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Stem–loop binding protein expressed in growing oocytes is required for accumulation of mRNAs encoding histones H3 and H4 and for early embryonic development in the mouse

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Abstract

Growing oocytes accumulate mRNAs and proteins that support early embryogenesis. Among the most abundant of these maternal factors are the histones. Histone mRNA accumulation and translation are mainly restricted to S-phase in somatic cells, and the mechanism by which oocytes produce histones is unknown. In somatic cells, replication-dependent histone synthesis requires the stem–loop binding protein (SLBP). SLBP is expressed during S-phase, binds to the 3′-untranslated region of non-polyadenylated transcripts encoding the histones, and is required for their stabilization and translation. SLBP is expressed in oocytes of several species, suggesting a role in histone synthesis. To test this, we generated transgenic mice whose oocytes lack SLBP. mRNAs encoding histones H3 and H4 failed to accumulate in these oocytes. Unexpectedly, mRNAs encoding H2A and H2B were little affected. Embryos derived from SLBP-depleted oocytes reached the 2-cell stage, but most then became arrested. Histones H3 and H4, but not H2A or H2B, were substantially reduced in these embryos. The embryos also expressed high levels of γH2A.X. Injection of histones into SLBP-depleted embryos rescued them from developmental arrest. Thus, SLBP is an essential component of the mechanism by which growing oocytes of the mouse accumulate the histones that support early embryonic development.

Keywords: SLBP; Histones; Oogenesis; Maternal control; Early embryogenesis

Introduction

Growing oocytes synthesize large quantities of mRNAs and proteins that support early embryonic development until transcription begins. Among the most abundant of these maternal factors are the histones. In the frog and the fly, enough histone to assemble the chromatin of several thousand nuclei must be produced by the oocyte (Anderson and Lengyel, 1980; Woodland, 1980). Oocytes of sea urchin and mouse accumulate smaller amount; nonetheless, in the mouse, where the major activation of embryonic transcription begins at the late 2-cell stage, one oocyte contains as much histone mRNA as a blastocyst (Graves et al., 1985) and its histone protein content has been estimated to be sufficient to assemble chromatin in about eight cells (Wassarman, 1988). In somatic cells, high levels of histone mRNA and protein synthesis occur only during S-phase, thus coordinating histone production with the need to assemble newly replicated DNA into chromatin. This indicates that oocytes, which remain arrested at late G2 of the cell cycle throughout their growth phase, have acquired the ability to accumulate large quantities of histones independently of DNA replication.

The restriction of histone production to S-phase in somatic cells is achieved primarily post-transcriptionally (reviewed by (Jaeger et al., 2005; Marzluff, 2005; Marzluff and Duronio, 2002; Osley, 1991)). The histone mRNAs that accumulate during S-phase are not polyadenylated. Instead, they carry a unique 3′-untranslated region that includes a highly conserved

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stem–loop sequence and a histone downstream element (HDE). During S-phase, a processing reaction cleaves the primary histone transcripts between the stem–loop and the HDE, and the cleaved transcripts are protected from rapid degradation, enabling them to accumulate. Processing does not occur at other stages of the cell cycle, so stem–loop histone mRNAs do not accumulate. Moreover, when DNA replication is inhibited, translation of the stem–loop histone mRNAs is arrested and the mRNAs become degraded. These regulatory mechanisms closely couple the accumulation and translation of stem–loop histone mRNAs to DNA replication. In addition to replication-dependent histone synthesis, a low level of histone synthesis persists throughout the cell cycle. These replication-independent histones, including the specialized histone variants H2A.Z, H3.3, $H1^0$ and the oocyte-specific H1, are encoded by polyadenylated mRNAs (Marzluff and Duronio, 2002).

The stem–loop binding protein (SLBP), also known as the hairpin-binding protein, plays a central role in the metabolism of stem–loop histone mRNAs (Martin et al., 1997; Wang et al., 1996). SLBP binds to the histone mRNA stem–loop through a highly conserved RNA-binding domain (Dominski et al., 2001). Histone stem–loop mRNAs are the only mRNAs known to bind to SLBP (Townley-Tilson et al., 2006). In the nucleus, SLBP binds to the stem–loop of newly produced transcripts and stabilizes a base-pairing interaction between the HDE and U7 snRNA. This enables factors associated with the U7 snRNA to perform the processing reaction that protects transcripts from degradation (Dominski et al., 1999, 2001, 2005; Kolev and Steitz, 2005). SLBP then accompanies the processed mRNAs to the cytoplasm where it bridges the 3′- and 5′-ends of the mRNA through an interaction with eIF4G and promotes their translation (Gorgoni et al., 2005; Ling et al., 2002; Sanchez and Marzluff, 2002; Whitfield et al., 2004). Thus, SLBP is necessary both for the accumulation of stem–loop histone mRNAs and for their translation. Importantly, although SLBP mRNA is constitutively present in mammalian somatic cells, the abundance of SLBP protein is cell cycle-regulated. It accumulates when cells enter S-phase and is degraded during G2 (Whitfield et al., 2000; Zheng et al., 2003). Thus, the high rate of histone synthesis during S-phase is due to the presence, at this stage of the cell cycle, of SLBP.

In view of the close link between SLBP and histone synthesis in somatic cells, it is possible that SLBP might also be required for the synthesis by oocytes of the histones that support early embryonic development. SLBP is present in the oocytes of the mouse (Allard et al., 2002), frog (Wang et al., 1999) and sea urchin (Robertson et al., 2004). As well, experimental manipulation of the quantity of SLBP in fully grown immature and maturing oocytes of the mouse affected the rate of translation of histone mRNAs in the oocytes and the timing of male pronuclear formation following fertilization (Allard et al., 2005). However, these studies did not address the role of SLBP in the accumulation of histone mRNAs during oogenesis nor the role of SLBP-dependent histone synthesis in supporting early embryonic development. To study these questions directly, we generated transgenic mice whose oocytes lack SLBP and examined whether these oocytes accumulated histone mRNAs and could develop as embryos following fertilization.

Materials and methods

Collection and culture of oocytes and embryos

Experiments were performed using CD-1 mice (Charles River Canada; Saint-Constant, QC). Growing oocytes were obtained 12-day old females as described (Eppig and Telfer, 1993) in the presence of 0.025% trypsin (Sigma Chemicals, Windsor, ON) to dissociate the oocytes from the granulosa cells. Ovulated eggs and embryos were obtained and incubated as described (Allard et al., 2002). Following appropriate treatments, oocytes or embryos were transferred to 0.5-ml microtubes and stored at −80 °C until analysis.

Production of SLBP dsRNA transgenic mice

A plasmid containing the zona pellucida glycoprotein-3 (*zp3*) promoter (a gift from Drs. Petr Svoboda and Richard Schultz (Svoboda et al., 2001)) was used to produce the SLBP dsRNA construct. Primers SLBP- *Nhe*I (CGGCTAGCCGCGACAACCTGGA) and SLBP- *Cla*I -I (CCATC-GATGGTCCCATAGACAC) or SLBP-*Cla*I-II (CCATCGATGAGAATTTATC) were used to amplify fragments spanning nt 539–1192 and 539–1236, respectively, of the cDNA encoding mouse SLBP (GenBank NM009193). The PCR-generated fragments were digested using *Cla*I and ligated to generate the SLBP inverted repeat (IR). SLBP IR was then digested using *Nhe*I and inserted into the *zp3* plasmid, which had been digested using *Xba*I, to produce pZP3-SLBP-IR. This plasmid contained a 654-nt inverted repeat of SLBP cDNA separated by a 50-nt spacer.

