

A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a *nanos*-like zinc finger domain

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SUMMARY

mRNAs concentrated in specific regions of the oocyte have been found to encode determinants that specify cell fate. We show that an intermediate filament fraction isolated from *Xenopus* stage VI oocytes specifically contains, in addition to Vg1 RNA, a new localized mRNA, Xcat-2. Like Vg1, Xcat-2 is found in the vegetal cortical region, is inherited by the vegetal blastomeres during development, and is degraded very early in development. Sequence analysis suggests that Xcat-2 encodes a protein that belongs to the CCHC RNA-binding family of zinc finger proteins. Interestingly, the closest known

relative to Xcat-2 in this family is *nanos*, an RNA localized to the posterior pole of the *Drosophila* oocyte whose protein product suppresses the translation of the transcription factor *hunchback*. The localized and maternally restricted expression of Xcat-2 RNA suggests a role for its protein in setting up regional differences in gene expression that occur early in development.

Key words: *Xenopus laevis*, localized maternal RNA, cortical cytoskeleton, zinc finger, oogenesis, *nanos*

INTRODUCTION

For more than a century, embryologists have known that the specification of cell type in the early embryo could be traced back to specific regions in the egg. These regions of the egg were believed to contain determinants of cell fate that became disproportionately segregated into blastomeres during cleavage (Davidson, 1990). However, until recently, the molecular nature of these determinants remained a mystery. There is now compelling evidence that in some cases regionalized determinants are localized mRNAs whose synthetic products act to alter gene activity (Lehmann and Nusslein-Volhard, 1986; Ephrussi et al., 1991; Wang and Lehmann, 1991; Cheung et al., 1992; Steward, 1989; Weeks and Melton, 1987). In a *Xenopus* or *Drosophila* egg, there are probably less than 20 mRNAs identified as localized to pole regions (Carpenter and Klein, 1982; King and Barklis, 1985; Rebagliati et al., 1985; Ephrussi et al., 1991; St. Johnston and Nusslein-Volhard, 1992). These transcripts have been implicated in early decisions involving anterior/posterior polarity (Lipshitz, 1991; Melton, 1991; St. Johnston and Nusslein-Volhard, 1992), dorsoventral patterning (Cheung et al., 1992) and the determination of the germ cell lineage (Ephrussi and Lehmann, 1992; Ephrussi et al., 1991). Decisions about body patterning are frequently governed by determinants that are expressed in a gradient (Macdonald and Struhl, 1986; Driever and Nusslein-Volhard, 1988; Smith et al., 1990).

Mutational analysis in *Drosophila* has identified at least 13 genetic loci involved in the establishment of two organizing centers, one at either end of the egg, which specify anteroposterior pattern (Nusslein-Volhard et al., 1987; Frohnhof and Nusslein-Volhard, 1986). The anterior pole is defined by a mRNA, *bicoid*, localized to the anterior cortex of the developing oocyte. The bicoid protein is a homeodomain protein required for head and thorax development (Driever and Nusslein-Volhard, 1989; Tautz, 1988). The posterior morphogen, *nanos*, is also encoded by a localized mRNA (Lehmann and Nusslein-Volhard, 1991; Wang and Lehmann, 1991). Nanos protein acts to direct abdomen formation by repressing the translation of *hunchback* (*hb*) mRNA (Struhl et al., 1992; Wharton and Struhl, 1991; Hulskamp et al., 1990; Tautz, 1988). *Hb*, one of the gap genes, codes for a transcriptional repressor that functions to determine the anterior and posterior borders of expression of the other gap genes (Hulskamp et al., 1990). The absence of *hb* protein permits the expression of the *knirps* gap gene, which is essential for abdomen formation. Thus, *nanos* specifies the abdominal region by acting regionally as a negative regulator of the uniformly distributed *hb* RNA.

In *Xenopus*, the identity of localized maternal mRNAs and their relationship to subsequent development remains to be elucidated. The animal-vegetal axis is determined very early in oogenesis by a process that is not understood, but is likely to involve selective transport of proteins and RNAs to the future vegetal pole (Gerhart and Keller, 1986). It is

clear, however, that many of the early developmental decisions of the embryo depend on maternal information localized to the vegetal region during oogenesis. For example, the dorsal-ventral axis of the embryo, specified by a 30° rotation of the cortex before first cleavage, depends on a maternal component at the vegetal pole (Elinson and Pasceri, 1989; Yuge et al., 1990). Embryological experiments have also mapped the specification of endoderm (Wylie et al., 1987), the germ cell lineage (Whittington and Dixon, 1975) and mesoderm (Nieuwkoop, 1973) to the vegetal hemisphere. Of all these processes, only mesoderm induction has been characterized at a molecular level. Members of the TGF- family, the activins, are potent inducers of mesoderm (Smith et al., 1990; Thomsen et al., 1990) as is bFGF (Kimelman and Maas, 1992). To date, only one member of these groups, Vg1, has been found in vegetal cells at the proper time of development, but it does not induce mesoderm in animal caps and its function in development remains unknown (Doug Melton, personal communication).

Are there other mRNAs localized to the vegetal pole of *Xenopus* oocytes that could function in pathways specifying cell fate? A reasonable hypothesis is that such RNAs would be tightly associated with the oocyte's cytoskeleton enabling them to remain anchored in place within the cell. We had shown that Vg1 RNA, which is localized to the oocyte cortex, is enriched in a cytoskeletal fraction (Pondel and King, 1988). In this paper, we describe a screening procedure that selects for other mRNAs highly enriched in this cytoskeletal fraction. Here we report on the identification and characterization of one such clone called Xcat-2, an acronym for *Xenopus* cytoskeletal associated transcripts. Xcat-2, like Vg1, is localized to the vegetal pole and appears to be strictly maternal, i.e., very abundant in the oocyte and early embryo and reduced to very low levels by gastrulation. Thus, Xcat-2 is a candidate for a determinant involved in processes mediated by the vegetal cells. We present evidence that Xcat-2 encodes a protein that belongs to the CCHC RNA-binding family of zinc finger proteins. Xcat-2's closest relative in this family is *nanos* (Wang and Lehmann, 1991). Therefore, the region of homology between *nanos* and Xcat-2 may be responsible for the RNA-binding activity suggested for *nanos*. We discuss possible roles for the Xcat-2 protein.

