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Novel genes expressed differentially in ascidians with alternate modes of development

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SUMMARY

We have used a subtractive procedure to isolate cDNA clones encoding genes expressed differentially in ascidian species with alternate modes of development. The ascidians used in this study were *Molgula oculata*, which develops a tailed (urodele) larva, and *Molgula occulta*, which develops a tailless (anural) larva. Two of the identified clones, *Uro-2* and *Uro-11*, are described. Southern blots show that the *Uro-2* and *Uro-11* genes are present in both species, but the corresponding mRNAs are expressed preferentially in the urodele species. In situ hybridization showed that *Uro-2* and *Uro-11* transcripts accumulate in small oocytes during oogenesis. The maternal *Uro-2* and *Uro-11* transcripts were distributed throughout the oocyte cytoplasm. Transcript concentrations declined during vitellogenesis, but mature eggs still contain detectable levels of *Uro-2* and *Uro-11* mRNA. After fertilization, the maternal *Uro-2* and *Uro-11* transcripts were localized in the ectoplasm of uncleaved zygotes and mostly entered the ectoderm cells during cleavage. The *Uro-2* gene appears to produce only maternal transcripts. In contrast, the *Uro-11* gene may also produce zygotic transcripts, which accumulate between gastrulation and neurulation in posterior epidermis, neural and tail muscle cells. Zygotic expression of the *Uro-11* gene was not detected in embryos of the anural species. The deduced amino acid sequences of the *Uro-2* and *Uro-11* cDNAs suggest that they encode novel basic proteins with distinctive structural features. The predicted Uro-2 protein contains a leucine zipper motif, suggesting that it may dimerize with another protein. The predicted Uro-11 protein contains a nuclear localization signal, a region with similarity to part of the DNA-binding motif in the bacterial histone-like HU and IHF proteins, 12 repeats of the proposed DNA-binding motif S(T)PXX, and a potential zinc finger of the C_6 or C_6H_2 class, suggesting that it may be a DNA-binding protein. The Uro-2 and Uro-11 proteins are candidates for regulatory factors involved in the evolutionary transition from urodele to anural development.

Key words: alternate modes of development, evolution, subtractive cloning, *Uro* genes, ascidians

INTRODUCTION

Little is known about the molecular mechanisms underlying evolutionary changes in development. Closely related species with different modes of development are attractive systems to investigate this problem (Raff, 1987). Ascidians are urochordates that have evolved alternate modes of development (reviewed in Jeffery and Swalla, 1992a). Most ascidian species form a tailed (urodele) larva, which is the dispersal phase in the life cycle. The urodele larva consists of a head, containing a brain with a pigmented sensory organ(s), and a tail, containing a notochord flanked by bands of striated muscle cells. The larval tail is formed by concerted movements of the prospective notochord (Cloney, 1964; Miyamoto and Crowther, 1985), muscle and epidermal cells in the posterior region of the embryo. As the tail elongates, myoblasts differentiate into muscle cells and a swimming tadpole larva is formed. The tail muscle cells develop autonomously, mediated by determinants segregated from the egg into the myoblast lineages (reviewed in Satoh et al., 1990; Swalla, 1992). In contrast, the brain sensory cells are specified by inductive signals (Nishida and Satoh, 1989), probably arising from presumptive notochord cells during gastrulation (reviewed in Venuti and Jeffery, 1989). The notochord cells themselves may be specified by an earlier induction during cleavage (Nishida, 1992).

Anural development is an alternate mode of development in ascidians, which results in the formation of a tailless (anural) embryo (reviewed in Jeffery and Swalla, 1990). The anural embryo lacks many of the typical urodele features

and forms an immotile larva, which develops directly into an adult. Fewer than 20 ascidian species have been shown to exhibit anural development and almost all of these species are members of the family Molgulidae (reviewed in Jeffery and Swalla, 1990). Urodele development is thought to be the ancestral mode of development in ascidians (Berrill, 1931). This viewpoint is supported by the retention of vestigial urodele features in embryos of some anural species (Whittaker, 1979a; Swalla and Jeffery, 1990, 1991). Anural ascidians appear to have evolved in habitats such as subtidal sand and mud flats in which there is less selective advantage for a larval dispersal phase.

The mechanisms underlying the transition from urodele to anural development have been investigated in two closely related species of ascidians with different modes of development (Swalla and Jeffery, 1990; Jeffery and Swalla, 1991). *Molgula oculata* shows conventional urodele development, whereas *Molgula occulta* shows anural development. Fertilization, cleavage, gastrulation and neurulation are similar in both species, but induction of brain sensory cells and tail morphogenesis does not occur in embryos of the anural species (Swalla and Jeffery, 1990). Remarkably, however, when eggs of the anural species are fertilized with sperm of the urodele species, hybrids develop some of the ancestral urodele features, including a brain sensory cell and a short tail containing a notochord (Swalla and Jeffery, 1990). Expression of the urodele genome in the anural zygote is responsible for restoring these urodele features (Jeffery and Swalla, 1992b). In contrast, the reciprocal cross, in which eggs of the urodele species are fertilized with sperm of the anural species results in hybrids with urodele development (Jeffery and Swalla, 1992a). These results suggest that loss-of-function mutations in zygotic genes are responsible for some of the developmental changes in the anural species. However, muscle cells did not differentiate in hybrids with restored urodele features, suggesting that maternal changes are also important in the transition to anural development. Consistent with this possibility, the myoplasm, an egg cytoplasmic region that is segregated to muscle lineages in ascidian embryos (see Swalla, 1992), is modified in eggs of anural species (Swalla et al., 1991; Jeffery and Swalla, 1992b).

The identification of regulatory genes expressed differentially in urodele and anural ascidians is needed to define the molecular mechanisms underlying the evolutionary transition to anural development. Here we identify and characterize two cDNAs cloned by subtractive procedures encoding *Uro* (urodele-specific) genes that are expressed during development in the urodele ascidian *M. oculata*, but are inactive or down-regulated in the anural ascidian *M. occulta*. The deduced Uro proteins contain potential leucine zipper and DNA-binding motifs and are candidates for regulatory factors involved in changing the mode of larval development.

MATERIALS AND METHODS

Biological materials

The urodele ascidian *Molgula oculata* and the anural ascidian *Molgula occulta* were obtained by dredging sand flats near Point de Bloscon, Roscoff, France. Animals were maintained in tanks of running sea water and gametes were obtained from dissected gonads. Cultures of mature eggs obtained in this way also contain large numbers of immature oocytes. Insemination of eggs, culture of embryos and developmental times have been described previously (Swalla and Jeffery, 1990).

Isolation of DNA and RNA

The method of Davis et al. (1986) was used to prepare genomic DNA for southern analysis. Total RNA was obtained by homogenization of dissected gonads or embryos using the guanidine isothiocyanate method (March et al., 1985). Poly(A)$^+$ RNA was isolated by purification with Oligotex-dT30 beads (Roche, Tokyo, Japan).

Preparation and PCR amplification of cDNA libraries

cDNA was synthesized from *M. oculata* and *M. occulta* gonad poly (A)$^+$ RNA using a Riboclone cDNA kit (Promega, Madison, WI). Double-stranded cDNA was size fractionated on a Sepharose CL-4B (Sigma Chemical Company, St. Louis, MO) column and fractions greater than 300 bp were collected. cDNA prepared from *M. oculata* gonad mRNA was directionally cloned into the *Eco*RI-*Xho*I site of a lambda Uni-ZAP vector (Stratagene, La Jolla, CA). *M. occulta* cDNA was ligated into the *Eco*RI site of a lambda ZAP-II vector (Stratagene) in either direction.

