**Drosophila Stem-Loop Binding Protein Intracellular Localization Is Mediated by Phosphorylation and Is Required for Cell Cycle-regulated Histone mRNA Expression**

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Stem-loop binding protein (SLBP) is an essential component of the histone pre-mRNA processing machinery. SLBP protein expression was examined during Drosophila development by using transgenes expressing hemagglutinin (HA) epitope-tagged proteins expressed from the endogenous Slbp promoter. Full-length HA-dSLBP complemented a Slbp null mutation, demonstrating that it was fully functional. dSLBP protein accumulates throughout the cell cycle, in contrast to the observed restriction of mammalian SLBP to S phase. dSLBP is located in both nucleus and cytoplasm in replicating cells, but it becomes predominantly nuclear during G2. dSLBP is present in mitotic cells and is down-regulated in G1 when cells exit the cell cycle. We determined whether mutation at previously identified phosphorylation sites, T120 and T230, affected the ability of the protein to restore viability and histone mRNA processing in vivo. The T120A SLBP restored viability and histone pre-mRNA processing. However, the T230A mutant, located in a conserved TPNK sequence in the RNA binding domain, did not restore viability and histone mRNA processing in vivo, although it had full activity in histone mRNA processing in vitro. The T230A protein is concentrated in the cytoplasm, suggesting that it is defective in nuclear targeting, and accounting for its failure to function in histone pre-mRNA processing in vivo.

**INTRODUCTION**

Replication-dependent histone mRNAs in animal cells are not polyadenylated but instead end in a conserved stem-loop. This unique 3′ end is formed by an endonucleolytic cleavage that requires two sequence elements, the stem-loop and a purine-rich histone downstream element (HDE) located just after the processing site (Dominski and Marzluff, 1999). The stem-loop structure binds the stem-loop binding protein (SLBP) and the HDE binds to the 5′ end of U7 small nuclear RNA. These factors recruit an endonuclease that cleaves the histone pre-mRNA. SLBP remains bound to the histone mRNA after processing, and accompanies the histone mRNA to the cytoplasm where it contributes to histone mRNA translation (Sanchez and Marzluff, 2002) and stability.

In mammalian cultured cells, histone mRNA expression increases 35- to 50-fold during S phase (Harris et al., 1991). Histone gene transcription increases only three- to fivefold at the G1-S transition, and much of the cell cycle regulatory mechanism that controls histone mRNA accumulation is posttranscriptional and mediated by the stem-loop at the 3′ end of histone mRNA (Stauber et al., 1986). SLBP protein expression is restricted to S phase of the cell cycle and accounts for the increase in histone pre-mRNA processing observed as mammalian cells enter S phase (Whitfield et al., 2000; Zheng et al., 2003). However, little is known about the coordination of SLBP protein expression and histone mRNA expression during the cell cycle in other organisms. Moreover, the regulatory signals and cis-acting sequences that control SLBP expression and localization in vivo are not known, especially in the context of animal development where changes occur in the regulation of histone mRNA at different stages of development (Marzluff and Duronio, 2002).

To address these questions, we have used Drosophila embryos to study the role of SLBP in cell-cycle regulation of histone mRNA accumulation. After fertilization, the Drosophila embryo undergoes 13 rapid S/M nuclear division cycles that lack gap phases and occur meta-synchronously in a syncytium (Foe et al., 1993). The nuclei enter G2 phase for the first time in cycle 14, at which time cellularization occurs. Subsequently, specific groups of cells referred to as mitotic domains enter M phase 14 at different times, resulting in a complex but stereotypic pattern of cell-cycle progression (Foe, 1989). Entry into mitosis 14 requires zygotic expression of the string (**stg**) gene, which encodes a CDC25-type phosphatase that activates the mitotic cyclin-dependent kinase (CDK1) (Edgar and O'Farrell, 1989). Cycles 15 and 16 lack G1 phase and are also regulated at the G2-M transition by **stg** expression (Edgar and O'Farrell, 1990). Most cells enter G1 for the first time in cycle 17, and subsequent cell cycle behavior depends on developmental information specified...
by cell type. Cells in the midgut no longer divide, but rather enter into S-G endocycles (Smith and Orr-Weaver, 1991; Edgar and Orr-Weaver, 2001). Cells in the central nervous system (CNS) continue to proliferate in G2-regulated cycles (Hartenstein et al., 1987; Weigmann and Lehner, 1995), and cells of the epidermis remain arrested in G1 of cycle 17 for the remainder of embryogenesis (Edgar and O’Farrell, 1989).

dSLBP is required during embryogenesis after cycle 14 for processing of histone pre-mRNA (Sullivan et al., 2001; Lanzotti et al., 2002). Histone mRNAs are expressed in Slbp mutant embryos, but these mRNAs are polyadenylated due to usage of cryptic polyA signals. These misprocessed mRNAs are not properly cell cycle regulated and accumulate during the entire cell cycle rather than only in S phase. The loss of dSLBP function causes lethality at the pupal stage.

