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A 3′ Exonuclease that Specifically Interacts with the 3′ End of Histone mRNA

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Summary

Metazoan histone mRNAs end in a highly conserved stem-loop structure followed by ACCCA. Previous studies have suggested that the stem-loop binding protein (SLBP) is the only protein binding this region. Using RNA affinity purification, we identified a second protein, designated 3′hExo, that contains a SAP and a 3′ exonuclease domain and binds the same sequence. Strikingly, 3′hExo can bind the stem-loop region both separately and simultaneously with SLBP. Binding of 3′hExo requires the terminal ACCCA, whereas binding of SLBP requires the 5′ side of the stem-loop region. Recombinant 3′hExo degrades RNA substrates in a 3′-5′ direction and has the highest activity toward the wild-type histone mRNA. Binding of SLBP to the stem-loop at the 3′ end of RNA prevents its degradation by 3′hExo. These features make 3′hExo a primary candidate for the exonuclease that initiates rapid decay of histone mRNA upon completion and/or inhibition of DNA replication.

Introduction

Metazoan histone mRNAs are not polyadenylated but end instead with a highly conserved stem-loop structure, a 6 bp stem and a 4 nucleotide loop, followed in vertebrates by an ACCCA sequence (Dominski and Marzluff, 1999). The stem-loop interacts with the stem-loop binding protein (SLBP) (Wang et al., 1996), also referred to as the hairpin binding protein (HBP) (Martin et al., 1997).

Histone mRNAs are generated from longer precursors (pre-mRNAs) by an endonucleolytic cleavage immediately downstream of the stem-loop and the ACCCA (Muller and Schumperli, 1997; Dominski and Marzluff, 1999). This 3′ end processing depends on the binding of SLBP to the stem-loop and is carried out by a multicomponent machinery containing U7 snRNP (Mowry and Steitz, 1987). Formation of histone mRNAs by 3′ end processing is most efficient during S phase coupling histone synthesis with DNA replication (Harris et al., 1991). Cell cycle regulation of histone pre-mRNA 3′ end processing in mammalian cells is largely if not entirely achieved by cell cycle regulation of SLBP, which is present only in S phase and is destroyed by the proteasome pathway after completion of DNA replication (Whitfield et al., 2000). Following processing, SLBP remains associated with the stem-loop structure and accompanies mature histone mRNA to the cytoplasm (Hanson et al., 1996) where it stimulates histone mRNA translation (Sanchez and Marzluff, 2002; Ling et al., 2002).

The half-life of histone mRNAs is greatly reduced when DNA synthesis is completed, resulting in disappearance of histone mRNAs from the G2 cells and cessation of histone production (Marzluff and Duronio, 2002). Rapid and selective degradation of histone mRNAs also occurs in the presence of agents inhibiting DNA replication. In both cases, the response depends on the presence of the terminal stem-loop in the mRNA (Pandey and Marzluff, 1987; Graves et al., 1987). However, the mechanisms and factors involved in coordinating histone mRNA stability with DNA replication have not yet been identified.

In yeast, the major pathway of mRNA degradation involves initial deadenylation by Ccr4p (Chen et al., 2002; Tucker et al., 2002) followed by mRNA decapping and degradation of the mRNA body in a 5′ to 3′ direction by Xrn1p 5′ exonuclease (Muhlrad et al., 1994). The 3′ exonucleolytic pathway catalyzed by the exosome plays a minor role in turnover of most mRNAs after deadenylation (Mitchell et al., 1997; Anderson and Parker, 1998). In mammalian cells, degradation of polyadenylated mRNAs begins with shortening of the poly(A) tail by a poly(A)-specific exoribonuclease, PARN (Dehlin et al., 2000; Gao et al., 2000). Deadenylation is followed by degradation of the mRNA body in a 3′ to 5′ direction by the exosome (Allmang et al., 1999). The components of the decapping and 5′ exonuclease degradation system are present in the genomes of higher eucaryotes and are also likely involved in degradation of many mRNAs following deadenylation.

We used RNA affinity purification and mass spectrometry to identify a human 3′ exonuclease, which has a very high affinity to the natural 3′ end of histone mRNAs and is related to PARN and one of the exosome subunits, PM-Scl 100 kDa. This protein, designated 3′hExo, binds very weakly to histone mRNAs containing substitutions within the 3′ terminal stem-loop and to the histone pre-mRNA, in which the wild-type stem-loop is located internally. The 3′hExo can bind to the stem-loop simultaneously with SLBP. Binding of these two proteins depends on both common and distinct specificity determinants in the stem-loop region. The possible consequences of tethering 3′hExo and SLBP to the stem-loop for selective rapid degradation of histone mRNAs are discussed.

Results

RNA-Mediated Pull-Down of Proteins Interacting with the Stem-Loop

SLBP was the only protein cloned by the RNA three-hybrid system that specifically interacted with the his-

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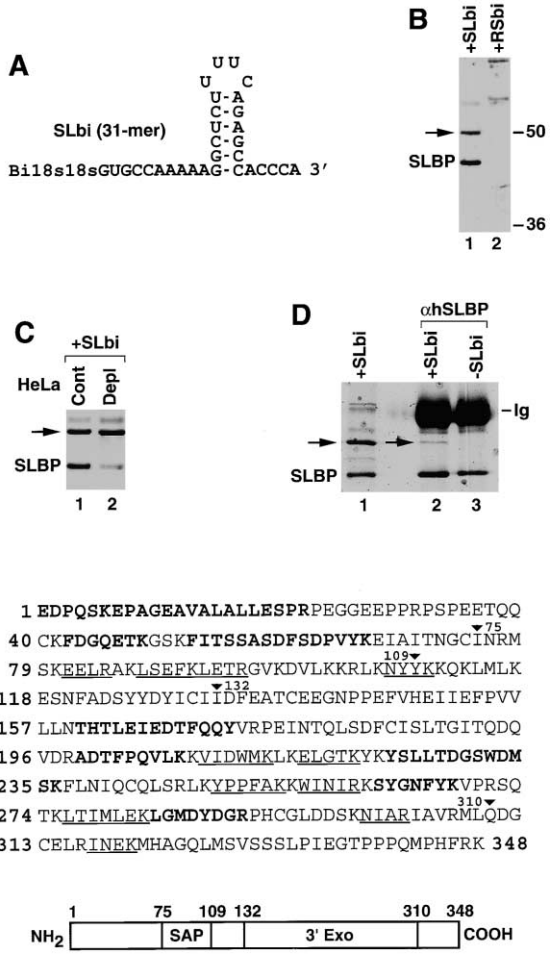

Figure 1. 3′hExo Specifically Binds the Histone mRNA 3′ End

(A) The sequence and secondary structure of the wild-type stem-loop RNA (SLbi) containing biotin (Bi) and two 18 atom spacers (18s) at the 5′ end is shown. In the reverse stem mutant RNA (RSbi), the sequence of the stem was reversed.

(B) Proteins bound to either SLbi (lane 1) or RSbi (lane 2) RNAs were resolved by gel electrophoresis and detected by staining with Coomassie blue. The unknown protein is indicated with an arrow and the position of 50 and 36 kDa size markers indicated.

(C) The unknown protein (arrow) was isolated with similar efficiency from both a control HeLa cell extract containing SLBP (lane 1) or from the same extract depleted of most of SLBP by anti-SLBP (lane 2).

