Transient polyadenylation of a maternal mRNA following fertilization of mouse eggs

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The molecular basis for the recruitment of maternal mRNAs after fertilization is poorly understood, since there is little information available regarding the identity of such mRNAs. An assay based on reverse transcriptase and PCR was used to identify a maternal mRNA that undergoes a transient polyadenylation after fertilization; the length of the poly(A) tail increases from 40–80 adenosine (A) residues in the unfertilized egg to approximately 250 3 h after insemination and then decreases to about 180 around the time of pronucleus formation, that is 7 h after insemination. The DNA sequence of this cDNA, which encodes the polyadenylation signal AAUAA, contains a uridine-rich sequence that may serve as the cytoplasmic polyadenylation element observed in other maternal mRNAs that are recruited after fertilization.

Introduction

After fertilization, there is a period of development in which the early embryo is essentially transcriptionally inactive. In the mouse, this period lasts about 18 h, which corresponds to a time during which a transcriptionally permissive state develops (Latham *et al.*, 1992) and the expression of both exogenous, plasmid-born, reporter genes (Ram and Schultz, 1993) and endogenous genes (Matsumoto, 1994; Bouniol *et al.*, 1995; Christians *et al.*, 1995) is detected. During this time, early development is directed by maternally inherited proteins, as well as maternal mRNAs that are recruited following fertilization. Although many of the changes in the pattern of protein synthesis that occur following fertilization of mouse eggs are due to post-translational modifications such as phosphorylation (Van Blerkom, 1981), the synthesis of many polypeptides is directed by recruited maternal mRNAs (see for example, Cascio and Wassarman, 1982). Nevertheless, the identity of these newly recruited maternal mRNAs, let alone the mechanism for their recruitment and function in the early embryo, is unknown. This situation stands in marked contrast to our understanding of the recruitment of mRNAs during oocyte maturation. For example, tissue plasminogen activator (tPA) is not synthesized in the fully grown, germinal vesicle, intact mouse oocyte (Huarte *et al.*, 1985). After germinal vesicle breakdown, mRNA for tPA is recruited by a mechanism that involves polyadenylation and that results in the synthesis of tPA (Huarte *et al.*, 1987; Vassalli *et al.*, 1989). The sequence that directs this cytoplasmic polyadenylation, the cytoplasmic polyadenylation element (CPE), has been identified (Sallés *et al.*, 1992).

An assay based on reverse transcriptase (RT) and PCR that permits the comparison of relative changes in mRNA abundance (oligo d(T) is used to prime the RT reaction) was used to

identify a cDNA, vi12H, the relative abundance of which transiently increases about twofold after fertilization; the amount of the PCR product of this cDNA was greater in the one-cell embryo than in either the egg or two-cell embryo (Temeles *et al.*, 1994). The DNA sequence for this cDNA is shown in Fig. 1. Since this increase probably occurs before the onset of zygotic gene activation, it is likely that the increase is due to a transient polyadenylation of the mRNA. The rationale for this interpretation is that an increase in the length of the poly(A) tail would increase the efficiency of the oligo-d(T)-primed RT reaction. Thus, vi12H could represent a maternal mRNA that is transiently polyadenylated following fertilization and could therefore serve as a molecular marker to study fertilization-induced mRNA recruitment.

An RT–PCR assay (Sallés *et al.*, 1992) was used in this study to show that vi12H is indeed transiently polyadenylated after fertilization; the size of the poly(A) tail increases from 40–80 to about 250 adenosine (A) residues and is then reduced to about 150 A residues. Nevertheless, the cDNA for this transcript does not contain a CPE element similar to those previously reported near the polyadenylation AAUAA sequence or anywhere in the cDNA, although it does possess a uridine(U)-rich tract situated 278 nucleotides from the polyadenylation sequence.

