A maternal mRNA localized to the animal pole of Xenopus eggs encodes a subunit of mitochondrial ATPase

(determinants)

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ABSTRACT We have previously isolated several cDNA clones of mRNAs that have the unusual property of being localized to either the animal pole or the vegetal pole of frog eggs. To gain insight into the function of these maternal mRNAs we have determined their DNA sequence and deduced the sequence of the proteins they encode. Here we report that An2, an mRNA localized to the animal pole of Xenopus oocytes and eggs, codes for the α chain of mitochondrial ATPase. Furthermore, we compare the intracellular localization of the An2 mRNA and mitochondria in oocytes and eggs and find that they do not have the same degree of localization. In the light of these results we discuss possible reasons for the maternal localization of the An2 mRNA.

Embryologists have often proposed that maternal components deposited in the egg cytoplasm are responsible for specifying cell fates during early development (1). In principle, the differential distribution of cytoplasmic factors to different blastomeres provides a mechanism for distinguishing one daughter cell from another. A variety of studies, including the isolation of blastomeres from different parts of embryos, demonstrate that different regions of the egg and embryo have distinct developmental fates. Though these studies provide indirect support for the existence of cytoplasmic determinants, there are still few examples of well-characterized, localized maternal components that might specify cell fate (reviewed in ref. 2).

We have examined the distribution of one type of potential determinant, maternal RNAs, along the animal–vegetal axis of Xenopus eggs. We identified a rare class of localized mRNAs and have obtained cDNA clones corresponding to some of these messages (3, 4). Four members of this class of maternal RNAs, three RNAs localized to the animal pole (An1-3) and one to the vegetal pole (Vg1) of unfertilized eggs, have been chosen for detailed study. Two of these RNAs (An1 and Vg1) are found only during oogenesis and pregastrula development, whereas An2 and An3 are present at least until the swimming tadpole stage. The isolation of these cDNA clones confirms and extends other studies showing that proteins (5) and translatable RNAs (6) are unevenly distributed along the animal–vegetal axis in frog eggs.

We are focusing on two aspects of these localized mRNAs. First, what, if any, developmental function do these mRNAs or their protein products have? Second, how are these mRNAs localized within a single cell? As a first step toward these goals, we have isolated and sequenced a nearly full-length cDNA clone for An2. The sequence of An2 reveals, somewhat surprisingly, that this localized mRNA codes for the α subunit of mitochondrial ATPase.

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MATERIALS AND METHODS

Materials. SP6 RNA polymerase and RQ1 RNase-free DNase were obtained from Promega-Biotec (Madison, WI). Ribonucleases A and T1 were obtained from Sigma. Klenow polymerase, DNA polymerase, and restriction enzymes were obtained from Promega-Biotec and New England Biolabs. Radioisotopes were purchased from Amersham. Xenopus laevis were purchased from Xenopus I (Ann Arbor, MI).

Sequencing. The DNA sequences of An2 cDNA clones were obtained by using the chain-termination method of Sanger et al. (7). restriction fragments were cloned into M13 vectors (8) and in all cases the sequence of both strands was determined.

Extraction of RNA and DNA from Oocytes and Embryos. Frozen oocytes and eggs were sectioned as described (3). RNA and DNA were extracted with proteinase K digestion and phenol/chloroform extraction as described (9). Large RNA (including mRNA) was separated from small RNAs (e.g., tRNA) and DNA (including mitochondrial DNA) by precipitation with 4.0 M LiCl on ice for 1 hr. After centrifugation for 10 min at 13,000 × g, the pellet containing RNA was washed with 70% ethanol and dissolved in water that had been treated with diethyl pyrocarbonate. The DNA in the supernatant was precipitated with ethanol, washed with 70% ethanol, and resuspended in sterile water.

Living oocytes were manually dissected with watchmaker's forceps to obtain preparations of nuclei and cytoplasm for analysis. In cases in which the nuclei were contaminated with yolk, this was eliminated by peeling off the nuclear membrane with forceps.