MATERIALS AND METHODS

Isolation of cDNA clones

Total RNA was recovered from the detergent soluble (soluble fraction; SF) and insoluble (intermediate filament fraction; IFF) fractions of defolliculated stage VI oocytes (60 ml) as previously described (Pondel and King, 1988). IFF poly(A)⁺ RNA was selected and copied into double-stranded cDNA using *NotI* primer-adapters and RNAase H-reverse transcriptase. After *SalI* adapter addition and *NotI* digestion, cDNA was directionally inserted into *NotI-SalI* arms of lambda gt22A and packaged using the Super Script Lambda system (Gibco-BRL). The unamplified library contained 7.0×10^5 independent clones with an average insert size of 1.3 kb. A total stage VI oocyte cDNA library had previously been made in Zap II (by Stratagene). The IFF library was screened with three different probes. ³²P-labeled cDNA

probes were prepared by oligo(dT)-primed synthesis of SF and IFF poly(A)⁺ RNA to yield specific activities of 1.5×10^8 cts/minute per µg. To prepare an IFF subtracted probe, a 10-fold excess of biotinylated SF poly(A)⁺ RNA was hybridized for 30 hours at 65°C corresponding to a *R₀* of 3000. After hybridization, streptavidin was added in five-fold excess to the RNA, the sample extracted with phenol/chloroform, and the unhybridized cDNA recovered in the aqueous solution (Sive and St John, 1988). 60% of the IFF poly(A)⁺ RNA was driven into hybrid under these conditions. ³²P-labeled anti-sense Vg1 RNA was synthesized from *DdeI*-cut pSP65-Vg1 DNA containing a 1.6 kb fragment of Vg1 (Rebagliati et al., 1985) to yield probes with a specific activity of 1×10^8 cts/minute per µg.

Triplicate nylon membranes (Biotrans, ICN, 15 cm) containing 1000 plaques from the unamplified IFF library were hybridized (Ausubel et al., 1987) with either $1-10 \times 10^6$ cts/minute of SF or IFF subtracted cDNA probe or 5×10^6 cts/minute pVg1 anti-sense riboprobe. Filters were washed with $0.2 \times$ SSC and 0.1% SDS at 45°C. Plaques that were detected exclusively or primarily by the subtracted IFF probe were isolated and one fifth of these rescreened. Clones exclusive to the IFF were isolated.

Northern blot analysis

RNA was extracted from the detergent soluble and insoluble fractions of oocytes, eggs and embryos as previously described (Pondel and King, 1988). Total RNA from each fraction was fractionated on a 1.2% agarose-formaldehyde gel, blotted to nylon membranes, and the blot hybridized with the appropriate ³²P-labeled DNA fragment (Sambrook et al., 1989). pSP65-Vg1 DNA containing a 1.6 kb insert; *NotI-SalI*, gel-purified insert of Xcat-2 (0.8 kb) DNA; and histone H3 DNA (0.6 kb; Pondel and King, 1988) were labeled by random priming to yield probes with a specific activity of $4-13 \times 10^8$ cts/minute/µg.

RNAase protection

RNAase protection assays were performed essentially as described by Krieg and Melton (1984). A Xcat-2 probe was made by digesting a Xcat-2 clone in pSPORT1 (BRL) vector with *DdeI*. Transcription with SP6 RNA polymerase yielded a probe of approximately 460 bases and a protected fragment of 410 bases. The fragment contains all of the 3' untranslated region (UTR) plus a few bases of the coding region. A probe for Vg1 was prepared by digesting a pSPT19 subclone of Vg1 with *XbaI*. After transcription with T7 RNA polymerase, a probe of approximately 350 bases was generated and a protected fragment of 320 bases. The fragment consisted of the middle region of the Vg1 3' UTR. A probe for EF1- was used as a control for RNA loading.

Sequencing and primer extension

DNA from lambda gt22A phage containing Xcat-2 was isolated using LambdaSorb from Promega. After *SalI/NotI* endonuclease digestion, the insert DNA was gel purified, subcloned into pSPORT1 and transformed into *E. coli* HB 101. To obtain a sequencing ready template, plasmid DNA was isolated by the alkaline SDS method followed by two cycles of PEG precipitation and RNAase treatment. The DNA sequence of Xcat-2 cDNA was obtained using Sequenase V.2 (United States Biochemical) and dideoxy chain termination (Sanger et al., 1977). In addition to the M13/pUC forward and reverse sequencing primers, three sequence-specific oligonucleotides primers were used to sequence Xcat-2: 5' GGGGATCCTCACTGTCTCAGCTTTGG3, 5' ATCC-CCAGAGTAACAG3, and 5' GTTGCTCCTGCAGAAC3. Sequence was confirmed on the opposite strand. Primer extension analysis was performed on RNA extracted from the IFF and SF of *Xenopus laevis* stage VI oocytes. The analysis was done as described in Sambrook et al. (1989) using a 30 nucleotide primer

[5 TGCTGATCAGACTGGAGAGCCCCAAGTAGT3] whose 3 end was 50 bases from the 5' end of the Xcat-2 cDNA clone.

Computer analysis

IntelliGenetics programs Gene and Gel were used for sequencing analysis. DNA homology analysis was done using FastDB, Seq, and Genalign. Protein analysis and comparisons were done using Pep, FastDB, IFind and Quest also from IntelliGenetics Inc.

In vitro translation

A pSPORT clone containing the full coding sequence of Xcat-2 was linearized with *Bam*HI or *Not*I and capped Xcat-2 RNA synthesized in vitro using T7 RNA polymerase (Krieg and Melton, 1984). Transcripts were ethanol precipitated three times and resuspended in distilled water. Synthetic transcripts were translated in vitro using a wheat germ extract according to the instructions provided by the manufacturer (Promega).