PCR reactions were performed in 100 μl volumes using Taq polymerase (Promega) and a Perkin-Elmer Thermal Cycler (Perkin-Elmer Corporation; Norwalk, Conn.). Final concentrations of the reaction mixture were 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 9 at 25°C), 0.1% Triton X-100 (Promega). A 68.5 μl aliquot of the *M. oculata* or *M. occulta* cDNA library was boiled in water and added to the PCR reaction at a final concentration of 10^5 pfu/μl. Each PCR reaction included 100 pmol of T3 primer (5′-ATTAACCCTCACTAAAGGGA-3′) and T7 primer (5′-GCG-TAATACGACTCACTATA-3′) for the *M. oculata* library or SK20 primer (5′-CCGCTCTAGAACTAGTGGAT-3′) and T7 primer for the *M. occulta* library. The primers were synthesized using an ABI 380B-02 DNA synthesizer (Applied Biosystems Inc., Foster City, CA). Amplification used 15 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C. One fifth aliquot of the amplified inserts was then reamplified in 100 μl of a fresh reaction mixture for 4 cycles and chased by 2 additional cycles that omitted the 94°C incubation (Timblin et al., 1990). After the final cycle, the sample was incubated at 72°C for 15 minutes, then chilled to 4°C.

Subtractive hybridization and library screening

To synthesize RNA from the amplified libraries, template DNA from each library was amplified as described above, extracted with phenol and precipitated with ethanol, and a quarter of the DNA was dissolved in 20 μl of RNA synthesis mixture according to Van Gelder et al. (1990). 80 units of T7 RNA polymerase (Bethesda Research Laboratories, Bethesda, MD) was used to synthesize antisense RNA from the *M. oculata* cDNA library. After incubation for 2 hours at 37°C, 1 unit of RQ1 DNase (Promega; Madison, WI) was added to digest the DNA template. The *M. oculata* library RNA was converted into cDNA using the T3 primer, and RNA was subsequently hydrolyzed in 1 N NaOH, 0.5% SDS. Because the *M. occulta* cDNA library was not directionally cloned, RNA prepared using the T7 promotor consisted of both sense and antisense messages. The *M. occulta* library RNA was labeled with photoactivatible biotin (Clontech Laboratories, Inc., Palo Alto, CA) by use of a PAB sunlamp (Clontech). Subtractive hybridization, in which 1.46 μg of *M. oculata* single-stranded cDNA and 20 μg of *M. occulta* library RNA were mixed, was performed according to the procedure of Sive and St. John (1988), except that 3 rounds of hybridization were done. After hybridization, 5 μg of streptavidin was added and the mixture was phenol extracted twice to remove the biotin-labeled transcripts. The subtracted cDNA (92 ng) was

then labelled with [alpha-^{32}P]dCTP (3000 Ci/mmole, Amersham, Arlington Heights, IL) by random primer labelling (BMB; Boehringer Mannheim Biochemicals, Indianapolis, IN) and used as a probe to screen the *M. oculata* cDNA library.

Screening was performed in 60 ml of hybridization solution (50% formamide, 6× SSPE, 5× Denhardt's, 0.5% SDS, 100 μg/ml salmon sperm DNA) with 2×10^6 cts/minute/ml probe. A total of 5×10^4 phage were screened at high stringency with the subtracted probe at low plaque density (5×10^3 per 10×14 cm plate). The 24 positive *M. oculata* clones obtained in the first screen were then hybridized with a cDNA probe prepared from RNA synthesized in vitro from the *M. occulta* cDNA library, according to the method described above, except that washes were at low stringency (5 washes at 2× SSC, 0.1% SDS for 30 minutes at 55°C; 4 washes at 0.1× SSC, 0.1% SDS for 25 minutes at 50°C), and 8 negative clones were selected. One of the 16 positive clones was also retained as a control for RNA slot blot hybridization.

The clones that screened positive with *M. oculata* cDNA but negative with *M. occulta* cDNA (*M. oculata* positive/*M. occulta* negative clones) were further characterized by RNA slot blot hybridization. RNA synthesized in vitro from the *M. oculata* and *M. occulta* cDNA libraries as described earlier was blotted onto Hybond N^+ filter paper (Amersham) using the formamide/formaldehyde method (Sambrook et al., 1989). The filters were hybridized separately in Rapid Hybridization Buffer (Amersham) with 1×10^6 cts/minute of random primed (BMB) ^{32}P-labelled (3000 Ci/mmole, Amersham) cDNA. Probes were prepared from templates obtained by PCR amplification of 7 *M. oculata* positive/*M. occulta* negative clones (1 insert did not amplify) and a control clone positive for both species. Three cDNA clones, *Uro 1*, *2* and *11* (see Results for terminology of *Uro* clones), hybridized preferentially to *M. oculata* RNA; the remaining clones hybridized to the same extent as the control clone to RNA of both species.

Clones encoding full-length cytoskeletal actin genes from *M. oculata* (*MocuCA-4*) and *M. occulta* (*MoccCA-4*) were used as controls in studies of the expression patterns of the mUro genes. The derived amino acid sequences of MocuCA-4 and MoccCA-4 correspond to previously described ascidian cytoskeletal actin genes (Beach and Jeffery, 1990).

Sequencing and computer analysis

Selected cDNA clones were sequenced on both strands using [^{35}S]-dATP (800 Ci/mmole; NEN; New England Nuclear, Boston, MA) by the dideoxy chain termination method (Sanger et al. 1977) with Sequenase (USB; United States Biochemical Corp., Cleveland, OH). Oligonucleotide primers were made on a Pharmacia LKB Gene Assembler Plus (Pharmacia Biosystems, Inc.; Piscataway, NJ) to create overlapping sequence information. Synthesized oligonucleotides were deprotected with NH_4OH and purified with Oligo-clean (USB), then diluted to 25 ng/ml with distilled water. The sequences were read and compared with the MacVector Program (IBI-A Kodak Company; New Haven, CT) on a MacIntosh IIci computer, and translated protein sequences were compared to sequences present in the National Biomedical Research Foundation protein database (IBI) by use of MacVector (IBI). Sequences were also compared by BLAST to the on-line GenBank protein sequences available through the National Library of Medicine. Sequences are available through GenBank by Accession no. L19340 (Uro-2) and Accession no. L19339 (Uro-11).

Filter hybridizations

Genomic DNA was exhaustively digested with *Eco*RI, *Hin*dIII, and *Bam*HI and blotted onto nylon membrane (MSI; Fisher Scientific; San Francisco, CA). After baking under vacuum for 2 hours, blots were hybridized at 42°C for 18-24 hours in a rotating hybridization chamber (Robbins Scientific Corporation, Sunnyvale, CA). Washes were done in decreasing concentrations of SET (1× SET=30 mM Tris, 2 mM EDTA, 150 mM NaCl) at increasing temperatures to obtain an appropriate signal-to-noise ratio. DNA probes for blot hybridizations were labelled with [^{32}P]dCTP (800-3000 Ci/mmole; NEN) using random primed labelling (USB) as described above. For some hybridizations, antisense RNA probes were synthesized in vitro with either T3 or T7 polymerase (Ambion; Austin, TX) and [^{32}P]UTP (3,000 Ci/mole; NEN) after linearizing the clone with the appropriate restriction enzyme.

In situ hybridization

Gonads and embryos were embedded in paraplast, sectioned at 8 μm, attached to subbed microscope slides and subjected to in situ hybridization (Swalla and Jeffery, 1990). Single-stranded RNA was synthesized from full-length *Uro* cDNA inserts using [^{35}S]ATP (1000-1500 Ci/mmole; NEN) and T7 RNA polymerase (MAXIscript Kit; Ambion). Approximately $1\text{-}5\times10^6$ cts/minute of labelled RNA probe was added to each pretreated slide in 50 μl of hybridization buffer (Swalla and Jeffery, 1990) containing 100 mM dithiothreitol (DTT). Coverslips were removed in 4× SSC with 10 mM DTT, and the slides were treated with 20 μg/ml pancreatic RNase A for 30 minutes at 37°C. Following RNase treatment, slides were washed at room temperature for 30 minutes with 2× SSC and then for 45 minutes with 1× SSC. Autoradiography was performed with NTB-2 emulsion (IBI-Kodak) and sections were stained through the emulsion with Harris hematoxylin and eosin (Humason, 1972). Background radioactivity was assessed from hybridizations carried out with sense RNA probes.