Drosophila SLBP is phosphorylated in vivo (Lanzotti et al., 2002). Mass spectrometry analysis of dSLBP purified from Sf9 cells after baculovirus expression revealed that four COOH-terminal serine residues are quantitatively phosphorylated (Dominski et al., 2002b; Raska et al., 2002). There was also substoichiometric phosphorylation of T120 (in the N-terminal domain) and T230 (in the RNA-binding domain). Interestingly, T120 is within a short stretch of amino acids (SFTP) similar in sequence and position to two threonine phosphorylation sites in human SLBP (SFTP) that are responsible for targeting the protein for degradation at the end of S phase (Zhang et al., 2003). T230 is located within a TPNK cyclin-dependent kinase consensus phosphorylation site that has been conserved in all known SLBP proteins.

Here, we show that, in contrast to mammalian cells, dSLBP protein persists throughout the cell cycle during development. Mutation of T120 to alanine causes no apparent phenotype, whereas a T230A mutation causes dSLBP to remain constitutively in the cytoplasm. As a result, the T230A mutant dSLBP does not complement the lethal Slbp mutant phenotype and cannot support processing of histone pre-mRNA in vivo, even though it functions in processing in vitro.

MATERIALS AND METHODS

Fly Stocks and Transgenes

Slbp is a null allele created by P-element excision, and Df(3R)3450 deletes the entire Slbp locus (Sullivan et al., 2001). stg78 and cyclin E (Cas) are previously described null alleles (Edgar and O’Farrell, 1989; Kneblitch et al., 1994). Transgenic Slbp constructs contain genomic sequence beginning at the SacI site 612 base pairs upstream of the Slbp start codon and continue to the BamHI restriction fragment containing the H3 gene (Lanzotti et al., 2002b). For the in vitro processing reaction, a Drosophila histone H3 pre-mRNA substrate (corresponding to 56 nt of H3 coding region followed by the stem-loop and 166 nt downstream) was 5'-end labeled. Then 1 ng of H3 pre-mRNA substrate (2 × 10⁶ cpm) was incubated for 2 h at 22°C with 1 µl of 200 mM EDTA, 2.5 µl of buffer D, and 5 µl of 50 µg of protein of line 2 Schneider cell (S2) nuclear extract (NE) in a total reaction volume of 10 µl as described previously (Dominski et al., 2002b). For complementation of processing, 250 ng of baculovirus expressed dSLBP for each of the three constructs was added to a 5-2 NE depleted of dSLBP with anti-dSLBP as described previously (Dominski et al., 2002b). The products of the reaction were treated with phenol-chloroform and resolved on a 7 M urea denaturing 6% polyacrylamide gel.

Western Blotting

One hundred 8- to 12-h-old dechorionated embryos were collected from homozygous transgenic lines, ground in 10 µl of SDS-containing sample buffer, boiled, and spun at 15,000 rpm for 1 min. Approximately 2 µg equivalents were loaded per lane and resolved on a 15% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, and the blots were blocked overnight at 4°C in 5% milk/phosphate-buffered saline/Tween 20. The blots were then probed with 1:500 mouse monoclonal anti-HA antibody (Covance, Denver, CO) followed by 1:500 fluorescein isothiocyanate-conjugated goat anti-rabbit.

Northern Blotting

Total RNA was isolated from 100 8- to 12-h-old dechorionated embryos by using TRIzol reagent (Invitrogen, Carlsbad, CA). Then 0.5 µg of RNA/lane was dissolved in denaturing 3% agarose gels, transferred to nylon membranes, and hybridized with random primed DNA-labeled probes (Roche Diagnostics, Indianapolis, IN). The H3-ds probe was generated from a polymerase chain reaction product corresponding to sequence downstream of the normal histone H3 processing site, and the H3 coding probe was generated from an Axl/BamHI restriction fragment containing the H3 gene (Lanzotti et al., 2002). Embryos containing different transgenic lines were obtained from the crosses: P[w*3nt]Slbp[w*3nt]/TM3 and P[w*3nt]Slbp[w*3nt] × P[w*3nt]Slbp[w*3nt]/TM3. Histone mRNA In Situ Hybridization

Dechorionated embryos were fixed in a 1:1 mixture of 37% formaldehyde/heptane for 5 min. Histone mRNA was detected by in situ hybridization using digoxigenin-labeled H3 coding or H3-ds RNA probes as described previously (Lanzotti et al., 2002).

Immunofluorescence Staining of Embryos

Transgenic embryos were fixed in a 1:1 mixture of 10% formaldehyde/heptane for 10 min. Embryos were labeled by 1:250 mouse anti-HA antibody (gift of Y. Xiong, University of North Carolina, Chapel Hill, NC) followed by 1:500 Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and 1:3000 rabbit anti-phosphohistone H3 (Upstate Biotechnology, Lake Placid, NY) followed by 1:2000 fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories). DNA was detected by treating embryos with 1.0 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 1 min. Images were obtained using a 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Cyclin E homozygous mutant embryos were distinguished from siblings by using the CyO/Y. Xiong, University of North Carolina, Chapel Hill, NC) followed by 1:500 mouse anti-β tubulin (Amersham Biosciences) and 1:8000 sheep anti-mouse horseradish peroxidase.
Threonine 230 Is Required for SLBP Function In Vivo

