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Rapid Analysis for the Isolation of Novel Genes Encoding Putative Effectors to the Position-specific Regulatory Element of Murine *Hoxa-7*

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***Hox* genes are known to play a critical role in pattern formation during vertebrate development by being expressed at the specific time and in the specific position along the antero-posterior body axis. In order to understand the regulatory mechanism for the position-specific expression of murine *Hoxa-7*, yeast one-hybrid system was applied. DNA fragment conferring a position specificity to the *Hoxa-7* gene was placed just upstream from the yeast *CYC1* promoter and *lacZ* gene in a reporter. Selection of LacZ positive clones after cotransformation of the reporter and mouse embryonic cDNA library as an effector, which was designed to be expressed as fusion proteins to the GAL4 activation domain, allowed us to isolate putative factors interacting with the position-specific regulatory element of murine *Hoxa-7*. A total of 28 positive clones were screened from 5×10^5 yeast transformants. About 70% of the clones turned out to be novel and most of the candidate clones selected in this study showed a temporally restricted expression pattern during embryonic development, suggesting that this method could provide an efficient way for isolating novel genes whose expressions are temporally regulated during embryogenesis.**

Hox genes, homeobox containing genes in vertebrates, play critical roles in pattern formation during embryonic development and their functions are performed in a coordinated fashion temporally as well as spatially (Krumlauf, 1994). So far, 39 *Hox* genes being clustered at 4 different loci (*HoxA*, *HoxB*, *HoxC*, and *HoxD*) of the chromosomes have been identified in both mice and humans. Their organization and nucleotide sequence showed strong conservation through an evolutionary process. Proteins encoded by these *Hox* genes have been identified as transcriptional factors controlling the expression of specific target genes during development (Kim and Kessel, 1993).

The *Hoxa-7* gene located at the middle of a *HoxA* cluster is expresssed in the restricted area along the antero-posterior axis during gastrulation; the anterior limit of expression at C5 in the ectoderm derived neural tube and spinal ganglia, and T3 to T4 in the mesoderm derived prevertebrae around day 12.5 p.c. Temporally, the expression started from the beginning of gastrulation around day 7.5 p.c., peaked at 12.5 p.c. and decreased since then (Mahon *et al.*, 1988; Puschel *et al.*, 1990). The upstream spatio-temporal regulatory regions have been cloned from both mice (Puschel *et al.*, 1990; 1991) and humans (Knittel *et al.*, 1995; Min *et al.*, 1996), and their roles have been analyzed in transgenic mice. This region showed a strong conservation in the nucleotide sequence level as well as in the function of directing a position-specific expression of the downstream reporter gene between two species (Knittel *et al.*, 1995; Min *et al.*, 1995, 1996). Although several position-specific *cis*-acting regulatory elements have been reported in many *Hox* genes, no definite correlation between regulatory factors and target sequences has been made except the interaction between *Krox-20* and *Hoxb-2* as well as the role of retinoic acid in establishing the segment restricted expression of *Hoxb-1* (Marshall *et al.*, 1994; Sham *et al.*, 1993; Studer *et al.*, 1994).

There are several methods to isolate genes encoding regulatory factors which bind to and activate a specific *cis*-element from the library, such as Southwestern blot-

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The abbreviations used are: bp, base pair(s); β-Gal, β-galactosidase; EST, expressed sequence tag; *Hox*, homeobox gene in mice related to the *Antennapedia* class gene of *Drosophila*; kb, kilobase(s); NGF, nerve growth factor; OD, optical density; ONPG, o-nitrophenyl-β-D-galactopyranoside; p.c., post coitum; PSRE, position-specific regulatory element; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

ting, affinity column chromatography, and one-hybrid assay. Especially the one-hybrid assay has been recently used successfully for identifying cDNA candidates which encode the DNA binding protein for a specific regulatory DNA element. These include the yeast origin recognition component ORC6 (Li and Herskowitz, 1993), the neuronal olfactory transcriptional factor Olf-1 (Wang and Reed, 1993), and the B cell specific factor Bob1 (Gstaiger *et al.*, 1995).

