

Drosophila stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression

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Replication-associated histone genes encode the only metazoan mRNAs that lack polyA tails, ending instead in a conserved 26-nt sequence that forms a stem-loop. Most of the regulation of mammalian histone mRNA is posttranscriptional and mediated by this unique 3' end. Stem-loop-binding protein (SLBP) binds to the histone mRNA 3' end and is thought to participate in all aspects of histone mRNA metabolism, including cell cycle regulation. To examine SLBP function genetically, we have cloned the gene encoding *Drosophila* SLBP (dSLBP) by a yeast three-hybrid method and have isolated mutations in *dSLBP*. *dSLBP* function is required both zygotically and maternally. Strong *dSLBP* alleles cause zygotic lethality late in development and result in production of stable histone mRNA that accumulates in nonreplicating cells. These histone mRNAs are cytoplasmic and have polyadenylated 3' ends like other polymerase II transcripts. Hypomorphic *dSLBP* alleles support zygotic development but cause female sterility. Eggs from these females contain dramatically reduced levels of histone mRNA, and mutant embryos are not able to complete the syncytial embryonic cycles. This is in part because of a failure of chromosome condensation at mitosis that blocks normal anaphase. These data demonstrate that dSLBP is required *in vivo* for 3' end processing of histone pre-mRNA, and that this is an essential function for development. Moreover, dSLBP-dependent processing plays an important role in coupling histone mRNA production with the cell cycle.

[Key Words: *Drosophila*; cell cycle; SLBP; histone; replication; RNA processing]

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The replication-dependent histone mRNAs are the only metazoan mRNAs that do not have polyA tails. Instead, they end in a conserved 26-nt sequence that contains a 16-nt stem-loop (Marzluff 1992; Dominski and Marzluff 1999). The only processing reaction necessary for formation of mature histone mRNA is an endonucleolytic cleavage to form the 3' end (Gick et al. 1986). This reaction is directed by two sequence elements, the stem-loop sequence (Vasserot et al. 1989; Melin et al. 1992) and a conserved region 3' of the stem-loop that binds to the U7 snRNP (Mowry and Steitz 1987; Cotten et al. 1988; Soldati and Schumperli 1988). The protein that binds the stem-loop, SLBP, has previously been cloned from vertebrates (Wang et al. 1996b; Martin et al. 1997). SLBP is required for histone pre-mRNA processing *in vitro* (Dominski et al. 1999) and accompanies the histone

mRNA to the cytoplasm, where it remains as part of the histone mRNP (Dominski et al. 1995; Hanson et al. 1996).

In cultured mammalian cells, accumulation of histone mRNA occurs only during S phase and requires ongoing DNA replication (Schumperli 1986; Osley 1991). Even though this much has been known for many years, it remains unclear how histone mRNA production is coupled to other regulatory events in the cell cycle. Much of the control of histone mRNA levels is mediated by the 3' end of histone mRNA (Stauber et al. 1986; Luscher and Schumperli 1987; Stauber and Schumperli 1988; Harris et al. 1991). Both pre-mRNA processing and mRNA half-life (Pandey and Marzluff 1987) are regulated by the sequence at the 3' end of the histone mRNA. SLBP protein also only accumulates during S phase of the mammalian cell cycle and is destroyed at entry into G2 phase, suggesting that regulation of SLBP is one of the major factors responsible for cell cycle regulation of histone mRNA in mammalian cells (Whitfield et al. 2000).

In early embryonic development of many species (e.g., frogs, sea urchins, and fruit flies), there are very high

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rates of DNA replication and, hence, a massive demand for histone proteins. During these cycles, histone mRNA is not cell cycle regulated and is present continuously, although these histone mRNAs end in the same stem-loop sequence as the cell cycle-regulated mRNAs expressed later in development. In frogs and *Drosophila*, histone mRNA is synthesized during oogenesis and deposited in large amounts in the egg (Woodland and Adamson 1977; Anderson and Lengyel 1980, 1984). Histone mRNA levels remain constant throughout the initial zygotic cycles when there is no transcription and then increase when transcription is activated at the midblastula transition.

The failure to coordinate histone mRNA accumulation with DNA synthesis during the initial rapid embryonic cell cycles is likely related to the differences in cell cycle regulation between early and late development. In many animals, the very first cell cycles lack gap phases and consist simply of a series of alternating S and M phases. The key regulators of the cell cycle, the cyclin-cdk complexes, are also differentially regulated between early and late development. For instance, in *Drosophila*, cyclin E/cdk2 is present constitutively during the first 16 embryonic cell cycles, and periodic activation of cyclin E/cdk2 does not occur until interphase 17, when G1/S control is introduced into the cell cycle (Richardson et al. 1993; Duronio and O'Farrell 1995; Sauer et al. 1995). At this stage, there is robust cell cycle-regulated histone expression, with histone mRNA accumulating only in replicating cells (Hassan and Vaessin 1997; R.J. Duronio, unpubl.).

We have initiated genetic analyses in *Drosophila* to examine the role played by SLBP in histone mRNA regulation during development. Here we report the isolation and characterization of mutations in the single *Drosophila* dSLBP gene. Our results demonstrate that pro-

duction of the normal histone mRNA 3' end in vivo is essential for development and requires dSLBP function. Moreover, dSLBP plays a major role in restricting histone mRNA accumulation to S phase.

Results

Isolation of *Drosophila* SLBP cDNA

We cloned the *Drosophila* stem-loop-binding protein (dSLBP) using the same three-hybrid strategy that was successful for cloning the vertebrate SLBPs (Wang et al. 1996a; Martin et al. 1997). Five positive clones were obtained from screening a *Drosophila* 0–4-h embryonic cDNA library (Dahanukar et al. 1999). Each of these clones encoded a fusion protein containing a region that was 75% identical to the 72-amino acid RNA binding domain (RBD) of the vertebrate proteins (Fig. 1B). Inspection of the *Drosophila* cDNA database resulted in the identification of ESTs that overlapped with the fusion protein clones, although the available sequences did not include the RBD. These cDNAs were obtained and sequenced, allowing identification of the predicted translation product (Fig. 1A). dSLBP is a 276-amino acid protein, slightly larger than the vertebrate SLBPs. The end of the dSLBP RBD is located 17 amino acids from the COOH terminus. Hydrophobic and basic residues in the RBD of vertebrate SLBP that are required for binding the stem-loop (Dominski et al. 2001) are conserved in dSLBP (Fig. 1B). There are few obvious similarities between dSLBP and the vertebrate or worm SLBPs other than the RBD.

dSLBP binds the histone mRNA 3' UTR stem-loop

Gel mobility shift assays were performed to test whether dSLBP is the major protein in *Drosophila* extracts that is

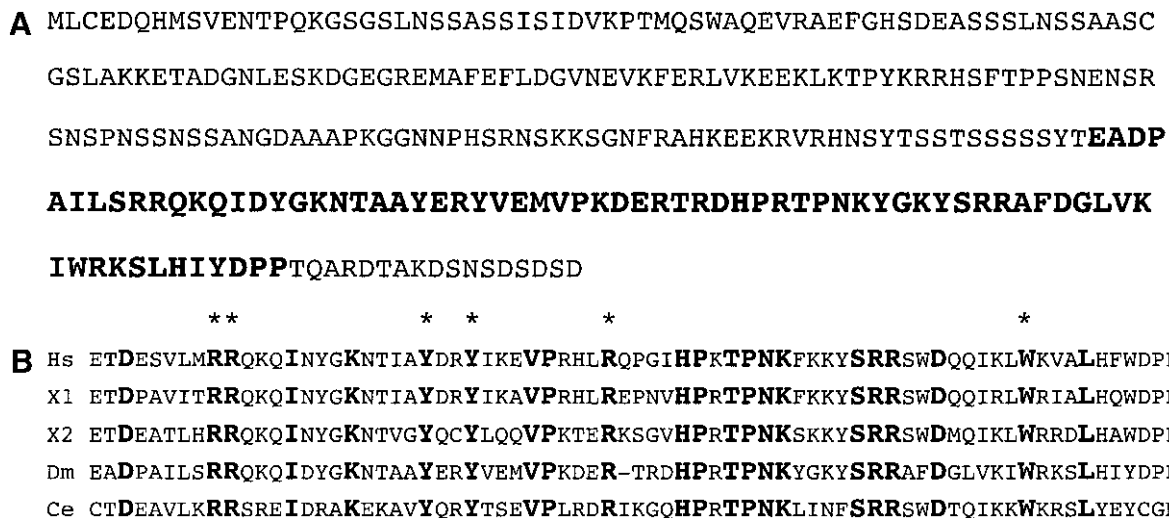


Figure 1. Cloning of dSLBP. (A) Predicted translation product of *Drosophila* SLBP cDNA. The 71-amino acid RNA binding domain is shown in bold. (B) Alignment of human, *Xenopus*, *Drosophila*, and *Caenorhabditis elegans* SLBP RNA-binding domains. Note that two SLBP proteins have been identified in *Xenopus*. The asterisks indicated the conserved hydrophobic and basic residues in vertebrate SLBP known to be required for RNA binding (Dominski et al. 2001).

capable of binding the histone RNA stem loop. When extract prepared from *Drosophila* ovaries (not shown), cultured SL2 cells (Fig. 2A, lanes 3,4), or 0–20-h *Drosophila* embryos (Fig. 2A, lane 5) was incubated with a radiolabeled stem-loop RNA probe, a single gel-shifted complex was detected. This complex specifically bound the stem-loop, as unlabeled wild-type stem-loop RNA could effectively compete for binding (Fig. 2A, lane 6) but a reversed stem-loop RNA sequence could not (Fig. 2A, lane 7). The complex comigrates with that formed between *in vitro* translated dSLBP and the stem-loop RNA (Fig. 2A, lane 1), suggesting that the complex detected in extracts contains dSLBP. To test this further, we performed supershift experiments. Polyclonal antibodies raised against recombinant dSLBP supershift the complex formed in *Drosophila* embryo extracts, whereas nonspecific antibodies have no effect (Fig. 2B, cf. lanes 3,4). Addition of purified dSLBP protein to the reaction effectively competes with antibody binding and blocks formation of the supershifted complex (Fig. 2B, lane 5). These results indicate that virtually all of the gel-shift activity in the embryo extracts is caused by the dSLBP we have cloned.