In situ hybridization

The hybridization procedure employed was a combination of published techniques using ³⁵S-radiolabelled antisense RNA probes (Jamrich and Sato, 1989; Kintner and Melton, 1987). Wild-type defolliculated oocytes and embryos were fixed in 95% ethanol, 5% acetic acid, 0.25% chromium trioxide for 1 hour on ice. Samples were dehydrated through an ice-cold ethanol series, cleared in xylene and embedded in paraffin (Fisher Tissue Prep 2). 6 µm sections were attached to silanated slides, the paraffin removed with xylene and the sections rehydrated through an ethanol series. The slides were treated with 2× SSC for 30 minutes at 68°C and digested with proteinase K (2 µg/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA) for 30 minutes at 37°C; the reaction was stopped with 2 mg/ml glycine. The sections were then post-fixed with 4% paraformaldehyde in PBS for 20 minutes, washed in PBS and acetylated. Antisense RNA probes were synthesized from Xcat-2 pSPORT using 50 µCi of ³⁵S-UTP (1000 Ci/mmol) in the transcription reaction. Probes were hydrolysed to a size of 150 bases with alkaline bicarbonate. Typically 10 µl hybridization solution containing 2×10⁶ cts/minute was used per slide. Hybridization was performed in 50% formamide, 5× SSC, 0.1 M NaPO₄ pH 7.0, 1× Denhardt's solution, 5% dextran sulfate, 100 mM DTT, 100 µg/ml tRNA at 50°C for 15 hours. The coverslips were then removed in 2× SSPE, 10 mM DDT and the slides treated with RNAase A (20 µg/ml in 4× SSPE for 40 minutes at 37°C). They were then washed in 2× SSPE, 50% formamide for 1 hour at 50°C, dipped in water and coated with emulsion (Kodak NTP-2 diluted 2:1 with water) and exposed for 2 to 3 weeks. They were developed in D-19 developer for 2.5 minutes, fixed in Kodak Fix for 2.5 minutes, dried and mounted in Canada balsam.

RESULTS

Isolation of Xcat-2

In a previous study, we showed by northern blot analysis that Vg1 RNA is concentrated between 10- and 60-fold in a high-salt, detergent-insoluble fraction that was essentially an intermediate filament fraction (IFF; Pondel and King, 1988). We reasoned that if the oocyte cytoskeletal matrix serves to anchor RNAs, then other localized maternal RNAs should also be highly enriched in the IFF. Therefore, our strategy was to make a cDNA library from IFF poly(A)⁺

RNA. Replica filters of the IFF cDNA library were screened with one of three different probes. We first tested for the frequency of Vg1 clones in this library by hybridization with a radiolabeled Vg1 antisense riboprobe. The frequency of Vg1 clones in the IFF cDNA library was six per thousand, or a 60-fold increase over that observed when a total oocyte library was similarly screened (data not shown). Therefore, the frequency with which Vg1 RNA was found in the IFF library was in good agreement with our previous results. As a control, one of the remaining two replica filters was screened with labeled cDNA made from poly(A)⁺ RNA isolated from a detergent soluble oocyte fraction (SF). After hybridization, only a few plaques produced a strong signal on autoradiograms, suggesting that the IFF library was significantly depleted in RNAs abundant in the SF. To select for cytoskeletally associated RNAs, a third filter was screened with a subtracted cDNA probe synthesized from IFF poly(A)⁺ RNA that had been hybridized with a ten-fold excess of poly(A)⁺ RNA from the SF. Approximately 60% of the IFF cDNA was driven into hybrid under the conditions used. Forty-five of the one thousand recombinants screened from the unamplified library were detected exclusively or primarily by the subtracted IFF cDNA probe. Eight showing the strongest signal were plaque purified and rescreened. Two of these continued to produce a strong signal after hybridization with the subtracted IFF cDNA probe and were designated as Xcat-2 and Xcat-3.

Northern blot analysis showed that each cDNA clone hybridized to a different RNA and almost exclusively to RNA isolated from the IFF. Only the results for Xcat-2 are presented in Fig. 1; studies on Xcat-3 will be reported elsewhere. In these and subsequent RNA blot analyses, histone H3 message is used as a control for poly(A)⁺ RNA loading. We know from ³H-poly(U) hybridization analysis that

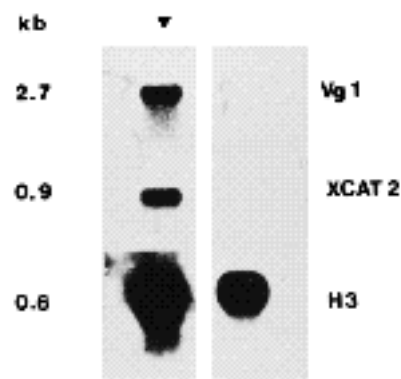


Fig. 1. Xcat-2 RNA is highly concentrated in the cytoskeletal fraction of stage VI oocytes. A northern blot of stage VI total RNA isolated from the IFF (▼) and SF was probed separately with Xcat-2, Vg1, and histone H3 ³²P-labeled DNA. In oocytes, IFF RNA is four-fold more concentrated in poly(A)⁺ material as determined by poly(U) hybridization (data not shown). Therefore, four-fold more SF RNA (16 µg) was loaded to allow comparison of mRNA concentration between the SF and IFF. Note that like Vg1, Xcat-2 (0.9 kb) is highly concentrated in IFF RNA whereas histone H3 RNA, the internal marker for poly(A)⁺ material, is of equal concentration in the two fractions.

