

Timely translation during the mouse oocyte-to-embryo transition

Bermseok Oh^{1,*}, Sue-Yun Hwang^{1,‡}, John McLaughlin^{2,§}, Davor Solter² and Barbara B. Knowles^{1,¶}

¹The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA

²The Max Planck Institute of Immunobiology, Stübeweg 51, Freiburg D79108, Germany

*Present address: Department of Biomedical Science, National Institute of Health, Seoul 122-701, Korea

‡Present address: Research Institute of Immunology, The Catholic University of Korea, Seoul 37-040, Korea

§Present address: University of Pennsylvania School of Veterinary Medicine, Unionville, PA, USA

¶Author for correspondence (e-mail: bbk@jax.org)

Accepted 13 June; published on WWW 9 August 2000

SUMMARY

In the mouse, completion of oocyte maturation and the initiation of preimplantation development occur during transcriptional silence and depend on the presence and translation of stored mRNAs transcribed in the growing oocyte. The *Spin* gene has three transcripts, each with an identical open reading frame and a different 3' untranslated region (UTR). β -galactosidase-tagged reporter transcripts containing each of the different *Spin* 3'UTRs were injected into oocytes and zygotes and β -galactosidase activity was monitored. Results from these experiments suggest that differential polyadenylation and translation occurs at two critical points in the oocyte-to-embryo transition – upon oocyte maturation and fertilization – and is dependent on sequences in the 3'UTR. The stability and mobility shifts of ten other maternal transcripts were monitored by reprobing a northern blot of

oocytes and embryos collected at 12 hour intervals after fertilization. Some are more stable than others and the upward mobility shift associated with polyadenylation correlates with the presence of cytoplasmic polyadenylation elements (CPEs) within about 120 nucleotides of the nuclear polyadenylation signal. A survey of the 3' UTRs of expressed sequence tag clusters from a mouse 2-cell stage cDNA library indicates that about one third contain CPEs. We suggest that differential transcript stability and a translational control program can supply the diversity of protein products necessary for oocyte maturation and the initiation of development.

Key words: Gene expression, Translational control, Preimplantation embryo, Mature oocyte, Stored maternal mRNA, Cytoplasmic polyadenylation, 3'UTR, dbEST

INTRODUCTION

The metazoan egg and newly formed embryo are transcriptionally inert. The completion of meiosis, initiation of the first mitoses and activation of transcription, which occur after visible differentiation in the invertebrate embryo, are under the control of genes whose mRNAs and protein products were synthesized and stored in the growing oocyte (Wilt, 1973; Rosenthal and Ruderman, 1987). In mammals, de novo synthesis of transcripts in the oocyte also ceases as it enters meiosis and begins maturation. Although new transcription from the embryonic genome is activated at the 2-cell up to the 8- to 16-cell stage, depending on the species (Telford et al., 1990), this process takes time. In mice, resumption of meiosis I, as measured by germinal vesicle breakdown (GVB), occurs approximately 12 hours before ovulation while complete activation of the embryonic genome has taken place within approximately 35 hours of fertilization, by the end of the 2-cell stage (Schultz, 1993; Wassarman et al., 1979). Taken together, this transcriptionally quiescent period can last almost 2 days. During this time the differentiated oocyte and sperm nuclei become reprogrammed to be the active genome of the now totipotent embryo.

Transcription and translation of maternal transcripts has been extensively investigated in *Xenopus*. In contrast to the mammalian egg, the frog egg contains localized cytoplasmic determinants and the frog embryo undergoes significant morphological differentiation and multiple cell divisions within the several hours that elapse between fertilization and activation of their embryonic genomes (Newport and Kirschner, 1982a,b). Translation of maternal transcripts in this organism during the time of transcriptional quiescence is associated with their polyadenylation in the cytoplasm, a process controlled by a protein-RNA complex assembled at an UA-rich sequence in the 3' untranslated regions (UTR) proximal to the nuclear polyadenylation signal, AAUAAA (Richter, 1996; Wickens et al., 1996). It has been suggested that cap ribose methylation is facilitated during the process of polyadenylation, and that the interaction between the modification machinery at the 3'- and 5'-UTRs of these transcripts underlies translational control at this time in development (Kuge and Richter, 1995).

Delayed translation of specific stored maternal messages could provide the temporal coordination of protein synthesis necessary for the mammalian oocyte-embryo transition. Compelling experiments performed in mice have shown

that polyadenylation of the mRNA encoding tissue-type plasminogen activator (tPA) accompanies translation (Huarte et al., 1987), and that interference with cytoplasmic polyadenylation of the *Mos* transcript prevents oocyte maturation (Gebauer et al., 1994). Controlled translation of both these messages is crucially dependent on the presence of the cytoplasmic polyadenylation element (CPE), originally defined as UUUUAU in the 3'UTR (Fox et al., 1989; McGrew et al., 1989). Indeed, it is phosphorylation of the CPE-binding factor that regulates translation of *c-Mos* (Mendez et al., 2000).

Up until now the scarcity and relative inaccessibility of mammalian eggs and preimplantation embryos precluded intensive study of RNA stability and protein synthesis at these developmental stages in mammals. In this report we have investigated a sufficient number of maternally transcribed mouse genes to allow a comprehensive look at the processes occurring at this time in development, and to define the consensus sequence of the mammalian cytoplasmic polyadenylation element and its placement within the 3'UTR. We demonstrate for the first time in mammals that maternal message polyadenylation and translation occur not only during oocyte maturation but also after fertilization. Furthermore, because we investigated a series of genes, and extended this study by analysis of Expressed Sequence Tags (ESTs) from our 2-cell stage embryo library (Rothstein et al., 1992), we suggest that the process of timely controlled translation is a general process, guiding the oocyte-embryo transition.

MATERIALS AND METHODS

Oocyte and embryo collection

Full-grown, germinal-vesicle (GV)-intact oocytes (FGO) were collected by puncturing ovarian follicles from 22- to 24-day-old female mice (B6D2 F1 or B6A F1) with a 30-gauge needle in HEPEs-buffered Eagle's MEM containing 3 mg/ml bovine serum albumin. The surrounding granulosa cells were separated from the oocytes by pipetting. Metaphase II-arrested ovulated oocytes (OO) were collected from the oviducts of female mice that had been superovulated by injecting human chorionic gonadotrophin (hCG) 48 hours after administering 5 units pregnant mare's serum gonadotrophin (PMSG), and killed 13 hours later. Cumulus cells were removed by a short incubation in medium containing 500 µg/ml hyaluronidase at 37°C and the oocytes were washed several times in fresh medium. To collect synchronized embryos, superovulated females were caged 13 hours after hCG injection with male mice for 1.5 hours, and vaginal plugs were checked. Zygotes (1-cell embryos) were collected from the oviducts 12 or 16 hours after mating. Early 2-cell, late 2-cell and 4- to 8-cell embryos were collected 24, 36 and 48 hours after mating, respectively.

