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Antisense RNA Directed Against the 3′ Noncoding Region Prevents Dormant mRNA Activation in Mouse Oocytes

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Primary mouse oocytes contain untranslated stable messenger RNA for tissue plasminogen activator (t-PA). During meiotic maturation, this maternal mRNA undergoes a 3′-polyadenylation, is translated, and is degraded. Injections of maturing oocytes with different antisense RNA's complementary to both coding and noncoding portions of t-PA mRNA all selectively blocked t-PA synthesis. RNA blot analysis of t-PA mRNA in injected, matured oocytes suggested a cleavage of the RNA·RNA hybrid region, yielding a stable 5′ portion, and an unstable 3′ portion. In primary oocytes, the 3′ noncoding region was susceptible to cleavage, while the other portions of the mRNA were blocked from hybrid formation until maturation occurred. Injection of antisense RNA complementary to 103 nucleotides of its extreme 3′ untranslated region was sufficient to prevent the polyadenylation, translational activation, and destabilization of t-PA mRNA. These results demonstrate a critical role for the 3′ noncoding region of a dormant mRNA in its translational recruitment during meiotic maturation of mouse oocytes.

DURING THE GROWTH PHASE OF OOGENESIS, MESSENGER RNA molecules accumulate that are not translated until after the oocyte enters the final phases of maturation or is fertilized (*1*). This posttranscriptional regulation allows rapid alterations in protein synthesis as maturation and embryogenesis are initiated. For this process to occur, the dormant mRNA's must have characteristics, such as structural determinants and subcellular location, that permit their selective and timely translational activation.

In mammals, little is known about mRNA recruitment during meiotic maturation, since specific dormant mRNA's have only recently been identified. The mRNA for tissue plasminogen activator (t-PA) is present in primary (germinal vesicle–containing) mouse oocytes, but the enzyme is not synthesized until resumption of meiosis, in the hours that precede ovulation. The dormant, stable mRNA accumulates during the oocyte's growth phase and is stored in the cytoplasm of the primary oocyte. After resumption of meiosis, the mRNA progressively acquires about 500 adenosine (A) residues at the 3′ end, and concomitantly it is translated. Later, this mRNA becomes unstable, so that it is undetectable in fertilized eggs (*2*, *3*). The expression of hypoxanthine phosphoribosyl transferase (HPRT) has similar characteristics in that an increase in enzyme activity during maturation and early embryogenesis may be due to the activation of a maternal mRNA (*4*), and HPRT mRNA is elongated during oocyte maturation and then degraded after the two-cell stage (*5*).

Regarding maternal mRNA's in mammals and other species, it is still not known (i) what molecular determinants specify the initial dormancy and then the temporally precise activation, structural alteration, and destabilization; (ii) whether these events are interrelated; and (iii) what roles are played in early development by the products of the genes whose expression is so exquisitely controlled.

Antisense RNA inhibition of gene expression (*6*) is well suited for addressing these questions in general, and the regulated expression of t-PA in mouse oocytes in particular. The amount of t-PA mRNA

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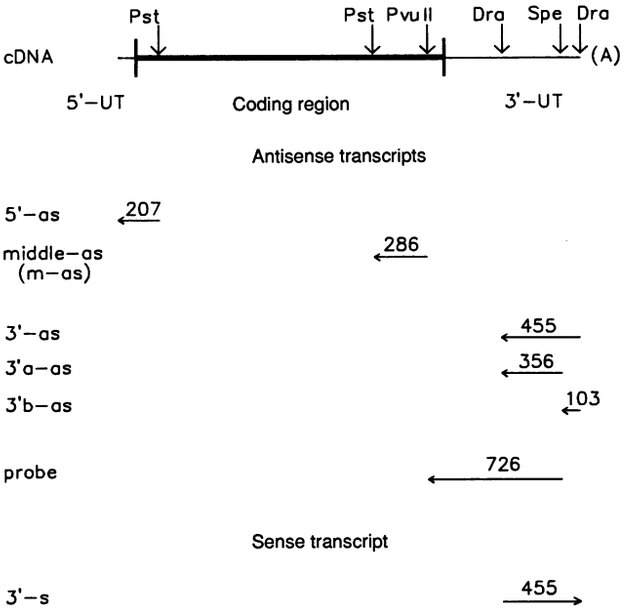