IFF prepared from oocytes is four-fold more concentrated in poly(A)⁺ RNA than is the SF (2% for the IFF versus 0.5% for the SF). Therefore, the concentrations of the various mRNAs which were tested in the two fractions are calculated relative to histone H3 RNA. Messages uniformly distributed in the oocyte such as those for cytokeratin, c-mos, actin, and histone H3 gave hybridization signals of equal intensity on northern blots indicating that they were equally concentrated in the two fractions (Table 1). Because the IFF contains only 2% of the total RNA in an oocyte (Pondel and King, 1988), but is four-fold more concentrated in poly(A)⁺, our results indicate that approximately 8% of these messages was actually recovered in the IFF. In sharp contrast, 80% or more of Xcat-2, Vg1 RNA (Fig. 1) and Xcat-3 (not shown) was found in the IFF. These results are in good agreement with the 80% figure reported by Yisraeli et al. (1990) for Vg1. From these experiments, we can conclude that the oocyte contains at least three RNAs specifically concentrated (> 80%) in the IFF. The results further suggest that such RNAs are uncommon with 90% of most mRNAs recovered in the SF. We next asked whether Xcat-2 RNA was spatially restricted in the oocyte as is Vg1 RNA.

Xcat-2 is localized to vegetal cortex in fully grown oocytes

The distribution of the 0.9 kb Xcat-2 in stage VI oocytes was first examined by northern blot analysis using total RNA isolated from the animal or the vegetal pole fifths (Fig. 2). Such an analysis does not examine the RNA distribution in the middle of the oocyte. Densitometer tracings of these and other blots show that Xcat-2 is 10- to 20-fold more abundant in the vegetal pole versus the animal pole RNA samples. The data shown in Fig. 2 emphasizes the asymmetry in Xcat-2 concentration at the two poles. Xcat-2 was detected only in the vegetal cap lane even though five times more RNA was inadvertently loaded for An caps (note the level of histone H3). To determine if this polarity was inherited by the embryo, 4- to 8-cell embryos were sectioned into equal fifths along the animal-vegetal axis, and the regional RNAs analyzed for Xcat-2 expression (King and Barklis, 1985). Through the ooplasmic rearrange-

Table 1. Relative concentration of localized and control mRNAs in the IFF and SF

RNA	Description	Ratio IFF:SF
An1	unknown	1.4
An2	subunit of mitochondrial ATPase	1.9
An3	RNA helicase	1.7
Vg1	TGF- member	12.0
Xcat-2	Zinc finger	45.0
Histone H3		1.0
cytokeratin (56 kb)		1.0
c-mos		1.0
actin		1.2

The IFF/SF ratio for each of the indicated RNAs was determined by comparing the relative intensities of hybridization of the respective cDNA probes to IFF and SF RNA from stage VI oocytes by densitometry. The amount of Histone H3 hybridization served as a control for poly(A)⁺ RNA loading differences between samples. Vg1 values were highly variable ranging from 12 to 60.

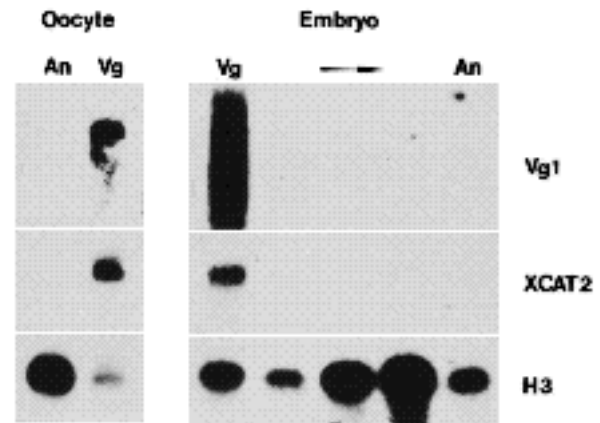


Fig. 2. Xcat-2 is localized to the vegetal pole in the stage VI oocyte and embryo. A northern blot of regional RNA extracted from cryostat-sectioned oocytes (King and Barklis, 1985) and embryos was hybridized separately with Xcat-2, Vg1, and histone H3 ³²P-labeled DNA. On the left blot, total RNA isolated from animal pole (An) and vegetal pole (Vg) fifths of stage VI oocytes is shown. Five-fold less vegetal pole than animal pole poly(A)⁺ RNA was loaded on the blot (note histone H3 levels), yet Xcat-2 was detected only in the vegetal fifth. On the right blot, each lane contains total RNA (15 µg) isolated from one fifth of 4- to 8-cell embryos sectioned along the animal/vegetal axis (An/Vg). Again, Xcat-2 and Vg1 are found only in the most vegetal fifth (200 µm) of the embryo.

ments that occur during the events of maturation, fertilization and cleavage, Xcat-2 remained highly concentrated in vegetal pole RNA (Fig. 2).

The apparent correlation between a RNA's enrichment in the IFF and its prevalence at the vegetal pole prompted us to ask whether RNAs localized to the opposite pole were also concentrated in this fraction. Three mRNAs designated An1, An2, and An3 have been shown to be localized to the animal hemisphere of the stage VI oocyte (Rebagliati et al., 1985). An2 encodes the chain of mitochondrial ATPase (Weeks and Melton, 1987) and An3 appears to encode a putative RNA helicase (Gururajan et al., 1991). Interestingly, at most 15% of the animal pole RNAs in an oocyte were in the IFF as compared to 8% of the control non-localized RNAs and 80-94% of Vg1 and Xcat-2 (based on the IFF/SF ratio in Table 1). From these results it can be concluded that the factors involved in retaining RNAs at the two poles differ as to their solubility characteristics, suggesting that different mechanisms may be involved in RNA retention at the animal pole than at the vegetal pole.