To produce transgenic mice, pZP3-SLBP-IR was double-digested using *Aas*I and *Ssp*I to release the 4.3 kb transgene cassette. DNA was isolated from a 0.7% agarose gel and purified using Geneclean (Q-BioGen, Carlsbad, CA) and eluted using injection buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA prepared using embryo-grade water (Sigma)). The DNA was diluted to a final concentration of 5 ng/μl in injection buffer and filtered through a 0.2 μm Ultrafree-CL filter (Millipore Canada, Mississauga, ON). Aliquots were stored at −80 °C until use. Fertilized eggs were collected and ∼5 pl of DNA was injected into one pronucleus using a Leica inverted microscope with Leica micromanipulators (Leica Canada, Montreal, QC) connected to a PLI-100 Pico-Injector (Harvard Apparatus Canada; St. Laurent, QC). Injected embryos were cultured for 4 days after which morula or blastocyst stage embryos were transferred into synchronized pseudopregnant mice.

Identification of transgenic mice

Founder animals were identified by Southern analysis of tail DNA. Genomic DNA was purified by phenol/chloroform extraction and isopropanol precipitation, then resuspended in water. Following digestion using *BspTI*, DNA fragments were separated in an agarose gel and transferred to a membrane (Hybond, GE Healthcare Biosciences; Baie D'Urfe, QC, Canada). Hybridization was performed using a 742-bp probe corresponding to the DNA sequence of enhanced green fluorescent protein (EGFP). The probe was PCR-labeled (primers: GCCGGTCGCCACCATGGTGAGC, GCGGCCGCTTTACTTGTA-CAGC using digoxigenin (Roche) and detected following the manufacturer's instructions. Transgenic lines were maintained by breeding hemizygous transgenic males. Offspring were screened using PCR to detect the EGFP sequence in the transgene (primers: GCACCATCTTCTTCAAGGACGAC, TCTTTGCTCAGGGCGGACTG). PCR was performed using *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) and a program of 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. PCR products were visualized on 1.5% agarose gels stained using ethidium bromide.

Fertility studies

Female transgenic mice were caged with males of proven fertility and checked daily for the presence of a vaginal plug. Following mating, they were housed in individual cages until they gave birth. To assess pre-implantation

development, they were sacrificed either on the morning that the plug was detected or the following morning. Embryos were recovered and incubated as above.

Anti-SLBP antibodies

Two antibodies against SLBP were used. One was provided by Dr. W.F. Marzluff (University of North Carolina) and has been previously used to identify SLBP in mouse oocytes and preimplantation embryos by immunoblotting and immunofluorescence (Allard et al., 2002, 2005). The second was obtained from rabbits immunized using the same peptide sequence, affinity-purified by the Marzluff laboratory, and recognizes the same species in immunoblots and shows the same pattern of staining in immunofluorescence as the first antibody (data not shown). The two antibodies were used interchangeably during the experiments.

RNA purification, cDNA synthesis and PCR amplification

RNA was extracted from oocyte or egg pools (PicoPure, Arcturus Biosciences Mountain View, CA) and reverse-transcribed using Murine Moloney Leukemia Virus (Invitrogen). PCR was performed using *Taq* DNA polymerase (2.5 units per reaction, Invitrogen, Burlington, ON) and one (egg) or two (oocyte) cell-equivalents of cDNA in 50 μl final volume. Primers were designed based on Genbank sequences (Table 1). Each PCR amplification cycle consisted of 94 °C for 30 s, primer-specific annealing temperature (Table 1) for 30 s and 72 °C for 30 s. Optimal cycle number for amplification during the exponential phase was determined for each gene. PCR products were analyzed in 1.5% agarose gels stained using ethidium bromide.

Immunoblotting, immunohistochemistry and immunofluorescence

Immunoblotting was performed as previously described (Allard et al., 2002, 2005). Anti-SLBP was used at a dilution of 1:2000, anti-ERK-1 (Santa Cruz Biotechnology; Santa Cruz, CA, sc-94) at 1:1000, and anti-tubulin (Cedarlane Laboratories) at 1:4000, and anti-histone H3 (Cell Signaling Technology, 9715) at 1:1000.

For immunohistochemical analyses, ovaries were collected from mice between 5 and 20 days of age and fixed in freshly prepared 4% *para*-formaldehyde in PBS for 4 h. Following dehydration and embedding in paraffin, 5-μm sections were cut and dried onto microscope slides. After rehydration, antigen recovery was performed by exposing the slides to 0.1% sodium citrate at 95 °C for 20 min. Slides were then incubated in blocking buffer (5% goat serum, 5% bovine serum albumin (Sigma) in PBST) for 30 min at room temperature, and then in affinity-purified anti-SLBP diluted 1:100 in blocking buffer overnight at 4 °C with gentle agitation. Following washing in PBST, they were incubated in HRP-conjugated anti-rabbit secondary antibody (diluted 1:200; Invitrogen) and YOYO-1 (diluted 1:20000; Invitrogen) for 1 h at room temperature. A tyramide-based signal amplification kit (Invitrogen) was used to enhance the fluorescent signal, after which the slides were mounted using Mowiol and covered with glass coverslips.

Immunofluorescence analysis was performed as previously described (Fu et al., 2003; Mohamed et al., 2001), except that a CY3-conjugated secondary antibody (diluted 1:400; Jackson ImmunoResearch; West Grove, PA) was used and DNA was stained using YOYO-1 diluted 1:20000. Fluorescence was visualized and images captured using a Zeiss LSM 510 Meta Confocal microscope. For each experiment, control and SLBP-depleted embryos stained with the same antibody were processed at the same time and mounted on the same microscope slide. Image-capture parameters below saturation were identified for each slide and images of each control and transgenic embryo were recorded under identical conditions. Fluorescence was quantified using ImageJ 1.36b software (National Institutes of Health, Bethesda, MD). For each slide, the mean value obtained for control embryos was set to 1 and the value for each control and transgenic embryo was normalized to this, enabling means and standard errors of the mean to be calculated. To label DNA synthesized during the first or second cell cycle, embryos were incubated in KSOM supplemented with 100 μM bromo-deoxyuridine (BrdU) from 20 to 26 h or 30 to 46 h, respectively, post-hCG. Embryos were processed as above, except that they were exposed to 2N HCl for 2 h prior to blocking.

Antibodies and suppliers were as follows. Cell Signaling Technology (Beverly, MA): H2A (2572), H4 (2592), acetyl-H2AK5 (2576), acetyl-H2BK20 (2571), acetyl-H3K9 (9671); Chemicon International (Temecula, CA): H2B (AB1623); Upstate Biotechnology (Charlottesville, VA): γ-H2A.X (05636), H3 (06755), di-methyl-H3K9 (07212), acetyl-H4K12 (06761); GE Healthcare Biosciences (Montreal, QC): BrdU (RPN202). All antibodies were affinity-purified and used at a dilution of 1:1000, except anti-H2A (1:400) and anti-SLBP (1:200).

Injection of histones

One-cell embryos were collected 14 h post-hCG as described above. They were injected with ~5 pl of either sterile H_2O or 10 mg/ml whole histone (Sigma) as described for DNA injection. Microinjected embryos were cultured in KSOM in a humidified incubator at 37 °C in 5% CO_2 in air. They were examined at regular intervals to assess developmental progression or were incubated in the presence of BrdU to assess DNA replication as described above.