(D) The unknown protein was coimmunoprecipitaed by anti-SLBP in the presence of the SLbi RNA (lane 2), but not in its absence (lane 3). Proteins bound by the SLbi are in lane 1. The heavy chain of the antibody is indicated with Ig.

(E) The amino acid sequence and schematic domain organization of 3′hExo. The sequenced peptides are in bold, and the peptides identified by molecular mass are underlined. The boundaries of the SAP domain (amino acids 75–109) and the exonuclease domain (amino acids 132–310) in 3′hExo are indicated with arrows and numbers.

tone stem-loop RNA (Martin et al., 1997; Wang et al., 1996). To determine whether there are other proteins that can interact with the stem-loop, we carried out RNA affinity experiments in HeLa whole-cell extract using a 31 nucleotide SLbi RNA containing the stem-loop structure and ending with ACCCA sequence, mimicking the 3′ end of mature histone mRNA (Figure 1A). The SLbi RNA contained biotin at the 5′ end, allowing isolation of bound proteins on streptavidin beads. Two proteins migrating at approximately 45 and 50 kDa bound to the SLbi RNA but not to the RSbi mutant RNA, in which the sequence of the stem was reversed (Figure 1B, lanes 1 and 2). The 45 kDa band was identified by Western blotting and mass spectrometry as SLBP (data not shown).

One possible explanation for concomitant isolation of SLBP and the unknown 50 kDa protein was that the two proteins form a tight complex together and binding to the RNA was mediated solely by SLBP. To test this possibility, the HeLa cell extract was depleted of SLBP using anti-SLBP antibody and the supernatant was incubated with the SLbi RNA. Despite the removal of more than 75% of SLBP from the extract, the amount of the 50 kDa protein isolated on streptavidin beads was comparable to that isolated from undepleted HeLa cell extract (Figure 1C, lanes 1 and 2). This result indicates the 50 kDa protein directly interacts with the stem-loop RNA. Next we addressed whether binding of SLBP and the 50 kDa protein to the stem-loop RNA is mutually exclusive or can occur simultaneously on the same RNA molecule. The whole-cell extract was incubated with anti-SLBP antibody both in the absence and in the presence of the SLbi RNA followed by absorption of complexes of the antibody and SLBP on protein A agarose beads and detection of coprecipitated proteins in SDS-polyacrylamide gels. While incubation of HeLa cell extract with anti-SLBP in the absence of the SLbi RNA resulted in isolation of only SLBP, the same antibody in the presence of the RNA also precipitated a small amount of the 50 kDa protein (Figure 1D, lanes 2 and 3). These experiments suggest that SLBP and the 50 kDa protein can bind simultaneously to the 31 nucleotide SLbi RNA.

The identity of the 50 kDa protein was determined by MS/MS electrospray mass spectrometry. Nine sequenced peptides were identified as peptides from a 348 amino acid protein deposited in Protein Information Resource (PIR) database (entry ID NF00830762). In addition, masses corresponding to ten more peptides were found in the sample giving an overall sequence coverage of 40% (Figure 1E).

Analysis of the 348 amino acid protein revealed the presence of two conserved motifs: a SAP domain located between amino acids 75 and 109, and a 3′ exonuclease domain located between amino acids 132 and 310 (Figure 1E). This protein, designated 3′hExo for histone mRNA 3′ end-specific exonuclease, has a predicted molecular weight of 40 kDa. The SAP domain (Aravind and Koonin, 2000), also described as a SAF-box (Kipp et al., 2000), has been defined as a 35 residue motif recognizing AT-rich regions in the chromosomal DNA known as scaffold-attachment regions (SARs). The exonuclease domain is similar to 3′ exonucleases of DEDD family characterized by the presence of four invariant acidic amino acids after which the family name has been originated (Zuo and Deutscher, 2001). The DEDD family includes both DNA- (deoxyribonucleases) and RNA-specific enzymes (ribonucleases).

Binding of 3′hExo to the Stem-Loop RNA in Yeast Cells and In Vitro

We cloned the cDNA for 3′hExo and determined whether 3′hExo binds the stem-loop RNA separately and/or si-

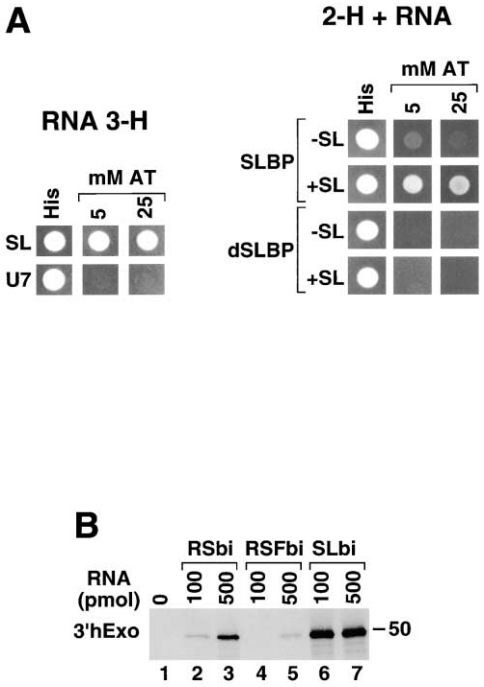

Figure 2. 3′hExo Expressed in Yeast Cells or In Vitro Binds the 3′ End of Histone mRNA

(A) Binding of 3′hExo to the stem-loop (SL) RNA was tested by the yeast RNA three-hybrid system (left), and binding of 3′hExo to SLBP alone (−SL) or the SLBP/SL complex (+SL) was tested using the RNA-supplemented two-hybrid system (right). Growth of yeast cells in the presence of 25 mM 3-AT is indicative of strong binding. In the yeast RNA three-hybrid system, the human U7 snRNA (U7) was tested for ability to bind 3′hExo as a negative control. In the RNA-supplemented two-hybrid system, human SLBP or *Drosophila* SLBP (dSLBP) were expressed in yeast cells together with 3′hExo either in the absence (−SL) or in the presence of the stem-loop RNA (+SL). (B) Binding of ^{35}S-labeled 3′hExo to the SLbi RNA (lanes 6 and 7) and two mutant RNAs; RSbi (lanes 2 and 3) and RSFbi (lanes 4 and 5). The sample in lane 1 contained no RNA.

multaneously with SLBP under in vivo conditions using the two previously described hybrid systems: the RNA three-hybrid system (RNA 3-H) and the RNA-supplemented two-hybrid system (2H+RNA). In the yeast three-hybrid system, developed to detect RNA-protein interactions (SenGupta et al., 1996), the DNA binding hybrid consisted of LexA DNA binding domain fused to the MS2 coat protein, while the activation hybrid consisted of the 3′hExo fused to the GAL4 activation domain. The bipartite RNA bait contained two MS2 binding sites at the 5′ end recognized by the MS2 coat protein and the histone stem-loop at the 3′ end (Wang et al., 1996). To test for nonspecific RNA binding by 3′hExo, we used a control RNA hybrid containing human U7 snRNA that also forms an extended stem-loop structure at the 3′ end. Yeast cells expressing the wild-type stem-loop RNA grew rapidly in the presence of 25 mM 3-AT (Figure 2A, left), indicative of a strong interaction between 3′hExo and the stem-loop structure. The yeast cells transformed with the U7 snRNA hybrid failed to grow in the presence of the lowest (5 mM) concentration of 3-AT, indicating that 3′hExo interacts with RNA in a sequence-specific manner.