Northern blot analysis

Oocytes and embryos were placed directly into lysis buffer containing 0.1 M Tris-HCl, pH 7.4, and 4 M guanidium thiocyanate. RNA was ethanol-precipitated after adding 20 µg *E. coli* tRNA (RNase-free, Roche Molecular Biochemicals). Northern blots were prepared from phenol/chloroform extracted, ethanol-precipitated RNA from 500 oocytes and embryos that had been run on a 0.9% formaldehyde agarose gel, transferred to a nylon membrane (Micro Separation, Inc.) and ultraviolet (u.v.)-crosslinked. The probes used for hybridization were: various *Spin* fragments including the 0.7 kb *Bam*HI-*Hind*III fragment spanning the *Spin* open reading frame, the 0.9 kb *Bgl*II-*Eco*RI *Spin* fragment spanning the 3' end of the 4.1 kb mRNA, the *Hind*III-digested pBlue-β-galactosidase vector containing either the

Spin 0.8 and 1.7 kb 3'UTR; a 1.8 kb full-length 18 S ribosomal RNA cDNA (Oh et al., 1997); β-*actin*, 1.8 kb full-length cDNA isolated from our blastocyst library (Rothstein et al., 1992); *Melk*, 1.2 kb partial cDNA probe isolated from our 2-cell cDNA library (Heyer et al., 1997); *tPA*, 2.5 kb full-length cDNA (Rickles et al., 1988; a kind gift from S. Strickland, SUNY, Stony Brook, NY, USA); *Maid*, 1.5 kb full-length probe isolated from our 2-cell cDNA library (Hwang et al., 1997); SSEC 51, 0.7 kb partial cDNA probe obtained from our 2-cell subtraction library; *Ptp4a1*, full-length 3.0 kb cDNA isolated from our 2-cell cDNA library (Diamond et al., 1994); β-*catenin*, 1.5 kb partial cDNA isolated from our ES cell cDNA library; SSEC D, 0.5 kb partial cDNA probe isolated from our 2-cell stage-specific subtraction library (Oh et al., 1999). The probes were stripped from membranes by boiling for 2 minutes in buffer containing 0.25% SDS and 0.05% 20× SSC before reprobing.

RNaseH treatment

Hybridization with oligo d(T)₁₅ (Roche Molecular Biochemicals, Indianapolis, IN, USA) or with specific antisense oligonucleotides and deletion of the resulting DNA/RNA hybrids was accomplished by RNaseH treatment. RNA, denatured by heating at 95°C for 5 minutes and cooling on ice, was incubated with 500 ng oligo d(T)₁₅, or with 500 ng of an antisense oligonucleotide (p3532, 5'-TCGGCGCTA-CGACTGCTTCA-3') specific to the 3' end of the 4.1 kb *Spin* mRNA, for 10 minutes at 25°C. The KCl concentration was adjusted to 50 µM and following a 10-minute incubation, 1 unit of RNaseH (Roche Molecular Biochemicals) was added and the reaction mixture was incubated for 30 minutes at 37°C. Phenol/chloroform-extracted ethanol-precipitated RNA was resuspended, separated on formaldehyde gels and analyzed by northern blotting as described (Mercer and Wake, 1985; Oh et al., 1999).

β-galactosidase reporter constructs and analysis

The coding sequence of β-galactosidase (*Hind*III 414-*Dra*I 3819) from pSV-β-galactosidase (Promega) was subcloned into the *Hind*III and *Sma*I sites of pBluescript SK (+) so that the T7 promoter was available for in vitro transcription (pBlue-β-galactosidase). The full-length *Spin* 3'UTR sequences were amplified with the *Bam*HI-anchored 5'-primer and *Xba*I-anchored 3'-primer from *Spin* cDNAs, digested with *Bam*HI and *Xba*I, and inserted into pBlue-β-galactosidase plasmid downstream of the coding sequence. Capped RNAs were transcribed in vitro from linearized reporter constructs using T7 RNA polymerase (Ambion, mMESAGE mMACHINE). These same reporter gene transcripts were also polyadenylated in vitro, as previously described (Vassalli et al., 1989).

Full-grown (GV-intact) oocytes were collected from 3-week-old B6D2 female mice that were not treated with PMSG, and β-galactosidase-reporter transcripts (0.2 µg/µl in 0.15 M KCl) were injected into their cytoplasm. For monitoring maturing oocytes that have undergone germinal vesicle breakdown (GVB), oocytes were cultured in modified MEM (Eppig and Wigglesworth, 1994) without supplementation. For monitoring germinal vesicle intact (GV)-oocytes the MEM was supplemented with 100 µM 3-isobutyl-1-methyl xanthine (IBMX) to prevent oocyte maturation. Oocytes were harvested 16-18 hours later for assay. Zygotes were injected 22-26 hours after hCG administration when the pronuclei became prominent, cultured for 16 hours, and β-galactosidase was analyzed in those that formed 2-cell stage embryos, by incubating with the luminescent β-galactosidase Genetic Reporter System (ClonTech) according to the manufacturer's instructions. Briefly, individual oocytes or zygotes were lysed by three freeze/thaw cycles in 5 µl 0.1 M potassium phosphate buffer, pH 7.8, mixed with 50 µl reaction buffer containing Galacton-Star, and substrate conversion was measured in an Auto Lumat LB953 (Berthold, Germany). The relative light units emitted by each oocyte or embryo was ranked, the median found, and the individual data points were ranked and tested for significance using the Kruskal-Wallis one-way analysis of variance.

The injection protocol and the time course of the assay have been previously described (Oh et al., 1999).

To assess the stability of the *Spin* 3'UTR reporter transcripts in the injected oocytes and embryos after injection, batches of 10 embryos from the following stages were harvested and the RNA extracted. Immature oocytes were harvested immediately and after culture for 14 hours in the presence of IBMX (GV), mature oocytes were harvested after 14 hours culture without IBMX (GVB), and zygotes were microinjected and immediately harvested or cultured for 8 hours. Northern blots of these extracted RNAs were hybridized to radiolabeled probes prepared by *in vitro* transcription of the *Hind*III-digested pBlue- β -galactosidase vector containing the *Spin* 0.8 and 1.7 kb 3'UTR, using T3 RNA polymerase (Clontech). To analyze the fate of the microinjected 4.1 kb *Spin* 3'UTR- β -galactosidase reporter gene, RNA was extracted from 10 microinjected oocytes, annealed with an antisense oligonucleotide complementary to the end of the β -galactosidase coding region (5'-CCACTACCATCAATCCGGTA-3'), and digested with RNaseH prior to gel electrophoresis so that the size of the RNA could be reduced and more accurately measured. The resultant northern blot was hybridized with an antisense RNA probe synthesized from *Mlu*I-linearized pBlue- β -galactosidase using T3 RNA polymerase.

Microinjected *Xenopus* eggs were lysed by three freeze-thaw cycles in 0.5 ml 0.1 M potassium phosphate solution, pH 7.8, and β -galactosidase activity was determined on one hundredth of the lysate volume (Oh et al., 1999).