To determine the biological effects of mutation at sites of dSLBP phosphorylation, transgenes were constructed that express either wild-type or threonine to alanine missense mutant HA-tagged dSLBP proteins from the endogenous Slbp promoter (see MATERIALS AND METHODS). Two single and one double mutant were made: T120A, T230A, and T120A/T230A (Figure 1A). At least four different insertions of each transgenic construct were tested for their ability at a single copy to complement the lethality caused by a hemizygous Slbpnull null allele (Table 1). Each of four wild-type lines was able to fully rescue viability, indicating that HA-tagged SLBP is functional in vivo. Three of the four T120A mutant lines tested were also able to fully rescue viability of the Slbp mutant. In contrast, none of the T230A or T120A/T230A mutant lines were able to rescue viability of Slbp mutant flies (p < 0.005). The few escaping Slbp mutant flies that contained a T230A transgene eclosed later than fully rescued flies, and most, but not all, died shortly after eclosion with no overt morphological defects. These data demonstrate that the T230A mutation impairs dSLBP function in vivo.

The hypomorphic Slbp10 allele is viable when hemizygous but causes maternal effect lethality due to a depletion of histone mRNA and protein supplied to the egg (Sullivan et al., 2001; Lanzotti et al., 2002). Slbp10 males are fertile. Male and female Slbp10 mutant flies rescued by a single copy of each of the transgenes were tested for fecundity and characterized qualitatively as fertile, moderately fertile, or sterile (Table 1). All rescued males from each of the wild-type and T120A lines as well as the few males recovered from the T120A/T230A#5 line were fertile. However, rescued females from the wild-type and T120A lines had variable degrees of fertility. For instance, the WT#16 and the T230A#5 lines were fertile; the WT#1, WT#11, T120A#15, and T120A#19 lines were moderately fertile; and the WT#29 and T120A#8 lines were sterile. The latter two lines expressed lower amounts of the transgene (our unpublished data), likely due to the site of P-element insertion, and this presumably compromised the ability to express sufficient histone mRNA and protein during oogenesis. A small number of rescued females from the T230A#22 and T120A/T230A#5 lines survived long enough to assess fecundity, and all were sterile.

The variation in female fertility among wild-type and T120A rescued flies likely results from insufficient expression of dSLBP. During the Drosophila life cycle, oogenesis likely requires the highest level of dSLBP expression to provide sufficient histones for the syncytial nuclear cycles of early embryogenesis (Lanzotti et al., 2002). We therefore tested whether two copies of each transgene would improve the fertility of rescued flies. Full viability and female fertility was provided by each of the wild-type and T120A lines examined (Table 2). Unlike the single copy transgene rescue experiments, two copies of the T230A and T120A/T230A transgenes partially rescued the lethality of the Slbp15 mutation (between 33 and 70% of the expected numbers were observed, depending on the line). However, rescued females from all of these crosses were completely sterile. We draw two conclusions from these genetic experiments: 1) both HA-tagged wild-type and T120A mutant dSLBP are able to fully restore viability and fertility when expressed at sufficient levels; and 2) the T230A mutant dSLBP protein can provide some level of in vivo activity, and therefore is not functionally null. In this respect, it behaves similarly to the Slbp10 mutation described previously, which retains ~5-10% of dSLBP expression (Sullivan et al., 2001).

Multiple HA-SLBP Isoforms Are Expressed from the Transgenes

To determine whether differences in the level of transgenic protein expression could account for phenotypic variation among different lines of the same construct, we analyzed the transgenic protein expression in embryos containing two copies of each transgene by Western blot analysis with an HA antibody (Figure 1B). HA-SLBP was detected in each of the transgenic lines examined, and the relative level of expression in each line correlates well with the genetic data. For example, one copy of the transgene in the higher expressing T120A#5 line was sufficient to restore viability and fertility, whereas two copies of the transgene in the lower expressing T120A#19 line was required to completely complement the Slbp15 mutant. The T230A mutant dSLBPs were expressed at or above the level detected in the functional wild-type HA-dSLBP lines (Figure 1B, lanes 6–8), demonstrating that this mutation reduces dSLBP function, but not expression. Several HA-dSLBP isoforms with slightly different electrophoretic mobilities (~2-3 kDa) were detected in...
both wild-type and mutant lines. In addition, a unique and slower migrating protein species was observed in flies expressing the T230A HA-dSLBP (arrow, Figure 1B, lanes 6–8). The molecular basis for these mobility differences is unknown.

