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Dual role for the RNA-binding domain of *Xenopus laevis* SLBP1 in histone pre-mRNA processing

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ABSTRACT

The replication-dependent histone mRNAs end in a conserved 26-nt sequence that forms a stem-loop structure. This sequence is required for histone pre-mRNA processing and plays a role in multiple aspects of histone mRNA metabolism. Two proteins that bind the 3′ end of histone mRNA are found in *Xenopus* oocytes. xSLBP1 is found in the nucleus, where it functions in histone pre-mRNA processing, and in the cytoplasm, where it may control histone mRNA translation and stability. xSLBP2 is a cytoplasmic protein, inactive in histone pre-mRNA processing, whose expression is restricted to oogenesis and early development. These proteins are similar only in their RNA-binding domains (RBD). A chimeric protein (1-2-1) in which the RBD of xSLBP1 has been replaced with the RBD of xSLBP2 binds the stem-loop with an affinity similar to the original protein. The 1-2-1 protein efficiently localizes to the nucleus of the frog oocyte, but is not active in processing of histone pre-mRNA in vivo. This protein does not support processing in a nuclear extract, but inhibits processing by competing with the active SLBP by binding to the substrate. The 1-2-1 protein also inhibits processing of synthetic histone pre-mRNA injected into frog oocytes, but has no effect on processing of histone pre-mRNA transcribed from an injected histone gene. This result suggests that sequences in the RBD of xSLBP1 give it preferential access to histone pre-mRNA transcribed in vivo.

Keywords: 3′ processing; histone mRNA; *Xenopus* oocyte

INTRODUCTION

Replication-dependent histone mRNAs are not polyadenylated; they end in a highly conserved 26-nt sequence containing a 16-nt stem-loop (Marzluff, 1992; Dominski & Marzluff, 1999). Histone pre-mRNAs do not contain introns, and only a single endonucleolytic cleavage to form the 3′ end (Gick et al., 1986) is necessary to generate a mature histone mRNA. This cleavage requires two *cis*-acting sequences in the histone pre-mRNA: the stem-loop and a purine-rich histone downstream element (HDE), which is located approximately 10 nt past the cleavage site. These elements are recognized by two *trans*-acting factors: the stem-loop binding protein (SLBP) (Wang et al., 1996c; Martin et al., 1997) and the U7 snRNP. The 5′ end of U7 snRNA base pairs with the HDE (Mowry & Steitz, 1987; Cotten et al., 1988; Soldati & Schümperli, 1988). Additional factor(s) that have not been well characterized, including a heat-labile factor (Gick et al., 1987), are also required. One role of the SLBP in the processing reaction is stabilization of binding of the U7 snRNP to the histone pre-mRNA (Spycher et al., 1994; Dominski et al., 1999), resulting in a stable complex containing histone pre-mRNA, SLBP, and U7 snRNP.

In mammalian cells, SLBP functions in the processing reaction in the nucleus and accompanies the histone mRNA to the cytoplasm, where it remains with the mRNA on polyribosomes as a component of the histone mRNP (Hanson et al., 1996). Recently we have cloned two SLBPs from frog oocytes (Wang et al., 1999). xSLBP1 is the homolog of the mammalian SLBP and is involved in histone pre-mRNA processing. The other protein, xSLBP2, is cytoplasmic and bound to histone mRNA in the frog oocyte. During oocyte maturation, xSLBP2 is destroyed and histone mRNA associates with xSLBP1 (Wang et al., 1999). These two SLBPs are similar only in their 73 amino acid RNA-binding domains (RBD) located in the center of each protein (Wang et al., 1999).

The availability of two SLBPs that bind the same RNA target has allowed us to construct and express chimeric proteins that retain the same RNA-binding specificity. Using complementation assays for histone pre-mRNA processing, we show that substitution of the RBD of xSLBP2 for the RBD of xSLBP1 results in a

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chimeric protein, 1-2-1, that does not function in processing in vivo or in vitro.

RESULTS

The 3′ end of histone mRNA and its bound SLBP play a critical role in multiple aspects of histone mRNA metabolism, including pre-mRNA processing (Dominski et al., 1995; Wang et al., 1996c; Martin et al., 1997), export (Eckner et al., 1991; Williams et al., 1994), translation (Sun et al., 1992; Gallie et al., 1996), and mRNA stability (Pandey & Marzluff, 1987; Williams et al., 1994). The frog oocyte is a convenient experimental system to study the many aspects of RNA metabolism because one can express or inject both specific RNAs and proteins and follow their subsequent metabolism.

Measurement of processing efficiency in *Xenopus* oocytes

When the mouse histone H2a-614 gene is injected into frog oocyte nuclei, properly processed histone mRNA is produced and the mature histone mRNA is efficiently transported to the cytoplasm (Williams et al., 1994). We developed an assay that detects both processed and unprocessed histone mRNAs generated from the injected mouse H2a-614 gene (Wang et al., 1999), allowing us to determine the efficiency of histone pre-mRNA processing in vivo (Fig. 1A). The 260-nt probe detects processed histone H2a-614 mRNA as a 183-nt fragment. Any unprocessed histone mRNAs (which would be expected to have heterogeneous 3′ ends) will protect a 217-nt fragment (Fig. 1A). Because the probe and each protected fragment has a single radioactive phosphate, the efficiency of processing can be determined by comparing the intensity of the two protected fragments. Plasmid DNA that is injected into the oocyte cytoplasm is not transcribed, so the results are not affected by the efficiency of injecting the DNA into the nucleus.

The efficiency of histone pre-mRNA processing from an injected gene was dependent on the amount of DNA injected (and hence the amount of pre-mRNA synthesized). When small amounts of DNA (150 pg) were injected, we often observed nearly 100% processing; larger amounts of injected DNA lowered processing efficiencies, as some of the factors for processing became limiting with the increase in rate of production of histone pre-mRNA. We chose a DNA amount (450 pg) that gave processing efficiencies between 20 and 80%, depending on the batch of oocytes.

Unprocessed histone mRNA is stable in *Xenopus* oocytes

For this assay to be a valid estimate of the efficiency of histone pre-mRNA processing, it is necessary that the unprocessed pre-mRNA not be rapidly degraded. In somatic mammalian cultured cells, unprocessed histone pre-mRNA is virtually undetectable (Stauber & Schümperli, 1988; Pandey et al., 1994). This is due both to the rapid processing of the histone pre-mRNA in vivo and the rapid degradation of any unprocessed pre-mRNA in the nucleus (Pandey et al., 1994). In contrast, unprocessed histone pre-mRNA is easily detectable in *Xenopus* oocytes (Williams et al., 1994), as are unprocessed or prematurely terminated transcripts (Bentley & Groudine, 1988; Bentley et al., 1989; Middleton & Morgan, 1990) from other genes. Thus it is likely that most RNAs, including histone pre-mRNA, are relatively stable in oocytes, unlike the situation in cultured cells.

To demonstrate the stability of histone pre-mRNA in oocytes, we did two different experiments. We carried out chase experiments and also expressed an uncleavable histone pre-mRNA in oocytes. In these experiments the oocytes were injected with blue Dextran, which remains in the nucleus, together with the DNA. Because only oocytes that were successfully injected in the nucleus were analyzed, we can make quantitative comparisons between samples. Oocytes from each female processed histone pre-mRNA to a different extent, and in each experiment the H2a-614 gene was injected as a control. Thus the oocytes used in Figure 1B and in lanes 3 and 4 of Figure 1C processed the H2a-614 pre-mRNA with over 90% efficiency, whereas the oocytes used in Figure 1C, lanes 1 and 2 only processed the H2a-614 pre-mRNA with 60% efficiency. Actinomycin D (200 μg/mL in the media) completely blocked expression of histone RNA in *Xenopus* oocytes (Fig. 1B), whereas another inhibitor of RNA polymerase II transcription, DRB, reduced but did not abolish transcription of the histone gene (not shown). The H2a-614 gene was injected into the oocyte nucleus and the oocytes incubated for 18 h, during which time the histone genes were transcribed and about 60% of the histone pre-mRNA was processed (Fig. 1C, lane 1). RNA was prepared from half of the oocytes and the other half of the oocytes were incubated with Actinomycin D to block further transcription during a 5-h "chase" period. During the chase in the absence of transcription almost all of the remaining histone pre-mRNA was processed (Fig. 1C, lane 2), demonstrating that the pre-mRNA was sufficiently stable to allow subsequent processing. This result also demonstrates that histone pre-mRNA processing does not require ongoing transcription, and that the processed histone mRNA is stable. As determined by PhosphorImager analysis, the total amount of histone mRNA was essentially identical in the two samples.

