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The stem-loop binding protein regulates translation of histone mRNA during mammalian oogenesis

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Abstract

Although messenger RNAs encoding the histone proteins are among the most abundant in mammalian oocytes, the mechanism regulating their translation has not been identified. The stem-loop binding protein (SLBP) binds to a highly conserved sequence in the 3′-untranslated region (utr) of the non-polyadenylated histone mRNAs in somatic cells and mediates their stabilization and translation. We previously showed that SLBP, which is expressed only during S-phase of proliferating cells, is expressed in growing oocytes at G2 of the cell cycle and accumulates substantially during meiotic maturation. We report here that elevating the amount of SLBP in immature (G2) oocytes is sufficient to increase translation of a reporter mRNA bearing the histone 3′-utr and endogenous histone synthesis and that this effect is not mediated through increased stability of the encoding mRNAs. We further report that translation of the reporter mRNA increases dramatically during meiotic maturation coincident with the accumulation of SLBP. Conversely, when SLBP accumulation during maturation is prevented using RNA interference, both translation of the reporter mRNA and synthesis of endogenous histones are significantly reduced. This effect is not mediated by a loss of the encoding mRNAs. Moreover, following fertilization, SLBP-depleted oocytes also show a significant decrease in pronuclear size and in the amount of acetylated histone detectable on the chromatin. These results demonstrate that histone synthesis in immature and maturing oocytes is governed by a translational control mechanism that is directly regulated by changes in the amount of SLBP.

Keywords

Oocyte; Histones; Translational control; SLBP; Meiotic maturation

Introduction

During growth, the mouse oocyte accumulates maternal products that are utilized during the late stages of oogenesis or after fertilization. For example, certain mRNAs synthesized by

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the growing oocyte are not translated immediately but instead are stored in a silent form until they are recruited for translation during meiotic maturation of the oocyte (Huarte et al., 1987; Vassalli et al., 1989; Stutz et al., 1998; Oh et al., 2000). Conversely, other mRNAs are actively translated during oocyte growth but undergo silencing during maturation (Bachvarova et al., 1989; Paynton and Bachvarova, 1994). The molecular mechanisms underlying silencing and activation of oocyte mRNAs are best understood in the context of the cytoplasmic polyadenylation element (CPE) and the CPE-binding protein (CPEB). The CPE is a U-rich sequence found in the 3′-untranslated region (utr) of certain mRNAs and is typically located within 100 nt of the polyadenylation signal (Oh et al., 2000). In *Xenopus* oocytes, CPEB binds to the CPE and also to a protein termed maskin. Maskin binds to eIF4E, and this prevents initiation of translation (Paris and Richter, 1990; Hake and Richter, 1994; Stebbins-Boaz et al., 1999). During maturation, phosphorylation of CPEB allows recruitment of the cleavage and polyadenylation specificity factor (CPSF), which contributes to the elongation of the polyA tail. This indirectly leads to the displacement of maskin from eIF4E, thus allowing translational activation of the mRNA (Mendez et al., 2000; Hodgman et al., 2001; Cao and Richter, 2002). Numerous maternally provided mRNAs contain CPEs and are differentially polyadenylated and translated during mouse oocyte maturation and early embryogenesis (Oh et al., 2000).

Among the most abundant mRNAs in the oocyte are those encoding the histones (Giebelhaus et al., 1983; Graves et al., 1985). A single oocyte contains as much histone mRNA as a blastocyst, despite the enormous difference in the number of nuclei (Graves et al., 1985), and synthesis of specific subtypes has been reported (Wiekowski et al., 1997; Fu et al., 2003). Some of the histone mRNA species in oocytes are likely polyadenylated; for example, the oocyte-specific linker histone, H1foo, contains a potential CPE (Tanaka et al., 2001). However, the bulk of the histone mRNAs in oocytes appears to be of the so-called replication-dependent class (Graves et al., 1985). These transcripts are not polyadenylated but instead carry a highly conserved 3′-utr that ends in a stem-loop structure (Birchmeier et al., 1982; Dominski and Marzluff, 1999). Thus, their translation in oocytes cannot be regulated by a CPE-based process but must be controlled through a different mechanism.

In somatic cells, several factors associate with the 3′-utr of replication-dependent histone mRNAs (hereafter termed stem-loop histone mRNAs): notably, the stem-loop binding protein (SLBP), which contains a unique RNA-binding domain and interacts with the stem-loop (Wang et al., 1996), and the U7 snRNP, whose RNA component associates with a purine-rich element, termed the histone downstream element (HDE), that is located 3′ of the stem-loop sequence on the histone pre-mRNA. In conjunction with SLBP and a zinc-finger protein termed hZPF100, the U7 snRNP directs cleavage within the nucleus of newly synthesized histone transcripts between the stem-loop and the HDE (Cotten et al., 1988; Dominski et al., 2002). This processing reaction protects the transcripts from rapid degradation. As well, an exonuclease, termed 3′hExo, also interacts with the stem-loop and is thought to function in histone mRNA degradation during G2 of the cell cycle (Dominski et al., 2003).

In addition to its nuclear role, SLBP is also associated with stem-loop histone mRNAs in the cytoplasm. Indeed, in myeloma cells, much of the SLBP is cytoplasmic and is associated

with polysomal histone mRNAs (Hanson et al., 1996; Whitfield et al., 2004). Furthermore, SLBP can activate the translation of a reporter mRNA carrying the histone stem-loop, both in vitro and in *Xenopus* oocytes (Sanchez and Marzluff, 2002). Moreover, SLBP co-purifies with translation initiation factors and physically interacts with eIF4G (Ling et al., 2002). These results suggest that, likely through interaction with factors bound to the 5′-end of the mRNA, SLBP stimulates translation of stem-loop histone mRNAs.

In somatic cells, SLBP is detectable only during S-phase of the cell cycle (Whitfield et al., 2000). To investigate whether SLBP might also play a role in regulating histone mRNA metabolism in mammalian oocytes, we previously characterized its expression in these cells (Allard et al., 2002). We found that SLBP is present in immature oocytes, which are at late G2 of the cell cycle, where it is enriched in the nucleus (germinal vesicle or GV). Upon initiation of meiotic maturation, SLBP begins to accumulate. This increase in SLBP begins shortly after germinal vesicle breakdown and continues throughout maturation, such that a mature meta-phase II oocyte contains 10- to 15-fold more SLBP than an immature prophase-I-arrested oocyte (Allard et al., 2002). Thus, in contrast to its S-phase-restricted expression in somatic cells, SLBP is present in oocytes at G2 and M-phase of the cell cycle. These observations raised the possibility that SLBP might play a central role in regulating translation of the stem-loop histone mRNAs in oocytes. We tested this by monitoring endogenous histone synthesis, injecting reporter mRNAs bearing the 3′-utr of histone stem-loop mRNAs and using RNA interference (RNAi) and mRNA injection to manipulate SLBP levels within the oocyte.