To test whether 3′hExo and SLBP can bind the stem-loop simultaneously, we utilized the RNA-supplemented two-hybrid system, an assay originally developed for cloning proteins that interact with SLBP bound to the histone stem-loop RNA (Dominski and Marzluff, 2001; Dominski et al., 2002). In this system, human SLBP was fused to the GAL4 DNA binding domain whereas 3′hExo was fused to the GAL4 activation domain. Both proteins were expressed in yeast cells either in the absence or in the presence of the stem-loop (SL) RNA. In the absence of SL RNA, the growth of yeast cells was completely inhibited by 25 mM 3-AT and severely slowed down by 5 mM 3-AT, indicating that SLBP and 3′hExo do not directly interact with each other (Figure 2A, right). When the two fusion proteins were expressed in the presence of the SL RNA, the yeast cells grew efficiently on a selective medium containing 25 mM 3-AT, proving that human SLBP and 3′hExo, each of which individually interacts with the histone SL RNA, can bind the SL RNA simultaneously. When *Drosophila* SLBP (dSLBP) was expressed in the system instead of human SLBP, no growth of yeast cells was detected at the least stringent concentration of 3-AT (5 mM) either in the presence or absence of the SL RNA (Figure 2A, right). dSLBP, the ortholog of human SLBP involved in processing *Drosophila* histone pre-mRNAs, binds the SL structure with affinity similar to that of human SLBP (Lanzotti et al., 2002). Human SLBP and dSLBP are similar only within the RNA binding domain, and the inability of dSLBP to bind to the stem-loop simultaneously with 3′hExo is likely a consequence of the structural differences between the two SLBPs.

The ^{35}S-labeled 3′hExo synthesized in the rabbit reticulocyte lysate migrates at 50 kDa, with the same mobility as the protein isolated from HeLa cells. Rabbit reticulocyte lysate containing ^{35}S-labeled 3′hExo was incubated with the SLbi RNA, and the bound protein was absorbed on streptavidin beads and analyzed by polyacrylamide gel electrophoresis and autoradiography. No protein bound to the beads in the absence of RNA (Figure 2B, lane 1). Increasing the amount of the SLbi resulted in an increase in the amount of the bound protein, reaching a maximum in the presence of 100 pmol of the RNA (data not shown). The use of 500 pmol (1 μM) SLbi RNA did not result in any further increase in bound 3′hExo (Figure 2B, compare lanes 6 and 7). The RSbi mutant RNA was much less efficient in binding 3′hExo than SLbi; in the presence of 100 pmol of RNA, the difference between the two RNAs was 100-fold (Figure 2B, lanes 2 and 6). A moderate affinity of 3′hExo for the RSbi RNA, possibly resulting from recognition of sequences flanking the stem-loop, was seen at 1 μM RNA (Figure 2B, lane 3). The RSFbi RNA containing the reverse stem mutation and base substitutions in both flanks only weakly bound to 3′hExo at this RNA concentration (Figure 2B, lane 5).

Binding Specificities of 3′hExo

To determine which residues of the stem-loop RNA are critical for interaction with 3′hExo, we introduced a number of alterations within the RNA and tested their ability to pull down the ^{35}S-labeled protein. As an alternative to chemical synthesis of a large number of biotinylated RNAs, we synthesized various RNA mutants using T7 RNA polymerase and annealed them to a 3′-biotinylated

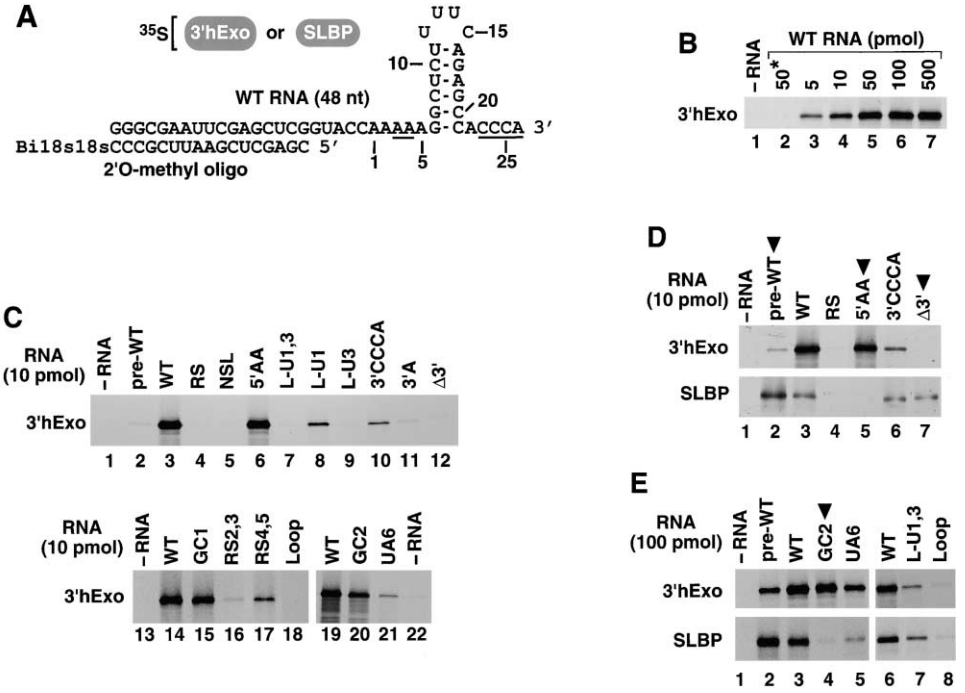

Figure 3. Binding Specificity of 3′hExo

(A) Diagram of the binding assay. The WT RNA was annealed to an excess of a complementary 2′O-methyl adaptor oligonucleotide containing biotin at the 3′ end. The duplex RNAs were used to assay binding of ^{35}S-labeled 3′hExo or SLBP. Nucleotides from the 3′ end of the H2a-614 mouse histone mRNA are numbered. The underlined nucleotides were changed to generate 5′AA and 3′CCCA RNAs.

(B) Increasing amounts of the WT RNA were added to a constant amount of ^{35}S-labeled 3′hExo. The star (lane 2) indicates the absence of the adaptor oligonucleotide.

(C) Ability of 10 pmol of indicated RNAs to bind ^{35}S-labeled 3′hExo.

(D and E) Ability of 10 pmol (D) and 100 pmol (E) of indicated RNAs to bind ^{35}S-labeled 3′hExo (top) or SLBP (bottom). The RNAs that selectively bound to one of the two proteins are marked with arrowheads.

2′ O-methyl oligonucleotide (an adaptor oligonucleotide) complementary to a common 17 nucleotide sequence at the 5′ end of each RNA (Figure 3A). The WT RNA was 48 nucleotides long and ended with the same 26 nucleotide stem-loop region present in the 31 nucleotide SLbi RNA (Figure 1A). We first established that the 48 nucleotide WT RNA annealed to the adaptor oligonucleotide binds 3′hExo with efficiency comparable to that of the SLbi RNA (Figure 3B, lanes 3–7).

