Requirement of *ets*-2 Expression for *Xenopus* Oocyte Maturation

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A molecular clone of the *Xenopus laevis ets*-2 gene was isolated from an oocyte complementary DNA library. The amount of messenger RNA (mRNA) in each oocyte or embryo was almost constant during oogenesis and was maintained until the blastula stage of embryonic development, indicating that the observed 3.2-kilobase transcript is a maternal message. The only normal adult tissue in which *ets*-2 mRNA was detected was the ovary. Injection of antisense oligonucleotides homologous to the *ets*-2 sequence into oocytes led to degradation of the mRNA and blocked hormone-induced germinal vesicle breakdown. The *ets*-2 product is thus required for the meiotic maturation of *Xenopus* oocytes.

THE *ets* FAMILY OF PROTO-ONCOgene sequences comprises sequences related to the v-*ets* oncogene sequence from avian erythroblastosis virus E26 (*1*), a retrovirus that causes erythroblastosis and myeloblastosis in chickens (*2*). The E26 v-*ets* transforming protein is expressed as a polyprotein (p135) containing *gag* and v-*myb* coded residues as well as v-*ets*. Beug and co-workers (*3*) have suggested that the *myb* sequences are responsible for myeloblastosis and that the *ets* sequences are responsible for erythroblastosis and fibroblast transformation. The proto-oncogene c-*ets* sequences are found in a wide variety of animals including *Drosophila* (*4*), sea urchins (*5*), chickens (*6*), mice (*7*), and humans (*7, 8*). In the human, mouse, and chicken, there are two *ets* genes, *ets*-1 and *ets*-2 (*7–9*). The function of the c-*ets* proto-oncogenes is unknown. We chose to study *ets* genes in the frog *Xenopus laevis* because of the ease of experimental manipulation of the frog's oocytes and embryos and because of its well-characterized developmental biology. This system allows one to perform in vitro fertilizations and to obtain oocytes and embryos that are suitable targets for microinjection.

Recombinant bacteriophages were isolated by screening a λgt10 library (*10*) derived from *Xenopus* oocyte RNA with an E26 v-*ets* probe (*11*). Inserts were subcloned into m13mp18 (*12*) and sequenced (*13*). A sequence derived from two overlapping clones contained a long open reading frame that can code for a 54-kD protein containing 472 amino acids starting from the first ATG. The predicted protein sequence is more similar to *ets*-2 than to *ets*-1 sequences, with the degree of amino acid identity with the human, mouse, and chicken *ets*-2 sequences being 69, 68, and 68%, respectively. The predicted initiation and termination codons are colinear with the human and chicken *ets*-2 sequences. This suggests that the cloned DNA is the *Xenopus ets*-2 homolog and that the presence of distinct *ets*-1 and *ets*-2 genes is a common feature of all vertebrates. The likelihood that *Xenopus* contains an *ets*-1 gene as well as an *ets*-2 gene is supported by the observation (*14*) that *ets*-1–specific sequences hybridize to *Xenopus* DNA.

To determine the expression pattern of the *Xenopus ets*-2 gene, we performed Northern (RNA) gel blot analysis on a variety of tissues obtained from an adult frog (Fig. 1). The only normal tissue in which *ets*-2 expression was detected was the ovary (Fig. 1, lane 2). The *ets*-2 RNA from this tissue was present as a single 3.2-kb band. During the dissection of the frogs, an ovarian tumor was detected and sampled. This tissue was found to have an extremely high level of *ets*-2 RNA expression (Fig. 1, lane 4). Northern blot analysis was also performed on RNA isolated from *Xenopus* oocytes, eggs, and embryos. As in the adult ovary, for each stage where *ets*-2 expression was detected, there was a single 3.2-kb band (Fig. 2). The amount of *ets*-2 RNA detected was approximately the same from oocyte stage II to the morula stage. Smaller but significant amounts of *ets*-2 RNA were de-

Table 2. Restoration of GVBD by *ets*-2 mRNA. Oocytes were microinjected with oligonucleotides and, where indicated, injected with mRNA 4 hours later. The human *ets*-2 mRNA was prepared by in vitro transcription of a full-length cDNA that had been subcloned into the vector pGEM-7Zf(+) (Promega). Transcription from the T7 promoter was performed with the Stratagene in vitro transcription and capping kit. The control mRNA was from the Stratagene in vitro translation kit. One hour after the second set of microinjections, the oocytes were treated with progesterone, incubated overnight, and examined for GVBD as in Table 1. A single dead oocyte resulting from the injection of oligonucleotides III + IV with the control mRNA was not included. This experiment has been repeated 2 times with similar results.