Misprocessing of Histone pre-mRNA in T230A, but Not T120A, Mutants

Loss of Slbp function causes histone mRNAs to be inappropriately polyadenylated at locations downstream of the normal processing site (Sullivan et al., 2001; Lanzotti et al., 2002). Misprocessed histone H3 mRNA can be specifically detected with a probe (H3-ds) that hybridizes to sequences between the normal site of processing and the cryptic downstream polyadenylation sites (Figure 2B) (Lanzotti et al., 2002). In Northern blot analysis, this probe does not hybridize to RNA isolated from wild-type embryos (Figure 2A, lane 1), but detects misprocessed histone H3 mRNA produced by Slbp15 mutant embryos (Figure 2A, lane 2). To test the ability of the various transgenes to restore normal processing of histone H3 mRNA to the Slbp15 mutants, RNA was isolated from embryos derived from Slbp15/TM3 heterozygous flies containing two copies of a particular transgene and analyzed by Northern blotting with the H3-ds probe. The amount of misprocessed histone mRNA was reduced dramatically relative to Slbp15/TM3 controls by expression of both the wild-type and T120A mutant transgenes (Figure 2A, lanes 3–6), indicating that the T120A mutant protein was as functional as wild-type in vivo. There was still a small amount of misprocessed RNA formed regardless of which transgene was expressed. In contrast, no reduction in misprocessing was observed for transgenes containing the

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P(Slbp)/P(Slbp); Slbp15/TM3 Sb × Df(3R)3450/TM3 Ser. Because TM3 Sb/TM3 Ser progeny die, eclosion of 20% of the P(Slbp)/+; Slbp15/Df(3R)3450 genotype represents full complementation.

a Rescued females fertile.
b Rescued females moderately fertile.
c Rescued females sterile.
d Rescued flies were late-eclosing.

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P(Slbp)/P(Slbp); Slbp15/TM3 Sb × P(Slbp)/P(Slbp); Df(3R)3450/TM3 Sb. Because TM3 Sb/TM3 Ser progeny die, full complementation occurs when the P(Slbp)/P(Slbp); Slbp15/Df(3R)3450 genotype represents 33% of the total eclosed flies.

a Rescued females fertile.
b Rescued females sterile.
T230A SLBP Binds the Stem-Loop In Vivo and Functions in Processing In Vitro

To participate in histone pre-mRNA processing, SLBP must bind the stem-loop and subsequently interact with other components of the processing machinery, including U7 small nuclear ribonucleoprotein (Dominski et al., 2002a). We previously demonstrated that SLBP’s ability to bind the stem-loop and to stimulate processing are molecularly distinct events (Dominski et al., 2001), and thus the T230A mutation, which is in the RNA binding domain, could disrupt either process. Mobility shift assays indicated that both the T120A and T230A mutant proteins expressed in a reticulocyte lysate bound the stem-loop as efficiently as wild-type dSLBP (Figure 3, A and B). We next tested whether the mutant SLBP proteins could function in processing a synthetic histone H3 pre-mRNA by using a Drosophila S2 nuclear extract that is dependent on exogenous dSLBP (Dominski et al., 2002b). Cleavage of the synthetic pre-mRNA is inhibited by the addition of excess cold stem-loop RNA (Figure 3C, lane 2) or depletion of dSLBP by using an anti-dSLBP antibody (Figure 3C, lane 3). Wild-type, T120A, and T230A mutant proteins expressed in baculovirus all restored processing to the depleted extract to a similar extent (Figure 3B, lanes 4–6, respectively). Although these data suggest that the T230A protein is nonfunctional in vivo for a reason other than a failure to bind the stem-loop and participate in pre-mRNA processing, it remained a possibility that T230A synthesized in vivo was incapable of binding the stem-loop. To test this, we used an affinity pull-down technique that uses a biotinylated stem-loop RNA to isolate endogenous dSLBP from embryo extracts with streptavidin-agarose beads (Figure 3D) (Dominski et al., 2003). Embryo extracts made from HA-dSLBP or HA-T230A dSLBP transgenic lines were incubated with the biotinylated stem-loop RNA, or a reverse stem-loop RNA as a negative control, and bound
SLBP Protein Expression during Embryogenesis