Using this approach, we tested a number of mutations within the stem, the loop, and the flanks (Table 1). In most experiments, we used a suboptimal amount of the RNA (10 pmol, 20 nM) to increase specificity of the assay and to allow detection of mutations which exert relatively small effects on binding. The RS RNA or a non-stem-loop mutant RNA (NSL), both containing extensive changes in the stem-loop region, did not bind detectable amounts of 3′hExo (Figure 3C, lanes 4 and 5). Residues in the 5′ flank of the stem-loop region are necessary for sequence-specific recognition of the RNA by SLBP (Williams and Marzluff, 1995; Battle and Doudna, 2001). Thus, we replaced two conserved adenosines located 2 and 3 nucleotides 5′ of the stem (Marzluff et al., 2002) with uridines (5′AA). The 5′AA mutation did not significantly affect interaction of the RNA with 3′hExo (Figure 3C, lane 6), suggesting that the 5′ flanking region of the stem-loop is not recognized by this protein. We next altered the two virtually invariant uridines at positions 1 and 3 of the loop, critical for binding of SLBP (Williams and Marzluff, 1995; Martin et al., 2000; Battle and Doudna, 2001). The double mutation (L-U1,3) replacing the two uridines with adenosines abolished binding to 3′hExo (Figure 3C, lane 7). The same effect was exerted by a single substitution of uridine to adenosine in position 3 of the loop (L-U3, lane 9) while substitution of the first uridine to adenosine (L-U1) reduced interaction with 3′hExo to less than 10% of the WT RNA (Figure 3C, lane 8). These results demonstrate that the loop plays a critical role in the interaction between the RNA and 3′hExo. We also substituted the CCCA at the 3′ end with AACC (3′CCCA) or deleted this sequence either entirely, creating a mutant RNA ending with the stem (Δ3′), or partially, leaving a single adenosine following the stem (3′A). All three mutations reduced the binding to 3′hExo, although the nucleotide substitution in the 3′CCCA mutant (Figure 3C, lane 10) was not as detrimental as the two deletions, which almost completely abolished binding (Figure 3C, lanes 11 and 12). The importance of the RNA 3′ end for binding 3′hExo was further confirmed using a histone pre-mRNA (pre-WT), containing 38 additional nucleotides downstream from the ACCCA. Binding of 3′hExo to the pre-WT RNA was 100 times less efficient than to the WT RNA (Figure 3C, lanes 2 and 3). These data suggest that a proper length and sequence of the single-stranded tail that follows the stem-loop is an important feature recognized by 3′hExo.

Table 1. Binding of 3′hExo and SLBP to Various RNAs

RNA	Position	Change	Binding (%WT) 3′hExo	SLBP
WT	–	–	100	100
Pre-WT	3′ end	extended	1.0	150[a]
NSL	all	see text	0.0	0.0
5′AA	nt 3 and 4	U, U	100	5.0[a]
RS	bp 1–6	reversed	0.5	0.5
GC1	bp 1	CG	100	100
GC2	bp 2	CG	50	1.0[a]
RS2,3	bp 2 and 3	reversed	5.0	1.0
RS4,5	bp 4 and 5	reversed	15	ND
UA6	bp 6	AU	5.0	1.0
L-U1,3	loop 1 and 3	A,A	0.5	10
L-U1	loop 1	A	10	30
L-U3	loop 3	A	0.5	10
Loop	loop 1–4	GUAA	0.0	0.0
3′CCCA	nt 22–25	AACC	10	75
3′A	nt 23–26	deleted	1.0	100[a]
Δ3′	nt 22–26	deleted	0.5	75[a]

All RNAs, with the exception of those containing deletions of the 3′ end and the pre-WT, were 48 nucleotides long. The base pairs are numbered starting from the bottom GC pair, and the loop nucleotides are numbered 5′ to 3′. The NSL RNA contains a number of mutations that disrupt the stem-loop and change the flanking regions. Each RNA (10 pmol, 20 nM) was tested for its ability to bind ^{35}S-labeled SLBP or 3′hExo. The binding values indicate a percentage of the WT RNA binding efficiency (100%) and are an average of at least two independent experiments. ND, not determined.
[a] RNAs that selectively bind one of the two proteins.

To determine which parts of the stem are required for interaction with 3′hExo, we inverted selected base pairs in the stem. The strongest reduction in binding to 3′hExo was caused by reversing the top UA base pair to AU (Figure 3C, lane 21). Reversing the second GC base pair to CG had only a moderate effect (GC2, lane 20), while the mutation of the bottom GC did not affect binding (GC1, lane 15). All three base pairs are highly conserved in vertebrate histone mRNAs, and the second GC and the sixth UA base pairs, but not the bottom GC base pair, were previously shown to be critical for binding of SLBP (Williams and Marzluff, 1995; Battle and Doudna, 2001). Reversing the second and third base pairs together (RS2,3) almost eliminated binding to 3′hExo (Figure 3C, lane 16), whereas reversing of the fourth and fifth base pairs (RS4,5) reduced binding to 10% of WT (Figure 3C, lane 17). We also completely altered the sequence of the loop by replacing the UUUC with the GUAA (Loop, lane 18), previously shown to abolish binding of SLBP to the stem-loop RNA (Battle and Doudna, 2001). This GNRA-type loop found in many RNA secondary structures (Moore, 1999) also abolished binding to 3′hExo, arguing that the loop nucleotides in the histone mRNA provide critical specificity determinants for binding SLBP and 3′hExo rather than solely stabilizing the double stranded stem.

We directly compared effects of selected mutations on binding 3′hExo and SLBP using two different amounts of RNA, 10 and 100 pmol (Figures 3D and 3E). The RS mutation (Figure 3D, lane 4) and the two loop mutations (Figure 3E, lanes 7 and 8) had a similar effect on binding of each protein. However, the 5′AA mutation had a selective effect, abolishing the binding to SLBP while having no effect on binding to 3′hExo (Figure 3D, lane 5). Mutations within the 3′ end had opposite effect; binding to 3′hExo was reduced to 10% of the WT efficiency by the 3′CCCA mutations and abolished by the Δ3′ mutation, while the binding to SLBP was either only slightly weakened or unaffected by these mutations (Figure 3D, lanes 6 and 7). 3′hExo bound much stronger to the WT RNA than to the pre-WT, while SLBP at both RNA concentrations had a moderate preference for the pre-WT (Figures 3D and 3E, lanes 2 and 3). At higher RNA concentration, binding of 3′hExo was unaffected by the GC2 mutation and reduced 2-fold by the UA6 mutation (Figure 3E, lanes 4 and 5). These two mutations nearly abolished binding to SLBP, indicating that the second and sixth base pairs are more important for interaction of the RNA with SLBP than with 3′hExo. The relative binding affinities of SLBP and 3′hExo to various RNAs at 20 nM are summarized in Table 1.

A Stable Ternary Complex Containing the SL RNA, SLBP, and 3′hExo

We determined whether SLBP and 3′hExo form a stable ternary complex with the 30 nucleotide stem-loop (SL) RNA (Figure 5C) that could be analyzed by a mobility shift assay. The RNA (50 fmol) labeled at the 5′ end was incubated on ice for 30 min in the presence of 20 mM EDTA and 25 pmol of the baculovirus-expressed 3′hExo and SLBP, either separately or together. Each protein individually formed a stable binary complex with the probe (Figure 4A, lanes 2 and 5). Both the SLBP-RNA complex and the 3′hExo-RNA complex were shifted to a slower mobility complex by specific antibodies targeted to the C-terminal peptide of each protein (Figure 4A, lanes 4 and 7). We tested specificity of formation of each complex by adding a large excess of unlabeled 5′AA mutant RNA which efficiently binds to 3′hExo but is inactive in binding to SLBP. Formation of the SLBP-RNA complex was only weakly competed by the 5′AA RNA (Figure 4A, lane 3), while formation of the 3′hExo-RNA complex was prevented by this RNA (Figure 4A, lane 6).