DNA and RNA Analysis. Mitochondrial DNA was analyzed by cutting at a unique BamHI site prior to electrophoresis in 1% agarose and Southern transfer (26) to GeneScreen (New England Nuclear-DuPont). Blots were hybridized under conditions already described (10) to nick-translated mitochondrial DNA isolated from pxIM31 (11), a clone containing the full mitochondrial genome of X. laevis. RNA analysis was performed as described (12) using SP6-generated antisense transcripts. The 32P-labeled RNA probes for histone H4, An2, and Vg1 transcripts were 450, 390, and 370 nucleotides long, respectively. Densitometry of the autoradiograms was performed with an LKB Ultrascan densitometer.

Computer Analysis. Sequence analysis was carried out on an IBM-AT computer using Microgenie software from Beckman. Protein data base from the National Biomedical Research Foundation was supplied with the Beckman software.

RESULTS

Sequence Analysis Suggests Homology to Mitochondrial ATPase. An2 mRNA isolated from Xenopus eggs was previously shown to be about 1.9 kilobases (kb) long (3). The original 1.6-kb An2 cDNA isolate was used as a probe to rescreen an oocyte cDNA library for longer clones of the same gene. One of the clones isolated, designated An2.1, is...
1.83 kb long or 96% of the estimated size of the full-length An2 mRNA.

The restriction map, DNA sequence, and protein encoded by An2 are presented in Fig. 1. We note that the cDNA clone lacks a poly(A) tract and the consensus 3' processing signal (AATAAA), though we have shown that An2 RNA is present in the poly(A)⁺ fraction of egg RNA (3). This suggests that at least part of the nucleotides missing from the An2.1 cDNA clone are from the 3' end. The proposed protein encoded by An2 begins at nucleotide 44 and ends at nucleotide 1679 to give a polypeptide with a deduced molecular mass of 58.9 kDa. There are no other open reading frames longer than 70 amino acids.

The An2 protein was compared to the protein sequences contained in the National Biomedical Research Foundation protein data bank. The only significant matches that appeared were with subunits of proton-translocating ATPases. Most striking were the homologies (>50%) between An2 and the α-chains of F₅/F₆-type ATPases from tobacco chloroplast and *Escherichia coli* (285/491 amino acids for tobacco chloroplast and 168/287 for *E. coli*). In addition, there are two regions that share >70% amino acid homology. These two highly conserved regions are thought to be involved in ATP binding (3), one of the activities of the α-subunit of mitochondrial ATPases. The specific residues conserved in ATP binding sequences are indicated by the boxed sequences in Fig. 2a.

The sequence data also reveal the presence of a putative mitochondrial transport signal in the An2 protein. These signals have no canonical amino acid sequence but seem to follow three rules: (i) the transport signals are enriched for positively charged residues, more or less periodically spaced, (ii) they are also enriched for hydroxylated amino acids, and (iii) they lack negatively charged amino acid residues (14).

In all animals so far studied, the α subunit of mitochondrial ATPase is encoded by the nuclear rather than the mitochondrial genome (14). An2 shows no sequence homology to the published *Xenopus* mitochondrial DNA sequence (15). Moreover, when An2 DNA is used to probe isolated *Xenopus* mitochondrial DNA no signal is detected. Finally, a genomic Southern blot using An2 as a probe confirms that An2 is represented in the *Xenopus* nuclear genome (data not shown).

**Comparative Localization of Mitochondria and An2 mRNA in Oocytes and Eggs.** Identification of the An2-encoded protein as the α subunit of a mitochondrial ATPase raises the question of whether mitochondria are similarly localized in the animal pole of oocytes and eggs. Coordinate localization of mRNAs encoding mitochondrial proteins with the mitochondria would help to explain the regional distribution of An2. To assay the distribution of mitochondria, frozen *Xenopus* oocytes and unfertilized eggs were manually dis-
BamHI site, Developmental Biology: was fractions was separated with bridized onto Vgl mRNAs. The results from presenten GeneScreen which examined homology (11). An2 Mitochondrial animal b by concentrated membranes. The results An2, E. coli ATPase a chain, and tobacco chloroplast ATPase a chain. Asterisks indicate shared homology between all three sequences. ATP binding domains (13) are enclosed in boxes. (b) Amino terminus of deduced An2 protein. Positively charged amino acids are enclosed in boxes; hydroxylated amino acids are underlined.

