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Interaction Of Histone mRNA Hairpin With Stem-Loop Binding Protein And Regulation Of The SLBP-RNA Complex By Phosphorylation And Proline Isomerization

Minyou Zhang^{1,2}, TuKiet T. Lam³, Marco Tonelli⁴, William F Marzluff⁵, and Roopa Thapar^{1,2,*}

¹Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY 14203, USA

²Department of Structural Biology, SUNY at Buffalo, 700 Ellicott Street, Buffalo, NY 14203, USA

³WM Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT 06511, USA

⁴National Magnetic Resonance Facility at Madison, University of Wisconsin-Madison, Madison, Wisconsin 53706

⁵Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Abstract

In metazoans, the majority of histone proteins are generated from replication-dependent histone mRNAs. These mRNAs are unique in that they are not polyadenylated but have a stem-loop structure in their 3' untranslated region. An early event in 3' end formation of histone mRNAs is the binding of Stem-Loop Binding Protein (SLBP) to the stem-loop. Here we provide insight into the mechanism by which SLBP contacts the histone mRNA. There are two binding sites in the SLBP RBD for the histone mRNA hairpin. The first binding site (Glu129-Val158) consists of a helix-turn-helix (HTH) motif that likely recognizes the unpaired uridines in the loop of the histone hairpin and upon binding, destabilizes the first G-C base-pair at the base of the stem. The second binding site lies between residues Arg180-Pro200 which appears to recognize the second G-C basepair from the base of the stem and possibly regions flanking the stem-loop. We show that the SLBP-histone mRNA complex is regulated by threonine phosphorylation and proline isomerization in a conserved TPNK sequence that lies between the two binding sites. Threonine phosphorylation increases the affinity of SLBP for histone mRNA by slowing the off-rate for complex dissociation whereas the adjacent proline acts as a critical hinge that may orient the second binding site for formation of a stable SLBP-histone mRNA complex. The NMR and kinetic studies presented here provide a framework for understanding how SLBP recognizes histone mRNA and highlight possible structural roles of phosphorylation and proline isomerization in RNA-binding proteins in remodeling ribonucleoprotein complexes.

*To whom correspondence should be addressed: Tel: 716-898-8687; Fax: 716-898-8660; rthapar@hwi.buffalo.edu.

AUTHORS CONFLICT OF INTEREST DISCLOSURE

All authors declare that they have no financial or other conflicts of interest associated with this work.

SUPPLEMENTAL INFORMATION

Supplementary data includes seven figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

SLBP; histone mRNA; stem-loop; phosphorylation; prolyl isomerization; protein-RNA recognition; kinetics; surface plasmon resonance; NMR spectroscopy; mass spectrometry

Replication-dependent histone genes encode the bulk of histone proteins in metazoans(1). Histone proteins are important for packaging DNA into chromatin as well as for regulating gene expression via the histone code(2, 3). The mRNAs that encode for all five core histone proteins (H1, H2A, H2B, H3, and H4) are unique in that they are the only naturally occurring eukaryotic mRNAs that are not polyadenylated. Instead of a poly(A) tail, the histone pre-mRNA 3' untranslated region (3'UTR) contains a conserved sixteen nucleotide stem-loop (Fig. 1A1) and a purine-rich sequence (termed the histone downstream element, HDE) located 12–19 nts downstream of the cleavage site (Fig. 1A2 and 2B). These are the major cis-elements that are required for histone pre-mRNA processing(4, 5). The highly conserved tetra-loop hairpin(6, 7) in the 3' UTR also includes a conserved five nucleotide AC rich sequence before the stem-loop, a sixteen nucleotide stem-loop with a six base stem and a four base loop, and an AC rich sequence of either four (in *Drosophila*, sea urchins) or five nucleotides (in vertebrates) after the stem loop (Fig. 1A1). This region is the binding site for Stem-Loop Binding Protein (SLBP)(8) or hairpin-binding protein (HBP)(9) (Fig. 2A) and the histone mRNA stem-loop-SLBP complex functions as the major regulatory element for coordinate regulation of histone mRNAs for all five histone proteins. In mammals, a number of proteins such as ZFP100(10), FLASH(11), symplekin(12), components of the CPSF complex(12, 13) as well as possibly the U2 snRNP(14) assemble at or near the histone 3' UTR and are involved in histone pre-mRNA processing. The molecular details of these protein-nucleic acid interactions, the mode of assembly and the dynamic rearrangements that likely occur in the ribonucleoprotein complex to catalyze endonucleolytic cleavage during 3' end formation remain unknown.