A slower migrating ternary complex was readily formed in the presence of both SLBP and 3′hExo, confirming that the two proteins can simultaneously bind the SL RNA (Figure 4A, lane 9). The presence of both proteins in the complex was confirmed by further retardation of the ternary complex by either anti-3′hExo or anti-SLBP antibodies (Figure 4A, lanes 10 and 11).

Collectively, these data provide compelling evidence that the highly conserved 26 nucleotide sequence at the 3′ end of replication-dependent histone mRNAs contains binding sites for two proteins, SLBP and 3′hExo. SLBP recognizes specific residues in the stem-loop and the 5′ flank of the stem-loop, with an important role played by the adenosines in positions 3 and 4. The binding site for 3′hExo is located at the 3′ end of the 26 nucleotide region and encompasses residues in the stem-loop and the terminal ACCCA sequence. A simplified model depicting proposed interaction of both proteins with the stem-loop region is presented in Figure 4B.

3′hExo Is an Active 3′ to 5′ Exonuclease

All known 3′ exonucleases from the DEDD superfamily, including both DNA- and RNA-specific enzymes, have

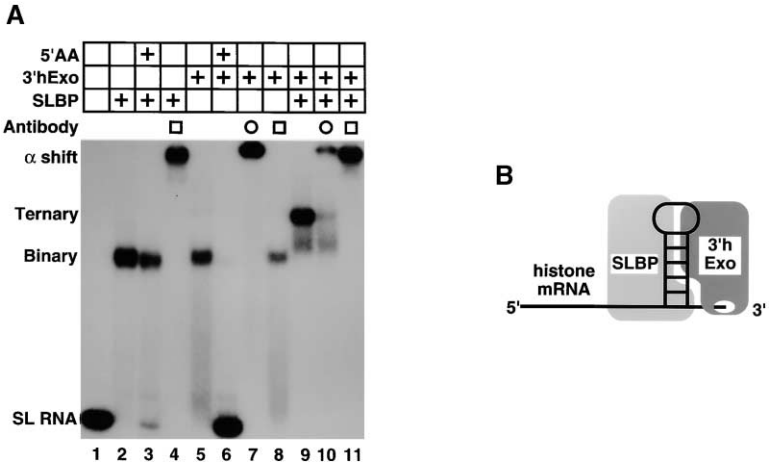

Figure 4. Purified SLBP and 3′hExo Can Simultaneously Bind the Stem-Loop RNA

(A) 25 pmol of SLBP (lanes 2–4), 3′hExo (lanes 5–8), or both proteins (lanes 9–11) expressed in the baculovirus system were incubated with 50 fmol of the 30 nucleotide stem-loop RNA (SL) labeled at the 5′ end (lane 1) and the complexes resolved in a 6% nondenaturing polyacrylamide gel. Each protein forms a binary complex with the RNA (lanes 2 and 5), and both proteins form a ternary complex with the RNA (lane 9). Samples in lanes 3 and 6 contain an excess of unlabeled competitor 5′AA RNA. Both the binary complexes and the ternary complex can be shifted to a higher position by specific antibodies against anti-SLBP (square) or anti-3′hExo (circle). The ternary complex associated with the 3′hExo antibody did not enter the gel, explaining the loss of radioactivity in the lane 10.

(B) A model depicting interaction of SLBP with the 5′ side of the stem-loop and 3′hExo with the RNA 3′ end.

a distributive mode of action and are dependent on divalent cations (Zuo and Deutscher, 2001). We tested the ability of 3′hExo to degrade several 5′-labeled RNAs in a buffer containing 2.5 mM Mg^{2+}. In the presence of excess amounts of 3′hExo (500 to 1 molar ratio of enzyme to substrate), the 48 nucleotide WT mRNA and the 86 nucleotide pre-WT were converted within 30 min at 37°C to the same major product which was several nucleotides shorter than the input WT RNA (Figure 5A, lanes 2 and 4). High-resolution gel electrophoresis revealed that the major product ended at the stem structure (data not shown) indicating that 3′hExo does not efficiently proceed into the stable double-stranded region of these RNAs.

We also determined the ability of 3′hExo to degrade a 30 nucleotide SL RNA. This RNA ends with the wild stem stem-loop followed by ACCCA but contains only 4 nucleotides 5′ of the consensus sequence (Figure 5C). In the presence of 0.25 pmol of the enzyme, 1–3 nucleotides were removed from the 3′ single-stranded end (Figure 5B, lanes 3 and 4). Surprisingly, degradation of the SL RNA in the presence of larger amounts of 3′hExo (25 pmol) progressed into the double-stranded region (Figure 5B, lanes 5 and 6), generating a major intermediate ending in the tract of uridines in the loop (Figure 5C). This intermediate is strikingly similar to the "−12" intermediate observed by Ross and coworkers in degradation of histone mRNA in vitro and in vivo (Ross et al., 1986). Apparently, the longer 5′ region preceding the stem-loop structure in the 48 nucleotide WT RNA has a stabilizing effect on the stem-loop, making it more resistant to 3′hExo activity. When we used the 48 nucleotide NSL RNA, which lacks any stable stem-loop structures, degradation in the presence of an excess of 3′hExo (25 pmol) rapidly preceded to a short RNA product, consisting of only 4–5 nucleotides (Figure 5D, lane 2, the short product is not shown). In the presence of 0.25 pmol of 3′hExo, the NSL RNA was converted to a ladder of multiple RNA intermediates differing in size by 1 nucleotide (Figure 5D, lane 3), indicative of a distributive mode of action.

Efficient degradation of the NSL RNA indicates that 3′hExo at high enzyme concentrations does not have inherent substrate specificity. We compared the ability of 3′hExo to degrade the WT RNA and the NSL RNA in the presence of limiting amounts of the enzyme. A 30 min incubation of the WT RNA in the presence of 2.5 fmol of 3′hExo (20-fold excess of the substrate over the enzyme) resulted in a complete disappearance of the substrate and generation of two major products, lacking two and three terminal nucleotides (Figure 5E, lane 2, top panel). After a 1 hr incubation, most of the input RNA was converted to the "−3" intermediate (Figure 5E, lane 3, top panel). The failure to degrade the substrate any further at this low enzyme concentration is likely due to the reduced affinity of 3′hExo for the WT RNA lacking the last 3 nucleotides. The same amount of the enzyme after 60 min incubation had no detectable activity toward the NSL mRNA (Figure 5E, lane 3, bottom panel).