Radiolabeling and immunoprecipitation

200 oocytes or zygotes were metabolically labeled in 50 μ l of methionine-free medium containing 1 mCi/ml 35 S-labelled methionine (specific activity >1,000Ci/mmol; Amersham) for 3 hours, lysed, and subjected to immunoprecipitation as previously described (Oh et al., 1997, 1998). Antibodies to E-cadherin and β -catenin were purchased from Transduction Laboratories, Inc.; rabbit antibody to SPIN has been previously described (Oh et al., 1997, 1999). Lysates were precleared of nonspecifically binding polypeptides by incubation with a slurry of protein A-Sepharose CL4B (Sigma) that was preincubated with normal rabbit immunoglobulins. The precleared supernatants were then incubated for 1 hour at 4°C with 50 μ l protein A beads that had been preincubated with immune Ig, and the antigen/antibody complexes were prepared for SDS-PAGE (Oh et al., 1999). Quantification of the Spindlin, β -catenin and E-cadherin

autoradiograms was performed using the quant mode of the FUJIX Bio-Imaging System (BAS 1000 MacBAS).

RESULTS AND DISCUSSION

Differential cytoplasmic polyadenylation of transcripts during the oocyte-to-embryo transition

Spindlin (*Spin*), a gene encoding a very abundant protein that undergoes cell cycle-dependent phosphorylation in the egg and early embryo, has three transcripts in the full-grown oocyte (Oh et al., 1997, 1998). Although each transcript contains the same open reading frame, each uses a different nuclear polyadenylation signal and consequently contains differing lengths of the 3'UTR (Fig. 1A). This finding afforded us the opportunity to study the effect of each 3'UTR on translation. The 0.8 kb *Spin* transcript uses an alternative polyadenylation

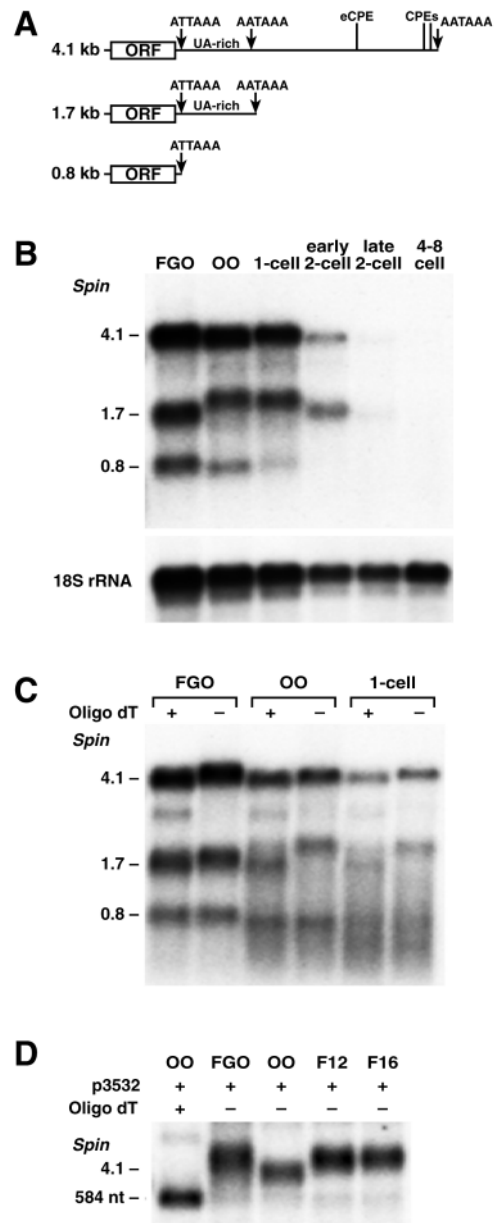


Fig. 1. Cytoplasmic polyadenylation of *Spin* maternal transcripts in oocytes and embryos. (A) Structure of the three *Spin* mRNAs. CPE, cytoplasmic polyadenylation element; eCPE, embryonic CPE-dodecauridine. (B) Northern blot analysis of the three *Spin* maternal mRNAs. Upper panel: a blot of RNAs from 500 oocytes or embryos was hybridized with a 0.7 kb *Bam*HI-*Hind*III *Spin* fragment spanning the open reading frame (ORF). Bottom panel: the same blot hybridized with a 1.8 kb full-length 18S ribosomal RNA cDNA. FGO, full-grown oocyte; OO, ovulated oocyte; 1-cell, early 2-cell, late 2-cell and 4-8-cell embryos. (C) Northern blot analysis of RNAs from 500 FGO, OO and 1-cell embryos that had been annealed with oligo d(T)₁₅ (oligo-dT⁺) or not (oligo-dT⁻) and treated with RNaseH to digest regions of DNA/RNA hybrids prior to electrophoresis, blotting and hybridization to the *Spin* ORF probe. (D) RNAs from 1-cell embryos collected 12 or 16 hours after fertilization (F12, F16), respectively, were annealed with an antisense oligo nt specific to the 3' end of 4.1 kb *Spin* mRNA (p3532), and then treated with RNaseH. RNAs in the first lane were also annealed with 500 ng of oligo d(T)₁₅. Hybridization was performed with the 0.9 kb *Bgl*II-*Eco*RI *Spin* fragment spanning the 3' end of the 4.1 kb mRNA. (The deadenylated fragment from the RNaseH-treated 4.1 kb transcript is 584 nt in length.) Blots from representative experiments are shown.

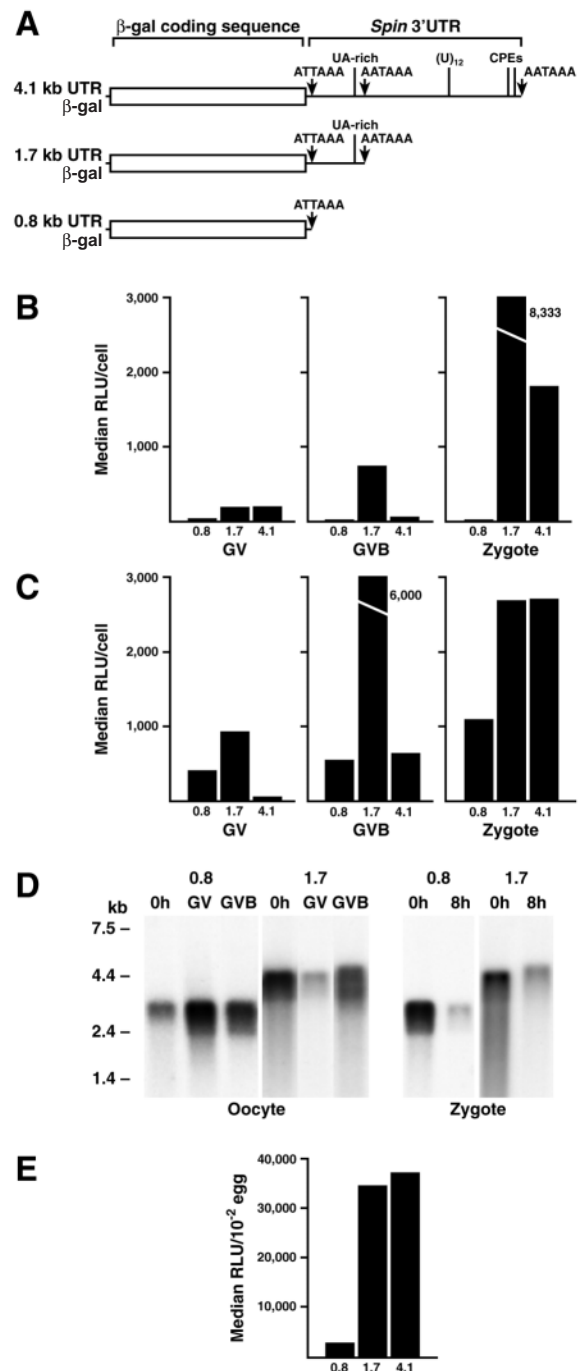
signal, AUUAAA, and contains a 40-nucleotide 3'UTR with no known motifs. Northern blot analysis shows that the size and abundance of this smallest transcript decreases in the ovulated oocyte, compared to that in the full-grown egg, until it is barely detected in the 1-cell embryo (Fig. 1B). In contrast, the relative amounts of the 1.7 kb and 4.1 kb transcripts do not diminish until early in the 2-cell stage. Furthermore, the 1.7 kb transcript undergoes an obvious size increase in the ovulated egg and 1-cell embryo (Fig. 1B).