Injection of *Xenopus* oocytes with synthetic mRNA encoding SLBP results in the synthesis of active SLBP that can be detected by gel-shift analyses using extracts prepared from the injected oocytes (Wang et al. 1999). When dSLBP is expressed in frog oocytes, there is accumulation of a large amount of a new complex that has the same mobility as the complex detected in *Dro-*

sophila embryo extract (Fig. 2C, cf. lanes 3 and 6). There is an equal amount of the dSLBP-RNA complex in nuclear (Fig. 2C, lane 4) and cytoplasmic (Fig. 2C, lane 5) fractions, indicating that dSLBP is imported into the nucleus. dSLBP is also detected in extract from both cytoplasmic and nuclear fractions of SL2 cells (Fig. 2A, lanes 3,4). These data are consistent with dSLBP being involved in histone pre-mRNA processing in the nucleus.

dSLBP mRNA is expressed in all stages of the *Drosophila* life cycle

We assayed a panel of first-strand cDNAs isolated from a variety of *Drosophila* tissues and developmental stages (Rapid Scan, ORIGEN) by PCR to get an estimate of the timing of expression of *dSLBP* mRNA (Fig. 3). The primers used amplify a 181-nt fragment from *dSLBP* mRNA. As a control, the RP49 ribosomal protein mRNA was assayed. The primers used for the RP49 mRNA generate a different size fragment with *Drosophila* genomic DNA because of the presence of a small intron. This confirms that the Rapid Scan panels are free of pre-mRNA or DNA contamination. *dSLBP* mRNA is expressed in all stages of the *Drosophila* life cycle (Fig. 3), suggesting that it may function in all cycling cells. The mRNA is most abundant in early (0–8-h) embryos and adult females. This is consistent with storage of maternal *dSLBP* mRNA in the developing oocyte to provide the dSLBP protein necessary for the accumulation of large amounts

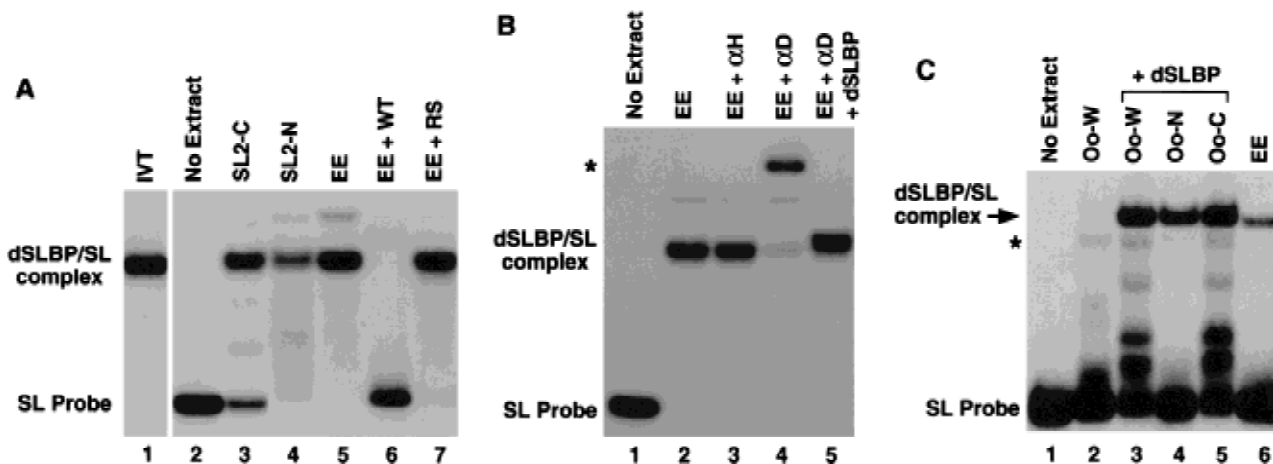


Figure 2. dSLBP binds the histone mRNA stem loop. (A) Detection of dSLBP by gel-shift assay. Incubation of a radiolabeled histone stem-loop probe (SL Probe) with *in vitro*-translated dSLBP (lane 1) retards the mobility of the free probe (lane 2). An identical gel shift is observed with SL2 cell cytoplasmic extract (lane 3), SL2 cell nuclear extract (lane 4), or 0–20-h embryo extract (EE; lane 5). Addition of unlabeled wild-type stem-loop RNA (WT) to the reaction effectively competes gel shift activity (lane 6), whereas addition of a reversed stem-loop sequence (RS) does not (lane 7). (B) Anti-dSLBP antibodies supershift the dSLBP/stem-loop complex. Addition of a nonspecific antibody (α H) to the binding reaction (lane 3) has no effect on the mobility of the embryonic dSLBP/stem-loop complex (lane 2). Addition of affinity purified anti-dSLBP antibody (α D) reduces the mobility of the dSLBP/stem-loop complex (lane 4, asterisk). Formation of the supershifted complex is prevented by inclusion of excess purified dSLBP protein in the reaction (lane 5). (C) Expression of dSLBP in *Xenopus* oocytes. Endogenous *Xenopus* SLBP can be detected by incubation of the stem loop probe (lane 1) with extract from uninjected oocytes (lane 2, asterisk). Whole-cell extracts made from oocytes (Oo-W) injected with a synthetic dSLBP mRNA result in the appearance of a new complex (lane 3, arrow) that has the same mobility as the complex formed with a *Drosophila* embryo extract (lane 6). Nuclear (lane 4) and cytoplasmic (lane 5) fractions of dSLBP-injected oocytes each contain gel-shift activity, indicating that dSLBP can enter the nucleus.

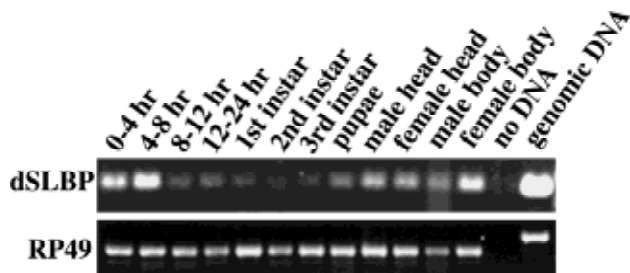


Figure 3. *dSLBP* is expressed throughout *Drosophila* development. The accumulation of *dSLBP* transcripts was monitored by PCR using Origen's Rapid-Scan Gene Expression Panels, which contain first-strand cDNAs derived from different tissues and developmental stages. The top panel shows the 181-nt *dSLBP* specific amplicon, and the bottom panel shows the 433-nt RP49-specific amplicon. Note that the RP49 primers span a small intron, which generates a larger amplicon from genomic DNA than from cDNA.

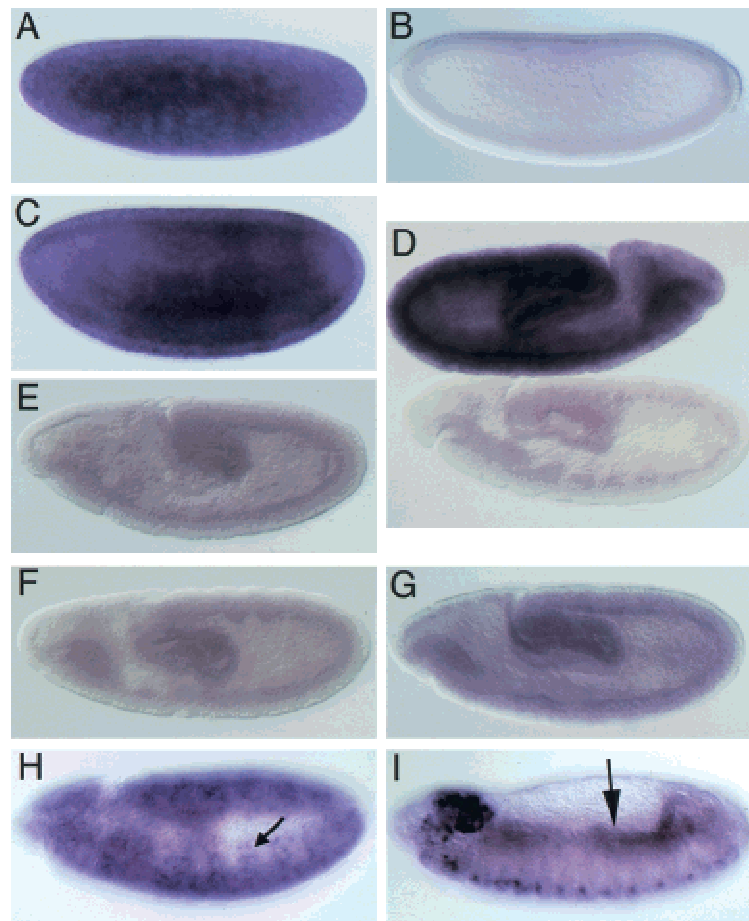
of histone mRNA required during the rapid cycles of early embryogenesis. Whole-mount in situ hybridization was used to examine embryonic *dSLBP* expression in more detail (Fig. 4). *dSLBP* RNA is indeed supplied maternally (Fig. 4A), but this RNA is destroyed by the end of the syncytial cycles (Fig. 4B) and replaced by ubiquitous,