Results

SLBP accumulates rapidly when oocytes enter the growth phase

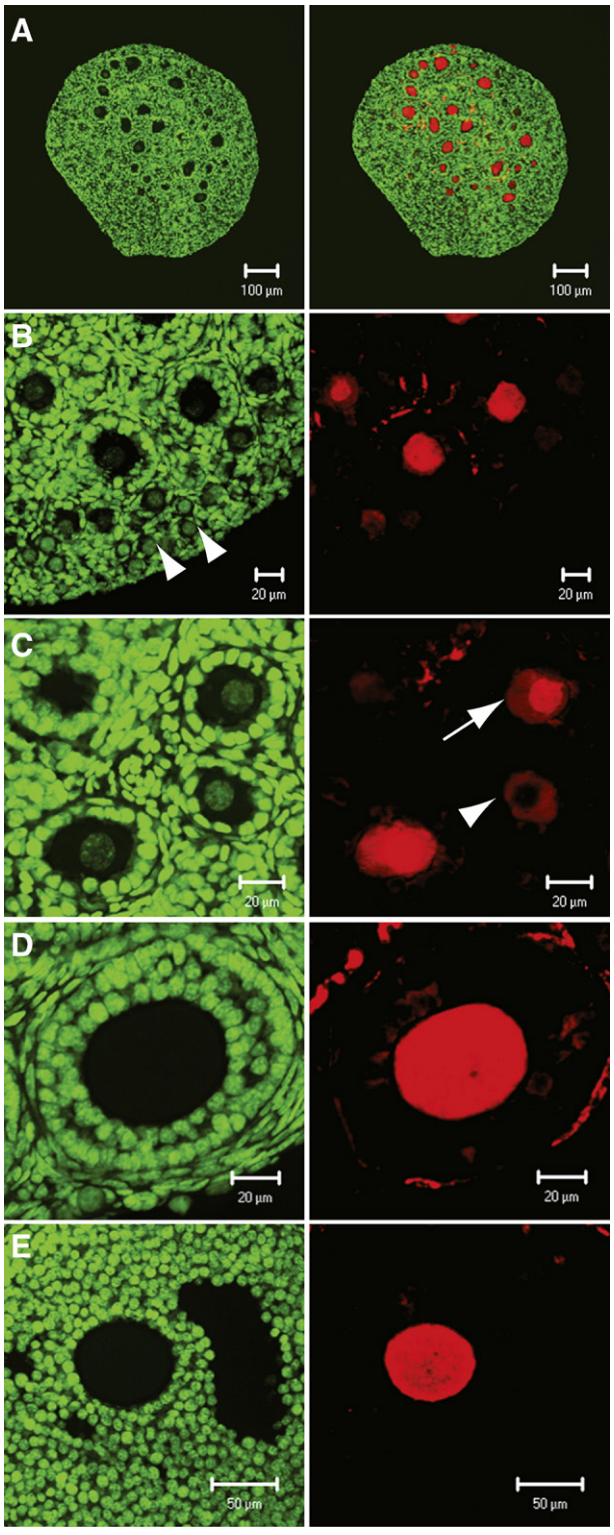
SLBP is known to be present in fully grown oocytes (Allard et al., 2002) but the timing of its accumulation during oocyte growth has not been described. To determine this, we took advantage of the fact that a large number of oocytes

Table 1
Primers used for RT-PCR analysis of gene expression

Protein	Gene[a]	Accession no.	Primer sequences[b]	Annealing temperature (°C)
SLBP	*Slbp*	NM009193	F- CAGCTGCAATGACTCTG R- GCAATTATCATTCCATAC	50
actin	*Actb*	NM007393	F- GCTGTGCTATGTTGCTCTAG R- ATCGTACTCCTGCTTGCTGA	55
c-mos	*Mos*	NM020021	F- CTCCGGAGATCCTGAAAGGA R- CAGTGTCTTTCCAGTCAGGG	58
Gdf9	*Gdf9*	NM008110	F- TGAGATTGATGTGACCTCCC R- AGTAGCTTCCTCTTTCACGG	58
Histone H2A	*Hist2h2aa2*	NM013549	F- GTTTGCGCTTTCGTGATG R- GGTTATATCACAGAGACG	50
Histone H2B	*Hist1h2bc* (common)	AY158937	F- AGAAGAAGGACGGCAAGAAG R- GGTCGAGCGCTTGTTGTAAT	58
Histone H3	*Hist1h3f* (common)	NM013548	F- TGGCTCGTACTAAGCAGACC R- AGGTTGGTGTCCTCAAACAG	56
	Hist2h3c2	NM054045	F- GGACTTCAAGACGGACCTG R- GCCAACTGGATGTCCTTG	54
Histone H4	*Hist1h4f*	AY158961	F- CATAGCCATGTCTGGTCGTG R- AGGGCCTTTGAGAAAGAAGC	50
	Hist1h4f (common)	AY158961	F- GGAGTGAAGCGCATCTCCGG R- CTGGCGCTTGAGCGCGTAGA	60

[a] Gene used to design primer is indicated. Where the primers match most genes encoding a particular histone subtype, these are further designated as 'common.'
[b] F: 5′-primer. R: 3′-primer. Both primers are written in 5′–3′ orientation.

synchronously enter the growth phase shortly after birth, reaching full size at about 3 weeks of age. Ovaries from mice between 5 and 20 days of age were fixed, sectioned and stained using an anti-SLBP antibody (Fig. 1). The specificity of the antibody for SLBP was confirmed by the absence of staining when the primary antibody was omitted (not shown) and in transgenic animals whose oocytes lack SLBP (Fig. 3). SLBP was not detectable in non-growing oocytes within primordial follicles (Fig. 1A, arrowheads in Fig. 1B). SLBP was clearly detected, however, in growing oocytes within primary follicles of these ovaries (Figs. 1A, B). In most oocytes within primary follicles, SLBP was more concentrated in the nucleus than the cytoplasm (arrow in Fig. 1C); however, in some cases it was predominantly cytoplasmic (arrowhead in Fig. 1C). SLBP remained detectable in growing oocytes in secondary follicles (Fig. 1D) and in fully-grown oocytes in antral follicles (Fig. 1E). Thus, SLBP is present throughout oocyte growth, when these cells are accumulating histone mRNAs.



SLBP does not accumulate in oocytes expressing double-stranded RNA targeting SLBP mRNA

To study the function of the SLBP produced during oocyte growth, we inhibited its accumulation, using a strategy based on generating mice that carry a transgene encoding double-stranded (ds) RNA corresponding to the target gene under the control of the *zp3* gene promoter (Stein et al., 2003), which is active specifically in growing oocytes (Philpott et al., 1987). This strategy has been used to deplete several gene products from oocytes (Fedoriw et al., 2004; Han et al., 2005; Ma et al., 2006; Stein et al., 2003). Diagrams of SLBP mRNA, the fragment that was used to generate the dsRNA sequence, and the construct into which the dsRNA was inserted are shown in Fig. 2A. Transgenic mice were generated by pronuclear injection and male founders were used to generate transgenic lines. Transgenic animals were obtained by mating transgenic males to wild-type females and were identified using Southern blotting (Fig. 2B) or PCR.