In an attempt to isolate *trans*-acting factors which interact with this position-specific regulatory element (PSRE) of the murine *Hoxa-7* gene, we applied the yeast one-hybrid system. This system allows the isolation of cDNAs encoding effectors which interact with the testing DNA element, PSRE, fused to the reporter *lacZ* gene by screening LacZ positive colonies. While analyzing the positive clones, this approach was proven to be an efficient way of screening novel genes whose expressions are temporally regulated during embryogenesis.

Materials and Methods

Construction of a reporter plasmid p360

The 357 bp *Bam*HI/*Nla*III fragment (from nt −1710 to −1354 of Parikh *et al.*, 1995) covering the control region of the murine *Hoxa-7* gene was isolated from the pAX-L680 (from Dr. Gruss, Max-Planck Institute, Germany) and cloned into pGEM3zf (Promega, Madison, WI, U.S.A.) digested with *Bam*HI and *Sma*I after the 3' overhanging end was eliminated with T4 DNA polymerase. An *Eco*RI/*Pst*I-digested fragment of the resulting plasmid was cloned into the pBluescript KS(+) (Stratagene, La Jolla, CA, U.S.A.), yielding the pBS-360. The 360 bp *Sal*I fragment of pBS360 was then cloned into the *Xho*I site of the pHY100 (Yoo and Cooper, 1989) and named p360.

Mouse embryo cDNA library construction

The effector, cDNA library, was either constructed in this laboratory or purchased from Clontech (Palo Alto, CA, U.S.A.). For construction, mouse embryos at day 11.5 p. c. were homogenized and subjected to RNA extraction based on the acid-guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified from total RNA according to the mRNA isolation kit (Pharmacia, Sweden). Double stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA with a cDNA synthesis kit from Stratagene. Fractions containing cDNA greater than 300 bp were ligated with *Eco*RI-*Xho*I digested pGAD-GH (Clontech). Ligated DNA was introduced into *E. coli* strain DH5α through electroporation using a Biorad electroporator and plasmid DNA was extracted from the transformants by a standard method (Sambrook *et al.*, 1989). Mouse 11-day Embryo MATCHMAKER cDNAs (Clontech) were also prepared in accordance with the manufacturer's guideline.

Yeast strain, transformation, plasmid extraction, and plasmid curing

S. cerevisiae L3262 (*leu*$^-$, *ura*$^-$, *his*$^-$, donated by Dr. Yoo in KRIBB) and its transformants were maintained on YPD (10 g yeast extract, 20 g peptone, 50 ml of 40% glucose per 1 l) or selective medium (Difco 0919-15-3) supplemented with appropriate amino acids. Plasmid DNA or cDNA library transformation in yeast strain was conducted by the lithium acetate method (Schiestl and Geitz, 1989). Plasmid DNA extraction from yeast transformants was carried out as described (Hoffman and Winston, 1987). Curing of a reporter plasmid which carries the *URA3* marker was performed by selection on the plate containing 5-fluoroacetic acid (Sigma) as described by Sikorski and Boeke (1991).

Screening the library

Reporter plasmid p360 and mouse embryo cDNA library (effector library) were cotransformed into *S. cerevisiae* L3262 and plated on the selective media lacking uracil and leucine (about 50 plates of 150 mm). After 50 h at 30 ℃, transformants (5×10^5) were subjected to the colony lift assay for selection of LacZ positive clones by the method of Breeden and Nasmyth (1985). Filters were incubated at 30 ℃ in the presence of X-gal and colonies which turned to blue within 2.5 h were selected. A single colony was purified and subjected to curing of the *URA3*$^+$ plasmid as described above. Effector plasmids were recovered and transformed into *E. coli* by electroporation.

Liquid β-galactosidase assay

For quantitation of β-Gal activity, cells grown in YPD medium to OD_{595} of 0.7-1.0 were subjected to the assay as described by Miller (1972) except that cells were lysed by placing tubes in liquid nitrogen. Units of β-Gal activities were expressed as OD_{420} U/OD_{595} of cells per min from duplicate assays.