SLBP is important for histone pre-mRNA processing(15), export(16), and translation of histone mRNA(17). The role of SLBP in histone mRNA processing has been proposed to be to stabilize the multi-protein complex with the U7 snRNP(15). SLBP has a small, highly basic RNA binding and processing domain (RPD) that bears no sequence similarity to any other RNA binding domain (RBD) in eukaryotic genomes and is expected to have a novel fold. The SLBP RBD is a subdomain of the RPD (Fig. 2A) as there are sequences in the RPD that are important for RNA processing but not RNA binding(18). The SLBP-histone mRNA complex functions as a coordinate unit. SLBP forms a stable complex with the 26 nucleotide binding site that includes the 16 nucleotide hairpin and the conserved single stranded 5' and 3' regions at the end of histone mRNAs *in vivo* and *in vitro*(19, 20). Our previous biophysical studies have shown that SLBP is an unstable protein with large segments of intrinsic disorder in the N-terminal domain(21) and the SLBP RPD has α -helical secondary structure but does not adopt a well-ordered tertiary structure in the absence of RNA(19). The intrinsic disorder and instability of the SLBP RPD has made structure determination of the SLBP-RNA complex particularly challenging by both NMR spectroscopy and X-ray diffraction. Previous top-down and bottom-up mass spectrometric analysis of the human and *Drosophila* SLBPs revealed that the protein is phosphorylated at an invariant threonine (in a sequence HPKTPNK) within its RNA binding domain(22). Phosphorylation increases the affinity of SLBP for the RNA substrate by approximately eight-fold *in vitro*. Phosphorylation of this threonine is also essential for viability of *Drosophila* embryos(23) and for histone pre-mRNA processing *in vivo*(23). Furthermore, mutation of the proline that flanks the phosphorylated Thr to a Ser in *C. elegans* SLBP results in loss of RNA binding and embryonic lethality(24).

In this paper we describe the structural determinants of the pre-mRNA and SLBP for sequence-specific recognition of histone mRNA. We use NMR spectroscopy to show that in the absence of bound proteins and U7 snRNA, the pre-mRNA has residual secondary structure that extends beyond the stem-loop. However the only stable element of secondary structure in the 3'UTR of the pre-mRNA is the 16-nt hairpin. Kinetic measurements using Surface Plasmon Resonance (SPR) show that the pre-mRNA interacts with SLBP with ten-fold higher affinity compared to the isolated stem-loop. Therefore the residual structure in the pre-mRNA may be important for interaction with SLBP and for assembly of the histone processing complex. Using baculovirus expressed SLBP proteins that were either fully phosphorylated or intentionally dephosphorylated at the conserved threonine in the TPNK sequence of the RNA binding domain, we show that phosphorylation of SLBP is important for the kinetics of association with the histone mRNA hairpin. The first thirty residues of the SLBP RPD have a number of arginines and tyrosines that are important determinants for sequence specific histone mRNA recognition(18). We show that this region forms a helix-turn-helix (HTH) in solution that by itself, has weak affinity for the histone mRNA stem-loop. NMR chemical shift mapping experiments and NOESY data on the HTH-RNA complex indicate that the HTH likely binds near the unpaired uridines of the loop, possibly via stacking interactions with conserved tyrosines in the SLBP HTH and this interaction may destabilize the first G-C basepair at the base of the stem. ^{31}P NMR spectra on the intact phosphorylated hSLBP RPD as well as 2D (^1H , ^1H) NOESY NMR spectra on SLBP peptides showed that the SLBP RPD interconverts between multiple conformations under physiological conditions that we attribute to proline isomerization. To provide unequivocal evidence that cis-trans proline isomerization about the Thr171/230-Pro172/231 bond is important for the instability of the SLBP RPD, we mutated Pro231 in the *Drosophila* SLBP (dSLBP) RPD to a glycine. The P231G dSLBP RPD showed a single conformer in solution and formed a stoichiometric complex with histone stem-loop RNA.