Materials and Methods

Oocyte and egg collection and in vitro fertilization

Fully grown mouse oocytes freed of attached cumulus cells were collected from CF-1 mice (Harlan) primed with pregnant mares' serum gonadotrophin, as described by Schultz *et al.* (1983); 0.2 mmol 3-isobutyl-1-methyl xanthine l^{-1} was present in the collection medium to inhibit resumption of meiosis. Ovulated eggs were collected from superovulated

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1
  GCTGAAATGT CACTTGCACA AGGACGACAC TATTTTAAAT AGTAGCTTCT
51
  GTTCACAGGG GAGCTTCTCA ACTTTGGTCA CGATCATTTA TCACCAACAA
101
  ACATACATGT ATGTAAC TTT ATACCATGTG TCTTTTCCTT CCTGGCCTCT
151
  TTGAACTAGT ACCTCCCTGT TTCCTAATGC ATACACAGAG TGTAGGTTGC
201
  ACAAGGACAG GACATTTTGG GTTTTTTTGT TTACTGTCTT CAGGGTAGGT
251
  CAGTAAGAGA AAGATGGGAT CAATACTTGT TGACTTGATA TAAAAACCAG
301
  CTTCTAGGA ATTGGTATAT AAGATGTAAG TTTAATAGCT GCTAATACTT
351
  AGAAAGTTGC TTAGTATATA GATGTTTTAG GTTGTTAAAA AAAACATTGA
400
  CTTGATGGAA GCAGTGT TTT GACAGACCTG CTTTAGGGAA AACAAAAATA
451
  TGCTTTCCTT GGATTAATTC ATACTACCCT TTGTCCTACT AAGTTATATC
501
  ATTCTTTaat aaaGATTTTA CTCCCAAAAA AAAAAAAAAA AAA

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Fig. 1. Nucleotide sequence of the cDNA from mouse encoding vi12H. The sequences for the gene-specific primers are underlined, the polyadenylation signal sequence is in lower-case letters, and potential cytoplasmic polyadenylation elements are double-underlined.

mice 12–13 h after hCG administration as described by Endo *et al.* (1987). One-cell embryos were collected from superovulated females that had been mated with B6D2F1/J males (Jackson Laboratory, Bar Harbor, ME) by flushing the oviduct as described by Manejwala *et al.* (1989). The collection medium (MEM/PVP) was bicarbonate-free minimal essential medium (Earle's salts) containing pyruvate ($100 \mu\text{g ml}^{-1}$), gentamicin ($10 \mu\text{g ml}^{-1}$), polyvinylpyrrolidone (3 mg ml^{-1}), and $25 \text{ mmol Hepes l}^{-1}$ at pH 7.2.

In vitro fertilization (IVF) was performed as described by Moore *et al.* (1993). Fertilization was performed in Whitten's medium containing 15% fetal calf serum and 200 000 spermatozoa ml^{-1} . The eggs were washed in CZB medium 3 h after fertilization (Chatot *et al.*, 1989) and transferred to CZB and cultured in CZB at 37°C in a humidified atmosphere of 5% CO_2 in air.

RT-PCR assay

RNA was isolated and 0.125 μg rabbit globin mRNA was added to each embryo, and the semi-quantitative RT-PCR assay for measuring relative amounts of specific mRNAs was performed as described in Temeles *et al.* (1994), except that the RT reactions always contained 50 egg or embryo equivalents; the gene-specific primers are underlined in Fig. 1. Some RT reactions were primed with a gene-specific 3' primer 5' TCCCCTAAAGCAGGTCTGG-3' for vi12H instead of the oligo d(T). The globin mRNA serves as an internal standard for

the efficiency of the RT-PCR reactions (Temeles *et al.*, 1994). For each set of gene-specific primers, the linear region of semi-log plots of the amount of PCR product as a function of cycle number was determined, and a cycle number for each primer pair was selected that was in this linear range; the amount of PCR product under these conditions is proportional to the number of cells used (Manejwala *et al.*, 1991). This method permits the comparison of relative changes in the abundance of a particular transcript (Latham *et al.*, 1994; Temeles *et al.*, 1994; Ho *et al.*, 1994, 1995).

PCR was performed in 100 μl volumes containing 10 mmol Tris-HCl l^{-1} at pH 8.3, 50 mmol KCl l^{-1} , 1.45 mmol MgCl_2 l^{-1} , 0.2 mmol of each of the four dNTPs l^{-1} , 5 μCi [α - ^{32}P] dCTP (specific activity is 3000 Ci per mmol; Amersham, Arlington Heights, IL), 2.5 U *Amplitaq* polymerase (Cetus-Perkin Elmer, Norwalk, CT), 20 pmol ($0.4 \mu\text{mol l}^{-1}$) each of the appropriate 5' and 3' primers, and a volume of the RT reaction equivalent to five embryos. The basic PCR programme consisted of a 95°C soak for 1 min, followed by a cycle programme of 95°C for 10 s, then 60°C for 15 s; the last cycle was followed by a 6 min soak at 60°C .

