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Two Isoforms of Orthodenticle-Related Proteins (HpOtx) Bind to the Enhancer Element of Sea Urchin Arylsulfatase Gene

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The sea urchin (*Hemicentrotus pulcherrimus*) arylsulfatase (*HpArs*) gene, expressed specifically in aboral ectoderm, contains a 229-bp enhancer in its first intron that is required for the activation of *HpArs* gene expression. Deletion analysis shows that a tandem repeat of orthodenticle-related protein (Otx) binding sites are responsible for the activity of this enhancer. Gel mobility shift analysis reveals that three types of Otx-proteins, which show different mobilities in gel shift assays, form complexes with the enhancer. Band I appears before hatching and gradually decreases by the gastrula stage. Band III appears at the blastula stage and Band II appears at the mesenchyme blastula stage; the levels of Band II and III remain constant until the gastrula stage. Two distinct types of HpOtx cDNA clones have been isolated from cDNA libraries of unfertilized eggs and gastrulae. Nucleotide sequences of the homeobox and downstream regions are well conserved in the two types of HpOtx cDNAs, while the region upstream from the homeobox has different nucleotide sequences. By genomic Southern blot analysis, only a single copy of *HpOtx* gene is detectable in the Hp genome, making it likely that two HpOtx isoforms are generated from the same gene. Results from Northern blot analysis confirm the presence of two types of HpOtx transcripts. Transcriptional regulation of the *HpArs* gene may, in part, be carried out through switching of Otx isoforms.

INTRODUCTION

Development is a complex process regulated by successive expression of various genes at appropriate stages and in appropriate embryonic regions. It is now believed that expression of genes during development is controlled by transcription-regulatory cascades consisting of various transcription factors and their genes. In sea urchin embryos upstream regulatory cascades have been studied in several structural genes, such as cytoskeletal actin genes, Spec genes, arylsulfatase gene, etc. With regard to one of the cytoskeletal actin genes, *CyIIIa,* many cis-active elements related to its temporal and spatial expression were identified, and protein factors that bind to cis-elements have been isolated and characterized (Theze *et al.,* 1990; Kirchhamer and Davidson, 1996). Cis- and trans-elements related to the regulation of *Spec2a* gene have also been studied in detail, and the SpOtx protein, a product of *Strongylocentrotus purpuratus* homologue of mouse Otx gene, has been identified as a transcription regulating factor (Mao *et al.,* 1994; Gan *et al.,* 1995).

Expression of arylsulfatase (*HpArs*) gene of the sea urchin (*Hemicentrotus pulcherrimus*) is also regulated in stage-specific and tissue-specific manners (Sasaki *et al.,* 1988; Akasaka *et al.,* 1990). Its transcription begins at the blastula stage and Ars mRNA accumulates as development proceeds. In the hatched blastula Ars mRNA is detectable in all embryonic cells, while at the mesenchyme blastula stage Ars mRNA becomes undetectable in the mesenchyme and vegetal plate cells. At the gastrula stage, it is totally restricted to the aboral ectoderm.

We have recently found that a 229-bp region in the first intron of *HpArs* gene has a remarkably high enhancer activity (Iuchi *et al.,* 1995). This region contains the tandem repeat of core consensus and closely related sequences of orthodenticle-related protein (Otx) binding sites. The activity of Otx and its binding sites has been reported to be indispensable for formation of the body plan of various animal species (Finkelstein and Boncinelli, 1994).

The *Otx* gene family is a member of the bicoid class homeobox genes, and its product contains a well conserved homeodomain which has a lysine at position 51 that confers

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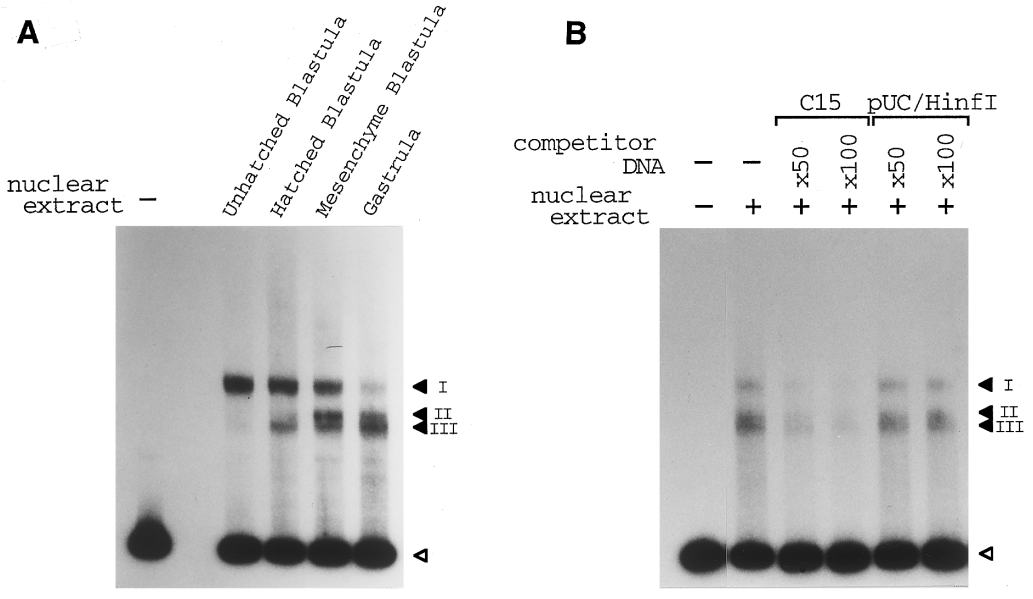

FIG. 1. Nuclear protein binding to C15 fragment. (A) Gel-mobility shift assay using nuclear extracts from various developmental stages of sea urchin embryos. Closed triangles, shifted bands; open triangle, free probe. (B) Competition analysis using mesenchyme blastula extract. Competitors were added in 50- or 100-fold excess. C15, nonlabeled C15 fragment; pUC/*Hin*fI, *Hin*fI-digested pUC119 plasmid DNA as a nonspecific competitor.

DNA-binding specificity for the sequence motif TAATCC/T (Hanes and Brent, 1989, 1991; Treisman *et al.*, 1989). Orthodenticle (Otd) cDNA was first isolated from *Drosophila* as a homeobox protein related to head formation (Finkelstein *et al.*, 1990; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein and Boncinelli, 1994). Subsequently, cDNA homologues (Otx) of Otd were isolated from various vertebrates. The mouse has two types of Otx cDNAs which are expressed in restricted regions of developing brain and are also responsible for head formation (Simeone *et al.*, 1992, 1993; Finkelstein and Boncinelli, 1994; Matsuo *et al.*, 1995). In zebrafish three types of Otx cDNAs are present in the developing diencephalon and midbrain (Mori *et al.*, 1994). Recently, Otx (SpOtx) cDNA was isolated from *S. purpuratus*, where it is involved in regulating the expression of *Spec2a* gene (Mao *et al.*, 1994; Gan *et al.*, 1995).

In the present study we demonstrate that a tandem repeat of Otx consensus sequence in the first intron of the HpArs gene acts as the enhancer element of this gene expression, and that two types of HpOtx cDNA isoforms are detected in cDNA libraries from unfertilized egg and gastrula.

MATERIALS AND METHODS

Sea urchins and embryo cultures. Gametes of the sea urchin (*H. pulcherrimus*) were obtained by coelomic injection of 0.55 *M* KCl, and fertilized eggs were cultured at 16°C with constant aeration and stirring.

Plasmid DNAs. The Ars gene enhancer fragment, C15 (229 bp in length), inserted into the *Sma*I–*Hin*dIII sites of pBluescript SK− vector (Stratagene Cloning System, U.S.A.) was progressively deleted at the downstream end by exonuclease III digestion using *Hin*dIII and *Apa*I sites. The deletion mutants were referred to as C15-ΔX, X indicating the length of remaining C15 fragments. For gel-mobility shift assay, C15 and deleted C15 fragments were excised with *Kpn*I and *Bam*HI and inserted into pGEM-7fz(+) vector (Promega Corp.).

