***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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Submitted September 7, 2003; Revised December 4, 2003; Accepted December 5, 2003
Monitoring Editor: Joseph Gall

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 to *dSLBP* null mutants. 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

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-09-0649. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-09-0649.

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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 were 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 (SFTTP) similar in sequence and position to two threonine phosphorylation sites in human SLBP (SFTTP) that are responsible for targeting the protein for degradation at the end of S phase (Zheng *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*¹⁵ is a null allele created by P-element excision, and *Df(3R)3450* deletes the entire *Slbp* locus (Sullivan *et al.*, 2001). *stg*^{7B} and *cyclin E*^{AR95} are previously described null alleles (Edgar and O'Farrell, 1989; Knoblich *et al.*, 1994). Transgenic *Slbp* constructs contain genomic sequence beginning at the *SacII* site 612 base pairs upstream of the *Slbp* start codon and continue to the *BamHI* site 605 base pairs downstream of the stop codon. This DNA fragment was cloned into the pCaSpeR4 P-element vector. Two tandem hemagglutinin (HA) epitope tags were engineered at the dSLBP NH₂ terminus. Four different versions of this basic construct were engineered: wild-type, T120A, T230A, and T120A + T230A.

Stem-Loop Binding Assays and In Vitro Processing Reaction

For gel-shift assays, 1 ng of a *Drosophila* histone H3 stem-loop (SL) RNA probe was 5'-end labeled with [γ -³²P]ATP and T4 polynucleotide kinase (NEB) and purified over a G-50 column. In a 10- μ l total volume assembled on ice, 5×10^3 cpm of SL probe was incubated with 1.0 μ l of 200 mM EDTA, 2 μ l of buffer D (20% glycerol, 0.2 M EDTA 8.0, 0.5 mM dithiothreitol [DTT], 100 mM KCl, 20 mM HEPES 7.9), and 5 μ l of *Drosophila* SLBP synthesized in a 30- μ l in vitro transcription/translation (TnT) reaction containing 1.0 ng of DNA for each of the four constructs, as described previously (Dominski *et al.*, 2001). The complexes were resolved by immediately loading the entire sample in a 7.5% native polyacrylamide gel. Increasing amounts of either wild-type or mutant versions of ³⁵S-labeled dSLBP were used in the band shift assay. dSLBP proteins were quantified using a PhosphorImager (Figure 3A). Negative controls included the absence of the TnT reaction or a TnT reaction that lacked substrate DNA.

For RNA affinity pull-down of dSLBP, ~250 μ l of packed volume of dechorionated *Drosophila* embryos was resuspended in 500 μ l of a low salt buffer containing 10 mM HEPES/KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT. The embryos were incubated on ice for 15 min and were broken in the Eppendorf tube using a Teflon plunger. The suspension was supplemented with 2 ml of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8), 10 mM sodium azide, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ protease inhibitor mix (Sigma-Aldrich, St. Louis, MO), 0.5% NP-40 and rotated for 30 min at 4°C. The resulting lysate was centrifuged 10 min at 15,000 rpm in a microcentrifuge, the supernatant adjusted to 10 mM EDTA (pH 8), and 0.5 ml incubated for 1 h at 4°C with 2.5 μ g of either a biotinylated histone stem-loop RNA (SLbi, GUGCCAAAAAGGCUCUUUU-CAGAGCCACCCA) or the reverse stem mutant RNA (RSbi, GUGCCAAAAACCGAGAUUUCUCUGGACCCA) (Pharmacia, Boulder, CO). The lysates were subsequently rotated for 2 h at 4°C with 25 μ l of streptavidin-agarose beads (Sigma-Aldrich), the beads rinsed for a total of 1 h with the NP-40 lysis buffer, and bound proteins were separated on a 15% SDS-polyacrylamide gel and detected by Western blotting with either anti-dSLBP or anti-HA.

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 (2×10^4 cpm) was incubated for 2 h at 22°C with 1 μ l of 200 mM EDTA, 2.5 μ l of buffer D, and 5 μ l (50 μ g of protein) of line 2 Schneider cell (S-2) nuclear extract (NE) in a total reaction volume of 10 μ l, 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 S-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 6–8% polyacrylamide gel.