To determine whether the electrophoretic mobility change is the result of transcript polyadenylation, RNAs from full-grown oocytes, ovulated oocytes and 1-cell embryos were annealed with oligo-d(T) and the DNA/RNA hybrid molecules were removed by treatment with RNaseH. As a result of this treatment the sizes of the 1.7 and 4.1 kb transcripts from the ovulated eggs and 1-cell embryos decrease (Fig. 1C), indicating that the differences in size of these transcripts in the ovulated oocytes and 1-cell embryos are the result of poly(A) tail elongation. In contrast, no difference in size of the 0.8 kb transcript is observed after RNaseH treatment in the ovulated

oocyte or zygote where it is already deadenylated and being degraded.

It is difficult to accurately assess gel-mobility shifts of the 4.1 kb transcript in the oocyte, egg and embryo because of its large size. Accordingly, we clipped this transcript into smaller fragments by annealing the RNA isolated from each stage with an oligonucleotide corresponding to a sequence 584 nucleotides (nt) from its 3' end, and then treating this RNA/DNA hybrid with RNaseH. Hybridization of a northern blot of these truncated transcripts with the 4.1 kb *Spin* 3'UTR probe shows this transcript is substantially polyadenylated in the full-grown oocyte, it decreases by 150 nt in the ovulated egg, and it regains its fully polyadenylated state (400 nt) in the

Fig. 2. Translation of reporter transcripts containing each of the *Spin* 3'UTRs. (A) Diagram of β -galactosidase reporter constructs used for microinjection. (B) A representative β -galactosidase assay of germinal vesicle (GV)-intact oocytes, oocytes that had undergone germinal vesicle breakdown (GVB), and zygotes microinjected with each non-polyadenylated transcript. Capped RNAs were transcribed in vitro from linearized reporter constructs. Data shown are medians of the relative light units (RLU) obtained from individual cell assays after incubation with the luminescent β -galactosidase Genetic Reporter System. In the experiments shown for the 0.8kb *Spin* 3'UTR reporter transcript, nine GV-intact oocytes, six GVB-oocytes and 67 2-cell embryos were evaluated, and for the 0.8 kb *Spin* 3'UTR polyadenylated reporter construct 10, 9 and 8 oocytes and embryos, respectively, were assayed. For the 1.7 kb *Spin* 3'UTR reporter transcript, $n=27$, 10 and 35 for each of the respective stages, and for the polyadenylated 1.7 kb *Spin* 3'UTR reporter transcript $n=8$, 2 and 9, respectively. For the 4.1 kb *Spin* 3'UTR reporter transcript, $n=20$ for the GV-intact oocytes, 15 for the GVB-oocytes and 14 for the 2-cell-stage embryos, and for the polyadenylated 4.1 kb *Spin* 3'UTR reporter transcript, $n=8$, 4 and 6, of the respective stages. These data were subjected to statistical analysis using the Kruskal-Wallis one-way analysis of variance. The results obtained are significantly different from each other. For GV-intact oocytes, $H=14.16$, $P<0.001$, $n=55$; for GVB oocytes, $H=19.74$, $P<0.001$, $n=91$; for zygotes, $H=17.58$, $P<0.001$, $n=76$. (C) A representative β -galactosidase assay of immature GV-intact oocytes, maturing GVB-oocytes, and zygotes injected with reporter gene transcripts that were polyadenylated in vitro. The results obtained, i.e. for GV-intact oocytes, $H=8.68$, $P<0.02$, $n=33$ and for GVB oocytes, $H=6.68$, $P<0.05$, $n=15$, are significantly different from each other, while those for zygotes, $H=0.67$, $P<0.80$, $n=23$, are not. (D) Status of *Spin* reporter transcripts after injection and incubation in oocytes and zygotes. In vitro synthesized *Spin*- β -galactosidase fusion mRNAs were microinjected into full-grown GV-intact oocytes. Representative experiments are shown in which ten immature oocytes were immediately harvested (0 h), ten were harvested after culture for 14 hours in the presence of IBMX (GV), and ten mature oocytes were harvested after a 14 hour culture without IBMX (GVB). Zygotes were microinjected and immediately harvested (0 h) or cultured for 8 hours (8 h). Northern blots of these RNAs were hybridized to radiolabeled probes of the *Spin* 0.8 and 1.7 kb 3'UTR. (E) β -galactosidase assay of *Xenopus* fertilized eggs after microinjection with each nonpolyadenylated transcript. The values vary significantly from each other, $H=6.5$, $P<0.05$, $n=15$.



zygote (Fig. 1D). This result suggests that the stable maternal *Spin* transcript is partially deadenylated during oocyte maturation and readenylated following fertilization.

The size changes and differential degradation of the three *Spin* transcripts in these gametes and embryos lead us to suggest that each is activated for translation at a specific time in the gamete to embryo transition. SPIN is a very abundant cytoplasmic and spindle-binding protein in the maturing oocyte and embryo that is differentially phosphorylated in a cell-cycle-specific fashion during the meiotic and first mitotic cell cycles in the MOS/MAP kinase pathway. At this time of transcriptional quiescence, translation from sequestered maternal transcripts would ensure a ready supply of de novo synthesized protein.

The 3'UTR alone drives differential translation of reporter transcripts

To determine whether the stage-specific polyadenylation of the transcripts observed by northern blot analysis correlates with their translation, β -galactosidase reporter constructs containing each of the different *Spin* 3'UTRs were prepared (Fig. 2A). Following in vitro transcription these β -galactosidase-tagged 3'UTR reporter RNAs were injected into, and β -galactosidase activity was analyzed from, immature oocytes (GV intact), maturing oocytes that had undergone germinal vesicle breakdown (GVB), and zygotes (1-cell embryos). The 0.8 kb *Spin* 3'UTR- β -galactosidase reporter transcript was not translated in any stage (Fig. 2B) unless the RNA was polyadenylated in vitro before injection. Then, a modest level of translation was detected in GV-intact oocytes and GVB-oocytes as well as in zygotes (Fig. 2C). The 1.7 kb *Spin* 3'UTR- β -galactosidase reporter transcript was translated in the maturing oocyte and in the zygote (Fig. 2B), but when this RNA was polyadenylated in vitro before injection, translation was detected in all tested stages (Fig. 2C). The 4.1 kb *Spin* 3'UTR- β -galactosidase reporter transcript was maximally translated in the zygote, but when it was polyadenylated before injection a modest amount of translation was also seen in the maturing but not the full-grown oocyte. Absence of translation of the fully polyadenylated reporter construct suggests that SPIN is also not translated from the fully polyadenylated, endogenous 4.1 kb *Spin* message present in full-grown oocytes (see Fig. 1D). These results suggest the presence of silencing factors in the full-grown oocyte cytoplasm that may bind to 3'UTR sequences to prevent message utilization. This mechanism has been suggested to control translational silencing of *tPA* mRNA in full-grown oocytes before the onset of their maturation (Stutz et al., 1998); however, unlike the 4.1 kb *Spin* mRNA, *tPA* mRNA is not extensively polyadenylated in the full-grown oocyte.

