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Isolation and Characterization of Two Novel

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Mouse Maternal RNAs

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During early development in mouse and *Xenopus,* translational activation of stored maternal mRNAs by cytoplasmic polyadenylation requires both the nuclear polyadenylation signal AAUAAA and U-rich *cis*-acting adenylation control elements (ACEs), also termed cytoplasmic polyadenylation elements, located in the 3′ UTR. Using an ACE-based PCR strategy (Sallés *et al.,* 1992) we have isolated two novel cDNAs from mouse oocytes: OM2a and OM2b (for Oocyte Maturation). Each message contains an ACE consensus sequence upstream of AAUAAA, is specifically transcribed in the growing oocyte, and is cytoplasmically polyadenylated upon oocyte maturation. Comparison of the mouse and rat homologs reveals considerable nucleotide sequence homology and conservation of overall gene organization. However, the predicted open reading frames are far less conserved, suggesting that these genes may not be functioning as proteins. The tissue specificity and tight temporal regulation of the RNAs suggest a role for these genes during early development.
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INTRODUCTION

During oocyte growth, many maternal mRNAs are produced and stored in a dormant state until their translation is triggered by specific developmental events (Davidson, 1986). One of the key mechanisms employed across the animal phyla for translational regulation of maternal mRNAs is cytoplasmic polyadenylation (Wickens, 1992). A number of maternal mRNAs essential for successful oogenesis and early embryogenesis, e.g., *bicoid* in *Drosophila* and *c-mos* in mouse and *Xenopus,* have been shown to require cytoplasmic polyadenylation for translational activation (Gebauer *et al.,* 1994; Sallés *et al.,* 1994; Sheets *et al.,* 1995). However, translation of *c-mos* alone is not sufficient for normal oocyte maturation in vertebrates (Yew *et al.,* 1992), suggesting that translational activation of additional maternal mRNAs is required for completion of oogenesis and early embryonic events. We were interested in identifying novel mouse maternal mRNAs that are translationally regulated by cytoplasmic polyadenylation and possibly required for oocyte maturation, fertilization, and early embryogenesis.

Analysis of maternal mRNAs regulated by cytoplasmic polyadenylation has led to the characterization of *cis*-acting sequences necessary for this type of translational regulation. Specifically, cytoplasmic polyadenylation in *Xenopus* and mouse requires the nuclear polyadenylation sequence AAUAAA and an adenylation control element (ACE) (Fox *et al.,* 1989; McGrew *et al.,* 1989; Vassalli *et al.,* 1989; McGrew and Richter 1990; Huarte *et al.,* 1992; Sallés *et al.,* 1992). Analysis of several U-rich ACEs has lead to the determination of an ACE consensus sequence (Fox *et al.,* 1989). We utilized the conserved nature of the ACE, in an anchored RT–PCR-based strategy (Sallés *et al.,* 1992), to identify novel maternal mRNAs from mouse oocytes that may be translationally regulated by cytoplasmic polyadenylation.

Here we describe the sequence, structure, and temporal and spacial expression of OM2a (Oocyte Maturation) and OM2b RNAs cloned by the ACE RT–PCR strategy. Each RNA contains the ACE consensus sequence upstream of the nuclear polyadenylation signal and is cytoplasmically polyadenylated significantly during oocyte maturation. Cytoplasmic polyadenylation of the OM2 RNAs is suggestive of translational activation. The two OM2 genes are very similar in length and have high homology at the nucleotide level, indicating that they arose from the duplication of a single ancestral gene. Both genes are transcribed specifically in the mouse oocyte and so represent two of a fairly small

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group of genes known to be transcribed in an oocyte-specific expression pattern (Richoux *et al.,* 1991; Schickler *et al.,* 1992; Lira *et al.,* 1993). Moreover, the mouse and rat OM2 genes share considerable nucleotide sequence homology and conservation of overall gene organization. However, the potential open reading frames are less conserved, suggesting that these genes may not be functioning as proteins.

MATERIALS AND METHODS

General Methods

All general subcloning and electrophoresis techniques were as described in Sambrook *et al.* (1989). Collection, culture, and injection of oocytes were performed as described (Sallés *et al.,* 1993). RNA was isolated from oocytes by a modification of the method of Chomczynski and Sacchi (1987) and reverse transcribed. Synthetic, capped RNAs were synthesized in either the sense or antisense orientation and prepared for injection as described by Strickland *et al.* (1988) except that either T7 or T3 RNA polymerase was used (Ambion MEGAscript kit).

Isolation and Sequence Analysis of cDNA and Genomic Clones

A 65-bp partial cDNA clone for OM2a was obtained as previously described (Sallés *et al.,* 1992). The full-length cDNA clone of OM2a was isolated from a mouse ovary library (donated by Paul Wassarman) using random-primed probes generated from the partial OM2a cDNA clone (Sambrook *et al.,* 1989). OM2b cDNA, rat cDNAs, and rat genomic clones were obtained by PCR amplification from primary oocyte cDNA or genomic DNA. Subsequently, the PCR products were cloned into the pCR II vector (Invitrogen). OM2a and OM2b P1-bacteriophage genomic clones were obtained using labeled, genomic-PCR probes (Genome Systems, Inc.). The Genetics Computer Group sequence analysis software package was utilized for sequence homology alignments and database searches (Devereux *et al.,* 1984).