Materials and methods

Oocyte collection and culture

Fully grown meiotically immature oocytes were collected from 21-day-old CD-1 female mice (Charles River Canada) by puncture of the ovarian antral follicles as previously described (Clarke et al., 1992). The oocytes were cultured in bicarbonate-buffered minimal essential medium (MEM) supplemented with sodium pyruvate, antibiotics, 3 mg/ml bovine serum albumin (BSA) and 0.1 mg/ml dibutyryl cyclic AMP (dbcAMP) at 37°C, in 5% CO_2 in air. Resumption of meiosis was initiated by transferring the oocytes into medium without dbcAMP.

SLBP overexpression

SLBP cDNA sequence corresponding to −2 to +884, encompassing the entire coding sequence, was excised by *Nco*I/*Stu*I digest, blunt-ended and cloned into the Cs2+ vector (gift from Dr. Mark Featherstone) that had been digested with *Bam*HI and blunt-ended. After linearization, SLBP cDNA was transcribed using the SP6 phage promoter and Ambion mMessage mMachine kit. The mRNA was purified by lithium chloride precipitation followed by three washes in 70% ethanol. The pellet was resuspended in Rnase-free water and stored at −80°C. SLBP mRNA was injected into immature oocytes at 1 μg/μl in Rnase-free water. The oocytes were incubated in culture medium for 16 h and collected for immunoblotting or radiolabeling.

Double-stranded RNA preparation and oocyte microinjection

Double-stranded RNA (dsRNA) was prepared from a pGEM cloning vector containing a 1.2 kb *Nco*I fragment of SLBP cDNA (−2 to +1191 relative to the coding sequence start) or 830 bp of LacZ cDNA (−10 to +840) and modified by insertion of two T7 phage promoters (prepared by Patricia Françon). After linearization of the constructs at either end of the insert, the dsRNA was synthesized and purified using Megascript RNAi kit (Ambion, Austin, Texas). The dsRNA was stored at −80°C in single-use aliquots at a concentration of 0.5 μg/μl.

Immature oocytes were microinjected in HEPES-buffered (pH 7.2) MEM supplemented with antibiotics, 3 mg/ ml BSA, 0.1 mg/ml dbcAMP and 3 mg/ml of polyvinyl pyrrolidone (PVP). Oocytes were microinjected in groups of 50 using a Leica inverted microscope equipped with Leica micromanipulators (Leica Canada, Montreal, Quebec). About 10 pl of double-stranded RNA (dsRNA) was micro-injected into each oocyte. Following microinjection, the oocytes were cultured for 7 h in the presence of dbcAMP. Oocytes were subsequently transferred to medium without dbcAMP to allow resumption of meiosis and were cultured for an additional 18 h.

Immunoblotting

Oocytes were collected and lysed in 10 μl of 2× Laemmli buffer. After denaturation, the samples were analyzed by SDS–Tris–glycine electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto PVDF membrane (Amersham, Montreal, Quebec) under constant voltage (100 V) for 1 h. The membrane was subsequently blocked in 5% non-fat milk in 0.1% Tween–PBS (PBST). The membrane was washed 3 times in PBST and incubated overnight at 4°C with anti-SLBP (Whitfield et al., 2000) or anti-ERK1 (Santa Cruz Biotechnology, Santa Cruz, California) antibody in blocking buffer at a dilution of 1/4000 or 1/1000 respectively. After washing, the membrane was incubated in secondary antibody conjugated to horseradish peroxidase (HRP, Promega, Montreal, Quebec) at a dilution of 1/5000 for 1 h at room temperature. After the final washes, the fluorescence was revealed using the ECL+ kit (Amersham).

Radiolabeling and histone extraction

Oocytes were incubated in KSOM (Lawitts and Biggers, 1993) supplemented with [^{3}H]-Alanine, [^{3}H]-Arginine and [^{3}H]-Lysine (Amersham) at 0.1 μCi/μl each for 8 h at 37°C in 5% CO_2 in air. Before collection, the oocytes were washed twice in 2 ml of PBS. Acid extraction of histones, electrophoresis and autoradiography were as previously described (Fu et al., 2003).

RNA isolation, reverse transcription, PCR and real-time PCR

Oocytes were deposited into a microfuge tube, and total RNA was isolated as described previously (Mohamed et al., 2001). In some experiments, 10 pg of EGFP mRNA was added to the tube containing the oocytes prior to the RNA purification step. First-strand cDNA was synthesized from RNA of 25–40 oocytes by using Superscript II RT (Invitrogen, Oakville, Canada) and random primers at 0.5 μg per 20 μl reaction. For each sample, an identical RT reaction was assembled but omitting the reverse transcriptase (RT minus). PCR was

performed using Taq DNA polymerase (Invitrogen) and 2 μl of reverse-transcription product in 50 μl of final volume. Each cycle consisted of 40 s at 94°C, 40 s at 50°C and 1 min at 72°C for a total of 27 cycles. The Alpha Innotech gel documentation system and AlphaEaseFC gel documentation software (Alpha Innotech, San Leandro, CA) were used to quantify the DNA signal following agarose gel electrophoresis and ethidium bromide staining.

For real-time PCR, cDNAs were amplified using Qiagen Quantitect SYBR-Green (Qiagen, Mississauga, Ontario) and a Roche Applied Science Lightcycler. For each PCR reaction, 2 μl of reverse-transcription product was used for 20 μl of final volume. The cycle conditions were as follows: 10 s at 94°C, 10 s at 50°C and 30 s at 72°C for a total of 50 cycles. Melting curve analysis as well as agarose gel electrophoresis were performed on each sample to assess for non-specific amplification and primer–dimer formation.

Primers were designed based on the corresponding published Genbank sequences: Actin forward (F)-GCTGTGCTATGTTGCTCTAG and reverse (R)-ATCG-TACTCCTGCTTGCTGA; Histone H2Aa2 F-GTTTGCG-CTTTCGTGATG and R-GGTTATATCACAGAGACG; Histone H4C F-GTAAAGGCCTTGGGAAAG and R-CCGTAGAGAGTGCGGCC; SLBP: F-CAGCTGCAAT-GACTCTGT and R-GCAATTATCATTCCATAC.

Luciferase assay

The luciferase-histone wild-type and reverse stem-loop constructs were prepared by Nancy Lewis and were generous gifts of Dr. D.R. Gallie (Gallie et al., 1996). Prior to in vitro transcription, the plasmids were linearized by *Afl*II restriction digestion. In vitro transcription was performed by using the plasmid T7 phage promoter and Ambion mMessage mMachine kit. The mRNA was purified by lithium chloride precipitation followed by three washes using 70% ethanol. The pellet was resuspended in Rnase-free water and stored at −80°C. The mRNA was injected into immature oocytes at 1 μg/μl in Rnase-free water. After 1 h of recovery, half of the oocytes were transferred to medium without dbcAMP to allow resumption of meiosis, while the other half was kept in medium with dbcAMP. The oocytes were further incubated for 18 h and were then collected for luciferase assay.