RESULTS

Isolation of *Uro* cDNA clones

Fig. 1 shows larvae of the urodele ascidian *M. oculata* and the anural ascidian *M. occulta*. The larva of the urodele species has a pigmented brain sensory cell and a tail, whereas the larva of the anural species lacks these features. A subtractive hybridization procedure was used to identify genes that are expressed preferentially in *M. oculata* oocytes, and thus are candidates for regulatory factors that initiate the changes leading to anural development. An *M. oculata* (urodele) gonad cDNA library was screened with a *M. oculata* subtracted cDNA probe and 24 positive clones were identified. Subsequent screening of the positive clones with an *M. occulta* (anural) gonad cDNA probe yielded 8 clones that were candidates for encoding differentially expressed genes. The PCR-amplified inserts of 7 of these clones were used to probe slot blots containing RNA synthesized from the urodele and anural gonad cDNA libraries, and 3 clones were identified that hybridized preferentially with *M. oculata* RNA (data not shown). These were designated *Uro* (urodele specific) clones because of their preferential expression in *M. oculata*, the urodele species. Here we describe two of these cDNA clones: *Uro-2* and *Uro-11*.

Identification of *Uro* transcripts

The *Uro-2* and *Uro-11* transcripts were identified by hybridizing northern blots containing RNA of the urodele and anural species with probes prepared from each insert. The *Uro-2* probe detected a single 1.8 kb mRNA (Fig. 2A; lane c) and the *Uro-11* probe a major 1.8 kb transcript and a minor 2.3 kb transcript (Fig. 2B; lane c) in gonad RNA from the urodele species. Neither the *Uro-2* nor *Uro-11*

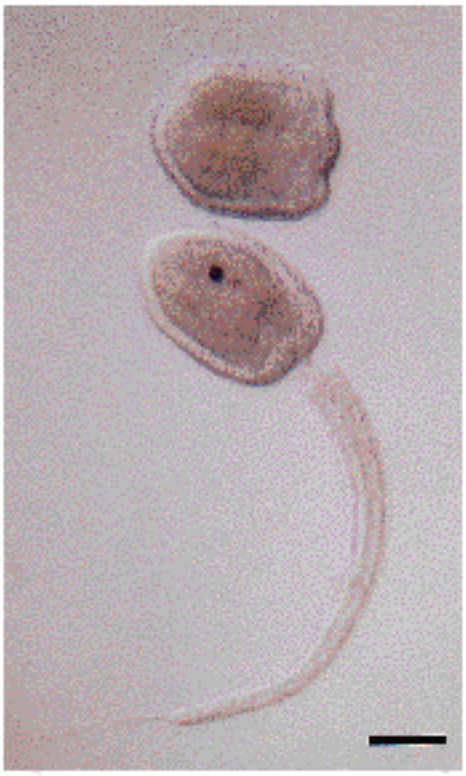


Fig. 1. A photomicrograph showing larvae of the anural ascidian *M. occulta* (top) and the urodele ascidian *M. oculata* (bottom). The anural larva lacks the neural sensory cell (melanin pigment spot) and the tail of the urodele larva. Scale bar is 50 μm.

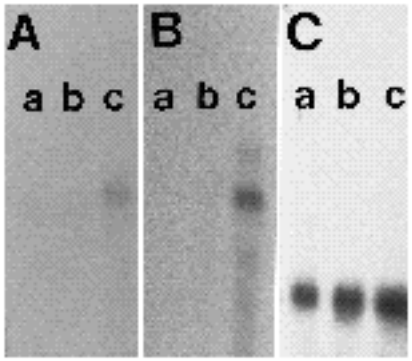


Fig. 2. Identification of *Uro* transcripts by northern blot hybridization. Each lane was loaded with 10 μg of total RNA isolated from *M. occulta* gonads (a), *M. occulta* 7.5 hour embryos (b), or *M. oculata* gonads (c) and hybridized with labeled RNA probes prepared from either *Uro-2* (A), *Uro-11* (B), or *MoccCA-4* (C) plasmids. The approximate size of the transcripts (see text) was determined by comparing the bands to RNA standards. Northern blots were washed first in 2× SET at 42°C and then at increasingly higher stringencies. The blots in this photograph were washed in 1× SET at 60°C. Exposure was 4 days in A,B and 1 day in C.

transcripts was detected in gonads or 7.5 hour embryos of the anural species (Fig. 2A, B; lanes a-b). Reducing the hybridization stringency to much lower levels did not result in detection of *Uro* mRNAs in gonads or zygotes of the anural species (data not shown). In addition, longer exposures of the blots shown in Fig. 2 failed to detect *Uro-2* transcripts in the anural species, although very weak 1.8 kb and 2.3 kb bands were sometimes observed in blots hybridized with the *Uro-11* probe. As a control, an identical northern blot was hybridized with a probe containing the coding region of an *M. oculata* cytoskeletal actin gene. The 1.4 kb cytoskeletal actin transcript was expressed in gonads of both species (Fig. 2C, lanes a, c), as well as in embryos of the anural species (Fig. 2C, lane b). Unfortunately, filter hybridization procedures could not be used to test for accumulation of zygotic *Uro* mRNAs in the urodele species because the embryo cultures also contain immature oocytes (see Materials and Methods; Fig. 6), which accumulate high levels of both maternal *Uro* transcripts. We conclude that the *Uro-2* and *Uro-11* clones encode transcripts that are preferentially expressed in gonads of the urodele species.

Uro genes are present in the urodele and anural species

The preferential accumulation of *Uro-2* and *Uro-11* transcripts in gonads of the urodele species could be caused by differential gene expression or by modification or deletion of the corresponding genes in the anural species. The presence of the *Uro-2* and *Uro-11* genes in the urodele and anural species was examined by Southern blot hybridization (Fig. 3). Only one or two labeled *M. oculata* DNA fragments were detected after hybridization with *Uro-2* (Fig. 3B) or *Uro-11* (Fig. 3D) inserts, suggesting that the corresponding genes are single copy in the urodele species. Hybridization was also observed in Southern blots containing genomic DNA fragments from the anural species (Fig. 3A,C). One or two labeled DNA fragments were observed in *M. occulta* DNA after hybridization with the *Uro-2* insert (Fig. 3A), suggesting that the *Uro-2* gene is also single copy in the anural species. However, two or more bands were detected in every lane in blots probed with the *Uro-11* insert (Fig. 3C), suggesting that multiple copies of *Uro-11*-related genes may be present in the anural species. The results suggest that homologous *Uro-2* and *Uro-11* genes may be present in the genome of the anural species.