To demonstrate that the reporter gene transcripts that are not translated are indeed present throughout the period after injection, RNA was isolated at appropriate times after microinjection into mouse oocytes and embryos, and the reporter transcript was identified by northern blot analysis. The *lacZ* reporter transcript containing the 0.8 kb *Spin* 3'UTR was intact, even 14 hours after injection in immature and maturing oocytes, and some remaining full-length transcript was detected in the zygote 8 hours after injection (Fig. 2D). We conclude that the 0.8 kb 3'UTR- β -galactosidase transcript is relatively stable after injection, and is simply not translated

unless it is polyadenylated in vitro before injection. The microinjected 1.7 kb 3'UTR- β -galactosidase reporter transcript was also still present at the time of harvest, and its size increased after incubation in both maturing oocytes and zygotes (Fig. 2D). This result confirms the studies showing that the 1.7 kb reporter transcript is maximally translated in the maturing oocyte and embryo, and correlates this translation with in vivo polyadenylation of the injected transcript. The 4.1 kb reporter transcript when microinjected in oocytes does not change in size (data not shown), mirroring the reporter gene assay.

To determine whether the robust regulation of translation by the diverse *Spin* 3'UTRs that we observed in mouse oocytes and embryos occurs in other species, we injected fertilized *Xenopus* eggs with these same *Spin* 3'UTR- β -galactosidase reporter RNAs. The 1.7 and 4.1 kb 3'UTR-containing RNAs were also abundantly translated in the frog embryo (Fig. 2E), suggesting, as found by others (Verotti et al., 1996), that these 3'UTRs contain *cis*-element(s) universally recognized by factors present in the cytoplasm of vertebrate embryos. These results support the theory that sequences in the 3'UTR of maternal mRNAs are a major factor contributing to polyadenylation and translation of stored mRNAs during the gamete to embryo transition in mice.

To determine whether the timing of endogenous SPIN biosynthesis correlates with the results obtained from the injection of *Spin* 3'UTR- β -galactosidase reporter transcripts, oocytes and embryos were pulse-labeled and SPIN was immunoprecipitated. As predicted, SPIN is biosynthesized in full-grown oocytes (GV) but more abundantly biosynthesized (and phosphorylated to higher molecular weight forms; Oh et al., 1997, 1998) in the ovulated oocyte (GVB) and embryo (Fig. 3). We suggest that the 0.8 kb form of *Spin* is translated in the growing oocyte, and that the 1.7 and 4.1 kb transcripts are sequentially translated in the maturing oocyte and zygote, respectively.

From this investigation of the *Spin* gene we find that the 3'UTR alone directs translation at particular stages in the oocyte-to-embryo transition and suggest that these multiple

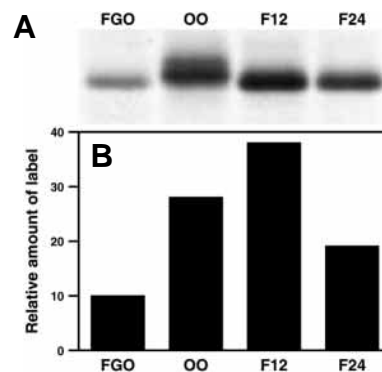


Fig. 3. SPIN biosynthesis in the oocyte-to-embryo transition. SPIN was immunoprecipitated from 200 oocytes or zygotes metabolically labeled with ^{35}S -methionine. (A) FGO (GV intact), OO (GVB), F12 (zygotes harvested 12 hours after fertilization) and F24 (early 2-cell stage embryos harvested 24 hours after fertilization). (B) Densitometric analysis of the relative amounts of SPIN label immunoprecipitated from oocytes and embryos.

transcripts of a single gene may ensure the continuous biosynthesis of its protein product in a changing cellular environment.

Cytoplasmic polyadenylation of maternal mRNAs is a general finding in the mouse oocyte and embryo

We expanded this study to a series of maternal mRNAs that are relatively abundant in the mouse oocyte and zygote. The presence of these maternal mRNAs in the transcriptionally active stages (the growing oocyte and the 4- to 8-cell-stage embryo) can be compared to their abundance in the transcriptionally inert stages (ovulated oocyte, 1-cell and early 2-cell embryo) by northern blot hybridization. This comparison reveals clear differences in the stability and consequent availability of these transcripts for translation. Transcripts of *β-actin*, *Melk* (Heyer et al., 1997), *β-catenin* and *Ptp4a1*, the mouse homologue of the rat *Prll* gene (Diamond et al., 1994), are degraded soon after fertilization and reappear in the 4- to 8-cell stage embryo, after the embryonic genome is activated (Fig. 4). Tissue-type plasminogen activator (*tPA*) mRNA (Rickles et al., 1988) is detectable in the oocyte and ovulated egg but, as previously described, it diminishes to undetectable levels after fertilization (Huarte et al., 1987). Most of the other mRNAs are still abundant in the 1-cell embryo and are then deadenylated and diminished in the 2-cell stage embryo.

These transcripts can also be grouped based on their sizes in the various stages. *β-actin* and *Melk* transcripts, like the 0.8 kb *Spin* transcript, undergo no apparent change in size in the oocyte-to-embryo transition. A second category, however, which includes *tPA*, *Maid* (Hwang et al., 1997) and a novel stage-specific embryonic cDNA clone (SSEC) 51, can be identified. These transcripts, like the 1.7 kb form of *Spin*, increase in size in the ovulated egg. In the case of *tPA*, *Maid* and *Spin* (see above), this size increase is known to result from cytoplasmic polyadenylation. If the transcript is stable, the size of these transcripts is sustained in the 1-cell embryo. The third category includes *Ptp4a1* and *β-catenin*. These transcripts appear to shift in size at two separate time points: once in the ovulated egg and again in the zygote (Fig. 4).