Fig. 1. Representation of mouse t-PA cDNA and of the RNA transcripts used. The restriction enzyme sites shown were used to subclone portions of the cDNA. (5′-UT and 3′-UT refer to the 5′ and 3′ noncoding regions. The numbers above the arrows indicate the number of t-PA cDNA-derived nucleotides contained in the transcripts; as indicates antisense, and s indicates sense orientation.) RNA's were transcribed from plasmids that contain the indicated inserts from mouse t-PA cDNA [numbering defined in (*7*)]. For 5′-asRNA, a 207-bp Eco RI–Pst I fragment (position 2 to 208) was cloned between the Eco RI and Pst I sites of pBS M13+ (Stratagene). The plasmid was linearized with Eco RI, and the antisense transcript was generated with T3 RNA polymerase. For middle-asRNA, a 286-bp Pst I–Pvu II fragment (position 1394 to 1679) was cloned between the Sma I and Pst I sites of pSP65. The plasmid was linearized with Pst I for transcription. For 3′-asRNA, 3′-sRNA, and 3′b-asRNA, a 455-bp Dra I fragment (position 2050 to 2504) was cloned in both orientations into the Hinc II site of pSP64. The plasmids were linearized with Xba I (3′-asRNA and 3′-sRNA) or Spe I (3′b-asRNA). The 3′b-asRNA transcript was gel-purified to remove trace amounts of unlinearized plasmid in the transcription reaction, which would give rise to a longer asRNA similar to 3′-asRNA. Both 3′a-asRNA and the cRNA probe were derived from pSP64-MT$_3$, which contains a Pvu II–Spe I insert of t-PA mRNA (*3*). The plasmid was linearized with Dra I (3′a-asRNA) or Eco RI (probe) for transcription. Transcriptions with SP6 (Promega) or T3 (BRL) RNA polymerases were performed as described (*20*). The transcripts were labeled ([α-^{32}P]UTP) to allow quantitation and were capped by inclusion of 500 μM m^{7}G(5′)ppp(5′)Gm and 50 μM guanosine triphosphate in the reaction (*21*). The free nucleotides were removed, the transcripts were precipitated with ethanol, dissolved in water, filtered through a 0.22-μm filter (Sartorius), lyophilized, and then dissolved in filtered 0.15M KCl. The size of all transcripts was verified by analysis on acrylamide-urea gels.

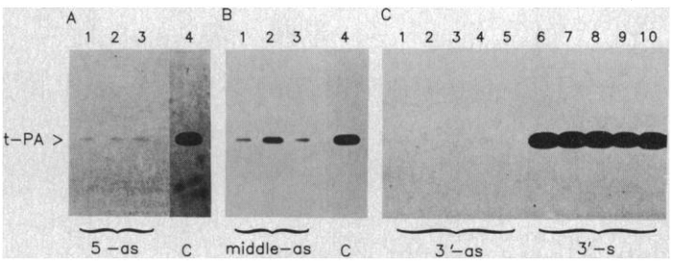


Fig. 2. Inhibition of t-PA expression in maturing oocytes by asRNA's. Primary oocytes injected with the indicated RNA were allowed to mature in vitro and collected as secondary oocytes. Each panel represents a single experiment, with injected and uninjected control (C) oocytes cultured in parallel, and then assayed by zymography on the same gel. In each lane the lysate of a single oocyte was analyzed. Oocyte collection (from B6/D2 F1 hybrid mice), oocyte culture, and analysis of t-PA activity by zymography were as described (*2, 3*). RNA was injected into the cytoplasm of primary oocytes as described (*22*); on average, 10 pl was injected into each oocyte. For all experiments (except Fig. 4), the concentration of the injected RNA was adjusted (100 ng/μl for a 500-nucleotide transcript) so that 10 pl would contain approximately 4×10^6 molecules. Approximately 50 percent of the oocytes survived the injection procedure and, of these, approximately 30 to 50 percent matured to secondary oocytes (as defined by the presence of the first polar body).