Our studies provide the first molecular insight into the mode of histone mRNA recognition by SLBP. The structural, kinetic and functional ramifications of phosphorylation and proline isomerization of the SLBP RPD in facilitating histone mRNA recognition and processing are discussed.

MATERIALS AND METHODS

Preparation of Proteins, Peptides, and RNA

Phosphorylated human and *Drosophila* SLBP proteins were expressed in baculovirus as previously described(25). The proteins used in this study were: full length *Drosophila* SLBP (dSLBP), full length dSLBP T230A mutant, the dSLBP RBD (residues 189–259), the dSLBP RPD (residues 189–276), and human SLBP RPD (residues 121–219). These SLBP proteins were subcloned into the *Nco*I and *Xho*I restriction sites of the baculovirus vector pFastBacTMHTA (Invitrogen) and were expressed using the Bac-to-Bac expression system (Invitrogen). All proteins have an N-terminal tag corresponding to the sequence MSYYHHHHHDY DIPTTENLYFQGAMA. After synthesis the methionine is removed and the α -amino group on the N-terminal serine acetylated(22). Sf21 cells were transfected with baculovirus (at a density of 1×10^6 cells/mL) in serum-free SF900 media (Gibco or Cyclone) and purified using nickel affinity chromatography. The identity of all proteins was confirmed by mass spectrometry. These dSLBP proteins gave a measured mass that corresponded to the removal of the N-terminal Met (−131), the acetylation of the Ser (+42) and phosphorylation of Thr230 (+80) as previously described(22) and the human protein gave similar results with T171 phosphorylated. For ^{31}P NMR studies a 128-residue pseudo-wild-type human SLBP (hSLBP) RPD construct was designed (residues 121–219) to insure a singly phosphorylated species at Thr171. The expressed protein consists of the following sequence:

**MSYYHHHHHDYDIP^{TENLYFQGAM}EAMAAVPADFETDESVLMRRQKQINY
GKNTIA YDRYIKEVPRHLRQPGIHPK(phospho)TPNKF^{KYSRRS}WDQQIKLW^K
VALHFWDPPAEEGCDLQE^I HPVDLE^{AAE}**