**Fig. 2.** Protein homology between An2 and ATPases. (a) Protein homology between An2, E. coli ATPase a chain, and tobacco chloroplast ATPase a chain. Asterisks indicate shared homology between all three sequences. ATP binding domains (13) are enclosed in boxes. (b) Amino terminus of deduced An2 protein. Positively charged amino acids are enclosed in boxes; hydroxylated amino acids are underlined.

sected into an animal pole third or a vegetal pole third or were undissected. In these three fractions, mitochondrial DNA was separated from large RNAs by precipitation of the RNA with 4 M LiCl. Mitochondrial DNA was cut at a unique BamHI site, fractionated by agarose gel electrophoresis, and blotted onto GeneScreen membranes. The filters were hybridized with a 32P-labeled probe made by nick- translating plasmid pXIM31, which contains the entire Xenopus mitochondrial genome (11). RNA isolated from the various fractions was examined by RNase protection assays using 32P-labeled antisense RNA probes for histone H4, An2, and Vgl mRNAs.

The results presented in Fig. 3a show that An2 mRNA is significantly more concentrated in the animal than are mitochondria. Quantitation of the autoradiograms by densitometer tracings (Fig. 3b) shows that the mitochondria are slightly concentrated in the animal pole with an animal pole/vegetal pole ratio of 1.5 in oocytes and 3 in eggs. This slight gradient closely parallels the distribution of histone H4 RNA. In fact, most cellular components other than yolk, including total poly(A)+ RNA (16), are slightly more concentrated in the animal pole. In contrast, An2 mRNA shows a 4- to 8-fold animal pole localization in oocytes and a 10- to 15-fold enrichment in the animal pole of eggs. The increased localization of mitochondrial DNA, An2 mRNA, and histone H4 mRNA in eggs relative to oocytes may be a consequence of oocyte maturation. A control for these experiments, Vgl mRNA, has the opposite orientation for its enrichment and is about 20 times more abundant in the vegetal pole in eggs and oocytes. We conclude that An2 mRNA, which most probably

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**An2**

| An2 | 185 | Ser Val Arg Glu Pro Met Gln Thr Gly Ile Lys Ala Val Asp Ser Leu Val Pro Ile Gly |
| Esherichia coli | 141 | Ser Val Asp Glu Pro Val Gln Thr Gly Tyr Lys Ala Val Asp Ser Met Ile Pro Ile Gly |
| Tobacco chloroplast | 142 | Ser Val Tyr Gln Pro Leu Gln Thr Gly Leu Ile Ala Ile Asp Ser Met Ile Pro Ile Gly |

**Esherichia coli**

| An2 | 263 | Tyr Thr Ile Val Val Ser Arg Thr Ala Ser Asp Ala Ala Pro Leu Gln Try Leu Ala Pro |
| Esherichia coli | 220 | Asn Thr Ile Val Val Ala Thr Ala Ser Glu Ser Ala Ala Leu Gln Try Leu Ala Pro |
| Tobacco chloroplast | 221 | Tyr Thr Ile Val Val Ala Glu Thr Ala Asp Ser Pro Ala Thr Leu Gln Try Leu Ala Pro |

**Tobacco chloroplast**

| An2 | 263 | Tyr Thr Ile Val Val Ser Arg Thr Ala Ser Asp Ala Ala Pro Leu Gln Try Leu Ala Pro |
| Esherichia coli | 220 | Asn Thr Ile Val Val Ala Thr Ala Ser Glu Ser Ala Ala Leu Gln Try Leu Ala Pro |
| Tobacco chloroplast | 221 | Tyr Thr Ile Val Val Ala Glu Thr Ala Asp Ser Pro Ala Thr Leu Gln Try Leu Ala Pro |

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codes for a subunit of mitochondrial ATPase, is more concentrated in the animal pole than are mitochondria.