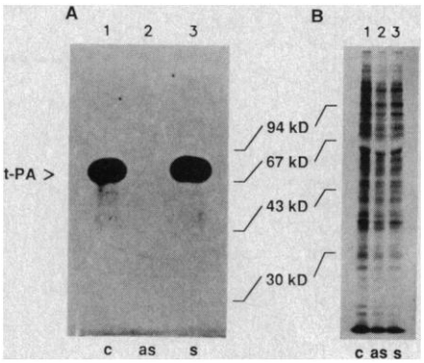
in the primary oocyte allows RNA blot analysis of individual oocytes, and the sensitivity of the enzyme assay makes it possible to analyze single oocytes for activity (*2, 3*). Also, a full-length mouse t-PA cDNA has been cloned and sequenced so that antisense probes that span the entire mRNA can be generated (*7*).

Antisense RNA inhibits translational activation of t-PA mRNA during oocyte maturation. Uninjected, explanted oocytes begin to synthesize t-PA approximately 5 hours after germinal vesicle breakdown (GVBD), and all secondary oocytes contain similar amounts of t-PA (*2*). To explore the possibility of inhibiting this translational activation of t-PA mRNA, we prepared antisense RNA's (asRNA's) directed against three different portions of the mRNA (Fig. 1). These capped, non-polyadenylated transcripts were injected into primary oocytes, which were then allowed to undergo meiotic maturation in vitro; individual secondary oocytes were assayed for t-PA activity by casein-agar zymography. The 5′-asRNA (complementary to part of the 5′ noncoding region and to 115 nucleotides of coding sequence, including the initiator AUG), the middle-asRNA (coding), and the 3′-asRNA (3′ noncoding) all inhibited the appearance of t-PA activity in maturing oocytes (Fig. 2). The 3′-asRNA was the most effective, causing more than 97 percent inhibition of enzyme production. In contrast, injection of a 3′ sense transcript (3′-sRNA) (Fig. 1) had no effect.

The specificity of this inhibition was analyzed by comparing the pattern of proteins synthesized in uninjected oocytes and in oocytes injected with antisense or sense RNA. After the maturing oocytes were injected, they were metabolically labeled with [^{35}S]methionine. Extracts of pooled secondary oocytes were prepared, and each extract was analyzed by zymography or electrophoresis with subsequent autoradiography. In contrast to the dramatic inhibition of t-PA synthesis by the asRNA (Fig. 3A), the overall amount and the general pattern of proteins synthesized were similar in sRNA- and asRNA-injected oocytes (Fig. 3B). Therefore, as judged by this analysis, the inhibition of t-PA synthesis is specific and does not reflect a generalized decrease in protein synthesis after asRNA injection.

In some cases, a large excess of asRNA is necessary to be effective (*8*). Primary oocytes contain about 10^4 copies of t-PA mRNA (*3*) and, in the experiments reported above, we estimated that approximately 4×10^6 molecules of asRNA had been injected. In order to address the efficiency of inhibition in this system, the 3′-asRNA was serially diluted and injected into oocytes, generating a dose-response relation for the inhibition; the remaining activity was compared to that expressed by varying amounts of oocyte extract (Fig. 4, lanes 1 to 4). A 40-fold excess of asRNA inhibited generation of t-PA activity by more than 90 percent (Fig. 4, compare lanes 5 to 7 with lanes 3 and 4), a fourfold excess inhibited by about 90 percent (lanes 8 to 10), and less than stoichiometric amounts still inhibited to some extent (lanes 11 to 13). These results demonstrate that, with mouse oocytes, the asRNA is an extremely effective inhibitor of t-PA mRNA translation (*9*).

Injection of asRNA induces cleavage of endogenous t-PA



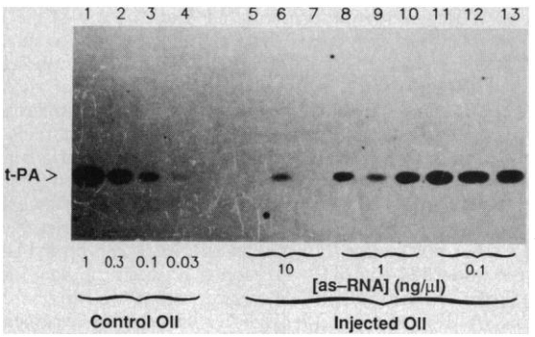


Fig. 3 (left). Pattern of protein synthesis. The maturing oocytes were injected with RNA and metabolically labeled (*3*) for 18 hours with [^{35}S]methionine (200 μCi/ml). Extracts of five secondary oocytes were prepared. (**A**) A portion (10 percent) of each extract was analyzed by zymography. (**B**) The remainder was subjected to electrophoresis and analyzed by autoradiography; c, uninjected oocytes; as, injected with 3′-asRNA; s, injected with 3′-sRNA. The migration of molecular-size markers is indicated. **Fig. 4 (right).** Dose dependence of 3′-asRNA inhibition of t-PA expression. Lane 1, uninjected oocyte; lanes 2 to 4, fraction of a single, uninjected oocyte for quantitative comparison; lanes 5 to 13, the 3′-asRNA was adjusted to the concentrations indicated, and injected into primary oocytes, which were then cultured overnight. Individual secondary oocytes were analyzed for t-PA activity.