To definitively demonstrate that histone pre-mRNAs were stable, we expressed a mutant histone pre-mRNA (H2a-4G) that is resistant to processing because it has Gs substituted at the cleavage site

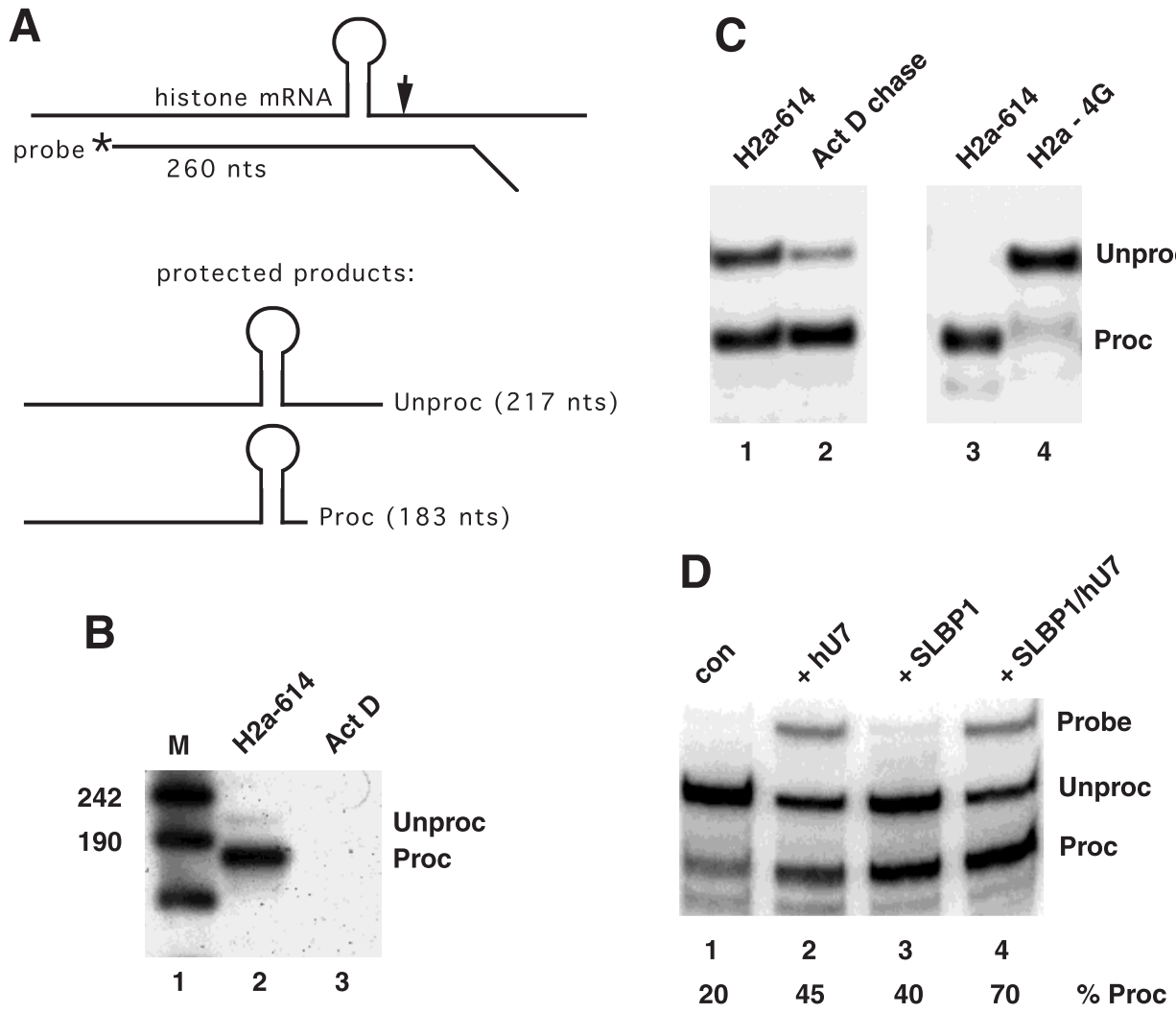

FIGURE 1. Characterization of the processing of histone pre-mRNA derived from injected DNA. **A**: The S1 nuclease protection assay used to measure histone pre-mRNA processing is diagramed. The 260-nt probe contains 43 nt that are not present in the H2a-614 histone pre-mRNA, but are derived from the vector pBluescript KS (zig-zag line). The unprocessed RNAs protect a 217-nt fragment regardless of their 3′ end and the processed histone mRNAs protect a 183-nt fragment. Because the probe is 3′-end labeled, the ratio of the intensity of the two fragments is a measure of processing efficiency. **B**: Frog oocytes were preincubated in buffer (lane 2) or with 200 μg/mL Actinomycin D (lane 3) for 1 h. They were then injected with the histone H2a-614 gene and blue Dextran, and incubated in the continued presence of the inhibitor for 18 h. RNA was prepared and then assayed for histone H2a-614 mRNA. Lane 1 is marker pUC18 digested with MspI. **C**: Oocytes were injected with the H2a-614 gene and blue Dextran, incubated for 18 h and then half of the oocytes were harvested (lane 1) and the other half were incubated for an additional 5 h in 200 μg/mL Actinomycin D (lane 2). A separate batch of oocytes was injected with either the H2a-614 gene (lane 3) or with the histone H2a-4G gene (lane 4) and incubated for 18 h. The H2a-4G gene produces a transcript that cannot be processed because of the insertion of four Gs at the cleavage site (Dominski et al., 1999). RNA was prepared from the oocytes and RNA from the equivalent of one oocyte analyzed by S1 nuclease mapping. **D**: Frog oocytes were injected with the histone H2a-614 gene and the levels of processed and unprocessed histone mRNAs measured 18 h later (lane 1). In lane 2, the human U7 gene (Jacobs et al., 1999) was coinjected into the nucleus with the H2a-614 gene. In lane 3 the oocytes were injected with synthetic xSLBP1 mRNA 30 h prior to injection of the histone H2a-614 gene. In lane 4, the oocytes were injected with the xSLBP1 mRNA 30 h prior to coinjection of the human U7 gene and the histone H2a-614 gene. The processing efficiency (percent processing) quantified using a PhosphorImager is indicated below each lane.

(Dominski et al., 1999). Histone pre-mRNAs expressed from this gene were not processed and the unprocessed pre-mRNA accumulated to high levels (Fig. 1C, lane 4). In a parallel batch of oocytes injected with the H2a-614 gene, more than 90% of the wild-type histone pre-mRNA was processed (Fig. 1C, lane 3). The amount of the histone pre-mRNA that accumulated from the H2a-4G gene was similar to the amount of processed mRNA accumulated from the H2a-614 gene (Fig. 1C, lanes 3 and 4). We conclude that the histone pre-mRNA is stable in the oocyte and that changes observed in the ratio of histone mRNA to histone pre-mRNA reflect differences in the efficiency of histone pre-mRNA processing.

xSLBP1 and U7 snRNP cooperate to process histone pre-mRNA in vivo

Histone pre-mRNA processing in oocytes requires both xSLBP1 (Wang et al., 1999) and the U7 snRNP (Scharl & Steitz, 1996) as well as other factors. A critical step in vitro is assembly of a stable complex containing SLBP and U7 snRNA on the histone pre-mRNA (Dominski et al., 1999), and efficient assembly of the complex requires both stable binding (high affinity) of SLBP to the stem-loop and base-pairing of U7 snRNP to the pre-mRNA (Dominski et al., 1999). To determine whether xSLBP1 or U7 snRNP were limiting for processing in vivo we increased the concentration of U7 snRNA by expressing U7 snRNA from the human U7 snRNA gene (Jacobs et al., 1999) and the concentrations of xSLBP1 by injecting synthetic xSLBP1 mRNA. There are excess snRNP proteins present in the frog oocyte that will assemble U7 snRNA into functional U7 snRNP particles (Strub & Birnstiel, 1986). In this experiment we used a batch of oocytes that processed the histone H2a-614 mRNA relatively inefficiently (Fig. 1D, lane 1). Overexpression of either U7 snRNA or xSLBP1 resulted in an increased efficiency of processing (Fig. 1D, lanes 2 and 3). Overexpression of both xSLBP1 and U7 snRNA in the same oocytes resulted in a further increase in processing (Fig. 1D, lane 4). Thus both U7 snRNP and xSLBP1 are present at suboptimal concentrations for processing the histone H2a-614 pre-mRNA expressed in the oocyte. This result is consistent with the suggestion that these two factors cooperate in histone pre-mRNA processing (Melin et al., 1992; Streit et al., 1993; Dominski et al., 1999).

Structure of xSLBP chimeras

The xSLBP1 and xSLBP2 proteins were divided into three domains, based on the definition of the 73 amino acids in the center of the protein as an RNA-binding domain (Wang et al., 1996c). We use the term RBD for the RNA-binding domain; this term simply reflects the ability of the 73-amino acid domain to bind RNA and is not meant to imply any similarity in structure to other RNA-binding proteins. This 73-amino acid region binds the stem-loop with an affinity similar to full-length SLBP (Wang et al., 1996c). The amino terminal and the carboxyl terminal regions were arbitrarily designated as other domains of the SLBPs. The amino acid sequences of the RBD of xSLBP1 and xSLBP2 are compared in Figure 2A. The terminal amino acids at each end of the RBD are conserved in both *Xenopus* SLBPs and in the mammalian SLBP. Only minimal changes were introduced at the amino terminal boundary of the domain during construction of the various chimeric proteins (Fig. 2B,C).