There are several non-native amino acids in this sequence. The first 25 residues at the N-terminus correspond to the vector sequence from pFastBac™HTA. The N-terminal (His)₆-tag does not contribute to RNA binding in *Drosophila* and human SLBPs and was retained for ³¹P NMR experiments. Several serine residues in the RPD (underlined) were mutated to alanines (and S122 was mutated to E122 to facilitate cloning). The minimal 73 amino acid RBD sequence (FET....DPP) that has been shown to be necessary and sufficient for RNA binding(18, 20) was all-native. In addition, 19 amino acids of hSLBP were included at the C-terminus. These residues are important for pre-mRNA processing(25) as well as for the stability of the RBD during protein expression (Thapar, R unpublished). The samples were concentrated and buffer exchanged using a G25 column into the NMR buffer (GE). Where indicated, baculovirus expressed SLBP proteins were dephosphorylated by treating the protein with calf intestinal phosphatase (CIP) at a molar ratio of 1:100 (SLBP:CIP) at 37°C for 12 hrs. Complete dephosphorylation was determined by mobility shift for the full length SLBP in SDS PAGE, in EMSA assays, and by mass spectrometry. The dSLBP P231G RPD (residues 173–276) mutant was generated using Quickchange mutagenesis (Stratagene) and was expressed from the vector pET15b. The mutant was labeled uniformly with ¹⁵N by growing *E. coli* in M9 minimal medium with ¹⁵NH₄Cl as the sole source of nitrogen. The dSLBP WT and P231G RPD recombinant proteins were expressed with an N-terminal His-tag and purified using nickel affinity and gel filtration chromatographies. Protein concentrations for full length and SLBP RPD samples were measured using extinction coefficients of 42060 M⁻¹ cm⁻¹ and 28420 M⁻¹ cm⁻¹ at 280 nm respectively. Threonine phosphorylated and unphosphorylated peptides corresponding to residues His161–Arg180 [¹⁶¹HLRQPGIHPKTPNFKKYS R¹⁸⁰ (WT_{20mer})] and [¹⁶¹HLRQPGI~~A~~PKTPNFKKYS R¹⁸⁰ (H168A_{20mer})] of human SLBP and a 30-residue peptide corresponding to residues Glu129–Val158 of human SLBP were synthesized either by Sigma-Genosys or Genscript. A 30mer mutant peptide where Y151 and Y154 were replaced with alanines and R137 and R138 were changed to lysines (MUT_{30mer}) [¹²⁹ETDESVLM~~K~~QKQINYGKNTIA~~DRA~~IKEV¹⁵⁸] was synthesized as a control to determine the specificity of the chemical shift titrations. All peptides were at least 90% pure as determined by reverse-phase HPLC and MALDI-MS. No tags were associated with the peptides.

The following RNAs were used in this study: SL₂₈-UA (5' GGCAAAGGCUCUUUCAGAGCCACCA 3'), SL₂₈-CG (5' GGCAAAGGCCCUUUCAGGCCACCA 3'), LSL₂₈-CG (5' GGCAAAGGCCUAUACAGGGCCACCA 3'), RSL₂₈-CG (5' GGCAAACCGGGUUUCAC CCGGACCCA 3') and the 65 nt pre-mRNA (5' CUCCCCAAAAGGCUCUUUCAGAGCCACCC ACUGAAUCAGAUAAAGAGUUGUGUCACGGUAGCCC 3'). Two different SL₂₈ RNAs with either a UA or a CG base-pair at fourth position of the stem were used as noted in the figure legends. In mammals, the fourth position is variable in that all H4 histone mRNAs have a CG at this position whereas all other histone mRNAs have a UA. Replacing a CG with UA has no effect on SLBP binding(20). The LSL₂₈-CG mutant has two unpaired uridines in the loop U7 and U9) mutated to adenosines. Stem-loop RNAs used in SPR measurements were biotinylated at the 5' end (Dharmacon, Inc) followed by a spacer sequence CUCCC to allow for optimal binding to the streptavidin chip. Stem-loop RNAs used in EMSA assays and NMR experiments lacked the biotinylated linker at the 5' end and were 5' end labeled using ³²P-γ-ATP. The SL₂₈ and 65-mer (SL+HDE) H2A-614 RNA samples used for NMR were synthesized, deprotected, and PAGE-purified by Dharmacon,

Inc. The RNA samples were taken up in 20 mM potassium phosphate buffer, 20 mM KCl, and 0.02 mM EDTA, pH 6.8. The RNA was folded by heating at 95°C for 2 min and snap-cooled on ice for 10 min to ensure the lowest energy hairpin conformer. The formation of the stem-loop (instead of a duplex) using this approach was confirmed by NMR. All RNA samples used for NMR were in 90% H₂O/10% D₂O in 240 µl. Sample concentration for the 65-mer (SL+HDE) was 50 µM and for the SL₂₈ RNAs was between 0.2–1 mM as noted.