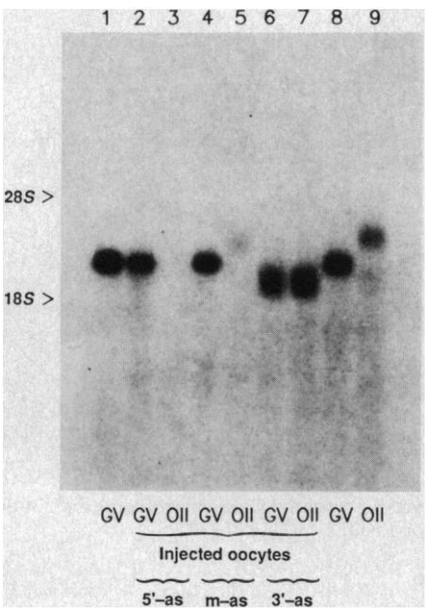


Fig. 5. RNA blot analysis of t-PA mRNA from primary and secondary oocytes injected with asRNA's. For each lane, ten oocytes were analyzed. Lanes 1 and 8, uninjected primary oocytes containing an intact germinal vesicle (GV); lane 9, uninjected secondary oocytes (OII). Control oocytes and oocytes injected with the indicated asRNA were cultured for 16 to 18 hours in the presence of Bt_2-cAMP at 100 μg/ml (maturation inhibited, GV) or in the absence of Bt_2-cAMP (oocytes that matured to OII were collected). RNA isolation and RNA blot analysis were performed (*3*) with the cRNA probe shown in Fig. 1.

mRNA. The mechanism of asRNA-mediated inhibition of t-PA synthesis was investigated by analyzing t-PA mRNA in injected oocytes. For RNA blot hybridization, a single-stranded cRNA probe was used (Fig. 1). Primary oocytes contain a high level of t-PA mRNA, which is approximately 2500 nucleotides long and has a stretch of less than 40 A residues at its 3′ end; secondary oocytes contain smaller amounts of a longer t-PA mRNA, which has had 400 to 600 A residues added to its 3′ extremity (Fig. 5, lanes 1, 8, and 9) (*3*). When primary oocytes were injected with the 5′-asRNA or the middle-asRNA, and maintained as primary oocytes by culture in the presence of dibutyryl cyclic adenosine monophosphate (Bt_2-cAMP), neither the amount nor the size of the mRNA changed (Fig. 5, lanes 2 and 4). In contrast, injection of the 3′-asRNA resulted in a decrease in size of t-PA mRNA suggesting that the 3′ terminal region complementary to the injected transcript had been removed (Fig. 5, lane 6). The kinetics of mRNA cleavage induced by the 3′-asRNA in primary oocytes was rather slow, with a pattern suggesting that the molecules were undergoing consecutive cleavages to yield progressively shorter products; cleavage was detectable at 3 hours and was almost complete 17 hours after injection.

If, after injection, the oocytes were allowed to mature to secondary oocytes, t-PA mRNA was not detected in cells that had been injected with the 5′-asRNA (Fig. 5, lane 3). In oocytes injected with middle-asRNA, a small amount of fully polyadenylated mRNA was detected (Fig. 5, lane 5), which probably accounts for the incomplete inhibition of t-PA synthesis by this antisense transcript. In the oocytes injected with 3′-asRNA, t-PA mRNA was present in the same amount and shortened to the same degree in secondary as in primary oocytes (Fig. 5, compare lanes 6 and 7).