To test whether SLBP accumulated in the oocytes of transgenic females, these animals and their wild-type littermates were superovulated and the eggs were subjected to either RT-PCR or immunoblotting. SLBP mRNA and protein were substantially depleted in eggs of transgenic females (Figs. 3A, B). We then determined at what stage of oocyte growth SLBP became depleted. Ovaries were collected from transgenic mice between 5 and 20 days of age, sectioned and stained using the anti-SLBP antibody as described for the wild-type animals. The transgenic ovaries contained many oocytes at different stages of growth and were morphologically indistinguishable from those of wild-type mice (Fig. 3C). Thus, there was no obvious effect of on the dynamics of oogenesis. However, SLBP was undetectable in oocytes at any stage of growth. These results

Fig. 1. Expression of SLBP in wild-type oocytes. Ovarian sections were stained using YOYO-1 to label DNA (left panels) and anti-SLBP (right panels), except in panel A where right panel shows overlaid images. (A) Ovary of 5-day animal showing primordial and primary follicles. SLBP is detectable in growing oocytes in primary follicles. Scale bar=100 μm. (B) Non-growing and growing oocytes. SLBP is detectable in growing oocytes in primary follicles but not in non-growing oocytes in primordial follicles (arrowheads). Bar=20 μm. (C). Growing oocytes in primary follicles. SLBP is mainly cytoplasmic is some oocytes (arrowhead) and mainly nuclear in others (arrow). Bar=20 μm. (D) Growing oocyte in secondary follicle. SLBP is present throughout the cytoplasm. Nucleus not visible in this section. Bar=20 μm. (E) Fully-grown oocyte in antral follicle. SLBP is present throughout the cytoplasm. Nucleus not visible in this section. Bar=50 μm.

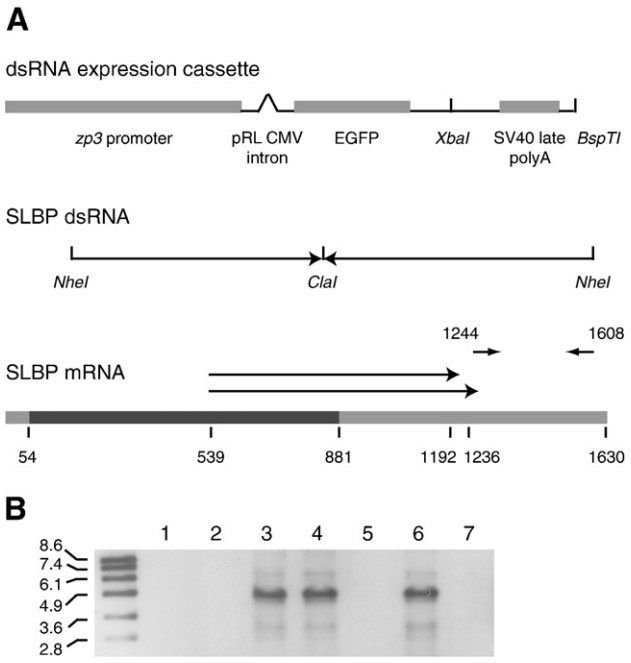


Fig. 2. (A) Construct encoding dsRNA targeting SLBP. At the bottom is shown the portion of the SLBP mRNA used to generate the dsRNA (long arrows) and the location of the primers used for RT-PCR. In the middle is shown the dsRNA construct and relevant restriction sites. At the top is shown the construct into which the dsRNA was inserted at the *Xba*I site. (B) Southern blot of transgenic (lanes 3, 4, 6) and non-transgenic (lanes 1, 2, 5, 7) animals.

demonstrate that SLBP fails to accumulate normally in growing oocytes of transgenic animals.

Embryos derived from SLBP-depleted oocytes arrest at the 2-cell stage

To test whether the SLBP in the oocyte is required for subsequent development, we tested the fertility of the transgenic females. Transgenic females mated with wild-type males produced an average of 2.45±0.92 pups per litter (6 females from 3 independent lines). In contrast, their wild-type littermates produced an average of 14.08±0.65 pups per litter. Thus, the failure to accumulate SLBP during oogenesis was associated with impaired fertility. To determine the basis of the impaired fertility, we mated the animals and flushed the embryos from the reproductive tract at 20 h post-hCG. Similar numbers of 1-cell embryos were recovered from wild-type and transgenic animals (Table 2). This suggests that the lack of SLBP did not impair oogenesis, consistent with the apparently normal morphology of the ovary as observed in histological sections (Fig. 3). When the embryos were placed in culture, most (81%) derived from wild-type mothers developed to the blastocyst stage (Table 2). In contrast, only 25% of embryos from transgenic females were able to cleave to the 4-cell stage. Almost all embryos that reached the 4-cell stage, however, subsequently developed to the blastocyst stage. These results establish that the SLBP mRNA and/or protein that accumulates during oogenesis is required for early embryonic development.

mRNAs encoding histones H3 and H4 are selectively depleted in SLBP-deficient oocytes

We then examined whether mRNAs encoding each of the four core histones accumulated in the oocytes and eggs of transgenic animals. As each core histone is encoded by numerous genes (Marzluff et al., 2002), primers were designed as follows (see Supplementary Table S1). Histone H2A is encoded by at least twenty genes; however, 90% of the histone H2A mRNA in the oocyte derives from the *Hist1h2aa2* gene (Graves et al., 1985) and primers were designed to match this gene. Histone H2B is encoded by seventeen genes. As no individual gene contributing more than 5% of the H2B-encoding mRNA in mouse oocytes has been identified (Graves et al., 1985), primers that match or nearly match all genes were designed. Histone H3 is encoded by thirteen genes that carry the stem–loop sequence and by two genes that encode the variant H3.3 and carry a polyadenylation signal. About 90% of the H3-encoding mRNA in mouse oocytes is transcribed from two genes, *Hist1h3f* and *Hist2h3c2* (Graves et al., 1985) and two sets of primers were designed. These primers also match the other stem–loop type H3-encoding genes, although they match those encoding H3.3 less well. Histone H4 is encoded by thirteen genes. As *Hist1h4f* is known to be expressed in oocytes (Allard et al., 2005; Graves et al., 1985), primers were designed to match this gene. As well, a second set was designed that matches or nearly matches the other H4-encoding genes.

mRNA obtained from ovulated eggs of transgenic and wild-type females was analyzed by RT-PCR using primers targeting the different histone subtypes and several non-histone gene products (Fig. 4A). Both types of eggs contained similar quantities of the non-histone mRNAs. Moreover, both contained similar quantities of histone H2A and H2B mRNAs. In contrast, histone H3 and H4 mRNAs were substantially depleted in eggs of transgenic females as compared to those of wild-type females. This difference was observed using two sets of primers for each subtype. As the mRNAs that are present in the ovulated egg accumulated during oocyte growth, we examined expression of the histone genes in growing oocytes (Fig. 4B). Oocytes of transgenic and wild-type females contained similar amounts histone H2A and H2B mRNAs. However, histone H3 mRNA was substantially depleted in oocytes of transgenic females. In the case of histone H4, oocytes of transgenic females produced little PCR product using primers that matched *Hist1h4f* specifically, but no difference between transgenic and wild-type oocytes was observed using the general H4 primers. This suggests that mRNAs derived from some histone H4-encoding genes began to accumulate during oocyte growth, although they apparently did not persist. Immunoblotting revealed that histone H3 was reduced by slightly more than half in ovulated eggs of transgenic as compared to wild-type females; we have not yet found antibodies with sufficient sensitivity to measure the other histones (data not shown). These results indicate that oocytes lacking SLBP are unable to normally accumulate mRNAs encoding histones H3 and H4, but those encoding histones H2A and H2B are much less affected.

