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8. The cell line OP9 was maintained in α-modified minimum essential media (α-MEM, Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Whittaker, Walkersville, MD). The same media was used for differentiation induction. The ES cells were maintained with standard procedures, on embryonic fibroblasts with recombinant leukemia inhibitory factor. For differentiation induction, ES cells were seeded onto confluent OP9 cell layers on six-well plates (Nunc, Roskilde, Denmark) at a density of 10^4 cells per well.
9. Both differentiated and undifferentiated cells were trypsinized with 0.25% trypsin for 10 min, suspended with α-MEM supplemented with 20% FCS, and pipetted vigorously until cells became single cells. Then the cells were seeded onto confluent OP9 cell layers on six-well plates at various cell densities.
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11. When the cells were transferred at low density, clusters consisting of even two cells did not appear 4 hours after plating, whereas many clusters emerged 1 day later (*10*).
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25. Preparation of high molecular weight DNA and Southern (DNA) blotting was carried out by standard procedures (*18*). DNA (10 μg) was digested with Eco RI, subjected to electrophoresis in 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled, 0.9-kb Hind III–Xba I JH4 probe (*19*).
26. RNA was prepared by using TRIzol (Life Technologies, Gaithersburg, MD). About 2 μg of total RNA was used for first strand synthesis by using random hexamer (*18*). The forward primer is VH1BACK, AGGT%G%CA%GCTGCAG%GAGTC%TG. Backward primers are CH1RC, AATGGGCACATGCAGATC-TC; and CH1HE, TCAGACAGGGGGCTCTCG (*20*). The complementary DNA was amplified with VH1BACK primer and CH1RC primer at first, and then the polymerase chain reaction (PCR) product was diluted 1000-fold and amplified with VH1BACK primer and internal primer CH1HE. Samples were amplified for 40 cycles under the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.
27. We cultured 10^4 D3 ES cells on OP9 cells for 5 days in the presence or absence of M-CSF as indicated. The numbers of differentiated and undifferentiated colonies were counted. Both differentiated and undifferentiated colonies were trypsinized, and 10^5 cells were transferred onto fresh OP9 cells and cultured for another 5 days in the presence or absence of M-CSF as indicated. Ten days after the initiation of the differentiation induction, the number of clusters were counted. After removal of OP9 cells the number of harvested cells were counted, and cytospin specimens stained with May-Grunwald Giemsa were examined to discriminate between macrophages and nonmacrophages. Data in Table 2 are shown as cumulative numbers yielded from 10^4 ES cells at the initiation of the induction.
28. We thank J. Frampton and T. Graf for critical reading of the manuscript, S. Okazaki for excellent technical assistance, K. Yamamura for the D3 ES cell line, T. Kina for TER-119, S. Nishikawa for IL-7 and S-i. Nishikawa and T. Suda for discussions and encouragement.

31 January 1994; accepted 27 June 1994

Delocalization of Vg1 mRNA from the Vegetal Cortex in *Xenopus* Oocytes After Destruction of Xlsirt RNA

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The Xlsirts are a family of transcribed repeat sequence genes that do not code for protein. Xlsirt RNAs become localized to the vegetal cortex of *Xenopus* oocytes early in oogenesis, before the localization of the messenger RNA Vg1, which encodes a transforming growth factor-β–like molecule involved in mesoderm formation, and coincident with the localization of Xcat2 transcripts, which encode a nanos-like molecule. Destruction of the localized Xlsirts by injection of antisense oligodeoxynucleotides into stage 4 oocytes resulted in the release of Vg1 transcripts but not Xcat2 transcripts from the vegetal cortex. Xlsirt RNAs, which may be a structural component of the vegetal cortex, are a crucial part of a genetic pathway necessary for the proper localization of Vg1 that leads to subsequent normal pattern formation.

Normal development of the vertebrate embryo is dependent on the proper spatial organization of maternally expressed macromolecules in the oocyte. In amphibian oocytes, mRNAs are localized at both the animal and vegetal regions (*1*). The vegetally localized mRNAs include Vg1 (*2*), which encodes a TGF-β–like molecule implicated in mesoderm formation, and Xcat2 (*3*), which encodes a nanos-like molecule. Based on the roles of their putative homologs in *Drosophila*, it is likely that these gene products are involved in axial patterning of the early amphibian embryo. Therefore, unraveling the genetic pathways involved in the localization of these transcripts would give greater insight into how the vertebrate body plan is established.