Western Blotting

One hundred 8- to 12-h-old dechorionated embryos were collected from homozygous transgenic lines, ground in 100 μ l of SDS-containing sample buffer, boiled, and spun at 15,000 rpm for 1 min. Approximately 20 embryo 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) and 1:2000 sheep anti-mouse horseradish peroxidase antibody (Amersham Biosciences, Piscataway, NJ) in 5% milk/phosphate-buffered saline/Tween 20. For loading control, blots were reprobbed with 1:5000 mouse anti- β tubulin (Amersham Biosciences) and 1:8000 sheep anti-mouse horseradish peroxidase.

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 μ g of RNA/lane was resolved in denaturing 1% 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 *Aval*/*BamHI* restriction fragment containing the H3 gene (Lanzotti *et al.*, 2002). Embryos containing different transgenic lines were obtained from the following crosses: P[*w*⁺ *Slbp*]/P[*w*⁺ *Slbp*]; *Slbp*¹⁵/TM3 $\times$ P[*w*⁺ *Slbp*]/P[*w*⁺ *Slbp*]; *Df(3R)3450*/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 by 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 with 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 (Jackson ImmunoResearch Laboratories, West Grove, PA), and 1:1000 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 μ g/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 P[wg-lacZ] balancer chromosome and staining with 1:500 rabbit anti- β -Gal and 1:1000 fluorescein isothiocyanate-conjugated goat anti-rabbit.

Figure 1. Expression of HA epitope-tagged dSLBP proteins. (A) Schematic representation of the SLBP protein encoded by the transgenes, with the RNA binding domain indicated by the shaded area. Two tandem HA tags were engineered at the start of translation. The location of the T120A and T230A mutations and the short highly conserved stretch in which they reside are indicated. (B) Western blot analysis of HA-SLBP expression in embryos. Proteins from 20 embryos were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose, and HA-dSLBP detected with an anti-HA antibody. The blot was reprobed with anti- β -tubulin antibody (bottom). Lane 1, extract from wild-type control embryos lacking a transgene. Lanes 2–8, extracts from embryos containing wild-type or mutant transgenic lines, indicated above each lane.

RESULTS

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 *Slbp*¹⁵ 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*¹⁵ 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 *Slbp*¹⁰ 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). *Slbp*¹⁰ males are fertile. Male and female *Slbp*¹⁵ 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 T120A#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 *Slbp*¹⁵ 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 *Slbp*¹⁰ 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 *Slbp*¹⁵ 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

Table 1. Single copy *Slbp* transgene rescue crosses

Transgene	Total no. of eclosed flies	<i>Slbp</i> ¹⁵ / TM3- <i>Ser</i>	<i>Df(3R)3450</i> / TM3- <i>Sb</i>	<i>Slbp</i> ¹⁵ / <i>Df(3R)3450</i>	% of expected	Chi-squared p value
WT#1	178	81	59	38 ^b	94.7	>0.1
WT#11	186	77	74	35 ^b	94.6	>0.5
WT#16	236	110	82	44 ^a	93.6	>0.1
WT#29	211	88	82	41 ^c	97.6	>0.5
T120A#5	151	57	57	37 ^a	123.3	>0.1
T120A#8	204	90	94	20 ^c	48.8	<0.01
T120A#15	76	33	33	10 ^b	66.6	>0.1
T120A#19	120	55	44	21 ^b	87.5	>0.1
T230A#6	131	68	61	0	0.0	<0.005
T230A#15a	54	27	27	0	0.0	<0.005
T230A#15b	104	63	44	2	9.5	<0.005
T230A#22	112	50	54	4 ^{c,d}	18.2	<0.005
T230A#23	64	38	26	0	0.0	<0.005
T230A#25	62	32	30	0	0.0	<0.005
T120A/T230A#1	55	22	31	2	18.2	<0.005
T120A/T230A#5	145	74	59	12 ^{c,d}	41.4	<0.005
T120A/T230A#10	136	70	65	15	3.7	<0.005
T120A/T230A#13	82	42	40	0	0.0	<0.005

P(*Slbp*)/+; *Slbp*¹⁵/TM3 *Sb* × *Df(3R)3450*/TM3 *Ser*. Because TM3 *Sb*/TM3 *Ser* progeny die, eclosion of 20% of the P(*Slbp*)/+; *Slbp*¹⁵/*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.