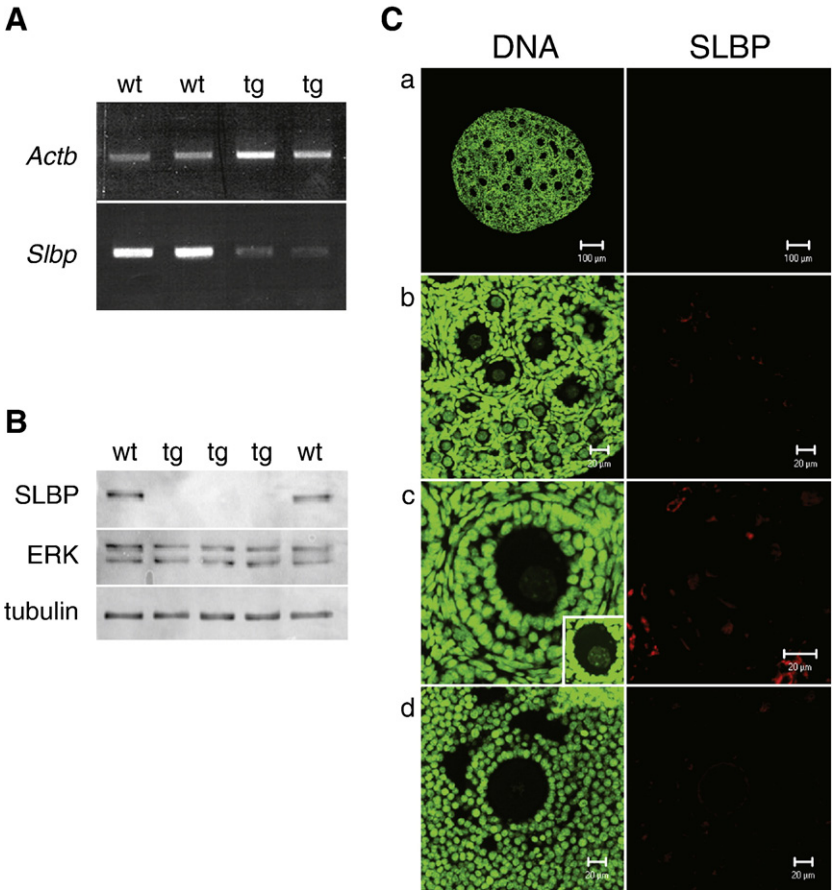


Fig. 3. Expression of SLBP in transgenic animals. (A) Ovulated eggs from wild-type (wt) or transgenic (tg) animals were analyzed using RT-PCR to detect mRNA encoding actin (*Actb*) and SLBP (*Slbp*). *Slbp* mRNA is depleted in transgenic oocytes. (B) Ovulated eggs from wild-type (wt) or transgenic (tg) animals were subjected to immunoblotting using the indicated antibodies. Each lane contains 25 eggs collected from an individual mouse. SLBP is depleted in eggs of transgenic mice. (C) Ovarian sections from transgenic mice were stained using YOYO-1 (left panels) and anti-SLBP (right panels). Images were collected using parameters identical to those in Fig. 1. (a) Ovary of 5-day animal showing primordial and primary follicles. (b) Higher magnification view of a 5-day ovary. (c) Ovary of 10-day animal showing oocyte in secondary follicle. Inset on left panel was obtained at higher-power to reveal the nucleus. (d) Ovary of a 20-day animal showing oocyte in antral follicle. SLBP is not detectable in oocytes at any stage of growth.

Embryos derived from SLBP-depleted oocytes do not complete the second round of DNA replication and lack histones H3 and H4

The results above suggested that the arrest of SLBP-depleted embryos at the 2-cell stage might be due to insufficient histones H3 and H4. We therefore examined the cell-cycle progression and histone content of these embryos. Embryos were incubated in the presence of BrdU during either the first or second round of embryonic DNA replication. Following fixation and staining with an anti-BrdU antibody, images were recorded using a confocal microscope and the fluorescent signal was quantified. BrdU incorporation during the 1-cell stage was slightly reduced in SLBP-depleted embryos as compared with wild-type embryos (Figs. 5A, B). It was substantially reduced, however, during the 2-cell stage. Thus, the second round of DNA replication was severely impaired in the SLBP-depleted embryos.

Table 2
Preimplantation development in vitro of embryos from transgenic and non-transgenic females

Genotype	No. of mice	No. of eggs	Number (%) that reached indicated stage[a]		
			2-cell	4-cell	morula/blastocyst
Non-transgenic	7	190	188 (99)	166 (87)	153 (81)
Transgenic[b]	7	148	133 (90)[c]	37 (25)[c]	32 (22)[c]

[a] Embryos were flushed from the oviduct at 20 h post-hCG and cultured in vitro for 4 days.
[b] Embryos from three independent lines were analyzed.
[c] Differs significantly from non-transgenic (Chi-square, $p<0.01$).

We then examined the abundance of each of the four core histones. The pool of histones in the mouse egg is sufficient to assemble about eight nuclei (Wassarman, 1988). Thus, 2-cell embryos contain both histone incorporated into chromatin and non-incorporated histone in the nucleoplasm. Wild-type and SLBP-depleted embryos were stained using antibodies recognizing each core histone and the signal was quantified as above. First, we used antibodies directed against unmodified forms of the histones. This immunofluorescent analysis indicated that SLBP-depleted embryos contained much less histones H3 and H4 than wild-type embryos (Figs. 6A, B). In contrast, histones H2A and H2B were not detectably or only slightly reduced in

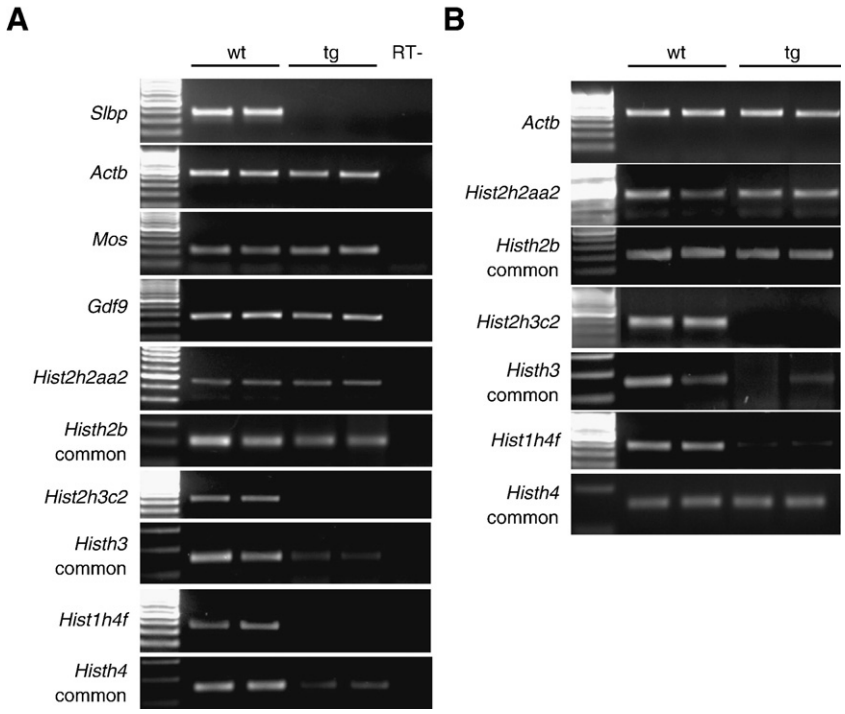


Fig. 4. Expression of histone and non-histone genes in oocytes of wild-type (wt) and transgenic (tg) animals. Ovulated eggs (A) and growing oocytes (B) were analyzed using RT-PCR to detect the indicated mRNAs. The experiments were performed twice using independent pools of eggs. Where primers match only one or a few genes encoding a histone subtype, the relevant gene is identified. Where the primers match most genes encoding a subtype, they are identified as 'common.' See Supplementary Table S1 for further details. mRNAs encoding histones H3 and H4 are depleted in ovulated eggs of transgenic females, but little difference is evident for H2A and H2B. mRNA encoding H3 is depleted in growing oocytes, whereas some mRNA encoding H4 is detectable at this stage.

the SLBP-depleted embryos. These results were consistent with the results of the mRNA analysis and indicated that the total amount of histone H3 and H4 was reduced in SLBP-depleted embryos, whereas the total amount of histones H2A and H2B in these embryos was similar to wild-type.