Preparation of nuclear extract. Nuclei were isolated according to the method of Hapgood and Patterton (1994) with a few modifications. Briefly, 20 ml of embryos were collected by centrifugation at various stages of development, washed twice with 0.5 *M* KCl, twice with buffer A containing 1 *M* hexylene glycol (Hex-A buffer), and resuspended in Hex-A buffer. The suspension was placed on ice for 2 hr to allow swelling of the cell membrane and homogenized by 15 strokes in a Dounce-homogenizer. Crude nuclei were pelleted by centrifugation and washed once in Hex-A buffer. The pellet was resuspended in Hex-A buffer and the suspension was adjusted to 1.8 M sucrose by adding 2.3 M sucrose in buffer A. The homogenate was centrifuged at 12,800 rpm for 45 min in an S28P rotor (HITACHI). The resultant pellet was washed with Hex-A buffer, suspended in the buffer containing 25% glycerol, 50 m*M* Tris–acetate (pH 8.0), 1 m*M* DTT, 1 m*M* EGTA, 1 m*M* spermidine, 5 m*M* magnesium acetate, and 0.1 m*M* PMSF, and stored at −80°C.

Nuclear extract was prepared as described by Barberis *et al.* (1987) except for buffer components and centrifugal conditions. Stored frozen nuclei were resuspended in the lysis buffer (25 m*M* Hepes–KOH (pH 7.5), 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 1 m*M* spermidine, 0.1 m*M* PMSF) and then extracted with 0.1 vol of satu-

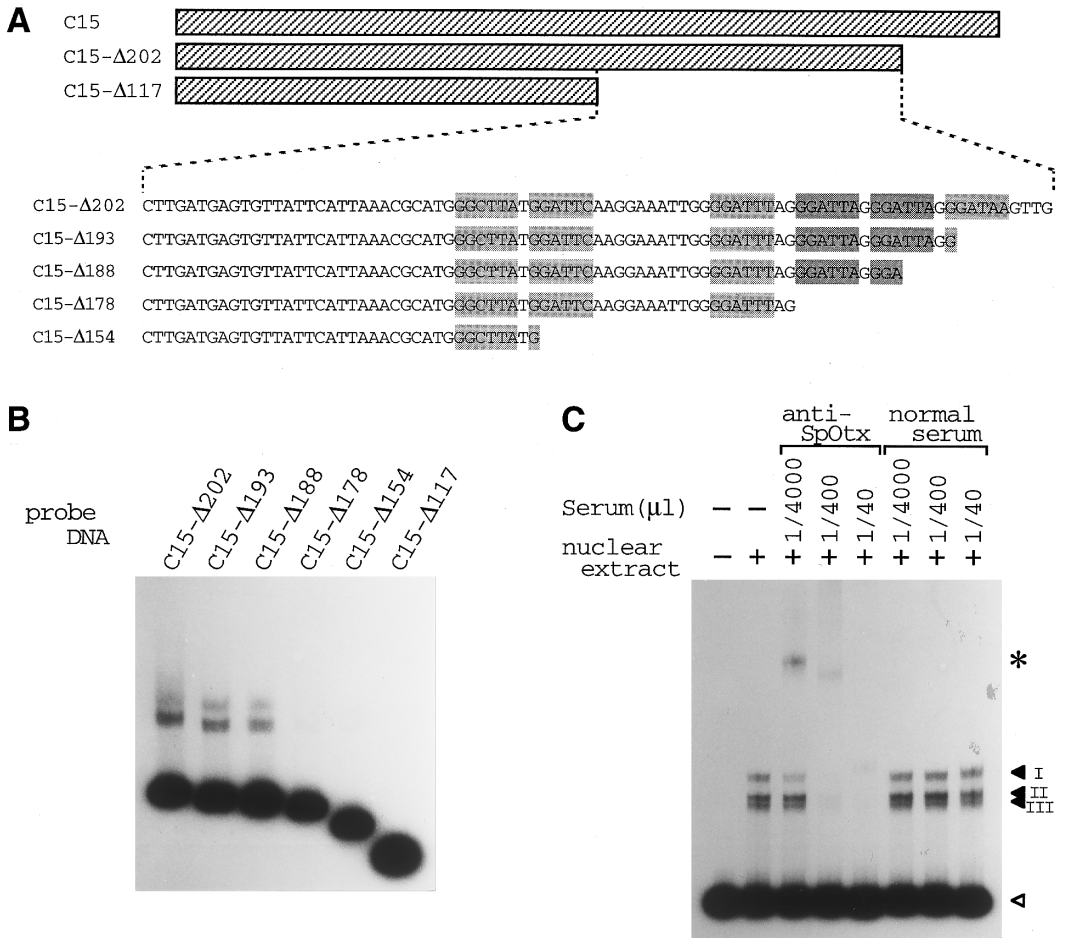

FIG. 2. Proteins extracted from mesenchyme blastula bind to Otx core consensus sequences in C15 fragment. (A) A schematic representation of the variously deleted C15 fragments. Dark shaded boxes, core consensus sequences of orthodenticle-related protein (Otx) binding site; light shaded boxes, Otx-like sequences. (B) Gel-mobility shift assay of deleted C15 fragments with mesenchyme blastula nuclear extract. (C) Supershift assay using the anti-SpOtx antibody. 1 μg of mesenchyme blastula nuclear extract was preincubated with indicated volumes of anti-SpOtx antibody or preimmune serum. Closed triangles, shifted bands; open triangle, free probe; asterisk, supershifted band.

rated ammonium sulfate (pH 7.9) for 30 min. The extract was centrifuged at 27,000 rpm for 60 min in an S28P rotor to pellet the chromatin, and proteins were precipitated from the supernatant by addition of 0.25 g $(NH_4)_2SO_4$ per milliliter. Proteins pelleted by centrifugation at 10,000*g* for 20 min were dissolved in buffer C (20 m*M* Hepes–KOH (pH 7.9), 40 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, 20% glycerol) and dialyzed against 3 liters of buffer C. Insoluble materials were removed by centrifugation in a microfuge, and the clear supernatant was stored in aliquots at −80°C.

Dephosphorylation of nuclear proteins was carried out by incubating 10 μg of nuclear extract with 1 unit of alkaline phosphatase (*Escherichia coli* C75) (TaKaRa) in a total volume of 20 μl of buffer C containing 50 m*M* Tris–HCl (pH 8.0) and 2 m*M* $MgCl_2$.

Gel-mobility shift assay. For preparation of DNA probes, C15 and C15-ΔX fragments inserted into pGEM7 fz(+) vector were digested with *Bam*HI and *Xho*I and then both 5′ overhanging termini were filled by use of Sequenase version 2.0 (USB) with [α-^{32}P]dCTP, dATP, dTTP, and dGTP. ^{32}P-labeled C15 and C15-ΔX fragments were electrophoresed on a 4% polyacrylamide gel, and DNA was extracted from excised gel fragments. Binding reactions were carried out by incubating 0.5 ng of ^{32}P-labeled DNA probe with 1 μg of the nuclear extract and competitor DNA in a total volume of 10 μl binding buffer (20 m*M* Hepes–KOH (pH 7.9), 5 m*M* $MgCl_2$, 100 m*M* KCl, and 10 m*M* DTT) at 13°C for 30 min. After incubation, 2 μl of Ficoll-dye was added to the reaction mixture and samples were analyzed by electrophoresis on a 4 or 5% native polyacrylamide gel in TBE buffer.

Gel band supershift assay by an Otx antibody was carried out as described by Gan *et al.* (1995).

Measurement of enhancer activity in C15 fragment. Procedures for introduction of genes into sea urchin eggs by a pneumatic particle gun and the luciferase and CAT assays were described elsewhere (Akasaka *et al.*, 1995).