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 *Slbp*¹⁵ mutant embryos (Figure 2A, lane 2). To test the ability of the various transgenes to restore normal processing of histone H3 mRNA to the *Slbp*¹⁵ mutants, RNA was isolated from embryos derived from *Slbp*¹⁵/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 *Slbp*¹⁵/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

Table 2. Two-copy *Slbp* transgene rescue crosses

Transgene	Total no. of eclosed flies	?/TM3- <i>Sb</i>	<i>Slbp</i> ¹⁵ / <i>Df(3R)3450</i>	% of expected	Chi-squared p value
WT#1	108	73	35 ^a	97.2	>0.5
WT#16	168	98	70 ^a	125.0	<0.1
T120A#19	231	156	75 ^a	97.4	>0.1
T230A#22	184	156	28 ^b	45.9	<0.005
T230A#23	90	80	10 ^b	33.3	<0.005
T120A/T230A#5	110	84	26 ^b	70.3	<0.05
T120A/T230A#13	107	86	21 ^b	58.3	<0.005

P(*Slbp*)/P(*Slbp*); *Slbp*¹⁵/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*); *Slbp*¹⁵/*Df(3R)3450* genotype represents 33% of the total eclosed flies.

^a Rescued females fertile.

^b Rescued females sterile.

Figure 2. HA-T230A mutant dSLBP does not support histone pre-mRNA processing in vivo. (A) Northern blot analysis of total embryonic RNA probed with the H3-ds probe, which only detects misprocessed, polyadenylated histone mRNA. Lane 1, RNA from wild-type. Lane 2, RNA isolated from embryos collected from a *Slbp*¹⁵/TM3 mutant stock. Lanes 3–9, RNA isolated from embryos collected from *Slbp*¹⁵/TM3 mutant stocks carrying two copies of the indicated transgenes. Lanes 2–9 contain RNA isolated from the entire population of embryos, which contains a mixture of TM3/TM3, *Slbp*¹⁵/TM3, and *Slbp*¹⁵/*Slbp*¹⁵ genotypes. The blot was stripped and reprobed with a histone H3 coding region probe to control for loading. Note that with this method we previously detected the longer, polyadenylated mutant mRNAs (Sullivan *et al.*, 2001), but in this particular gel these species remained unresolved from wild-type H3 mRNA. (B) Diagram of the H3-ds probe, which hybridizes to sequence downstream of the normal processing site (arrow) and therefore detects only misprocessed H3 mRNA. (C–F) show a stage 13 embryo (ventral at bottom, and anterior at left) hybridized with the H3-ds probe, which only detects misprocessed, polyadenylated histone mRNA. (C) Wild-type embryo. (D) *Slbp*¹⁵ homozygous null mutant embryo. Misprocessed histone H3 message is present in all tissues that normally express histone mRNA, including endocycling midgut cells (arrow) and proliferating CNS cells (arrowhead). (E–I) *Slbp*¹⁵ homozygous null mutant embryos carrying two copies of the indicated transgenes. Note that phenotypic rescue of histone mRNA processing seems somewhat better in the CNS (arrowhead in E and G).

T230A mutation (Figure 2A, lanes 7–9). This result indicates that the T230A SLBP protein has very low activity in histone pre-mRNA processing in vivo.

Because the Northern blot analysis used RNA prepared from a mixed population of embryos, we measured the degree of histone pre-mRNA processing in individual em-

bryos by whole mount in situ hybridization using the H3-ds probe. We have shown previously that the H3-ds probe does not hybridize to wild-type embryos (Figure 2C) but detects misprocessed histone H3 mRNA in *Slbp*¹⁵ mutants in replicating cells that normally express histone mRNA, including those in the endocycling midgut and the proliferating CNS (Figure 2D) (Lanzotti *et al.*, 2002). Both wild-type and T120A mutant transgenes greatly reduced the amount of misprocessed histone mRNA throughout the *Slbp*¹⁵ mutant embryo (Figure 2, E–G). The degree of phenotypic rescue correlates well with the level of protein expression as shown in Figure 1B. For instance, the T120A#5 transgene nearly eliminated misprocessing (Figure 2F), whereas the WT#16 and T120A#19 embryos, which produce less HA-dSLBP than the T120A#5 line (Figure 1B), still contained detectable amounts of misprocessed histone H3 mRNA (Figure 2, E and G). We routinely observed greater rescue of processing in the CNS than in the endocycling cells (arrowhead, Figure 2, E and G), perhaps because dSLBP is expressed at a higher level in this tissue relative to the others (Sullivan *et al.*, 2001). Consistent with the Northern blot experiments, very little reduction in the amount of misprocessed histone mRNA was observed in *Slbp*¹⁵ mutant embryos expressing the T230A#22 (Figure 2H) or the T120A/T230A#5 (Figure 2I) transgenes, confirming that the T230A mutant protein is defective for histone pre-mRNA processing in vivo. In addition, no misprocessed H3 mRNA was detected in embryos genotypically wild-type for the *Slbp* locus and expressing T230A (i.e., the transgenic lines), suggesting that the T230A mutant protein does not act as a dominant negative by competing with endogenous dSLBP.