Injected material	Number GVBD/ number injected	GVBD (%)
None	17/21	81.0
Sense oligonucleotide I	12/15	80.0
Antisense oligonucleotides III + IV	0/17	0.0
Antisense oligonucleotides III + IV + *ets*-2 mRNA	6/17	35.3
Antisense oligonucleotides III + IV + control mRNA	0/12	0.0

Table 1. Effect of *Xenopus ets*-2–specific oligonucleotides on germinal vesicle breakdown after treatment with progesterone. Stages VI oocytes were collected from *Xenopus* ovaries and microinjected with oligonucleotides as in Fig. 3. Four hours after microinjection, the oocytes were treated with progesterone (5 μg/ml) in 1X MBS. After incubation overnight at 22°C, they were fixed in 5% trichloroacetic acid and examined for GVBD (*16*). Dead oocytes were not included in the analysis. The nucleotide positions are relative to the initiation codon.

Oligonucleotide	Description	Oligonucleotide position	Number GVBD/ number injected	GVBD (%)
Sense				
I	5′-Coding	177–192	90/104	86.5
II	3′-Coding	1076–1093	61/89	68.5
Antisense				
III	5′-Coding	192–177	29/88	33.0
IV	3′-Coding	1092–1076	41/118	34.7
III + IV			23/81	28.4
V	ATG-Spanning	12–(−7)	11/65	16.9
VI	3′-Coding	1309–1280	10/38	26.3
VII	3′-Noncoding	1661–1637	22/29	75.9
Controls				
No injection			168/205	82.0
Injection with 88 mM NaCl			65/92	70.7

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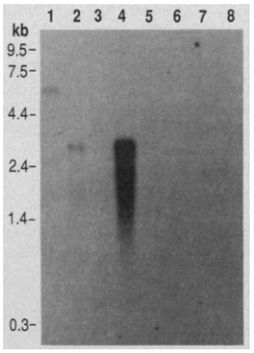

Fig. 1. *Xenopus ets*-2 RNA expression in adult tissues. RNA was extracted from various tissues (*19*). Northern blot analysis was performed as described (*5*) with 10 μg of total RNA per lane. A 1.5-kb *Xenopus ets*-2 cDNA Eco RI fragment labeled with ^{32}P by nick translation was used as the probe. The RNA in lane 1 was from pooled 16-cell-stage embryos of the sea urchin *Strongylocentrotus purpuratus* (*5*). The RNA used in the remaining lanes was obtained from the following *Xenopus* tissues: 2, ovary; 3, oviduct; 4, ovarian tumor; 5, lung; 6, liver; 7, heart; 8, brain.

tected in oocyte stage I and the blastula stage, but not at the later stages analyzed. The *ets*-2 mRNA thus represents a maternal RNA. The *ets*-2 mRNA was not localized to either the animal or vegetal pole of oocytes or embryos when analyzed as described (*10*).

The injection of antisense oligonucleotides into *Xenopus* oocytes results in the degradation of homologous messages and the inhibition of the expression of the corresponding proteins (*15, 16*). To determine if *Xenopus ets*-2 antisense oligonucleotides affect mRNA levels, we injected sense and antisense oligonucleotides into immature oocytes and performed Northern blot analysis on RNA extracted from these cells. We detected little *ets*-2 RNA in oocytes injected with *ets*-2 antisense oligonucleotides (Fig. 3, lanes 3 and 4) but normal levels in oocytes that were not injected or were injected with an *ets*-2 sense oligonucleotide (Fig. 3, lanes 1 and 2). Therefore, *ets*-2 antisense oligonucleotides specifically induce the degradation of *ets*-2 mRNA.

An important indicator of meiotic maturation of the *Xenopus* oocyte is germinal vesicle breakdown (GVBD), the dissolution of the nuclear membrane. To determine if the *Xenopus ets*-2 gene product is necessary for maturation, we microinjected oocytes with *ets*-2 sense or antisense oligonucleotides and examined for GVBD after treatment with progesterone. In the absence of microinjection, progesterone induced GVBD in 82.0% of the oocytes. Antisense, but not sense, oligonucleotides inhibited GVBD in the hormone-treated oocytes, reducing its occurrence to 16.9% of the oocytes (Table 1). To study the specificity of the reaction, we injected human *ets*-2 mRNA into oocytes that had previously been injected with antisense oligonucleotides and observed that this led to a partial restoration of GVBD (Table 2). This result demonstrated that the inhibition of GVBD was specifically due to the breakdown of the *ets*-2 message and that *ets*-2 expression is required for *Xenopus* oocyte maturation. The fact that human mRNA restored GVBD indicates that the human and *Xenopus ets*-2 products have homologous functions.