Groups of 10–20 oocytes were lysed on ice in 45 μl of lysis buffer (0.01% Triton X-100, 15 mM magnesium acetate, 4 mM EGTA and 1 mM DTT) for 5 min. Immediately prior to the luciferase assay, 5 μl of assay buffer (1 mM ATP, 15 mM KH_2PO_4, 15 mM magnesium acetate) was added to each sample. Luciferin solution (0.1 M KH_2PO_4, 15 mM magnesium acetate, luciferin 0.22 mM, co-enzyme A 0.2 mg/ml) was added to each sample, and activity was recorded using a microtiter plate EG&G Berthold LB96 luminometer.

In vitro fertilization (IVF) and immunofluorescence

Sperm used for IVF was collected from CD-1 and B6D2F1 male (Charles River) mice by squeezing the caudae epididymi. The sperm mass was transferred to an 800-μl drop of human tubal fluid (HTF) medium (Quinn et al., 1985) and capacitated for 1 h. Then, 40 μl of the capacitation-HTF drop was added to a 20 μl IVF drop of HTF. Metaphase II oocytes

were treated with acid Tyrode's solution (Sigma, Oakville, Ontario) and transferred to the IVF-HTF drop.

Six or 7.5 h after insemination, the oocytes were washed in HTF medium, fixed for 15 min in 4% *para*-formaldehyde in phosphate-buffered saline (PBS) and blocked overnight at 4°C in 3% bovine serum albumen (BSA), 0.1% Triton-X-100 in PBS. For immunofluorescence, they were incubated overnight at 4°C with a polyclonal antibody directed against H2A-Ac-K5 (Cell Signaling, Pickering, Ontario) or against H4-Ac-K12 (Upstate, Charlottesville, Virginia) diluted 1:100 in blocking solution. After 3 washes in blocking solution, the oocytes were incubated for 1 h at room temperature with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon) diluted 1:100 in blocking solution. DNA was stained by incubating the oocytes in blocking solution supplemented with 2.5 μg/ml DAPI (Roche Diagnostics, Laval, Quebec) for 15 min.

Fluorescence was visualized by a Leica DM1L microscope (Leica Canada, Montreal, Quebec) coupled to a Hamamatsu ORCA II ER digital camera (Hamamatsu, Japan). Pronuclear measurements and fluorescence quantification were performed using the Metamorph imaging software (Universal Imaging Corporation, Downingtown, Pennsylvania).

Results

Histones are synthesized in immature and mature oocytes

The mammalian oocyte contains considerable amounts of stem-loop histone mRNAs (Graves et al., 1985). Little is known, however, concerning their expression in the immature and mature oocyte. To examine whether histone mRNAs are translated in these cells, histone synthesis was assessed by radiolabeling. Immature (GV stage) oocytes and mature (metaphase II or MII) oocytes were incubated in medium supplemented with ^{3}H-radiolabeled amino acids, after which they were lysed and the histones were acid-extracted and separated by PAGE. Coomassie staining of the gel before drying revealed the position of the carrier histones added to the cellular histones during the acid-extraction procedure and allowed us to identify the corresponding histone bands on the autoradiogram. As shown in Fig. 1, radiolabeled proteins co-migrating with all four core histones were detectable in both immature and mature oocytes. This result indicates that histone mRNAs transcribed during oocyte growth are translationally active before and during meiotic maturation.

SLBP overexpression in the immature oocyte increases histone synthesis

SLBP is present in immature oocytes, albeit at low levels compared to mature oocytes (Allard et al., 2002). To test whether SLBP could regulate histone mRNA translation in immature oocytes, we overexpressed SLBP in these cells. mRNA encoding SLBP was microinjected into fully grown immature oocytes, which were then incubated for 18 h in medium supplemented with dbcAMP to prevent maturation and subsequently collected. SLBP protein was significantly increased in SLBP mRNA microinjected cells as compared to controls (Fig. 2A).

We then examined the effect of overexpressing SLBP on the expression of a reporter mRNA consisting of the luciferase coding sequence upstream of a 32-bp fragment including the consensus histone stem-loop sequence. This construct has been extensively used to study the role of the histone stem-loop sequence and of SLBP in mediating translation of stem-loop histone mRNAs (Gallie et al., 1996; Sanchez and Marzluff, 2002). SLBP mRNA and the luciferase reporter mRNA were co-injected into fully grown immature oocytes, which were subsequently processed for luciferase activity. SLBP mRNA microinjected oocytes showed a 3-fold increase in luciferase activity compared to oocytes microinjected with the reporter mRNA alone (Fig. 2B, $P \leq 0.05$).

Next, we tested the effect of SLBP overexpression on endogenous histone synthesis. Following SLBP or control mRNA injection into immature oocytes and overnight incubation, the oocytes were incubated for 8 h in medium supplemented with ^{3}H-labeled amino acids and processed for histone extraction as previously described (Fu et al., 2003). After PAGE and autoradiography, the activity incorporated into the histone bands was normalized against the activity incorporated into non-histone protein bands. The quantification of the gel revealed that the synthesis of all four core histones, H2A, H2B, H3 and H4, increased by 30% compared to control (Fig. 2C, $P \leq 0.05$). Thus, endogenous histone synthesis was also increased in the oocytes overexpressing SLBP.

To test whether the increase in histone synthesis was due to an increase in histone mRNA stability, we then measured the relative levels of two stem-loop histone mRNAs, H2Aa2 and H4c mRNAs, in immature oocytes following SLBP or control mRNA injection. After microinjection and overnight incubation, the immature oocytes were collected, and their RNA content was extracted. Reverse transcription was performed, and the template cDNA was amplified by real-time PCR using primers specific for histone H2Aa2, H4c and also actin for normalization. The amplification efficiency of each pair of primers was determined based on the standard curve and integrated in the calculation of the relative change in template amount as described in Pfaffl (2001). Three independent experiments were performed, each one in triplicate.

As shown in Fig. 2D, there was no significant difference in the amount of PCR product corresponding to each histone mRNA between the SLBP and control mRNA-injected samples. This implies that increasing SLBP levels in immature oocytes did not increase histone mRNA stability. Taken together, these results demonstrate that increasing the quantity of SLBP in the immature oocyte causes an increase in histone mRNA translation.