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

Figure 3. T230A and T120A dSLBPs are active in RNA binding and pre-mRNA processing. (A) A rabbit reticulocyte lysate was programmed with synthetic mRNA encoding dSLBP, and the T230A and T120A mutant dSLBPs (lanes 2–4, respectively) in the presence of [35 S]methionine, and analyzed by SDS-gel electrophoresis and autoradiography to quantify the amount of dSLBP proteins used for the gel shift shown in B. Lane 1 is the lysate incubated in the absence of synthetic mRNA. (B) The in vitro-translated wild type (lanes 4–6), T230A (lanes 7–9), and T120A (lanes 10–12) dSLBPs were incubated with the radiolabeled stem-loop RNA. Equal amounts of dSLBP were used in lanes 4, 7, and 10; twice as much in lanes 5, 8, and 11; and four times as much in lanes 6, 9, and 12. Lane 1 is the probe alone, lane 2 is a *Drosophila* embryo nuclear extract and lane 3 is the unprogrammed reticulocyte lysate. The positions of the dSLBP/stem-loop complex and free probe (SL Probe) are indicated. (C) In vitro histone pre-mRNA processing in S2 nuclear extract. Lane 1, processing of a synthetic radiolabeled H3 pre-mRNA (“input”) in an S2 nuclear extract. “Proc” indicates the cleavage product. Lane 2, H3 pre-mRNA plus excess unlabeled SL RNA. Lane 3, processing of H3 pre-mRNA after immunodepletion of endogenous SLBP. Lanes 4–6, addition of baculovirus expressed wild type (lane 4), T120A mutant (lane 5), or T230A mutant (lane 6) SLBP protein to immunodepleted extract. All three proteins restore processing to the depleted extract. (D) An extract from 0- to 24-h-old *Drosophila* embryos was incubated with a biotinylated stem-loop RNA (SLbi, lanes 1 and 4) as a negative control, and bound proteins were recovered with streptavidin-agarose beads. Equal amounts of the input extract (lane 3), and the supernatants (lanes 1 and 2), and the bound proteins from the equivalent of 100 $\times$ as much extract (lanes 4 and 5) were analyzed by SDS-gel electrophoresis and the proteins transferred to nitrocellulose. The dSLBP was detected by Western blotting with anti-dSLBP antibodies (Sullivan *et al.*, 2001). The * indicates a cross-reacting protein that serves as an internal control. The multiple “dSLBP” bands in lanes 1–3 and 5 are due to different endogenous dSLBP isoforms (Figure 1). (E) HA-T230A produced in vivo binds the stem-loop. Extracts were prepared from transgenic embryos expressing either HA-dSLBP (top) or HA-T230A mutant (bottom). Lane 1, an aliquot of each extract analyzed by gel electrophoresis. The extracts were incubated with the SLbi RNA (lane 3) or the RSbi RNA (lane 2) and the bound proteins from equivalent amounts of extract, 20 $\times$ the amount analyzed in lane 1, were analyzed by gel electrophoresis. The HA-tagged SLBPs were detected by Western blotting with an anti-HA antibody. Note that the HA-SLBP in the input lanes does not give sharp bands because of the presence of large amounts of comigrating yolk protein.

proteins analyzed by SDS-electrophoresis and Western blotting. In this assay, the endogenously synthesized HA-T230A mutant dSLBP bound the stem-loop similarly to HA-tagged wild-type dSLBP (Figure 3E). These data indicate that the inability of the T230A mutant form of dSLBP to rescue viability and support processing in vivo is not due to an intrinsic inability of the protein to bind and process histone pre-mRNA.