Nucleotide sequencing was performed as described using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, OH) or the Taq DyeDeoxy Terminator cycle sequencing kit with a Model 370A automatic DNA sequencer (Applied Biosystems, Inc., CA). All cDNA regions were sequenced at least three independent times, and exon regions from genomic clones were sequenced at least two times.

PCR Poly(A) Test

The PCR poly(A) test was performed as described (Sallés and Strickland, 1995). The following primers were used in this experiment: OM2b forward primer, 5′-TTTGAGGCCGGAACTATTGCTATAC; OM2a forward primer, 5′-AGGATTGCGTGGTCAGCATGATTCG; reverse oligo(dT) primer/adapter, 5′-GCGAGCTCCGCGGCCGCGT$_{(12)}$.

Northern Analysis and Transcript Synthesis

Northern analysis (Carroll *et al.,* 1993) was performed using radiolabeled antisense transcripts prepared (Strickland *et al.,* 1988) from 500 bp of the 3′ end of the OM2a cDNA clone. Transcripts with a specific activity greater than 10^8 cpm/μg were used in Northern analysis.

Isolation and RT–PCR Amplification of Tissue RNA

RNA was isolated (Chomczynski and Sacchi, 1987) from various tissues and reverse transcribed. Tissue cDNAs were amplified with forward (5′-AGGATTGCGTGGTCAGCATGATTCG) and reverse (5′-CCCCACTTAAAAGCTGGACATGGCA) primers to OM2a, or with forward (5′-TCATTTAAGAGGACGCCTGTCCAGG) and reverse (5′-CTGGTAAAACCCCCTCCCCACCTGTCC) primers having 100% homology to both OM2a and OM2b. Control actin primers: 5′-AAGAGGAGGATGGTCGCGGTCCATGC, 5′-CTGCCTCAACACCTCAACCCCCTCC.

In Situ Hybridization

In situ hybridization on 6-μm paraffin sections of mouse tissues (Frohman *et al.,* 1990) was performed using a ^{33}P-labeled antisense RNA probe corresponding to the entire cDNA of OM2a.

Interspecies Southern Blot Analysis

Southern blot hybridization was performed to determine conservation of the OM2 genes through evolution. Ten micrograms of each genomic DNA sample was digested with both *Eco*RI and *Pst*I restriction enzymes and analyzed by Southern analysis (Sambrook *et al.,* 1989).

Chromosomal Mapping and Organization

Chromosomal mapping and Southern analysis were performed using random-primed probes to OM2a. PCR confirmation of the distance between the two OM2 genes was performed with Stratagene's Taq Extender Additive.

Antisense Injections

The following are the antisense oligodeoxynucleotides used in oocyte injections: 5′-CTGGTAAAACCCCCTCCCCACCTGTCC, 5′-GATCTGGAGCTTGGCATCATGGTCA, 5′-TGGCAAAGGACATGTGAACAACGGG, 5′-CAGGCTCTGGCTCGATTTCGCTTGGTCCGGAG, 5′-AGCATTGTAGACTCCAGGTACAGAC.

RESULTS

Cloning of Maternal mRNAs from the Mouse

A primer homologous to the ACE was used in an anchored PCR strategy to amplify partial cDNAs from mouse oocytes that contain this sequence (Frohman *et al.,* 1988; Loh *et al.,* 1989; Sallés *et al.,* 1992). One of the clones was a 65-nucleotide partial clone of the OM2a 3′ UTR. This partial clone was utilized to obtain the full-length OM2a cDNA