We searched the 3'UTR of these transcripts for potential CPEs, as defined by a working consensus sequence (A)UUUU(U)AU(AA) derived from the *Xenopus* consensus sequence (Fox et al., 1989; McGrew et al., 1989) and from our own data (see Table 1). Of the transcripts in the first category the 0.8 kb *Spin* transcript contains no CPE. Although both *β-actin* and *Melk* transcripts contain potential CPEs in their 3'UTR, more than 120 nt separate the 3' end of the CPEs in *Melk* and *β-actin* from the 5' end of the AAUAAA nuclear polyadenylation signal. In the remainder of the transcripts analyzed, a potential CPE is located no more than 60 or 70 nt away from the nuclear polyadenylation signal (Table 1). There is also a report of two other mouse maternal cDNAs containing potential CPEs in the literature. One, in which the distance from the potential CPEs to the AAUAAA was 95 nt, exhibited cytoplasmic polyadenylation while the other, in which the potential CPE was 145 nt distant from the AAUAAA, did not (West et al., 1996). In *Xenopus*, the distance between the reported CPEs and nuclear polyadenylation signal ranges from 4 to 13 nt, although the addition of 23 more nt did not affect the ability of these transcripts to undergo cytoplasmic polyadenylation (McGrew et al., 1989; McGrew and Richter,

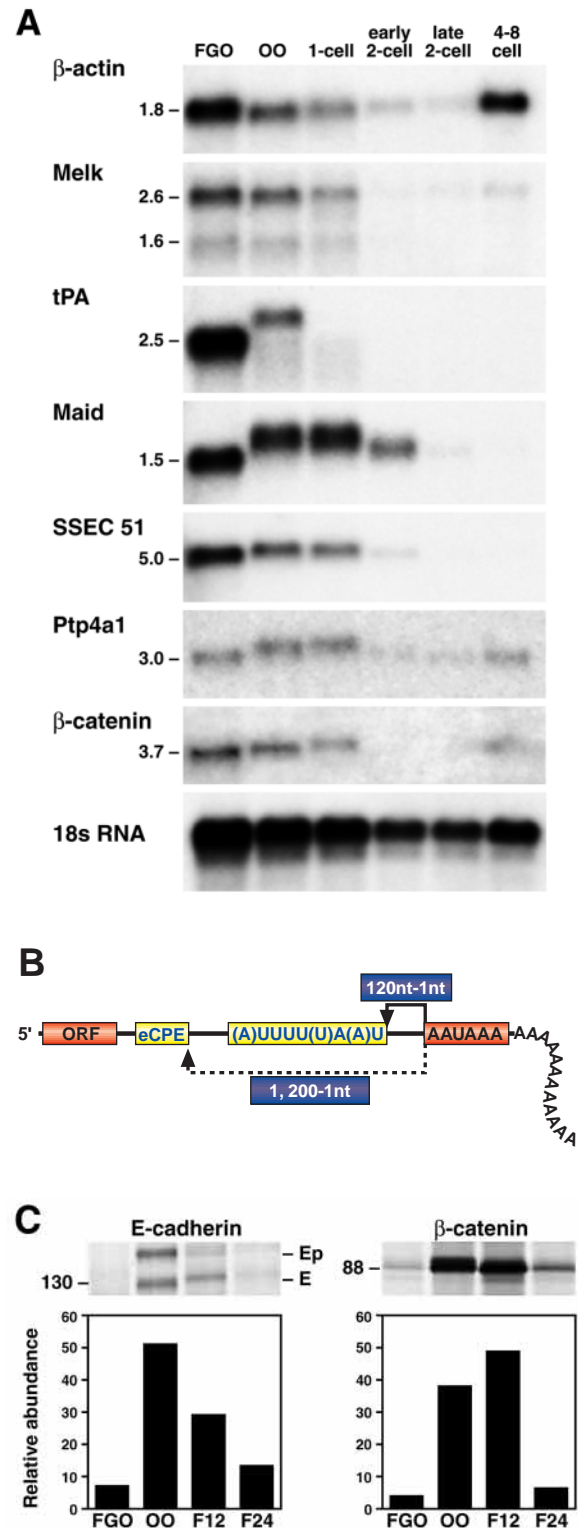


Fig. 4. Differential stability, cytoplasmic polyadenylation and translation from stored maternal mRNAs during the transition from oocyte to embryo. (A) Northern blot of RNAs from 500 oocytes probed, stripped and reprobed as noted. (B) Sequences in the 3'UTR that might regulate translation during the oocyte-to-embryo transition. (C) Immunoprecipitation of pulse radiolabeled E-cadherin and *β-catenin* (upper panel). Densitometric analysis of the relative amounts of each labeled protein immunoprecipitated from eggs and embryos (lower panel). The results from representative experiments are shown.

Table 1. Potential cytoplasmic polyadenylation elements (CPE) in maternal transcripts

cDNA clone	Full-length (kb)	Potential CPE		Polyadenylation
		Sequence	nt from nuclear poly(A) signal	
β -actin	1.8	UUUUUAAU	132	None
<i>Melk</i>	2.6	UUUUUAAU UUUUAAU	120 152	None
<i>Spin</i>	0.8	None	–	None
tPA	2.5	AUUUUAAU AUUUUAAU	53 89	Ovulated egg
<i>Maid</i>	1.5	AUUUUAAA UUUUAAU	6 17	Ovulated egg
SSEC 51	5.0	AUUUUUA AUUUUUAAA	21 56	Ovulated egg
<i>Spin</i>	1.7	UUAUUUUUU	70	Ovulated egg/ zygote
β -catenin	3.7	UUUUAAU	66	Ovulated egg/ zygote
<i>Ptp4a1</i>	3.0	AUUUUUAAA (U) ₈ A(U) ₁₁	66 1059	Ovulated egg/ zygote
<i>Spin</i>	4.1	AUUUUUAAU AUUUUUAAA (U) ₁₂	37 119 1101	Ovulated egg/ zygote

nt, nucleotides.

1990). These data suggest that the distance between the CPE and the AAUAAA may place an inherent constraint on the secondary structure necessary for effective cytoplasmic RNA polyadenylation, and in mice the maximal distance ranges between 95 and 120 nt 5' of the nuclear poly(A) signal (Table 1, Fig. 4B).

Each of the mouse mRNAs investigated that undergoes a second distinct change in the degree of polyadenylation in the embryo, i.e. *Ptp4a1*, β -catenin and the 4.1 kb form of *Spin*, have consensus CPEs in the right range of proximity to the AAUAAA. In addition, *Ptp4a1* and the 4.1 kb *Spin* transcript contain a polyuridine stretch, similar to that described for the *Xenopus* 'embryonic' CPE (Simon et al., 1992; Simon and Richter, 1994). Timely translation of maternal messages containing this consensus sequence is achieved by preventing premature polyadenylation of messages in the frog egg. In these two mammalian transcripts the poly(U) sequences in the 3'UTR are approximately 1,000 nt from the nuclear polyadenylation signal (Table 1, Fig. 4B); however, the presence or absence of this potential 'embryonic' CPE sequence varies between

homologues in different mammalian species. The *Ptp4a1* mouse transcript contains a polyuridine tract in the range of 1,000 nt from the poly(A) signal but the rat homologue does not, and the human β -catenin transcript contains a poly(U) tract but the homologous mouse transcript does not. Polyuridine tracts may be 'embryonic' CPEs in mammals or they may be retained in these genes as a reflection of their evolutionary history.