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 transgenes (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 dSLBP 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 4, 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). dSLBP was 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

Figure 4. dSLBP intracellular localization at the onset of zygotic gene expression. Each panel contains a fixed embryo fluorescently stained with anti-HA antibody (red), anti-phosphohistone H3 (green) to detect mitotic cells, and DAPI (blue), and visualized by confocal microscopy. (A and B) Wild-type embryos lacking a transgene. No staining was detected throughout development, including in the epidermis (A) and the CNS (B) (asterisk in B indicates the brain). (C–I) Transgenic embryos containing two copies of the wild-type HA-SLBP construct. (C) A syncytial preblastoderm embryo. Note the lack of HA-SLBP. For the images in D–I, HA-SLBP alone is shown on the left, DAPI plus anti-phosphohistone H3 staining are shown in the middle, and HA-SLBP plus anti-phosphohistone H3 is shown on the right. (D–F) The head region of a ~3-h-old postblastoderm embryo (anterior at top left). HA-SLBP accumulates in all nuclei during G2 phase of cycle 14 “G2(14)”. HA-SLBP protein spreads throughout the cell as mitosis of cycle 14 “M(14)” occurs in the mitotic domain pattern (domains 1, 2, and 5 are indicated). (G–I) The same region of a slightly older postblastoderm embryo (anterior at left). During S phase of cycle 15 “S(15)” HA-SLBP is detected throughout the cell, in both cytoplasmic and nuclear compartments.

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-SLBP 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). Zy-

gotic HA-dSLBP expression occurs in the *stg* mutant, and the HA-SLBP 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-SLBP throughout embryogenesis (our unpublished data), consistent with it complementing the *Slbp*¹⁵ 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-SLBP protein is detected in both nucleus and cytoplasm throughout the cell cycle, as shown for

Figure 5. During the embryonic postblastoderm cycles, SLBP is predominantly nuclear during G2. For the images in A–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 in the right. (A–C) A germ band extended, postblastoderm embryo undergoing cycle 15 in the lateral epidermis. SLBP is predominantly nuclear in G2 of cycle 15 “G2(15)”. (D–F) SLBP again becomes localized to the nucleus during G2 phase of cycle 16 “G2(16)”. Cells near the tracheal placodes (TP) enter mitosis 16 (arrowhead) before the surrounding epidermal cells, which are in cycle 16 G2. (G–I) Whole mount embryo in situ hybridization by using a histone H3 coding region probe (ventral is down, and anterior is to the left). (G and H) Germ band extended postblastoderm embryos. The cell cycle is indicated in parentheses. Histone mRNA is present throughout cycle 15 and through early interphase of cycle 16, with an elevated level during S phase; e.g. “S(16)”. (I) In G2 of cycle 16 “G2(16)”, histone mRNA declines sharply. (J) Histone mRNA is absent in cells that arrest in G1 of cycle 17 “G1(17)”. PNS and CNS cells continue to divide and express histone mRNA. Histone expression persists in a patch of cells in the first thoracic segment that are known to enter S phase of a 17th cell division cycle (Sauer *et al.*, 1995). Embryos successively increase in age from G to J.

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.

Figure 6. SLBP is enriched in the nucleus of *cyclin E* and *stg* mutant cells. SLBP staining (red) is shown on the left, DAPI (blue) plus anti-phosphohistone H3 (green) staining is shown in the middle, and SLBP plus DAPI staining is shown on the right. (A–C) A *stg*^{7B} null mutant embryo containing 2 copies of the HA-SLBP#16 transgene. All cells in this embryo arrest in G2 of cycle 14, and SLBP is located in the nucleus (arrowhead). (D–F) Stage 13 *Cyclin E*^{AR95} null mutant embryo containing two copies of the HA-SLBP#16 transgene. At this stage, all cells are arrested in G1. Some cells contain predominantly nuclear SLBP (arrowhead), and in others cells SLBP is distributed between nucleus and cytoplasm (arrow). *Cyclin E*^{AR95} mutant embryos were identified by lack of β -gal expression driven by a *wg-lacZ* transgene present on the balancer chromosome.