To examine histones within chromatin specifically, we used antibodies that recognize modifications that may be imposed on histones during or following their assembly into chromatin (Nightingale et al., 2006; Sobel et al., 1995). First, we used antibodies recognizing acetylated forms of each of the core histones. Acetylated histones H3 and H4 are associated with transcriptionally active chromatin, which is replicated during early S-phase (Belyaev et al., 1996; Gilbert, 2002). Although 1- and early 2-cell mammalian embryos are only weakly transcriptionally active (reviewed by (Kanka, 2003; Schultz, 2002)), their pattern of DNA replication implies the existence of transcriptionally competent and incompetent domains (Ferreira and Carmo-Fonseca, 1997). Thus, antibodies recognizing acetylated histones may preferentially label early-replicating chromatin. As a complementary approach, we used an antibody recognizing K9-methylated histone H3, a modification associated with transcriptionally inactive late-replicating chromatin (Mcnairn and Gilbert, 2003; Wallace and Orr-Weaver, 2005).

Acetylated forms of all four histones were significantly reduced in the SLBP-depleted embryos as compared with controls (Fig. 6C). The degree of reduction was similar for all four antibodies, which is consistent with the equimolar representation of histones in chromatin. However, K9-methylated histone H3 was reduced to a much greater degree in SLBP-depleted embryos. These results suggest that, as compared with controls, the chromatin of the SLBP-depleted embryos contained moderately less acetylated histone and substantially less K9-methylated histone H3. These results are consistent with a developmental arrest during S-phase. To further test whether developmental arrest occurred during S-phase, we stained 2-cell embryos using an antibody against phosphorylated (γ-) H2A.X, which is present at sites of double-stranded DNA breaks (Wurtele and Verreault, 2006) and might be expected to accumulate if DNA replication was impaired. Consistent with this prediction, SLBP-depleted embryos displayed a considerably higher level of γ-H2A.X than wild-type embryos (Fig. 6D). Taken together, these results strongly suggest that SLBP-depleted embryos fail to complete the second round of DNA replication and that this is mainly due to an insufficient supply of histones H3 and H4.

Exogenous histones rescue development of SLBP-depleted embryos

The results described above suggested that the developmental arrest of SLBP-depleted embryos was due to a lack of histones. To test this hypothesis, we examined whether development of the SLBP-depleted embryos could be rescued by exogenous histones. SLBP-depleted one-cell embryos were injected with 50 pg of mixed histones or with water. To determine whether the defect in the second round of DNA

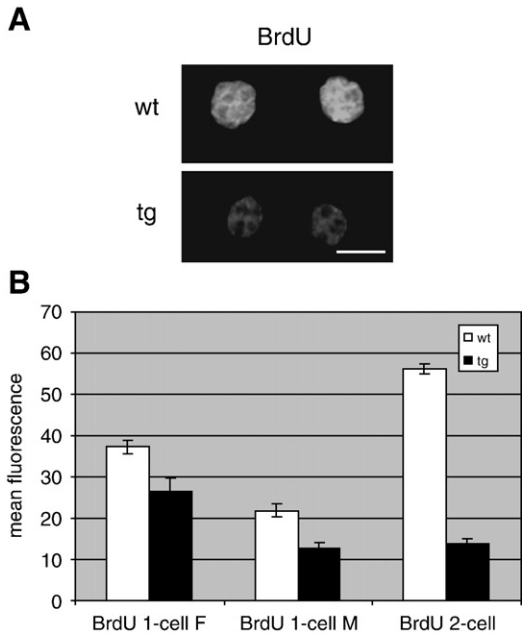


Fig. 5. (A) DNA replication in embryos derived from wild-type (wt) or transgenic (tg) eggs. Embryos were incubated in the presence of BrdU, then stained using anti-BrdU. Images are of 2-cell embryos. Staining is much weaker in embryos derived from tg eggs. Bar=25 μm. (B) Quantification of images from wild-type (white bars) and transgenic (black bars) embryos. Images were recorded using a confocal microscope and analyzed using ImageJ. Female (F) and male (M) pronuclei were analyzed separately in the 1-cell embryos. Each bar shows the mean of at least 15 cells. The difference between the wild-type and transgenic groups is significant in all three groups (*t*-test, $p<0.05$).

replication was rescued, injected embryos were incubated in the presence of BrdU during the second round of DNA replication and analyzed as above. BrdU incorporation was significantly elevated in histone-injected embryos as compared with controls (Fig. 7A). Thus, supplying exogenous histones enabled SLBP-depleted embryos to continue the second round of DNA replication. We then tested whether injection of histones enabled embryos to develop beyond the 2-cell stage. Injected embryos were incubated for 4 days and development was assessed at regular intervals. Most (60%) of the histone-injected embryos reached the 4-cell stage and the majority of these subsequently became blastocysts (Fig. 7B). In contrast, only about one-quarter of the control embryos progressed beyond the 2-cell stage. This frequency of development is the same as observed in non-injected SLBP-deficient embryos (see Table 2). We conclude that the failure of SLBP-depleted embryos to develop beyond the 2-cell stage is due to an insufficient supply of histones.

Discussion

Growing oocytes accumulate large quantities of histone mRNAs and proteins that are required for chromatin assembly in early embryos until transcription begins. We have studied the role of SLBP in this process. We report that SLBP begins to accumulate when mouse oocytes enter the growth phase and remains present throughout growth. Oocytes that lack SLBP fail to accumulate mRNAs encoding histones H3 and H4. In contrast, accumulation of mRNAs encoding H2A and H2B is little affected. Embryos derived from SLBP-depleted oocytes show markedly impaired DNA replication at the 2-cell stage and contain less histones H3 and H4 than normal embryos, and most become developmentally arrested at this stage. Both the DNA replication defect and the developmental arrest of SLBP-deficient embryos are rescued by injection of histones. These results establish that SLBP is an essential component of the mechanism by which growing oocytes of the mouse accumulate the histones that support early embryogenesis.

Histones H3 and H4 are encoded by thirteen and twelve genes, respectively. If our methods measured expression from only a subset of the encoding genes, then the reduced quantities that we observed in the SLBP-depleted oocytes and embryos might not reflect the actual quantity of each subtype that is present in the cells. This is unlikely to be the case. Regarding histone H3, the primers selected match perfectly the two genes that produce ~90% of the mRNA in the oocyte, as well as the nine other stem–loop bearing genes. These genes encode two forms of histone H3 that differ at only one amino acid position and would likely each be recognized by the antibody. We cannot entirely exclude that histone H3.3, which is expressed in oocytes (Torres-Padilla et al., 2006; Van Der Heijden et al., 2005), was not recognized by our primers or antibodies. However, H3.3 differs at only five and four amino acids from H3.1 and H3.2, respectively, and contains the K9 that is recognized by the anti-methyl-K9-H3 antibody. Regarding histone H4, the primers used for PCR match ten of the twelve genes exactly, and the proteins encoded by all twelve are identical and thus would be recognized by the antibody. Thus, there are unlikely to be forms of histone H3 or H4 that are present in significant quantities in the oocyte or early embryo but are undetected by our methods. We conclude that SLBP is required for the accumulation of histones H3 and H4 in mouse oocytes.