PCR products were analysed on 4% agarose gels and the amount of radiolabelled material in the ethidium-bromide-stained bands was quantified as described by Temeles *et al.* (1994). For each developmental stage, the amount of radioactivity in the vi12H product relative to that of the globin product was expressed as a ratio; the value for the one-cell stage was set at 100% and those of the other stages were expressed relative to it.

Polyadenylation assay

The RT-PCR assay for polyadenylation developed by Sallés *et al.* (1992) was used essentially as described. Briefly, RNA corresponding to 50 embryo equivalents was reverse-transcribed in a reaction that contained 200 ng of the oligo d(T) primer/adaptor of the sequence 5'-GCGAGCTCCGCGCCGCG-(T)₁₂-3'. The reaction conditions were as described by Temeles *et al.* (1994) except that the RT was Superscript II (BRL, Gaithersburg, MD). PCR was carried out exactly as described by Temeles *et al.* (1994) on five embryo equivalents of the resulting cDNAs in a 100 µl reaction that contained 25 pmol gene-specific 5' primer l⁻¹ and 25 pmol primer/adaptor l⁻¹. The reactions were run on a PEC 9600 thermocycler on the following programme: 95°C soak for 1 min; 40 cycles of a cycle programme of 95°C for 10 s; 60°C for 15 s; and a 6 min extension soak at 60°C.

Acid-precipitable radioactivity was determined by spotting duplicate 1 µl aliquots of the PCR reaction on 0.45 µm HA filters (Whatman), washing the filters three times in ice-cold 5% trichloroacetic acid in 20 mmol sodium pyrophosphate l⁻¹ and once in 70% ethanol. Equal numbers of acid-precipitable counts from each PCR sample were subjected to electrophoresis in a 5% non-denaturing polyacrylamide gel in a Tris-borate buffer system (Sambrook *et al.*, 1989). The gel was dried under a vacuum and subjected to autoradiography using Reflection film (Dupont-New England Nuclear, Boston, MA) and the manufacturer's supplied intensifying screen.

Results

Polyadenylation of vi12H mRNA after fertilization

We had previously isolated a cDNA whose abundance apparently increased after fertilization. This increase in the amount of the PCR product could be due either to an increase in the abundance of the transcript or to an increase in the length of the poly(A) tail. A lengthening of the poly(A) tail was the more likely, since this increase occurs during a time when the one-cell embryo is transcriptionally inactive. As a first attempt to distinguish between these two possibilities, the relative yield of PCR product obtained with RNA from eggs and one-cell embryos in RT-PCR reactions that had been primed with oligo d(T) were compared with the relative yield of PCR product obtained with egg and one-cell RNA in RT-PCR reactions that had been primed with a gene-specific 3' primer for vi12H. The rationale for this is that if the number of transcripts increased, both methods would reveal an increase between the egg and one-cell stages, whereas if an increase in the length of the poly(A) tail were responsible, no increase in the amount of the PCR product would be obtained when the gene-specific 3' primer was used to prime the RT reaction. Results of these experiments indicated that whereas there was a 75% increase in the amount of the PCR product between the egg and one-cell stages when oligo d(T) was used to prime the RT reaction, there were essentially equal amounts of the PCR product when the gene-specific 3' primer was used (Fig. 2). These data strongly suggest that the observed increase was not due to an increase in the amount of the vi12H transcript but rather due to an increase in the length of the poly(A) tail.

Fig. 2. Relative amount of the product of the mouse cDNA vi12H obtained using PCR after either priming of the reverse transcription reaction with oligo d(T) or a gene-specific primer. The RNA was prepared from eggs and one-cell embryos and aliquots were reverse-transcribed with either oligo d(T) (□) or a 3' primer specific for the gene encoding vi12H (■). The samples were then subjected to PCR using a vi12H gene-specific primer. In parallel, aliquots from the oligo d(T) primer samples were subjected to PCR using globin-specific primers. After electrophoresis, the ethidium-bromide-stained gel bands were excised and the radioactivity quantified by Cerenkov counting. The ratios of c.p.m. in the experimental bands versus those in the globin bands were calculated. In each case, the value obtained for the one-cell stage was set at 100% and that for the egg was expressed relative to it.