A group of nontranslatable interspersed repeat transcripts, Xlsirts, is also localized to the vegetal cortex of *Xenopus* oocytes during the early stages of oogenesis and may be a structural component of the cortex involved in the localization of other RNAs (*4*). To determine whether Xlsirts function to localize other RNAs at the vegetal cortex, we injected anti-sense oligodeoxynucleotides (AS ODNs) into stage 4 oocytes (*5*) to destroy the endogenous localized Xlsirt RNAs and analyzed the subsequent distribution of Vg1 and Xcat2 RNAs. Because Xlsirt RNAs do not code for protein, destruction of the RNA with AS ODNs should create a null mutant (*6*, *7*).

The Xlsirt AS ODNs consisted of a mixture of two different phosphothiolated 17-mers. We determined that the optimal dosage was 50 ng [that dose destroyed the Xlsirts without causing nonspecific toxic effects (*8*)]. Xlsirt RNA that was localized at the vegetal cortex was detected in oocytes injected with a control ODN for another maternally transcribed RNA, Xlcaax (formerly Xlgv 7) (*6*, *9*) (Fig. 1A). However, we did not detect any Xlsirt RNA localized at the vegetal cortex by in situ hybridization in oocytes that were injected with Xlsirt AS ODNs and cultured for 3 days (Fig. 1B). Xlcaax ODNs do not produce any nonspecific toxic effects when injected into oocytes (*6*–*8*).

Xlsirts are a heterogeneous population of RNAs consisting of transcripts from both strands of the genes (*4*). Those molecules found localized at the vegetal cortex consist of transcripts from one strand that are referred to as sense strand transcripts. Other Xlsirt RNAs are found throughout the cytoplasm and in the germinal vesicle (GV) and may exist as double-stranded molecules. Xlsirt RNAs appear as a smear on Northern (RNA) blots (*4*, *10*). On Northern blots of RNA isolated from oocytes injected with Xlsirt AS ODNs, we detected a small decrease in the amount of Xlsirt RNA but not the loss of any specific RNA species. This result made it difficult to determine the effi-

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ciency of Xlsirt AS ODNs in destroying the transcripts. We suspected that the residual Xlsirt RNAs were distributed throughout the oocyte and were probably double-stranded forms that were resistant to Xlsirt AS ODN destruction. Therefore, to determine the efficiency of Xlsirt AS ODN destruction of Xlsirt RNA, we tested the ability of AS ODNs to destroy exogenous Xlsirt transcripts. We injected ^{35}S-labeled Xlsirt RNA made from two different Xlsirt complementary DNA (cDNA) clones into stage 4 oocytes along with Xlsirt AS or Xlcaax ODNs. The two exogenous Xlsirt transcripts were destroyed after injection of Xlsirt AS ODN but were unaffected by injection of control Xlcaax ODNs (Fig. 1C). We conclude that the localized endogenous Xlsirt RNAs of stage 4 oocytes were also destroyed by the Xlsirt AS ODNs. This finding was further substantiated by the demonstration that oocytes injected with the Xlsirt AS ODNs showed a loss of the endogenous Xlsirt RNA in their GVs as determined by whole-mount in situ hybridization (Fig. 1D).

We also found that Vg1, but not Xcat2, transcripts were delocalized after destruction of the Xlsirts. Stage 4 oocytes were injected with Xlsirt AS ODNs, cultured for 2 to 5 days, and analyzed by in situ hybridization of oocyte sections with Xlsirt, Vg1, and Xcat2 probes. Oocytes injected with control ODNs showed normal localization of Xlsirt, Xcat2, and Vg1 transcripts at the vegetal cortex (Fig. 2, A, C, E), but in oocytes injected with the Xlsirt AS ODNs there were no detectable Xlsirt and Vg1 transcripts at the vegetal cortex (Fig. 2, B and G). However, injection of the Xlsirt AS ODN did not affect the localization of Xcat2 RNA (Fig. 2D). A time course shows the dispersal of Vg1 transcripts from the vegetal cortex into the cytoplasm after 2 days (Fig. 2F) and 5 days (Fig. 2G). Northern blot analysis of polyadenylated (poly A^+) RNA from Xlsirt AS ODN–injected oocytes showed that the total amount of Vg1 and Xcat2 mRNAs per oocyte was unaffected, indicating that Vg1 mRNA was not degraded after its release from the vegetal cortex (Fig. 2H).