The translation of a luciferase reporter mRNA ending with histone 3′-utr consensus stem-loop sequence is up-regulated during meiotic maturation

During meiotic maturation, there is a substantial increase in the quantity of SLBP. It is also known, however, that while polyadenylated mRNAs carrying a CPE are activated for translation, those lacking a CPE become translationally repressed (Oh et al., 2000). We therefore examined whether stem-loop histone mRNAs, which are not polyadenylated, might also be subject to translational regulation during maturation. Immature oocytes were microinjected with the luciferase-stem-loop reporter mRNA described above and were either kept at the immature state or allowed to undergo maturation. Oocytes were incubated for 18

h, and luciferase activity from the two groups was subsequently recorded. As shown in Fig. 3A, immature oocytes generated only moderate luciferase activity. By contrast, oocytes that underwent meiotic maturation accumulated substantial amounts of luciferase activity, showing a 20-fold increase compared to the immature oocytes ($P \leq 0.01$).

To confirm that the behavior of the reporter mRNA is mediated by the histone stem-loop sequence, we also followed during maturation the expression of a mutant luciferase-stem-loop reporter mRNA, in which the stem- loop sequence has been reversed. SLBP has only a weak affinity for the reverse stem-loop sequence (Gallie et al., 1996; Sanchez and Marzluff, 2002), allowing us to assess the involvement of SLBP in the regulation of the reporter mRNA behavior. Both immature and mature oocytes contained only a minimal amount of luciferase activity (Fig. 3A), demonstrating that the presence of a wild-type histone stem-loop is necessary for the full increase in expression of the reporter mRNA. This suggests that binding of SLBP to the reporter mRNA stem-loop sequence is required for a significant accumulation of luciferase activity during meiotic maturation.

The relatively low luciferase activity detected in immature oocytes could be caused by a decrease in the quantity of the reporter mRNA in oocytes that were kept in an immature state as compared to those allowed to mature. To test this possibility, we measured the reporter mRNA levels by semi-quantitative RT-PCR in fully grown oocytes and mature oocytes after 18 h of incubation. After collection of the immature and mature oocytes, equal amounts of a standard RNA (EGFP) were added to each sample to control for variation in RNA extraction and reverse transcription between samples. As shown in Fig. 3B, there was no significant difference in the levels of the luciferase reporter mRNA in immature versus mature oocytes ($P = 0.075$). This indicates that the increase in luciferase activity during meiotic maturation cannot be due to a relatively higher stability in the maturing oocyte. Taken together, these results show that the presence of the wild-type histone stem-loop promotes an increase in mRNA translation during meiotic maturation.

SLBP accumulation during meiotic maturation is necessary for normal histone synthesis in the oocyte

The increased translation during meiotic maturation of the reporter mRNA bearing the histone stem-loop together with the observation that SLBP accumulates during maturation (Allard et al., 2002) suggested that this accumulation is required to sustain normal histone synthesis during maturation. To test this, we specifically prevented the accumulation of SLBP during maturation by performing RNA interference (RNAi) in the oocyte (Svoboda et al., 2000). SLBP dsRNA was microinjected into immature oocytes that were allowed to recover for 7 h. The oocytes were then allowed to undergo maturation, and those that reached metaphase II after 18 h were collected for analysis. The same experiments were carried out in parallel using LacZ dsRNA as a control. As shown by RT-PCR, following SLBP dsRNA delivery in the oocytes, there was a significant reduction in SLBP mRNA compared to control (Fig. 4A). In addition, these oocytes failed to accumulate SLBP protein during meiotic maturation; rather, they showed a level comparable to that of immature oocytes (Fig. 4B). In contrast, the rate of total protein synthesis and the frequency of maturation did not differ between experimental and control groups (data not shown). Thus,

using RNAi, we were able to specifically and selectively prevent the increase in SLBP during meiotic maturation.

To test whether decreased SLBP levels in mature oocytes affected histone synthesis, we first examined the behavior of the luciferase-stem-loop reporter mRNA. Immature oocytes were co-microinjected with the luciferase mRNA and either SLBP dsRNA or control LacZ dsRNA. Following injection, the oocytes were processed as described above. Oocytes co-injected with SLBP dsRNA and the luciferase mRNA showed only one-third of the increase in luciferase activity during meiotic maturation relative to the LacZ dsRNA control (Fig. 5A, $P \leq 0.01$). This result indicates that SLBP accumulation is required for the increase in luciferase activity seen during meiotic maturation.

To examine whether the behavior of the luciferase-stem-loop reporter mRNA reflected endogenous histone protein synthesis, we directly measured histone synthesis in oocytes injected with SLBP or LacZ dsRNA. After injection and maturation, oocytes were incubated with radiolabeled amino acids, and the histones were acid-extracted, separated by PAGE and examined by autoradiography as described above. As shown in Fig. 5B, SLBP dsRNA microinjected oocytes showed a decrease in the synthesis of the core histones compared to the LacZ dsRNA control. After quantification of the gel, we found that oocytes with low SLBP levels showed only one-half of the histone protein synthesis of the control oocytes (Fig. 5C, $P \leq 0.005$). These results demonstrate that SLBP accumulation during maturation is required for quantitatively normal histone synthesis in the oocyte.

SLBP accumulation during maturation is not required for histone mRNA stability

Since in somatic cells SLBP mediates stability as well as translation of stem-loop histone mRNAs (Gallie et al., 1996; Marzluff and Duronio, 2002), preventing SLBP accumulation during maturation could potentially affect histone synthesis through an effect on mRNA stability. We therefore measured the relative amounts of the two stem-loop histone mRNAs, H2Aa2 and H4c, in oocytes injected with SLBP or LacZ dsRNA. Following dsRNA delivery to the immature oocyte, recovery and maturation as described above, metaphase II oocytes were collected, and total RNA was extracted. Efficient depletion of SLBP mRNA in each SLBP dsRNA microinjected sample was confirmed by RT-PCR (data not shown).

As shown in Fig. 6, following real-time PCR amplification of cDNAs from SLBP dsRNA-injected oocytes and lacZ dsRNA control oocytes, we found that there was no significant difference in the relative amounts of histone H2Aa2 and H4c mRNAs between the two groups. This result demonstrates that histone mRNA stability is not affected by a failure to accumulate SLBP during meiotic maturation. Therefore, when SLBP accumulation during maturation is prevented, the decrease in the rate of histone synthesis is a direct consequence of the reduced capacity of these oocytes to translate histone mRNAs.