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 (Schumperli, 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-phosphohistone 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 stem-loop and an exonuclease 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-phosphohistone 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 16 and G1 of cycle 17 when 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

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-stained mitotic chromosomes in green. (A) Blastoderm embryo containing two copies of the HA-T230A#22 transgene. Note the exclusion of the mutant protein from the nucleus, unlike the nuclear accumulation of wild-type SLBP shown in Figure 4D. (B) Postblastoderm embryo of the same genotype. The aberrant localization of HA-T230A persists through the cell cycle. (C) Dorsal view of the brain lobes in a stage 13 embryo containing two copies of the HA-SLBP#16 transgene. In these rapidly dividing neuronal cells that have a very short G2 phase, SLBP is found throughout the cell during interphase and mitosis. (D) Brain lobe neuroblasts expressing HA-T230A#22. SLBP is localized predominantly near the cell periphery of this stage 14 embryo. (E) T120A/T230A#5 mutant SLBP is similarly localized predominantly near the cell periphery in this cross section of a stage 12 embryo brain. (F) Higher magnification view of epidermal cells along the ventral midline further showing the concentration of HA-T230A#22 at the cell periphery.

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 during S phase, although 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 its 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 T120A/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.

T230 Is Necessary for Location of SLBP in the Nucleus and Hence Is Required for Histone pre-mRNA Processing In Vivo

SLBP is a phosphoprotein in both mammalian and *Drosophila* cells. 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 Marzluft, 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

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.

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

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).

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 mis-processing 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-T230 dSLBP 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

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/cdk2) 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 production of replication dependent histone mRNAs during the cell cycle.

ACKNOWLEDGMENTS

We thank Judy Erkmann for critical reading of the manuscript. This work was supported by National Science Foundation Career Award 9984068 (to R.J.D.) and National Institutes of Health grant GM-58921 (to W.F.M.).