On the basis (i) of our current understanding of mRNA metabolism and (ii) of experiments described below, we can interpret our results as follows. There exists in primary and secondary oocytes a ribonuclease activity (dsRNase) that can cleave the asRNA·mRNA duplex region. Such a cleavage generates two fragments of t-PA mRNA: a stable 5′ fragment that retains the cap structure and an unstable 3′ fragment. In the primary oocyte, only the 3′ portion of t-PA mRNA is accessible for hybrid formation, and thus only the 3′-asRNA induces cleavage, leading to an mRNA shorter by about 500 nucleotides (the region complementary to the injected antisense transcript plus the short stretch of 3′ A residues). In secondary oocytes, the mRNA is fully exposed, and all three antisense transcripts can form hybrids, which are then cleaved. The probe used in our RNA blot hybridizations recognizes the 5′ mRNA fragment generated by 3′-asRNA–directed cleavage, but not the 5′ fragments that are generated by 5′-asRNA and middle-asRNA directed cleavages (Fig. 1). Thus, stable t-PA mRNA fragments would not be detected in secondary oocytes injected with the 5′ and middle antisense transcripts. Finally, since the size of t-PA mRNA is the same in primary and secondary oocytes after injection of 3′-asRNA, the shortened mRNA clearly does not undergo the polyadenylation that normally accompanies maturation.

Antisense RNA to the 3′ terminal 103 nucleotides prevents t-PA mRNA translation, polyadenylation, and destabilization. Several facts suggested that the 3′ noncoding region of t-PA mRNA participates in the control of t-PA synthesis during oocyte maturation. First, the 3′-asRNA was more effective than the 5′- or middle-asRNA's (Fig. 2), even though the 5′- and middle-asRNA's are complementary to coding sequences (Fig. 1). Second, the 3′ noncoding region of the mRNA appeared to be more exposed in primary oocytes than the other regions (Fig. 5). To refine our analysis, two other asRNA's were injected into primary oocytes: 3′a-asRNA and 3′b-asRNA (Fig. 1); each of these transcripts represents a portion of the 3′-asRNA. Both were as effective as the originally used 3′-asRNA in preventing t-PA synthesis in maturing oocytes (Fig. 6). Injection of poly(U), which could hybridize to poly(A) tails and possibly interfere with translation, had no detectable effect.

Analysis of t-PA mRNA in secondary oocytes after injection of the various 3′-asRNA's provided further evidence for an RNase cleavage in the hybridized regions. Injection of the 3′-asRNA and the 3′a-asRNA yielded an mRNA shortened to the same extent

(Fig. 7, lanes 3 and 4); this result was expected since these two antisense transcripts have identical termini, and would therefore generate, after hybridization to the mRNA and cleavage, the same 5′ fragment of t-PA mRNA. Injection of 3′b-asRNA, which is complementary to only the last 103 nucleotides of the mRNA, yielded an mRNA shortened by about 100 nucleotides (Fig. 7, lane 5). After poly(U) was injected, some t-PA mRNA molecules were not elongated (Fig. 7, lane 6); this could result from cleavage of poly(A)·poly(U), or from a reduction in the rate of polyadenylation of the mRNA.

Since the 3′b-asRNA directed against the last 103 nucleotides of t-PA mRNA inhibits translational activation and polyadenylation, it

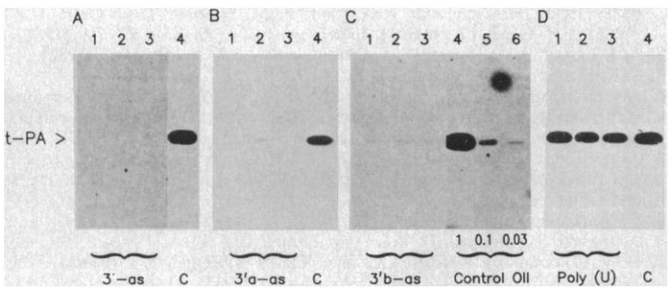


Fig. 6. Inhibition of t-PA production in maturing oocytes by different 3′-asRNA's. Primary oocytes were injected and allowed to mature in vitro for 16 to 18 hours; individual secondary oocytes were then assayed. (**A** to **D**) Each panel represents a single experiment, with oocytes injected with the the indicated asRNA and control (C, lanes 4) uninjected oocytes cultured in parallel, and then assayed by zymography on the same gel. (**C**) Lanes 5 and 6, fractions of a single, uninjected OII for quantitative comparison. (**D**) Lanes 1 to 3, injected with poly(U) (2 μg/μl); average size, 200 nt).