Isolation of HpOtx cDNA clones. Isolation of HpOtx cDNA

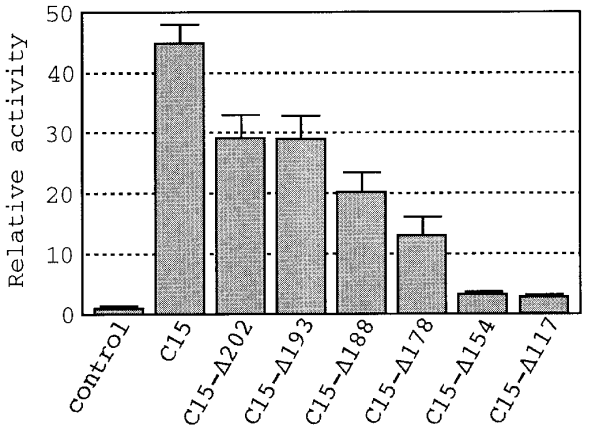

FIG. 3. Sites of enhancer activity in the C15 fragment. The deleted C15 fragments (see Fig. 2) were ligated to the luciferase gene which is drived by HpArs promoter. These constructs were introduced into sea urchin fertilized eggs by particle gun method. The enhancer activities were represented as the luciferase activities relative to that of HpArs promoter construct at the prism stage (48 hr postfertilization). Bars indicate standard errors of 4 independent experiments.

from the unfertilized egg cDNA library, constructed in λgt10 vector, were performed by using *Eco*RI fragments of SpOtx cDNA (Gan *et al.,* 1995) as the probe. Positive clones were subcloned into the pBluescript SK− vector. Sequence analysis of the isolated HpOtx cDNA was performed by DNA digestion with restriction endonucleases and by the chain termination method (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit, Perkin–Elmer).

To isolate the HpOtx cDNA clone from the gastrula library, a probe DNA fragment was generated by polymerase chain reaction (PCR) using Primer 1, 5′-GARMGNACNACNTTYACNMGNGC-3′; Primer 2, 5′-GCGGATCCGCNCARYTNGAYGTNYTNG-ARAC-3′; and Primer 3, 5′-GCAAGCTTNGCNCKNCKRTTY-TTRAACCA-3′ to amplify the 134-bp HpOtx homeobox-containing DNA fragment. Primers 2 and 3 contain *Bam*HI and *Hin*dIII cleavage sites at their 5′ ends, respectively.

Primers 1 and 3 were used in the first PCR reaction, and the 150-bp DNA fragment obtained was purified and used for a second PCR with Primer 2 and 3. The product of the second PCR was cleaved with both *Bam*HI and *Hin*dIII and subcloned into the pBluescript SK− vector. PCR-amplified HpOtx homeobox-containing fragment was used as a probe to screen the *H. pulcherrimus* gastrula cDNA library in λgt10. Positive clones were subcloned into pBluescript SK− vector. Sequence analysis was performed by Exonuclease III deletion and by the chain termination method (Taq DyeDeoxy Terminator Cycle Sequencing kit and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit, Perkin–Elmer).

The 5′ terminal regions of HpOtx cDNAs were obtained by the 5′-RACE method using RNA PCR kit (AMV) Ver.2, TaKaRa. Following primers were used: 5′RACE-1 primer, 5′-CTTTGCTCTCCTAT-TCTTG-3′; 5′RACE-2 primer, 5′-CTTCATAGCAACTTCCTCC-3′; 5′RACE-Otx$_E$, 5′-TTCACTGGTCATTGGACC-3′; 5′RACE-Otx$_L$, 5′-TGTATATGACATAGCGGGAT-3′; Oligo $(dT)_{20}$-Adaptor primer, 5′-GTTTTCCCAGTCACGACTTTTTTTTTTTTTTTTTTT-3′; M13-M4 primer, 5′-GTTTTCCCAGTCACGAC-3′. Five micrograms of total mesenchyme blastula RNA was used as a template, and the resultant PCR products were subcloned into pBluescript SK− vector. Sequence analysis was performed by the chain termination method (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit, Perkin–Elmer).

In vitro transcription and translation of HpOtx$_E$ and HpOtx$_L$. To generate *in vitro* translation products of HpOtx$_E$ and HpOtx$_L$, the 5′ untranslated regions of the HpOtx cDNAs were truncated and were subcloned into the pBluescript SK− vector. One microgram of purified closed circular plasmids was used as templates. The *in vitro* transcription and translation reactions were carried out in the TNT Coupled Reticulocyte Lysate System (Promega). Two micrograms of reaction products were used in gel-mobility shift assay.

Northern hybridization analysis. The total RNA of *H. pulcherrimus* embryos was extracted as described by Chomczynski and Sacchi (1987). To distinguish expression pattern of HpOtx$_E$ from that of HpOtx$_L$, specific probes to each of HpOtx mRNA were generated by PCR. PCR products of 5′ region of each HpOtx cDNA were prepared by using following primers: HpOtx$_E$NS primer, 5′-GCTAGTCGTGAAATCAAG-3′; HpOtx$_E$NA primer, 5′-TTCAC-TGGTCATTGGACC-3′; HpOtx$_L$NS primer, 5′-GTTCGTGAGG-CGGTTCG-3′; HpOtx$_L$NA primer, 5′-TGTATATGACATAGCG-GGAT-3′.

RESULTS

Nuclear Protein Binding to C15 Region

We previously demonstrated that the 229-bp fragment (C15 fragment) in the first intron of *HpArs* gene has a strong enhancer activity (Iuchi *et al.,* 1995). To detect the nuclear protein that binds to this enhancer region, gel-mobility shift assays were carried out with nuclear proteins extracted from embryos at various stages of development by using the C15 fragment as the probe (Fig. 1A). Three distinct bands, referred to as Bands I, II, III, were detected. Band I, the uppermost band in Fig. 1A, appeared before hatching and gradually decreased by the gastrula stage. Band II, the middle band, and Band III, the lowermost band, appeared at the mesenchyme blastula and at the hatched blastula stages, respectively, and their levels remained constant until the gastrula stage.

The specificity of the nuclear protein binding to the C15 fragment was demonstrated by the competitive gel-mobility shift assay (Fig. 1B). Band shifts were not affected by addition of nonspecific competitor, *Hin*fI-cleaved pUC119 fragment (compared with lanes 2, 5, and 6), while addition of nonlabeled C15 fragments resulted in disappearance of all three bands (compare with lanes 2, 3, and 4).

Otx Consensus Motifs in C15 Enhancer Are Protein Binding Sites

In order to locate the binding sites of nuclear proteins in the 229-bp C15 fragment, the C15 fragment was variously deleted from its 3′ end, labeled, and used in gel mobility shift assay with protein fractions extracted from the mesenchyme blastula. The naming and features of these deletion

A

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GAACAGTGATCAACAGCACCGGTCAATCAATGGCACATGATTACTCACTCTCACAAATCGCATTCTCCGCAGCGTCGATCCGACATTGAA  90
CAGCGTTATCAGCTGGACTTCAAACTGGTATCTCCTCGGCTGGACATTGATAGCTTCACAATACACTTTCTACTTGTAACTTGTAAACAT 180
                                                                                        M   1

GGAAGCACTTTCTGATCTTGCTAGTCGTGAAATCAAGATGGAATCACATTCTCCACAGGATTCCAAGAATTTGAATGTCAAGCCCATGAA 270
 E  A  L  S  D  L  A  S  R  E  I  K  M  E  S  H  S  P  Q  D  S  K  N  L  N  V  K  P  M  K  31

GCTGGAGCGGGTGGGGATGTCGTCATCGCCGCCGAGGTTGACCATCGACTGCGGCAACACCGGTCGATCACCGGTACCATCACACATGGA 360
 L  E  R  V  G  M  S  S  S  P  P  R  L  T  I  D  C  G  N  T  G  R  S  P  V  P  S  H  M  E  61

