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CANADA

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Summary

We have cloned and characterized a *Xenopus* member of the *CAP* (*cyclase associated protein*) gene family. *xCAP1* is expressed as a maternal transcript, but is up-regulated prior to gastrulation and subsequently localizes to head mesenchyme, lens, otic vesicle, and trunk

mesoderm including the pronephros. At different stages, the gene also appears to differentiate surface from deep (sensorial) ectoderm. As in *Drosophil*a, *Xenopu*s *CAP1* is expressed in the developing eye, specifically in the differentiating lens. However in distinction to *Drosophila, Xenopus CAP1* does not express in periodically arrayed neural bands.

1. Results and Discussion

The genes that encode cyclase-associated proteins (CAP) are conserved across organisms as divergent as plants, yeast, worms, flies, and mammals (Baum et al., 2000; Benlali et al., 2000; Fedor-Chaiken et al., 1990; Field et al., 1990; Gottwald et al., 1996; Kawai et al., 1998; Matviw et al., 1992; Swiston et al., 1995; Vojtek and Cooper, 1993). CAPs are monomeric actin sequestering proteins that are thought to play a pivotal integrative role in linking cytoskeletal modifications with signal transduction pathways. Recently, a *CAP* homolog, *act up*, was shown to be necessary for normal oocyte polarity and imaginal eye furrow formation and differentiation in *Drosophila* (Baum et al., 2000; Benlali et al., 2000).

We have isolated a *Xenopus* clone, *xCAP1,* which possess the entire conceptual open reading frame, and which would encode a protein with 80%, 79%, 79%, 54% amino acid identity to human, mouse, rat, and *Drosphila* CAP1 respectively. RT-PCR reveals that *xCAP1* is detectable at low levels as a maternal transcript, but subsequently expresses at higher levels during blastula and later stages (Fig. 1). Whole-mount riboprobe *in situ* hybridization reveals the progressive restriction of *xCAP* to the animal pole with a presumptive dorsal bias (Fig. 2A, B). This bias, though hard to detect in early stages, is absent in sense controls, and is not seen when we probe with other faint maternally expressed probes such as *Pitx1* and *Pitx3*. Dorsal restriction ensues (Fig. 2C, D), followed by expression in developing head (Fig. 2E,F,G,H). Ectoderm layers express in a differentially dynamic manner, with expression eventually restricting to the sensorial (deep) ectoderm (Fig.3A-D). *xCAP1* appears to differentiate between surface and sensorial ectoderm earlier than previously recognized (Hausen and Riebesell, 1991) (Fig. 3A,D), and the period during which both layers express correlates well with the period when sensorial ectoderm cells migrate up into the surface ectoderm to give rise to ciliated cells through to late neurulation (Fig. 3B,C). During gastrulation and early neurulation (Fig. 2C,D) *xCAP1* is expressed in antero-dorsal mesoderm, and later in branchial arch and optic mesenchyme, lens vesicle, otic vesicle, and lateral mesoderm, but not endoderm (Fig. 2 E-H, Fig. 3). By stage 37, *xCAP1* is expressed in the pronephros, rhombencephalon, and persists in the branchial arches, periocular mesenchyme, and lens through to late organogenesis. Sense controls were consistently clear of staining.

In *Drosophila*, CAP-modulated actin polymerization plays a fundamental role in eye differentiation (Benlali et al., 2000). Vertebrate eye development is also a multi-step process that requires specific inductive signals, morphogenetic movements, and dramatic cytoskeletal rearrangements (Jean et al., 1998). When lens ectoderm invaginates to form lens vesicles, the posterior lens epithelium cells lose their nuclei and elongate to produce primary lens fibers which then synthesize lens-specific proteins such as the crystallins. The *xCAP* – expressing anterior lens epithelial cells are fated to proliferate in the equatorial region of the lens and give rise to secondary lens fiber cells which eventually elongate and form lamellae surrounding the embryonic nucleus. The role of *xCAP1* in this differentiation is currently being investigated.

2. Materials and Methods

2.1 Cloning. A partial fragment of *xCAP1* was obtained using degenerate primers. The fragment was then subcloned and used to screen a *Xenopus* head and heart cDNA library (stages 28-35) which was constructed in commercially prepared vector (Stratagene). Dye terminator and dye primer chemistries were employed to bi-directionally cycle-sequence the largest obtained clone (2212 bp) which encompassed a complete open reading frame encoding a conceptual protein similar to CAP1 found in other species (*xCAP1* accession #AF411959). Amino acids sequences were compared using the Clustal method.