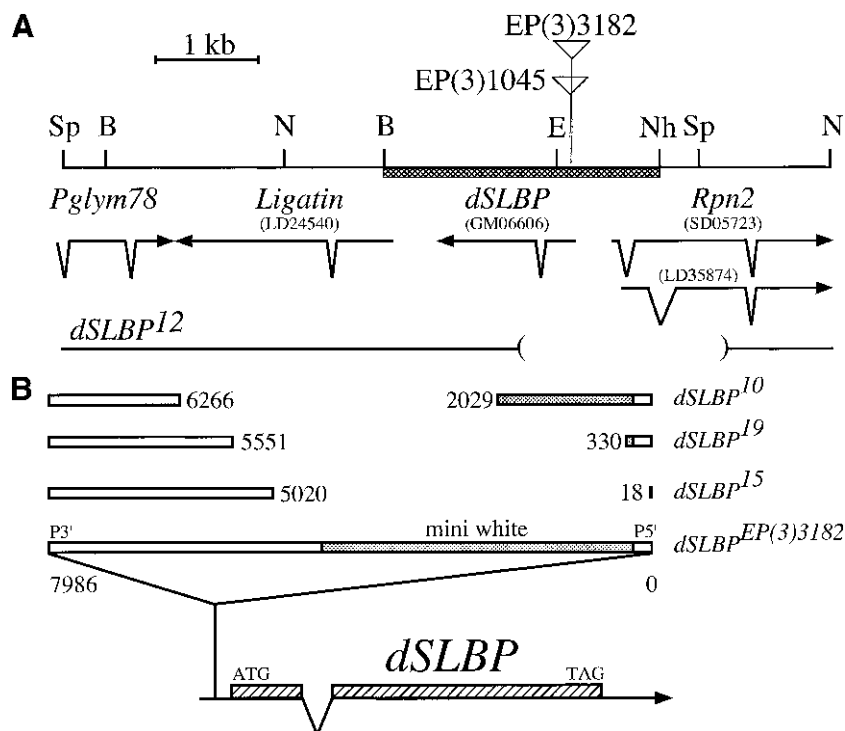
zygotically expressed *dSLBP* RNA at the cellular blastoderm stage (Fig. 4C). During early germ-band extended stages, when rapid cell division occurs throughout the embryo, ubiquitous *dSLBP* expression is maintained (Fig. 4D, top). At later stages, *dSLBP* expression is down-regulated in G1 arrested cells (e.g., the epidermis) and restricted to proliferating (e.g., CNS and PNS) and endoreduplicating (e.g., midgut) cell populations (Fig. 4H,I). Taken together with the gel-shift analyses and the fact that there is not another SLBP-like sequence in the *Drosophila* genome, these expression data suggest that we have cloned the only histone mRNA stem-loop binding protein in *Drosophila*.

Generation of null and hypomorphic *dSLBP* mutations

The Berkeley *Drosophila* Genome Project (BDGP) maintains a collection of *P*-element insertion lines from which flanking chromosomal DNA has been isolated and sequenced to determine the precise location of the insertion. BLAST searching of this database revealed two independent *P*-element insertions into *dSLBP*. EP(3)1045 and EP(3)3182 are inserted in the 5' UTR 54 and 68 nt upstream of the *dSLBP* initiator methionine, respectively (Fig. 5A,B). Both *P*-element lines are viable when homozygous or in trans to a large deficiency

Figure 4. *dSLBP* mRNA expression in wild-type and *dSLBP* mutant embryos. RNA was detected by in situ hybridization of whole embryos using a *dSLBP* probe. (A–C, D [top], H, I) *yw*⁶⁷ wild-type embryos; (D [bottom], E–G) *dSLBP* mutant embryos. All embryos are oriented with anterior to the left and dorsal to the top, except the wild-type embryo in panel D, which has anterior to the right. *dSLBP* RNA is supplied maternally to the embryo (A). By the cellular blastoderm stage, maternal *dSLBP* RNA is destroyed (B) and subsequently replaced by ubiquitous zygotic *dSLBP* expression (C), which is maintained during germ band extension (D, top). During stage 11 (H), *dSLBP* RNA becomes restricted to cells that are replicating, including those of the central and peripheral nervous system (arrow in H). Replication-associated *dSLBP* expression continues in dividing cells of the CNS (ventral part of the embryo in I) and in endoreduplicating midgut cells (arrow in I) of stage 13, germ-band-retracted embryos. G1-arrested epidermal cells do not express *dSLBP*. This program of gene expression is very similar to other genes encoding factors required for S phase (Duronio and O'Farrell 1994). (D–G) Little to no *dSLBP* RNA accumulates in embryos that are genotypically *dSLBP*¹²/*dSLBP*¹² (E), *dSLBP*¹⁵/*dSLBP*¹⁵ (D, bottom), or *dSLBP*¹⁹/*dSLBP*¹⁹ (F). In contrast, *dSLBP* RNA is detected in *dSLBP*¹⁰/*dSLBP*¹⁰ embryos (G) but at a level much reduced relative to their phenotypically wild-type siblings. Note that phenotypically wild-type and mutant sibling embryos of identical stage are shown within the same photomicrograph in panel D.





tom line indicates the location of the deletion breakpoints in the *dSLBP*¹² excision allele. B = *Bam*HI, E = *Eco*RI, Sp = *Sph*I, N = *Nco*I, Nh = *Nhe*I. (B) Diagram of *dSLBP* excision alleles. The *dSLBP* transcript and coding region (hatched box) is shown at the bottom. The 7986-bp EP(3)3182 *P* element is located in the 5' UTR 68 bp upstream of the AUG. The breakpoints of each excision allele are indicated relative to the full-length EP *P* element. Note that the miniwhite gene (shaded) has been disrupted in each w⁻ excision.

(*Df(3R)3450*) that deletes the entire *dSLBP* region. Although viable, these flies are female sterile (but male fertile). Because of the critical role SLBP plays in histone mRNA accumulation in vertebrates, we were somewhat surprised to find that both *P*-element insertions in the 5' UTR of *dSLBP* were homozygous viable. A possible explanation for this result is that SLBP function is not essential in *Drosophila*. Alternatively, these alleles may be hypomorphic and not completely devoid of *dSLBP* function, which is a common occurrence for *P*-element insertions into a noncoding region. We therefore mobilized the EP(3)3182 and EP(3)1045 *P*-element lines to isolate additional alleles of *dSLBP* via imprecise excision (see Materials and Methods for details). Of the new *dSLBP* excision alleles that we recovered, four (*dSLBP*¹⁰, *dSLBP*¹², *dSLBP*¹⁵, *dSLBP*¹⁹) were subjected to detailed analysis.

The precise breakpoints of these four excision alleles were determined by PCR analysis and direct sequencing of PCR products. The structure of each mutant is diagrammed in Figure 5. Three of the mutants (*dSLBP*¹⁰, *dSLBP*¹⁹, and *dSLBP*¹⁵) have internal deletions within the *P* element, and the fourth (*dSLBP*¹²) contains a small genomic deletion. The *dSLBP*¹² deletion removes the entire *P* element, the promoter of *dSLBP* and the adjacent *Rpn2* gene, and a portion of the *dSLBP* and *Rpn2* coding region (Fig. 5A). Consequently, *dSLBP*¹² is a molecular null for both genes. As three of the four excision alleles do not delete any *dSLBP* coding sequence, we performed

Figure 5. The *dSLBP* locus located at 98F/99A on the right arm of chromosome 3. (A) Restriction map of a 6.5-kb fragment containing *dSLBP*. The direction and intron/exon structure of *dSLBP* and surrounding transcripts are indicated below the restriction map. Within this 6.5 kb of sequence, three genes in addition to *dSLBP* were identified: *RPN2*, encoding the 110-kD proteasome subunit (Adams et al. 2000); *Ligatin*, encoding a neuronal phosphoglycoprotein receptor (Jakoi et al. 1989); and *Pglym78*, encoding the glycolytic enzyme phosphoglycerate mutase (Currie and Sullivan 1994). cDNA clones LD24540 (*Ligatin*), GM06606 (*dSLBP*), and LD35874 (*Rpn2*) were sequenced in their entirety. The indicated splice variants of *Rpn2* were identified by comparing the sequence of clone LD35874 to the 5' EST sequence from clone SD05723 obtained by the BDGP. These transcripts utilize the same AUG codon but generate proteins that differ in primary sequence near the amino terminus. The location of the EP(3)3182 and EP(3)1045 *P* elements is indicated by triangles. The *Bam*HI-*Nhe*I restriction fragment used in the transgene that complements *dSLBP* mutant alleles is indicated by a hatched box. The bot-

tom line indicates the location of the deletion breakpoints in the *dSLBP*¹² excision allele. B = *Bam*HI, E = *Eco*RI, Sp = *Sph*I, N = *Nco*I, Nh = *Nhe*I. (B) Diagram of *dSLBP* excision alleles. The *dSLBP* transcript and coding region (hatched box) is shown at the bottom. The 7986-bp EP(3)3182 *P* element is located in the 5' UTR 68 bp upstream of the AUG. The breakpoints of each excision allele are indicated relative to the full-length EP *P* element. Note that the miniwhite gene (shaded) has been disrupted in each w⁻ excision.

in situ hybridization on embryos collected from heterozygous parents to determine if the mutations reduce expression of *dSLBP*. Little if any *dSLBP* RNA was detected in homozygous *dSLBP*¹⁵, *dSLBP*¹², and *dSLBP*¹⁹ germ-band extended embryos (Fig. 4D bottom, 4E, and 4F, respectively). This is consistent with the observation that maternal *dSLBP* RNA is quantitatively destroyed before the start of zygotic gene expression (Fig. 4B). Moreover, *dSLBP*¹⁵ and *dSLBP*¹⁹ homozygous embryos are indistinguishable from *dSLBP*¹² embryos, which contain a molecular null for *dSLBP*. In contrast, a low but detectable level of *dSLBP* RNA was observed in homozygous *dSLBP*¹⁰ mutant embryos (Fig. 4G), indicating that this allele is hypomorphic.