One possible explanation for the different results obtained in the in vivo and in vitro assays is that the T230A mutation interferes with the normal localization of SLBP during embryogenesis. To evaluate this possibility, we first used anti-HA antibodies to characterize the expression and intracellular localization of wild-type HA-SLBP during embryogenesis. The anti-HA antibody does not stain wild-type embryos lacking the HA-tagged SLBP transgene (Figure 4, A and B). In these experiments, mitotic cells were detected using an anti-phospho H3 antibody, which stains mitotic chromosomes. We previously demonstrated by Western blot analysis that endogenous SLBP protein is not present during the syncytial stages but that it begins to be expressed in blastoderm embryos around 2 h after egg deposition as a result of zygotic Slbp transcription (Lanzotti et al., 2002). In accordance with these data, HA-SLBP protein is not detected in syncytial preblastoderm embryos (Figure 4C) but accumulates in the nuclei of blastoderm embryos, which cellularize during interphase of cell cycle 14 (Figure 4, D–F). When the nuclear envelope breaks down as cells enter mitosis of cycle 14 in the mitotic domain pattern, HA-SLBP is detected throughout the cell (Figure 4F, arrows). After mitosis, HA-SLBP is present in both cytoplasmic and nuclear compartments during S phase of cycle 15 (Figure 5, G–I). HA-SLBP becomes concentrated in the nucleus during G2 of cycle 15 (Figure 5, A–C), when histone mRNA levels are reduced (Figure 5G) and then it distributes throughout the cell during mitosis of cycle 15. This dynamic pattern of HA-SLBP localization during the cell cycle is repeated in the subsequent cycle, with HA-SLBP again becoming concentrated predominantly in the nucleus during G2 of cycle 16 (Figure 5, D–F). Note that this behavior is different than that of mammalian SLBP, which is degraded at the end of S phase in cultured mammalian cells and in mouse embryos after the four-cell stage (Whitfield et al., 2000; Allard et al., 2002; Zheng et al., 2003). SLBP is clearly present in mitotic cells in both embryos (Figure 4) and imaginal disks (Figure 8), suggesting that unlike mammalian SLBP, dSLBP protein levels are not tightly regulated during the cell cycle. SLBP accumulates in the cytoplasm during S phase most likely because it is part of the histone mRNP and stimulates the translation of histone mRNA. Conversely, SLBP may be...
imported into the nucleus when histone mRNA levels are relatively low or absent, in both G1 and G2. To compare SLBP localization with the pattern of histone mRNA expression during cycles 15 and 16, in situ hybridization was performed with a probe derived from the histone H3 coding region. Late in G2 of cycle 14 histone H3 transcription occurs in the mitotic domain pattern in anticipation of S phase 15 (Lanzotti et al., 2002). During cycles 15 and 16, H3 mRNA is detected throughout the cell cycle (e.g., during S and G2 phase), although more mRNA is present during S phase than G2 (Figure 5, G and H). However, during G2 of cycle 16, the level of histone mRNA declines rapidly in cells that are committed to arrest in G1 of the next cycle (Figure 5, I and J). At this stage, proliferating cells in the CNS and peripheral nervous system (PNS) as well as the endocycling cells continue to express histone mRNA in a cell cycle-regulated manner (Sullivan et al., 2001; Lanzotti et al., 2002). These data suggest that SLBP is predominantly nuclear when histone mRNA is low (e.g., G2 of cycle 14 and 15) or absent (e.g., G2 of cycle 16 and G1 of cycle 17; Figure 9A) and present in both the nucleus and cytoplasm when the level of histone mRNA is high during S phase.

Mutations in the cell cycle regulators stg and cyclin E result in cell cycle arrest during embryogenesis (Edgar and O’Farrell, 1989; Knoblich et al., 1994). We determined the localization of the HA-SLPB in stg mutant embryos, which arrest in G2 of cycle 14, and cyclin E mutant embryos, which arrest in G1 of cycle 17. These embryos contain very low levels of histone mRNA due to failure to initiate transcription of the histone genes in the stg mutant and as a result of cessation of histone mRNA expression in the cyclin E mutant (Lanzotti, Marzluff, and Duronio, unpublished data). Zygotic HA-dSLBP expression occurs in the stg mutant, and the HA-SLPB accumulates in the nucleus of these G2-arrested cells (Figure 6, A–C). In the cyclin E mutant embryos, which undergo G1 cell cycle arrest at a later stage due to the time of depletion of maternally supplied cyclin E, HA-dSLBP is nuclear in most cells (Figure 6, D–F, arrowhead). In some of the neuroblasts, which are the last cells to stop replicating in cyclin E mutant embryos (Knoblich et al., 1994), HA-dSLBP is found in both the nucleus and cytoplasm, consistent with these cells still containing histone mRNA (Figure 6, D–F, arrow; Lanzotti, Marzluff, and Duronio, unpublished data). Thus, in both G1 and G2 dSLBP is predominantly nuclear.

**T230A Mutant dSLBP Is Constitutively Cytoplasmic**

We next examined the localization of T120A and T230A mutant proteins. HA-T120A was expressed essentially identically to HA-SLPB throughout embryogenesis (our unpublished data), consistent with it complementing the Slb15 mutant. In marked contrast, HA-T230A and HA-T120A/T230A were dramatically mislocalized relative to wild type and are predominantly cytoplasmic in all cells regardless of cell cycle stage (Figure 7). For instance, HA-T230A is first detected in late blastoderm stages, much like the wild-type protein, but it is restricted to the cytoplasm rather than the nucleus (Figure 7A). The mutant protein remains cytoplasmic throughout the postblastoderm cell cycles (i.e., cycles 15 and 16; Figure 7B). The mislocalization also occurs in cell types that continue to proliferate and to express high levels of SLBP late in embryogenesis, such as the CNS. In this tissue, wild-type HA-SLPB protein is detected in both nucleaus and cytoplasm throughout the cell cycle, as shown for
the large brain lobe neuroblasts in Figure 7C. In contrast the HA-T230A and HA-T120A/T230A mutant proteins are mislocalized relative to wild type and concentrated in the cytoplasm of these cells (Figure 7, D and E). Higher magnification of epidermal cells on the ventral side of the embryo further shows the concentration of HA-T230A near the cell periphery (Figure 7F). A similar phenotype is observed in the T230A mutant at postembryonic stages in eye imaginal discs (Figure 8). HA-SLBP protein is detected in both compartments of cycling cells, as well as in mitotic cells (arrow, Figure 8, A–C), whereas HA-T230A SLBP is more restricted to the cytoplasm in these cells (Figure 8, D–F). These data suggest that SLBP proteins containing the T230A phosphorylation site mutation do not function efficiently in vivo because they are improperly localized.