ACCGCCCGGTGGCGCCAGGGTACCATACCCGATGCACCTTTACCCTTACCAGTATGCCTACAGCAACCCCATGTATGGAGAGGGGGCCTT 450
 P  P  G  G  A  R  V  P  Y  P  M  H  L  Y  P  Y  Q  Y  A  Y  S  N  P  M  Y  G  E  G  A  L  91

ACCAGCACCTGATCGCCACGTTCCACCAACCCAACAGCACCCCATGTTCCAGCCCCAGGTCCTTGGTCCAATGACCAGTGAAAGGCCGCA 540
 P  A  P  D  R  H  V  P  P  T  Q  Q  H  P  M  F  Q  P  Q  V  L  G  P  M  T  S  E  R  P  H  121

TTCGAATGGCGTCGATCCTCCTCGGAAACAGCGAAGAGAAAGAACAACGTTTACCCGAGCCCAGCTCGACGTCCTCGAGACCCTTTTCAG 630
 S  N  G  V  D  P  P  R  K  Q  R  R  E  R  T  T  F  T  R  A  Q  L  D  V  L  E  T  L  F  S  151

CAGGACCAGATACCCAGATATCTTCATGAGGGAGGAAGTTGCTATGAAGATTAATCTACCAGAGTCGAGAGTACAGGTCTGGTTCAAGAA 720
 R  T  R  Y  P  D  I  F  M  R  E  E  V  A  M  K  I  N  L  P  E  S  R  V  Q  V  W  F  K  N  181

TAGGAGAGCAAAGTGTAGGCAACAGCAGCAACAGCAACAGAACGGTCCCAACAGCAACAACACCCCCAACAAGCCACGTCCTGCTAAGAA 810
 R  R  A  K  C  R  Q  Q  Q  Q  Q  Q  Q  N  G  P  N  S  N  N  T  P  N  K  P  R  P  A  K  K  211

GAAGACGCCACCACCCACGCCACGTGAGAATGATGCCCCCACCACTACATCAAGCGATACACCACCGTTCAAGGCATCGCCGTCCGTCTC 900
 K  T  P  P  P  T  P  R  E  N  D  A  P  T  T  T  S  S  D  T  P  P  F  K  A  S  P  S  V  S  241

ATCGAGTATGCCCAATAACAACAGTATTTGGAGCCCGGCGTCGATAGCCCCTCAACCCATGTCAAGCGATCATTTAGCAGAGAACATGAG 990
 S  S  M  P  N  N  N  S  I  W  S  P  A  S  I  A  P  Q  P  M  S  S  D  H  L  A  E  N  M  S  271

TAATAACAGTTGTATGCAGCATTCCTACACCATGCCCAACGCACAGCCTGCTGCAGGCTACACAGCGCAGGGCTACCAATCACCGTACTT 1080
 N  N  S  C  M  Q  H  S  Y  T  M  P  N  A  Q  P  A  A  G  Y  T  A  Q  G  Y  Q  S  P  Y  F  301

CGGCGCCGGTCTCGACTACCTCTCTCACATGCCGCAATTCCCCGGCTCCATCAACCACCAGATGGCAGCGAGTGCTATGAACAACGGACC 1170
 G  A  G  L  D  Y  L  S  H  M  P  Q  F  P  G  S  I  N  H  Q  M  A  A  S  A  M  N  N  G  P  331

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 M  T  T  M  A  S  Q  L  P  P  P  H  H  A  H  M  P  M  G  A  M  S  S  A  E  C  I  D  G  K  361

AGAACAGCCGCAATGGAAGTTCCAGTCATTGTAACCCCCTACGGCTTATCTCAAGAAGACGATCCCATCGACTCCATCGAAATCAATGGG 1350
 E  Q  P  Q  W  K  F  Q  S  L  *                                                           371

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CACTCCCAAATATGACAATGAAAGGACCAATGACATTATTGGTAGGAAGCATTTTTAAAATTTTAATTTATCACAATATTTTTTGGAAAG 1530
GAATCATACGATACAGTCTAGAGATAATGGTTTTCATCAGTTTTGTTATGGCGCCAATCTGAGACGCAAGACATTGCCGTTCGGACAATC 1620
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GGGCAACTTCATTATTTTTTCCTTGATTCTGTTCATTCCATGTTTAAAGTAGTGTGACACAAATCATGTAGTATGTAGGTTTATAGATAT 3150
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GCACGAAATATAGTAAACGTAAAAAAAAAAAAA                                                          3273
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FIG. 4. Structure of orthodenticle-related cDNAs of *H. pulcherrimus*. (A) Nucleotide sequence of $HpOtx_E$ cDNA and deduced amino acid sequence. (B) Nucleotide sequence of $HpOtx_L$ cDNA and deduced amino acid sequence. The asterisk marks the translational termination codon.

B

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AGACTTGACCGGTACTCGCCGCTTGGCCAGCACAAACCCGATTTTCCTCTCCGACTGTCCGCTAATATTAATACCTGACTTAATCCGAAT  90
ATGTGCGCTTAACGAGTAATCTCATAACGTCTATAGAACATCGCTTAATTTATTATCGTCAGATTATCCTATCACACTTGCCGTTCATTG 180
GCATTTTTAGGAACGTCGCTGGAACATTTCTGCACTTCTTGTCTACTTTTGATTTTAGTAAGACTTGCTTTGTAATTGGACATGCATATC 270
TCTTGATAAGAACATCTCCTAAACTTCTAACTTCAAGCAAAAATCAACAAAGTGAGATATCTATAGAGTGAAATATTGTGAATAACAGCC 360
CTAGAAGAGCATTCCAAACTAAATATTTTCACCACTTCGAGAATCAGTACTAATACATTTTAACGAAGATAGAAATCTTCCTATGATCTC 450
TAAGTGAATCGGTGACAGTAGAGCAGCTTTGGGACCAGACATTGACAGGGTGTTCTCATCACGGAATCTTATCCTATCTTTCAGTTCGTG 540
AGGCGGTTCGACCCTACCTCCGCCAAGCTGGATCATTCTGCCTTGACAGGAGGTCCCCGGACCTTCCCGGTAGCGAGCATGGCTTACACC 630
                                                                  M  A  Y  T                4

ATTCCGCCCGTGCCTCCCCAACATCACCACCACCTTCAGAACAAGATGAACGCACTCGGCTCTCCCTACTCCGTCAACGGCCGTTCGCTG 720
I  P  P  V  P  P  Q  H  H  H  H  L  Q  N  K  M  N  A  L  G  S  P  Y  S  V  N  G  R  S  L   34

GCGTCGCCGAACGTTGAGCTCATGCATCCCGCTATGTCATATACAAATCCTCCTCGGAAACAGCGAAGAGAAAGAACAACGTTTACCCGA 810
A  S  P  N  V  E  L  M  H  P  A  M  S  Y  T  N  P  P  R  K  Q  R  R  E  R  T  T  F  T  R   64

GCCCAGCTCGACGTCCTCGAGACCCTTTTCAGCAGGACCAGATACCCAGATATCTTCATGAGGGAGGAAGTTGCTATGAAGATTAATCTA 900
A  Q  L  D  V  L  E  T  L  F  S  R  T  R  Y  P  D  I  F  M  R  E  E  V  A  M  K  I  N  L   94

CCAGAGTCGAGAGTACAGGTCTGGTTCAAGAATAGGAGAGCAAAGTGTAGGCAACAGCAGCAACAGCAACAGAACGGTCCCAACAGCAAC 990
P  E  S  R  V  Q  V  W  F  K  N  R  R  A  K  C  R  Q  Q  Q  Q  Q  Q  Q  N  G  P  N  S  N   124

AACACCCCCAACAAGCCACGTCCTGCTAAGAAGAAGACGCCACCACCCACGCCACGTGAGAATGATGCCCCCACCACTACATCAAGCGAT 1080
N  T  P  N  K  P  R  P  A  K  K  K  T  P  P  P  T  P  R  E  N  D  A  P  T  T  T  S  S  D   154