These observations were confirmed by immunoblot analysis of homozygous mutant embryos. In SL2 nuclear and cytoplasmic extract, a protein that comigrates with in vitro-translated dSLBP is detected with anti-dSLBP antibodies (Fig. 6A). This protein can be specifically precipitated by a biotinylated stem-loop RNA bound to streptavidin agarose but not by control reverse stem-loop RNA (Fig. 6B). Thus, we can measure dSLBP abundance by Western blotting. In addition, we can obtain pure collections of homozygous mutant embryos by hand sorting using GFP expressing balancer chromosomes (see Materials and Methods). dSLBP is readily detected in late stage (i.e., 13–16-h AED) wild-type embryos (Fig. 6C, lane 1). In contrast, equivalently staged hemizygous *dSLBP*¹⁵/*Df(3R)3450* mutant embryos con-

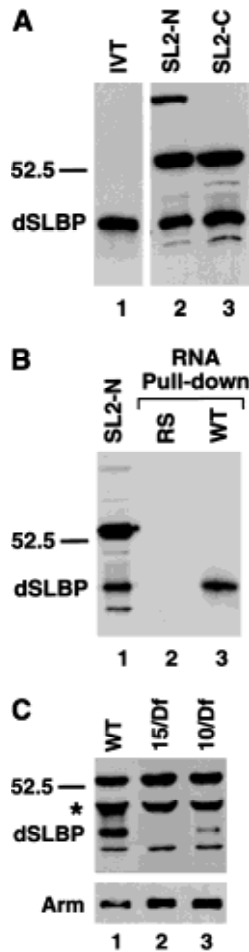


Figure 6. dSLBP protein expression is reduced in *dSLBP* mutant embryos. Each panel shows a Western blot using affinity purified dSLBP antibodies. (A) The antibody recognizes a protein in SL2 cell nuclear (lane 2) and cytoplasmic (lane 3) extracts that comigrates with the protein translated in vitro from dSLBP cDNA (lane 1). Note that the antibody cross reacts with several unknown polypeptides, including one at 53 kD that appears in both embryo and SL2 cell extracts and one at 50 kD (asterisk in C) that appears only in embryo extracts. One cross-reacting protein is found in nuclear but not cytoplasmic SL2 cell extract (A), indicating successful fractionation. (B) RNA/protein complexes were precipitated from SL2 nuclear extract incubated with wild-type (WT) biotinylated stem-loop RNA or biotinylated RNA containing a reverse stem-loop sequence (RS). The protein in the extract that comigrates with in vitro translated dSLBP (lane 1) but not the cross-reacting proteins, is specifically precipitated by the wild-type (WT) stem-loop RNA (lane 3) and not by the control RS RNA (lane 2). (C) Extracts were made from hand-selected 13–16-h *yw⁶⁷* wild-type embryos (lane 1) or *dSLBP* homozygous mutant embryos (*dSLBP¹⁵/Df(3R)3450*, lane 2; *dSLBP¹⁰/Df(3R)3450*, lane 4). Mutant embryos were distinguished from their phenotypically wild-type siblings using balancer chromosomes that express GFP. Little if any dSLBP is produced by the *dSLBP¹⁵* allele, whereas drastically reduced but detectable levels of dSLBP are produced by the hypomorphic *dSLBP¹⁰* allele. Anti-armadillo antibodies were used as a loading control (Arm).

tain very little if any detectable dSLBP (Fig. 6C, lane 2). Similar to the in situ hybridization results, a low amount of dSLBP is observed in *dSLBP¹⁰/dSLBP¹⁰* and *dSLBP¹⁰/Df(3R)3450* mutant embryos (Fig. 6C, lane 3). These molecular data strongly indicate that the *P*-element excision mutations are simple loss of function alleles of *dSLBP*.

Genetic analysis of the four excision alleles corroborates the molecular studies. We performed reciprocal crosses among each of the four excision alleles and the *Df(3R)3450* deficiency (Table 1). *dSLBP¹⁰* behaves much like the EP(3)3182 chromosome from which it was derived: *dSLBP¹⁰/Df* flies are viable but female sterile and male fertile (Table 1). Unlike *dSLBP¹⁰*, *dSLBP¹⁹* and *dSLBP¹⁵* have a dramatic effect on viability. Adult *dSLBP¹⁵/Df* flies are very rarely recovered (1/2621; Table 1), while 7%–18% of the expected number of *dSLBP¹⁹/Df* flies are recovered. For both alleles, the lethal phase is very late in development, with many flies dying at eclosion. The few surviving *dSLBP¹⁹/Df* individuals have no overt morphological defects but are female sterile and male fertile. The *dSLBP¹²* allele is fully lethal in trans to *Df(3R)3450* and behaved like the null *Df(3R)3450* deletion in each cross, as expected (Table 1). Together, the molecular and genetic data suggest that the four excision mutations represent an allelic series for *dSLBP* function, with *dSLBP¹⁰* > *dSLBP¹⁹* > *dSLBP¹⁵* ≥ *dSLBP¹²* = *Df(3R)3450* = null. The precise reason why internal deletion of the EP *P* element affects dSLBP mRNA accumulation is not known. One possibility is that these alleles remove cryptic splice and/or transcription start sites from within the *P* element, thereby reducing production of dSLBP mRNA.

We tested whether a *P*-element transgene containing only the full-length *dSLBP* transcription unit would rescue the mutant phenotypes. A *Bam*HI–*Nhe*I genomic restriction fragment containing *dSLBP* (Fig. 5A) complemented the lethality of *dSLBP¹⁵/Df(3R)3450* flies (Table 1) and restored fertility to *dSLBP¹⁰/Df(3R)3450* adult females. Therefore, these alleles do not disrupt the function of the neighboring genes but only mutate *dSLBP*. In contrast, the same transgene could not rescue the lethality of *dSLBP¹²/Df(3R)3450* flies, which lack both *dSLBP* and *Rpn2* (Table 1). This demonstrates that *Rpn2* is also an essential gene.

Analysis of histone mRNA expression in dSLBP mutant embryos

The homozygous lethality caused by the *dSLBP¹⁹* and *dSLBP¹⁵* mutations suggested that histone mRNA accumulation may be disrupted in the mutants. We chose to initially examine histone mRNA at embryonic stages when replication-associated histone expression is known to occur (i.e., post-germ-band retraction, cycle 17 and later). In situ hybridization of wild-type embryos with a histone H4 antisense RNA probe results in a stereotypic but dynamic pattern of expression that is essentially identical to that obtained by pulse labeling with BrdU (Fig. 7A–D). This includes dividing cells of the

Table 1. Complementation testing of dSLBP excision alleles

Female ¹	Male ¹	Number of heterozygous flies	Number of homozygous mutant flies ²	Percentage of expected if fully viable ³
<i>Df(3R)3450</i>	<i>dSLBP</i> ¹⁰	462	115	60
<i>dSLBP</i> ¹⁰	<i>Df(3R)3450</i>	709	282	86
<i>Df(3R)3450</i>	<i>dSLBP</i> ¹²	279	0	0
<i>dSLBP</i> ¹²	<i>Df(3R)3450</i>	1715	0	0
<i>Df(3R)3450</i>	<i>dSLBP</i> ¹⁵	455	0	0
<i>dSLBP</i> ¹⁵	<i>Df(3R)3450</i>	596	1	0.5
<i>Df(3R)3450</i>	<i>dSLBP</i> ¹⁹	404	10	7
<i>dSLBP</i> ¹⁹	<i>Df(3R)3450</i>	675	42	18
<i>dSLBP</i> ¹⁰	<i>dSLBP</i> ¹²	853	145	44
<i>dSLBP</i> ¹²	<i>dSLBP</i> ¹⁰	653	157	58
<i>dSLBP</i> ¹⁰	<i>dSLBP</i> ¹⁵	870	216	60
<i>dSLBP</i> ¹⁵	<i>dSLBP</i> ¹⁰	640	128	50
<i>dSLBP</i> ¹⁰	<i>dSLBP</i> ¹⁹	714	104	38
<i>dSLBP</i> ¹⁹	<i>dSLBP</i> ¹⁰	777	94	32
<i>dSLBP</i> ¹²	<i>dSLBP</i> ¹⁵	819	0	0
<i>dSLBP</i> ¹⁵	<i>dSLBP</i> ¹²	751	0	0
<i>dSLBP</i> ¹²	<i>dSLBP</i> ¹⁹	679	28	12
<i>dSLBP</i> ¹⁹	<i>dSLBP</i> ¹²	847	28	10
<i>dSLBP</i> ¹⁵	<i>dSLBP</i> ¹⁹	802	1	0.3
<i>dSLBP</i> ¹⁹	<i>dSLBP</i> ¹⁵	1009	30	9
Rescue crosses:				
<i>Df(3R)3450</i>	<i>P{w⁺, dSLBP}/+;</i> <i>dSLBP</i> ¹⁵ /TM3 <i>Sb</i>	358	126 ⁴	78
<i>Df(3R)3450</i>	<i>P{w⁺, dSLBP}/+;</i> <i>dSLBP</i> ¹² /TM3 <i>Sb</i>	156	0	0
<i>dSLBP</i> ¹² /TM3 <i>Sb</i>	<i>P{w⁺, dSLBP}/+;</i> <i>Df(3R)3450</i> /TM3 <i>Sb</i>	278	0	0

¹Balancer chromosome is TM3 Ser P[w⁺; act-GFP] for all flies, unless otherwise indicated.

²All viable flies are female sterile.