To obtain more exact information on the spatial distribution of Xcat-2, *in situ* hybridizations were performed on stage VI oocytes. Xcat-2 RNA was not uniformly distributed throughout the vegetal hemisphere but was tightly localized in a cortical shell at the vegetal pole (Fig. 3). The hybridization signal extended as far as the oocyte's equator and appeared evenly distributed radially about the A/V axis (arrows in Fig. 3). Positive controls using Vg1 anti-sense strand showed a hybridization pattern strikingly similar to that observed for Xcat-2 (not shown) and dissimilar to that of histone RNA (Melton, 1987). The main conclusion to be drawn from these experiments is that an inter-

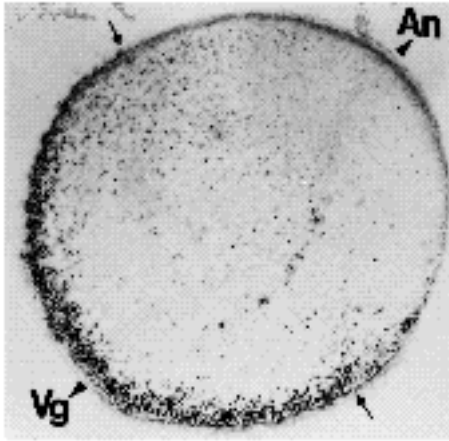


Fig. 3. In situ hybridization of Xcat-2 in oocyte. Paraffin sections along the animal-vegetal axis of stage VI oocytes were hybridized with ^{35}S -labeled Xcat-2 antisense RNA probes. The A/V axis is indicated by arrowheads with the animal (An) pole at the top and the vegetal pole (Vg) at the bottom of the figure. The hybridization signal is represented by silver grains (black) in this bright-field photograph. The small arrows mark the extent of cortical hybridization. Autoradiographic exposure was for 14 days.

mediate filament fraction prepared from stage VI oocytes is specifically enriched in mRNAs localized to the vegetal pole. Furthermore, Vg1 and Xcat-2 RNA appear to co-distribute to the cortex, a region particularly concentrated in cytoskeletal elements which include intermediate filaments.

Developmental expression of Xcat-2

To begin to understand the function of Xcat-2, we examined when during development its RNA was present. Total RNA was isolated from the IFF and SF of oocytes, ovulated eggs, gastrula and neurula and blots of these RNAs were hybridized with labeled DNA for Xcat-2, Vg1 and histone H3 (Fig. 4A). Xcat-2 appeared to be strictly maternally expressed with transcript levels highest in oocytes and falling to barely detectable levels by gastrulation. Xcat-2 transcript levels appeared to remain constant through maturation relative to histone H3 or EF-1 RNA levels (Fig. 4). RNAs from nine adult tissues (testis, brain, heart, skeletal muscle, skin, spleen, kidney, liver, gut) were also tested for Xcat-2 accumulation. Even after a lengthy exposure time, no signal was detected for any of these tissues in sharp contrast to an actin probe that served as a positive control (data not shown). In summary, Xcat-2 is most abundant in the oocyte and cleavage stages. If it is expressed elsewhere, it is at very low levels.

An unexpected result from these experiments was that, unlike Vg1, Xcat-2 RNA remained highly concentrated in the cytoskeletal fraction of ovulated eggs (Fig. 4A). Previous experiments had shown that the transition from oocyte to egg is accompanied by the loss of Vg1 RNA from the IFF into the SF (Pondel and King, 1988; Yisraeli et al., 1990). In contrast, Xcat-2 remains associated with the IFF after ovulation. The ratio of Xcat-2 RNA levels in oocyte IFF to that of ovulated egg IFF was 1.23, indicating no change in concentration (average of three determinations

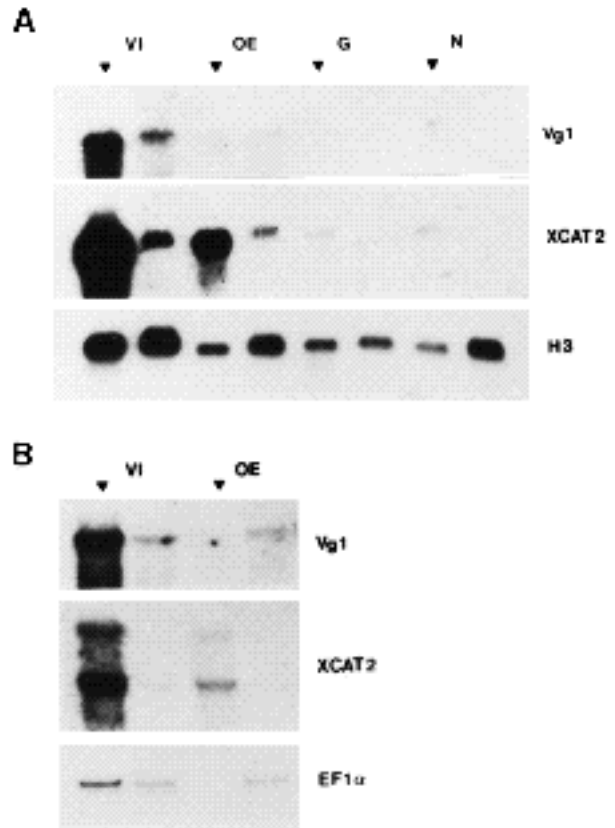


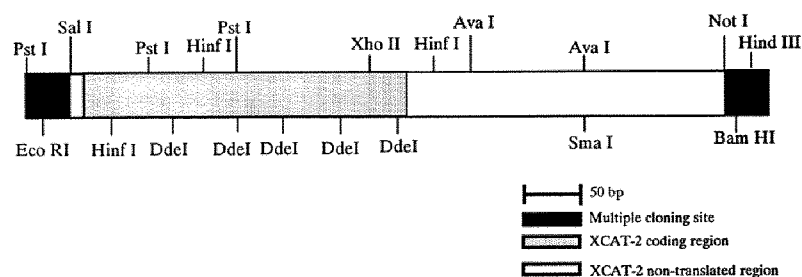
Fig. 4. Xcat-2 expression is developmentally regulated. (A) RNA was extracted from the IFF (▼) and SF (unlabeled) of stage VI oocytes (VI), ovulated eggs (OE), gastrulae (G), and neurulae (N). The same filters were successively hybridized with ^{32}P -labeled Vg1, Xcat-2, and H3 histone DNA. Xcat-2 is barely detected by gastrulation. Note: Autoradiograms were over-exposed to show any weak responses at later stages of development. (B) RNAase protection analysis showing Vg1, Xcat-2 and EF1 α levels in the IFF and SF for stage VI oocytes (VI) and ovulated eggs (OE). Xcat-2 RNA is not released from the IFF at maturation as is Vg1 but remains concentrated in that fraction.

from three different blots normalized to H3 values). RNAase protection analysis confirmed these observations (Fig. 4B). Taken together, our findings indicate that at the end of maturation, Xcat-2 RNA remains associated with a complex that sediments as $>6000\text{ S}$ while Vg1 RNA does not. The shift in Vg1 solubility is correlated with its loss from its cortical location (Weeks and Melton, 1987). Perhaps Xcat-2 remains in the cortex during maturation and displays a different pattern of inheritance from that of Vg1 RNA in the embryo. In situ hybridization analysis will resolve this question.