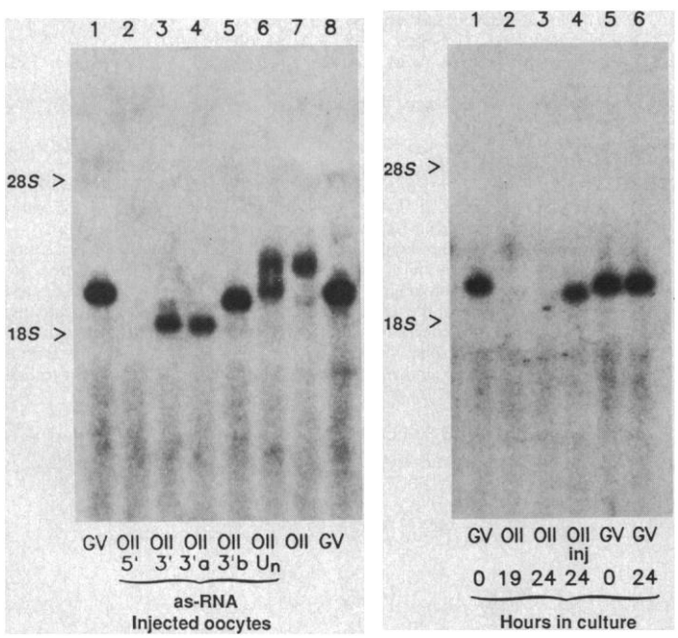


Fig. 7 (**left**). RNA blot analysis of t-PA mRNA in secondary oocytes injected with different 3′-asRNA's. Primary oocytes were injected, cultured for 16 to 18 hours, and then secondary oocytes were processed for RNA analysis. Each lane contains RNA extracted from ten oocytes. Lanes 1 and 8, uninjected primary oocytes (GV); lane 7, uninjected secondary oocytes (OII); lanes 2 to 6, oocytes injected with the indicated asRNA. U_n indicates poly(U). **Fig. 8** (**right**). Stability of t-PA mRNA determined by RNA blot analysis after injection of 3′b-asRNA. Each lane contains RNA extracted from nine oocytes. Uninjected primary oocytes immediately after isolation from the ovary (lanes 1 and 5) or after 24 hours culture in the presence of Bt_2-cAMP at 100 μg/ml (lane 6); uninjected secondary oocytes after 19 hours (lane 2) or 24 hours (lane 3) in culture. (Lane 4) Secondary oocytes collected 24 hours after injection of 3′b-asRNA into primary oocytes.

was of interest to determine whether this asRNA could also prevent the destabilization of the mRNA. The t-PA mRNA was stable in primary oocytes cultured for 24 hours under conditions that prevent resumption of meiosis (Fig. 8, compare lanes 5 and 6) (*3*). When oocytes were collected after 19 hours of maturation, the level of t-PA mRNA was drastically reduced (Fig. 8, lane 2), and after 24 hours, the mRNA was no longer detectable (Fig. 8, lane 3). In contrast, after injection of the 3′b-asRNA, most of the shortened mRNA was still present after 24 hours of maturation (Fig. 8, lane 4). Therefore, cleavage within the terminal 103 nucleotides prevents t-PA mRNA degradation as maturation proceeds.

The effectiveness of injections of asRNA at preventing t-PA synthesis in maturing oocytes has made it possible to assess the consequences of this deficiency. Primary oocytes injected with 3′-asRNA mature to secondary oocytes with a frequency similar to that of oocytes injected with saline or "irrelevant" RNA's; at the resolution of the light microscope, these secondary oocytes are indistinguishable from controls. Thus, t-PA synthesis is not required for in vitro meiotic maturation. A more extensive analysis is necessary to define exactly the phenotype of t-PA–deficient oocytes.

Translational control of oocyte mRNA's. Our present information makes it possible to exclude a number of potential control mechanisms for t-PA mRNA translational activation. (i) The absolute rate of protein synthesis (*10*) is similar in primary oocytes, which do not synthesize t-PA, and maturing oocytes, which do, indicating that the activation of this message does not result from a general increase in translational activity. (ii) t-PA mRNA in primary oocytes is cytoplasmic (*3*), and therefore dormancy is not due to nuclear compartmentalization. (iii) The dormant mRNA is not structurally unusual: its size is comparable to that of the molecule in somatic cells, its 3′ terminal sequence appears to be identical to somatic t-PA mRNA (*11*), and it has had approximately 40 A residues added (*3*).