EMSA assays

Wild type and mutant SLBP proteins were incubated with 5'-³²P end labeled stem-loop probe for 10–20 min on ice in binding buffer (20 mM Tris pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mg/mL BSA). The total reaction volume (10 µl) was analyzed on an 8% native polyacrylamide Tris-borate gel run in Tris-borate buffer at 150 V for 1–1.5 hr. The gel was dried at 80°C for 2 hr and then exposed to film.

Mass spectrometry

Baculovirus expressed phosphorylated and dephosphorylated SLBP proteins were treated with chloroform:water:methanol to remove trace amounts of detergent as previously described(26). The samples were reconstituted with 40 µl 2% ACN/97.9% H₂O/0.1% formic acid. 20 µl of the dissolved sample was de-salted using either a C4 (for phosphorylated and dephosphorylated FL-dSLBP) or C18 (for the dSLBP-RBD) ZipTip (Millipore). Bound proteins were eluted with 10 µl 60% ACN/39.9% H₂O/0.1% formic acid. The de-salted samples were then loaded onto a metal coated glass capillary tip for direct infusion into a Waters Qtof-Micro mass spectrometer. Calibration of the instrument was carried out over a wide mass range (200–2200 Da in positive ion mode) using Sodium Iodide standard prior to infusion of the sample. Positive mode ESI was set with capillary, sample cone, and extraction voltages at 3kV, 25V, and 2V, respectively. Scanning was set at 0.6 sec per scan with 100 msec interscan time. The Analyser and Tof Penning pressure were at 4.07×10⁻⁹ and 5.17×10⁻⁷, respectively. Data were collected and analyzed with MassLynx(version 4.0). Collected raw data were smooth twice with a mean smoothing method consisting of a smoothing window of +/− 3, and then deconvoluted with MaxEnt 1 algorithm in the appropriate mass range.

Measurement of off rates by competition experiments and analyzed by EMSA

Drosophila SLBP proteins were incubated with 5' end labeled ³²P stem-loop probe for 30 min on ice in binding buffer (20 mM Tris pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mg/ml BSA). The total reaction volume was 100 µl that included 5 nM γ -³²P-ATP-labeled probe, and 0.5 mM protein. At the 0 min time point, 10 µl of the reaction was removed and frozen at −80°C as the reference, and the dissociation reaction was initiated by the addition of 100-fold excess cold unlabeled RNA stem-loop. The reaction was kept at 37°C and 10 µl aliquots were removed at varying time points over a period of 2.5 days and kept at −80°C. Time points were analyzed on a 8% native gel then exposed to a PhosphorImager.

Surface Plasmon Resonance (SPR)

Kinetic SPR studies were performed on a BIACore T100 (Biacore, Inc., Piscataway, NJ) instrument. Biotinylated RNAs were chemically synthesized (Dharmacon Research, Boulder, CO) with a 5' biotin tag and the hairpins were prepared by heating to 95°C for 5 min followed by snap cooling on ice. The RNAs were immobilized on BIACore SA (streptavidin-coated) sensor chips. Prior to coupling the 5' biotinylated RNA ligand onto the sensor chip, the chip was conditioned by three sequential injections (1 min each) of 1M NaCl in 50 mM NaOH at a flow rate of 50 µl/min. A 1 µM solution of either the pre-mRNA

or the RNA hairpin was used as a stock solution for immobilization onto the chip. The binding of RNA was monitored as the change in response units (RUs) and 60–70 RUs were captured onto the sensor chip. No RNA ligand was coupled to the first and third channels that were maintained as reference flow-cells. The chip was primed in BIAcore HBS-P buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Surfactant P20) until the drift in the baseline was no greater than \pm 5 RUs. The SLBP analyte concentrations were in the range of 2 nM–18 μ M and all samples were prepared by dialyzing the SLBP stock solution against HBS-P buffer for 24 hrs.