Uro gene expression during development

Further information on the developmental expression of the *Uro* genes was obtained by in situ hybridization. Figs 4 and 5 show sectioned gonads of the urodele and anural species hybridized with *Uro-2* (Fig. 5A,B), *Uro-11* (Figs 4A,B, 5C, D) and actin (Figs 4C,D, 5E,F) RNA probes. The hermaphroditic gonads of each species contain sperm and oocytes at various stages of development. Consistent with the northern blot results (Fig. 2), *Uro-2* and *Uro-11* transcripts were present in gonads of the urodele species (Fig. 5A,C), but were undetectable (Fig. 5B) or present at very low levels (Fig. 5D) respectively in the anural species. In contrast, actin mRNA was observed in gonads of both species (Figs 4C,D, 5E,F). The *Uro-2* and *Uro-11* transcripts showed the same temporal and spatial expression patterns in gonads. Transcripts were observed in oocytes but not in accessory cells

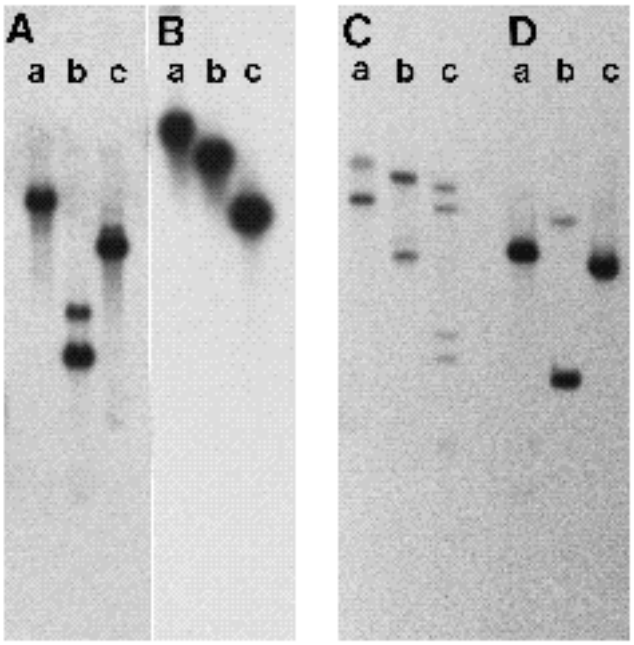


Fig. 3. Southern blots of *M. occulta* (A,C) and *M. oculata* (B,D) genomic DNA digested with *Bam*HI (a), *Eco*RI (b), or *Hin*dIII (c). 10 μg of digested DNA from each species was loaded in each lane of the gels and blots were hybridized with labelled *Uro-2* (A,B) or *Uro-11* (C,D) probes and washed in 2× SSC at 50°C. Exposure was 2 days for A,C and D and 1 day for B.

surrounding the oocytes (data not shown) or in developing spermatocytes within *M. oculata* gonads (Fig. 4A). The highest concentration of *Uro-2* and *Uro-11* transcripts was seen in small previtellogenic oocytes (Fig. 5A,C). The concentration of *Uro-2* and *Uro-11* mRNA gradually declined during vitellogenesis (Fig. 5A,C). A similar decline in actin transcripts was observed in vitellogenic oocytes of both species (Fig. 5E,F). The *Uro-2* and *Uro-11* transcripts were distributed uniformly in the cytoplasm of both previtellogenic and vitellogenic oocytes (Fig. 5A,C). The results are consistent with the northern analysis described above and confirm that maternal *Uro* mRNAs accumulate preferentially in oocytes of the urodele species.

Although the concentration of *Uro* transcripts declined during oogenesis, signal above background was still present in mature oocytes (Fig. 5A,C), implying that maternal mRNA may persist in eggs and embryos. The expression and distribution of *Uro* mRNA in eggs and embryos of the urodele species was examined by in situ hybridization. The results are shown in Fig. 6. In this figure, developing embryos are shown mixed with immature oocytes (see Materials and Methods), which served as internal controls. Maternal transcripts of both *Uro* genes were present in unfertilized eggs. After fertilization and ooplasmic segregation, the maternal *Uro-2* and *Uro-11* mRNAs were localized in the ectoplasm (Conklin, 1905) of uncleaved zygotes and distributed to ectodermal blastomeres in the animal hemisphere during cleavage (Fig. 6A-D). The maternal *Uro*-2

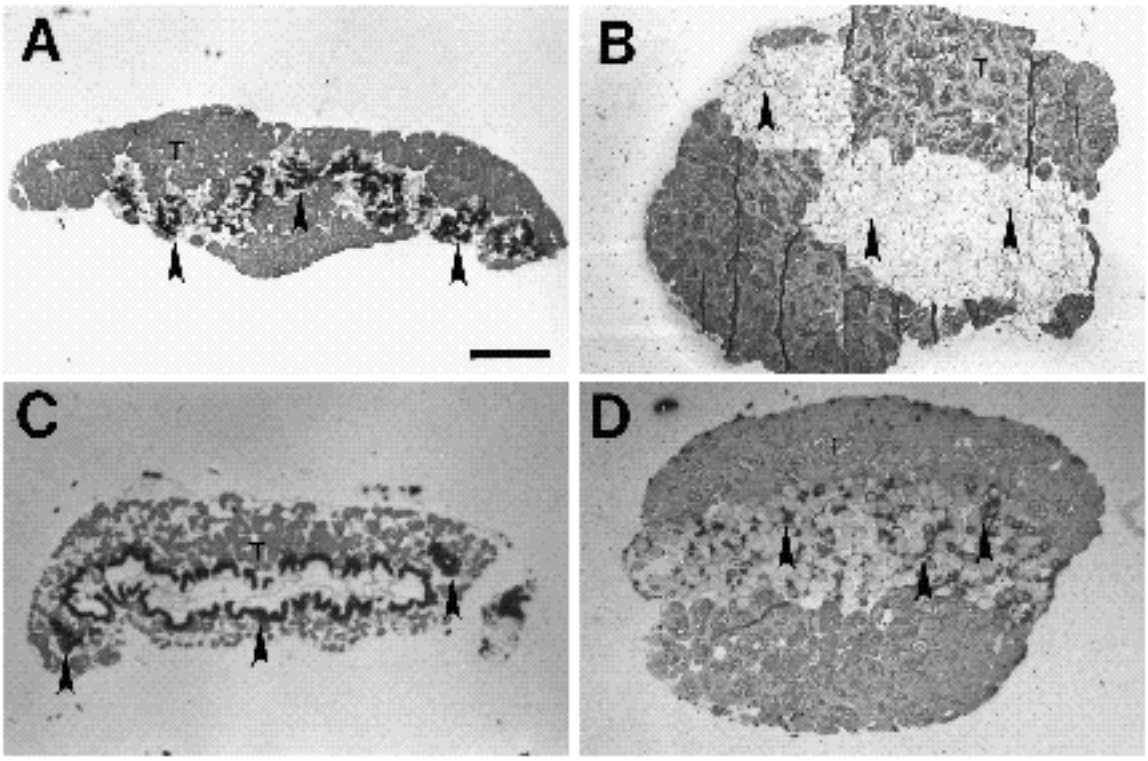


Fig. 4. Expression of *Uro-11* and actin transcripts in *M. occulta* and *M. oculata* gonads. Sections of gonads from each species were hybridized in situ with radioactive RNA probes and are shown here at low magnification. Sections of entire *M. oculata* (A,C) and *M. occulta* (B,D) gonads hybridized with *Uro-11* (A,B) or *MocuCA-4* (C,D) RNA. In A-B, *Uro-11* transcripts are expressed in small oocytes of the urodele (A) but not the anural (B) species (arrowheads) and are absent from the testes (T) of both species. In C-D, actin transcripts are present in oocytes (arrowheads) of both species. Scale bar in A represents 500 μm; magnification is the same in A-D.

transcripts could no longer be detected by the late cleavage stages, and did not accumulate to levels above background during the remainder of embryogenesis (Fig. 6E,G,I). Thus, expression of the *Uro-2* gene appears to be strictly maternal. In contrast, *Uro-11* transcripts were detectable through the neurula stage (Fig. 6F,H), but could not be observed at later stages of embryogenesis (Fig. 6J). In other ascidian species, maternal transcripts disappear during cleavage and embryonic transcription begins by the early gastrula stage (Beach and Jeffery, 1990, 1992; Kusakabe et al., 1991). Therefore, it is likely that the *Uro-11* gene shows both maternal and zygotic expression. The putative zygotic *Uro-11* transcripts accumulated primarily in the posterior epidermis, the developing nervous system (neural folds and tube) and the presumptive myoblasts (Fig. 6D,F,H). However, zygotic *Uro-2* and *Uro-11* transcripts were not detected in embryos of the anural species (data not shown; Fig. 2B, lane b). The results suggest that maternal *Uro-2* and *Uro-11* transcripts are localized in the zygote and segregated to ectodermal cells during cleavage. Afterwards, expression of the *Uro* genes appears to diverge: the *Uro-2* gene appears to be zygotically inactive, whereas the *Uro-11* gene may produce zygotic transcripts in posterior tissues of gastrulae and neurulae.

