments.

Generation and Analysis of Transgenic Mice

Transgenic mice were produced according to the method previously published (Min *et al.,* 1998). To confirm the transgenic mice harbored NM307 and NR271, a small amount of tissue was taken from the ears, and digested in 20 μl of digestion buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, and 1% SDS), and 1 μl of proteinase K (20 mg/ml) at 55°C for 3 hour with vortexing every hour. DNA was purified by centrifugation at 15,000 rpm after adding 180 μl of distilled water and boiling for 5 min. PCR reactions were carried out with *lacZ* specific sense (5'-GAA GCC AAT ATT GAA ACC CA) and anti-sense (5'-GCA AAG ACC AGA CCG TTC AT) primers.

Histological analyses and X-Gal staining of transgenic embryos were performed as described previously (Min *et al.,* 1998).

Cell Culture, Transfection, β-Galactosidase and Luciferase Assay

F9 teratocarcinoma cells were grown in Dulbecco's modified Eagles medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS; Gibco, BRL) in Gelatin-coated (0.1%) culture dishes. Transient transfections were performed using LipofectAMINE PLUS reagent (GibcoBRL) with pGL2-px construct and the β-gal reporter plasmid was used as an internal control. After 3 hours incubation, the media was replaced with the one containing RA (final concentration of 10^{-7} M of all trans-RA, Sigma Chemical Co.), and cultured for a further 24 hour before cell harvesting. The luciferase assay was performed using the Promega's Luciferase Assay System.

Sequence Analysis

Sequence alignment of the *HOXA-7* enhancers from human (GenBank accession number, X81029), mouse (U15972), and chick (Y18147) was undertaken with the multiple sequence alignment program developed by Huang (1994). Gaps were introduced to maximize sequence alignment.

Acknowledgments

This work was supported by the Ministry of Science and Technology (00, 01-J-LF-01-B-61) and in part by grant No. R04-2001-00156 from the KOSEF.

References

CHEN, Y., HUANG, L., RUSSO, A.F. and SOLURSH, M. (1992). Retinoic acid is enriched in Hensen's node and is developmentally regulated in the early chicken embryo. *Proc. Natl. Acad. Sci. USA* 89, 10056-10059.

DESCHAMPS, J., VAN DEN AKKER, E., FORLANI, S., DE GRAAFF, W., OOSTERVEEN, T., ROELEN, B. and ROELFSEMA, J. (1999). Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. *Int. J. Dev. Biol.* 43, 635-650.

DOERKSEN, L.F., BHATTACHARYA, A., KANNAN, P., PRATT, D. and TAINSKY, M.A. (1996) Functional interaction between a RARE and an AP-2 binding site in the regulation of the human HOX A4 gene promoter. *Nucleic Acids Res.* 24, 2849-2856.

GUDAS, L.J.(1994). Retinoids and vertebrate development. *J. Biol. Chem.* 269: 15399-15402.

HAACK, H. and GRUSS, P. (1993). The establishment of murine Hox-1 expression domains during patterning of the limb. *Dev. Biol.* 157, 410-422.

HOFMANN, C. and EICHELE, G. (1994) Retinoids in development. *In* The retinoids: Biology, Chemistry and Medicine. (Eds. Sporn, M.B., Roberts, A.B., and Goodman, D.S.). pp.387-441, Raven Press, New York, NY.

HORARD, B., TATOUT, C., POUX, S. and PIRROTTA, V. (2000). Structure of a polycomb response element and in vitro binding of polycomb group complexes containing GAGA factor. *Mol. Cell. Biol.* 20: 3187-3197.

HUANG, D., CHEN, S.W., LANGSTON, A.W. and GUDAS, L.J. (1998). A conserved retinoic acid responsive element in the murine Hoxb-1 gene is required for expression in the developing gut. *Development* 125, 3235-3246.

HUANG, X. (1994) On global sequence alignment. Computer Applications in the Biosciences 10, 227-235

KESSEL, M. and GRUSS, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* 67, 89-104.

LANGSTON, A.W., THOMPSON, J.R. and GUDAS, L.J. (1997). Retinoic acid-responsive enhancers located 3' of the Hox A and Hox B homeobox gene clusters. *J. Biol. Chem.* 272, 2167-2175.

MADEN, M. (1985) Retinoids and the control of pattern in regenerating limbs. *Ciba Found Symp.*113:132-155.

MARSHALL, H., NONCHEV, S., SHAM, M.H., MUCHAMORE, I., LUMSDEN, A. and KRUMLAUF, R. (1992) Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* 360, 737-741.

MIN, W., WOO, H.J., LEE, C.-S., LEE, K.-K., YOON, W.K., PARK, H.W. and KIM, M.H. (1998). 307-bp fragment in HOXA7 upstream sequence is sufficient for anterior boundary formation. *DNA and Cell Biol.* 17, 293-299.

POPPERL, H. and FEATHERSTONE, M. S. (1993). Identification of a retinoic acid response element upstream of the murine Hox-4.2 gene. *Mol. Cell. Biol.* 13: 257-265.

SIMEONE, A., ACAMPORA, D., ARCIONI, L., ANDREWS, P.W., BONCINELLI, E. and MAVILIO, F. (1990) Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* 346, 763-766.

SUBRAMANIAN, V., MEYER, B.I. and GRUSS, P. (1995). Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* 83, 641-653.

TABIN, C.J. (1992). Why we have (only) five fingers per hand: hox genes and the evolution of paired limbs. *Development* 116: 289-296.

WAGNER, M., HAN, B. and JESSELL, T.M. (1992). Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development* 116: 55-66.

Received: April 2001
Reviewed by Referees: May 2001
Modified by Authors and Accepted for Publication: January 2002 and March 2002