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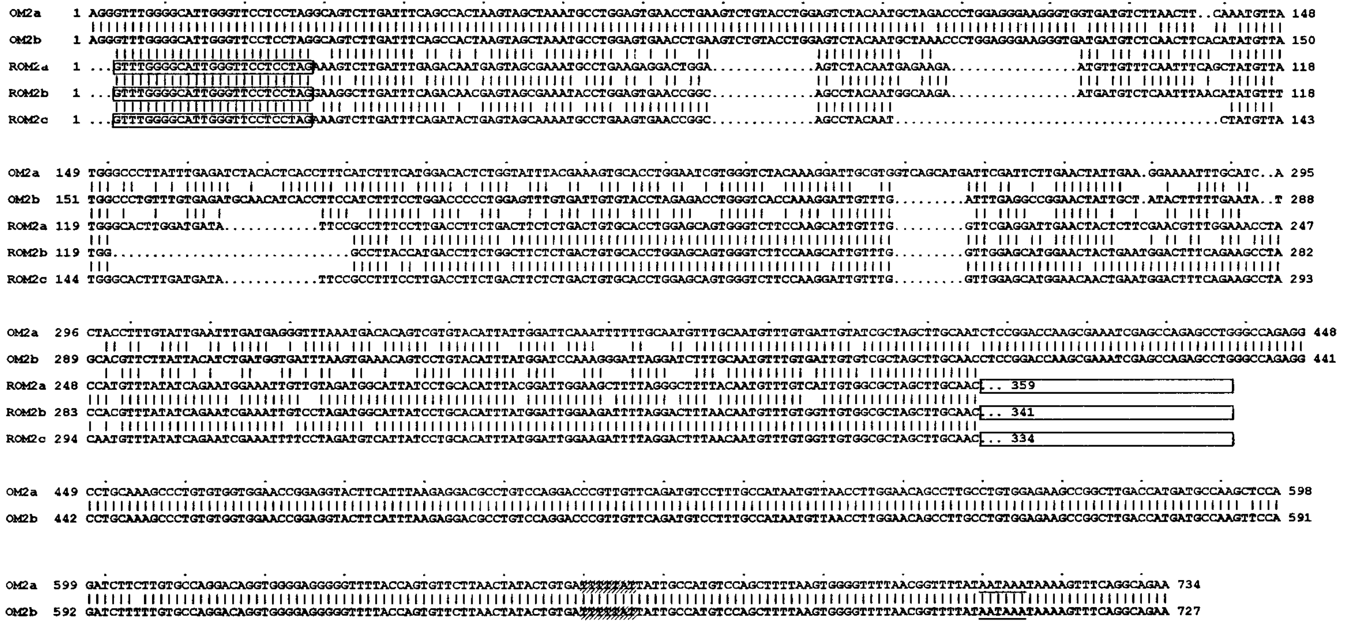

FIG. 1. Alignment of mouse and rat OM2 transcripts. Vertical lines (|) represent identical residues between the transcripts. Dots (.) represent gaps placed in the sequence to optimize alignment. ACE homologous sequences are shown in stippled boxes. Nuclear polyadenylation signals are underlined. Mouse primers utilized to amplify the rat sequences are boxed and may not be the actual sequence of the rat genes in this region.

clone by screening an ovary cDNA library (provided by Paul Wassarman). Clones for both OM2a and OM2b were also obtained by cloning RT–PCR products (see Materials and Methods). cDNA clones of 734 and 727 bp were obtained for OM2a and OM2b, respectively. The clones are the products of different, yet homologous genes (Fig. 1). Each clone contains the canonical nuclear polyadenylation signal AAUAAA downstream of a U-rich sequence identical to previously characterized ACE elements. The clones are 90% identical at the nucleotide level. Additionally, genomic clones for both genes were obtained by screening a P1-bacteriophage vector library (Genome Systems, Inc.) and by cloning mouse genomic PCR products (see Materials and Methods). Comparison of the genomic and cDNA sequences revealed that each gene contains four exons (see Fig. 2).

Genomic Organization of OM2 Genes

Analysis of OM2 genomic clones indicated that both OM2a and OM2b are contained within one bacteriophage P1 vector clone (Genome Systems, Inc.). P1 clones typically contain inserts between 75 and 100 kilobase pairs (kb), suggesting that both OM2 clones are within this distance of each other. Southern and PCR analysis of these clones and mouse genomic DNA (data not shown) indicates the two genes (each with four exons) are separated by approximately 11 kb and are arranged in a head-to-head orientation (Fig. 2).

PCR Test for the Polyadenylation State of the OM2 Transcripts

To determine whether OM2a or OM2b exhibits changes in poly(A) status during oocyte maturation, the PCR poly(A) test (Sallés and Strickland, 1995) was utilized on cDNA from primary (O_I) and secondary (O_{II}) oocytes. The test was applied to each message individually with message-specific, forward primers and oligo(dT) primer/adapter. The size of the PCR products in this assay reflects the poly(A) tail length of the RNA. In primary oocytes, both OM2a and OM2b have a poly(A) tail of approximately 70 nucleotides (nt). In secondary oocytes the PCR poly(A) test detects adenylation of greater than 500 nt for both RNAs (Fig. 3A).

Northern Analysis of OM2

To further establish elongation of the OM2 RNAs upon oocyte maturation, Northern analysis with a radiolabeled antisense transcript to OM2a was performed. A single band of 0.75 kb could be detected in O_I RNA. This species exhibited slower mobility in the O_{II} lane (~1.4 kb) (Fig. 3B). Due to the high percentage of identity between OM2a and OM2b, the probe is likely to have hybridized to both RNAs.