SLBP Facilitates Binding of 3′hExo to the SL RNA

It is likely that binding of SLBP to the stem-loop structure is a major mechanism preventing the degradation of histone mRNAs by cellular 3′ exonucleases, including the exosome (Ford et al., 1997; Allmang et al., 1999) and 3′hExo. We tested whether SLBP can protect RNA against the exonucleolytic activity of 3′hExo. As a substrate we used the 30 nucleotide SL RNA since it is degraded by 3′hExo past the stem structure. The RNA substrate in each reaction was analyzed in denaturing polyacrylamide gels for the extent of degradation by 3′hExo and in native polyacrylamide gels for ability to form RNA-protein complexes. Samples containing 25 pmol of SLBP, 3′hExo, or both proteins were mixed on ice with 50 fmol of the SL RNA and 2.5 mM $MgCl_2$ and after 30 min incubation on ice were transferred to 37°C for 5 or 30 min. After a 5 min incubation with 3′hExo in the absence of SLBP, 3 nucleotides were removed from most of the input RNA (Figure 6A, lane 2). The same product was generated in the presence of SLBP (Figure 6A, lane 4). The protective function of SLBP on RNA

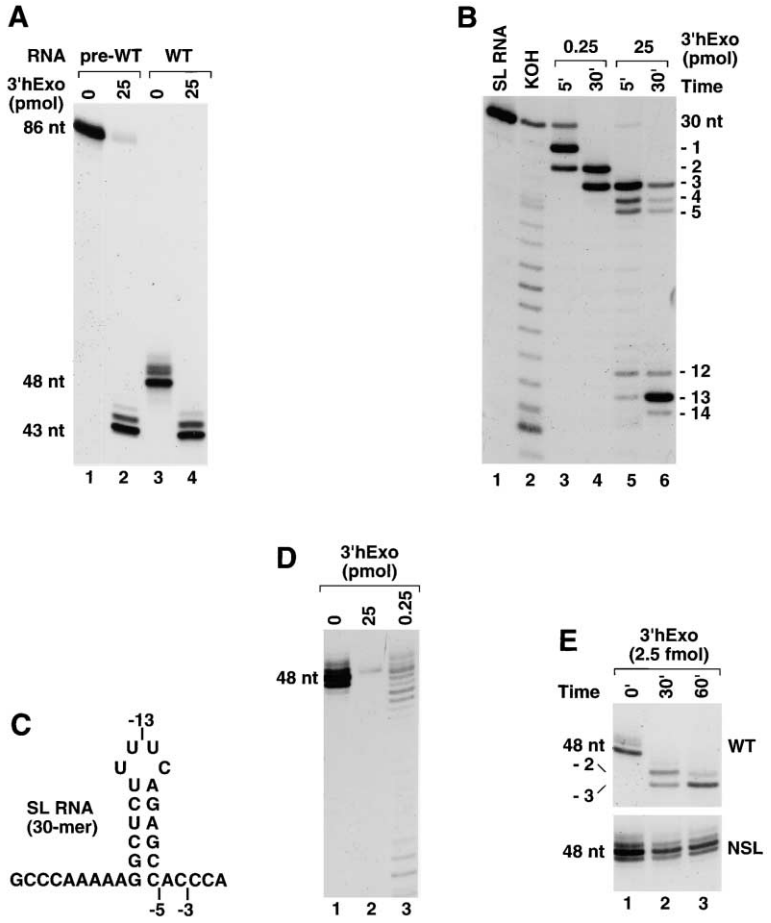

Figure 5. Baculovirus-Expressed 3′hExo Has Exonucleolytic Activity

Various RNAs (50 fmol) were labeled at the 5′ end and incubated with the indicated amounts of 3′hExo at 37°C in the presence of Mg^{2+}. The degradation products were analyzed in 8%–12% denaturing polyacrylamide gels and detected by autoradiography.

(A) In vitro degradation of the 86 nucleotide pre-WT (lane 2) and 48 nucleotide WT (lane 4) RNAs in the presence of 25 pmol of 3′hExo during 30 min incubation at 37°C. Lanes 1 and 3 contain the input RNAs.

(B) Progressive degradation of the 30 nucleotide SL RNA by 3′hExo (lanes 3–6). Lane 1 contains the input RNA and lane 2 products generated by partial KOH hydrolysis of the SL RNA.

(C) The sequence of the SL RNA and position of the degradation products detected in (B).

(D) In vitro degradation of an unstructured RNA (NSL) by 3′hExo during 30 min incubation at 37°C. Lane 1 contains the input RNA.

(E) Comparison of degradation of the WT and NSL RNAs in the presence of a limiting amount of 3′hExo.

degradation was clearly visible after 30 min incubation. In the absence of SLBP, most of the RNA substrate was degraded to short fragments, with the most predominant product having 13 nucleotides removed from the 3′ end (Figure 6A, lane 5), while degradation in the presence of SLBP did not advance further than during the 5 min digestion (Figure 6A, lane 7). These experiments demonstrate that binding of SLBP to the SL RNA efficiently protects the adenosine nucleotide directly following the stem. The mobility shift assay confirmed that all the substrate RNA was bound to SLBP (Figure 6B, lane 3). Consistent with Figure 3, removal of the last 3 nucleotides from the ACCCA sequence by 3′hExo during a 5 min incubation prevents formation of a stable complex between the enzyme and the stem-loop (Figure 6B, lane 2). Strikingly, 3′hExo can efficiently bind the same "−3" intermediate if SLBP is also bound to the stem-loop (Figures 6A and 6B, compare lane 2 with lanes 4 and 7). Thus, SLBP associated with the RNA must stabilize interaction of 3′hExo with the "−3" intermediate, suggesting that the two proteins directly interact with each other upon binding the RNA. Alternatively, the binding

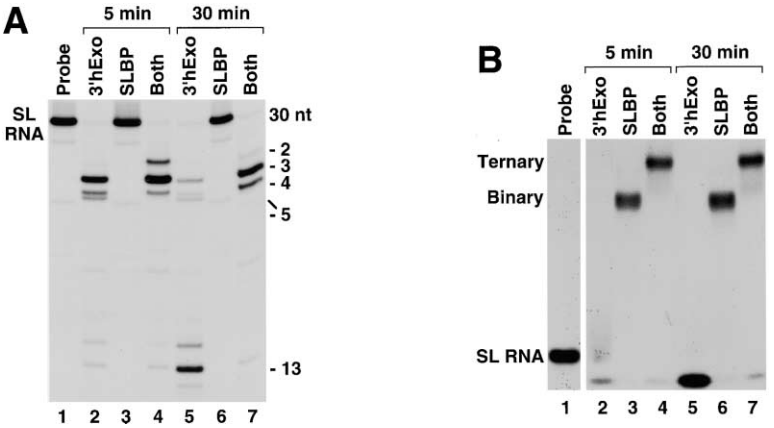

Figure 6. SLBP Both Facilitates Binding of 3′hExo to the SL RNA and Prevents Its Degradation

The 30 nucleotide SL RNA (50 fmol) was incubated with 25 pmol of 3′hExo (lanes 2 and 5), 25 pmol of SLBP (lanes 3 and 6), or both proteins together (lanes 4 and 7) in the presence of 2.5 mM Mg^{2+} at 37°C. Lane 1 contains the input RNA. An aliquot of the same reaction was analyzed in a 12% denaturing gel (A) for extent of RNA degradation and in a 6% native gel in the presence of EDTA (B) to detect formation of the binary or the ternary complexes. Note that the ternary complex is detected in lanes 4 and 7, while the same RNA does not form a complex with 3′hExo alone (lane 2).

of SLBP to the RNA results in structural rearrangements of the RNA making binding of 3′hExo more efficient. We conclude that association of SLBP with the stem-loop structure helps recruit 3′hExo to its binding site but at the same time protects the 3′ end of histone mRNA against extensive degradation.