Each construct was transcribed with SP6 RNA polymerase to produce a synthetic capped polyadenylated mRNA that was injected into stage VI oocytes. Expression of the protein product was detected by its ability to bind a radiolabeled histone mRNA stem-loop (Williams & Marzluff, 1995). The oocytes were subsequently injected with the mouse histone H2a-614 gene and the ability of the protein(s) to affect histone pre-mRNA processing determined.

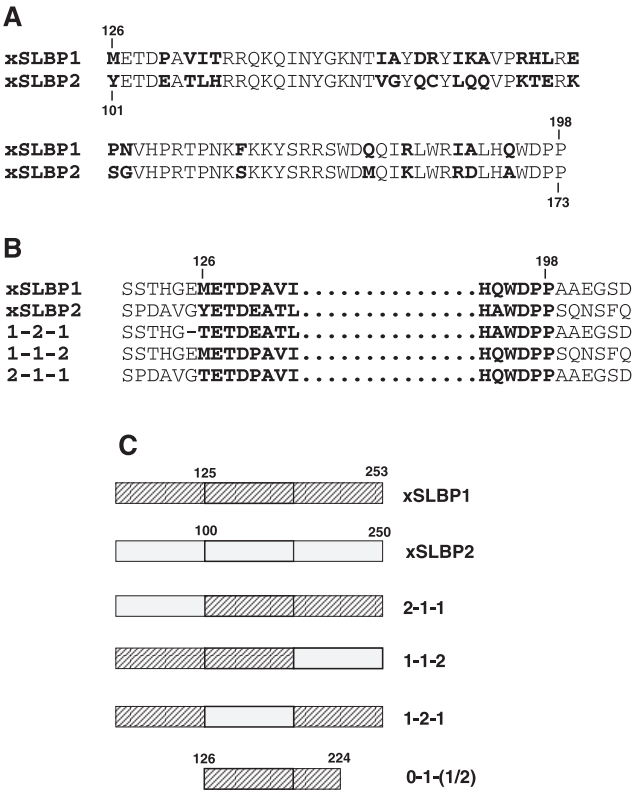

FIGURE 2. Chimeric clones of the SLBPs. **A**: The amino acid sequence of the RBDs of xSLBP1 and xSLBP2 is shown. The numbers indicate the position of each region in the amino acid sequence of the protein and the differences between the RBD of xSLBP1 and xSLBP2 are indicated in bold. **B**: The amino acid sequence immediately flanking the RBD of xSLBP1 and xSLBP2 is shown. The amino acid sequences at the junctions of chimeric SLBP clones are shown (the RBD is in bold). The same sequences are found in all the chimeric clones at the junctions of the SLBP1 and SLBP2 domains. **C**: The chimeric SLBPs, constructed from the three domains from xSLBP1 and xSLBP2, and the deletion clone 0-1-(1/2) used in this study are diagramed.

The xSLBP2 RBD cannot substitute for the xSLBP1 RBD in vivo

The RBD of SLBP1 and SLBP2 are 70% identical and bind the stem-loop with similar affinity (Wang et al., 1999). To determine if the only role of the RBD in histone pre-mRNA processing was to bind the protein to the pre-mRNA, we tested the ability of the chimeric 1-2-1 SLBP to complement processing. The same batch of oocytes was injected with a control solution or mRNAs encoding one of the following proteins: xSLBP1, *Xen-*

opus hnRNP A1 (as a control RNA binding protein), or 1-2-1. About 30% of the histone H2a-614 pre-mRNA was processed in this batch of oocytes (Fig. 3A, lane 2). Overexpression of xSLBP1 increased processing to >85% (Fig. 3A, lane 4), whereas expression of hnRNP A1 had no effect on processing (Fig. 3A, lane 3). Expression of 1-2-1 also had little effect on processing (Fig. 3A, lane 5). Similar results were obtained with oocytes from a different frog, where we injected xSLBP2 mRNA as well as xSLBP1 or 1-2-1 mRNAs. Again expression of xSLBP1 increased processing efficiency to >85% (Fig. 3B, lane 3), whereas expression of 1-2-1 (Fig. 3B, lane 4), or xSLBP2 (Fig. 3B, lane 2) did not have a significant effect on processing.

Because histone pre-mRNA processing occurs in the nucleus, we tested the ability of 1-2-1 to efficiently localize to the nucleus. The injection of synthetic mRNA encoding the various SLBPs resulted in at least a 10–20-fold increase in active (based on RNA binding) SLBP in the frog oocytes (Wang et al., 1999; see Fig. 4C, lanes 2–3 and 9–10). Synthetic mRNA encoding the relevant clone was injected into oocytes. Two days later, oocytes were fractionated into nuclear and cytoplasmic components and the fractions assayed by the mobility shift assay. As we have previously shown (Wang et al., 1999), overexpressed SLBP1 is found in both the nucleus and the cytoplasm in similar proportions as the endogenous SLBP1, and overexpressed xSLBP2 is

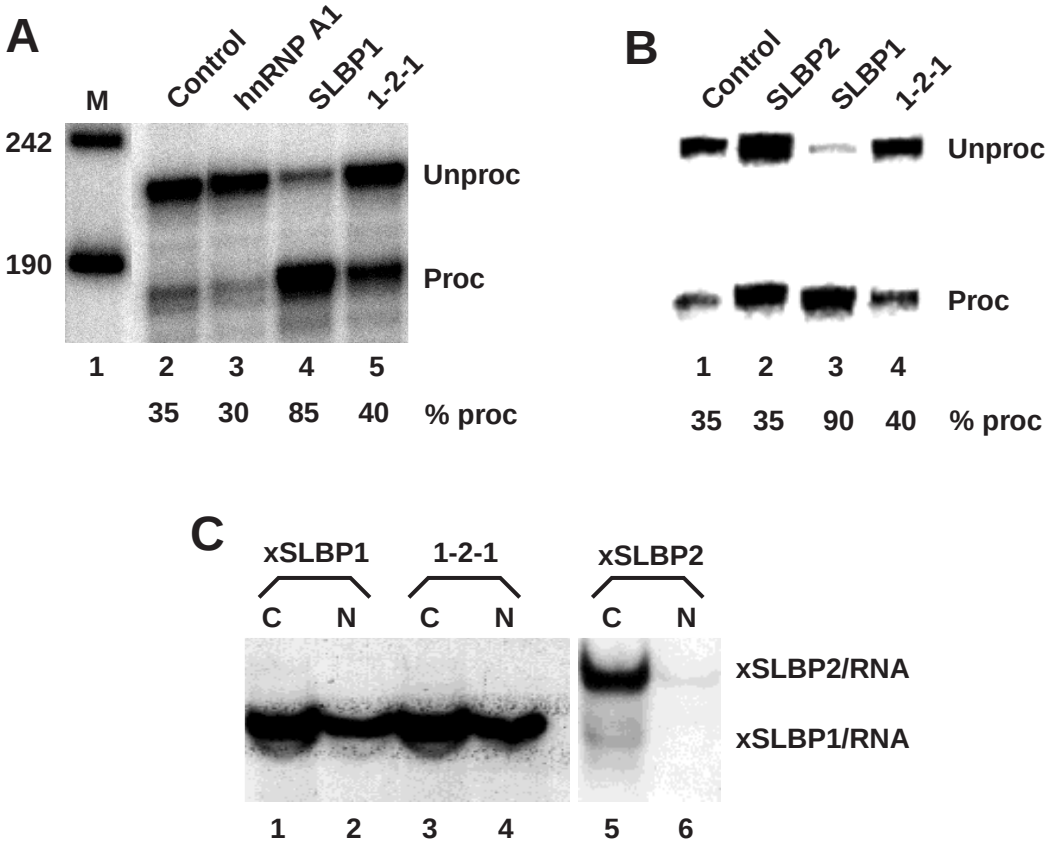

FIGURE 3. The xSLBP2 RNA binding domain does not function in histone pre-mRNA processing in vivo. **A**,**B**: *Xenopus* oocytes were injected with synthetic mRNAs encoding the indicated RNA-binding proteins. Forty-eight hours later the oocyte nuclei were injected with a plasmid encoding the mouse histone H2a-614 gene. Eighteen hours later RNA was prepared and assayed for processed (Proc) and unprocessed (Unproc) mouse histone H2a-614 mRNA using the S1 nuclease assay diagramed in Figure 1A. Lane labeled M is pUC18 DNA digested with *Msp*I. The results are representative of at least three independent experiments with each construct. **A**: Oocytes were injected with buffer (lane 2), synthetic mRNA encoding *Xenopus* HnRNP A1 (lane 3), xSLBP1 (lane 4), or the 1-2-1 chimeric protein (lane 5). The percent processing determined using a PhosphorImager is indicated below each lane. **B**: Oocytes were injected with buffer (lane 1), synthetic mRNA encoding xSLBP2 (lane 2), xSLBP1 (lane 3), or the chimeric protein 1-2-1 (lane 4) and assayed for the processing of the H2a-614 pre-mRNA. The processing efficiency (percent processing) quantified using a PhosphorImager is indicated below each lane. **C**: Oocytes were injected with synthetic mRNA encoding the indicated protein. Forty-eight hours later the oocytes were fractionated into cytoplasmic and nuclear fractions (C and N), and the localization of the proteins determined using the mobility shift assay. Each lane represents material from one oocyte. Only the complexes formed (designated xSLBP1/RNA and xSLBP2/RNA) are shown; the unbound probe is not shown in this experiment. Lanes 1 and 2: xSLBP1; lanes 3 and 4: 1-2-1; lanes 5 and 6: xSLBP2.