SLBP-dependent histone synthesis is required for normal pronuclear development following fertilization

To assess whether histones synthesized during maturation were required for pronuclear development following fertilization, we inseminated oocytes that had been depleted of SLBP by RNAi. Inseminated oocytes were incubated for 6 or 7.5 h and then fixed. The size of the

pronuclei was measured and the quantity of pronuclear histones determined by immunofluorescence. We chose to detect acetylated histone H2A-K5 and acetylated histone H4-K12, as the corresponding antibodies produce a strong signal in the pronuclei, whereas antibodies recognizing unmodified histones produce only a weak signal (Spinaci et al., 2004, Q.Y. and H.J.C., unpublished results). At both 6 and 7.5 h after insemination, oocytes injected with SLBP dsRNA contained significantly smaller pronuclei than injected controls (Fig. 7A, $P \leq 0.01$ and $P \leq 0.005$ respectively). Furthermore, these pronuclei showed a significant reduction in the amount of detectable acetylated histone H2A-K5 and histone H4-K12 compared to controls (Figs. 7B, C, $P \leq 0.01$). Although this experiment did not directly detect total histones, the results suggest that SLBP-dependent histone synthesis during maturation is required for normal pronuclear development following fertilization.

Discussion

We have investigated the role of SLBP in regulating the synthesis of histones in the mouse oocyte. We demonstrate that overexpression of SLBP in immature oocytes leads to an increase in the translation both of a reporter construct bearing the stem-loop histone 3′-utr and of endogenous histone mRNAs. Moreover, concomitant with the accumulation of SLBP that occurs during meiotic maturation, there is an increase in the translation of a reporter mRNA bearing the stem-loop histone 3′-utr. Conversely, preventing the accumulation of SLBP during maturation through the use of RNAi largely abolishes this increased translation. SLBP-depleted mature oocytes also manifest lower levels of endogenous histone mRNA translation as compared to control oocytes, as well as a reduced presence of acetylated histones on pronuclear chromatin following insemination. Taken together, these experiments demonstrate that changes in the SLBP quantity play a central role in regulating histone synthesis in mouse oocytes.

In somatic cells, SLBP has been implicated in both stabilization and translation of stem-loop histone mRNAs (Gallie et al., 1996; Marzluff and Duronio, 2002). Thus, it was conceivable that the increased synthesis of both the reporter protein and endogenous histones in immature oocytes overexpressing SLBP, and their decreased synthesis in maturing oocytes that failed to accumulate SLBP, was due to an effect on mRNA stability. We observed, however, that neither increasing SLBP in immature oocytes nor decreasing SLBP in maturing oocytes affected the quantity of histone stem-loop mRNAs. We conclude that SLBP regulates histone synthesis in both immature and mature oocytes by controlling the translational activity of stem-loop histone mRNAs.

In somatic cells, SLBP is thought to remain stably associated with a newly transcribed histone mRNA during both its processing in the nucleus and its translation in the cytoplasm. As the histone mRNAs that accumulate in oocytes are likely to have been processed (Graves et al., 1985) and therefore to be associated with SLBP, it may be questioned how an increase in SLBP would promote their translation. We postulate that there is equilibrium between free and mRNA-associated SLBP in the oocyte. According to this model, in the immature oocyte, mRNA-associated SLBP is favored. Thus, most endogenous histone mRNAs would be associated with SLBP. Experimental elevation of the SLBP level would increase the fraction of histone mRNAs bound to SLBP, leading to increased histone synthesis; however,

only a modest increase in translational activity might be achieved. This is consistent with the results that we observed. In the mature oocyte, by contrast, the equilibrium shifts to favor unbound SLBP. Interestingly, activation of SLBP-regulated translation requires the cap complex (Gallie et al., 1996; Ling et al., 2002), and there is a general repression of cap-dependent translation at M-phase of the cell cycle (Pyronnet et al., 2001). Moreover, SLBP is modified by phosphorylation during maturation, which may affect its activity (Allard et al., 2002). Hence, an accumulation of SLBP might be required to keep sufficient SLBP associated with histone mRNAs and to overcome the general decline in mRNA translation, thus maintaining normal histone synthesis during maturation.

The proposal that there is a dynamic association between SLBP and histone mRNAs is supported by results obtained using frog oocytes, which contain two SLBPs—xSLBP1 and xSLBP2. In immature oocytes, xSLBP2, which is inactive in processing and translation, is the only SLBP associated with histone mRNAs. However, at maturation, xSLBP1 replaces xSLBP2 on histone mRNAs leading to their translational activation (Wang et al., 1999; Sanchez and Marzluff, 2002). Furthermore, as xSLBP2 is solely cytoplasmic, histone mRNAs must have been processed with the help of xSLBP1 that was then exchanged for xSLBP2 in the cytoplasm. How the switch between the two xSLBPs is accomplished is unknown. However, together with our results, it establishes that, at least in oocytes, SLBP is not always tightly associated with histone mRNAs.

Although histone synthesis was reduced when the accumulation of SLBP was prevented during oocyte maturation, some synthesis continued. Therefore, at least during maturation, histone synthesis is not entirely dependent on the accumulation of SLBP. Several possible explanations may be envisioned. First, the SLBP that was present in the immature oocyte and remained during maturation presumably can support histone synthesis. Second, stem-loop histone mRNAs may continue to be translated, albeit inefficiently, when SLBP is depleted. Third, the oocyte may contain a population of polyadenylated histone mRNAs, whose translation would be independent of SLBP.

However, when we inseminated the SLBP-depleted oocytes in which this sub-normal histone synthesis occurred, they developed relatively small pronuclei that contained a reduced quantity of detectable acetylated histone. One possibility is that the reduced level of histones present in the oocyte leads to a slower replacement of protamines by histones onto the sperm DNA that occurs following fertilization (Nonchev and Tsanev, 1990). This is consistent with the development of *Drosophila* eggs that lack SLBP, which arrest at an early stage of embryonic development and display obvious chromosome condensation defects (Sullivan et al., 2001). In any case, these results suggest that SLBP-dependent histone synthesis in the maturing oocyte is required for normal pronuclear development.

Our results have identified a component of the molecular mechanism governing histone synthesis during oogenesis in the mouse. Specifically, it is the quantity of SLBP that regulates the translation of stem-loop histone mRNAs in both immature and mature oocytes. This mechanism is clearly distinct from that described in oocytes of other species. As discussed above, *Xenopus* oocytes express two SLBP species, and translational control is affected by a change in the type of SLBP bound to the histone mRNAs. Translational

activation of histone stem-loop mRNAs during oocyte maturation in *Xenopus* also requires removal of a short polyA tail that is present just 3′ of the stem-loop sequence (Sanchez and Marzluff, 2004). *Drosophila* contain a single species of SLBP. The eggs contain abundant histone stem-loop mRNAs, and while these fail to accumulate in eggs of mutant flies that lack SLBP, suggesting a key role for SLBP, SLBP itself is not detectable in the egg (Sullivan et al., 2001; Lanzotti et al., 2002). Thus, in *Drosophila*, the timing of translational activation of these mRNAs remains to be determined. In sea urchins, the maturing oocyte completes both meiotic divisions and arrests at the female pronucleus stage until fertilization. Both SLBP and the histone mRNAs are localized in the female pronucleus until first embryonic mitosis when they are released into the cytoplasm and histone synthesis begins (Robertson et al., 2004). Our work taken together with these results demonstrates that, while a role for SLBP in regulating histone synthesis in the oocyte is widely conserved among species, the mechanism by which this is achieved varies widely.