For determination of SLBP-RNA binding kinetics, SLBP was injected onto the chip in the “Kinetics” mode at a flow rate of 30 μ L/min for 2 min followed by a 10 minute dissociation phase during which only the HBS-P buffer was injected. All binding experiments were performed at 25°C. The bound protein was removed by two 60 s injection bursts of regeneration buffer (3M NaCl) to restore the baseline. Five sensorgrams were collected with varying protein concentrations. The data were analyzed using BiaEvaluation® T100 software. The control channel was subtracted, the sensorgrams were aligned, and the data was globally fit by least-squares analysis to a 1:1 Langmuir binding model. Both the association and dissociation phases were fit simultaneously. The best fit of the data was determined by the randomness of the residuals and the lowest value of χ^2 obtained. Linear errors in the determination of k_{on} and k_{off} are reported from the best fits of the data and standard error propagation methods were used for the determination of errors for K_a and K_d . At least two independent sets of measurements were performed for each protein.

Fluorescence Measurements

Fluorescence experiments were performed on a SPEX Fluorolog-3 Spectrofluorometer at 25°C. The association of SLBP with either stem-loop RNA or a non-specific RNA that corresponded to a 30 nucleotide HDE sequence was monitored by following a decrease in intrinsic tryptophan fluorescence ($\lambda_{excitation}=295$ nm; $\lambda_{emission}$ between 300–500 nm; $\lambda_{max,emission}=385$ nm) as a function of increasing [RNA]. The concentration of SLBP was 5 μ M and the concentration of RNA varied from 5 μ M to 200 μ M. After the last titration point, the samples were stored at room temperature and the change in fluorescence intensity was monitored over a period of six days, which was determined to be the optimal time period for the reaction to reach equilibrium.

NMR Spectroscopy

1D 1H NMR experiments for detection of RNA imino protons were collected either on a Varian Inova 600 MHz spectrometer or a Varian VNMRS 900 MHz spectrometer with a 5 mm cryogenic $^1H\{^{13}C/^{15}N\}$ probe. Each experiment was collected with both a binomial 1-1 echo pulse with maximal excitation at 12.5 p.p.m for solvent suppression as well as an SS-pulse for water suppression as a separate experiment. This was done to unambiguously determine whether intensity changes observed for the imino protons were real due to addition of the wild type peptide, or could be attributed due to baseline distortions or the mode of water suppression. RNA samples were in 20 mM potassium phosphate buffer, 20 mM KCl, and 0.02 mM EDTA, pH 6.8. SLBP protein and peptides used for homonuclear NMR experiments were dissolved in 20 mM Tris acetate buffer, 50 mM NaCl, and 90% $H_2O/10\% D_2O$. The pH or pD* of samples varied according to the experiment and is noted in the figure legends. 2D (1H , 1H) NOESY spectra were collected on a 0.3 mM SL₂₈ RNA, free and in the presence of a six-fold excess of the 30 residue SLBP peptide at NMRFAM on the VNMRS 900 MHz spectrometer with a cryogenic probe. The NOESY data were collected with a mixing time of 125 ms at 1°C with a binomial 1-1 echo pulse for solvent suppression. The exchangeable imino protons for the SL₂₈-UA RNA were assigned using 2D (1H, 1H) NOESY spectra (mixing times of 125 ms and 250 ms) collected in H_2O at 1°C.