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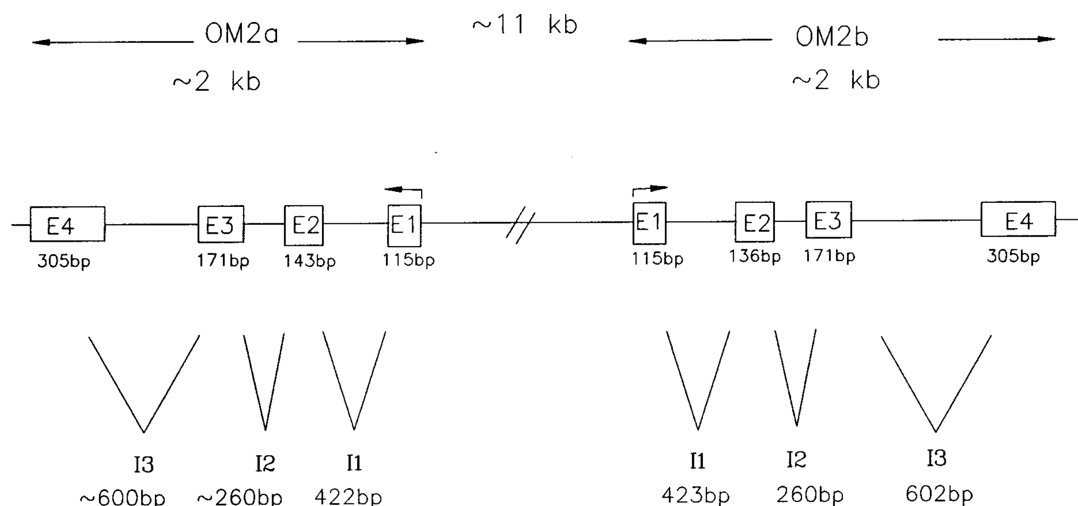

FIG. 2. Schematic representation of the genomic organization of OM2a and OM2b derived from cDNA and genomic clones. Exons are boxed and labeled E1 through E4. Introns are represented by single lines and labeled I1 through I4. The arrows above E1 indicate direction of transcription of the genes. Note the genes are transcribed away from each other on different DNA strands.

Additionally, due to the similar size of the clones, detection of only one band was expected. These data suggest that the OM2 clones are full length and become polyadenylated by 600–700 residues, confirming the significant polyadenylation seen with the PCR poly(A) test. No hybridization was seen with Northern analysis of granulosa, cumulus, and

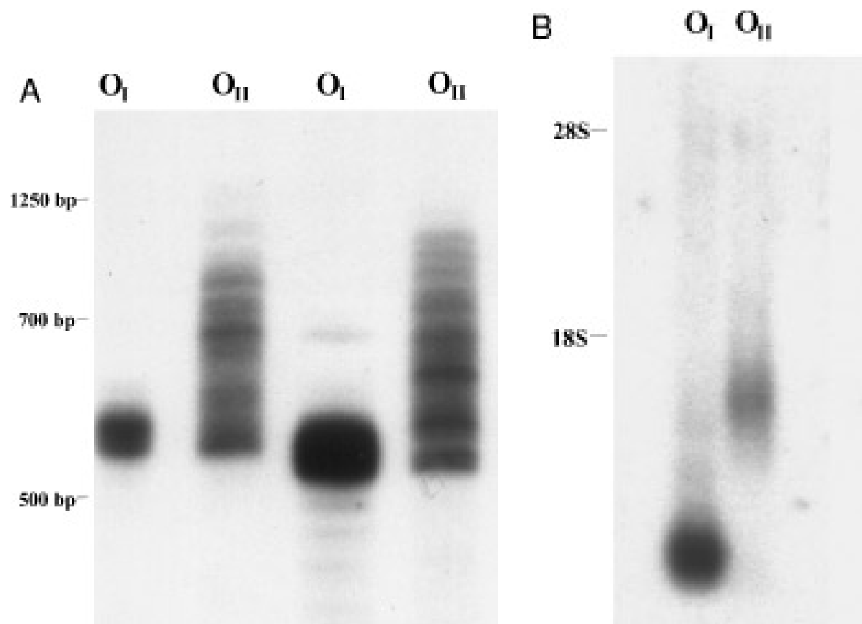

FIG. 3. Poly(A) length and Northern blot analysis of OM2 RNAs: (A) The radiolabeled PCR poly(A) test of the OM2 transcripts. Shown is an autoradiograph of PCR-amplified products demonstrating polyadenylation of OM2a and OM2b upon maturation. (O_I) Primary oocytes; (O_{II}) secondary oocytes. One oocyte equivalent of PAT cDNAs was amplified per time point. Each lane contains 25% of the respective PCR products. The no-template controls were negative in all cases (data not shown). The minimum expected size of the amplified products is 504 bp [474 bp OM2b 3′ end + 30 bp oligo(dT)-anchor] for OM2b and 532 bp for OM2a. (B) Northern analysis of OM2 RNA. RNA preparations were from 370 primary oocytes (O_I) and 370 secondary oocytes (O_{II}). The migrations of the 28S and 18S RNAs are indicated.