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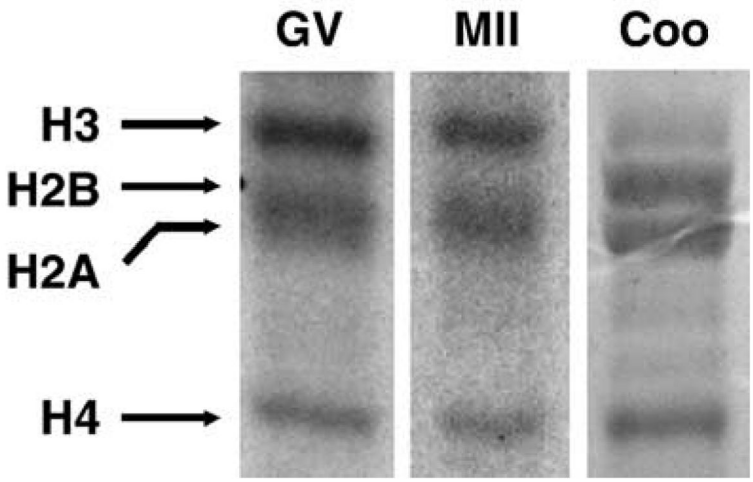

Fig. 1.

Histone synthesis in immature and mature oocytes. GV-stage oocytes (immature) or MII-stage oocytes (mature) were cultured in medium supplemented with ^{3}H-labeled alanine, arginine and lysine for 8 h. Histone proteins were acid-extracted using unlabelled histone proteins as carrier and separated by PAGE. After drying, the gel was exposed for autoradiography for 2 weeks. The position of the histone bands was determined by Coomassie staining of the gel (Coo). In this gel, representative of three independent experiments, 35 oocytes at each stage were used.

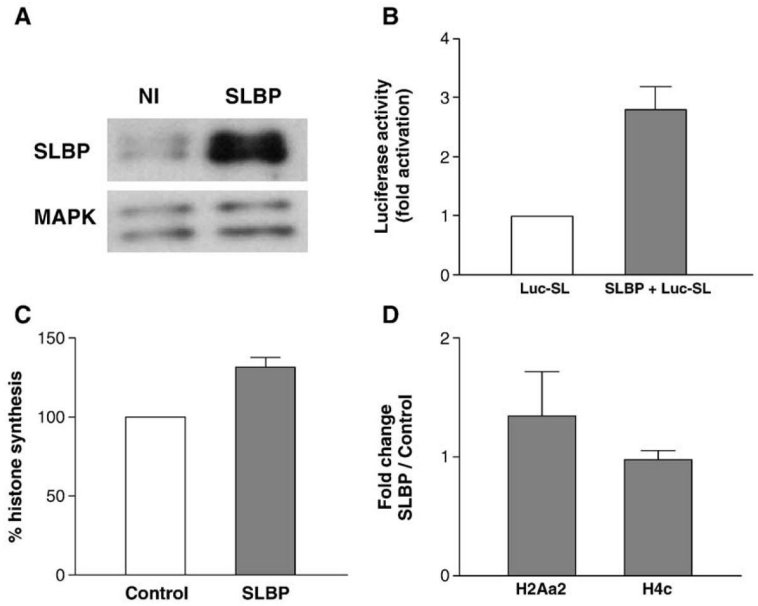

Fig. 2.

SLBP overexpression in GV-stage oocytes increases histone mRNA translation. (A) GV-stage oocytes were injected with in vitro synthesized SLBP mRNA (SLBP) or not injected (NI) and maintained at the GV stage for 18 h. The oocytes were then collected for Western blotting. MAP kinase (MAPK) was used as a loading control. Each lane contains 20 oocytes. (B) GV-stage oocytes were either injected with luciferase wild-type histone stem-loop mRNA alone or co-injected with SLBP mRNA along with the luciferase wild-type histone stem-loop mRNA. The oocytes were then incubated for 18 h, and pools of 10–20 were assayed for luciferase activity. $P \leq 0.05$, $n = 3$, paired Student's t test. (C) GV-stage oocytes were injected with SLBP mRNA or with luciferase-polyA mRNA as a control. The oocytes were incubated for 18 h and then transferred to radiolabeled medium. The histones were then acid-extracted and separated by PAGE. For quantification, the bands corresponding to the core histones were cut from the gel, and the amount of radioactivity was measured with a β-counter. The experiment was performed three times, using the same number of oocytes (40–60) per lane. $P \leq 0.05$, paired Student's t test. (D) H2Aa2 and H4c mRNA levels following SLBP mRNA microinjection compared to control and normalized to actin levels. mRNA was prepared from the same number of oocytes (25–30) in each group, and one-fifth oocyte-equivalent was used for each PCR reaction. The mean and variation of three independent experiments, each one done in triplicate, are represented. $P = 0.4$ (H2Aa2), $P = 0.7$ (H4c), $n = 3$, paired Student's t test.

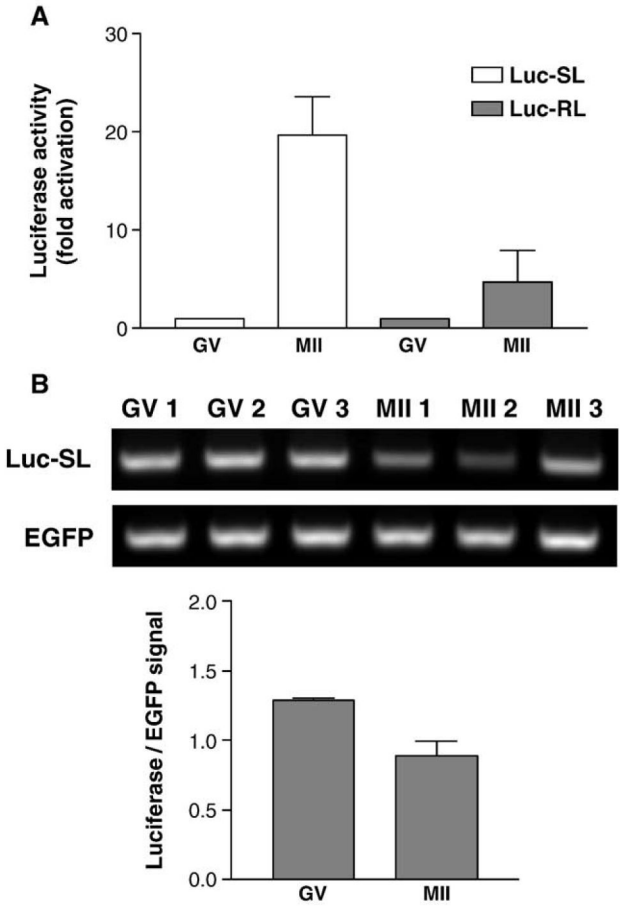

Fig. 3.