Oncogenes are often involved in differentiation and proliferation. It is hoped that the function of the *ets*-2 oncogene can be elucidated with the use of the well-characterized developmental biology of *Xenopus*. The *Xenopus ets*-2 gene shares many properties with the c-*mos* gene, which also is expressed as a maternal message and is required for GVBD (*16*). The v-*mos* product, and thus presumably the c-*mos* product, is a soluble cytoplasmic protein (*17*), whereas the *ets*-2 product is nuclear (*18*).

Cancer is generally considered to be caused by aberrant control in the mitotic division of somatic cells. An oocyte is arrested at the G2-M border of the cell cycle. Since meiosis is very similar to mitosis, these processes may require common products. Thus, overexpression of this protein in a somatic cell may trigger inappropriate cell

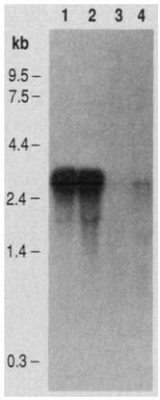

Fig. 3. Effects of microinjected oligonucleotides on *Xenopus ets*-2 expression. Stage VI oocytes were collected from a *Xenopus* ovary by dissection and microinjected with 60 nl of a solution containing one or two oligonucleotides (1 mg/ml each) in 88 mM NaCl. The injected oocytes were then incubated at 22°C in MBS for 4 hours. For each lane, total RNA was extracted from a pool of 14 oocytes as described (*5*). The RNA was then subjected to Northern blot analysis with radiolabeled *ets*-2 cDNA as the probe as in Fig. 1. The RNA was from oocytes injected with the following: lane 2, oligonucleotide I (sense); lane 3, oligonucleotide IV (antisense); and lane 4, a mixture of oligonucleotides III and IV (antisense). The oligonucleotides are listed in Table 1. The RNA used in lane 1 was from oocytes that were not microinjected.

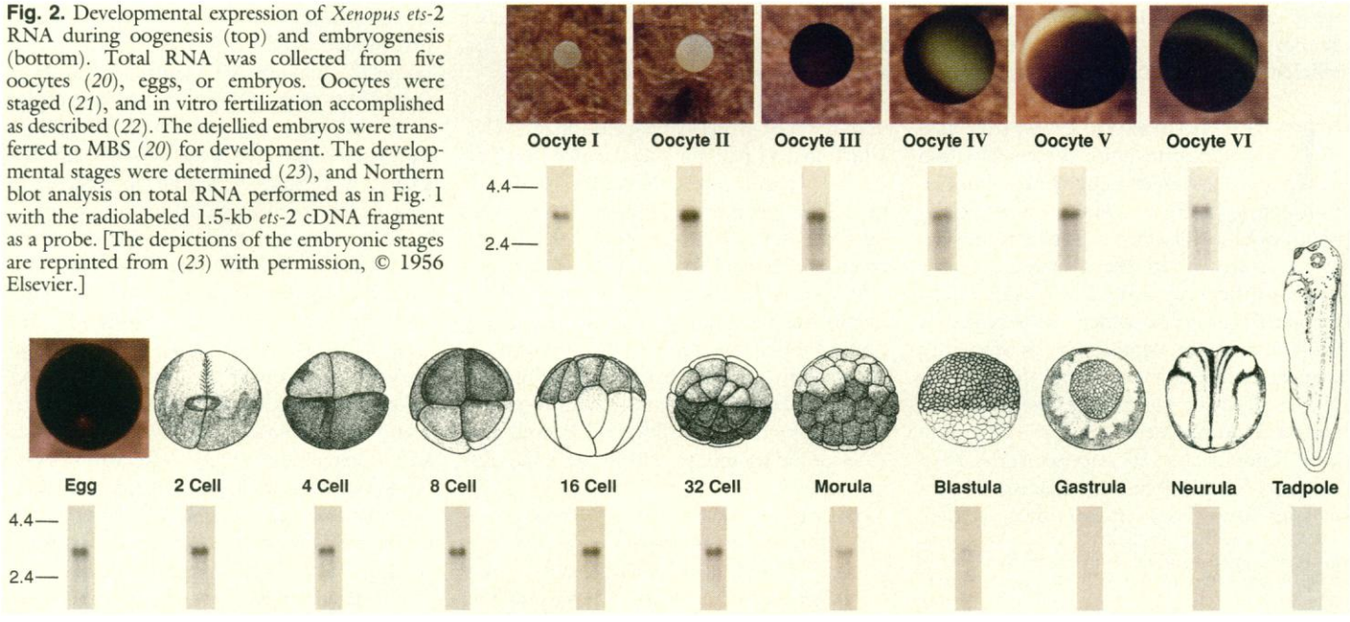

Fig. 2. Developmental expression of *Xenopus ets*-2 RNA during oogenesis (top) and embryogenesis (bottom). Total RNA was collected from five oocytes (*20*), eggs, or embryos. Oocytes were staged (*21*), and in vitro fertilization accomplished as described (*22*). The dejellied embryos were transferred to MBS (*20*) for development. The developmental stages were determined (*23*), and Northern blot analysis on total RNA performed as in Fig. 1 with the radiolabeled 1.5-kb *ets*-2 cDNA fragment as a probe. [The depictions of the embryonic stages are reprinted from (*23*) with permission, © 1956 Elsevier.]