In many cases, a correlation has been observed between the extent of polyadenylation of oocyte or embryo mRNA's and their translation (*12*). In mice, this correlation applies to the expression of actin (*13*), whose mRNA becomes deadenylated as its translation decreases, and t-PA (*3*) and HPRT (*5*), whose mRNA's are adenylated concomitant with translational activation. The mRNA for c-Mos also undergoes elongation during meiotic maturation (*14*), and may be another example of this phenomenon. In spite of the association between polyadenylation and translation, nothing is known about what determines message-specific elongation, and, most importantly, whether this modification is sufficient or even necessary for message activation. However, this structural change points toward the 3′ end of the molecule as a region of particular interest in the context of translational control.

Injection of asRNA's complementary to different regions of t-PA mRNA has uncovered two striking properties of its 3′ noncoding region. (i) It is a region of t-PA mRNA that is exposed in primary oocytes: in contrast to the 5′ and middle antisense transcripts, the 3′-asRNA induces cleavage of the mRNA in primary oocytes. The high inhibitory efficiency of the 3′-asRNA may be due to the accessibility of the region in the primary oocyte, that is, before translation begins. Other asRNA's may have to compete with the translational machinery as the mRNA is unmasked during maturation. (ii) The presence of the terminal 3′ noncoding sequences is crucial for regulated expression of t-PA mRNA; removal of the last 103 nucleotides generates a stable mRNA that is neither polyadenylated nor translated. Whether there is a "maternal" sequence imbedded in this portion, or whether it is the classical cleavage and polyadenylation sequence (AAUAAA) which resides in this 103-nucleotide segment that is exclusively responsible, remains to be determined. Although in general the 3′ untranslated regions of the

human and mouse t-PA mRNA's have little homology, the terminal 100 nucleotides are very similar, possibly reflecting functional significance for this region. Comparison of the 3′ noncoding regions of the mRNA's for t-PA, HPRT, and c-Mos, all of which may have comparable regulation, reveals short stretches of similarity. The significance of these similarities should become clearer as the sequences required for translational activation are better defined.

The shortening of t-PA mRNA after injection of 3′-asRNA prevents the polyadenylation, translational activation, and destabilization of the mRNA that normally occur in concert during maturation. This fact suggests that these three events are related. Translation of t-PA mRNA is not necessary for polyadenylation; treatment of maturing oocytes after GVBD with inhibitors of protein synthesis prevents t-PA production, but the mRNA undergoes full elongation (*3*). One possibility is that polyadenylation may be necessary to assemble the mRNA into polysomes, thus allowing its translation and eventual degradation.

There is increasing evidence for posttranscriptional control of gene expression in somatic cells (*15*), both through varying rates of translation of a given mRNA and selective mRNA degradation (*16*). A role of 3′ untranslated regions in mRNA translation (*17*) and stability (*16*) has been demonstrated by in vitro translation, mRNA injection, or transfection of recombinant genes. We have shown here the importance of the 3′ untranslated region of an endogenous, oocyte mRNA in the control of its translation and subsequent degradation. The mechanism of controlled maternal mRNA expression may thus be generally relevant to posttranscriptional regulation of gene expression.

Use of asRNA to study mammalian oocytes and early embryos. Analysis of t-PA mRNA in oocytes injected with different antisense transcripts showed that the duplex region is progressively cleaved. The presence, in both primary and maturing oocytes, of a dsRNase could account for our observations. The stability of t-PA mRNA in uninjected primary oocytes in the presence of a dsRNase activity implies that there are no exposed intramolecular or intermolecular double-stranded segments in the mRNA. Unlike *Xenopus laevis* maturing oocytes and early embryos (*18*), mouse oocytes in our experiments do not exhibit an RNA duplex unwinding activity that would preclude the formation of double-stranded regions; similarly, preimplantation mouse embryos do not contain dsRNA unwinding activity (*19*).

The effect of asRNA injection on t-PA synthesis indicates that this approach may have general applicability in studies on mammalian oocyte biology, and that asRNA's complementary to 3′ noncoding regions may be particularly valuable. Cleavage of asRNA·mRNA duplexes by the oocytic dsRNase should allow the selective destruction of individual mRNA's, and help to explore the function of maternal mRNA products in meiosis and fertilization.

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