³Expected is 33% of the total eclosed flies, since balancer homozygotes die.

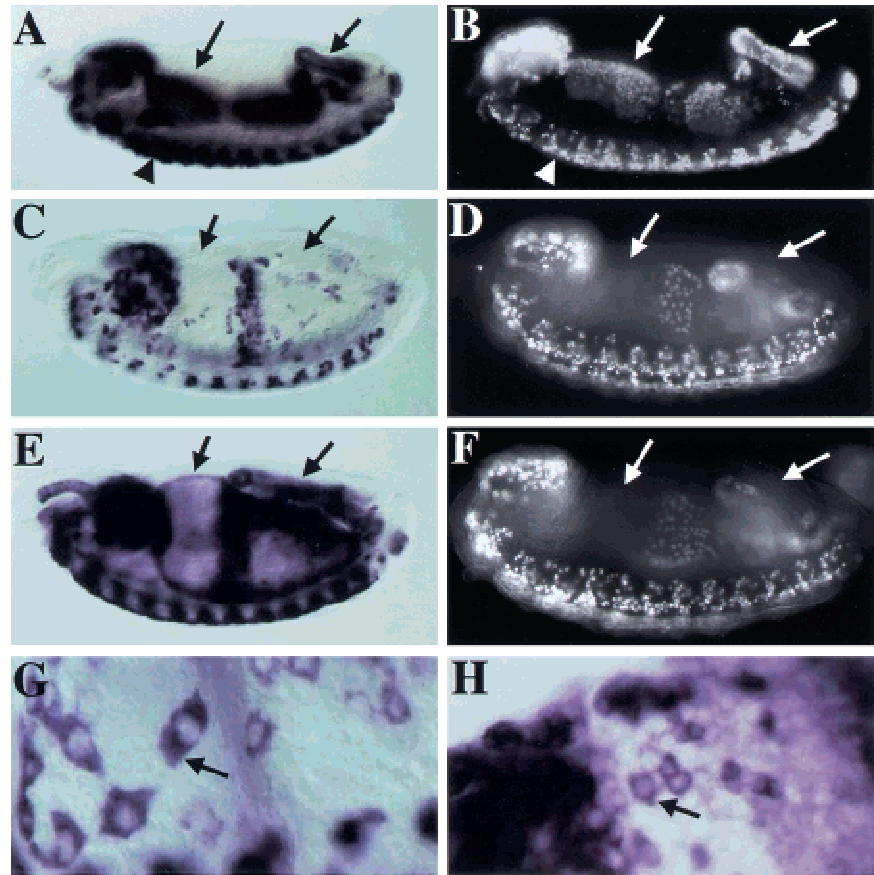
⁴Female fertile.

CNS (Fig. 7A,B, arrowheads) and endoreduplicating cells of the alimentary canal (e.g., midgut and hindgut; Fig. 7A–D, left and right arrows, respectively). We then examined H4 expression in mutant embryos. *dSLBP*¹⁹, *dSLBP*¹⁵, and *dSLBP*¹² mutations each caused a similar, recessive phenotype, whether as homozygotes or in trans to *Df(3R)3450*. Contrary to our expectations for a gene product suspected of being required for production of a mature mRNA, histone H4 RNA still accumulates to high levels in cells of *dSLBP* mutant embryos (Fig. 7E). In addition, the pattern of staining in the mutant embryos differed from wild-type embryos at the same stage. In particular, histone transcripts were present in cells that were not replicating, as judged by pulse labeling with BrdU (Fig. 7E,F, arrows). As in wild type, the histone mRNAs in mutant embryos were present in the cytoplasm (Fig. 7G,H, arrow), consistent with their being translated at this stage. A similar aberrant accumulation of RNA was observed using a probe derived from histone H3 (not shown). The altered pattern of histone RNA accumulation suggests that endoreduplicating cells that normally down-regulate histone expression after each endocycle S phase no longer do so in the mutant (e.g., the anterior midgut and hindgut; cf. arrows in Fig. 7C,E, and 7D,F). Therefore, mutation of *dSLBP* uncoupled histone

RNA accumulation from ongoing replication in cycling cells.

To determine whether the accumulated histone mRNAs present are processed normally, we prepared total RNA from embryos collected from heterozygous parents and analyzed them by Northern blotting. Wild-type embryos contained only the 500-nt H3 transcript that has been described previously (Fig. 8A, lane 6). In embryos derived from stocks of mutants *dSLBP*¹⁰, *dSLBP*¹², *dSLBP*¹⁵, *dSLBP*¹⁹, and *dSLBP*⁴⁷, we detected larger histone H3 transcripts in addition to the wild-type mRNA (asterisks, Fig. 8A, lanes 1–5, respectively; see Materials and Methods for a description of *dSLBP*⁴⁷). The largest of these was ~1 kb longer than the wild-type transcript. A similar result was obtained using RNA extracted from *dSLBP*¹⁵ embryos and a histone H4 probe, although the mutant transcript detected was not as long (Fig. 8C, lane 3, asterisk). To further characterize these transcripts, RNA extracted from embryo collections of *dSLBP*¹⁵ heterozygous parents were fractionated on oligo(dT) magnetic beads, and the polyA⁺ RNA was analyzed by Northern blotting. Whereas very little wild-type H3 RNA was found in the polyA⁺ fraction (Fig. 8B, lanes 1,2), the longer, aberrant H3 RNA species were readily detected in the polyA⁺ fraction (Fig. 8B, lane 1, asterisks).

Figure 7. Altered histone RNA expression in *dSLBP* mutant embryos. (A,B) Stage-13 wild-type embryos stained for histone H4 expression by in situ hybridization (A) or DNA synthesis by pulse labeling for 15 min with BrdU (B). Note that the histone expression and replication patterns are identical, indicating that histone mRNA accumulates only in cells that are synthesizing DNA. This includes proliferating cells in the CNS (arrowhead) and endoreduplicating cells in the anterior midgut (left arrow) and the hindgut (right arrow). (C) H4 expression in a stage 14 *dSLBP¹⁹/+* embryo. (D) BrdU-labeled stage 14 *dSLBP¹⁵/+* embryo. These heterozygous embryos are phenotypically wild type. Again, histone expression and replication are coincident, but the pattern has changed from stage 13. Anterior midgut and hindgut cells have exited the endocycle S phase and do not express histone H4 mRNA. (E) H4 expression in a *dSLBP¹⁹/dSLBP¹⁹* mutant sibling of the embryo in C. (F) DNA replication in a *dSLBP¹⁵/dSLBP¹⁵* mutant sibling of the embryo in D. In the mutant embryos, histone H4 mRNA persists (arrows in E) in cells that have already finished replicating (arrows in F). The histone H4 expression pattern is identical in *dSLBP¹⁹* and *dSLBP¹⁵* homozygous embryos (not shown). Histone H4 mRNA accumulates in the cytoplasm of cells in both *dSLBP¹⁹/+* wild-type (arrow in G) and *dSLBP¹⁹/dSLBP¹⁹* (arrow in H) mutant embryos.



Similar results were obtained using H4 probes (not shown). The binding of these transcripts to oligo dT suggests that they are polyadenylated. Therefore, mutation of *dSLBP* resulted in the synthesis of abnormal histone mRNA, probably because of a failure to properly generate the usual 3' end. As polyadenylated histone mRNAs are stable at the end of S phase or when DNA replication is inhibited (Levine et al. 1987; Harris et al. 1991), it is likely that the continued expression of histone mRNA in cells that have completed S phase is because of a failure to degrade much of the histone mRNA expressed in the mutant embryos. Consistent with this hypothesis, we have shown previously that accumulation of mammalian histone mRNAs containing the stem-loop internally in the 3' untranslated region, rather than at the 3' end, is not coupled to DNA replication (Levine et al. 1987; Pandey and Marzluff 1987).

dSLBP is required very early in embryonic development

The first ~2.5 h of *Drosophila* embryogenesis consist of 13 rapid, nuclear division cycles that occur in a syncytial cytoplasm (Fig. 9A–C), lack G1 and G2 phases and are driven by large stores of maternally provided cell cycle components, including histone RNA and protein. Rather

than being synthesized only during S phase, maternally provided histone mRNA and protein are present continuously during these cycles (Anderson and Lengyel 1984). *dSLBP* mRNA is also present at this stage (Fig. 4A), suggesting that *dSLBP* may function during the cleavage divisions. The *dSLBP¹⁰* allele is viable in trans to a deficiency but causes female sterility when crossed with wild-type males (Table 1). These females lay a normal amount of eggs that are wild type in appearance. Essentially, none of these eggs complete embryogenesis (99% failed to hatch; $n = 777$), suggesting that the defect occurs during the earliest syncytial cycles before the activation of the paternal genome, which would provide wild-type *dSLBP*. To test this, embryos collected 0–4 h AED from *dSLBP¹⁰/Df(3R)3450* mothers were pulse labeled with BrdU to monitor DNA synthesis and visualize syncytial stage nuclei. The mutants had obvious disruptions of the nuclear cleavage cycles and accumulated nuclei that had aberrant morphologies (Fig. 9D–I). The earliest cycles appeared somewhat normal (Fig. 9D,E), but we could never identify a blastoderm stage embryo with the typical uniform arrangement of dividing nuclei (cf. Fig. 9C with 9F–H).