Discussion

The highly conserved 26 nucleotide sequence at the 3′ end of metazoan replication-dependent histone mRNAs is specifically recognized by the stem-loop binding protein, SLBP. Interaction between SLBP and its RNA target plays an essential role in histone pre-mRNA 3′ end processing in the nucleus and stimulates translation of mature histone mRNAs in the cytoplasm. The stem-loop region is also required for regulation of histone mRNA stability but whether SLBP participates directly in this process is not known. Here we describe identification of a second protein that specifically interacts with the 3′end of histone mRNAs. This protein, referred to as 3′hExo, contains a conserved 3′ exonuclease domain and has 3′ exoribonuclease activity in vitro. The properties of 3′hExo suggest that it may play an important role in initiating the rapid decay of histone mRNAs that occurs in response to completion or inhibition of DNA replication.

The 3′ End of Histone mRNA Contains a Binding Site for Two Proteins

Tight binding of SLBP to the stem-loop requires the highly conserved second and the sixth (top) base pairs of the stem, GC and UA, respectively. Also important are the two virtually invariant uridines in positions 1 and 3 of the loop and the 5 nucleotides 5′ to the stem-loop (Williams and Marzluff, 1995; Martin et al., 2000; Battle and Doudna, 2001). The mutations in the loop had a comparable effect on interaction of the RNA with either SLBP or 3′hExo. The most notable difference were observed for the GC2 mutation changing the second GC base pair to CG and mutation upstream of the stem-loop replacing the two invariant adenosines, each of which abolished binding of the RNA to SLBP but did not significantly affect the interaction with 3′hExo. In contrast, mutations in the ACCCA sequence 3′ of the stem-loop that either deleted or substituted this region with other nucleotides did not affect interaction of the RNA with SLBP but abolished or severely reduced binding to 3′hExo. Significantly, the wild-type RNA terminating with the stem-loop followed by the ACCCA was approximately 100 times more efficient in binding 3′hExo than a pre-mRNA containing 38 additional nucleotides downstream from the mature 3′ end. SLBP had a reverse preference interacting slightly stronger with the pre-mRNA than with the mature mRNA (Battle and Doudna, 2001). This property allows SLBP to efficiently recognize the histone pre-mRNA prior to 3′ end processing and then to remain associated with mature mRNA after completion of processing. The last adenosine of the ACCCA sequence plays an important role in 3′ end processing by allowing the correct positioning of the cleavage site (Scharl and Steitz, 1994). The four remaining nucleotides located directly 3′ of the stem are highly conserved in mammalian histone mRNAs yet do not play any role in SLBP binding or in 3′ end processing (Scharl and Steitz, 1994; Furger et al., 1998; Battle and Doudna, 2001). Our results suggest that interaction of 3′hExo with the 3′ end of histone mRNA may be an important reason for the high conservation of these nucleotides.

Particularly surprising was the finding that 3′hExo and SLBP can form a stable complex on the same RNA molecule. In the presence of excess of SLBP and 3′hExo, the SL probe was quantitatively converted to the ternary complex indicating that each molecule of the RNA bound both SLBP and 3′hExo. Binding of the two proteins to the same RNA was also observed in whole-cell extracts containing excess RNA and low concentrations of each protein. Detailed mutagenesis studies confirmed that the conserved 3′ end of histone mRNA, previously viewed as a unique and exclusive binding site for SLBP, contains in fact an additional site for 3′hExo. Since binding of SLBP and 3′hExo to the stem-loop has both distinct and overlapping structural requirements, the two proteins must rely at least in part on separate specificity determinants provided by the same nucleotides or base pairs of the structure. The fact that SLBP facilitates binding of 3′hExo to the stem-loop RNA lacking the last 3 nucleotides suggests that binding of the two proteins to the 3′ end of histone mRNA is in part cooperative. We favor a model in which binding of 3′hExo and SLBP to their respective targets results in direct protein-protein contacts that additionally stabilize the ternary complex.

3′hExo Is a 3′ Exoribonuclease

3′hExo belongs to the DEDD family of 3′ exonucleases (Zuo and Deutscher, 2001). This family includes such RNA-specific 3′ exonucleases as RNase D required for 3′ end maturation of small stable RNAs, the vertebrate poly(A)-specific ribonuclease (PARN), and the human exosome component, PM-Scl 100 kDa autoantigen. The family also includes DNA-specific bacterial Exo I and Exo X, both involved in DNA repair, and the proofreading subunits of DNA polymerases. 3′hExo, as other 3′ exonucleases of the DEDD family, is magnesium dependent, acts in a distributive fashion, and does not require inorganic phosphate for activity indicating that it is a hydrolytic exonuclease. At higher concentrations, 3′hExo can nonspecifically remove single-stranded sequences but is not efficient in degrading RNA duplexes. In its ability to remove a CCA sequence adjacent to a stem structure, 3′hExo resembles another member of the DEDD family, *E. coli* RNases T (Deutscher and Li, 2001; Zuo and Deutscher, 2002). However, unlike RNase T, which can function as both DNase and RNase (Zuo and Deutscher, 1999; Viswanathan et al., 1999), 3′hExo does not degrade single-stranded DNA substrates (data not shown).

The most striking property of 3′hExo, not shared by any other known 3′ exonuclease, is the presence of the SAP domain, a 35 residue DNA binding motif recognizing AT-rich regions in the chromosomal DNA known as scaffold-attachment regions, SARs (Aravind and Koonin, 2000). The SAP domain has been identified in over 150 eukaryotic proteins including poly(ADP-ribose) polymer-

ase (PARP) and Ku70 subunit of DNA-dependent protein kinase (Aravind and Koonin, 2000). It is unclear what function is played by the SAP domain in 3′hExo, an exclusively RNA-specific exonuclease. Possibly, the SAP domain is a general nucleic acid recognition motif which can have specificity toward either DNA or RNA and in 3′hExo contributes to the sequence-specific recognition of the stem-loop.

A Role of 3′hExo in Histone mRNA Degradation

Although there are exonucleases with preferences for certain RNA substrates, to our knowledge 3′hExo is the first example of an exonuclease interacting with its substrate in a sequence specific manner. It is possible that the high-affinity binding of 3′hExo to the terminal stem-loop allows selective degradation of histone mRNAs at the low enzyme concentrations likely to exist in the cell. In vitro 3′hExo can readily degrade single-stranded RNA regions but stalls at the histone stem-loop and other stable secondary structures. Progression of the enzyme is additionally hampered by formation of a complex between the histone stem-loop and SLBP. Therefore, complete degradation of histone mRNA in vivo likely requires other factors. The fact that SLBP facilitates binding of 3′hExo to the stem-loop suggests that tethering of both proteins to the 3′ end of histone mRNA is a step in formation of a larger degradation complex. This complex could include an RNA helicase capable of displacing SLBP and melting the stem-loop structure (Tanner and Linder, 2001).