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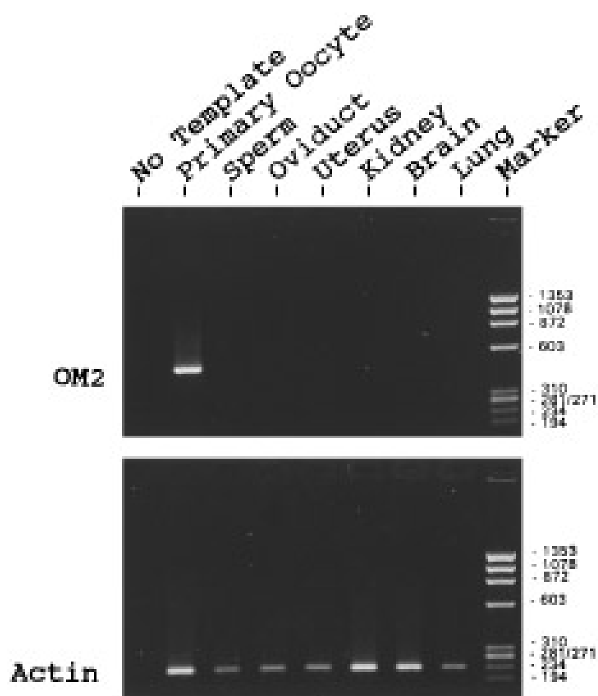

FIG. 4. RT–PCR analysis of OM2 RNA expression from various mouse tissues. PCR products are visualized by ethidium bromide staining during agarose gel electrophoresis. (Top) Tissue cDNAs were amplified with primers to OM2a. A 457-bp PCR product is expected. (Bottom) The same tissue samples amplified in parallel with actin primers.

undifferentiated or differentiated F9 teratocarcinoma stem cells (data not shown), demonstrating that the signal detected in the oocyte samples is not the result of follicle cell contamination.

Tissue Expression Analysis of OM2 RNA

The expression pattern of OM2a in several major tissue types was further characterized using RT–PCR analysis with OM2a-specific primers (Fig. 4). The expression of OM2a was restricted to the oocyte; based on comparison with the mRNA for tissue plasminogen activator (Huarte *et al.,* 1987), we estimate approximately 5000 to 10,000 molecules of OM2 RNA per oocyte. As shown, there was no detectable OM2a amplification from adult sperm, oviduct, uterus, kidney, brain, or lung cDNA. Additionally, no amplification was seen from muscle, spleen, heart, pancreas, liver, seminal vesicle, or testis cDNA (data not shown). Identical results were obtained with primers directed to the OM2b cDNA (data not shown). As an internal control for reverse transcription, the various tissue cDNAs were amplified in parallel with actin primers.

To confirm the oocyte-specific expression pattern of OM2, *in situ* RNA hybridization was performed on adult mouse ovary sections. OM2 transcripts were highly expressed in the cytoplasm of growing or fully grown oocytes and are not apparent in nongrowing oocytes (Fig. 5). This observation suggests that the OM2 genes are transcribed during the growth phase of primary oocytes. Hybridization was slightly above background in the immediately surrounding follicle cells; however, we have not been able to detect OM2 RNA by Northern analysis from granulosa cells, so that the significance of this signal is unclear. No specific hybridization to any tissue type was observed by *in situ* analysis on whole 17-day mouse embryo sections. OM2 transcripts were not detectable in testes sections by *in situ* analysis (data not shown). These *in situ* hybridization results combined with the PCR data suggest oocyte-specific transcription of the OM2 genes.

Cloning of the Homologous Rat OM2 Genes

Analysis of the cDNA clones for the OM2 genes did not reveal a conserved open reading frame (ORF). Sequences surrounding the first ATGs do not conform to the Kozak consensus and thus are probably not the translation start sites, while the nucleotides surrounding the putative translational initiation sites, on downstream ATGs, partially conform to the Kozak consensus (Kozak 1991). *In vitro* transcription/translation was used to confirm the predicted open reading frames of the OM2 cDNA clones. Only OM2a produced a detectable product. The size of the product (data not shown) is consistent with a 59-aa open reading frame starting from the second ATG, which conforms to the Kozak consensus better than the first ATG (Kozak, 1991). However, this OM2a polypeptide does not have a homologous OM2b counterpart. The cDNA sequence for OM2b gave a predicted open reading frame of 119 aa. The hypothetical polypeptides for OM2a and OM2b are not homologous due to translation from start codons in different reading frames (Fig. 1).

To shed light on the differences between these sequences, we evaluated the conservation of these genes through evolution. An interspecies Southern blot was hybridized with a mouse OM2a probe (Fig. 6). This analysis revealed hybridization to mouse, rat, and *Drosophila pseudoobscura* DNA. No hybridization was detected in any other species tested. The presence of a homologous rat gene offered the opportunity to analyze conservation of the sequence in detail.

Three genes were isolated from rat genomic DNA by PCR using mouse OM2 primers. Additionally, RT–PCR was utilized to obtain the cDNA clones from rat oocytes. The sequences of two of the rat cDNA clones show open reading frames that encode hypothetical polypeptides with minor homology to the one predicted for mouse OM2b (Fig. 7). The identity between rat and mouse genes is higher at the nucleotide (~70%) than the amino acid level (~35%). No homologues of any of the OM2 cDNAs or ORFs were apparent in the major data banks, nor do any of the ORFs contain any obvious peptide motifs suggestive of function.