These nuclear cycle defects appear to result, at least in part, from improper chromosome condensation during

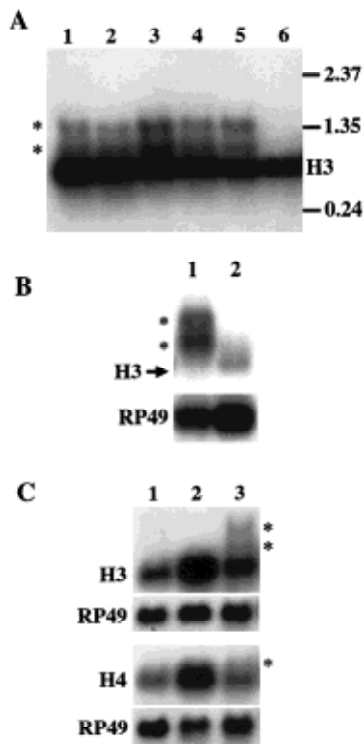


Figure 8. Altered RNA processing in *dSLBP* mutants. (A) Total RNA extracted from 7–22-h embryos collected from *dSLBP*¹⁰ (lane 1), *dSLBP*¹² (lane 2), *dSLBP*¹⁵ (lane 3), *dSLBP*¹⁹ (lane 4), *dSLBP*⁴⁷ (lane 5) heterozygous mutant stocks, or from *yw*⁶⁷ wild-type flies (lane 6) and was blotted and probed with radiolabeled histone H3 coding region. Longer H3 transcripts are detected only in the mutant samples (asterisks). Note that three-quarters of the embryos in each preparation are phenotypically wild type, accounting for the RNA of wild-type length in the mutant lanes. (B) *dSLBP*¹⁵ mutant (lane 1) and *yw*⁶⁷ wild-type (lane 2) embryonic RNA samples prepared as in (A) were fractionated with oligo dT magnetic beads, and the polyA⁺ RNA was subjected to Northern analysis with the H3 coding region probe, or an RP49 probe as loading control. The arrow indicates migration of the wild-type H3 mRNA. The aberrant, long H3 transcripts (asterisks) bind to oligo dT, whereas wild-type H3 mRNA does not. (C) The maternal effect lethal allele of *dSLBP* causes reduced deposition of maternal histone mRNA. Total RNA extracted from 0–2-h embryos collected from *dSLBP*¹⁰/*Df*(3R)3450 (lane 1) or *yw*⁶⁷ wild-type (lane 2) females was blotted and probed with a histone H4 or H3 coding region probe or RP49 probe as loading control. Lane 3 contains total RNA prepared from 7–22-h embryos collected from *dSLBP*¹⁵/+ flies as a comparison. At these later embryonic stages, there is less total histone RNA expressed per embryo when compared to 0–2-h embryos, accounting for the reduced intensity of the wild-type band. Note that the longer histone transcripts (asterisks) are not nearly as abundant in the mutant maternal RNA samples (lane 1).

mitosis. This was revealed by simultaneous detection of microtubules with anti β -tubulin antibodies and DNA with propidium iodide (Fig. 10). While mitotic nuclei in wild-type embryos contain fully condensed chromosomes that align at the metaphase plate (Fig. 10A,B), embryos with a similar nuclear density collected from *dSLBP*¹⁰/*Df*(3R)3450 mutant females never formed nor-

mal metaphase spindles (Fig. 10C–F). Microtubules assemble around improperly condensed DNA (Fig. 10E,F), causing the formation of spindles with dramatically altered morphologies (Fig. 10C,D). Spindle-pole fusion is also frequently observed (Fig. 10C, arrow). Improper chromosome condensation prevents anaphase, and core histones are needed to recruit condensation factors (Saka et al. 1994; Strunnikov et al. 1995; Sutani et al. 1999; Wei et al. 1999; de la Barre et al. 2000; Freeman et al. 2000). A failure of anaphase could contribute to the large chromosome masses sometimes observed (Fig. 9G and 10 E,F). Indeed, dramatic anaphase bridges were detected in some embryos (Fig. 9H,I). As such nuclei were labeled with BrdU, the condensation failure did not block DNA synthesis.

One possible explanation for the failure of proper chromosome condensation is insufficient histone biosynthesis. We therefore examined histone H3 and H4 RNA levels in the maternally mutant embryos by Northern blot analysis. Mutant embryos contained about 10-fold less histone H3 and H4 RNA than wild-type embryos (Fig. 8C, cf. lanes 1 and 2). Unlike embryos mutant for zygotic *dSLBP* function (Fig. 8C, lane 3), very little of the longer histone mRNAs were detected in eggs laid by *dSLBP*¹⁰/*Df*(3R)3450 females. This suggests that the mechanism for forming these longer transcripts was not as efficient during oogenesis and that any unprocessed histone mRNA is degraded during oogenesis. Perhaps in the embryo all the controls of histone mRNA production are not yet in place, allowing polyadenylation of the unprocessed histone pre-mRNA. Production of lower amounts of histone mRNA suggests that insufficient synthesis of histone protein prevents proper chromatin structure, leading to the observed defects in chromosome condensation.

Discussion

The replication-associated histone mRNAs are unique in that they are the only metazoan mRNAs lacking polyadenylated tails. Their mature 3' end contains a sequence that forms a stem-loop to which SLBP binds. Here we have shown that in *Drosophila* SLBP is required for proper cell cycle regulation of histone mRNA in vivo. Mutation of *dSLBP* causes the synthesis of histone mRNAs with aberrant 3' ends that accumulate in non-replicating cells. This is not well tolerated, as both maternal and zygotic *dSLBP* function is essential for development.

The role of dSLBP in histone mRNA production

Before cell cycle 16, histone H4 expression in *dSLBP* mutant embryos appears like wild type (not shown), suggesting that it is directed by maternal supplies of *dSLBP* protein. We first observed a defect in histone mRNA expression during cell cycle 17 of *dSLBP* mutant embryos, which display an altered pattern of histone mRNA accumulation that is most consistent with expression of

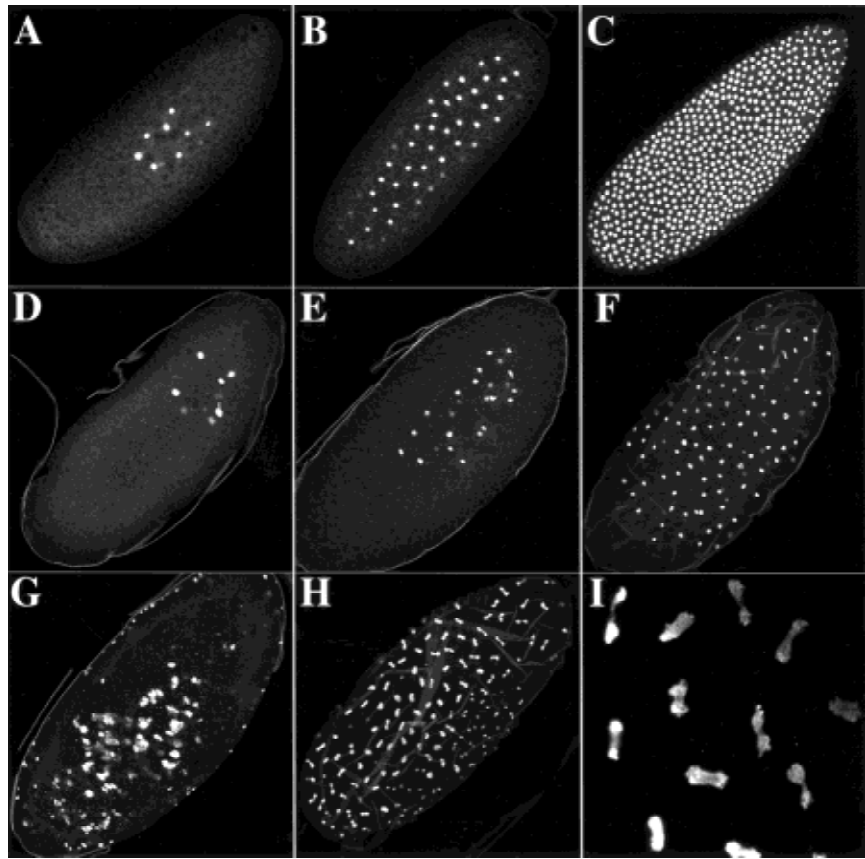


Figure 9. The *dSLBP¹⁰* maternal effect lethal allele causes disruption of the early nuclear cleavage cycles. Confocal images of syncytial stage embryos collected from *yw⁶⁷* wild-type (A–C) and *dSLBP¹⁰/Df(3R)3450* mutant (D–I) females. These embryos were pulse labeled for 10 min with BrdU to detect replicating nuclei. A, B, and C show wild-type embryos of increasing age, with a nuclear cycle 13 syncytial blastoderm shown in panel C. Panels D–I show representative mutant embryos. Panel I is a high-magnification view of panel H. Most embryos (e.g., G) were impossible to stage for a particular nuclear cycle because of the accumulation of defects with increasing nuclear density. The vitelline membrane was inefficiently removed from the mutant embryos by our standard procedure and is, therefore, visible in these images.

a stabilized histone message: The mRNA accumulates in cells that are replicating during an endocycle S phase but fails to disappear when the cells exit S phase. At the end of S phase, histone mRNA is normally destabilized, and this requires the 3' end of the mature message (Harris et al. 1991), which is thought to provide a substrate for exonucleases (Ross et al. 1986, 1987). Consistent with this model, the 3' end of histone mRNA is altered in the *dSLBP* mutant. The transcripts are longer than normal and bind to oligo(dT), suggesting that they are polyadenylated. In the absence of sufficient dSLBP, it is likely that transcription continues past the normal 3' end of the histone mRNA, resulting in subsequent processing at cryptic polyA sites (Akhmanova et al. 1997). In mammalian cells, artificially polyadenylated histone mRNA has an increased half-life and is not subject to normal posttranscriptional regulation (Pandey and Marzluff 1987). We therefore conclude that dSLBP is required for efficient processing of the histone pre-mRNA in vivo. This is consistent with the known role of mammalian SLBP in processing histone pre-mRNA substrates in a reconstituted in vitro processing assay (Dominski et al. 1999) and following injection of histone genes into frog oocytes (Wang et al. 1999).