DNA manipulation, sequence determination, and Northern hybridization

Recombinant DNA techniques were carried out mainly as described by Sambrook *et al.* (1989). Nucleotide sequences were determined by the chain termination method using a Sequenase 2.0 kit (USB, Cleveland, OH, U.S.A.) and a homology search was done by the Fasta program. For Northern blotting of mouse embryo stage-specific RNA, Multiple Embryo Tissue Northern Blot (Clontech) was probed with a ^{32}P-labeled DNA fragment under conditions recommended by the manufacturer.

Results and Discussion

Screening of effectors interacting with PSRE of murine Hoxa-7

Previous studies have shown that the 5' upstream

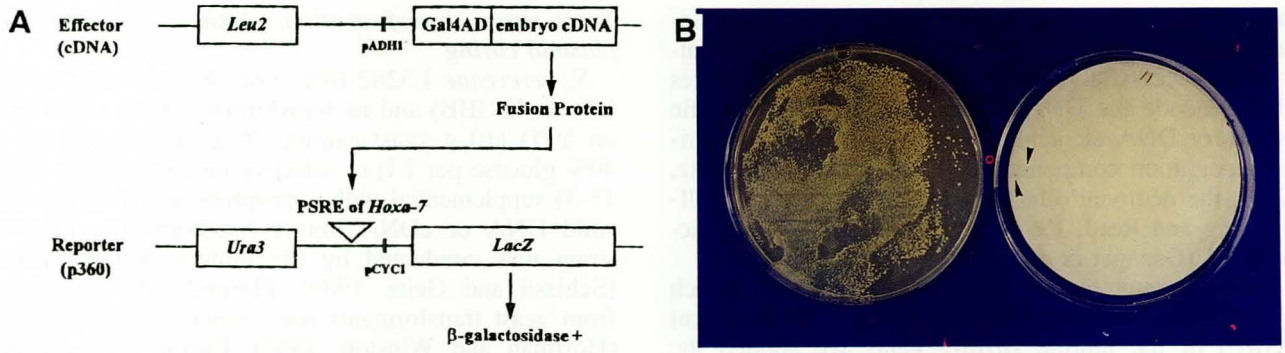

Figure 1. Strategy for screening effectors through their interaction with the PSRE of the *Hoxa-7* gene. A) Schematic drawing of effector and reporter construct. The effector fusion proteins, yeast GAL4 activation domain (GalAD) and embryonic cDNA fused in frame, which interact with the PSRE of *Hoxa-7* were designed to be selected by activating *lacZ* expression in the reporter. Effector and reporter (p360) contain an *LEU2* and *URA3* marker for selection, respectively. The promoters, pADH1 (alcohol dehydrogenase) and p*CYC1* (iso-1-cytochrome c) used in each effector and reporter construct are written. B) Screening LacZ positive clones from cDNA transformants. Colonies were lifted on filter paper and exposed to substrate X-gal after the filter was soaked into liquid nitrogen. Colonies showing a blue color were selected for further analysis.

region of murine *Hoxa-7* specifies an anterior boundary of expression. The minimal region sufficient for directing the position specific expression of murine *Hoxa-7* has been mapped to be a 470 bp long fragment (AX470) (Knittel *et al.*, 1995). Recently, we have cloned and determined the nucleotide sequence of a 3.9 kb human homologue from a human genomic library and revealed that the most 3' 1.1 kb fragment in this region had a similar function in transgenic mice (Min *et al.*, 1996). This portion has a sequence homology of over 70% of that of the murine sequence, indicating that the regulatory mechanism for the position-specific expression of *Hoxa-7* is conserved between two species, which strongly suggests the existence of regulatory factors having a conserved DNA binding motif. We further deleted the 5' region of the AX470 based on Min *et al.* (1996) and used a 357 bp DNA fragment (from nt −1710 to nt −1354 of Parikh *et al.*, 1995) as the minimal portion of the position-specific regulatory element (PSRE) required to set the anterior boundary of the *Hoxa-7* expression and applied for screening the regulatory factors.