**An2 mRNA Is Not Localized in the Germin al Vesicle.** We have begun to investigate how the animal localization of An2 RNA might be accomplished. One possibility is that the An2 RNA is sequestered in the oocyte’s nucleus or germin al vesicle, as has been demonstrated for specific histone mRNAs in sea urchin oocytes (17). It has been proposed that the nuclear location of the histone mRNAs may prevent translation until the nuclear membrane is dissolved. Because the nucleus of full-grown frog oocytes lies in the animal pole, localization of An2 mRNA inside the nucleus could account for its animal pole localization. We have dissected stage VI oocytes and analyzed the distribution of the An2 mRNA in nuclei and cytoplasm. The RNase protection assays (Fig. 4) reveal that the An2 mRNA is found almost entirely in the cytoplasm. In situ hybridization experiments (18) have already shown that histone mRNA is mainly found in the cytoplasm of oocytes as opposed to the nucleus. Similarly, using RNase protection assays (Fig. 4), histone H4 mRNA is predominantly found in the cytoplasm. We note a faint band detected with the histone probe in the nuclear RNA. This may be due to unprocessed histone transcripts and/or readthrough transcripts known to occur in lampbrush chromosomes (19). In any case, we conclude that the animal pole localization of the An2 mRNA is not due to sequestration in the nucleus.

**DISCUSSION**

The sequence of the An2 cDNA allows us to identify its protein product as a Xenopus mitochondrial ATPase α chain. The mitochondrial ATPase, of which the α chain is a part, is responsible for the generation of ATP from ADP and P_i and also for the generation of an ATP-driven electrochemical gradient (20). The protein encoded by An2 is the proper size to be the α chain of this mitochondrial ATPase, is strikingly homologous to other α-chain sequences, and has a suitable amino terminus for a mitochondrial import signal sequence. Final proof of this assignment must await the production of antibodies to assay for the An2 protein in mitochondria.

The reason for the strong animal pole localization of the An2 mRNA is not clear. As noted above, An2 mRNA is localized in a much steeper gradient than are mitochondria. In this regard, it may be helpful to recall some details about the biogenesis of mitochondria in frog development. All of the mitochondria found in eggs and early embryos are of maternal origin; indeed they are synthesized in pre-stage IV oocytes (21). There is no new synthesis of mitochondria until the tadpole stage (stage 32, ref. 22), some 2 days after fertilization. In oocytes, mitochondria appear to be synthesized in the so-called mitochondrial cloud, which is located on what will eventually become the vegetal pole side of the nucleus. In addition, some mitochondria are produced elsewhere in the cytoplasm at discrete “foci of proliferation” (23). We do not know if the An2 mRNA is translated or localized in early oocytes. Nevertheless, one can speculate that mRNAs coding for mitochondrial proteins would be positioned near the mitochondrial cloud but excluded from the vegetal pole side by the mass of the mitochondrial cloud itself.

Another possibility is that An2 mRNA is localized to accomplish the generation of a respiration gradient during development. There is an animal–vegetal gradient of respiration in developing embryos (24). By middle to late blastula, cells in the animal pole of the embryo are more actively respiring than those in the vegetal pole, and dorsal cells are more active than ventral cells. In axolotls, changes in the structure of mitochondria, including expansion of inner mitochondrial surface area during early embryogenesis, can be correlated with the spatial differences in respiration described by Brachet (24). The largest inner mitochondrial surface areas are found in the animal pole cells (25). Regional differences in respiration, and therefore ATP synthesis, correspond to the higher rate of cell division in the animal pole and also with the prospective energy needs related to motility of these cells during gastrulation. Neither new mitochondria nor new mRNA for the α subunit of mitochondrial ATPase (An2) is synthesized while this gradient forms (3, 22). In light of previous reports (23) and the mitochondrial localization data presented above, differences in mass of mitochondria seem an unlikely mechanism for the establish-
ment of the respiration gradient. We have previously shown that the animal localization of An2 mRNA is maintained during the cleavage divisions and the message is preferentially segregated to the animal pole cells of blastula. One can therefore speculate that the regional activation of mitochondria that is responsible for the respiration gradient is mediated by translation of localized An2 mRNA. This regional production of the α subunit of the ATPase could preferentially activate mitochondria in the animal region.

One tends to think that likely candidates for maternal cytoplasmic determinants are factors that directly affect gene expression. These would include factors that affect gene transcription, mRNA translation, or protein processing and activities in a region-specific manner. And perhaps examples of these types of factors will be identified as the characterization of localized molecules continues. At the same time, the identification of the protein product of An2, the α subunit of the mitochondrial ATPase, provides an example of another type of molecule that may serve as a local determinant. These molecules, by affecting processes like respiration, ion flow, or metabolism, might regionally alter the physiological state of blastula cells. Such changes in cell physiology may enable this type of localized molecule to exert an effect on prospective cell fate.

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