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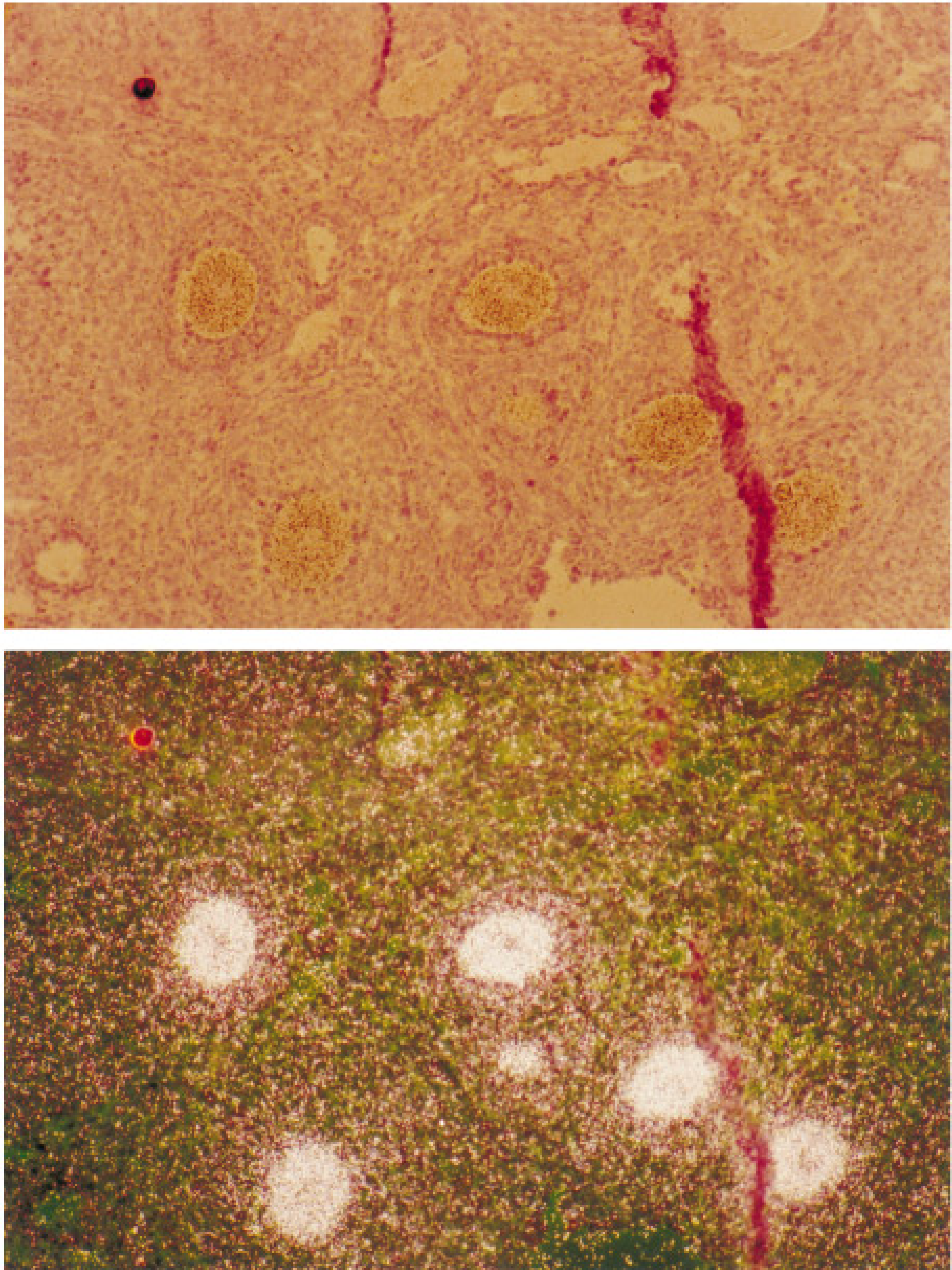

FIG. 5. *In situ* RNA hybridization on mouse ovaries probed with antisense OM2a RNA. (Top) Light-field photomicrograph of a sectioned adult ovary. (Bottom) Dark-field photomicrograph of the same ovary section. Points of light are silver grains that have been exposed to the radiolabeled antisense transcript.