found exclusively in the cytoplasm (Fig. 3C, lanes 1, 2, 5, and 6). The 1-2-1 protein is imported into the nucleus as efficiently as the xSLBP1 (Fig. 3C, lanes 3 and 4), indicating that its inability to enhance processing was not a result of failure to localize to the nucleus.

The 1-2-1 chimeric protein has a similar RNA-binding affinity to SLBP1 in oocyte extracts

One possible explanation for the failure of the 1-2-1 protein to efficiently complement processing is that it might have a reduced affinity for the stem-loop. We compared the ability of xSLBP1 and the 1-2-1 protein to bind the 3′ end of histone mRNA using three different approaches.

We compared the binding affinity of the two proteins by expressing them from the synthetic mRNA in the rabbit reticulocyte lysate. To determine the amount of each protein synthesized, the proteins were labeled with ^{35}S-methionine (Fig. 4A, left panel). The lysates were then tested for their ability to bind the stem-loop using equal amounts of radiolabeled protein. Competition experiments were done using increasing amounts of competitor RNA (Fig. 4A, right panel). The two proteins bound the RNA probe to a similar extent and showed identical patterns of competition, demonstrating that they had similar affinities for the stem-loop RNA.

Each of the proteins was overexpressed in oocytes to similar levels by injection of synthetic pre-mRNA. To demonstrate that the two proteins were expressed at similar levels, we performed western blots using an antibody that recognized the N and C terminal domains of xSLBP1 (Fig. 4B, left panel). At the exposure shown in the western blot the endogenous xSLBP1 in uninjected oocytes is barely detectable (not shown), demonstrating the high level of expression relative to the endogenous protein. Similar amounts of SLBP–stem-loop complex were formed in oocytes expressing xSLBP1 (1-1-1) and 1-2-1 proteins (Fig. 4B, right panel).

We also compared the relative affinities of the SLBP in extracts from oocytes expressing the same proteins. The level of overexpression of SLBP active in RNA binding is high in all of these experiments and is clearly seen by comparing lanes 2 and 9 of Figure 4C with lanes 3 and 10, consistent with the results obtained from the western blot. Extracts were prepared from the oocytes and the affinity of the proteins for the stem-loop measured by the amount of competitor RNA necessary to compete formation of the stem-loop RNA complex. Because the concentrations of xSLBP1 and 1-2-1 are similar in the two extracts, this provides an estimate of the relative affinities of the two proteins under relatively physiological conditions in the presence of the total proteins from *Xenopus*. Each of the proteins also bound the stem-loop with a similar affinity by this criterion (Fig. 4C).

To further demonstrate the similar affinity of 1-2-1 and xSLBP1 for the stem-loop, three proteins, myc-tagged xSLBP1 (which is larger than xSLBP1 and can be distinguished from 1-2-1), 1-2-1, and a 94 amino acid protein 0-1-(1/2), were overexpressed in the same oocyte by injecting a mixture containing equal amounts of the three synthetic mRNAs. When these three proteins were expressed simultaneously in oocytes, three distinct complexes were formed with the stem-loop RNA in oocyte extracts (Fig. 4D). To assess the relative affinity of the three proteins, mobility shift assays were done using varying amounts of radiolabeled probe. At low probe concentrations, the three proteins are competing for limiting probe. At the three concentrations of the probe used, all three proteins formed complexes with the probe. The complexes are present in similar relative proportions (Fig. 4D, lanes 1–3), indicating that all three proteins bound the probe with similar affinity. If there were any slight differences in affinity, the 1-2-1 and 0-1-(1/2) proteins had a slightly higher affinity than the myc-SLBP1 protein, because at the lowest probe concentrations (Fig. 4D, lanes 2 and 3), these complexes were overrepresented compared with the amount of complex formed by the full-length xSLBP1. Using similar approaches we have previously shown that the full-length endogenous xSLBP1 and xSLBP2 proteins have similar affinities for the stem-loop (Wang et al., 1999).

Both the 1-2-1 protein and xSLBP1 (1-1-1) form very stable complexes with the stem-loop RNA. When the extracts from oocytes overexpressing these two proteins were incubated with the probe for 20 min followed by addition of excess unlabeled competitor RNA, the unlabeled competitor did not displace the pre-bound radiolabeled probe (Fig. 4E, lanes 3 and 5). In contrast, addition of the same amount of unlabeled probe together with the labeled probe completely prevented formation of the labeled complexes (Fig. 4E, lane 6). Thus, like the mammalian SLBP (Williams & Marzluff, 1995), the complexes between the stem-loop and the frog SLBP (and the 1-2-1 protein), are extremely stable. By all of these criteria, the 1-2-1 protein and the xSLBP1 have similar affinities for the stem-loop.

The 1-2-1 protein is also inactive in histone pre-mRNA processing in vitro

We next tested the ability of the 1-2-1 protein to function in an in vitro processing reaction. A nuclear extract from mouse myeloma cells efficiently processes a synthetic substrate containing the histone H1t downstream element. Processing of this substrate is completely dependent on the presence of SLBP (Dominski et al., 1999; Fig. 5A, lanes 1 and 3). The histone H1t pre-mRNA is also processed in *Xenopus* oocytes as effi-