It is likely that 3′hExo only initiates degradation of histone mRNAs by destroying the binding site for SLBP and thus exposing the body of histone mRNA for subsequent degradation by the exosome. A related nuclease, PARN, serves a similar function in degradation of all other mRNAs by removing the polyA tail (Dehlin et al., 2000; Gao et al., 2000). It is also possible that the initial degradation of the stem-loop by 3′hExo is followed by decapping and degradation of the histone mRNA body by a 5′ exonuclease. However, this 5′ to 3′ degradation pathway, predominant in degradation of polyadenylated mRNAs in yeast, may be less important in mammalian cells (Wang and Kiledjian, 2001). We assume that SLBP and 3′hExo are tightly bound to two closely spaced motifs at the 3′ end of histone mRNA but form only weak protein-protein contacts that do not prevent subsequent displacement of SLBP from the RNA and further degradation of the stem-loop by 3′hExo.

Rapid decay of histone mRNAs at the end of S phase coincides with proteolytic degradation of SLBP by proteasome (Whitfield et al., 2000). Since it is unlikely that SLBP is targeted to the proteasome once bound to the histone mRNA, it must be first released from the complex by an active mechanism. Therefore, recruitment of a potential RNA helicase and 3′hExo to the stem-loop region may play a critical role not only in rapid degradation of histone mRNAs but also in timely proteolysis of SLBP during the cell cycle.

In conclusion, we identified a 3′ exonuclease, 3′hExo, with several unusual properties, including the presence of the SAP domain, sequence-specific and tight binding to the 3′ end of histone mRNA, and ability to coexist on the same RNA molecule in an intimate complex with SLBP, a major regulator of histone mRNA biogenesis, and metabolism. These properties make 3′hExo a primary candidate for an exonuclease initiating rapid degradation of histone mRNAs that occurs in the presence of DNA synthesis inhibitors and /or at natural completion of DNA replication at the end of S phase.

Experimental Procedures

RNA

The sequence of the SLbi RNA is shown in Figure 1. In the RSbi RNA (CCAAAAACCGAGAUUUCUCUCGGACCCA), the sequence of the stem was reversed. The RSFbi RNA (GUGCGAUUUGCCGAGAU UUCUCUCGGAGCUU) additionally contained substitutions within the flanking regions. All three RNAs had biotin at the 5′ end followed by two 18 atom spacers and were purchased from Dharmacon Research (Colorado). Nonbiotinylated RNAs, in most cases 48 nucleotides long, were synthesized with T7 RNA polymerase and started at the 5′ end with a common sequence of 17 nucleotides complementary to the 2′O-methyl adaptor oligonucleotide containing biotin and two 18 atom spacers at the 3′ end (Figure 3A). The sequence of the first 22 nucleotides of the non-stem-loop mutant RNA (NSL) was the same as in the 48 nucleotide WT RNA (Figure 3A) and was followed by AGAGCGAAUGAACACGAACCUAGUAG. Changes made in the WT RNA to generate other mutant RNAs are listed in Table 1. The 86 nucleotide WT histone pre-mRNA (referred to as pre-WT) was identical to the 48 nucleotide WT RNA but contained an additional sequence after the ACCCA including the histone downstream element (HDE) from the mouse H2a-614 gene (Dominski et al., 1999).

RNA Affinity Purification of Proteins Interacting with the Histone Stem-Loop

Approximately 2.5×10^9 HeLa cells (NCCC, Minneapolis, MN) were lysed in 25 ml of NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 10 mM sodium azide, 1 mM DTT, 1mM PMSF, 1× protease inhibitor mix, 0.5% NP40) and centrifuged. The supernatant was adjusted to 20 mM EDTA (pH 8) and incubated for 2 hr at 4°C with 5 μg of a biotinylated RNA containing the wild-type histone stem-loop (SLbi, Figure 1A) or the reverse stem mutation (RSbi). The samples were subsequently rotated for 2 hr in 4°C in the presence of 50 μl of streptavidin agarose beads (Sigma). The beads were rinsed several times with the NP-40 lysis buffer and bound proteins separated on a 15% SDS-polyacrylamide gel and detected by staining with Coomassie blue.

Protein Identification by Mass Spectrometry

The gel slice containing the 50 kDa unknown protein was digested with trypsin following standard protocols. Peptides eluted from the gel were HPLC separated prior to analysis by ESI-MS/MS on a Q-Tof (Micromass) mass spectrometer working in the regime of data-dependent MS to MS/MS switch.

Expression of 3′hExo

3′hExo was expressed in rabbit reticulocyte lysate (Promega) and in the baculovirus system (GIBCO-BRL) as previously described (Dominski et al., 1999).

Immunodepletion and Immunoprecipitation of SLBP

For depletion of SLBP, the whole-cell lysate from 2.5×10^9 HeLa cells (25 ml) was incubated with 25 μg of affinity-purified anti-SLBP followed by 2 hr incubation with 50 μl of protein A agarose beads (Pierce). For coprecipitation of 3′hExo by anti-SLBP antibody in the presence of the stem-loop RNA, the whole-cell extract from 2.5×10^9 HeLa cells was adjusted to 20 mM EDTA (pH 8), supplemented with 5 μg of the SLbi RNA, and incubated 30 min at 4°C. The sample was next rotated with 25 μg of anti-SLBP for 2 hr followed by 2 hr rotation with 50 μl of protein A agarose beads. The beads were washed several times with the NP-40 lysis buffer and resuspended in SDS-loading dye. Bound proteins were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie blue.

The ^{35}S Protein-RNA Binding Assay

The in vitro synthesized RNAs were annealed with the 17 nucleotide adaptor oligonucleotide and mixed with 400 μl of the NP 40 lysis buffer, 20 μl of 0.5 M EDTA (pH 8), and 5–15 μl of reticulocyte lysate containing ^{35}S-labeled 3′hExo or SLBP. Each sample was rotated at 4°C for 2 hr, then transferred to a new tube containing 25 μl of streptavidin agarose beads (Sigma) and rotated for an additional 2 hr. The beads were rinsed several times with the NP-40 lysis buffer, resuspended in SDS sample buffer, and the bound proteins were resolved on a 15% SDS-polyacrylamide gel and detected by autoradiography and/or phosphorimaging.

The Yeast Hybrid Systems

The yeast three-hybrid system (SenGupta et al., 1996; Zhang et al., 1999) and the yeast RNA-supplemented two-hybrid system (Dominski et al., 2002) were used as previously described (Dominski and Marzluff, 2001). In both systems, 3′hExo was fused to the GAL4 activation domain in the pGAD GH plasmid (Clontech).

In Vitro 3′ Exonuclease Assay and Mobility Shift Assay

The RNAs were labeled at the 5′ end as described (Dominski et al., 1999). The in vitro degradation assay was carried out in a total volume of 10 μl containing 135 mM KCl, 50 mM Tris [pH 8], 2.5 mM $MgCl_2$, 2.5% glycerol, 1 μg/μl bovine serum albumin, 50 fmol ^{32}P-labeled RNA (1×10^3 cpm), and a variable amount of the baculovirus-expressed proteins. Samples were assembled on ice and incubated for indicated time at 37°C. The reaction was stopped by addition of 4 vol of 7 M urea loading dye. The RNA was separated in 8%–12% denaturing polyacrylamide gels and detected by autoradiography and/or phosphorimaging. Samples for parallel analysis of RNA degradation and complex formation were kept on ice for 30 min to allow formation of the RNA-protein complex prior to transfer to 37°C. At the end of the incubation at 37°C, a small portion of each sample was supplemented with 7 M urea loading dye and analyzed on a denaturing gel while the remainder of the sample was adjusted to 20 mM EDTA and analyzed for complex formation in a 6% native gel.

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Accession Numbers

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