To test whether the different patterns of polyadenylation of maternal transcripts are reflected in their translation we quantitated the relative amount of protein immunoprecipitated from metabolically pulse-labeled oocytes and embryos (Fig. 4C). The same radiolabeled extract was used for each immunoprecipitation so that quantitative comparisons between the various proteins of specific stages could be made. β -catenin, a member of the third category of transcripts (see Fig. 4A), is as abundantly translated in the zygote as in the ovulated oocyte, whereas E-cadherin, a member of the second category in that its transcripts are equally polyadenylated in the ovulated oocyte and zygote (data not shown), is minimally translated in the immature oocyte, abundantly translated in the ovulated oocyte and less-well translated in the zygote. These data suggest that a detectable increase in transcript polyadenylation presages maximal translation after fertilization.

As an extension to this study we also examined the proportion of transcripts containing CPE consensus sequences in the 1,000 most abundant ESTs from the 2-cell and blastocyst stages (S.-Y. Hwang and B. B. Knowles, in preparation). Over 30% of the clustered ESTs from the 2-cell stage library contain CPEs that are marked for preferential translation in the maturing oocyte and zygote/2 cell embryo (Table 2). Overall the differential stability and clear polyadenylation patterns we have observed in cDNAs from the mouse egg and zygote suggest that metabolism of this unique and constantly changing single cell is actively regulated by differential transcript stability and differential translation of maternal mRNAs.

How are maturation and the initiation of embryo development in mammals controlled by differential mRNA stability and timely translation? The idea that some maternal transcripts are stored in the egg in a dormant state and activated at a later time to initiate development is part of our biological intellectual heritage (Davidson, 1986). In the polarized eggs of species where the embryonic genome is quiescent until

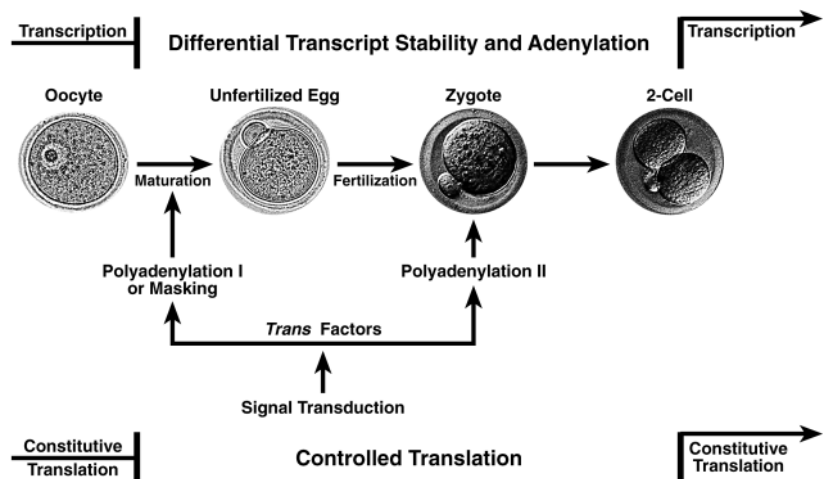


Fig. 5. Diagrammatic representation of the control of the oocyte-to-embryo transition. Stored maternal transcripts are both differentially stable and temporally translated. During the period of transcriptional silence, which in mammals can last several days, the complex temporal control of new protein synthesis is mediated in part by mechanisms which regulate accessibility of stored maternal mRNAs to the translational machinery.

Table 2. Abundance of cytoplasmic polyadenylation elements in 2-cell and blastocyst cDNA libraries

	Clusters with CPE*	Clusters with poly(A) signal‡	Percentage
2-cell	75	249	30
Blastocyst	74	429	17

*As determined by automated pattern matching of individual full-length cluster sequences to consensus CPE motifs within 100 nt of the polyadenylation signal AAUAAA (<http://www.bigarea.area.ba.cnr.it:8000/Biowww>).

‡Libraries were sequenced by the WashU/HHMI mouse EST project from the 5'-end. The average insert size in the 2-cell library is larger than that in the blastocyst. Cluster sequences were prepared from approximately 10⁴ ESTs (of a total of 10⁶ ESTs) from each library.

substantial morphological development has occurred it is well accepted that these maternal transcripts are set down in the egg to initiate and guide development until the embryonic genome is activated. In the *Drosophila* egg this initial control is achieved by localization of protein-protein/protein-mRNA complexes (Driever and Nüsslein-Volhard, 1988; Casanova and Struhl, 1989; Gavis and Lehmann, 1994) as well as by controlled translation.

Totipotency of the early mammalian blastomeres argues against the likelihood that mRNA localization and determinative polarity play an important role in the development of the mammalian embryo. Temporal translation of stored maternal messages, however, has been evolutionarily conserved as a mechanism to ensure a continuing supply of protein to the actively metabolizing ovulated oocyte and embryo, pacing the oocyte-to-embryo transition. Timely translation during transcriptional silence, coupled with turnover and disappearance of some proteins synthesized in the growing oocyte, can significantly alter the protein content of the maturing egg and embryo. Even in the absence of transcription this combination of message lability and timely translation of multiple messages can give rise to a changing environment in which the gametic genomes are reprogrammed for embryogenesis (Fig. 5). The emerging picture is that multiple mRNAs containing translational control elements are silenced and preserved from degradation in the oocyte cytoplasm by the binding of trans-factors (Stutz et al., 1998). They are made available when these binding factors are modified, perhaps as the result of their phosphorylation in the signal transduction pathways activated by ovulation or fertilization (Richter, 1996; Wickens et al., 1996; Mendez et al., 2000; Tay et al., 2000).

Prior to this investigation it was not known whether controlled translation was applicable to just a handful of mouse maternal mRNAs or was a more general process. Furthermore, controlled translation after fertilization had not been demonstrated in mammals. We show on the one hand that sequential translation of these multiple transcripts can ensure continuous synthesis of a protein product throughout this time in development; on the other hand, we demonstrate that transcript stability ensures a changing protein milieu within the egg and embryo. Our results demonstrate how these two elements can orchestrate the changing environment needed for reprogramming of the zygotic nucleus and activation of the mammalian embryonic genome. Understanding the molecules and molecular mechanisms that control the mammalian

oocyte-to-embryo transition takes on a new light when we consider that they also control the reprogramming of somatic cell nuclei transferred to the enucleated oocyte (Wilmot et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998).

We thank Dr Mimi DeVries for her advice, reading, rereading and experimental input, Dr Reinhard Korn for *Xenopus* fertilized egg injections, and Drs Susan Ackerman and Achim Gossler for their comments. Humane animal care was overseen by an institutional review board according to guidelines set by AALAC. Supported by NIH grant HD 35252 and 37102, The Max Planck Society and a postdoctoral fellowship from The Lalor Foundation (B. O.).