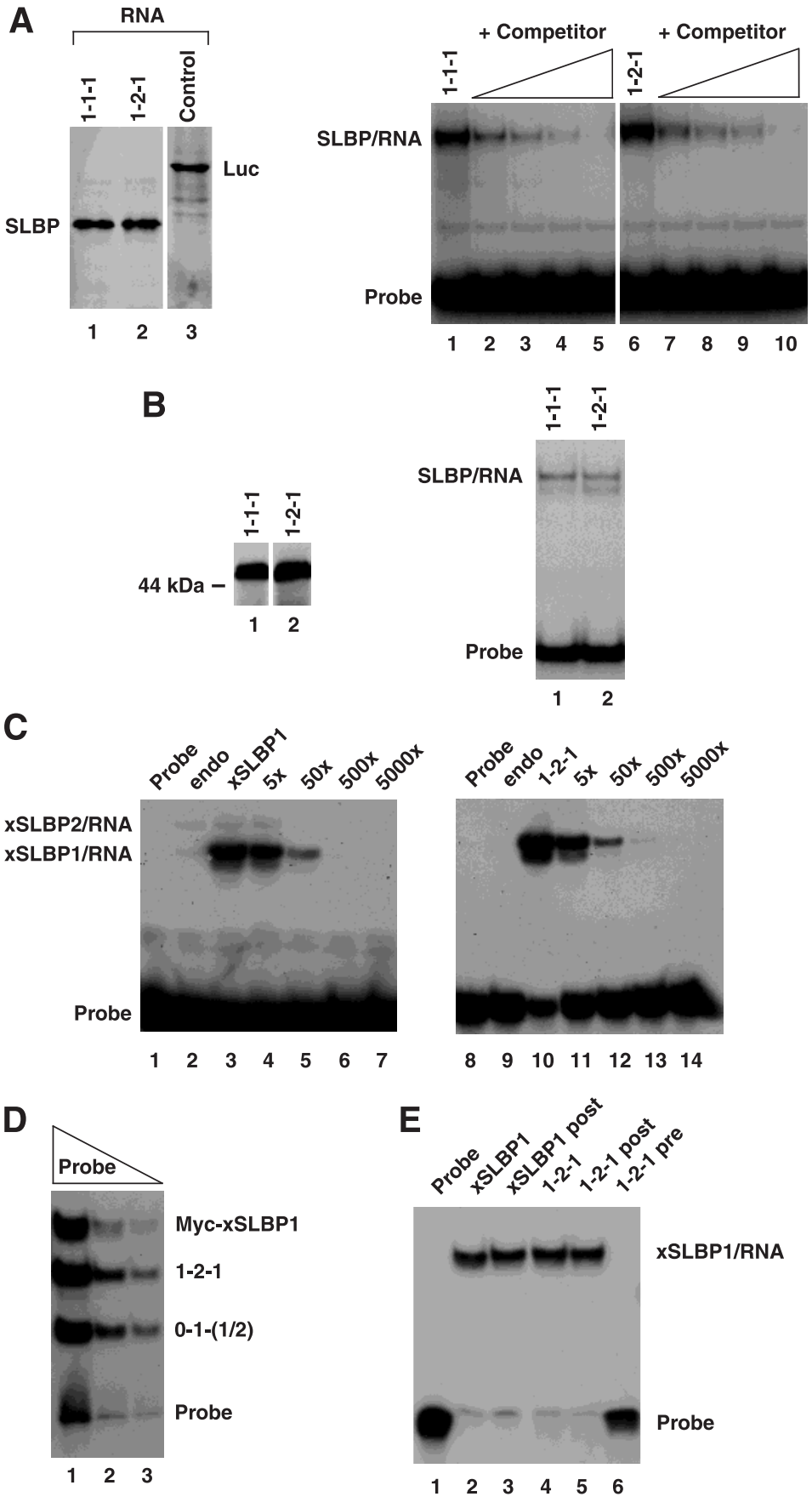

FIGURE 4. Relative binding affinities of xSLBP1 and the mutant SLBPs. **A**: xSLBP1 (1-1-1) and 1-2-1 were expressed from the same synthetic mRNAs used for microinjection in the reticulocyte lysate in the presence of ^{35}S-methionine. The proteins were resolved on a 10% SDS-polyacrylamide gel and detected by autoradiography (left panel). Lane 3 is a control luciferase mRNA. Equal amounts of the reticulocyte lysates were tested for the ability to bind the stem-loop using a mobility shift assay (lanes 1 and 6, right panel), in the presence of increasing amounts of competitor stem-loop RNA (lanes 2–5 and 7–10, right panel). **B**: Expression of xSLBP1 (1-1-1) and 1-2-1 detected by western blotting. Equal amounts of synthetic mRNAs encoding xSLBP1 (1-1-1) and 1-2-1 protein were injected into oocytes. Extracts were prepared from oocytes after 10 h and the proteins detected by western blotting (left panel) using an antibody that reacts with the N- and C-terminal regions of xSLBP1. The same extracts (1/60 oocyte) were used in a mobility shift experiment using the stem-loop as a probe (right panel). **C**: xSLBP1 (1-1-1) (lanes 3–7) and 1-2-1 (lanes 10–14) were overexpressed in stage VI *Xenopus* oocytes. Extracts from these oocytes were incubated with radiolabeled probe and increasing amounts of unlabeled stem-loop RNA. Complexes (xSLBP2/RNA and xSLBP1/RNA) were resolved by nondenaturing gel electrophoresis. Lanes 2 and 9 show the endogenous levels of xSLBP1 and xSLBP2. Lanes 1 and 8 show the probe. **D**: A myc-tagged xSLBP1 (myc-xSLBP1), 1-2-1, and 0-1-(1/2) were coexpressed in *Xenopus* oocytes by injection of a mixture of the three synthetic mRNAs. An extract from these oocytes was incubated with decreasing amounts of radiolabeled stem-loop RNA. The complexes were resolved by gel electrophoresis. **E**: xSLBP1 (1-1-1) (lanes 2 and 3) and 1-2-1 (lanes 4–6) were overexpressed in stage VI *Xenopus* oocytes. Oocyte extracts were incubated with the radiolabeled probe (lanes 2 and 4) or preincubated for 15 min with the probe before addition of a 1,000-fold excess of cold competitor (lanes 3 and 5) before analysis of the complexes by gel electrophoresis. In lane 6, the same amount of competitor was mixed with the probe and added. Lane 1 is the probe.

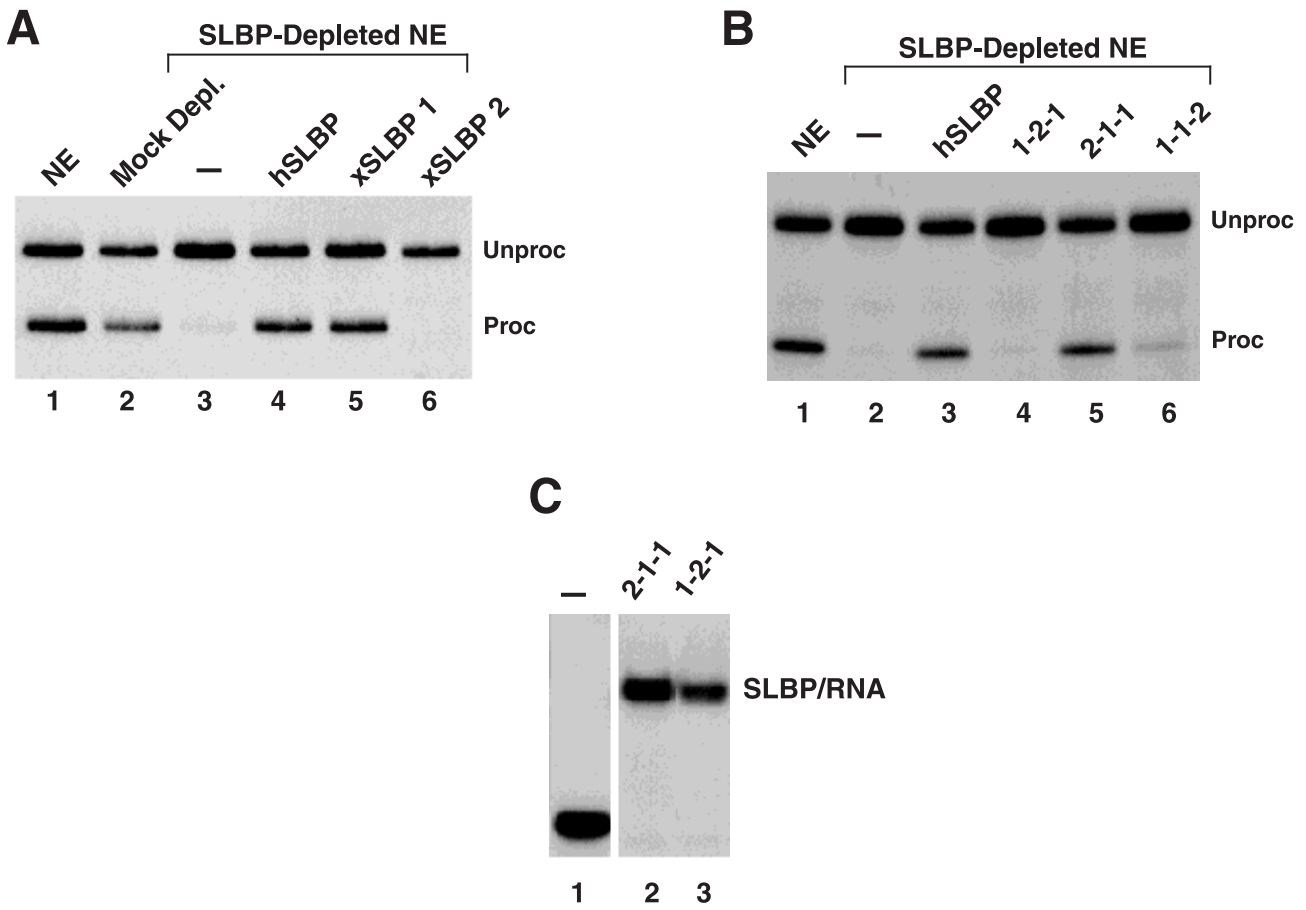

FIGURE 5. Complementation of processing of histone mRNA in vitro with chimeric proteins. **A**: A nuclear extract active in histone pre-mRNA processing was prepared from mouse myeloma cells (lane 1). Removal of the mouse SLBP from the extract using an antibody to its C-terminus abolished processing (lane 3), whereas depletion with IgG from preimmune serum had little effect on the activity (lane 2). The extract was supplemented with 20 ng of human SLBP (lane 4), frog xSLBP1 (lane 5), or frog xSLBP2 (lane 6). A radiolabeled synthetic histone pre-mRNA was added to the extract, incubated for 60 min at 32 °C and RNA prepared. The RNAs were resolved by gel electrophoresis and detected by autoradiography. The processed (Proc) and unprocessed (Unproc) mRNAs are indicated. **B**: The depleted extract (lane 2) was supplemented with 20 ng of the indicated recombinant proteins expressed in baculovirus (lanes 3–6). hSLBP is human SLBP. The processing reaction was carried out as in **A**. Lane 1 is undepleted nuclear extract. **C**: Twenty nanograms of the recombinant proteins 2-1-1 or 1-2-1 were incubated with the radiolabeled stem-loop and the complexes resolved by native gel electrophoresis. Lane 1 shows the free probe.

ciently as the H2a-614 pre-mRNA (data not shown). The mouse SLBP was removed from the extract using an antibody against the C-terminus of the protein (Wang et al., 1996c), abolishing processing of this histone pre-mRNA substrate (Dominski et al., 1999; Fig. 5A, lane 3). The depleted extract was complemented with various recombinant SLBPs expressed in baculovirus. Addition of the recombinant human SLBP expressed and purified from baculovirus restored processing to control levels (Fig. 5A, lane 4). Addition of the xSLBP1 protein also restored processing, whereas the xSLBP2 protein was inactive (Fig. 5A, lanes 5, 6; Wang et al., 1999). The chimeric protein 1-2-1 bound the stem-loop efficiently (Fig. 5C, lane 3), but was not able to complement processing in vitro (Fig. 5B, lane 4), clearly demonstrating a role for the RBD in the processing reaction in addition to its ability to bind the pre-mRNA. Consistent with our results with mammalian SLBP (Dominski et al., 1999), the 2-1-1 chimeric protein complemented processing (Fig. 5B, lane 5), whereas the 1-1-2 protein had very little activity (Fig. 5B, lane 6). This suggests that there is a requirement for the C-terminal domain as well as the RBD of xSLBP1 in processing.