Xcat-2 and Nanos share a region of homology

We isolated what appeared to be a full-length clone for Xcat-2 in our initial screen. Primer extension analysis revealed that 11 nt were missing from the 5' end of the Xcat-2 cDNA clone (data not shown) indicating that the authentic mRNA contains a 26 base 5' leader sequence. The sequence for the 0.8 kb cDNA clone is presented in Fig. 5. An open reading frame begins 15 nts from the 5' end of

A.



B.

1	CAG AAC AAT TCC AAC ATG GAT GGC GGT CTC TGG TTT GAG TCA TGG	45
	M D G G L C F D S W	
46	AGC GAC TAC TTG GGG CTC TCC AGT CTG ATC AGC AGG GGG CTG CAG	90
	Ⓢ D Y L G L S S L I S R G L Q	
91	CCT CAG AGA GAA GGC GAA AGG CCA AGA TGG GAC GTT TTA TCT CCG	135
	P Q R E G E R P R W D V L S P	
136	GCC TCC GCG GAA CCG TTA CCC AGT AAT GAG TCT GTG GGA CAC AAA	180
	A S A E P L P S N E S V G H K	
181	GGC TGC GGG TTC TGC AGG AGC AAC AGG GAG GCG CTG TCT CTA TAC	225
	G C G F C R Ⓢ N R E A L S L Y	
226	ACG TCT CAC CGT CTG AGG GCG CTG GAT GGC CGG GTG CTG TGT CCA	270
	T Ⓢ H R L R A L D G R V L C P	
271	GTA CTA AGA GGC TAC ACT TGC CCT TTG TGT GGG GCT AAT GGC CAC	315
	V L R G Y T C P L C G A N G D	
316	TGG GCC CAC ACC ATG CCG TAC TGC CCC CTC CGT CCG CTC CTC AGA	360
	W A H Ⓢ M R Y C P L R R L L R	
361	GAT CCC CAG AGT AAG AGT AAT AAG CCA AAG CTG AGA CAC TGA ACT	405
	D P Q S N S N N P K L R H	
406	GGAAGGAACCCCTGGGATTCTGCA CCACTAATGGGCTCCTCGGGGACTGGC	457
458	CTTTTGACATCAGACTGACCTTTTCTAATGTTTGTGCTTTATTGCAAC	510
511	TGTTCCCTATTGACACTTTAACTAAAGAATAATGATTGAGTGATCACCAGCC	562
563	CGTGACAGACACTGATCTCTCACTCGGCACCCACGTTGCACCTCATTGTTC	614
615	CACTTGTTCCTGTCCCGGGGCAAGCCGTCGCCATGCCCATGTACCCGACA	666
667	TTCCAGCTCTCCAGAGACGCTCCAGCCTGGGCCCGCCCGCCTGTAATTATT	721
722	TTATTTACTGTTTAAACAGCATTAAATTTGTATTTTATTAAATGAAAAAAT	774
775	TCCGGAAAAAATAAAAAAAAAA 795	

Fig. 5. Diagram and Sequence of the Xcat-2 Transcript. (A) Map of Xcat-2 cDNA (B) DNA sequence of Xcat-2 with amino acid single letter translation. The numbering begins with the 5' end of the cDNA clone. The start and stop codons are in bold type and the TAAAT polyadenylation signal is underlined. Potential phosphorylation sites are circled.

the cloned sequence and continues for another 384 before reaching a stop codon. A number of stop codons in all reading frames then follow. The remaining 391 nts constitute a 3 untranslated region that contains an acceptable polyadenylation site (TAAAT) and a poly(A) tail of 16 residues. The first methionine codon (ATG) is in a favorable context for translation initiation (Kozak, 1987). Conceptual translation of the cDNA sequence gives a 128 amino acid protein with a predicted relative molecular mass of 14.3×10^3 . In vitro translation of the synthetic Xcat-2 transcript confirmed that this first AUG was used. The synthesized polypeptide species migrated with an apparent relative molecular mass of 15×10^3 on a denaturing gel, in good agreement with the predicted value (data not shown).

As a first step towards identifying Xcat-2, a small protein data bank was compiled by translating published cDNA sequences for localized *Drosophila* transcripts. Searches of this data bank for related sequences revealed significant homology to a 58 amino acid region in nanos (Wang and Lehmann, 1991; Fig. 6A). The putative Xcat-2 protein is

less than a third the length of nanos which has 400 amino acids. Like nanos, the Xcat-2 polypeptide is moderately basic with a pI of 8.68 and lacks a signal sequence. The region of homology lies near the carboxy terminus of both proteins; the amino-terminal regions are not related. In the common domain of 58 amino acids, 29 showed identity (50.0%) and 7 represented conservative substitutions (62.1%) with no gaps introduced (Fig. 6B).