We do not know the efficiency of forming the polyadenylated 3' ends in the *dSLBP* mutants. In mammalian cells, histone mRNAs are transcribed in G1 phase, but the pre-mRNAs are not processed and are rapidly degraded (DeLisle et al. 1983; Stauber and Schumperli

1988). A similar situation is seen when one mutates the 3' end of histone mRNA; the genes are still transcribed, but no histone mRNA accumulates (Pandey et al. 1994). This suggests that mammalian cells have an efficient mechanism for destroying unprocessed histone pre-mRNA. Perhaps *Drosophila* embryos do not have such a mechanism.

The role of SLBP in coupling histone mRNA production with the cell cycle

Changes in the rate of pre-mRNA processing play a major role in coupling the synthesis of mature histone mRNA with the cell cycle. There is evidence that histone pre-mRNA processing is activated at the G1–S transition in mammalian cells (Luscher and Schumperli 1987; Stauber and Schumperli 1988; Harris et al. 1991). If one introduces a gene that contains both a histone 3' end followed by a strong polyadenylation site, then polyadenylated histone mRNA accumulates in G1 cells, while mRNAs ending in the stem-loop end accumulate in S-phase cells (Harris et al. 1991). Recent data suggest that this increase in processing is probably a result of increased synthesis of SLBP at the G1/S transition. SLBP is cell cycle regulated in mammalian cells, accumulating just before the accumulation of histone mRNA and disappearing at the end of S phase (Whitfield et al. 2000). Thus, SLBP could be the limiting component for histone pre-mRNA processing in vivo both in G1 and G2, with

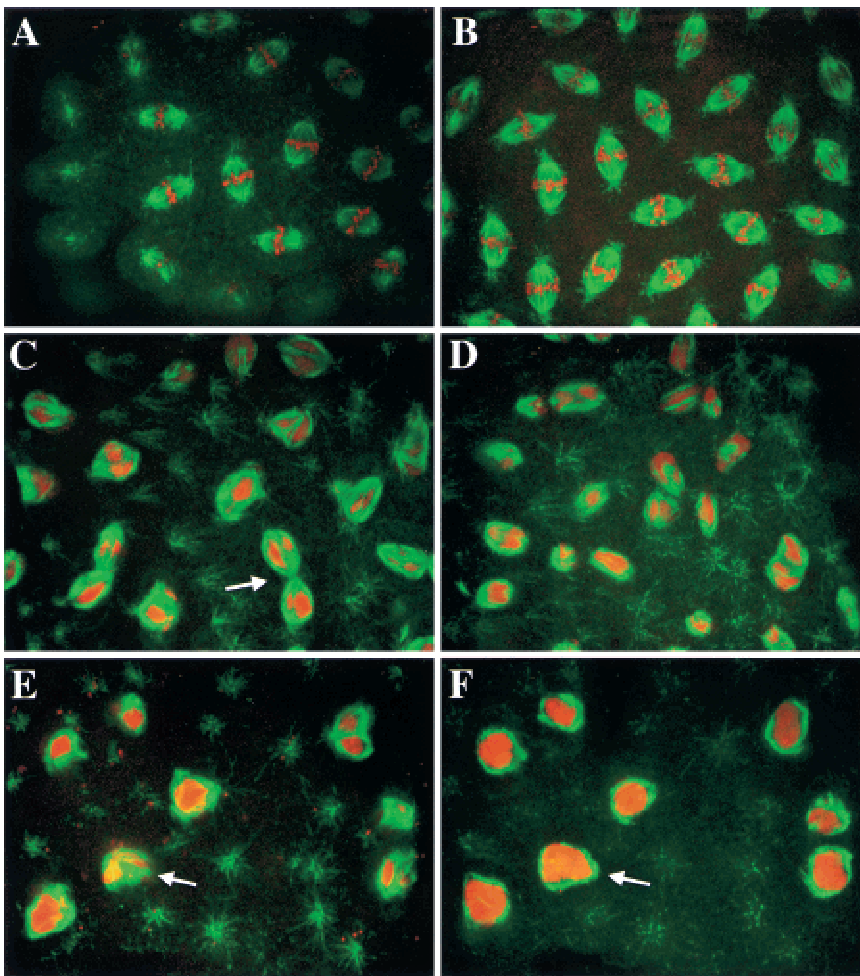


Figure 10. Aberrant spindle morphology caused by *dSLBP*¹⁰ maternal effect allele. Syncytial stage embryos were collected from *yw*⁶⁷ wild-type (A,B) or *dSLBP*¹⁰/*Df(3R)3450* mutant females (C–F), fixed, and stained with anti- β -tubulin antibodies to detect mitotic spindles (green) and propidium iodide to detect chromosomes (red). Each panel shows a merged confocal image. Note the regular array of spindles with properly condensed chromosomes that have congressed to the metaphase plate in wild type (A,B). The normal regular array of mitotic spindles is lost in mutant embryos (C–F). Nuclear DNA fails to condense into discrete chromosomes (best seen in F), but microtubules nevertheless form around the improperly condensed DNA generating misshapen spindles. These spindles sometimes appear fused to one another (e.g., arrow in C). Panels E and F show different focal planes of the same visual field to illustrate how the spindle microtubules encapsulate the DNA (arrow).

production of SLBP at the G1/S transition stimulating processing and destruction of SLBP at the end of S phase inactivating processing (Whitfield et al. 2000). We postulate that SLBP provides a direct link between the cell cycle machinery and histone mRNA biosynthesis. When cells are blocked at the G1/S transition by DNA synthesis inhibitors, there are normal S-phase levels of SLBP, but very little histone mRNA accumulates (Whitfield et al. 2000). Therefore, while the levels of mammalian histone mRNA are coupled to the rate of DNA replication and not only to the phase of the cell cycle, SLBP levels are coupled to the phase of the cell cycle. We are currently investigating whether dSLBP protein is cell cycle regulated in *Drosophila*.

Cell cycle regulation of histone mRNA accumulation also is a result of an increased rate of histone gene transcription as cells enter S phase (DeLisle et al. 1983; Heintz et al. 1983). A number of elements in histone gene promoters have been implicated in this transcriptional increase (LaBella et al. 1988; Heintz 1991), and a sequence shared among all the histone-coding regions has also been implicated in histone gene transcription (Hurt et al. 1991; Bowman and Hurt 1995) through the transcription factor YY1 both in mammals (Eliassen et al. 1998) and in *Drosophila* (K. Eliassen, M. Hurt, and D.

Garza, pers. comm.). Moreover, recent evidence suggests that the mammalian cyclin E/cdk2 substrate NPAT directly couples the G1–S cell cycle machinery with histone gene transcriptional activation (Ma et al. 2000; Zhao et al. 2000). Transcription of *Drosophila* histone genes appears to be unaffected by mutation of *dSLBP*. As in wild type, *dSLBP* mutant embryos express histone mRNA only in tissues that contain cycling cells (either dividing, like the CNS, or endoreduplicating, like the midgut) and do not express histone mRNA in G1-arrested cells (e.g., the epidermis). Thus, transcriptional regulation alone is insufficient to provide the tight coupling of histone mRNA accumulation with S phase.

The role of SLBP in early embryonic development

Early embryonic development is one exception to the tight coupling between histone mRNA levels and S phase. Histone mRNA persists at a constant level during the initial, rapid S–M cycles of *Drosophila* embryos (Anderson and Lengyel 1980, 1984). Our results indicate that *dSLBP* function is required maternally for these cycles. Eggs laid by females hemizygous for a viable, hypomorphic allele (*dSLBP*¹⁰) display defects in the syncytial nuclear division cycles. Rather than the rapid expo-

ponential increase in the number of nuclei characteristic of normal development, there is production of a reduced number of nuclei that are abnormal in arrangement and morphology. The amount of wild-type histone H4 and H3 mRNA is drastically reduced in these embryos. Consequently, while replication still occurs, the DNA probably cannot be properly packaged into normal chromatin because of limited histone biosynthesis. This likely causes the observed failure of chromosome condensation during mitosis, leading to impaired nuclear division. Thus, the demand for dSLBP function appears to be highest in the earliest stages of embryogenesis. However, we have not yet formally determined whether this represents a requirement for dSLBP during oogenesis (e.g., for production of mature histone mRNA) and/or a requirement during the cleavage cycles themselves (e.g., for translation of the maternally provided histone mRNA). Perhaps the ~100 copies of the tandemly repeated *Drosophila* histone genes are only needed to provide the stored histone mRNA in the oocyte and for production of histones in the early embryo. In subsequent developmental stages there will be a lower demand for histone mRNA as the cell cycles are slower (and S phase is longer), and the reduced level of dSLBP produced in the hypomorphic mutant is sufficient to support development.

What is the purpose of restricting histone synthesis to S phase?

Replication-associated histone gene expression is evolutionarily conserved, occurring from yeast to metazoans (although in yeast the regulation is primarily at the transcriptional level; Osley 1991). This conservation of regulation presumably has important functional significance. The tight regulation may have evolved to ensure production of histones when they are needed, to achieve the proper stoichiometry of each histone protein, and to prevent production of histones when they are not needed. Proper regulation is likely to be important for normal nucleosome formation, and hence, proper chromosome function. Consequently, the overaccumulation of individual histone proteins, or even all histones, when cells are not synthesizing DNA may be deleterious. Thus, the lethality of *dSLBP* mutants is likely caused by the misexpression of replication-associated histone genes, although we cannot rule out that dSLBP functions in the regulation of other mRNAs, the absence of which could cause the deleterious effects.