2.2 Embryos and in situ hybridization. Embryos were fertilized, dejellied in 2% cysteine, cultured and staged as previously described (Drysdale and Elinson, 1991; Nieuwkoop and Faber, 1967). Wholemount in situ hybridizations were performed according to Harland (1991). Digoxygenein labeled sense and antisense riboprobes for *CAP* were generated from full-length linearized template. Dorsoventral dispositions of early cleavage stage blastomeres were identified and followed using regular furrow and colour determinants according Sive et al., (2000) and in situ hybridizations were thrice repeated in embryos derived from different egg clutches, and using different batches of riboprobe.

2.3 RT-PCR Total RNA from ten pooled embryos of each developmental stage was passed over oligo dT-polystyrene beads (Sigma DMN-10). mRNA equivalent to one embryo was withdrawn and reverse transcribed in the presence on RNasin (Promega) using reverse transcriptase (Omniscript, Qiagen). One fifth volume of this reaction was employed as template for amplification. PCR conditions were determined empirically to establish the

linear range of amplification for *xCAP1* using a thermo-stable polymerase in 10mM Tris (pH 9.0), 50mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1mM [^{32}P]dCTP, and 1 ug of each primer (*CAP1* – CCACATCCTCAGAGATGAA and GGCTCTATACCCTTTATTAC; *EF1-α* – CAGATTGGTGCTGGATATG and ACTGCCTTGATGACTCCTA; ODC – GTCAATGATGGAGTGTATG and TCCATTCCGCTCTCCTGA). Following denaturation (3 minutes at 94^{o}C), ODC and *EF1-α* were cycled 29x(94^{o}C for 45^{o}C seconds; 57^{o}C, and 74^{o}C for 45 seconds each). *xCAP1* assays were denatured (94^{o}C for two minutes) and cycled 23x(94^{o}C then 55^{o}C for 45 seconds each; 72^{o}C for 30 seconds). One tenth of each reaction was run out on 4% polyacrylamide in 0.5 x TBE, and then monitored by autoradiography.

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Figure Legends

Fig. 1. Temporal expression of *xCAP1* analyzed by RT-PCR. Although *xCAP1* mRNA is discernable at the limit of detectability as a maternal transcript, its expression is progressively up-regulated from blastula through to neurula stages. Ornithine decarboxylase (ODC) and elongation factor 1 alpha are provided as controls.