Particularly striking is the fact that all the cysteines in either protein are found within the homology region and in the same positions. This cysteine-rich region for Xcat-2 and nanos can be represented in the form Cys-X2-Cys-X12-His-X10-Cys-X7-Cys-X2-Cys-X7-His-X4-Cys and bears resemblance to three zinc finger families (reviewed by Berg, 1990). The best fit is with the Cys-X2-Cys-X4-His-X4-Cys (CCHC) RNA-binding zinc finger family of proteins found in retroviruses and first described by Green and Berg (1989; Fig. 6C). However, other possibilities that cannot be ruled out are the zinc finger proteins that bind damaged DNA (Uchida et al., 1987) and those involved in protein-protein interactions (Berg, 1990). Also within this region are three

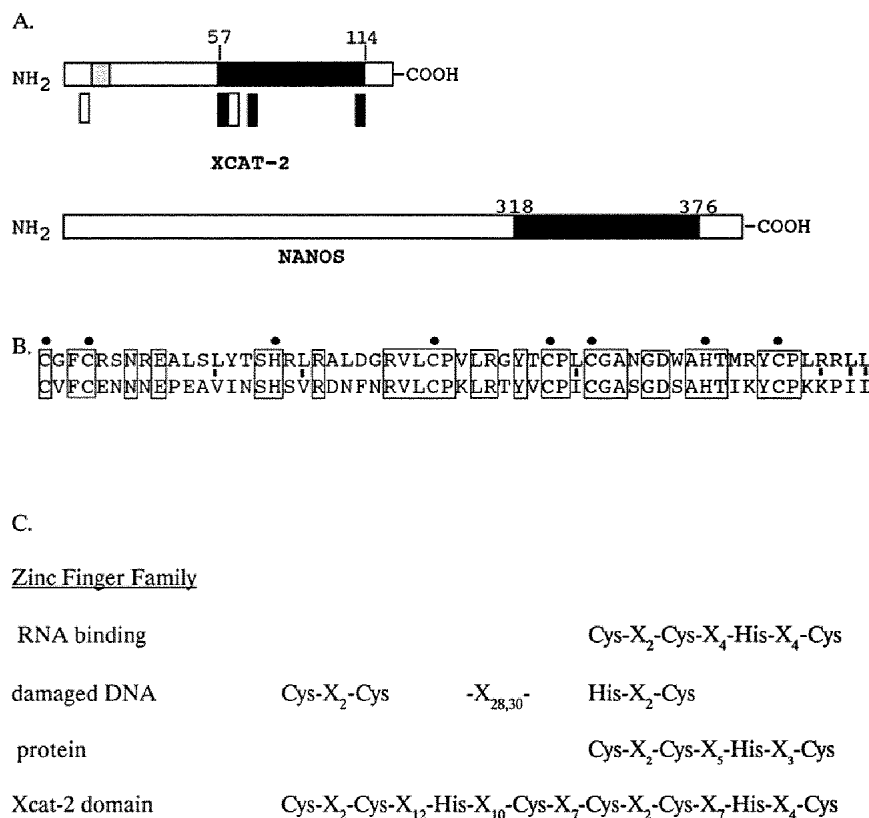


Fig. 6. Comparison between the Xcat-2 and nanos derived protein sequence. (A) Cartoon of Xcat-2 and nanos protein. Numbers refer to amino acid position. The region of homology between the two proteins is black and the histone-like region in Xcat-2 is shaded. Putative casein kinase II sites are indicated by open rectangles; protein kinase C sites are denoted by black rectangles. (B) Region of homology. Identical amino acids are enclosed in boxes in the two proteins. Conserved substitutions are marked as a vertical bar. The black dots mark the cysteines and histidines within this region that are hypothesized to form a zinc finger motif present in both proteins. (C) Comparison of the Xcat-2 zinc finger motif with three other zinc finger families: RNA-binding family (Green and Berg, 1989); damaged DNA family (Uchida et al., 1987) and the protein-protein family (Berg, 1990).

XCAT-2	L G L S S L I S R G
Histone H1	L G L K S L V S K G
Dpn I	L R K S S L S S R G
AT-BP1	L G H S S L I S Y L
EF-Tu	L R L S S V I S R T
LCVLA L Protein	L C L S S L T K R S
NADH(Ubiquinone)	L I L S S L I S W S

Fig. 7. Comparison of a short sequence found in the Xcat-2 protein and selected proteins that interact with nucleic acids. A 10 amino acid stretch in Xcat-2 showed identity with 7 amino acids (9 allowing conserved substitutions) with a region in histone H1 located within the DNA-binding loop. A search of protein sequence banks revealed a short list of other proteins 70% identical with this region; none were completely identical. Many of these proteins interact with nucleic acids or nucleotides. Selected examples of such proteins are listed. Amino acids identical to the Xcat-2 region are boxed, conserved substitutions are marked with a black dot. The histone H1 sequence is identical in cow, human, rabbit, chicken, mouse and rat (Allan et al., 1980). DpnI is a restriction enzyme, endonuclease from *Streptococcus pneumoniae*. Also listed are AT-BP1, a zinc finger protein found in rats (Mitchellmore et al., 1991); EF-Tu, an elongation factor (Jurnak, 1985); LCVLA L protein, a RNA-dependent RNA polymerase (Singh et al., 1987); NADH dehydrogenase (ubiquinone).

possible protein kinase C phosphorylation sites and one casein kinase II site, none of which appear to be conserved in nanos (Fig. 6A).

A serine-rich region at the amino terminus (position 14-23) appears to be related to a segment of histone H1 found in the loop domain, but its significance is not clear because of its very small size (Fig. 6A). Here seven out of ten amino acids are identical and this increases to nine with conservative substitutions. A search limited to these ten amino acids revealed other proteins similarly related, many of which recognize nucleic acids or nucleotides (Fig. 7). A perfect match for these ten amino acids was not found in any known protein.

3'UTR of Xcat-2 and nanos RNA

A very striking similarity between Xcat-2 and *nanos* RNA is that both are localized to specific poles of their respective oocytes. Because the 3' UTR has been implicated in the localization of other maternal RNAs (Mowry and Melton, 1992; Cheung et al., 1992), we searched for stretches of identity between the two RNAs in this region. As a comparison, we also examined the 3' UTR of Vg1. There were no statistically significant regions of identity between any of the three RNAs.