Up-regulation of luciferase-histone stem-loop mRNA translation during meiotic maturation. (A) GV-stage oocytes were injected with either luciferase wild-type stem-loop mRNA (Luc-SL) or luciferase-reverse stem-loop mRNA (Luc-RL). The oocytes were then kept at the GV stage for 18 h or allowed to undergo maturation and collected 18 h later as metaphase II oocytes. Pools of 10–20 oocytes were assayed for luciferase activity that was expressed on a per-oocyte basis. For each experiment, GV activity was set to a value of one. Luc-SL: $P \leq 0.01$, Luc-RL: $P = 0.4$, $n = 3$, paired Student's t test. (B) Oocytes treated as above were collected and subjected to RT-PCR using two oocyte-equivalents per reaction. Three independent groups of GV-stage oocytes and the corresponding groups of metaphase II oocytes, all injected with the luciferase wild-type stem-loop mRNA, are shown. EGFP RNA was added before the RNA extraction procedure to control for variability between samples. $P = 0.075$, $n = 3$, paired Student's t test.

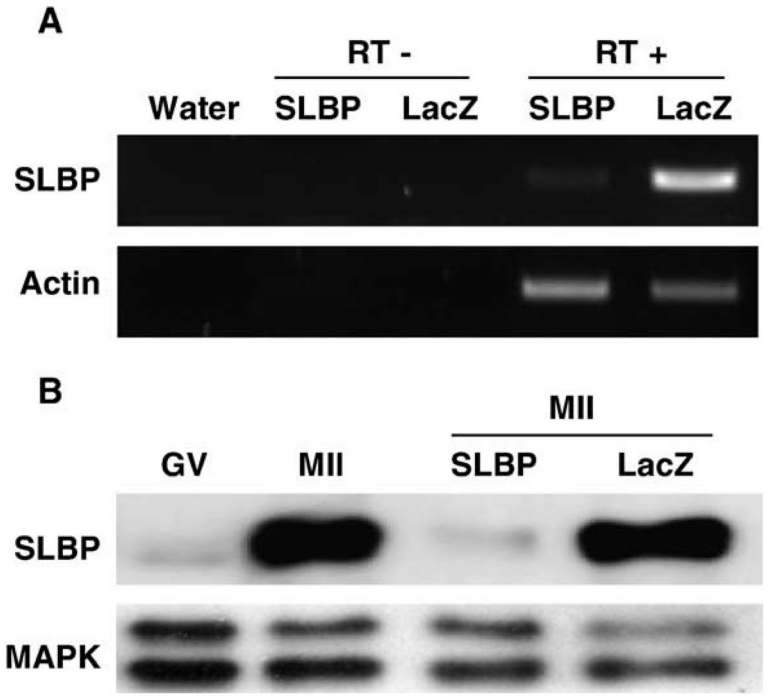

Fig. 4.

Accumulation of SLBP during meiotic maturation can be specifically prevented by dsRNA. GV-stage oocytes were injected with SLBP dsRNA or LacZ dsRNA as control. Following in vitro maturation, the resulting metaphase II (MII) oocytes were collected for RT-PCR (A) or used for Western blotting (B). Two oocyte-equivalents were used per reaction for PCR; actin was used as endogenous control. For Western blotting, 20 oocytes were loaded into each well. After probing for SLBP, the blot was stripped and reprobed for MAP kinase (MAPK) as a loading control.

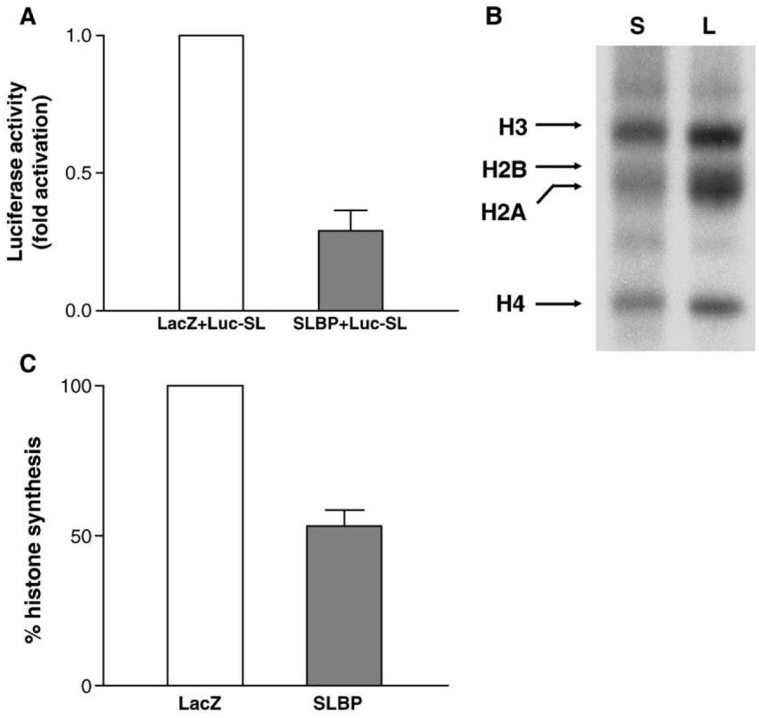

Fig. 5.

Oocyte histone synthesis is reduced following SLBP dsRNA injection. (A) GV-stage oocytes were co-injected either with SLBP dsRNA or LacZ dsRNA along with luciferase wild-type stem-loop mRNA. Following injection, the oocytes were allowed to recover and then were matured for 18 h. The resulting MII oocytes were collected, and pools of 10–20 were assayed for luciferase activity. For each experiment, activity of the LacZ group was set to a value of one. $P \leq 0.01$, $n = 3$, paired Student's t test. (B) GV-stage oocytes were injected with SLBP (S) or LacZ (L) dsRNA and processed as described in panel A. After 18 h, the MII oocytes were further cultured in radiolabeled medium. The histones were acid-extracted and separated by PAGE. Position of the histone bands was determined by Coomassie staining of the gel. (C) For quantification, the bands corresponding to the core histones were cut from the gel, and the amount of radioactivity was measured with a β-counter. The experiment was performed five times, using the same number of oocytes (35–50) per lane. $P \leq 0.005$, paired Student's t test.