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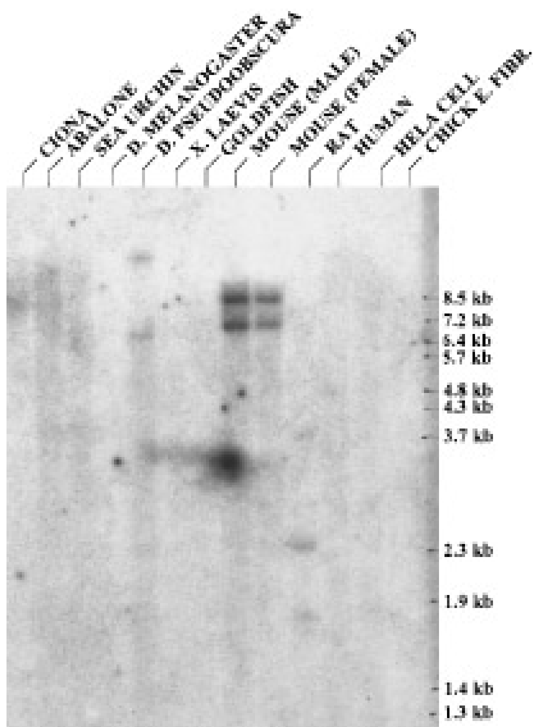

FIG. 6. Interspecies hybridization of genomic DNA to OM2 cDNA (see Materials and Methods).

Functional Analysis of OM2 RNAs

Chromosomal mapping was performed with a full-length OM2a probe in order to determine if OM2a and OM2b comap with any previously known mutations in the mouse (D. Gilbert and N. A. Jenkins, unpublished observations). Both genes appear to be located in the middle region of mouse chromosome 9 distal to dilute (*d*) and proximal to Snell's Waltzer (*sv*), a region homologous to human chromosomal segment 6q. These genes do not appear to comap with any previously characterized mutations.

A routine method to analyze loss of function of maternal mRNAs in the oocyte is antisense oligodeoxynucleotide or RNA-mediated mRNA destruction (Sagata *et al.,* 1988; Strickland *et al.,* 1988). Microinjection of antisense oligodeoxynucleotides or RNA into fully grown oocytes leads to hybrid formation and RNAse mediated cleavage of the endogenous message (Strickland *et al.,* 1988; Sallés *et al.,* 1992). Such modifications of the mRNA in oocytes are irreversible and sufficient to prevent polyadenylation and translational activation of the message during meiotic maturation of oocytes. If cleavage is successful, the endogenous mRNA cannot be replenished due to the lack of transcription in maturing oocytes. The efficiency of mRNA destruction may vary for different transcripts, depending on the accessibility of a given antisense sequence to its complementary region on the RNA. To further address the role of OM2, primary oocytes were injected individually with one of five different antisense oligodeoxynucleotides or a full-length, capped, antisense RNA. Four of the oligodeoxynucleotides were 100% identical to conserved regions in both OM2a and OM2b. The antisense RNA was 100% identical to the full OM2a transcript and 100% identical to long stretches at the 5′ and 3′ termini of OM2b. Subsequent *in vitro* culture of the injected oocytes to secondary oocytes or blastocysts (K. Wigglesworth and J. Eppig, unpublished observation) did not show any visible phenotypes at the light-microscopic level. Further analysis of antisense injected oocytes utilizing RT–PCR indicated that the targeting antisense was not degrading the endogenous OM2 transcripts. It appears that secondary structure, association with protein, or compartmental sequestration of the RNA may be preventing the antisense from working as expected. Additionally, capped sense transcripts were injected into primary oocytes in attempts to induce a phenotype due to overexpression of the RNA. No phenotype was detectable at the light-microscopic level in cultured sense injected oocytes (data not shown).

DISCUSSION

Translational activation of key dormant mRNAs by cytoplasmic polyadenylation is essential for completion of oogenesis and early embryogenesis. In both *Xenopus* and

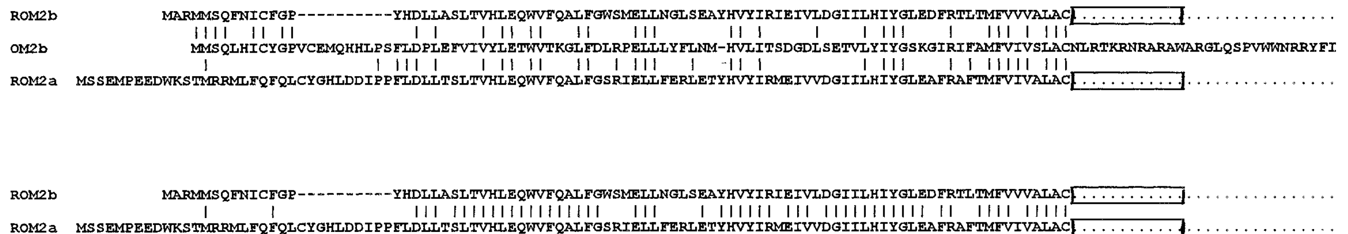

FIG. 7. Alignment of putative mouse and rat OM2 polypeptides. Identity between adjacent polypeptides is denoted by vertical lines (|). The region spanned by the reverse primer used in amplification of the rat clones is boxed. In all cases, the identity is higher at the nucleotide level than the amino acid level (compare to Fig. 1).