ACACCACCGTTCAAGGCATCGCCGTCCGTCTCATCGAGTATGCCCAATAACAACAGTATTTGGAGCCCGGCGTCGATAGCCCCTCAACCC 1170
T  P  P  F  K  A  S  P  S  V  S  S  S  M  P  N  N  N  S  I  W  S  P  A  S  I  A  P  Q  P   184

ATGTCAAGCGATCATTTAGCAGCGAACATGAGTAATAACAGTTGTATGCAGCATTCCTACACCATGCCCAACGCACAGCCTGCTGCAGGC 1260
M  S  S  D  H  L  A  A  N  M  S  N  N  S  C  M  Q  H  S  Y  T  M  P  N  A  Q  P  A  A  G   214

TACACAGCGCAGGGCTACCAATCACCGTACTTCGGCGCCGGTCTCGACTACCTCTCTCACATGCCGCAATTCCCCGGCTCCATCAACCAC 1350
Y  T  A  Q  G  Y  Q  S  P  Y  F  G  A  G  L  D  Y  L  S  H  M  P  Q  F  P  G  S  I  N  H   244

CAGATGGCAGCGAGTGCTATGAACAACGGACCGATGACCACCATGGCCTCGCAGCTGCCACCTCCCCACCACGCCCACATGCCCATGGGC 1440
Q  M  A  A  S  A  M  N  N  G  P  M  T  T  M  A  S  Q  L  P  P  P  H  H  A  H  M  P  M  G   274

GCCATGTCGAGTGCAGAGTGTATCGACGGCAAAGAACAGCCGCAATGGAAGTTCCAGTCATTGTAACCCCCTACGGCTTATCTCAAGAAG 1530
A  M  S  S  A  E  C  I  D  G  K  E  Q  P  Q  W  K  F  Q  S  L  *                          295