Mislocalized T230A SLBP Protein Is Rapidly Degraded during G1 Arrest

In epidermal cells that have just completed mitosis 16 and arrested in G1 of cycle 17, HA-dSLBP is detected predominantly in the nucleus (Figure 9A). Because Slbp transcription is terminated in these cells (Sullivan et al., 2001), HA-dSLBP levels gradually decline through subsequent stages of embryogenesis (our unpublished data). Proliferating cells in the CNS and PNS continue to express high levels of dSLBP protein (Figure 9A, arrow), whereas epidermal cells express some dSLBP in the nucleus (Figure 9A, arrowhead). Like wild-type, T230A HA-dSLBP is expressed at a high level in the cytoplasm of proliferating PNS cells (arrow, Figure 9, B and C). But unlike wild type, the T230A protein is rapidly destroyed when epidermal cells enter G1 of cycle 17 (arrowhead, Figure 9, B and C). Similarly, the T230A protein is also rapidly destroyed in PNS cells after they cease dividing in later embryos, whereas expression continues in the proliferating CNS cells (Figure 9D). These results indicate that the cytoplasmically localized T230A mutant SLBP is more vulnerable to degradation after G1 arrest than the nuclear localized wild-type SLBP. Moreover, because T230A is stable in the cytoplasm before cycle 17, this observation suggests the activation of a mechanism that degrades cytoplasmically localized SLBP when cells enter G1 arrest. The limiting step for SLBP degradation in wild-type embryos may be the relocalization of SLBP from the nucleus to the cytoplasm, which would allow its degradation.
DISCUSSION

SLBP is a major regulator of replication-dependent histone mRNA metabolism. SLBP is best characterized as an essential component of the pre-mRNA processing machinery, but it also plays a role in histone mRNA translation and stability (Dominski and Marzluff, 1999; Dominski et al., 2002b; Marzluff and Duronio, 2002; Sanchez and Marzluff, 2002). Thus, SLBP functions in both the nucleus and the cytoplasm, and characterizing the regulation of its intracellular localization is important for understanding its mechanism of action in the control of histone mRNA levels. Here, we have described the expression and localization of SLBP during the rapid cell cycles of Drosophila embryonic development and have demonstrated that a conserved, phosphorylated threonine is essential for proper SLBP intracellular localization.

Drosophila SLBP Is Expressed throughout the Cell Cycle

The restriction of histone mRNA accumulation to S phase in mammalian cells occurs through the control of transcription, pre-mRNA processing, and message stability (Schumberl, 1988; Harris et al., 1991). Control of SLBP production and degradation provides the mechanism by which mammalian histone pre-mRNA processing is regulated during the cell cycle (Zheng et al., 2003). Mammalian SLBP accumulates predominantly during S phase via an increase in translation efficiency of SLBP mRNA at the G1-S transition and quantitative destruction in a phosphorylation-dependent manner at the completion of S phase (Whitfield et al., 2000; Zheng et al., 2003). In contrast, our data demonstrate that Drosophila SLBP protein is expressed throughout the cell cycle in both embryos and eye imaginal discs. dSLBP is detected when cells have very low or no histone mRNA, such as during G2 of embryonic cycle 16 and G1 of cycle 17. dSLBP was also prevalent in all mitotic cells, as revealed by anti-phospho histone H3 staining. Therefore, the regulatory mechanisms that affect the cellular level of dSLBP and histone mRNA are distinct, and dSLBP synthesis and destruction is not the primary means by which histone mRNA accumulation during the cell cycle is controlled in Drosophila.

The steady-state level of SLBP and histone mRNA can also be experimentally uncoupled in mammalian cells. When S-phase cells are treated with inhibitors of DNA synthesis, histone mRNA is rapidly destroyed, whereas SLBP remains stable (Whitfield and Marzluff, unpublished data). Similarly, at the end of S-phase histone mRNA is destabilized and depleted from the cell even in the presence of a stable, mutant version of SLBP that is not destroyed at the end of S phase (Zheng et al., 2003). Therefore, even though histone mRNA destruction requires the 3'-untranslated region and an exonulease that is recruited to the message via SLBP (Dominski et al., 2003), the signals that trigger this process are different than the signals that trigger SLBP destruction.