division leading to malignancy. Our data are thus consistent with the hypothesis that *ets* has an important function in the control of cell proliferation. Further study of the role of the *ets*-2 protein in *Xenopus* oocyte maturation may lead to an understanding of the involvement of this protein in oncogenesis.

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The Function of a Leader Peptide in Translocating Charged Amino Acyl Residues Across a Membrane

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Insertion of bacteriophage coat proteins into the membrane of infected bacterial cells can be studied as a model system of protein translocation across membranes. The coat protein of the filamentous bacteriophage Pf3—which infects *Pseudomonas aeruginosa*—is 44 amino acids in length and has the same basic structure as the coat protein of bacteriophage M13, which infects *Escherichia coli*. However, unlike the Pf3 coat protein, the M13 coat protein is synthesized as a precursor (procoat) with a typical leader (signal) sequence, which is cleaved after membrane insertion. Nevertheless, when the gene encoding the Pf3 coat protein is expressed in *E. coli*, the protein is translocated across the membrane. Hybrid M13 and Pf3 coat proteins were constructed in an attempt to understand how the Pf3 coat protein is translocated without a leader sequence. These studies demonstrated that the extracellular regions of the proteins determined their cellular location. When three charged residues in this region were neutralized, the leader-free M13 coat protein was also inserted into the membrane. Differences in the water shell surrounding these residues may account for efficient membrane insertion of the protein without a leader sequence.

THE INFORMATION SPECIFYING membrane insertion is located within proteins as transmembrane integration signals (*1*). For example, leader (signal) peptides are NH_2-terminal extensions that target a protein to the membrane. Some leader peptides are removed after membrane integration, whereas others are retained as uncleaved leader peptides or as start-stop sequences that anchor the protein in the bilayer. Each of these leader peptides contains a hydrophobic stretch of ~20 amino acid residues that is important for their function. The molecular mechanism of protein insertion has been extensively studied with the coat protein of bacteriophage M13, which inserts into the plasma membrane of *E. coli*. This protein is synthesized as a precursor (procoat) with a typical 23-residue NH_2-terminal leader peptide. Both the positively charged region of the leader peptide (*2*) and the apolar core are required for membrane insertion (*3*). In addition, features of the mature region of the protein are also essential (*4*). The hydrophobic domains of the leader and the mature region form a looped structure that allows the central segment of the protein to translocate across the membrane (*5*).

The major coat protein of bacteriophage Pf3, which infects *Pseudomonas aeruginosa*, is synthesized without a leader peptide (*6*) but has other structural features similar to the M13 coat protein (Fig. 1A). Both proteins have a hydrophobic region of ~20 amino acids that is flanked by an upstream acidic region and a short downstream basic region. However, there is no sequence similarity, either at the nucleotide or amino acid level (*7*).

To study membrane insertion of the Pf3 coat protein, we cloned into an expression vector (pUC9) a 184–base pair (bp) fragment of the Pf3 genome that encodes the major coat protein, and we used the resulting construct to transform *E. coli* (strain JM103). Protein synthesis was induced with isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) and the cells were labeled with [^{35}S]methionine for 3 min. Detergent-solubilized proteins were immunoprecipitated and separated by polyacrylamide gel electrophoresis (PAGE). The Pf3 coat protein was immunoprecipitated with antibodies to Pf3 phage (anti-Pf3) but not with antibodies to M13 (anti-M13) (Fig. 1C). In a pulse-chase experiment, the apparent molecular size (4625 daltons) of the Pf3 coat protein did not change with time—as would be expected in view of the absence of a leader peptide (*8*).

To determine the cellular location of the Pf3 coat protein, we induced its expression from the plasmid pAN-1, which also overproduces a cytoplasmic fragment of ribulokinase (AraB). Cells induced to synthesize the coat protein were labeled with [^{35}S]methionine for 3 min and their outer membrane was permeabilized. Proteinase K was added externally to the cells for various times, and samples were analyzed by PAGE. Outer membrane protein A (OmpA) was digested by the protease as would be expected (Fig. 2A), whereas the cytoplasmic AraB fragment was only digested when the inner

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