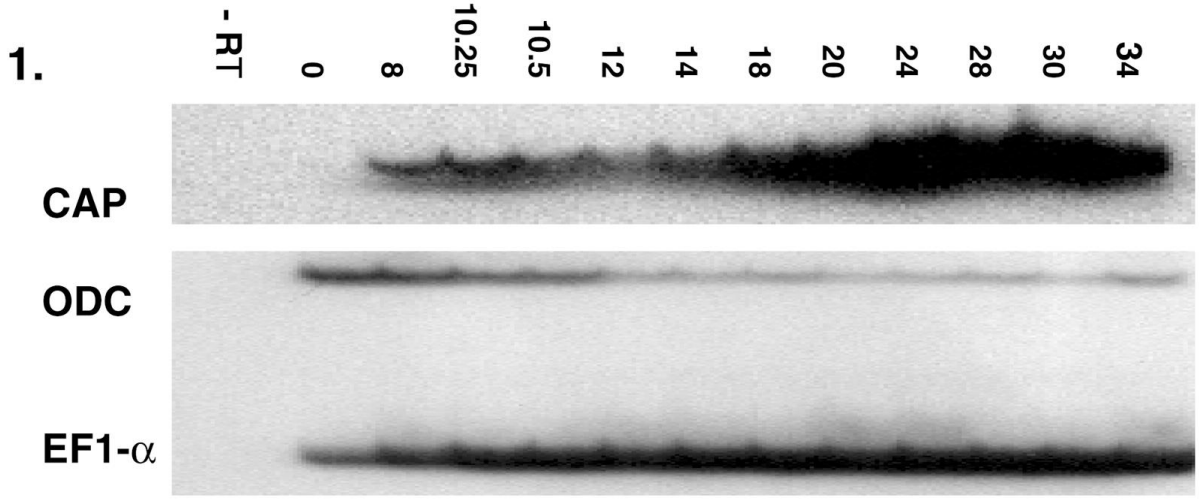

Fig. 2. *xCAP1* expression during embryogenesis. Maternal *xCAP1* transcript becomes localized to the animal pole during the early stages of cleavage (fig. A), and progressively concentrates on the presumptive dorsal (d) side (figs. A, B). As the ectoderm thickens, surface ectoderm down-regulates *xCAP1* at the extreme animal pole, while expression ensues in sensorial ectoderm, marginal zone and dorsal mesoderm during gastrulation, so that cells immediately above and passing through the dorsal lip (dl) express (fig. C). Late in gastrulation, presumptive neurectoderm is devoid of *xCAP1* expression anteriorly (not shown), but by mid neurulation, the gene expresses in neurectoderm up until the neural folds have sutured. Both layers of dorsal ectoderm express during neurulation, as well as dorsal mesoderm and neurectoderm. Dorsal expression extends in a comparable pattern from the yolk plug to the anterior end (a), but is absent from endoderm from neural plate (stage 12) through to neural tube suturing (in this dorsal view of a stage 15.5 embryo)(fig. D). During elaboration of the head, *xCAP1* is expressed in the branchial arches (ba), otic vesicle (o), lens (l), and peri-optic mesenchyme (pom) (figs. E, F). Branchial arch expression is predominantly mesenchymal. By stages 36 to 37, olfactory placodes (op) and pronephric structures (pn) express transcript (figs. G, H).

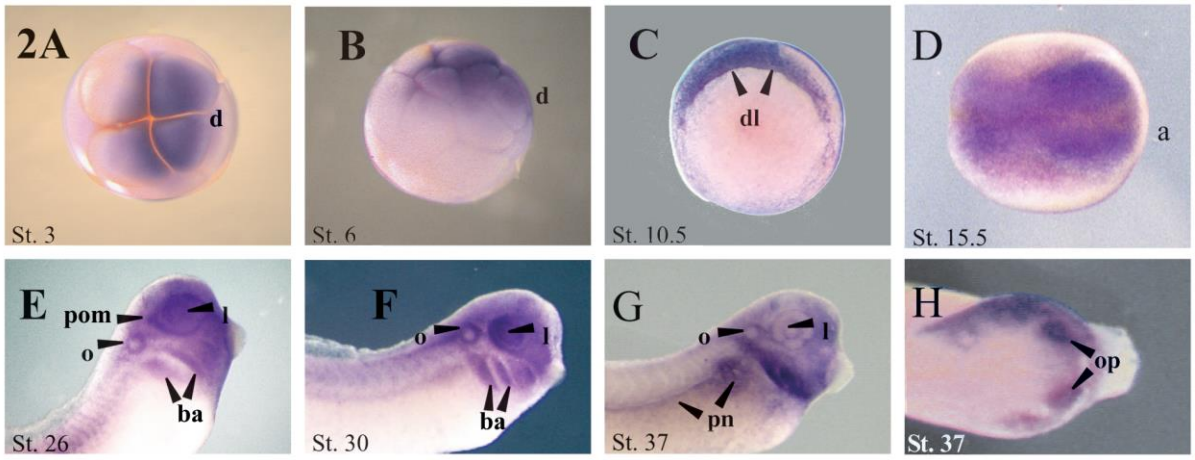

FIG 3. *xCAP1* expression revealed in section. Animal pole (an) and surface ectoderm (se) do not express *xCAP1* during gastrulation, though both mesoderm (m) and sensorial ectoderm (sn) do (fig. A). Cells passing through the dorsal lip (dl) appear to temporarily down-regulate *xCAP1* indicating that expression patterns of this transcript can be rapidly altered. By late neurulation (fig B), both layers of ectoderm express transcript, but expression in surface ectoderm begins to diminish, while sensorial ectoderm (sn) and, to a lesser extent) lateral plate mesoderm (lpm) continue to express. Neither notochord (n) nor somites (s) express *xCAP1,* however a low level of expression can be seen in the lateral ventral aspect of the neural tube (nt). During tail bud stages (fig.C), transcript is detectable in the ventral rhombencephalon (r), the otic vesicle (o), and the lateral plate mesoderm (lpm), and expression in surface ectoderm has down-regulated. When eye begins to form, *xCAP1* expression is detectable in the lens, particularly at the margins (fig.D). By this stage *xCAP1* expression is entirely lacking in the surface ectoderm.