Drosophila SLBP Intracellular Location Changes during the Cell Cycle

By correlating HA-dSLBP staining with anti-phospho histone H3 staining and the well known, stereotypic program of cell cycle progression in the epidermal cells of the embryo, we observe that dSLBP is predominantly nuclear in G2 and G1 phase and present in both the nucleus and cytoplasm during S phase. After pre-mRNA processing, mammalian SLBP protein remains bound to histone mRNA and accompanies it to the cytoplasm where it contributes to translation of the message (Sanchez and Marzluff, 2002). This suggests a "passive" model of dSLBP localization in which the level of cytoplasmic dSLBP depends entirely on the level of histone mRNA in the cytoplasm. Some of our observations fit this model. For instance, during G2 of cycle 14 when histone mRNA is very low, the newly synthesized dSLBP is efficiently imported into and accumulates in the nucleus. This ensures an ample supply of dSLBP to participate in the processing of nascent histone mRNA, whose transcription is activated in late G2 in the mitotic domain pattern in response to the activation of cdc2 by stg (Lanzotti, Marzluff, and Duronio, unpublished data). Similarly, in G2 of cycle 15 histone mRNA is undetectable, dSLBP is nuclear. This is also true in stg and cyclin E mutant embryos, which arrest in G2 and G1 phase, respectively, with low levels of histone mRNA. However, in G2 of cycle 15 histone mRNA is not completely absent from the cell, most likely because this G2 phase is very short, yet most of the dSLBP seems nuclear (Figure 5). Thus, the redistribution of dSLBP to the nucleus in G2 probably occurs after release.
of dSLBP from the histone mRNA at the end of S phase. This allows dSLBP to be imported into the nucleus and is consistent with the idea that removal of SLBP from histone mRNA is necessary for its degradation.

In mammalian cells, SLBP is also present in both the nucleus and the cytoplasm. SLBP is normally not present during G1 and G2. The nuclear SLBP is the pool of SLBP active in histone pre-mRNA processing. Some of the cytoplasmic SLBP is associated with histone mRNA (Hanson et al., 1996). It is likely that SLBP is imported into the nucleus during S phase, but it may also contain nuclear export signals that result in it shuttling between the nucleus and the cytoplasm. Similarly in frog oocytes, xSLBP1, the orthologue of mammalian SLBP, is present in both the nucleus and the cytoplasm (Wang et al., 1999). Mammalian SLBP can be stabilized, either by mutation of the phosphorylation sites required for SLBP degradation or by inhibiting protein degradation with proteasome inhibitors (Zheng et al., 2003). The mutant stable mammalian SLBP is found predominantly in the cytoplasm of both G1 and G2 cells (Zheng et al., 2003), suggesting degradation of SLBP requires location of SLBP in the cytoplasm. Consistent with this interpretation, the phosphorylated SLBP, which is the immediate signal for SLBP degradation, is found exclusively in the cytoplasm in cells treated with proteasome inhibitors (Zheng et al., 2003).

In mammalian cells, the rapid degradation of SLBP at the end of S phase prevents processing of histone mRNA outside of S phase (Whitfield et al., 2000; Zheng et al., 2003), even though the histone genes are transcribed outside of S phase (DeLisle et al., 1983). The restriction of histone mRNA to S-phase cells in Drosophila clearly does not involve regulating the levels of dSLBP, because dSLBP is present throughout the cell cycle and is found in the nucleus during G1 and G2. Therefore, very stringent regulation of transcription of the histone genes, and/or regulation of histone pre-mRNA processing by a mechanism other than SLBP synthesis and destruction, likely accounts for the tight cell-cycle regulation of histone mRNA in Drosophila.

Both the cytoplasmically localized T230A and T210A/T230A mutant dSLBPs are rapidly degraded when cells exit the cell cycle, whereas wild-type dSLBP is largely present in the nucleus and is degraded more slowly. This suggests that dSLBP may need to be exported to the cytoplasm for degradation. Thus, it is likely that in both mammalian cells and Drosophila cells, SLBP is degraded in the cytoplasm. In mammalian cells, this occurs after completion of DNA replication, and in Drosophila after exit from the cell cycle.

In contrast to the epidermis, we did not detect predominantly nuclear staining of HA-dSLBP at any time in the dividing embryonic neuroblasts of the CNS and PNS. dSLBP was always detected throughout these cells, including constitutively high levels in the cytoplasm. Neuroblast cell cycles lack a G1 phase and have a very short G2 phase. Thus, it is possible that the preponderance of time spent in S phase in these rapid cycles precludes nuclear accumulation of dSLBP and/or our ability to detect this. Alternatively, the active mechanism that targets SLBP to the nucleus in G2-phase in the epidermal cells may not exist in neuroblasts.

**Figure 7.** T230A SLBP is mislocalized throughout development and is enriched at the cell periphery. In all panels, HA-SLBP is shown in red, DAPI-stained DNA in blue, and anti-phospho H3-phosphorylated in baculovirus expressed dSLBP (Raska et al., 2002). The T120A mutant SLBP was correctly localized in Vivo. In Vivo phosphorylation of T58 and T59 of human SLBP triggers the protein for destruction at the end of S phase (Zheng et al., 2003), and there are additional phosphorylation sites whose function is not known (Whitfield and Marzluff, unpublished data). dSLBP is stoichiometrically phosphorylated on four serine residues at the very COOH terminus of the protein (Raska et al., 2002). These phosphates are essential for dSLBP to function in pre-mRNA processing in vitro (Dominski et al., 2002b). In this study, we characterized the in vivo consequence of mutating two threonine residues (T120 and T230) that are substoichiometrically phosphorylated in baculovirus expressed dSLBP (Raska et al., 2002). The T120A mutant SLBP was correctly localized in vivo.
within the cell and behaved identically to wild-type in all of our in vivo and in vitro assays, rescuing viability and histone pre-mRNA processing in Slbp null mutants. Although T120 is located in an analogous position to two threonine residues that when phosphorylated trigger destruction of human SLBP at the end of S phase, we have no evidence for similar cell cycle regulated destruction of dSLBP, and thus T120A phosphorylation plays an unknown role, if any, in the biological function of SLBP in Drosophila.

