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 CYCl promotor 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 $5 \times 10^6$ yeast transformants. About 70% of the clones turned out to be novel and most of the candidate clones selected in this study showed a temporarily 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 expressed 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.

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.

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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 Olfp1 (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 BamHI/NlaIII 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 BamHI and Smal after the 3' overhanging end was eliminated with T4 DNA polymerase. An EcoRI/PstI-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 SalI fragment of pBS360 was then cloned into the XhoI 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 EcoRI-Xhol 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 (lei, ura, his) donated by Dr. Yoo in KIRIBB 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-fluorooracetic 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 °C, transformants (5 × 10⁵) 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 °C 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 OD₅₆₅ 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 OD₄₂₀ U/OD₅₆₅ 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 32P-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
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 $5 \times 10^7$ 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-
of c153 protein or existing in crude yeast extract or
ever, the signal was not strong enough to be seen
Therefore, clone c153 showing the highest
phoretic mobility shift assay. Unfortunately, how-
activity (about 36 fold) was chosen for electro-

~Gal

Merck

~Gal

man fetal liver and spleen cDNA library (The
bosomal protein clone were given as ratios to the value of strain carrying
onl y a reporter plasmid (C). Clone cl53 showed a 36-fold
higher activity than that of the control.

The yeast cells harboring a reporter
effectors were lysed by immersin g the cell pellet into
liquid nitrogen and sub jecting them to the enzyme assay as

described in Materials and Methods. Activities of each
effectors. Activities of each clone were given as ratios to the value of strain carrying
only a reporter plasmid (C). Clone cl53 showed a 36-fold higher activity than that of the control.

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

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<th>Putative identification</th>
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<th>Size (bp)</th>
<th>Accession No.</th>
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a: ribosomal protein S3; b: ribosomal protein S9 (Lee et al., 1995); c: ratus sp. cDNA (Lee et al., 1995); d: human fetal cDNA; e: 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

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 im-
portant 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 con-
tantly throughout the development in the case of
c134, or gradually increased in the case of c131.
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 rat tus cDNA isolated from 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 strategies 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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