We also analyzed the effect of Xlsirt AS ODN injection using whole-mount in situ detection of Xlsirts, Vg1, and Xcat2 RNAs (Table 1 and Fig. 3). In stage 4 oocytes injected with control Xlcaax ODN, both Xlsirts and Xcat2 transcripts were localized in the cortex as a disk at the apex of the vegetal pole (Fig. 3, A and E), whereas Vg1 transcripts were more dispersed across the vegetal hemisphere extending from the vegetal pole to the marginal zone (Fig. 3C). In oocytes in-

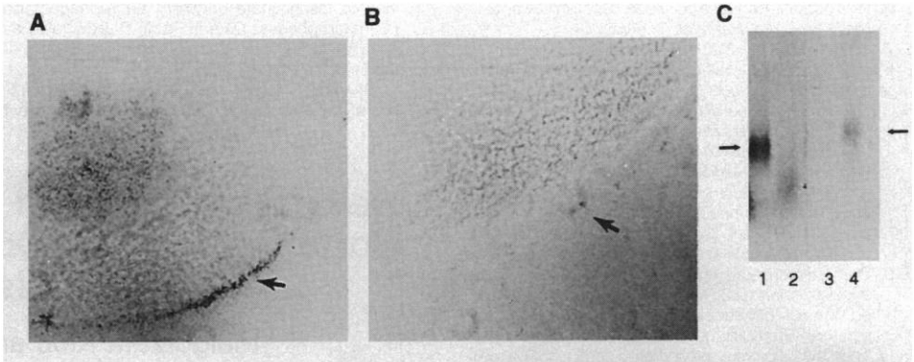

Fig. 1. Xlsirt transcripts localized at the vegetal cortex destroyed by the injection of AS ODNs. In situ hybridization analysis of sectioned stage 4 oocytes that were injected with control ODN (Xlcaax) or Xlsirt AS ODNs by use of a probe against the Xlsirts (*18*). (**A**) Xlcaax ODN injected control oocyte; the arrow indicates the localized Xlsirt RNA. (**B**) Oocyte injected with Xlsirt AS ODNs; the arrow indicates the expected position of the transcripts. (**C**) Autoradiograph of ^{35}S-labeled Xlsirt RNA from oocytes that were injected with Xlsirts AS ODNs (lanes 2 and 3) and from oocytes injected with control Xlcaax ODN (lanes 1 and 4); the arrows indicate the position of the exogenous Xlsirt transcripts. (**D**) Whole-mount in situ hybridization to oocytes injected with Xlcaax ODN (left) and with Xlsirt AS ODN (right). The arrow indicates the position of the GV that was visible after the oocyte was dissected.