In contrast to its essential role with respect to histones H3 and H4, SLBP is not required for the accumulation of histones H2A and H2B in oocytes. The major H2A-encoding gene expressed in oocytes (*Hist1h2aa2*) and the known H2B-encoding genes all bear a stem–loop sequence. Thus, the SLBP-independent accumulation of H2A and H2B and their encoding mRNAs was unexpected. It has previously been observed that *Hist1h2aa2* gives rise to a small quantity of polyadenylated mRNA in mouse spermatids (Moss et al., 1994) and in tissue culture cells (Levine et al., 1987), and a possible polyadenylation signal lying 3′ to the stem–loop and U7 RNA-binding sites has been identified (Moss et al., 1994). Spermatids also transcribe a polyadenylated mRNA from the *Hist1h2bp* gene (Moss et al., 1989). Moreover, in *Drosophila* embryos carrying mutant alleles of *dSLBP*, the histone genes that normally generate stem–loop mRNAs now generate polyadenylated mRNAs in somatic cells, although these are not found in the oocytes (Lanzotti et al., 2002; Sullivan et al., 2001). These results suggest that, in the absence of SLBP, transcriptional elongation at histone genes continues until a polyadenylation signal is encountered. Studies using HeLa tissue-culture cells, however, are not entirely consistent with this explanation.

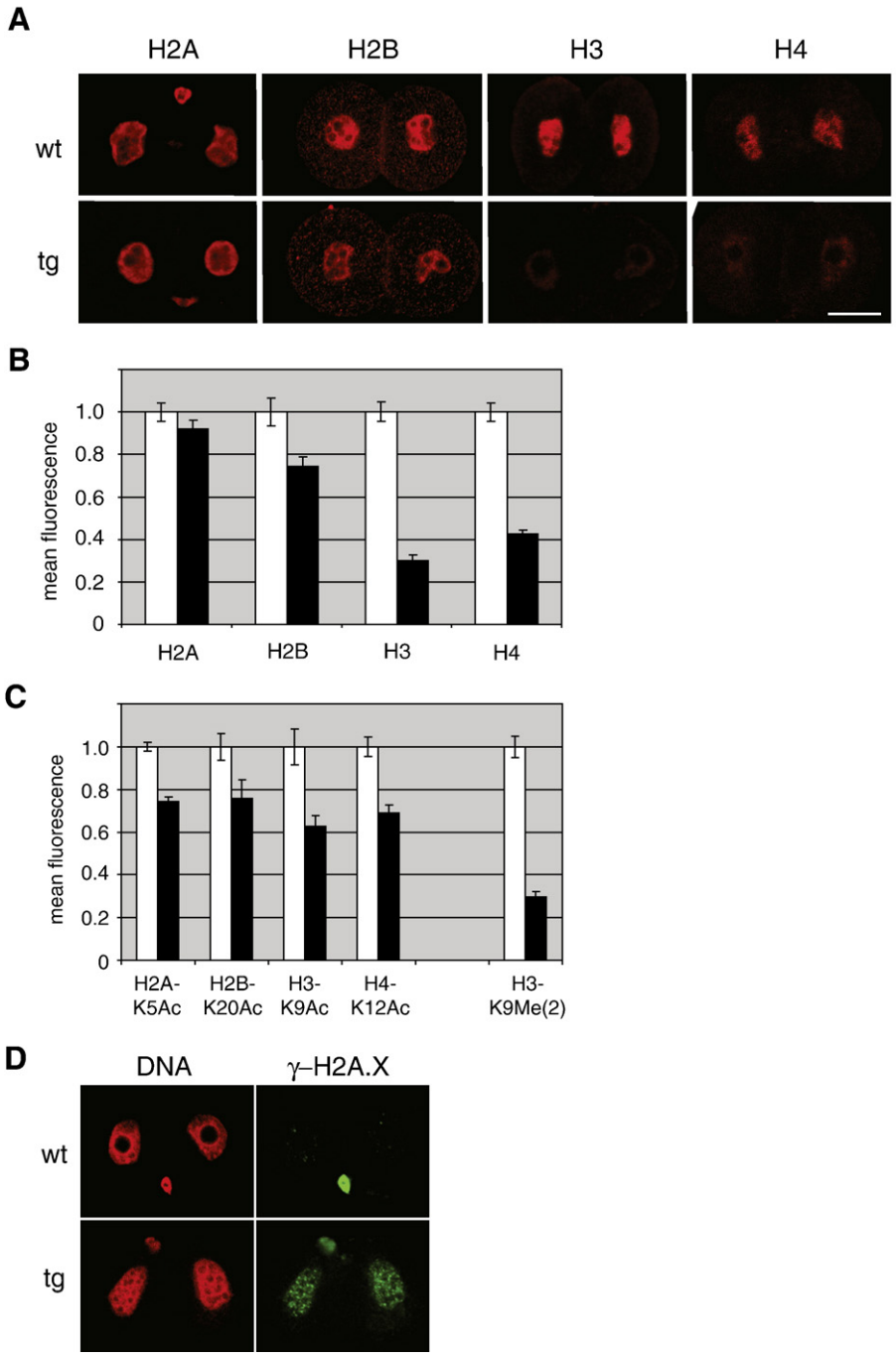


Fig. 6. Expression of histones in 2-cell embryos derived from wild-type (wt, white bars) and transgenic (tg, black bars) eggs. (A) Representative images of embryos stained using antibodies against the indicated histones. Embryos derived from transgenic eggs show reduced H3 and H4 staining. Bar=25 μm. (B, C) Quantification of images obtained using antibodies recognizing unmodified histones (B) or specific histone modifications (C). Each bar shows the mean of at least 18 cells. In all groups except H2A, the difference between the wild-type and transgenic groups is significant (*t*-test, $p<0.05$). (D) Expression of γ-H2A.X. Embryos were stained using propidium iodide to label the DNA (left) and with anti-γ-H2A.X (right). Embryos derived from transgenic eggs show much more intense γ-H2A.X staining. The small stained structure in each panel is the second polar body. Twenty-five embryos from each group were examined; all showed the same pattern of staining. Bar=25 μm.

Following RNAi-mediated depletion of SLBP, Narita et al. (2007) observed polyadenylated mRNA derived from the *HIST2H2AA* gene; however, most of the *HIST2H2AA* mRNA was not polyadenylated, suggesting that it had been processed normally despite the absence of SLBP. On the other hand, another study reported a reduction in the quantities of mRNAs encoding all four core histones (Zhao et al., 2004). Thus, the relative amounts of polyadenylated and non-polyadenylated mRNAs encoding histones H2A and H2B in SLBP-depleted oocytes needs to be determined.