The predicted *Uro-2* protein contains a leucine zipper motif

The nucleotide and predicted amino acid sequences of the *Uro-2* clone are shown in Fig. 7A. The cDNA clone contains a single opening reading frame of 1092 nucleotides which

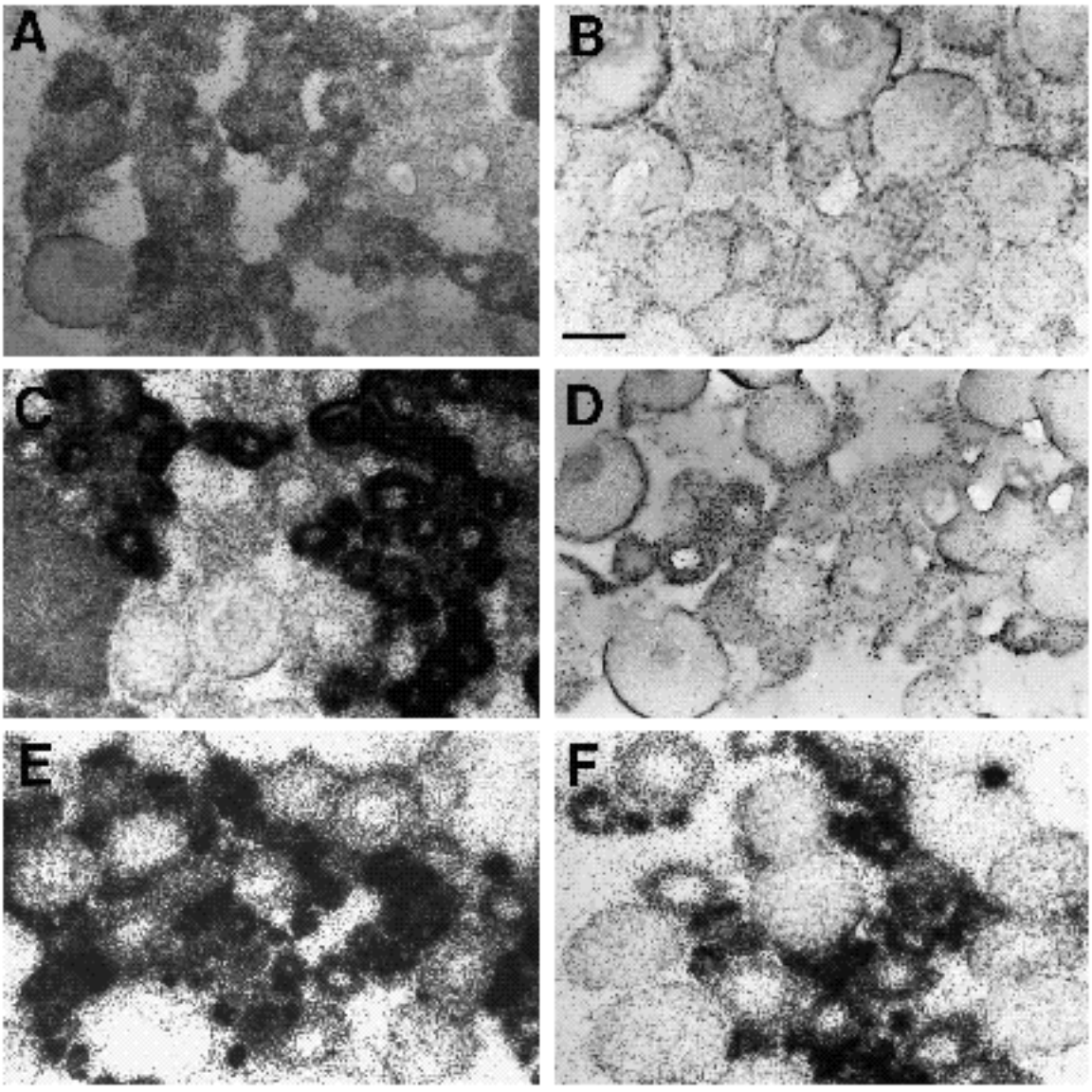
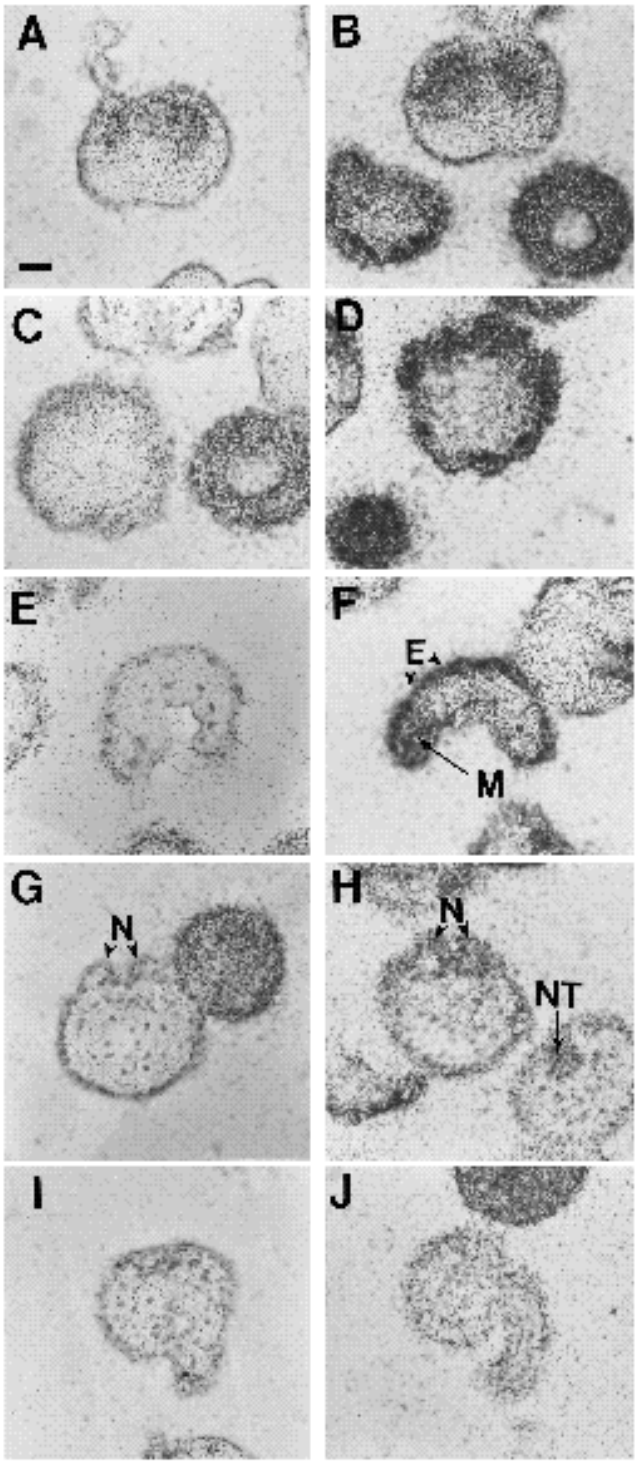


Fig. 5. Expression of *Uro-2, Uro-11* and actin transcripts in developing oocytes in *M. oculata* (A,C,E) and *M. occulta* (B,D,F) gonads hybridized in situ with *Uro-2* (A,B), *Uro-11* (C,D) or *MocuCA-4* (E,F) RNA probes. The *Uro-2* and *Uro-11* transcripts are distributed throughout the cytoplasm of small previtellogenic oocytes in the urodele (A,C) but not the anural (B,D) species, and gradually decrease in concentration in larger vitellogenic oocytes. The actin transcripts are present in previtellogenic oocytes of both species (E,F). Scale bar in E represents 50 μm; magnification is the same in A-F.

is flanked by a 5′ leader of 61 nucleotides and a 3′ untranslated region of 684 nucleotides. The first ATG triplet is in the context most closely resembling the consensus eukaryotic translational initiation site (Kozak, 1991), and the 3′ non-coding region contains a putative polyadenylation signal. The size of the *Uro-2* cDNA clone (1836 bp) is consistent with the 1.8 kb *Uro-2* transcript identified in northern blots (Fig. 2A, lane c).