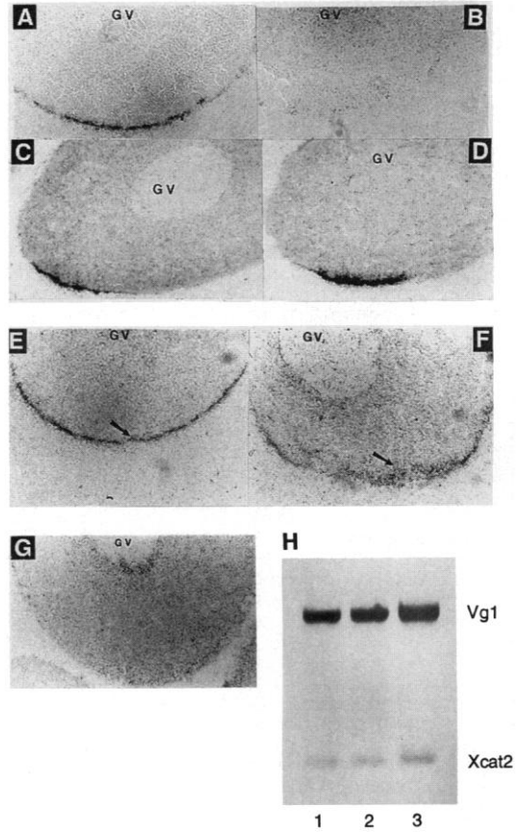

Fig. 2. The destruction of Xlsirt RNA, resulting in delocalization of Vg1 but not Xcat2 RNA. Stage 4 oocytes were injected with Xlsirt AS ODNs. After 2 to 5 days in culture, oocytes were fixed, embedded in paraffin, and sectioned. Sectioned oocytes were hybridized with Xlsirt, Vg1, and Xcat2 probes as described in the legend to Fig. 1 (*18*); GV, germinal vesicle. (**A**) Control oocytes injected with Xlcaax ODN and hybridized with Xlsirt probe. (**B**) Oocytes injected with Xlsirt AS ODNs and hybridized with Xlsirt probe. (**C**) Oocytes injected with Xlcaax AS ODN and hybridized with Xcat2 probe. (**D**) Oocytes injected with Xlsirt AS ODNs and hybridized with Xcat2 probe. (**E**) Oocytes injected with control Xlcaax AS ODN and hybridized with Vg1 probe. (**F**) Oocytes injected with Xlsirt AS ODNs and hybridized with Vg1 probe after 2 days in culture. (**G**) Oocytes injected with Xlsirt AS ODNs and hybridized with Vg1 probe after 5 days in culture. (**H**) Northern blot of Vg1 and Xcat2 mRNA in oocytes injected with Xlsirt AS ODNs. Polyadenylated RNA from Xlcaax and Xlsirt ODN AS–injected oocytes was extracted and separated by electrophoresis on formaldehyde denaturing agarose gel and blotted to the membrane. The blot was probed with digoxigenin-labeled probes against Vg1 and Xcat2 (*19, 20*). The lanes are as follows: lane 1, RNA from oocytes injected with control sham; lane 2, RNA from oocytes injected with Xclaax ODN; lane 3, RNA from oocytes injected with Xlsirt AS ODNs. The Vg1 RNA (2.7 kb) and the Xcat2 mRNA (0.9 kb) are shown. The amount of RNA loaded onto the gel was determined by EtBr staining.

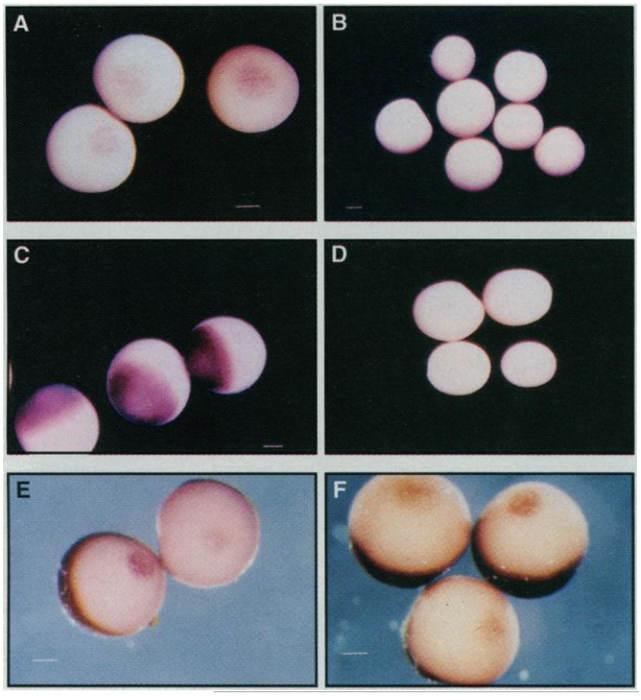

Fig. 3. Whole-mount in situ analysis of Vg1, Xlsirt, and Xcat2 in oocytes injected with Xlsirt AS ODN. Oocytes injected with Xlcaax and Xlsirt AS ODNs were cultured for 3 days and analyzed by whole-mount in situ hybridization with the use of digoxigenin-labeled probes (*20*). (**A, C,** and **E**) Oocytes injected with Xlcaax ODN and probed with Xlsirt, Vg1, and Xcat2 probes, respectively. (**B, D,** and **F**) Oocytes injected with Xlsirt AS ODNs and probed with Xlsirt, Vg1, and Xcat2 probes, respectively. Oocytes in (A) to (D) are albino oocytes; oocytes in (E) and (F) are pigmented. The bars represent 200 μm.

Table 1. The effect of Xlsirt AS ODN injection on the localization of Vg1 and Xcat2. Data are based on three independent experiments. The plus and minus signs indicate oocytes showing and lacking signal at the vegetal cortex, respectively.

ODN injected	In situ probe	Signal at vegetal cortex	
		+	−
Sham injection	Xcat2	27	2
	Xlsirt	41	2
	Vg1	75	2
Xlcaax	Xcat2	31	0
	Xlsirt	23	2
	Vg1	35	0
Xlsirt	Xcat2	24	3
	Xlsirt	0	27
	Vg1	8	63

jected with Xlsirts AS ODNs, no localization of Xlsirts or Vg1 transcripts was apparent (Fig. 3B and D), whereas localization of Xcat2 transcripts was unaffected (Fig. 3F). These data demonstrate that the destruction of Xlsirt RNA by AS ODNs resulted in the delocalization of the Vg1 mRNA but did not affect Xcat2 localization.