ACGATCCCATCGACTCCATCGAAATCAATGGGATATCTTCTCCAGTCACCATGTTTTTGCGAAATGTGAAAAAGGACACTCATGGTAATA 1620
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GTGACCTCGTGATGAATACATACAACTTGCAATTTGGCAAAGAACTTTTGCATCAGAATGTATTCTCATTTGACAAATAGCGTCACTAGT 2070
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AAAGGACCTTGTTAAAATGAACCACCACCAAACTTCCCACCGAAAATGGACAAAAAAAGAATGACTGATTTATCTATCATGCAATGTAAA 2340
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CACAAATCATGTAGTATGTAGGTTTATAGATATCTGAATATCTTCGTTATTTTTTCCAGTTGTTTCTAGCCTGACGCGATGTTAGGTACA 3420
TTATGCACAGTTGAATGAAAATTATTAAAAATTGCACGAAATATAAAAAAAAAAAAAA                                 3478
```

FIG. 4—*Continued*

mutants are described in Fig. 2A. The C15 fragment possesses two adjacently aligned core consensus sequence of orthodenticle-related protein (Otx) binding sites surrounded by four Otx-like sequences which have a single base pair difference from the Otx consensus. As shown in Fig. 2B, removal of an Otx-like motif at the 3′ end of Otx consensus

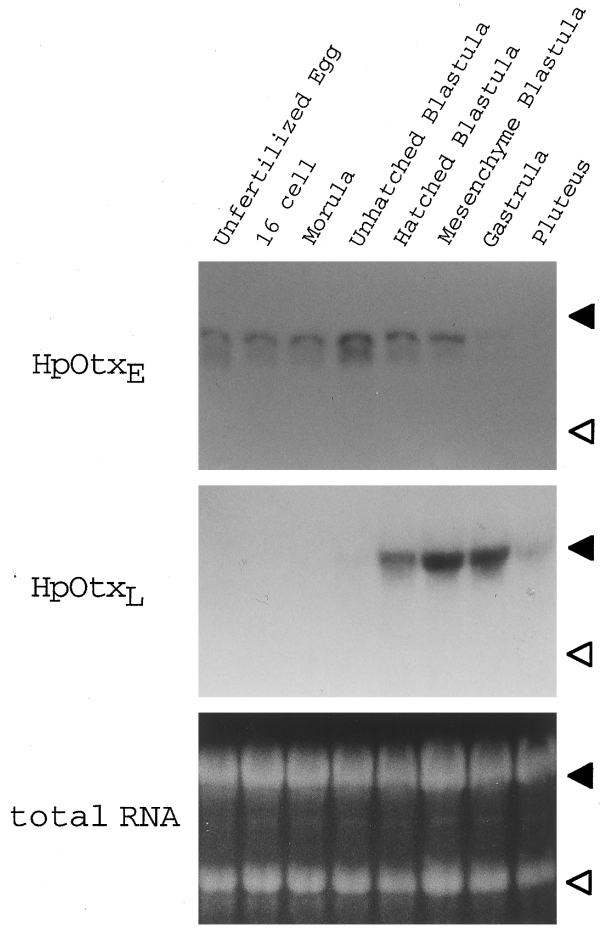


FIG. 5. Accumulation of HpOtx$_E$ and HpOtx$_L$ transcripts during embryogenesis. 10 μg of total RNA extracted from sea urchin embryos at various developmental stages was run in parallel. Northern hybridization analysis was performed by using specific probes for each HpOtx cDNA synthesized as described under Materials and Methods. Closed triangles, 26S rRNA (4.2 kb); open triangles, 18S rRNA (2 kb).

(C15-Δ193) did not have any effect on the band shift pattern, while deletion of one of the core Otx consensus resulted in nearly 50% reduction in intensity of three shift bands. No gel shift bands were observed after removal of both Otx consensus motifs.

To ensure that Otx proteins are responsible for appearance of gel shift bands, we carried out a supershift assay by adding anti-SpOtx antibody (Gan *et al.*, 1995) to the reaction system for gel mobility shift assay of C15 fragment. As shown in Fig. 2C, addition of anti-SpOtx clearly supershifted the gel bands, while normal serum had no such effect. By increasing the amount of anti-SpOtx in the reaction mixture, inhibition of gel retardation was observed, indicating that it is orthodenticle-related proteins that bind to C15 fragment producing gel shift bands.

Otx Is an Enhancer Element of C15 Fragment

Various deletion mutants of the C15 fragment were inserted into the upstream region of the pArs252-Luc reporter (Iuchi *et al.*, 1995), introduced into fertilized eggs of *H. pulcherrimus*, and assayed for expression of luciferase 48 hr postfertilization. Deletion of the Otx consensus caused a significant decrease in the enhancer activity (Δ188 and Δ178 in Fig. 3) as compared to full-length C15. This indicates that two core Otx consensus motifs are intimately related to the enhancer activity of the C15 fragment. However, other regions in C15 also seem to have significant enhancer activities, though these regions lack the Otx consensus and DNA binding sites are barely detectable in these regions by gel mobility shift assay, since a deletion up to 202 bp from the 3′ end of C15 caused clear reduction of the enhancer activity and C15-Δ178 fragment still considerably enhanced expression of the pArs252-Luc.

Cloning of HpOtx cDNA

The first screening of the *H. pulcherrimus* unfertilized egg cDNA library (8×10^4 plaques) by an SpOtx cDNA fragment produced approximately 100 positive clones. The two longest positive clones were subcloned into pBluescript SK− vector and sequenced. The 5′ terminal region of this cDNA was isolated by the 5′RACE method using 5 μg of total RNA isolated from the mesenchyme blastula embryos. The nucleotide sequence of this cDNA (referred to as HpOtx$_E$) and the deduced amino acid sequence are shown in Fig. 4A.

To isolate the cDNA(s) for the HpOtx mRNA(s) which are expressed in the later stages of development (hatching) (Fig. 1A), the HpOtx homeobox-containing a 134-bp DNA fragment generated by PCR from *H. pulcherrimus* gastrula cDNA library was used to probe the same gastrula cDNA library (approximately 5×10^4 plaques). Two positive clones obtained were subcloned into pBluescript SK− vector and sequenced. The 5′ end of this cDNA was also isolated by the 5′RACE method. Its nucleotide sequence and deduced amino acid sequence are shown in Fig. 4B, and this cDNA is referred to as HpOtx$_L$.

The analysis of HpOtx transcripts was performed using specific probes for each HpOtx cDNA, as described under Materials and Methods (Fig. 5). HpOtx$_E$ transcript is present in the unfertilized egg and gradually decreases in amount after hatching; HpOtx$_L$ transcript first appears after hatching, increases gradually until the gastrula stage, and then decreases to a very low level in the pluteus.

To confirm whether the different gel shift bands represent complexes produced by these HpOtx transcripts, both HpOtx proteins were synthesized, *in vitro*, and used in a gel shift analysis (Fig. 6A). HpOtx$_E$ and HpOtx$_L$ proteins bound to the C15 fragment (Fig. 6A, lanes 3 and 4). While the complex of HpOtx$_L$ protein and Band II migrated with the same mobility (compare with lanes 4 and 5), the complex of HpOtx$_E$ protein migrated faster than Band I (compare