REFERENCES

- Casanova, J. and Struhl, G. (1989). Localized surface activity of torso, a receptor tyrosine kinase, specifies terminal body pattern in *Drosophila*. *Genes Dev.* **3**, 2025-2038.
- Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F. A. and Robl, J. M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256-1258.
- Davidson, E. H. (1986). *Gene Activity in Early Development*. New York: Academic Press.
- Diamond, R. H., Cressman, D. E., Laz, T. M., Abrams, C. S. and Taub, R. (1994). PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. *Mol. Cell. Biol.* **14**, 3752-3762.
- Driever, W. and Nüsslein-Volhard, C. (1988). A gradient of bicoid protein in *Drosophila* embryos. *Cell* **54**, 83-93.
- Eppig, J. J. and Wigglesworth, K. (1994). Atypical maturation of oocytes of strain I/LnJ mice. *Human Reprod.* **9**, 1136-1142.
- Fox, C. A., Sheets, M. D. and Wickens, M. P. (1989). Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUU. *Genes Dev.* **3**, 2151-2162.
- Gavis, E. R. and Lehmann, R. (1994). Translational regulation of *nanos* by RNA localization. *Nature* **369**, 315-318.
- Gebauer, F., Xu, W., Cooper, G. M. and Richter, J. D. (1994). Translational control by cytoplasmic polyadenylation of *c-Mos* mRNA is necessary for oocyte maturation in the mouse. *EMBO J.* **13**, 5712-5720.
- Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B. and Ackerman, S. L. (1997). New member of the Snf1/AMPK kinase family, *Melk*, is expressed in the mouse egg and preimplantation embryo. *Mol. Reprod. Dev.* **47**, 148-156.
- Huarte, J., Belin, D., Vassalli, A., Strickland, S. and Vassalli, J.-D. (1987). Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. *Genes Dev.* **1**, 1201-1211.
- Hwang, S. Y., Oh, B., Johnson, K., Solter, D. and Knowles, B. B. (1997). Maid: a maternally transcribed novel gene encoding a potential negative regulator of bHLH proteins in the mouse egg and zygote. *Dev. Dyn.* **209**, 217-226.
- Kuge, H. and Richter, J. D. (1995). Cytoplasmic 3' poly(A) addition induces 5' cap ribose methylation: implications for translational control of maternal mRNA. *EMBO J.* **14**, 6301-6310.
- McGrew, L. L., Dworkin-Rastl, E., Dworkin, M. B. and Richter, J. D. (1989). Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev.* **3**, 803-815.
- McGrew, L. L. and Richter, J. D. (1990). Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: characterization of cis and trans elements and regulation by cyclin/MPF. *EMBO J.* **9**, 3743-3751.
- Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V. and Richter, J. D. (2000). Phosphorylation of CPE binding factor by Eg2 regulates translation of *c-mos* mRNA. *Nature* **404**, 302-307.
- Mercer, J. F. and Wake, S. A. (1985). An analysis of the rate of metallothionein mRNA poly(A)-shortening using RNA blot hybridization. *Nucl. Acids Res.* **13**, 7929-7943.
- Newport, J. and Kirschner, M. (1982a). A major developmental transition in early *Xenopus* embryos: 1. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.

- Newport, J. and Kirschner, N.** (1982b). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30**, 687-696.
- Oh, B., Hwang, S.-Y., Solter, D. and Knowles, B. B.** (1997). Spindlin, a major maternal transcript expressed on the mouse during the transition from oocyte-to-embryo. *Dev.* **124**, 493-503.
- Oh, B., Hampl, A., Eppig, J. J., Solter, D. and Knowles, B. B.** (1998). SPIN, a substrate in the MAP kinase pathway in mouse oocytes. *Mol. Reprod. Dev.* **50**, 240-249.
- Oh, B., Hwang, S.-Y., De Vries, W. N., Solter, D. and Knowles, B. B.** (1999). Identification of genes and processes guiding the transition between the mammalian gamete and embryo. In *Advances in Molecular Biology: A Comparative Method Approach to the Study of Oocytes and Embryos* (ed. J. Richter), pp. 101-126. New York: Oxford University Press.
- Richter, J. D.** (1996). Dynamics of poly(A) addition and removal during development. In *Translational Control* (ed. J. Hershey, N. Sonnenberg and M. Matthews), pp. 481-503. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Rickles, R. J., Darrow, A. L. and Strickland, S.** (1988). Molecular cloning of complementary DNA to mouse tissue plasminogen activator mRNA and its expression during F9 teratocarcinoma cell differentiation. *J. Biol. Chem.* **263**, 1563-1569.
- Rosenthal, E. T. and Ruderman, J. V.** (1987). Widespread changes in the translation and adenylation of maternal messenger RNAs following fertilization of *Spisula* oocytes. *Dev. Biol.* **121**, 237-246.
- Rothstein, J. L., Johnson, D. K., DeLoia, J. A., Skowronski, J., Solter, D. and Knowles, B. B.** (1992). Gene expression during preimplantation mouse development. *Genes Dev.* **6**, 1190-1201.
- Schultz, R. M.** (1993). Regulation of zygotic gene activation in the mouse. *BioEssays* **15**, 531-538.
- Simon, R., Tassan, J. P. and Richter, J. D.** (1992). Translational control by poly(A) elongation during *Xenopus* development: differential repression and enhancement by a novel cytoplasmic polyadenylation element. *Genes Dev.* **6**, 2580-2591.
- Simon, R. and Richter, J. D.** (1994). Further analysis of cytoplasmic polyadenylation in *Xenopus* embryos and identification of embryonic cytoplasmic polyadenylation element-binding proteins. *Mol. Cell. Biol.* **14**, 7867-7875.
- Stutz, A., Conne, B., Huarte, J., Gubler, P., Volkel, V., Flandin, P. and Vassalli, J. D.** (1998). Masking, unmasking, and regulated polyadenylation cooperate in the translational control of a dormant mRNA in mouse oocytes. *Genes Dev.* **12**, 2535-2548.
- Tay, J., Hodgman, R. and Richter, J. D.** (2000). The control of cyclin B1 in mRNA translation during mouse oocyte maturation. *Dev. Biol.* **221**, 1-9.
- Telford, N. A., Watson, A. J. and Schultz, G. A.** (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol. Reprod. Dev.* **26**, 90-100.
- Vassalli, J. D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O'Connell, M. L., Parton, L. A., Rickles, R. J. and Strickland, S.** (1989). Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. *Genes Dev.* **3**, 2163-2171.
- Verrotti, A. C., Thompson, S. R., Wreden, C., Strickland, S. and Wickens, M.** (1996). Evolutionary conservation of sequence elements controlling cytoplasmic polyadenylation. *Proc. Natl. Acad. Sci. USA* **93**, 9027-9032.
- Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R. and Yanagimachi, R.** (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369-374.
- Wassarman, P. M., Schultz, R. M., Letourneau, G. E., LaMarca, M. J., Josefowicz, W. J. and Bleil, J. D.** (1979). Meiotic maturation of mouse oocytes in vitro. *Adv. Exp. Med. Biol.* **112**, 251-268.
- West, M. F., Verrotti, A. C., Sallés, F. J., Tsirka, S. E. and Strickland, S.** (1996). Isolation and characterization of two novel cytoplasmically polyadenylated, oocyte-specific, mouse maternal RNAs. *Dev. Biol.* **175**, 132-141.
- Wickens, M., Kimble, J. and Strickland, S.** (1996). Translational control of developmental decisions. In *Translational Control* (ed. J. Hershey, N. Sonnenberg and M. Matthews), pp. 411-415. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. S.** (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810-813.
- Wilt, F. H.** (1973). Polyadenylation of maternal RNA of sea urchin eggs after fertilization. *Proc. Natl. Acad. Sci. USA* **70**, 2345-2349.