In order to isolate factors which interact with PSRE, we used a yeast one-hybrid system in which a reporter and an effector are present in a single cell. The reporter plasmid p360 contains the bacterial *lacZ* gene under the yeast *CYC1* promoter as well as *Hoxa-7* PSRE (Fig. 1A). Since no β-Gal activity was observed with the reporter plasmid alone, it was assumed that the endogenous yeast proteins do not bind to or activate PSRE. The effector embryonic cDNA library was designed to be expressed as fusion proteins to the transcription activation domain of yeast GAL4 (Fig. 1A), so that any fusion protein which can interact with *Hoxa-7* PSRE could be isolated after X-gal staining. Since the transcriptional activation ability is supplied by the N-terminal GAL4 activation domain in the fusion protein, a fused gene encoding a protein which can interact with the PSRE element subsequently activates the *lacZ* gene expression in a reporter, no matter whether it is a repressor or an activator. We screened 5×10^5 yeast transformants and selected 28 positive clones (Fig. 1B).

Sequence analysis of PSRE responsive inserts

To determine the nature of the proteins encoded in the positive effector plasmids, reporter plasmids were cured out as described in Materials and Methods. The effector plasmids were then transferred into *E. coli* and partial nucleotide sequences were determined using the dideoxynucleotide sequencing method. Most of the *Hoxa-7* PSRE responsive cDNAs were identified to be novel genes when compared with the EMBL data bases (Table 1); 19 out of 28 clones (68%) were previously unreported sequences and GenBank accession numbers of these clones are shown in Table 1. Five clones, c134, c143, c161, c166, and c168, showed a strong homology to the reported cDNA sequences whose functions have been partially characterized, and four clones showed significant sequence homologies to some of the ribosomal protein coding genes.

Effector responsiveness to PSRE

In order to quantitate the interaction between the effector fusion protein and the PSRE of *Hoxa-7*, β-Gal activity was measured (see Materials and Methods). Six LacZ positive clones (c124, c131, c134, c143, c153, and c171), which turned blue a short time after X-gal treatment during screening, were chosen. As shown in Figure 2, most of the clones tested exhibited rather low β-Gal activities, about 1.5 to 3.5-fold higher than that of the control cells except for c153. When these clones were cotransformed with a plasmid lacking the PSRE, no activities were observed (data not shown). The clones c131, c124 having a sequence homology with EST107511 (H 34975; Lee *et al.*, 1995) which is similar to the ri-

Table 1. Sequence analysis of Hoxa-7 PSRE response cDNAs

No.	Size (bp)	Accession No.	Putative identification	No.	Size (bp)	Accession No.	Putative identification
c101	157	Z82914	unknown	c158	137	Z82924	unknown
c104	175	Z82915	98% homology to X76772[a]	c159	137	Z82925	unknown
c114	137	Z82916	unknown	c160	227	Z82926	unkown
c117	73	Z82913	unknown	c161	102	Z82927	80% homology to M26651[e]
c124	92	Y09519	90% homology to H34975[b]	c162	179	Z82934	unknown
c131	181	Z82917	unknown	c163	133	Y09520	94% homology to U13369[f]
c134	201	Z82918	80% homology to H36700[c]	c165	138	Y09521	84% homology to N39311[g]
c137	209	Z82919	unknown	c166	107	Y09522	83% homology to N20634[h]
c143	181	Z82920	70% homology to H72555[d]	c167	163	Z28928	unknown
c151	215	Z82921	unknown	c168	164	Y09523	88% homology to D18777[i]
c153	221	Z82933	unknown	c170	146	Z82929	unknown
c154	160	Z82922	unknown	c171	215	Z82930	unknown
c156	147	Z82923	unknown	c172	136	Z82931	unknown
c157	218	Z82935	unknown	c175	67	Z82932	unknown

[a]ribosomal protein S3; [b]ribosomal protein S9 (Lee *et al.*, 1995); [c]rattus sp. cDNA (Lee *et al.*, 1995); [d]human fetal cDNA; [e]5-region of mouse epo gene (Bera *et al.*, 1989); [f]ribosomal DNA repeating unit (Gonzalez *et al.*, 1985); [g]ribosomal protein S6; [h]human EST; [i]mouse EST