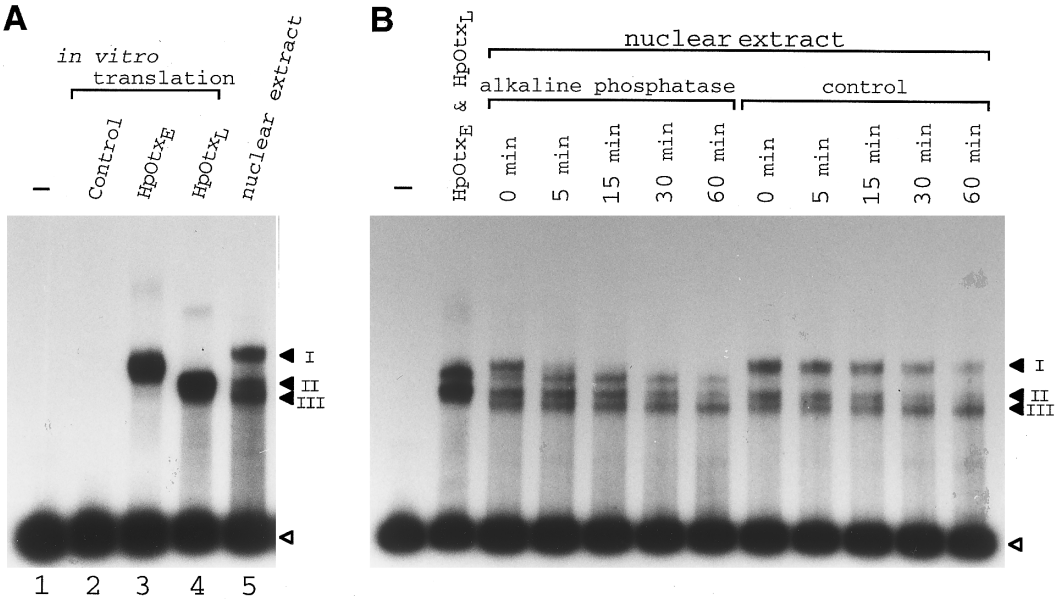


FIG. 6. HpOtx$_E$ and HpOtx$_L$ isoforms represent the Bands I and II, respectively. (A) Gel-mobility shift assay using *in vitro*-translated HpOtx$_E$ and HpOtx$_L$ proteins. (B) Dephosphorylation of nuclear proteins from the mesenchyme blastula stage embryos. 10 μg of nuclear extracts was treated with or without 1 unit of alkaline phosphatase at 30°C for indicated time. 1 μg of treated proteins was used in gel-mobility shift assay. Closed triangles, shifted bands; open triangle, free probe.

with lanes 3 and 5). This difference in mobility between the HpOtx$_E$ protein synthesized, *in vitro*, and the nuclear extract disappeared when nuclear extracts were treated with alkaline phosphatase (Fig. 6B). Thus, it is possible that the transcripts of HpOtx$_E$ and HpOtx$_L$ cDNA encode the proteins that produce Bands I and II, respectively, and that HpOtx$_E$ protein(s) contained in nuclear extracts are phosphorylated.

As shown in Fig. 7A, the nucleotide sequences of the two HpOtx cDNAs are almost identical from the 5′ end of the homeobox to the 3′ end of the cDNA including the 3′ UTR sequence. However, the nucleotide sequence in the region upstream of the homeobox are different in HpOtx$_E$ and HpOtx$_L$.

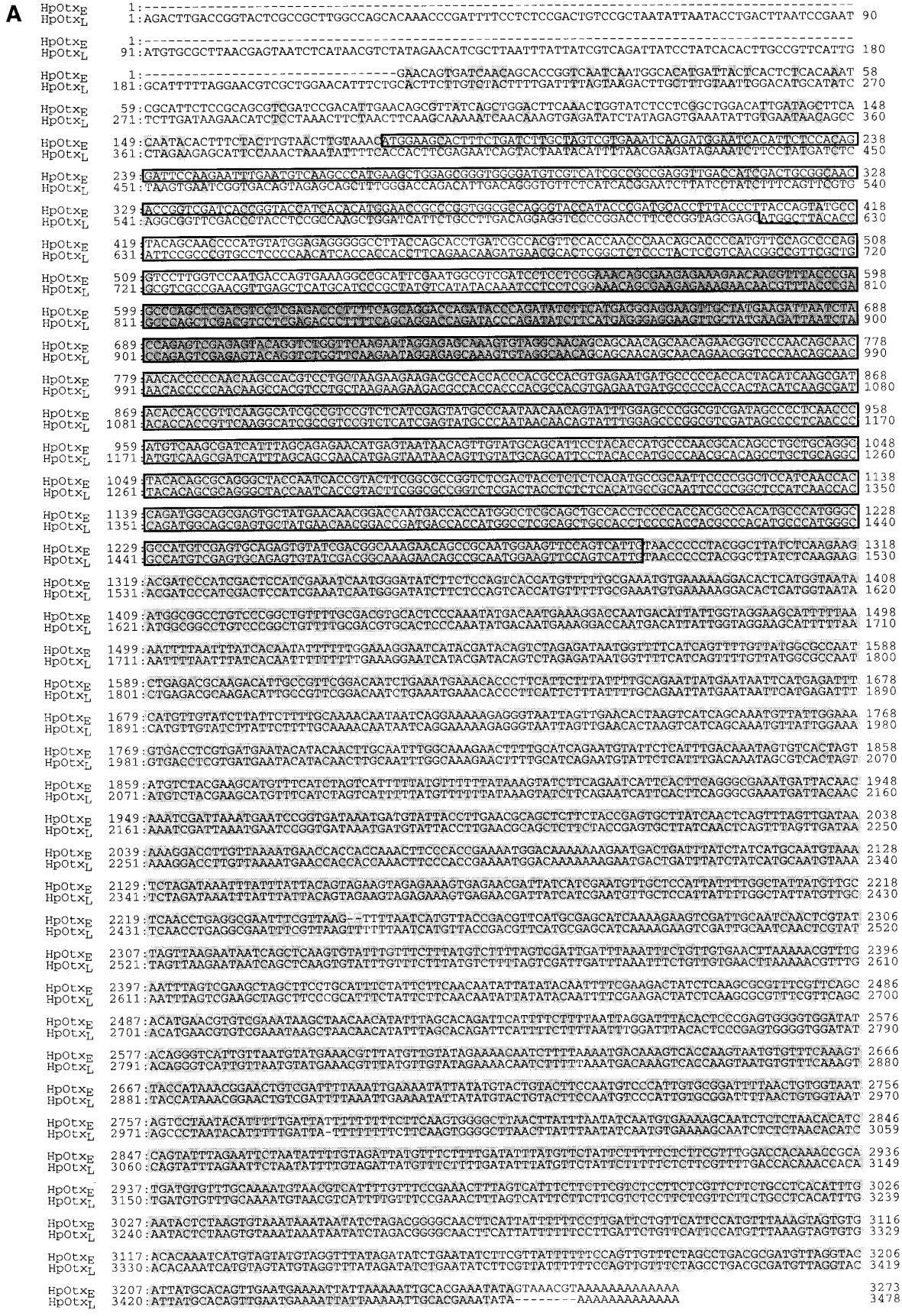
Comparison of the amino acid sequences encoded by HpOtx$_E$, HpOtx$_L$, and SpOtx (Fig. 7B) reveals that HpOtx$_E$ and SpOtx are almost identical. Thus, HpOtx$_E$ is a homologue of SpOtx, while HpOtx$_L$ is somewhat different from them. The amino acid sequence of HpOtx$_L$ from its N terminal to the 5′ end of the homeodomain is different from HpOtx$_E$ and SpOtx, though the amino acid sequences of the homeodomain and the region downstream to the C terminal are almost identical.

A genomic Southern analysis using a 386-bp region in 3′ UTR of HpOtx$_E$ as the probe (which has a nucleotide sequence highly homologous to that of HpOtx$_L$) yielded a single band after treatment with three different restriction endonucleases (Fig. 7C). This suggests that only a single copy of the *HpOtx* gene is present in the Hp genome. The two types of HpOtx mRNAs (HpOtx$_E$ and HpOtx$_L$) may, therefore, be generated from same *HpOtx* gene.

DISCUSSION

It has been reported that Otx protein acts as a positive regulatory transcription factor of the *Spec2a* gene in *S. purpuratus* embryos (Mao *et al.*, 1994). In this study, we found that a cluster of Otx sites in the first intron of *HpArs* gene act as a major enhancer element of this gene and that three types of Otx proteins, which bind to the clustered Otx sites, appear under different temporal regulation during development. Among these three Otx proteins of *H. pulcherrimus*, we have cloned cDNA of two of them, HpOtx$_E$ and HpOtx$_L$. The amino acid sequence of HpOtx$_E$ is almost identical to that of SpOtx, suggesting that HpOtx$_E$ is a homologue of SpOtx. Supershift of the HpOtx band by addition of anti-SpOtx to gel shift assay confirms identity of HpOtx$_E$ to SpOtx.

Results of gel shift assay and Northern blot experiments show that HpOtx$_E$ is an early acting factor (maternal factor), while HpOtx$_L$ is a later acting factor that is produced after hatching. Since transcription of the *HpArs* gene begins in the hatching blastula, maternal existence of HpOtx$_E$ and its gradual decrease after hatching suggest that transcription of the *HpArs* gene is suppressed by binding of HpOtx$_E$ to the Otx sites in the C15 region. Transcription of the *Ars*


A
HpOtx$_E$ 1:----------
HpOtx$_L$ 1:AGACTTGACCGGTACTCGCCGCTTGGCCAGCACAAACCCGATTTTCCTCTCCGACTGTCCGCTAATATTAATACCTGACTTAATCCGAAT 90
HpOtx$_E$ 1:----------
HpOtx$_L$ 91:ATGTGCGCTTAACGAGTAATCTCATAACGTCTATAGAACATCGCTTAATTTATTATCGTCAGATTATCCTATCACACTTGCCGTTCATTG 180
HpOtx$_E$ 1:----------GAACAGTGATCAACAGCACCGGTCAATCAATGGCACATGATTACTCACTCTCACAAAT 58
HpOtx$_L$ 181:GCATTTTTAGGAACGTCGCTGGAACATTTCTGCACTTCTTGTCTACTTTTGATTTTAGTAAGACTTGCTTTGTAATTGGACATGCATATC 270
HpOtx$_E$ 59:CGCATTCTCCGCAGCGTCGATCCGACATTGAACAGCGTTATCAGCTGGACTTCAAACTGGTATCTCCTCGGCTGGACATTGATAGCTTCA 148
HpOtx$_L$ 271:TCTTGATAAGAACATCCTCTAAACTTCTAACTTCAAGCAAAATCAACAAAGTGAGATATCTATAGAGTGAAATATTGTGAAATAACAGCC 360
HpOtx$_E$ 149:CAATACACTTTCTACTTGTAACTTGTAAACATGGAAGCACTTTCTGATCTTGCTAGTCGTGAAATCAAGATGGAATCACATTCTCCACAG 238
HpOtx$_L$ 361:CTAGAAGAGCATTCCAAACTAAATATTTTCACCACTTCGAGAATCAGTACTAATACATTTTAACGAAGATAGAAATCTTCCTATGATCTC 450
HpOtx$_E$ 239:GATTCCAAGAATTTGAATGTCAAGCCCATGAAGCTGGAGCGGGTGGGGATGTCGTCATCGCCGCCGAGGTTGACCATCGACTGCGGCAAC 328
HpOtx$_L$ 451:TAAGTGAATCGGTGACAGTAGAGCAGCTTTGGGACCAGACATTGACAGGGTGTTCTCATCACGGAATCTTATCCTATCTTTCAGTTCGTG 540
HpOtx$_E$ 329:ACCGGTCGATCACCGGTACCATCACACATGGAACCGCCCCGGTGGCGCCAGGGTACCATACCCGATGCACCTTTACCCTTACCAGTATGCC 418
HpOtx$_L$ 541:AGGCGGTTCGACCCTACCTCCGCCAAGCTGGATCATTCTGCCTTGACAGGAGGTCCCCGGACCTTCCCGGTAGCGAGCATGGCTTACACC 630
HpOtx$_E$ 419:TACAGCAACCCCATGTATGGAGAGGGGGCCTTACCAGCACCTGATCGCCACGTTCCACCAACCCAACAGCACCCCATGTTCCAGCCCCAG 508
HpOtx$_L$ 631:ATTCCGCCCGTGCCTCCCCAACATCACCACCACCTTCAGAACAAGATGAACGCACTCGGCTCTCCCTACTCCGTCAACGGCCGTTCGCTG 720
HpOtx$_E$ 509:GTCCTTGGTCCAATGACCAGTGAAAGGCCGCATTCGAATGGCGTCGATCCTCCTCGGAAACAGCGAAGAGAAAGAACACAACGTTTACCCGA 598
HpOtx$_L$ 721:GCGTCGCCGAACGTTGAGCTCATGCATCCCGCTATGTCATATACAAATCCTCCTCGGAAACAGCGAAGAGAAAGAACACAACGTTTACCCGA 810