Because the 1-2-1 protein binds the histone pre-mRNA with the same affinity as xSLBP1, we tested the ability of this protein to compete with mouse SLBP in the processing reaction in the nuclear extract. The nuclear extract processed about 70% of the synthetic histone H2a-614 pre-mRNA in 30 min (Fig. 6A, lanes 1 and 4). Addition of human SLBP to the extract increased the processing efficiency to $>$90% (Fig. 6A, lanes 2 and 5) whereas addition of the same amount of recombinant xSLBP2 reduced the processing efficiency to $<$10% (Fig. 6A, lane 3). A similar inhibition of pro-

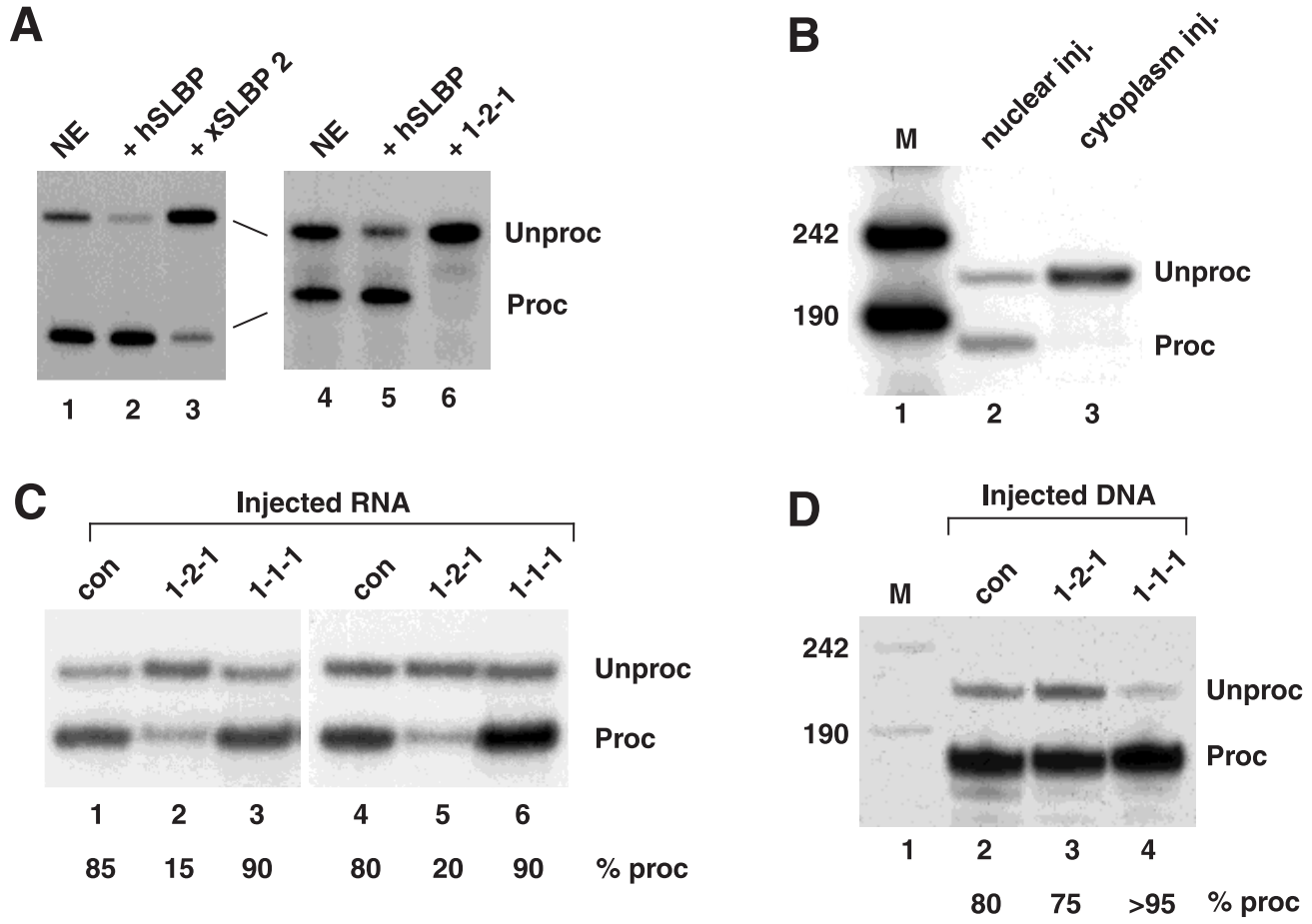

FIGURE 6. Processing of injected synthetic pre-mRNA in *Xenopus* oocytes. **A**: A nuclear extract prepared from mouse myeloma cells was incubated with the radiolabeled H1t pre-mRNA for 30 min (lanes 1 and 4). Seventy-five nanograms of human SLBP (lanes 2 and 5), xSLBP2 (lane 3), or 1-2-1 protein (lane 6) was added to the extract prior to addition of the H1t pre-mRNA. The RNA was resolved by gel electrophoresis and detected by autoradiography. **B**: Histone H2a-614 pre-mRNA together with blue Dextran was injected into the nucleus (lane 2) or the cytoplasm (lane 3) of *Xenopus* oocytes. RNA was prepared after 2 h and assayed as described in Figure 1. Lane 1 is marker pUC18 digested with *Msp*I. **C**: Two separate batches of frog oocytes were injected with buffer or with synthetic mRNA encoding either the 1-2-1 protein (lanes 2 and 5) or the full-length xSLBP1 (lanes 3 and 6) and incubated for 30 h. They were then injected in the nucleus with synthetic H2a-614 pre-mRNA plus blue Dextran. Two hours later RNA was prepared and analyzed as in **B**. **D:** The same batch of ooctyes as in **C**, lanes 1–3, were injected with the indicated synthetic mRNAs and incubated for 30 h, and then injected with the H2a-614 gene and blue Dextran in the nucleus. Eighteen hours later RNA was prepared and analyzed as in **B**. Lane M is marker pUC18 digested with *Msp*I.

cessing was obtained by addition of the 1-2-1 protein (Fig. 6A, lane 6). Thus recombinant proteins containing the xSLBP2 RNA binding domain compete for binding to the pre-mRNA substrate and act as dominant-negative proteins in the processing reaction.

Different requirements for processing of injected histone pre-mRNA and in vivo-transcribed histone pre-mRNA in *Xenopus* oocytes

Strikingly, the overexpressed 1-2-1 protein did not block the processing of histone pre-mRNA by the endogenous xSLBP1 (Fig. 3A, lane 5; Fig. 3B, lane 4), although the 1-2-1 protein was localized to the nucleus and was overexpressed at least 10-fold relative to the endogenous SLBP1 (Fig. 4). It was not a dominant-negative protein for processing pre-mRNA transcribed from a histone gene in vivo, although it acted as a dominant-negative protein in vitro.

One difference in these two systems is that in vivo the histone pre-mRNA was produced by transcription from an injected gene. It is possible that there was preferential recruitment of xSLBP1 to the histone pre-mRNA during transcription. We reasoned that injection of histone pre-mRNA (rather than histone genes) into *Xenopus* oocyte nuclei, might reveal different effects of the 1-2-1 protein on histone pre-mRNA processing, because, in that case, both the exogenously expressed and the endogenous proteins might have similar ac-

cess to the pre-mRNA in the nucleoplasm. Cleavage of a synthetic histone pre-mRNA injected into *Xenopus* oocyte nuclei was one of the initial lines of evidence for formation of the 3′ end of histone mRNA by an RNA processing reaction (Krieg & Melton, 1984).