DISCUSSION

Isolation of localized mRNAs

In the absence of genetics as a tool to identify genes whose

activity is essential in early development, we have turned to selecting for maternal messages that are asymmetrically distributed in the frog oocyte. Localized messages in the frog oocyte have been difficult to isolate because they are only a minor component of the 40-80 ng of mRNA that the oocyte has stored to carry it through early development. A reasonable assumption was that localized RNAs would be predominantly found associated with a cytoskeletal compartment in the oocyte which would serve to anchor them in place. Previously, we showed that 80-90% of Vg1 RNA was found in an intermediate filament fraction (Pondel and King, 1988). Therefore, we made a cDNA library from IFF RNA and selected for and isolated other RNAs predominantly found in this cytoskeletal fraction. Using this strategy, we have identified a novel mRNA, Xcat-2, which is localized to the vegetal cortex. Preliminary results indicate that at least two other messages selected from the IFF library are also localized to the vegetal pole. In contrast, three animal pole-specific RNAs were predominantly found in the detergent soluble fraction along with histone and other non-localized messages. These results indicate that a dual system operates to retain localized RNAs at opposite poles. The correlation between vegetal pole localization and a cytoskeletal fraction is consistent with the vegetal cortex containing receptors for specific RNAs (Jeffery, 1989; Yisraeli et al., 1990). A 625 nucleotide stretch in the 3' UTR is both required and sufficient for anterior localization of the *bicoid* message in the *Drosophila* oocyte (Macdonald and Struhl, 1988). The sequence of this functional domain however, has diverged considerably when compared with *bicoid* in six other species of *Drosophila* (Macdonald, 1990). In Vg1 RNA a single 340 nucleotide region in the 3' UTR has recently been identified as being required for vegetal pole localization (Mowry and Melton, 1992). By determining the RNA sequence required for targeting each of the vegetal specific RNAs, it may be possible to identify a common localization signal for the vegetal pole. It is likely that this signal will involve RNA secondary structure. A comparison between the 3' UTR for Vg1, Xcat-2 and *nanos* did not reveal any regions that showed significant sequence identity. Characterization of the RNA-binding proteins that specifically recognize the 3' UTR domains required for localization is now under investigation and should yield insights into the mechanism for docking RNAs at the vegetal pole.

The biological significance of the retention of Xcat-2 in the >6,000S cytoskeletal fraction after ovulation is not clear. Two possibilities are that release of Vg1 RNA initiates a change in translation or message stability. However, Vg1 protein continues to be synthesized after ovulation despite its release (Tannahill and Melton, 1989) and Vg1 and Xcat-2 RNA levels decline in a similar pattern (Fig. 4; Rebagliati et al., 1985). An interesting third possibility is that the different associations of RNAs with the cytoskeleton determine the patterns of inheritance of those RNAs and their protein products. For example, Vg1 RNA is released and becomes restricted to the vegetal tier of the 32-cell embryo (Weeks and Melton, 1987). Because Xcat-2 is not released, its inheritance may be more restricted to the vegetal blastomeres exposed to the outside environment.

Xcat-2 contains a zinc finger motif in a region homologous with *nanos*

Xcat-2 encodes a protein that appears to be a member of an RNA-binding family of zinc finger proteins (Green and Berg, 1989). Other members of this family include the retroviral nucleocapsid proteins which contain either one or two CCHC boxes depending on the type of virus (Berg, 1990). Xcat-2's closest relative in this family is *nanos* where a 58 amino acid domain is 50% identical. The homology domain covers 45% of the Xcat-2 protein, but only 14.5% of *nanos*, making it unlikely that Xcat-2 is a *nanos* homolog. But strong evolutionary conservation around the zinc finger motif and genetic data argue that this region is a functional domain. Overall, the *nanos* amino acid sequence has not been well conserved between species. *D. melanogaster* and *D. virilis*, two very closely related species, share only limited identity within the amino half of the protein. The carboxyl terminus is conserved to a significantly higher extent (Curtis and Lehmann, personal communication). This region is highly conserved between *Drosophila* and *Xenopus*, unusual for two species of such evolved divergence. In light of the proposed mechanism of action for *nanos* as a translational repressor of hunchback (Irish et al., 1989; Wharton and Struhl, 1991) and the evidence linking the zinc finger motif with nucleic acid binding (Green and Berg, 1989; Hattman et al., 1991), we would suggest that both proteins bind RNAs whose expression they regulate. This regulation appears negative for *nanos*, but there is no reason to rule out the possibility that Xcat-2 may act as a positive regulator. Taken as a whole, the evidence strongly suggests that the homology domain is a functional domain critical to both *nanos* and Xcat-2. The histone related-region identified in Xcat-2 was not found in *nanos* but may play a role in facilitating RNA binding.

Possible functions for Xcat-2 protein

Based on Xcat-2 RNA's location at the vegetal pole cortex, several different developmental roles can be envisioned. Determinants that have been functionally localized to this region include those that are involved in the specification of the germ cell lineage, endoderm, mesoderm, and Spemann's organizer. One possibility is that Xcat-2 might act as an RNA-binding protein in presumptive endodermal cells to suppress a response to cues that trigger mesodermal induction. However, preliminary experiments designed to test whether Xcat-2 protein could suppress mesoderm induction in animal caps failed to support this hypothesis. Another possibility is that Xcat-2 functions in establishing the vegetal dorsalizing region or Nieuwkoop center, by controlling the translation of a maternal dorsal mesoderm signal, perhaps *noggin* (Smith and Harland, 1992), or a member of the *wnt* family (Smith and Harland, 1991; Sokol et al., 1991). Phosphorylation of the target sites within the zinc finger domain of Xcat-2 could function to disrupt nucleic acid binding, thus allowing the translation of the dorsal signal. Consistent with this hypothesis is the observation that all three possible protein kinase C sites and one casein kinase II site are found within the zinc finger domain in Xcat-2 or next to the histone H1 region. It is worth noting that none of these putative phosphorylation sites are con-

served in nanos which is believed to remain bound to *hb* RNA.

It will be of considerable interest to determine when and where the Xcat-2 protein is expressed and if it is a RNA-binding protein. The isolation and characterization of any such bound RNA should clarify the developmental function of Xcat-2

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