In contrast, the T230A mutation dramatically disrupted the in vivo function of dSLBP. Transgenes that express this protein at a level similar to wild-type were unable to efficiently rescue the lethal phenotype or histone mRNA misprocessing caused by null mutations of the Slbp gene. T230A protein was constitutively mislocalized to the cytoplasm and concentrated near the cell periphery. Several observations suggest that this aberrant localization is not simply a non-specific artifact. First, the T230A protein has normal biochemical function: it binds the stem-loop when synthesized either in vitro or in vivo and reconstitutes a dSLBP-depleted and defective processing extract as well as wild-type protein. Second, HA-T230A is hypomorphic in vivo, and two copies of a T230A transgene can partially rescue the lethal Slbp null phenotype, although the resulting females are sterile. Because weak T230A nuclear staining is observed in this genotype, it is likely that some T230A protein enters the nucleus and remains there long enough to participate in processing of some histone pre-mRNA. Third, the mislocalized T230A protein is subject to developmental regulation, because it is rapidly destroyed only in cells that have entered G1 arrest (e.g., cycle 17 epidermal cells). Presumably, the wild-type protein is not rapidly degraded at this time because it relocates to the nucleus in G1 of cycle 17. These data suggest that T230A is not functional in histone pre-mRNA processing in vivo because it is effectively excluded from its site of action at the histone locus in the nucleus.

What could the aberrant localization represent biologically? One possibility is that T230A protein is "trapped" in a cellular location that wild-type SLBP only occupies transiently as part of its normal trafficking in the cell. Consequently, phosphorylation of T230 could promote the release from this location. Alternatively, T230A phosphorylation at the end of S phase may promote the release of dSLBP from the histone mRNA and its subsequent reaccumulation in the nucleus. Because SLBP may shuttle between the nucleus and

Figure 8. HA-SLBP and HA-T230A localization in eye imaginal cells. For both sets of images (i.e., A–C and D–F), HA-SLBP staining (red) alone is shown on the left, DAPI (blue) plus anti-phosphohistone H3 (green) staining is shown in the middle, and HA-SLBP plus anti-phosphohistone H3 is shown on the right. (A–C) Section of an eye disk from an animal containing two copies of HA-SLBP#16. dSLBP protein is expressed throughout the cell and accumulates to high levels in mitosis (arrowhead). (D–F) HA-T230A#22 is also expressed throughout the cell cycle and accumulates to high levels in mitotic cells (arrowhead). However, the protein is more restricted to the cytoplasmic compartment. The morphogenetic furrow is indicated with asterisks, and anterior is to the left in these images.

Figure 9. T230A mutant SLBP protein becomes immediately degraded as soon as cells enter quiescence. In all panels, HA-SLBP is shown in red and DAPI-stained DNA in blue (ventral is down, and anterior is to the left). (A) A stage 13 germ-band retracted embryo containing two copies of the HA-SLBP#16 transgene. The arrowhead indicates epidermal cells arrested in G1 of cycle 17 with nuclear HA-SLBP. A slightly higher level of HA-SLBP is present in cells of the PNS that continue to divide at this stage (arrow) with the protein localized in both compartments of these cells. (B and C) Stage 12 and 13 embryos, respectively, containing two copies of the HA-T230A#22 transgene. T230A mutant protein is predominantly localized in the cytoplasm of PNS cells (arrows) but is absent in quiescent cells of the epidermis (arrowheads). (D) A stage 14 T230A#22 embryo. HA-T230A SLBP is rapidly destroyed when the PNS cells cease dividing (arrow).
the cytoplasm, it is also possible that the T230A mutation promotes export of SLBP from the nucleus.

There are many precedents for phosphorylation affecting either import or export of proteins from the nucleus (Alt et al., 2000). For example, cyclin B is cytoplasmic before initiation of mitosis, as a result of rapid export of cyclin B from the nucleus (Hagting et al., 1998; Yang et al., 1998, 2001). Phosphorylation of cyclin B results in an inhibition of export (crm1 no longer recognizes the nuclear export signal), and the phosphorylation results in accumulation of cyclin B in the nucleus, an essential step in the initiation of nuclear envelope breakdown.

T230 is located in a highly conserved CDK consensus phosphorylation site, TPNK. Therefore, it is interesting to speculate that cell cycle kinases are regulating the activity and/or localization of dSLBP. Cyclin E/cdk2 cannot be solely responsible for T230 phosphorylation, because dSLBP accumulates in the nucleus of cyclin E mutant cells. Other types of kinases or other CDKs (e.g., cyclin A/cdc2) could phosphorylate T230 and perhaps also act redundantly in this regard.

In conclusion, our data suggest that T230 phosphorylation is part of the intricate mechanism that controls SLBP function and its ability to contribute to the production of replication dependent histone mRNAs during the cell cycle.

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