A synthetic histone H2a-614 pre-mRNA identical in sequence to the pre-mRNA synthesized from the histone gene was injected into *Xenopus* oocyte nuclei or cytoplasm. The histone pre-mRNA injected into the nucleus was processed with about 60% efficiency in this experiment (Fig. 6B, lane 2), whereas the histone pre-mRNA injected into the cytoplasm was not processed, but was stable (Fig. 6B, lane 3). We injected about 10% as much histone pre-mRNA as was synthesized from the injected histone genes in 18 h and assayed processing after a 2-h incubation. Thus the concentration of histone pre-mRNA in the nucleus in the RNA injection and the histone gene injection experiments are likely to be similar. As with the histone gene injections, the efficiency of processing of the injected histone pre-mRNA varied between batches of oocytes.

To see whether there was a difference in the ability to affect processing on the same substrate introduced into nuclei by two different methods, oocytes were injected with synthetic mRNAs encoding the xSLBP1 and 1-2-1 proteins. They were subsequently injected in the nucleus with either the histone H2a-614 gene or with the synthetic histone H2a-614 mRNA. Because any histone pre-mRNA injected into the cytoplasm will not be processed (Stefanovic et al., 1995; Fig. 6B, lane 3), only oocytes that had been successfully injected in the nucleus, as determined by coinjection of blue Dextran, were analyzed. Overexpression of full-length xSLBP1 stimulated processing in the DNA-injected oocytes as shown previously (Fig. 6D, lane 4) and had a smaller stimulatory effect in the RNA-injected oocytes (Fig. 6C, lanes 3 and 6). In contrast overexpression of the 1-2-1 protein had little effect on processing of the in vivo generated histone pre-mRNAs (Fig. 6D, lane 3) consistent with the results shown previously in Figure 3. However, expression of the 1-2-1 protein inhibited processing about 80% in oocytes injected with the histone pre-mRNA (Fig. 6C, lanes 2 and 5).

The 1-2-1 protein can block processing, presumably by binding to the histone pre-mRNA in a nonproductive complex, in the nuclear extract and in oocytes into which histone pre-mRNA has been injected into the nucleoplasm of the germinal vesicle. In contrast, the histone pre-mRNA expressed from the histone DNA in oocytes is insensitive to the presence of the 1-2-1 protein, suggesting that xSLBP1 (and not the chimeric protein 1-2-1) is preferentially recruited to the site of histone gene transcription, where it then stimulates processing of histone mRNA transcribed from the injected gene.

DISCUSSION

The only processing event required for formation of histone mRNA is cleavage of the nascent transcript to form the 3′ end of histone mRNA. In the cell, this reaction probably also serves to release the nascent RNA from the chromatin template. Processing of histone pre-mRNA requires assembly of both SLBP and U7 snRNP on the pre-mRNA (Dominski et al., 1999). Increased expression of either of these factors in frog oocytes stimulates processing of histone pre-mRNA transcribed from an injected gene (Fig. 1D; Jacobs et al., 1999; Wang et al., 1999). We have previously shown that the two *Xenopus* SLBPs have different functions. xSLBP1 is orthologous to human and mouse SLBP and participates in histone mRNA 3′ end formation (Wang et al., 1999). xSLBP2 is expressed only during oogenesis, is degraded after oocyte maturation (Wang et al., 1999), and may be involved in storing maternal histone mRNA in a translationally inactive form.

The xSLBP1 RNA binding domain has multiple functions

Both xSLBP1 and xSLBP2 bind to the same *cis*-acting sequence with similar affinity, and contain similar RBD (Wang et al., 1999). Substitution of the RBD of xSLBP2 into xSLBP1 resulted in a protein, 1-2-1, that differs from xSLBP1 in only 22 of the 73 amino acids in the RBD. Surprisingly, this protein is totally inactive in processing both in vivo and in vitro even though it retains the ability to bind RNA. This result demonstrates that xSLBP1 does not contain distinct RNA-binding and -processing domains, unlike transcription factors, many of which have structurally distinct DNA-binding and transcription activation domains that are often interchangeable. Instead, specific sequences in the RBD of xSLBP1 are critical for processing, although they are not required for RNA binding. Specific amino acids in the RBD of xSLBP1 probably interact directly with other components of the processing machinery, possibly assisting the SLBP in stabilizing the binding of U7 snRNP to the histone pre-mRNA (Dominski et al., 1999). Interactions between the RBD of xSLBP1 and the U7 snRNP could help explain the strict dependence of processing on the distance between the stem-loop and the U7 snRNP binding site (Scharl & Steitz, 1994; Cho et al., 1995).

It is formally possible that the RBD of xSLBP2 contains a sequence that actively inhibits processing. If this were the case, then the sequences in the RBD of xSLBP1 would not actively participate in the processing reactions. We think this possibility is unlikely for two reasons. First, the 1-2-1 protein is not active in a mammalian extract. Mammals do not contain an SLBP2 and hence would not be expected to contain a putative inhibitor. Second, we have recently cloned the *Drosoph-*

ila SLBP, and constructed a similar hybrid protein in which the *Drosophila* RBD replaces the mammalian RBD (E. Sullivan and W.F. Marzluff, unpubl. results). This hybrid protein, 1-D-1, binds the stem-loop with high affinity and is also inactive in processing in both a mammalian extract and in frog oocytes (T.C. Ingledue, Z. Dominski, and E. Sullivan, unpubl.) although there is only one SLBP in *Drosophila* and this protein participates in histone pre-mRNA processing in *Drosophila*. These results strongly argue for a positive role of the RBD of xSLBP1 in histone pre-mRNA processing.

Different effects of the 1-2-1 protein on processing of histone pre-mRNA transcribed in the nucleus and histone pre-mRNA injected into the nucleus

One might expect that proteins that bind to the pre-mRNA but are not capable of processing would have a dominant-negative effect on the processing reaction, particularly when they are present in excess. This is true for both xSLBP2 and 1-2-1 when they were added to a nuclear extract. xSLBP2 is present exclusively in the cytoplasm (Wang et al., 1999), and so would not be able to affect histone pre-mRNA processing in vivo. However, the 1-2-1 protein is imported into the nucleus as efficiently as the xSLBP1 (Fig. 3C). When the 1-2-1 protein was expressed in large (>20-fold) excess over the endogenous xSLBP1, there was little effect on processing of histone pre-mRNA expressed from a histone gene, even though much of the 1-2-1 protein was present in the nucleus. In contrast, the overexpressed 1-2-1 inhibited processing of the injected histone pre-mRNA, although it had little effect on the processing of the same pre-mRNA generated in the oocyte nucleus. The failure of the overexpressed 1-2-1 protein to significantly affect the processing of the in vivo-synthesized pre-mRNA, suggests that the smaller amount of xSLBP1 (relative to 1-2-1) in the nucleus is preferentially recruited to the site of gene mRNA transcription, whereas the abundant 1-2-1 protein in the nucleus clearly does not have access to the histone pre-mRNA transcribed from the injected gene. A specific sequence in the RBD must be part of this selective recruitment mechanism. In contrast, when the histone pre-mRNA is injected into the nucleus, the 1-2-1 protein can then compete with the xSLBP1 for binding to the protein, as it can in the in vitro system.

In the cell, much of the U7 snRNP is found in Cajal (coiled) bodies, "spheres" in *Xenopus* oocytes (Wu & Gall, 1993; Wu et al., 1996), and these nuclear organelles are located near the histone genes in oocytes (Callan et al., 1991) and in mammalian cells (Frey & Matera, 1995; Matera, 1999), suggesting a role for the coiled bodies in histone pre-mRNA processing. Some of the xSLBP1 in the frog oocyte is also located in the spheres (Abbott et al., 1999). One possibility is that xSLBP1 may function in a distinct subnuclear compartment. The histone genes have remained tightly clustered throughout evolution, suggesting that there is selective pressure keeping them closely linked (Wang et al., 1996a, 1996b). One reason for this tight clustering may be that the genes are localized in a region of the nucleus specific for histone mRNA processing. Ninety-five percent of the U7 snRNA in the *Xenopus* oocyte nucleus is present in spheres (Wu & Gall, 1993). Spheres are found dispersed in the nucleoplasm and some are physically attached to the chromosomes at histone gene loci in amphibian oocyte lampbrush chromosome spreads (Callan et al., 1991). Recently Gall and coworkers have shown that xSLBP1 is present in the spheres of the amphibian germinal vesicle (Abbott et al., 1999). A region of xSLBP1, including part of the RBD and the C-terminal region, is capable of directing the xSLBP1 to coiled bodies (Abbott et al., 1999). The spheres also contain RNA polymerase II and coilin (Bellini & Gall, 1998), which has also been shown to associate with the U7 snRNP (Bellini & Gall, 1998).