The *Uro-2* nucleotide sequence predicts a basic protein (pI 10.0) of 364 amino acids with a calculated molecular mass of 42 kD (Genbank Accession no. L19340). No significant matches were revealed between the Uro-2 amino acid sequence and proteins represented in the protein databases. The Uro-2 protein contains a tyrosine kinase phosphorylation motif (KKIDPHSY) and a region containing mostly hydrophobic amino acids (Fig. 7A). The hydrophobic region lacks charged amino acids and is extensive enough to be a transmembrane domain, although a conventional signal sequence (von Heijne, 1986) is not present at the N terminus (Fig. 7B). The most notable feature of the Uro-2 protein is a heptad repeat of four leucine residues in the C-terminal region (Fig. 7A). This region may be a leucine zipper, the dimerization motif present in certain transcription factors (Landschultz et al., 1988) and integral membrane proteins (Buckland and Wild, 1989; McCormack et al., 1989; White and Weber, 1989; Inoue et al., 1991). Although a short basic region precedes the putative leucine zipper in the Uro-2 protein, the sequence lacks the conserved basic domain typical of the bZIP family of transcription factors. Thus, the *Uro-2* gene may encode a novel leucine zipper protein.



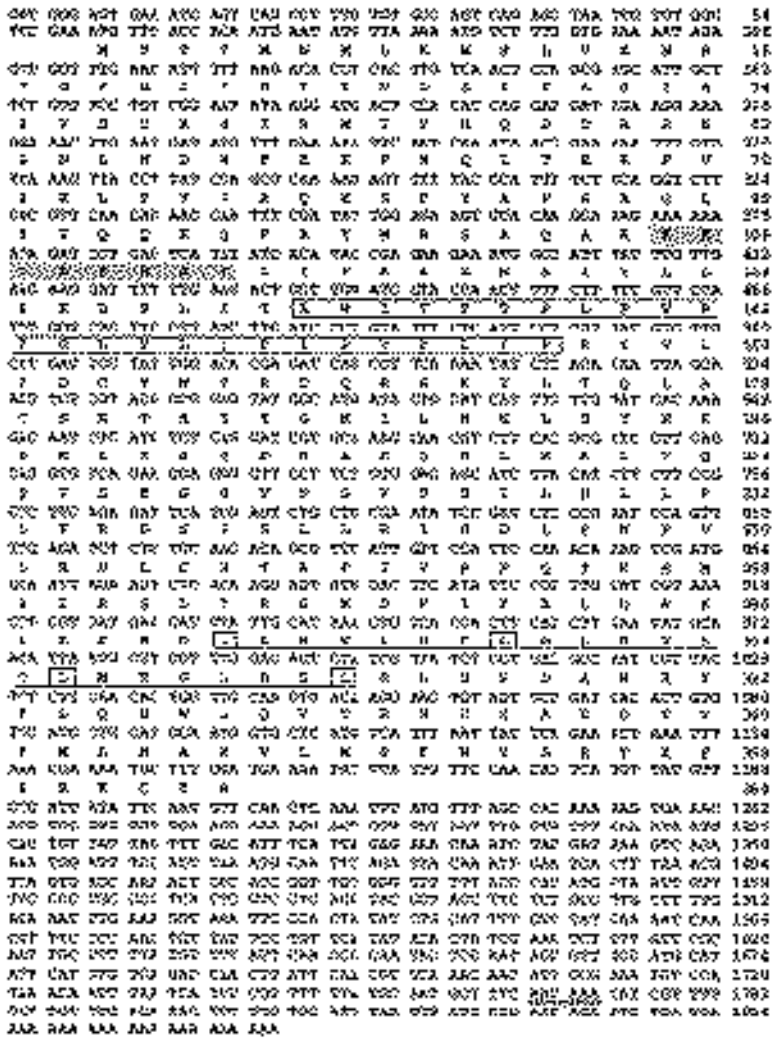
The predicted *Uro-11* protein contains DNA-binding motifs

The *Uro-11* nucleotide and predicted amino acid sequences are shown in Fig. 8. The cDNA clone contains an open reading frame of 1674 nucleotides, a 5′ leader of 428 nucleotides, and a 3′ untranslated region of 192 nucleotides (GenBank Accession no. L19339). The first ATG is in the most reasonable context for translational initiation (Kozak, 1991) and a polyadenylation signal is present in the 3′ untranslated region. The inferred *Uro-11* mRNA (2294 nucleotides) is sufficient to encode either the 1.8 or 2.3 kb transcripts identified by northern analysis (Fig. 2B, lane c).

The *Uro-11* sequence predicts a basic protein (pI 10.0) of 558 amino acids with a calculated molecular mass of 62 kD.

Fig. 6. Expression of *Uro-2* and *Uro-11* transcripts in *M. oculata* embryos hybridized in situ with *Uro-2* (A,C,E,G,I) and *Uro-11* (B,D,F,H,J) RNA probes. (A,B) Sections of 2-cell embryos showing *Uro-2* and *Uro-11* transcripts localized in the ectoplasm. The animal pole is at the top in both figures. In B, the 2-cell embryo is flanked by a labeled immature oocyte (lower right). (C,D) Sections through the animal hemisphere and parallel to the anteroposterior axis (top to bottom) of 32-64 cell embryos showing *Uro-2* and *Uro-11* transcripts in ectoderm cells. In C, the concentration of *Uro-2* mRNA has decreased relative to A and the adjacent (right) labeled immature oocyte. In D, the concentration of *Uro-11* mRNA has increased relative to B, suggesting that zygotic *Uro-11* transcripts have started to accumulate. (E,F) Parasagital sections through the anteroposterior axis (right to left) of mid-gastrulae. In E, *Uro-2* transcripts are undetectable at the gastrula stage. In contrast, in F, *Uro-11* transcripts are located in the posterior epidermis (E) and presumptive muscle (M) cells of a gastrula. (G,H) Cross-sections through the dorsoventral axis (top to bottom) of neurulae showing lack of *Uro-2* transcripts (G) and *Uro-11* transcript accumulation in the neural folds (N) and neural tube in two neurulae (H). In G, there is a labeled immature oocyte at the upper right side of the neurula. (I,J) Parasagittal sections through early tailbud stage embryos showing lack of *Uro-2* (I) and *Uro-11* (J) transcripts. Scale bar in A represents 20 μm; magnification is the same in each frame.

A



B

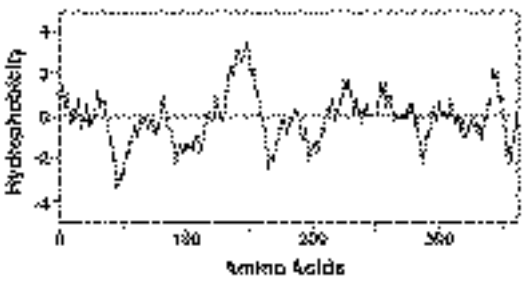


Fig. 7. (A) The nucleotide and predicted amino acid sequence of *Uro-2*. The putative tyrosinse kinase phosphorylation site is shaded, the hydrophobic domain is boxed, the leucine zipper motif is indicated by a single underline with boxed heptad leucine residues, and the polyadenylation signal is indicated by a double underline. GenBank Accession no. L19340. (B) Hydrophobicity plot (Kyte and Doolittle, 1982) of the predicted Uro-2 protein showing the central hydrophobic domain. The window size is 11.