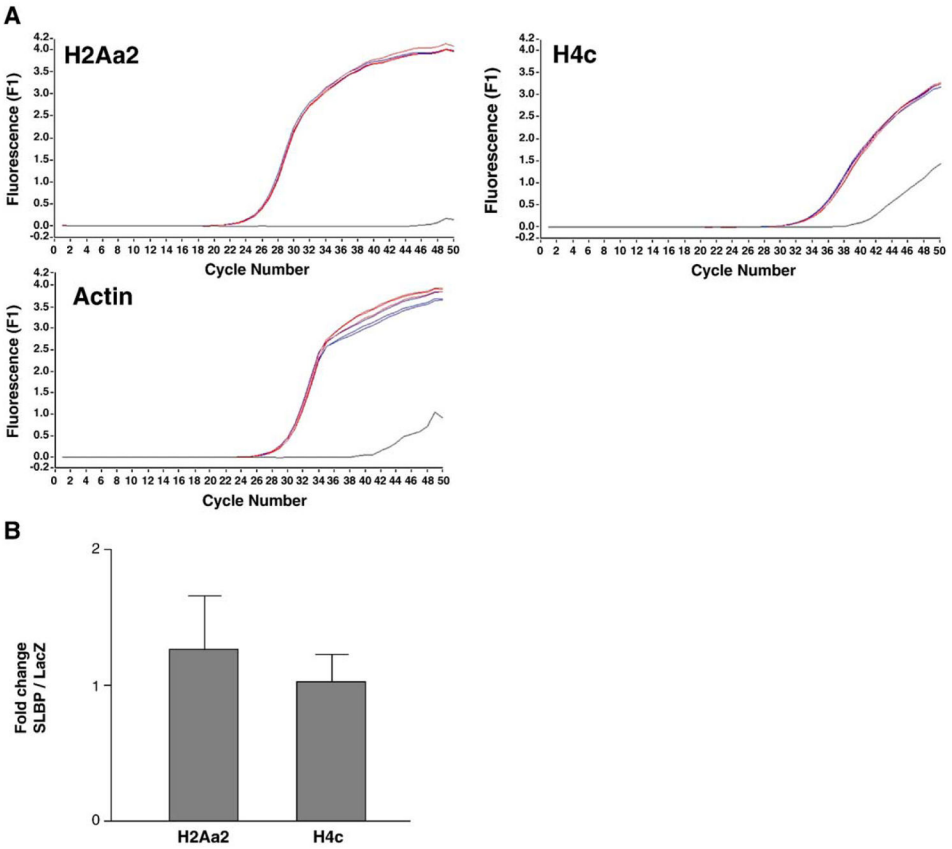

Fig. 6.

Histone mRNA levels remain unchanged following SLBP dsRNA injection. (A) GV-stage oocytes were injected with SLBP or LacZ dsRNA, allowed to recover, matured and collected. After RNA extraction and reverse transcription from pools of 25–30 oocytes, quantitative PCR was performed on one-fifth oocyte-equivalent using primers specific to histone H2Aa2 and H4c and actin as a control. Three independent experiments were performed. One representative experiment for each pair of primers is shown. Amplification curves in red or blue correspond to SLBP or LacZ dsRNA-injected samples, respectively. (B) Graphic representation of the fold-change in H2Aa2 and H4c levels following SLBP dsRNA compared to LacZ dsRNA and normalized to actin levels. The mean and variation of three independent experiments, each done in triplicate, are represented. $P = 0.6$ (H2Aa2), $P = 0.9$ (H4c), $n = 3$, paired Student's t test.

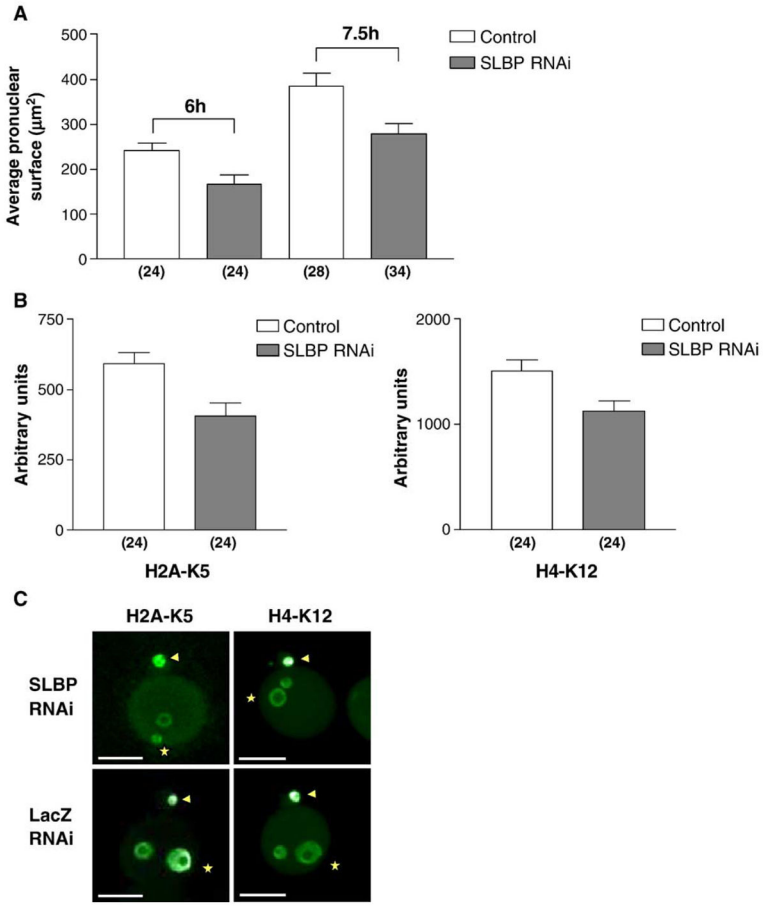

Fig. 7.

Eggs injected with SLBP dsRNA show defects in pronuclear formation. GV-stage oocytes were injected with SLBP or LacZ dsRNA, allowed to recover for 7 h and matured for 18 h. The mature oocytes were inseminated, incubated for either 6 h or 7.5 h then fixed. Acetylated histones H2A-K5 and H4-K12 were detected by immunofluorescence. (A) The combined two-dimensional surface area of the pronuclei in SLBP dsRNA and LacZ dsRNA (control) embryos was measured at 6 h and 7.5 h post-insemination. The experiment was performed three times, and the total number of oocytes examined is shown at the base of each bar. $P \leq 0.01$ (6 h), $P \leq 0.005$ (7.5 h), unpaired Student's *t* test. (B) The amount of acetylated histones H2A-K5 and H4-K12 present in the pronuclei was quantified using Metamorph imaging software. Shown are arbitrary units of fluorescence recorded for SLBP dsRNA eggs and LacZ dsRNA controls. Number of oocytes examined is shown at the base of each bar. $P \leq 0.01$ (H2A), $P \leq 0.01$ (H4), unpaired Student's *t* test. (C) Example of pronuclear H2A-K5 and H4-K12 as detected by immunofluorescence in 1-cell embryos obtained from oocytes injected with LacZ dsRNA or SLBP dsRNA. Star indicates the male pronucleus and arrowhead the second polar body. Scale bar = 50 μm.