Localization of the Vg1 mRNA at the vegetal cortex is a two-step process involving a microtubule-dependent translocation step and a microfilament-dependent anchoring step (*11*). We demonstrated that the Xlsirt RNA was involved in the anchoring of Vg1 transcripts at the vegetal cortex and that AS ODN destruction of these RNAs resulted in the release of Vg1 from the cortex. This result suggests that the Xlsirt RNA and microfilament components of the cytoskeleton are involved in a genetic pathway that is required for the proper anchoring of Vg1 at the cortex.

We do not believe that there is a direct interaction between Xlsirts and Vg1, although Xlsirts may play a fundamental role in the organization of the microfilament cytoskeletal network at the vegetal cortex, perhaps by anchoring cytoskeletal filaments in the disk-like structure formed by Xlsirt RNA at the vegetal pole apex. The destruction of Xlsirts did not affect the anchoring of Xcat2 mRNA, suggesting that this mRNA was anchored by a different mechanism and that the release of Vg1 was not due to nonspecific toxic effects.

Our results are consistent with recent evidence showing that Xcat2, unlike Vg1, is not released from the cortex during oocyte maturation (*12*). They are also consistent with recent findings in *Drosophila* showing that there are different genetic pathways controlling the localization and anchoring of individual mRNAs at the posterior pole of the oocyte (*13–15*).

We recently found that the Xlsirt, Xcat2, and Xwnt11 transcripts are translocated to the vegetal cortex through a mechanism dependent on the Balbiani body (*4*) while Vg1 uses a different mechanism of translocation, suggesting the existence of two independent pathways for translocation of RNAs to the vegetal cortex (*16*). The release of Vg1 by destruction of the Xlsirt RNAs demonstrates a cross talk between the two genetic pathways involved in the localization and anchoring of mRNAs at the vegetal cortex. Because Vg1 is important for mesoderm induction during embryogenesis, it is likely that mislocalization of the Vg1 mRNA by aberrant expression or localization of Xlsirts would have severe consequences for dorsal-ventral patterning of the embryo (*17*).

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18. Stage 4 *Xenopus* oocytes were injected with 50 ng of a mixture of two different anti-sense ODNs or 50 ng of Xlcaax ODN as a control. Xlsirt AS ODN (i): 5′CAGGTATAGTAGGGAGA 3′ and (ii) 5′ TCTCTGGGAAGGGAGTG3′, Xlcaax ODN: 5′CTGCGCTTAGAGAACCC 3′. Oocytes were incubated for various periods of time (*4*) and fixed in 100% methanol overnight, cleared with xylene, embedded in paraffin, and sectioned. De-paraffinated sections were hybridized according to Kloc *et al.* (*4*). Exogenous Xlsirt RNAs were synthesized from two different constructs: Xlsirt p11R-U and Xlsirt p11-R (*4*) in the presence of ^{35}SUTP as described previously (*4*). RNA (1×10^5 counts per minute) in 5 nl was injected into each oocyte along with 50 ng of the two different Xlsirt AS ODNs or 50 ng of the Xlcaax ODN. After overnight culture, total RNA was extracted from 50 oocytes and separated on formaldehyde agarose gels. The gels were incubated in Amplify (Amersham), dried, and exposed to x-ray film.
19. Northern blots were performed with polyA$^+$ RNA (10 μg per lane). Anti-sense RNA probes to Vg1 and Xcat2 were synthesized in vitro with T7 and Sp6 RNA polymerase, respectively, in the presence of digoxigenin-11 uridine triphosphate (UTP) (Boehringer) according to R. Harland [*Methods Cell Biol.* **36**, 685 (1991)].
20. Whole-mount in situ hybridization was done according to Harland (*19*). Anti-sense RNA probes to Xlsirts, Vg1, and Xcat2 were synthesized in vitro with the T3, T7, and Sp6 polymerases, respectively, in the presence of digoxigenin-11 UTP. After alkaline phosphatase staining, the oocytes were post-fixed in 4% formaldehyde and stored in 70% methanol.

21. Supported by grants from the National Science Foundation and the National Institutes of Health (L.D.E.).

25 March 1994; accepted 7 July 1994