No significant similarity was revealed by comparing the Uro-11 amino acid sequence to proteins in the protein databases. The Uro-11 protein contains a putative nuclear localization signal (KKKTK; Kalderon et al., 1984) and several potential DNA-binding motifs (Fig. 8). First, an N-terminal region of the protein matches the consensus of a predicted DNA-binding motif (Fig. 9) present in the bacterial histone-like proteins HU (Goshima et al., 1990) and IHF (Nash, 1990). HU and IHF are small proteins that bind to DNA as dimers via an extended arm structure consisting of a two-stranded antiparallel β ribbon (reviewed in Dijk and Reinhardt, 1986). The turns (GFG and PXT) separating the strands of the β ribbons as well as critical basic and hydrophobic residues in the N-terminal strand are conserved in the Uro-11 protein. Second, the Uro-11 protein contains 12 repeats of S(T)PXX, a proposed DNA-binding motif in certain transcription factors (Suzuki, 1989) and histone H2B (Lindsey and Thompson, 1992) (Fig. 8). Finally, the C-terminal region of the Uro-11 protein contains a potential zinc finger (or cluster; Vallee et al., 1991) of the form $CX_2CX_{12}CX_2CX_4CX_3C$ (Fig. 10). Although the number of cysteine residues would place the Uro-11 protein in the C_6 family of zinc finger proteins (Evans and Hollenberg, 1988), the cysteine positions are not conserved relative to the C_6 fungal transcription factors (Johnston, 1987). Histidine residues are present in the cysteine rich region of the Uro-11 protein that could also be involved in coordination of zinc moieties. In this way, Uro-11 resembles some of the C_6H_2 B box-containing zinc finger proteins (Reddy et al., 1992). Thus, the *Uro-11* gene may encode a DNA-binding protein.

DISCUSSION

Previous studies have shown that changes in maternal and zygotic factors are involved in the evolution of alternate modes of development in ascidians (reviewed in Jeffery and Swalla, 1992a). Here we have identified and characterized candidates for regulatory genes governing the transition from urodele to anural development. Our approach for isolating the *Uro* clones was based on the existence of two closely related ascidian species exhibiting urodele and anural development. The *Uro* clones were isolated from a

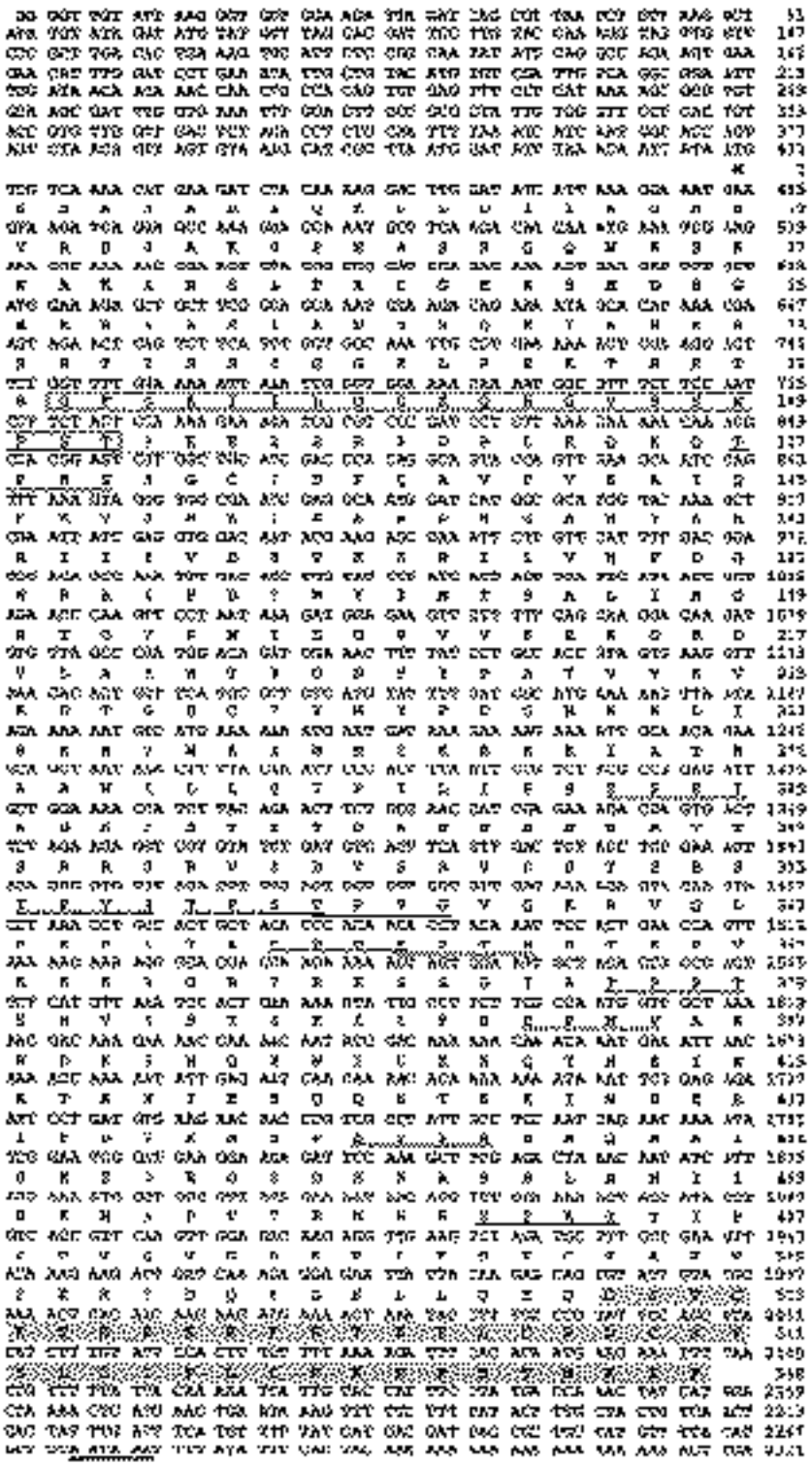


Fig. 8. The nucleotide and predicted amino acid sequence of *Uro-11*. The region with homology to part of the DNA-binding motif of the bacterial HU and IHF proteins is boxed, the cysteine-rich region containing a potential zinc finger is indicated by shading, the S(T)PXX repeats are indicated by a single (sometimes overlapping) underline and the polyadenylation signal is indicated by a double underline. The nuclear localization signal KKKTK (Kalderon et al., 1984) begins at residue 528 within the shaded region. GenBank Accession no. L19339

gonad cDNA library of the urodele species, *M. oculata*, after subtraction with a gonad cDNA library from the anural species, *M. occulta*. The *M. oculata* cDNA library was screened with a subtracted probe enriched in urodele-specific sequences to isolate clones encoding genes expressed preferentially in the urodele species. In this investigation, we have described two of these clones: *Uro-2* and *Uro-11*.

The temporal and spatial expression patterns of *Uro-2* and *Uro-11* genes are identical during oogenesis of the urodele species. Transcripts of each gene accumulate to their highest levels in previtellogenic oocytes and gradually decrease in concentration during vitellogenesis. The expression of *Uro-2* and *Uro-11* mRNA during oogenesis is similar to that of actin transcripts in the urodele and anural species and may be characteristic of messages synthesized in previtellogenic

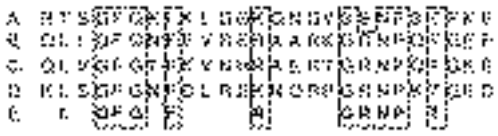


Fig. 9. Comparison of the Uro-11 protein to part of the DNA-binding region in the bacterial HU and IHF proteins. Amino acids 90-121 of Uro-11 sequence (A) are compared to part of the DNA-binding domain of the *B. stearothermophilus* HBs (B), *E. coli* NS-2α (C) and *E. coli* IHFα (D) proteins. (E) The consensus amino acids in part of the DNA-binding motif of the bacterial HU and IHF proteins are shown in boldface. Boxed residues indicate identical and conserved substitutions in the protein sequences. Bacterial sequences are from Dijk and Reinhardt (1986) and Miller (1984).

oocytes. A single *Uro-2* transcript was detected on northern blots, but two distinct *Uro-11* transcripts were present. The size of the *Uro-11* opening reading frame is consistent with expression of either transcript, and further experiments will be necessary to determine the relationship between the *Uro-11* transcripts and how they may be generated during RNA transcription or processing.