Misexpression of histone genes could disrupt development in many ways. For instance, in *Saccharomyces cerevisiae*, the overexpression of H2A/H2B or H3/H4 histone dimer pairs causes an increased frequency of chromosome loss (Meeks-Wagner and Hartwell 1986), and changes in the dose of histone H2B alter transcription of certain genes (Clark-Adams et al. 1988). Chromosome segregation during cell division may also be affected. There are several variant histone proteins that are expressed at low levels throughout the cell cycle (Wells and Kedes 1985; Fretzin et al. 1991; van Daal and Elgin 1992).

These variants substitute for the core histones to form specialized nucleosomes at particular loci that perform specific functions. In *dSLBP* mutant cells, the overexpression or mistimed expression of replication-associated histones during the cell cycle may affect the formation of these specialized nucleosomes. For instance, the *Drosophila* H3-like protein Cid accumulates in nucleosomes only at centromeric DNA, presumably as a critical component of centromere identity and function (Henikoff et al. 2000). Perhaps continuous overexpression of H3 during the cell cycle in *dSLBP* mutants competes with Cid for centromere binding, thereby disrupting centromere function and chromosome transmission during cell division. Whatever the basis is for the lethality, *dSLBP* mutants survive until very late in development, long after the first evidence of misexpressed histone mRNA. Therefore, any deleterious effects from histone misexpression are not acute but, rather, may eventually be harmful by causing the accumulation of defects throughout development. Our current efforts are focused on addressing many of these issues.

Materials and methods

Yeast three-hybrid screen

Yeast strains expressing either wild-type or control bait that does not bind SLBP have been described previously (Wang et al. 1996b). Thirty-seven His⁺ positives that also activated the *lacZ* reporter were identified from ~3 × 10⁶ colonies obtained from a 0–4-h embryonic cDNA library (gift of A. Dahanukar and R. Wharton, Duke University). Only five of these required the hybrid RNA expressing plasmid for growth in His⁻ medium. Plasmid DNA was isolated from each of these colonies, and all five contained clones with *dSLBP* sequence. One corresponding cDNA, GM06606, was sequenced in its entirety (GenBank AF258617).

Protein expression and antibody production

The coding region of dSLBP was engineered by PCR to contain 5' *NcoI* and 3' *XbaI* sites and expressed in Sf9 insect cells using the Bac-TO-Bac baculovirus expression system (GIBCO BRL). His-tagged full-length dSLBP was purified by affinity chromatography on Ni-NTA agarose (QIAGEN) and used as an antigen to generate polyclonal antibodies in rabbits (Pocono Rabbit Farms). Anti-dSLBP antibody was affinity purified using nitrocellulose-mobilized full-length dSLBP protein as described (Robinson et al. 1988).

Gel shift assays

Mobility shift assays were carried out as previously described (Dominski et al. 1999). dSLBP was synthesized in vitro with a TnT kit (Promega) using cDNA subcloned into the pSP64T vector. Embryo extract (gift of K. Williamson and L. Searles, University of North Carolina) for gel-shift experiments was made as previously described (Rio 1988).

Preparation of Drosophila cell extract and RNA-pull down of dSLBP

SL2 cells were grown in suspension at room temperature using Shield's and Sung M3 medium (Sigma) supplemented with

12.5% heat-inactivated fetal bovine serum and streptomycin/penicillin mix. Nuclear and cytoplasmic extracts from 5 L of culture (1×10^6 cells/mL) were prepared as previously described (Dominski et al. 1995, 1999). dSLBP was precipitated from the SL2 nuclear extract by binding to synthetic histone stem-loop RNA biotinylated at the 5' end (5'BiWT RNA; 5' Biotin-18s-18s-CCAAAAAGGGUCUUUCAGAGCCACCCA 3', where 18s refers to an 18-atom spacer) as described in Dominski et al. (1995). Protein bound to 5'BiWT RNA was recovered from streptavidin agarose beads with SDS-PAGE loading buffer and analyzed by immunoblotting with anti-dSLBP antibody. A non-SLBP-binding biotinylated RNA (5'BiRS; 5'-Biotin-18s-18s-CCAAAAACCGAGAUUUCUCUCGACCCA-3') was used to monitor nonspecific binding to the agarose beads. Both 5'BiWT and 5'BiRS RNAs were synthesized by Dharmacon Research.

Expression of dSLBP in *Xenopus* oocytes

Healthy stage V–VI oocytes were injected with 45 nL of a solution containing 0.5 $\mu\text{g}/\mu\text{L}$ of synthetic dSLBP mRNA transcribed from a modified version of pSP64T (Promega) as previously described (Wang et al. 1999). Whole-cell, nuclear, and cytoplasmic extracts were prepared from injected oocytes that had been incubated for 48 h at 18°C. dSLBP RNA-binding activity in the extracts was detected by gel mobility-shift assays as described above.

Cosmid analysis

Cosmid clones 144B6, 36E5, and 1H12 kindly provided by EDGP were confirmed to contain dSLBP by Southern blotting. A 4.5-kb *NcoI* fragment and a 5.3-kb *SphI* fragment from cosmid 144B6 that hybridized to the probe were isolated in pGEM5Zf (Promega). DNA sequence analysis indicated that a 6465-nt region containing the entire dSLBP gene is contained in these overlapping subclones (shown in Fig. 5A). Database searches using this sequence identified ESTs corresponding to the *Ligatin* and *Rpn2* transcription units on either side of dSLBP. The insert of a single cDNA clone representing each of these genes was sequenced and the data deposited in GenBank: AF260235 for *Ligatin* and AF312231 for *Rpn2*. To generate the dSLBP-complementing transgene, a 2.3-kb *NheI*–*Bam*HI genomic dSLBP fragment was cloned into *SpeI*/*Bam*HI cut pCaSpeR4 transformation vector.

P element excision mutagenesis

Male flies of the genotype w^{1118}/Y ; EP(3)1045 or EP(3)3182/TM6B were crossed to *Sb*, P[ry⁺, Δ 2, 3]/TM6, P[ry⁺, Δ 2, 3] females, and the resulting EP(3)/TM6, P[ry⁺, Δ 2, 3] male progeny were subsequently crossed to yw^{67}/yw^{67} ; *D*/TM3, *Sb* females. w^- revertant male flies of genotype Δ EP(3)/TM3, *Sb* were identified and backcrossed to yw^{67}/yw^{67} ; *D*/TM3, *Sb* to create a balanced stock of each P-element excision chromosome having the genotype yw^{67}/yw^{67} ; Δ EP(3)/TM3, *Sb*. Five homozygous lethal chromosomes (four from EP(3)3182 and one from EP(3)1045) that affect dSLBP were identified from among 64 w^- lines by PCR, Southern blot, and inter se complementation analysis. Two probes were used for Southern analysis of genomic DNA prepared from flies heterozygous for the excision chromosome: the *EcoRI*–*Bam*HI fragment containing only dSLBP and the *SphI*–*Bam*HI fragment containing dSLBP and the 5' part of *Rpn2* (see Fig. 5A). The dSLBP¹⁹, dSLBP¹⁵, and dSLBP¹² mutations were identified this way. Two lethal lines (dSLBP³⁵ and dSLBP⁴⁷) within the dSLBP complementation

group showed no changes by Southern analysis. These lines may have suffered a deletion that extends beyond the region we probed into the *Rpn2* gene (i.e., past the *SphI* site). dSLBP¹⁰ was identified because it is female sterile over *Df(3R)3450* and each of the other members of the dSLBP complementation group. A series of primers within the P element and in the adjacent genes were used to identify the breakpoints of excision alleles. Because these were large PCR products (2.5–10 kb), we used Boehringer Mannheim's Expand Long Template PCR System and high-molecular weight genomic DNA. Once fragments were successfully amplified, they were sequenced directly. Detailed PCR conditions for all experiments are available on request.

RNA analysis

Total RNA was prepared from 0–2-h- or 7–22-h-old fly embryos collected at 25°C using the TRIzol Reagent (GIBCO BRL) protocol. The only modification was the need to homogenize the flies in a frosted glass homogenizer (10 strokes). The RNA was fractionated into polyA⁺ and polyA⁻ fractions using Promega's PolyATtract mRNA isolation system. An expression profile for dSLBP mRNA was obtained by the PCR-based Rapid-Scan System (Origene Technologies). Hybridization probes were generated from a clone (gift of T. Shermoen, University of California, San Francisco) containing the coding region of the H3 and H4 genes from the histone gene cluster (GenBank X14215).

Embryo analysis

In situ hybridization was done as previously described (Duronio and O'Farrell 1994) using probes containing the coding region of H3, H4, and dSLBP. Embryos were collected at room temperature and aged at 18°C or 25°C (for histone experiments) or room temperature (for dSLBP experiments) until the desired stage. The dSLBP mutant histone expression phenotype was the same at 18° and 25°C. BrdU pulse labeling was performed exactly as described (Duronio and O'Farrell 1994). Mutant germ-band-extended embryos were distinguished from wild-type siblings with a balancer chromosome expressing β -galactosidase (TM3 P[*Ubx-lacZ*]). Microtubules were detected in embryos fixed for 5 min in 37% formaldehyde using anti- β -tubulin mouse monoclonal (Amersham) and goat antimouse Oregon Green (Molecular Probes) antibodies. Live mutant embryos were sorted from their wild-type siblings using stocks containing a TM3 Ser P[act-GFP] balancer chromosome. At room temperature, 1–2-h egg collections were aged for 13–14 h, dechorionated, and then hand selected by visualization using a Zeiss stereomicroscope equipped with epifluorescence. Hemizygous embryos (Fig. 6, lanes 2,3) were obtained using *Df(3R)3450*/TM3 P[act-GFP] as the mothers. Batches of hand-sorted embryos were frozen at –80°C and then combined and solubilized in SDS-PAGE gel loading buffer (at a ratio of 1 μL /embryo) by boiling for 5 min. For Westerns, 20 embryo equivalents were loaded per lane.

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