Following fertilization, SLBP-depleted eggs began embryonic development but most became arrested at the 2-cell stage. The ability of the fertilized eggs to assemble the male pronucleus and undergo the first round of DNA replication

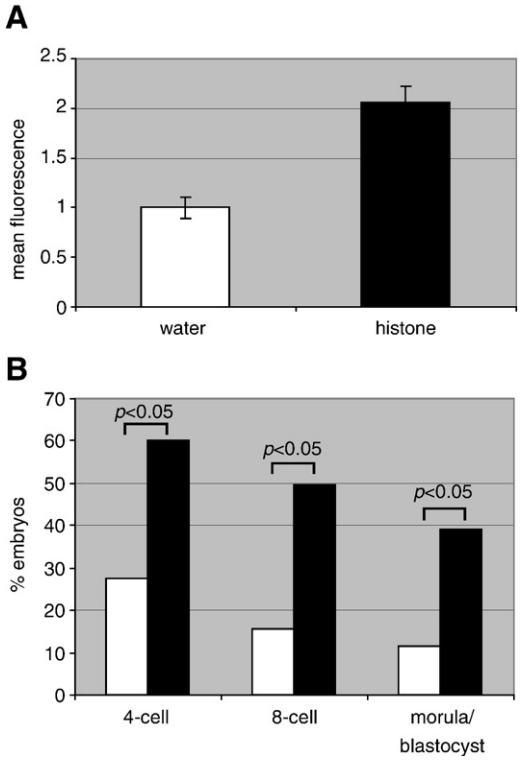


Fig. 7. Rescue of DNA replication and embryonic development by injected histone. (A) One-cell embryos derived from transgenic eggs were injected with water (white bars) or with 50 pg of histone (black bars). At the 2-cell stage, they were incubated in the presence of BrdU and then stained using anti-BrdU. Fluorescent signals were recorded and quantified. Each bar represents the mean of at least 43 cells. BrdU incorporation is significantly higher in the histone-injected embryos (*t*-test, $p<0.05$). (B) Embryos injected as in panel A were examined at regular intervals to assess developmental progression. The number reaching each of the indicated stages is shown. The experiment was performed 6 times with a total of 95 embryos in each group. At all stages, the difference between the two groups is significant (χ^2, $p<0.05$).

implies that they had produced enough histone H3 and H4 to support these events. Several factors may contribute to this limited histone production. First, as the dsRNA strategy may not inactivate all copies of its target mRNA, it is reasonable to suppose that a small amount of SLBP is present in the oocytes of transgenic females. Second, it is possible the mRNAs encoding histones H3 and H4 can be processed and translated, albeit inefficiently, in oocytes lacking SLBP. Third, histone H3.3, which is encoded by a polyadenylated mRNA, may provide a small quantity of this subtype. Nevertheless, most embryos failed to complete the second round of DNA replication and became developmentally arrested. Both the DNA replication defect and developmental arrest were rescued by injected histone, indicating that they were due to a lack of histones. In tissue-culture cells, insufficient histones trigger arrest of DNA replication, DNA damage and activation of the S-phase checkpoint (Nelson et al., 2002; Ye et al., 2003). Our observation that SLBP-depleted embryos contained high levels of γ-H2A.X suggest that a similar process occurs in these cells. Taken together, these results suggest that the developmental arrest of the SLBP-depleted embryos may be due to activation of an S-phase checkpoint mechanism.

Some embryos derived from SLBP-depleted eggs were able to reach the 4-cell stage. These may have failed to activate the S-phase checkpoint or may have contained sufficient histone to complete the second S-phase. Interestingly, most of these embryos subsequently developed to the blastocyst stage. This suggests that the SLBP produced in the oocyte is required only to support development to about the 4-cell stage. As the major transcriptional activation occurs at the late 2-cell stage in mice, the embryonic genes encoding SLBP and the histones presumably are transcribed beyond this point. Although the stability of the dsRNA targeting SLBP is unknown, expression from the *zp3* promoter stops before oocyte meiotic maturation (Philpott et al., 1987). Thus, SLBP embryos that reach the 4-cell stage may subsequently be able to produce normal quantities of SLBP and histones, which in turn support continued cycles of DNA replication and chromatin assembly. In support of this idea, 1-cell embryos injected with about 50 pg of histone, an amount sufficient to package chromatin in only about 8 cells, could develop to the blastocyst stage, strongly suggesting that the injected embryos synthesized histones.

The role of SLBP in the accumulation of histones in oocytes has previously been experimentally studied in *Drosophila*, using mutants that carry mutant alleles of *dSLBP* (Lanzotti et al., 2002; Sullivan et al., 2001). Two key differences are apparent as compared with our studies using the mouse. First, mRNAs encoding all four histones are reduced in the oocytes of the mutant flies, whereas we observed a selective effect on histones H3 and H4. This may reflect the different mechanisms by which the oocytes of the two species accumulate histones. In the fly, the histone mRNAs that accumulate in the oocyte are synthesized by the nurse cells (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). These undergo several rounds of endoreduplication prior to synthesizing the oocyte-bound histone mRNAs (Lilly and Duronio, 2005). Low levels of SLBP in the mutant animals would likely impair the endoreduplication cycles, which in turn might impair the ability of the nurse cells to support a high rate of histone gene transcription. Thus, the reduced quantity of histone mRNAs in oocytes of flies carrying hypomorphic *dSLBP* alleles may reflect a function for this gene in the nurse cells, a view supported by the observation that SLBP has not been detected in oocytes (Lanzotti et al., 2002). In contrast, in the mouse, our experiments identified a role for SLBP that is expressed in oocytes and independently of DNA replication.

Second, unlike the case in the mouse, *Drosophila* embryos derived from females carrying mutant *dSLBP* alleles do not become arrested during S-phase. Instead, they undergo multiple rounds of DNA replication and chromatin assembly, ultimately becoming arrested owing to defects in chromosome condensation and organization on the mitotic spindle (Lanzotti et al., 2002; Sullivan et al., 2001). Similar chromosomal defects occur in *C. elegans* embryos that lack SLBP (Kodama et al., 2002; Pettitt et al., 2002). As discussed above, the developmental arrest of SLBP-depleted mouse embryos is likely due to activation of the S-phase checkpoint. In flies, however, it has been suggested that an S-phase checkpoint may not become

functional until cycle 10 of embryogenesis (Crest et al., 2007). Thus, it is possible that the early rounds of DNA replication and chromatin assembly are impaired in *Drosophila* embryos, as in the mouse, but this does not directly trigger developmental arrest.

SLBP is also expressed in the oocytes of frogs (Sanchez and Marzluff, 2004; Wang et al., 1999) and sea urchins (Robertson et al., 2004) and is likely required for the accumulation of maternal histones as it is in the mouse. In somatic cells, although SLBP is likely required for the high rate of replication-coupled histone synthesis (Zhao et al., 2004; Narita et al., 2007), it is not expressed outside of S-phase (Whitfield et al., 2000; Zheng et al., 2003) and hence plays no role in histone synthesis in non-replicating cells. Thus, it may be questioned why accumulation of histones in non-replicating oocytes requires SLBP. One possibility is that SLBP-independent histone synthesis may occur in oocytes, but the rate is too low to enable sufficient accumulation of the proteins. This is consistent with the fact that somatic cells synthesize histones at a far higher rate during S-phase than at other stages of the cell cycle. On the other hand, oocyte growth is a slow process – requiring about 3 weeks in the mouse – and it cannot be assumed that this period is too brief to permit sufficient accumulation of histones. Alternatively, it may be that for certain histone subtypes none of the encoding genes can give rise polyadenylated mRNAs, so SLBP is needed to support production of these subtypes. Finally, even where genes that can produce a polyadenylated transcript are known, such as those encoding histone H3.3, perhaps the differences in amino acid sequence of the variant mean that it cannot be used to assemble normal chromatin in early embryos (Henikoff and Ahmad, 2005). In any case, the activity of SLBP in growing oocytes implies that these cells, which are at late G2 of the cell cycle, have evolved mechanisms that enable SLBP to accumulate and function in a replication-independent manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.032.

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