HpOtx$_E$ 599:GCCCAGCTCGACGTCCTCGAGACCCTTTTCAGCAGGACCAGATACCCAGATATCTTCATGAGGGAGGAAGTTGCTATGAAGATTAATCTA 688
HpOtx$_L$ 811:GCCAGCTCGACGTCCTCGAGACCCTTTTCAGCAGGACCAGATACCCAGATATCTTCATGAGGGAGGAAGTTGCTATGAAGATTAATCTA 900
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HpOtx$_E$ 869:ACACCCACCGTTCAAGGCATCGCCGTCCGTCTCATCGAGTATGCCCAATAACAACAGTATTTGGAGCCCGGCGTCGATAGCCCCTCAACCC 958
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HpOtx$_E$ 1139:CAGATGGCAGCGAGTGCTATGAACAACGGACCAATGACCACCATGGCCTCGCAGCTGCCACCTCCCCACCACGCCCACATGCCCATGGGC 1228
HpOtx$_L$ 1351:CAGATGGCAGCGAGTGCTATGAACAACGGACCGATGACCACCATGGCCTCGCAGCTGCCACCTCCCCACCACGCCCACATGCCCATGGGC 1440
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HpOtx$_L$ 3330:ACACAAATCATGTAGTATGTAGGTTTATAGATATCTGAATATCTTCGTTATTTTTTCCAGTTGTTTCTAGCCTGACGCGATGTTAGGTAC 3419
HpOtx$_E$ 3207:ATTATGCACAGTTGAATGAAAATTATTAAAAATTGCACGAAATATAGTAAACGTAAAAAAAAAAAAA 3273
HpOtx$_L$ 3420:ATTATGCACAGTTGAATGAAAATTATTAAAAATTGCACGAAATATA--------AAAAAAAAAAAA 3478

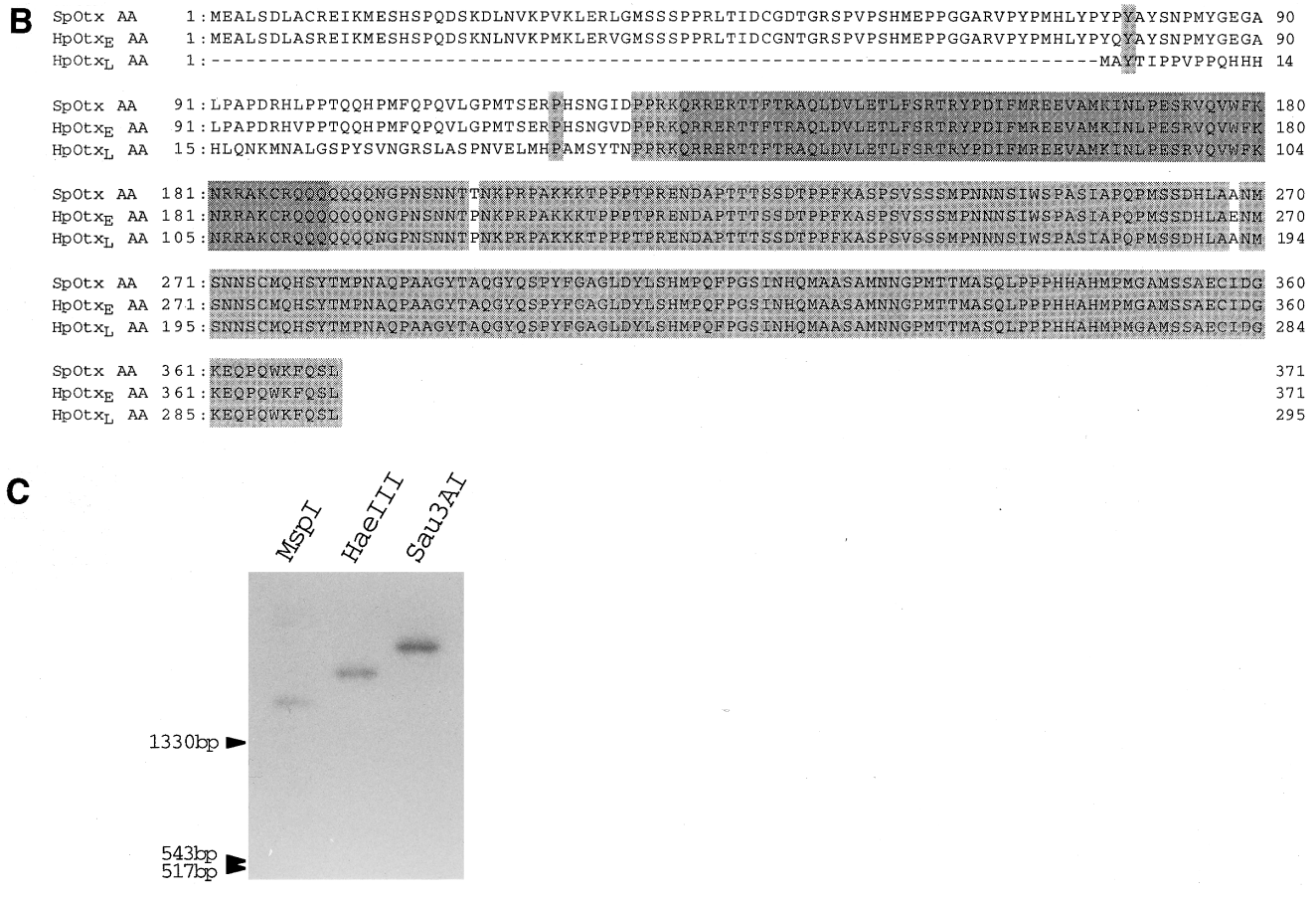

FIG. 7. (A) Comparison of nucleotide sequences of two types of HpOtx cDNAs. A dark shaded box indicates the sequence for well conserved homeodomain, and light shaded boxes show the nucleotides identical between two HpOtx cDNAs. Boxed regions represent the coding region of both HpOtx cDNA. (B) Comparison of amino acid sequences of sea urchin Otx family proteins. A dark shaded box indicates the sequence of well conserved homeodomain, and light shaded boxes show the amino acids identical among these Otx proteins. (C) Genomic Southern analysis using a 386-bp region in 3′UTR of HpOtx$_E$ as the probe. 1 μg of sea urchin sperm DNA digested with restriction endonucleases was run. The sizes of marker, *Hin*fI-digested pUC119 plasmid DNA, are indicated on the left side.

gene may be commenced by displacement of HpOtx$_E$ on Otx sites by HpOtx$_L$.

The alkaline phosphatase treatment of nuclear extracts showed that HpOtx$_E$ proteins in the nuclear extracts are phosphorylated (Fig. 6B). It should be noticed that the reduction of intensity of gel-shift bands during the long incubation time employed in Fig. 6B may be due to endogenous proteolytic enzymes because these bands were also reduced by a long incubation in the buffer without alkaline phosphatase. A signal transduction pathway containing protein phosphorylation/dephosphorylation may be related to the function of HpOtx$_E$ proteins. The protein(s) giving Bands II and III seem to be the product of HpOtx$_L$ since the expression of HpOtx$_L$ coincides with the appearance of Bands II and III; the protein obtained by *in vitro* translation of HpOtx$_L$ mRNA gives a band with a mobility similar to endogenous late type Otx proteins. However, the relationship between Bands II and III remains to be clarified.

The result of genomic Southern blot shows that only a single copy of the *HpOtx* gene is detected in the *H. pulcherrimus* genome; this suggests that two different types of HpOtx mRNA are produced from the same gene. Since the nucleotide sequence from the homeobox to 3′ end of cDNA and the deduced amino acid sequence from homeodomain to C-terminal are both almost identical between HpOtx$_E$ and HpOtx$_L$, two types of mRNA may be produced by alternative splicing of a common precursor RNA or they may be produced by transcription using different promoter sites in the same gene. The small number of differences in the compared nucleotide sequences may be attributable to polymorphism of the *HpOtx* gene.

During development of *H. pulcherrimus* embryos ex-

pression of the *Ars* gene begins at the time of hatching in the aboral ectoderm (Akasaka *et al.*, 1990). Cis-elements related to temporal regulation of the *Ars* gene transcription seems to be located within the proximal promoter region and those for spatial regulation also seem to be present in the 5′ upstream of the gene (our unpublished data). DNA–protein interactions between Otx sites in the first intron and a group of HpOtx proteins seem to be related to the quantitative regulation of the transcription of the *Ars* gene; in this respect the role of SpOtx in *Spec2a* gene expression and that of the HpOtx proteins in the regulation of *Ars* gene expression appears similar.

Luciferase assays for the enhancer activity of variously deleted C15 fragments indicate the existence of other cis-elements besides Otx sites because a sharp drop in the enhancer activity was detected by deleting a 27-bp sequence from the downstream end of the complete C15 fragment and by deleting a 24-bp sequence between C15-Δ178 and C15-Δ154 (Fig. 3). Since these two regions contain CAAT sequences, these enhancer activities may be related to CAAT motifs which are also present in the Spec2a RSR enhancer element (Mao *et al.*, 1994). CAAT is also known as an active cis-element in various eukaryotic genes (Wingender, 1993), including the sea urchin histone gene (Barberis *et al.*, 1987). Though we have not yet detected the nuclear protein binding to these, two other enhancer regions in C15, it is likely that CAAT motifs also act as enhancer elements regulating *Ars* gene expression.

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