To provide more direct evidence for the polyadenylation of vi12H, we used an anchored RT-PCR-based assay that permits the rapid assessment of the polyadenylation state of a particular mRNA (Sallés *et al.*, 1992). In this assay, the RT reaction was primed with an oligo d(T) primer with a GC-rich anchor sequence. The PCR reaction was then performed with a gene-specific 5' primer and the 3' GC-rich primer. If an mRNA has a short poly(A) tail, the PCR fragment would resolve after electrophoresis as a narrow band about the same size as predicted from the sequence. However, if the mRNA was polyadenylated to any extent, the resolved products would appear as a broad smear, since the anchored 3' primer used for the initial RT reaction could prime at a number of different sites along the poly(A) tail. A schematic diagram of this assay is shown Sallés *et al.* (1992).

When this assay was used, fertilization resulted in an increase in the length of the PCR product (Fig. 3). There are 346 nucleotides between the 5' primer and the last nucleotide before the poly(A) tail. For both the egg and one-cell embryo, the leading edge of the PCR product corresponded to 380–390 nucleotides, which suggests that the length of the poly(A) tail of vi12H was at least 40–50 A residues. More important,

Fig. 3. Autoradiogram of the PCR product of the mouse cDNA vi12H before and after fertilization. RNA samples collected from unfertilized eggs and one-cell embryos were subjected to the PCR polyadenylation assay. Equal amounts of acid-precipitable material from each were subjected to electrophoresis on a 5% polyacrylamide gel that was then subjected to autoradiography. Lane A, RNA from unfertilized eggs; lane B, RNA from one-cell embryos 7 h after insemination. The experiment was conducted three times and similar results were obtained in each case; this figure shows a representative example. Molecular size markers (in base pairs) are indicated to the left.

however, was the observation that the trailing edge of the PCR product increased in the fertilized eggs. Whereas the trailing edge obtained from eggs corresponded to about 430 base pairs, the product obtained from one-cell embryos obtained about 7 h after insemination extended to over 550 base pairs. Results of these experiments suggest that vi12H mRNA in the egg has a poly(A) tail of 40–80 nucleotides, and that after fertilization the length of this tail increases by some 180 A residues.

The possibility that the observed size heterogeneity that occurred after fertilization was not due to an increase in the length of the poly(A) tail but due to spurious mispriming at the 5' end was excluded by restricting the PCR products with *AvrII*. Without taking the length of the poly(A) tail into account, restriction of the PCR product with *AvrII* should yield a 126 base pair 5' fragment and a 220 base pair 3' fragment. As expected, a sharply defined product of 126 base pairs was obtained from eggs and one cell embryos, as well as a broader, slower migrating product the leading edge of which corresponded to 260 base pairs, which is consistent with a minimum length of the poly(A) tail of about 40 nucleotides (see above) (Fig. 4). This result strongly suggested that the increase in the size heterogeneity observed following fertilization resided at the 3' end of the transcript and was therefore due to polyadenylation. Consistent with this interpretation was the additional observation that the length of this fragment derived from the 3' end of the transcript was more heterogeneous in the one-cell embryos when compared with that in the egg. The results of these experiments indicate that vi12H mRNA was polyadenylated following fertilization.

Transient polyadenylation of vi12H after fertilization

Polyadenylation of mRNA has been well documented for several mRNAs, including messages encoding tPA, hypoxanthine ribosyl transferase (HPRT) and actin, during oocyte maturation in the mouse (Vassalli *et al.*, 1989; Sallés

Fig. 4. Autoradiogram of the PCR product of the mouse cDNA vi12H obtained from either unfertilized eggs or one-cell embryos restricted with *AvrII*. The RNA samples from unfertilized eggs and one-cell embryos were subjected to the PCR polyadenylation assay. An aliquot from each was restricted with *AvrII*, and equal amounts of trichloroacetic-acid-precipitable material were subjected to electrophoresis in a 10% polyacrylamide gel that was then autoradiographed. Lane A, RNA from unfertilized eggs; lane B, RNA from one-cell embryos. The arrows indicate the position of the portion of the PCR product derived from either the 5' or 3' end. Molecular size markers (in base pairs) are indicated to the right.