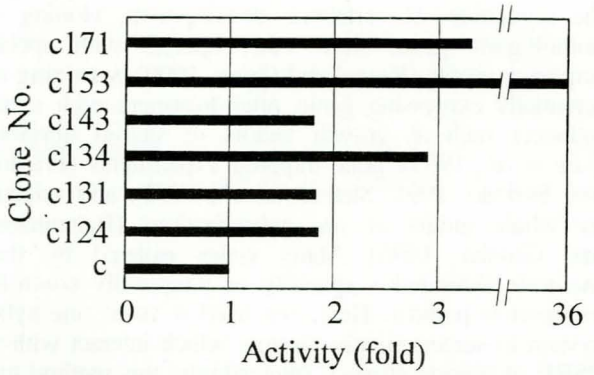

Figure 2. Transactivation of the *lacZ* gene by interaction between the PSRE of *Hoxa-7* and putative effectors. Expression of the *lacZ* gene directed through effector interaction with PSRE was monitored by the β-Gal assay using ONPG as a substrate. Yeast cells harboring a reporter and effector were lysed by immersing the cell pellet into liquid nitrogen and subjecting them to the enzyme assay as described in Materials and Methods. Activities of each clone were given as ratios to the value of strain carrying only a reporter plasmid (C). Clone c153 showed a 36-fold higher activity than that of the control.

bosomal protein S9, and c143 which has a sequence homology with an EST (H72555) isolated from human fetal liver and spleen cDNA library (The WashU-Merck EST project, Unpublished) showed rather low β-Gal activity compared to other tested clones.

It is generally believed that a clone having higher β-Gal activity has a higher binding affinity to PSRE. Therefore, clone c153 showing the highest β-Gal activity (about 36 fold) was chosen for electrophoretic mobility shift assay. Unfortunately, however, the signal was not strong enough to be seen (data not shown). It could be due to the low amount of c153 proteins existing in crude yeast extract or that the binding protein is provided by the host and the fusion protein which associates with it by protein-protein interaction is selected through this method. Although the latter case is very unlikely since we did not see any shifted band with the host cell extract, this problem could be solved by purification of the fusion protein.

Stage-specific expression of PSRE responsive inserts

Since it is known that the expression of *Hoxa-7* is temporally regulated during embryogenesis like other *Hox* genes, it was of particular interest to characterize the expression mode of positive clones during early mouse development. Therefore, 7 clones (6 tested β-Gal activity plus c158) were chosen and Northern hybridization was conducted with the mouse embryonic stage-specific RNA blot of Clontech.

As shown in Figure 3, all the clones except c124 exhibited temporally restricted expression patterns. Clone c124, which has sequence homology with ribosomal protein S9, did not show any significant difference (or slight difference, if any) at the level of expression during development.

On the other hand, for example, clones c158 and c171 showed an extreme stage-specific expression mode, such as a restricted expression, at day 17.0 and 7.0 p.c., respectively. A protein like c171, which is restrictively expressed at day 7.0 p.c., might be important for an onset of initial *Hox* gene expression. Since *Hoxa-7* gene starts expression at the beginning of mesoderm formation around day 7.5 p.c., the regulatory protein necessary for initial expression somehow should be provided before gastrulation.

Clones c131 and c134 are also interesting. They were not expressed at day 7.0 p.c., but abruptly synthesized at day 11.0 p.c. and were maintained constantly throughout the development in the case of c134, or gradually increased in the case of c131.