Localization of xSLBP1 to the spheres (coiled bodies) may be important for efficient histone pre-mRNA processing in vivo. The 1-2-1 protein may be deficient in localizing to the coiled bodies or may not interact with unknown factor(s) necessary for processing in vivo, but not in vitro. In nuclear extracts, any nuclear substructures are disrupted allowing interaction of factors that may be physically separated in the nucleus in vivo. It is also likely that histone pre-mRNA processing is coupled to histone gene transcription in vivo, as is polyadenylation (McCracken et al., 1997). Thus in vivo there may be a defined pathway by which the histone pre-mRNA processing machinery is assembled and this pathway could be disrupted in nuclear extracts.

Differences in metabolism of the same RNA molecule transcribed from a gene and injected into the nucleoplasm are likely to be observed in many cases. Differences in the "masking" of mRNAs have been observed in *Xenopus* oocytes depending on whether synthetic pre-mRNA was injected or whether the pre-mRNA was expressed from an injected gene (Meric et al., 1996; Matsumoto et al., 1998). It is likely that many steps in nuclear RNA metabolism are tightly coupled in vivo, and can only be accurately reproduced starting with RNAs transcribed from the appropriate gene.

The histone genes represent a distinct class of genes transcribed by RNA polymerase II, and it is likely that there are novel pathways, distinct from those involved in metabolism of polyadenylated mRNAs, for histone mRNA metabolism. SLBP plays a central role in all aspects of histone mRNA metabolism and may interact with numerous potential binding partners, both in the nucleus and the cytoplasm.

MATERIALS AND METHODS

Subcloning of xSLBP1 and xSLBP2 and construction of chimeric proteins

PCR was used to amplify xSLBP1 and xSLBP2 cDNAs from the two-hybrid vector pGAD10, removing the 5′ and 3′ UTRs and inserting an *Nco*I site (xSLBP1) or *Bsp*H1 site (xSLBP2) at the initiation codon. The PCR products were digested with either *Nco*I/*Xba*I or *Bsp*HI/*Xba*I and subcloned into a modified p64T vector digested with *Nco*I or *Bsp*HI and *Xba*I, which provides 5′ and 3′ β-globin UTRs and a poly(A) tail for efficient translation of in vitro-transcribed RNA in oocytes (Wang et al., 1999). PCR was used to insert a *Kpn*I site at the 5′ end of the RBD of both xSLBP1 and xSLBP2, and a *Bam*H1 site into the 3′ end of the RBD of xSLBP2. There was a preexisting *Bam*H1 site at the 3′ end of the RBD of xSLBP1. The chimeric clones were then made by combining the fragments from the N-terminal domain (*Nco*I-*Kpn*I), the RBD (*Kpn*I-*Bam*H1), and the C-terminal (*Bam*H1-*Xba*I) domain. The sequence of all clones was confirmed at the University of North Carolina, Chapel Hill sequencing facility. The 0-1-1/2 clone was constructed by cloning the 100 amino acid fragment of SLBP1 into the same vector using PCR and introducing an *Nco*I site to provide an initiation codon at the beginning of the RBD.

Synthesis of mRNA and injection into *Xenopus* oocytes

The plasmids were linearized with *Eco*RI and used to make capped RNA with SP6 RNA polymerase (Wang et al., 1999). Ovaries were removed from female frogs and treated with 0.2% collagenase in OR-2 for 2 h (Kay, 1991). They were rinsed profusely with OR-2 and stored in OR-2 overnight for recovery. Healthy stage V-VI oocytes were injected with 45 nL of a solution containing 0.5 μg/μL of the in vitro-synthesized capped RNA encoding the SLBP constructs. The oocytes were incubated for 48 h at 18 °C in OR-2. RNA was prepared from pools of 30 oocytes for each point.

Histone pre-mRNA processing in oocytes

Forty-eight hours after the injection of the synthetic mRNA encoding SLBP proteins, healthy oocytes were then injected in the nucleus with 15 nL of mouse H2a-614 DNA, 0.03 μg/μL (450 pg), and incubated for 18 h in OR-2 prior to preparation of the RNA from pools of 30 oocytes for each point. For experiments with human U7 snRNA, 450 pg of a plasmid containing the human U7 gene was coinjected with the mouse H2a-614 gene (Jacobs et al., 1999).

The transcription inhibitor Actinomycin D (Sigma) was used at 200 μg/mL in the media to completely block transcription (Longo et al., 1996). Actinomycin D was dissolved in ethanol and the final concentration of ethanol in the media was less than 0.5%. Oocytes were treated with Actinomycin D for 1 h prior to H2a-614 gene injection and maintained in inhibitor in the dark. For the chase experiment, oocytes were injected with the H2a-614 gene, allowed to transcribe RNA for 18 h and subsequently incubated with Actinomycin D for 5 h in the dark before preparation of RNA.

Processing of synthetic pre-mRNA in oocytes

The mouse histone H2a-614 gene was amplified using PCR and oligonucleotides, with the 5′ oligonucleotide containing a T7 RNA polymerase promoter. A synthetic 640-nt capped pre-mRNA that contained the histone 3′ processing signal was synthesized as described above using T7 RNA polymerase. The pre-mRNA contained the entire H2a-614 mRNA and extended 100 nt past the 3′ end of the histone mRNA. Eight hundred picograms of the pre-mRNA were injected into the oocyte nucleus (together with blue Dextran to identify those oocytes that had been successfully injected in the nucleus). Two hours after injection of the synthetic pre-mRNA, the oocytes with blue nuclei were harvested. RNA was prepared from pools of 30 oocytes and the amount of processed histone mRNA was measured using an S1 nuclease protection assay described below.

RNA extraction and analysis

Oocytes (30 oocytes were pooled for each sample) were homogenized in 300 mM NaCl, 50 mM Tris, 10 mM EDTA, 2% SDS, and 5 μg/mL Proteinase K (Boehringer-Mannheim) and incubated for 1 h at 37 °C. The homogenate was extracted with water-saturated phenol/chloroform and then with chloroform. The RNA was precipitated from the aqueous phase by addition of 1/10 vol of 3 M NaOAC and 0.6 vol of isopropanol.

An oocyte equivalent of RNA was used to perform S1 nuclease analysis. A *Pst*I/*Hpa*II fragment of the mouse H2a-614 gene, extending from amino acid 85 in the coding region to 34 nt past the 3′ end of histone mRNA, was cloned into the *Pst*I/*Cla*I sites of BlueScript II SK (Stratagene) to generate pSL3. A 260-nt fragment was amplified by PCR from pSL3 using an oligonucleotide complementary to the T7 promoter and an oligonucleotide (5′-GCTGCTGGGCAAAGTGAC-3′) complementary to the coding region of the mouse H2a-614 gene. This fragment ends in a 3′ deoxycytidine followed by a random, terminal 3′ unpaired nucleotide from the PCR reaction. The PCR fragment was 3′-end labeled with the Klenow fragment of DNA polymerase I (New England Biolabs) and [α-^{32}P]-dCTP, using the 3′ exonuclease activity to exchange the terminal paired deoxycytidine. Ten nanograms of probe were hybridized with an oocyte-equivalent of RNA. The conditions of hybridization and digestion have been previously described (Wang et al., 1999). The protected fragments were resolved on a 7.5% polyacrylamide-7 M urea gel, detected by autoradiography and quantified on a PhosphorImager (Molecular Dynamics). Properly processed RNA protected a fragment of 183 nt and unprocessed RNA protected a fragment of 217 nt, extending to the region of divergence between the probe and the injected gene.

Mobility shift assays

Mobility shift assays were performed using a 30-nt synthetic RNA as a probe essentially as previously described (Williams & Marzluff, 1995). Oocyte extracts were prepared and extracted with freon to remove the yolk. For the nuclear localization experiments, oocytes were fractionated into nucleus and cytoplasm as previously described and the overexpressed

SLBPs detected by mobility shift assays (Wang et al., 1999). Competition experiments were done as previously described (Williams & Marzluff, 1995).

Baculovirus protein production

The chimeric and deletion constructs were subcloned into the baculovirus pFastBacHTb (Gibco BRL). SF9 cells were infected with the baculovirus for 96 h and the recombinant proteins purified on nickel affinity columns (Dominski et al., 1999). The proteins were checked for purity by SDS-gel electrophoresis and for RNA binding activity in a gel shift assay (Williams & Marzluff, 1995).

Complementation of histone pre-mRNA processing with baculovirus expressed proteins

A nuclear extract active in histone pre-mRNA processing was prepared from mouse myeloma cells as previously described (Dominski et al., 1995; Marzluff et al., 1997). SLBP was removed from this extract by incubation with SLBP antibody followed by removal of the antibody-SLBP complex by protein A agarose (Wang et al., 1996c, 1999; Dominski et al., 1999). The purified SLBPs expressed in baculovirus (20 ng) were added to the extract. A radiolabeled synthetic 86 nt RNA, including a stem-loop and the U7 binding site from the mouse histone H1t gene (Dominski et al., 1999), was incubated in this extract for 60 min at 32 °C. The RNA was purified and analyzed by polyacrylamide gel electrophoresis and the products detected by autoradiography.

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