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mouse, *cis*-acting, U-rich elements (ACEs) controlling cytoplasmic polyadenylation have been identified. We have utilized the conserved nature of ACE sequences in an anchored, RT–PCR strategy (Sallés *et al.,* 1992) to clone two novel, cytoplasmically polyadenylated, maternal mRNAs: OM2a and OM2b. These two RNAs are elongated with 600–700 adenosine residues upon meiotic maturation. To our knowledge, this elongation is the greatest extent of polyadenylation for a stored maternal RNA, exceeding the 500–600 A's added to tissue-type plasminogen activator (tPA) mRNA (Huarte *et al.,* 1987; Sallés and Strickland, 1995). The significance of the extensive polyadenylation of the OM2 RNAs is unknown. The OM2 genes are transcribed specifically in the oocyte and, therefore, fall into a small class of identified mammalian, oocyte-specific transcripts. The transcripts are not detected in the testes, suggesting that these genes are not a general germ cell requirement. By *in situ* analysis, the transcripts appear to accumulate during oocyte growth, suggestive of a role after the onset of meiosis. Cytoplasmic polyadenylation during oocyte maturation has typically been associated with translational regulation. Translational regulation of the OM2 genes, at this time, would suggest a functional role after oocyte maturation.

Homologs of the mouse OM2 genes were cloned from the rat. Unexpectedly, the identity between the rat and mouse genes is higher at the nucleotide than the amino acid level. Higher homology at the nucleotide level suggests that these genes may not be functioning as proteins. If the genes are functioning at the RNA level, these genes would also be members of a small group of identified functional, nonribosomal, cytoplasmic RNAs. To address the role of the OM2 genes, antisense oligodeoxynucleotides, a full-length antisense RNA, and full-length sense transcripts were utilized for oocyte microinjection. No phenotypes were observed after culture of the injected oocytes.

Do the OM2 genes encode a protein in the oocyte? They share features in common with other translated mRNAs: they are processed by RNA splicing, they are polyadenylated, and at least OM2a is translated *in vitro.* If the OM2 genes are not translated *in vivo,* it would be the first example of cytoplasmically polyadenylated RNAs that are not translated. Analysis of putative ORFs from the two OM2 genes did not support a clear candidate for a conserved OM2 protein. To address this question, the rat OM2 genes were cloned and compared to the mouse genes. Even though the mouse and rat genes were fairly well conserved at the nucleotide level (up to 72%), the predicted ORFs were not significantly conserved, the highest conservation being 36% identity between one of the predicted rat polypeptides and the OM2b 119-aa ORF.

There are several possibilities for why the OM2 genes do not have well-conserved open reading frames: (1) The genes may encode substantially different proteins making them one of the few examples of homologous genes producing less homologous proteins (Fini *et al.,* 1989). An explanation for this is that the protein coding regions have evolved faster than expected from random drift (Tucker and Lundrigan, 1993; Whitfield *et al.,* 1993; Hughes, 1988; Hill and Hastie, 1987). (2) A common ORF may be generated by a splicing pattern different from the one revealed by the mouse and rat cDNAs. If this is the case, then the alternate RNA must be rare, as no other RNA species are detected in mouse oocytes by Northern blotting. (3) The OM2 genes may be functioning as RNA molecules. Examples of functional RNA molecules include RNAs that are required for, and probably catalyze reactions involved in, RNA processing, protein synthesis, and transport of proteins. In particular, meiRNA acts as an RNA and is essential for meiosis in *Schizosaccharomyces pombe* (Watanabe and Yamamoto 1994). However, there are no striking conservations between OM2 and meiRNA. Two other mouse genes, Xist and H19 are spliced, polyadenylated, and thought to function as neither messenger RNA nor regulators of RNA processing (Brannan *et al.,* 1990; Brockdorff *et al.,* 1992; Brown *et al.,* 1992). (4) It is possible that one or all of the mouse and rat OM2 genes have become pseudogenes. Arguments against both the mouse OM2 genes having suffered deleterious mutations are reflected in their identical transcriptional control, maintenance of splicing, and regulation of cytoplasmic polyadenylation. The maintenance of transcriptional competence is not normally associated with the loss of function of genes. Additionally, the mouse genes show long stretches of 100% nucleotide identity at their 5′ and 3′ ends suggesting a selective pressure. Taken together, these conservations argue that the cell is preserving the capacity to express these RNAs.

Future studies may include comparison of the OM2 promoters with those of other oocyte-specific genes such as mZP3 (mouse Zona Pellucida) to gain insight into factors regulating oocyte-specific transcription (Philpott *et al.,* 1987; Roller *et al.,* 1989; Kinloch and Wassarman, 1989). Additionally, transgenic mice overexpressing either sense or antisense transcripts for the creation of maternal effect mutations (Richards *et al.,* 1993) may be useful in addressing OM2 function. Transgenically expressed antisense transcripts, unlike injected transcripts, will be present at the time endogenous transcripts are being produced. This early synthesis may allow antisense transcripts to work prior to protein masking or sequestration of the endogenous transcripts. Additionally, transgenically expressed transcripts will allow analysis, in the maternal environment, of phenotypes that may not be seen during the brief window of development in a culture system.

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