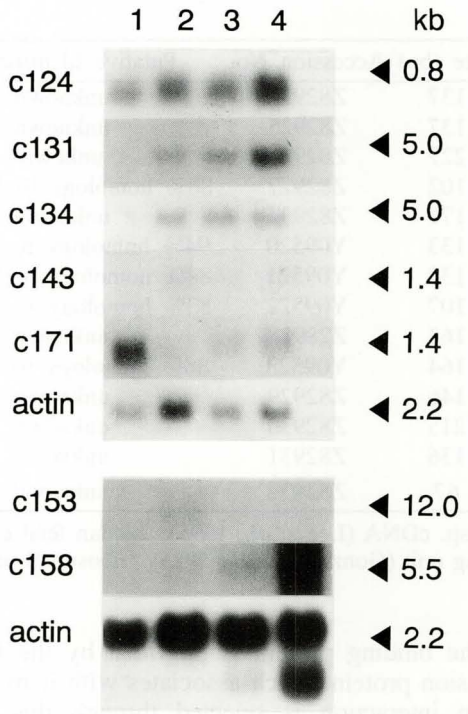

Figure 3. Stage-specific expression of putative factors interacting with PSRE. Northern blot analysis of poly(A)$^+$ RNA isolated at different stages of mouse embryo was performed with several LacZ positive clones. The filter was hybridized with *Eco*RI inserts isolated from each positive clone and labeled by a random priming method. Clone numbers are shown on the left side and the size of the transcripts are given in kb on the right side. Human β-actin gene was used as a control probe. Lane 1, poly(A)$^+$ RNA from day 7 p.c.; lane 2, poly(A)$^+$ RNA from day 11 p.c.; lane 3, poly(A)$^+$ RNA from day 15 p.c.; and lane 4, poly (A)$^+$ RNA from day 17 p.c.

Because gastrulation starts around day 7.5 p.c., it is reasonable to postulate that these are likely to be the mesoderm or neuroectoderm specific genes which are expressed after gastrulation. Especially c134, which has some sequence homology with one of the *rattus* cDNA isolated from 9 day NGF treated PC12 cells (Lee *et al.*, 1995), might be a regulatory signal for *Hoxa-7* expression in the ectoderm-derived tissue, since PC12 cells differentiate into sympathetic-like neurons after NGF induction. Since *Hox* genes have a different anterior boundary of expression between ectoderm- and mesoderm-derived tissues, it has been thought that the regulatory mechanism for position-specific expression could be different between two tissue layers. It is also interesting to point that the PSRE used here has been postulated as being a position-specific regulatory element in the ectoderm derived neural tube and spinal ganglia (Min *et al.*, 1996).

Most transcripts of the clones c143 and c153 were detected at day 11.0 p.c., the mid-gestation stage, during which most *Hox* genes are strongly expressed. The proteins expressing later in gastrulation might be involved in the maintenance mechanism for *Hox* expression, for which an autoregulatory mechanism has been known in the case of *Ultrabithorax* and *Deformed* genes of *Drosophila* (Beachy *et al.*, 1988; Regulski *et al.*, 1991). Among these, clone c143 has some sequence homology with an EST (H72555) isolated from a liver and spleen cDNA library made with a 20 week-post conception male fetus. Since lineage-specific expression of *Hox* genes has been reported in human hematopoietic systems (Vieille-Grosjean *et al.*, 1992), it is interesting to presume that clone c143 could be a *Hox* gene regulator involving murine erythropoiesis, which actively occurs in 20-week old fetal liver, in the case of humans.

It was surprising that most of the positive clones analyzed here showed a temporally restricted expression pattern during mouse development, suggesting that this method could provide an efficient method for isolating novel genes whose expressions are temporally regulated during embryogenesis. So far, various stragtegies have been developed to isolate novel genes participating in the regulation of vertebrate development; cloning the homologous genes from other species with specific sequence motifs (Kessel and Gruss, 1990), screening differentially expressing genes after treatment with certain inducers such as growth factors or steroid hormones (Lee *et al.*, 1995), gene trapping experiments (Friedrich and Soriano, 1991; Skarnes *et al.*, 1992), and selection by whole mount *in situ* hybridizations (Bettenhausen and Gossler, 1995). Many genes isolated by these methods exhibited a spatially or temporally controlled expression pattern. Here, we used a yeast one-hybrid system to screen putative factors which interact with the PSRE of murine *Hoxa-7*. Interestingly, this method turned out to be a rapid and efficient way not only for isolating novel factors associated with the specific motif but also for screening novel genes whose expressions are temporally regulated during development. Comprehensive characterization of these genes and factors in the future will help us to understand the molecular mechanism underlying vertebrate development, including the regulatory cascade for *Hox* gene expression.

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