Fig. 5. Time course of polyadenylation of the mouse cDNA vi12H after insemination. Unfertilized eggs were collected, fertilized *in vitro*, and incubated for the times (h) indicated above each lane; 0 refers to the time of insemination. At the indicated times, the one-cell embryos were removed and the extent of polyadenylation of vi12H mRNA was determined. The experiment was performed three times and similar results were obtained in each case; the figure shows a representative example. Molecular size markers (in base pairs) are indicated to the left.

et al., 1992; Paynton and Bachvarova, 1994). In contrast, the polyadenylation of vi12H mRNA takes place after fertilization. For a more precise definition of the temporal pattern of adenylation, eggs were fertilized *in vitro* and samples for RNA preparation were removed 3, 5, 7, 9, 12, 15, 18 and 21 h after insemination, and the polyadenylation status of vi12H was measured as described above.

Results of these experiments indicate that the maximum amount of polyadenylation occurred within 3 h of insemination and corresponded to a poly(A) tail of about 250 residues (Fig. 5). The polyadenylation status remained relatively constant up to 5 h after insemination; however, around the time of pronuclear formation, 7 h after insemination, it has declined to the 150 base pairs seen in RNA obtained from one-cell embryos fertilized *in vivo*. Thus, vi12H mRNA was transiently polyadenylated after fertilization.

Discussion

This study reports the identification of a maternal mRNA that is transiently polyadenylated after fertilization of mouse eggs. This increase is observed by 3 h after insemination, and since fertilization *in vitro* occurs between 1 and 2 h after the addition of spermatozoa, this increase is likely to occur within 1 h after fertilization. Although the function of vi12H is unknown, the identification of this cDNA provides a molecular marker to study further aspects of the recruitment of maternal mRNA following fertilization.

There are now several examples of mRNAs that are polyadenylated during mouse oocyte maturation, such as tPA and HPRT. Although the best studied to date is tPA, the function of the recruited tPA mRNA is unknown since ablation of the maternal mRNA with antisense oligonucleotides does not inhibit either oocyte maturation or preimplantation development (Strickland *et al.*, 1988). Nevertheless, the sequence element responsible for directing the maturation-associated polyadenylation of tPA mRNA, which then becomes translationally active, has been identified (Sallés *et al.*, 1992). This CPE is a U-rich sequence AUUUUAAU near the polyadenylation signal. The CPE for HPRT mRNA is UUUUAAA (Paris and Richter, 1990). Thus, these CPEs resemble other CPEs, the hallmark feature of which is a U-rich track that is usually located within 100–200 nucleotides of the polyadenylation signal AAUAAA (Richter, 1991).

The clone for vi12H, which is 543 base pairs in length, does not contain an open reading frame; northern blotting analysis reveals that the greatest expression of the 3.9 kilobase transcript is in the testes (data not shown). The cDNA displays no significant homologies to any sequence in the Genbank files, and contains a poly(A) tail as well as the polyadenylation signal sequence AAUAAA that is required for both nuclear and cytoplasmic polyadenylation (Wickens, 1992). Unlike mRNAs that are polyadenylated during oocyte maturation, sequence analysis of the cDNA for vi12H does not reveal the presence of a CPE resembling CPEs that have previously been reported and shown to be involved in translational control. The minimum CPE element identified to date is UUUUAAU; for example, the *c-mos* CPE (Paris and Richter, 1990). Examination of the sequence for vi12H reveals a U-rich tract at nucleotides 221–232 (UUUUUUUGUUU) that could serve as a CPE. In addition, there are other regions that contain five U residues within a six nucleotide stretch; for example, nucleotides 373–378 and nucleotides 415–420 (UGUUUU). It should be noted, however, that the established CPEs do not contain internal G residues and terminate with AU (Richter, 1991). Thus, the mRNA for vi12H may contain a novel CPE element. We are currently constructing a chimaeric mRNA transcribed *in vitro* that contains the coding sequence of the gene for luciferase and the vi12H sequence. This aim is to ascertain whether the vi12H sequence is sufficient to direct the polyadenylation of and an increase in luciferase activity after insemination of eggs microinjected with this transcript and, if so, to define the element(s) required for its mobilization after insemination.

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