Ascidian eggs contain distinct cytoplasmic localizations (Conklin, 1905) and there is previous evidence that a subtype of actin mRNA is localized in the myoplasm (Jeffery et al., 1983), an egg cytoplasmic region inherited by the larval tail muscle cells. Although the maternal *Uro-2* and *Uro-11* transcripts are uniformly distributed in the oocyte cytoplasm throughout oogenesis, they become localized in the ectoplasm after fertilization and are segregated to the ectodermal cells during cleavage. This pattern of localization and segregation is similar to that of a maternal cytoskeletal actin mRNA in the ascidian *Styela clava* (Beach and Jeffery, 1990). The maternal *Uro-2* mRNA disappeared during cleavage and no further accumulation was detected during embryogenesis, indicating that this *Uro* gene shows exclusively maternal expression. In contrast, the *Uro-11* gene is present in embryos of the urodele species during gastrulation and neurulation. This suggests that there is also a zygotic expression period of the *Uro-11* gene. The accumulation of *Uro-11* transcripts in the posterior epidermis, the neural cells and the presumptive muscle cells suggests a possible function for this gene during urodele development.

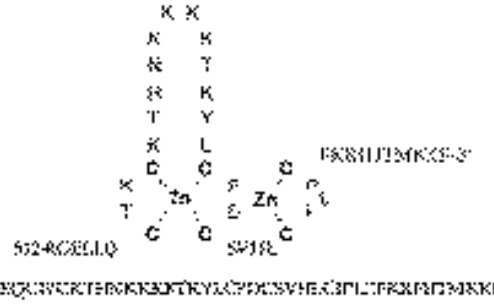


Fig. 10. The proposed structure of a C_6 zinc finger in the Uro-11 protein. This structure assumes that the two histidine residues in the cysteine-rich region of the protein do not serve as zinc ligands.

After gastrulation, the larval tail is extended by concerted morphogenetic movements and differentiation of cells in the posterior region of the embryo. Interference with this process could lead to anural development. Thus, the temporal and spatial expression pattern of the *Uro-11* gene in some of the posterior embryonic cells is consistent with a role in tail formation in the urodele species.

Although *Uro* transcripts accumulate preferentially in the urodele species, the *Uro 2* and *Uro-11* genes are present in the genomes of both *M. oculata* and *M. occulta*. Therefore, the simplest explanation for lack of *Uro* transcripts in the anural species is that gene expression has been suppressed. The longer exposure times required to distinguish the *Uro-2* bands in Southern blots containing DNA from the anural species suggests that the single copy *Uro-2* gene has diverged in the anural species. An extreme possibility is that the *Uro-2* sequence detected in the genome of the anural species by Southern analysis represents hybridization with a pseudogene. In addition, the increased number of bands detected in genomic DNA of the anural species suggests that the *Uro-11* gene may have duplicated or undergone a chromosomal rearrangement. It will be interesting to investigate the sequence divergence and organization of the *Uro-2* and *Uro-11* genes in the urodele and anural species.

The *Uro-2* gene appears to encode a novel protein containing a leucine zipper motif. Leucine zippers promote protein-protein interactions in certain dimeric transcription factors (Landschultz et al., 1988) and channel-forming, integral membrane proteins (McCormack et al., 1989; Buckland and Wild, 1989; White and Weber, 1989; Inoue et al., 1991). Although a basic region precedes the leucine zipper in the Uro-2 protein, it is not as extensive as the conserved basic domain characteristic of the bZIP family of transcription factors. The presence of a potential membrane spanning domain in the Uro-2 protein brings up the alternate possibility that it may represent a leucine-zipper transmembrane protein. It should be pointed out, however, that the hydrophobic region and leucine zipper motif are widely separated in the Uro-2 protein, whereas these regions are adjacent in the leucine zipper transmembrane proteins. If Uro-2 is a transmembrane protein, its absence in eggs of the anural species could lead to structural changes in the myoplasm, a unique cytoskeletal domain linked to the egg plasma membrane (Jeffery and Meier, 1983). It has been shown previously that the organization of the myoplasm is modified in the eggs of anural ascidian species (Whittaker, 1979b; Swalla et al., 1991; Jeffery and Swalla, 1992b). Direct information on localization of the Uro-2 protein using antibodies will be necessary to determine whether it is a nuclear protein or a component of the egg plasma membrane.

The *Uro-11* gene appears to encode a novel protein containing a potential nuclear localization signal and several different DNA-binding motifs. A region in the N-termimal portion of the predicted protein shares a part of the consensus of the DNA-binding arm in the bacterial histone-like HU and IHF proteins (Dijk and Reinhardt, 1986). Structural and biochemical studies suggest that the HU and IHF proteins interact with DNA via a two-stranded β-ribbon (Tanaka et al., 1984; Yang and Nash, 1989). Although Uro-11 shows homology only to the N-terminal half of the HU-IHF β-ribbon, this is precisely the region known to be

important for DNA binding (Goshima et al., 1992). This is the first report of a bacterial histone-like DNA-binding motif in a eukaryotic protein, although similarities between the DNA-binding domains of the IHF protein and transcription factor TFIID have previously been recognized (Nash and Granston, 1991). Recent structural analysis of the TFIID protein indicates that its DNA-binding site forms a multi-stranded antiparallel β-sheet, which resembles a molecular 'saddle' sitting astride the DNA helix (Nicolov et al., 1992).

The Uro-11 protein also contains a potential C_6 zinc finger in its C-terminal region. Although this structure has 6 cysteine (and 2 histidine) residues, which could form a coordinated finger or cluster with 2 zinc moieties (Vallee et al., 1991), the cysteine positions are not conserved relative to known members of the C_6 zinc fingers, such as the GAL4 fungal transcription factor (Johnston et al., 1987), or the B box class of zinc fingers, some of which contain 6 cysteine and 2 histidine metal-binding ligands (Reddy et al., 1992). Therefore, the Uro-11 protein could represent a new subclass of zinc finger proteins in the C_6 or C_6H_2 family. Twelve repeats of the motif S(T)PXX are positioned between the regions of HU-IHF similarity and the potential zinc finger in the Uro-11 protein. The S(T)PXX motif has been proposed to form β turn I structures that interact with the minor groove of the DNA helix (Suzuki, 1989). S(T)PXX repeats are frequently observed in transcription factors with zinc finger DNA-binding motifs, such as the *Drosophila krüppel* protein and the steriod hormone receptor (Suzuki, 1989), or in other DNA-binding proteins, such as histone H2B (Lindsey and Thompson, 1992), but are much less common in general proteins. Although further studies will be necessary to determine whether Uro-11 is a DNA-binding protein, it is interesting to note that this clone was identified in a library screen for mRNAs that are down regulated during development of the anural species. This result is consistent with the possibility that anural development is mediated by eliminating the expression of a transcription factor(s).

In summary, we have described potential genes that are expressed during the ontogeny of an ascidian with urodele development but not in a closely related species with anural development. Since our library screening was not exhaustive, it is possible that other genes showing this pattern of expression remain to be identified. Indeed, we have recently determined that another *Uro* clone (*Uro-1*) encodes a putative tyrosine kinase (Makabe et al., unpublished data). Interestingly, the Uro-2 and Uro-11 proteins contain potential phosphorylation sites, raising the possibility that they function in a signal transduction pathway downstream from the Uro-1 tyrosine kinase.

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