REFERENCES

- Allard, P., Champigny, M.J., Skoggard, S., Erkmann, J.A., Whitfield, M.L., Marzluff, W.F., and Clarke, H.J. (2002). Stem-loop binding protein accumulates during oocyte maturation and is not cell-cycle-regulated in the early mouse embryo. *J. Cell Sci.* 115, 4577–4586.
- Alt, J.R., Cleveland, J.L., Hannink, M., and Diehl, J.A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev.* 14, 3102–3114.
- DeLisle, A.J., Graves, R.A., Marzluff, W.F., and Johnson, L.F. (1983). Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. *Mol. Cell. Biol.* 3, 1920–1929.
- Dominski, Z., Erkmann, J.A., Greenland, J.A., and Marzluff, W.F. (2001). Mutations in the RNA binding domain of stem-loop binding protein define separable requirements for RNA binding and for histone pre-mRNA processing. *Mol. Cell. Biol.* 21, 2008–2017.
- Dominski, Z., Erkmann, J.A., Yang, X., Sanchez, R., and Marzluff, W.F. (2002a). A novel zinc finger protein is associated with U7 snRNP and interacts with the stem-loop binding protein in the histone pre-mRNP to stimulate 3'-end processing. *Genes Dev.* 16, 58–71.
- Dominski, Z., and Marzluff, W.F. (1999). Formation of the 3' end of histone mRNA. *Gene* 239, 1–14.
- Dominski, Z., Yang, X.C., Kaygun, H., Dadlez, M., and Marzluff, W.F. (2003). A 3' exonuclease that specifically interacts with the 3' end of histone mRNA. *Mol. Cell* 12, 295–305.
- Dominski, Z., Yang, X.C., Raska, C.S., Santiago, C., Borchers, C.H., Duronio, R.J., and Marzluff, W.F. (2002b). 3' end processing of *Drosophila melanogaster* histone pre-mRNAs: requirement for phosphorylated *Drosophila* stem-loop binding protein and coevolution of the histone pre-mRNA processing system. *Mol. Cell. Biol.* 22, 6648–6660.
- Edgar, B.A., and O'Farrell, P.H. (1989). Genetic control of cell division patterns in the *Drosophila* embryo. *Cell* 57, 177–187.
- Edgar, B.A., and O'Farrell, P.H. (1990). The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by string. *Cell* 62, 469–480.
- Edgar, B.A., and Orr-Weaver, T.L. (2001). Endoreplication cell cycles: more for less. *Cell* 105, 297–306.
- Foe, V.E. (1989). Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development* 107, 1–22.
- Foe, V.E., Odell, G.M., and Edgar, B.A. (1993). Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In: *The Development of Drosophila melanogaster*, vol 1, ed. M. Bate and A. Martinez Arias, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 149–300.
- Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998). MPF localization is controlled by nuclear export. *EMBO J.* 17, 4127–4138.
- Hanson, R.J., Sun, J., Willis, D.G., and Marzluff, W.F. (1996). Efficient extraction and partial purification of the polyribosome-associated stem-loop binding protein bound to the 3' end of histone mRNA. *Biochemistry* 35, 2146–2156.
- Harris, M.E., Bohni, R., Schneiderman, M.H., Ramamurthy, L., Schumperli, D., and Marzluff, W.F. (1991). Regulation of histone mRNA in the unperturbed cell cycle: evidence suggesting control at two posttranscriptional steps. *Mol. Cell. Biol.* 11, 2416–2424.
- Hartenstein, V., Rudloff, E., and Campos-Ortega, J.A. (1987). The pattern of proliferation of the neuroblasts in the wild-type embryo of *Drosophila melanogaster*. *Wilhelm Roux Arch. Dev. Biol.* 196, 473–485.
- Knoblich, J.A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C.F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* 77, 107–120.
- Lanzotti, D.J., Kaygun, H., Yang, X., Duronio, R.J., and Marzluff, W.F. (2002). Developmental control of histone mRNA and dSLBP synthesis during *Drosophila* embryogenesis and the role of dSLBP in histone mRNA 3' end processing in vivo. *Mol. Cell. Biol.* 22, 2267–2282.
- Marzluff, W.F., and Duronio, R.J. (2002). Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences. *Curr. Opin. Cell Biol.* 14, 692–629.
- Raska, C.S., Parker, C.E., Dominski, Z., Marzluff, W.F., Glish, G.L., Pope, R.M., and Borchers, C.H. (2002). Direct MALDI-MS/MS of phosphopeptides affinity-bound to immobilized metal ion affinity chromatography beads. *Anal. Chem.* 74, 3429–3433.
- Sanchez, R., and Marzluff, W.F. (2002). The stem-loop binding protein is required for efficient translation of histone mRNA in vivo and in vitro. *Mol. Cell. Biol.* 22, 7093–7104.
- Sauer, K., Knoblich, J.A., Richardson, H., and Lehner, C.F. (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of *Drosophila* embryogenesis. *Genes Dev.* 9, 1327–1339.
- Schumperli, D. (1988). Multilevel regulation of replication-dependent histone genes. *Trends Genet.* 4, 187–191.
- Smith, A.V., and Orr-Weaver, T.L. (1991). The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development* 112, 997–1008.
- Stauber, C., Luscher, B., Eckner, R., Lotscher, E., and Schumperli, D. (1986). A signal regulating mouse histone H4 mRNA levels in a mammalian cell cycle mutant and sequences controlling RNA 3' processing are both contained within the same 80-bp fragment. *EMBO J.* 5, 3297–3303.
- Sullivan, E., Santiago, C., Parker, E.D., Dominski, Z., Yang, X., Lanzotti, D.J., Ingledue, T.C., Marzluff, W.F., and Duronio, R.J. (2001). *Drosophila* stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. *Genes Dev.* 15, 173–187.
- Wang, Z.F., Ingledue, T.C., Dominski, Z., Sanchez, R., and Marzluff, W.F. (1999). Two *Xenopus* proteins that bind the 3' end of histone mRNA: implications for translational control of histone synthesis during oogenesis. *Mol. Cell. Biol.* 19, 835–845.
- Weigmann, K., and Lehner, C.F. (1995). Cell fate specification by even-skipped expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development* 121, 3713–3721.
- Whitfield, M.L., Zheng, L.X., Baldwin, A., Ohta, T., Hurt, M.M., and Marzluff, W.F. (2000). Stem-loop binding protein, the protein that binds the 3' end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. *Mol. Cell. Biol.* 20, 4188–4198.
- Yang, J., Bardes, E.S., Moore, J.D., Brennan, J., Powers, M.A., and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev.* 12, 2131–2143.
- Yang, J., Song, H., Walsh, S., Bardes, E.S., and Kornbluth, S. (2001). Combinatorial control of cyclin B1 nuclear trafficking through phosphorylation at multiple sites. *J. Biol. Chem.* 276, 3604–3609.
- Zheng, L., Dominski, Z., Yang, X.C., Elms, P., Raska, C.S., Borchers, C.H., and Marzluff, W.F. (2003). Phosphorylation of stem-loop binding protein (SLBP) on two threonines triggers degradation of SLBP, the sole cell cycle-regulated factor required for regulation of histone mRNA processing, at the end of S phase. *Mol. Cell. Biol.* 23, 1590–1601.