and 25°C at 900 MHz and 600 MHz. 2D NOESY spectra (125 ms and 250 ms) in D₂O at 25°C and a 50 ms homonuclear 2D TOCSY spectrum at 600 MHz were also collected to assign the nonexchangeable protons of SL₂₈-UA RNA. One-dimensional ³¹P NMR experiments were performed on a Varian Inova 500 MHz spectrometer using a broadband probe operating at a phosphorus frequency of 202 MHz. All ³¹P NMR measurements were made at 25°C. For each experiment 1000–20000 transients were collected with a 65° excitation pulse, a recycle delay of 3 s, and a sweep width of 98.7 p.p.m. with proton decoupling. ³¹P chemical shifts were referenced to 85% phosphoric acid. 2D (¹H, ¹⁵N) HSQC spectra were collected for uniformly ¹⁵N-labeled dSLBP P231G SLBP at 900 MHz with cryogenic probe at 25°C and pH 6.0 in the presence and absence of RNA. The concentration of the protein and the SL₂₈-UA RNA were 80 μM and 0.1 mM, respectively. (¹H, ¹⁵N) HSQC spectra on the 30-mer and 20-mer peptides were collected at natural abundance on 40–50 mM samples (corresponding to a 0.15–0.2 mM uniformly ¹⁵N isotopically labeled sample) in 20 mM Tris pH 5, 50 mM NaCl, 10% D₂O on an Inova 700 MHz spectrometer equipped with a cryoprobe at 283 K to decrease solvent exchange of backbone amides. No evidence of aggregation was observed for the peptides under these conditions. The 30-mer and 20-mer peptides were assigned using standard 2D homonuclear NOESY (τ_m 300 ms), TOCSY (isotropic mixing time using MLEV-17 was 100 ms), and DQF-COSY spectra acquired in States-TPPI mode with a 2s steady-state relaxation delay on 3–7 mM samples, 32–64 scans per t_1 , with a matrix size of 1024 complex points in t_2 and 512–600 points in t_1 , using WATERGATE for water suppression. NMR data were processed using a square shifted sinebell (72–90°) and analyzed using Felix (Felix NMR) or NMRPipe. The tautomeric states of the histidines in the WT₂₀mer peptide were determined from an (¹H, ¹⁵N)-HMQC-J experiment that used a 22 ms ¹H-¹⁵N evolution period to refocus magnetization arising from the ¹J_{NH} couplings and to ensure that direct ¹H-¹⁵N couplings were minimized(27). The ¹⁵N carrier was set to 205 ppm. For structure calculations, interproton distance restraints were obtained from 2D NOESY spectra (τ_m of 80 ms, 110 ms, 250 ms) acquired at 700 MHz on a 7 mM sample of the 30-mer peptide at 5°C and dihedral angle restraints obtained from ³J_{NHα} couplings obtained from a high resolution 2D DQF-COSY spectrum collected at 700 MHz. Structure calculations were performed using torsional dynamics simulations with CYANA 2.1 using standard simulated annealing protocols. NOE-based restraints were obtained from the CYANA automated NOE assignment protocol.

RESULTS

Secondary structure in the mouse histone H2A-614 pre-mRNA 3'UTR

Several mutagenesis studies have previously shown that the histone mRNA stem-loop has all the sequence determinants for specific and high affinity recognition by SLBP(20, 28). However, SLBP plays an active role in stimulating histone pre-mRNA processing, particularly in histone genes that have a weaker HDE that cannot efficiently base-pair with the U7 snRNP(15), by either inducing structural rearrangements in the 3'UTR that facilitate U7 snRNP binding and/or by promoting protein-protein interactions in the processing complex. Early on, Steitz and coworkers showed that proper processing of the histone mRNA requires a fixed distance between the stem-loop and the HDE(29, 30). One explanation for this molecular ruler hypothesis for precise cleavage after the stem-loop and the role of SLBP is that there may be other secondary structure elements in the 3'UTR that dictate processing. Recent biochemical experiments using enzymatic and chemical footprinting(31) suggest that the histone 3'UTRs of H4-12, H1t, H2A-614 genes are structured such that the HDE sequence lies either in a second RNA hairpin or in a duplex and SLBP binding induces structural rearrangements in the 3'UTR. NMR studies(6) show that in the context of a 28 nucleotide stem-loop, the 5' and 3' flanking nucleotides are

unstructured and have long C¹³ relaxation times indicating that it is unlikely that the stem-loop is part of a pseudo-knot structure(6).

To determine whether the 3' UTR has other secondary structural elements besides the stem-loop, we collected 1D imino proton NMR spectra for a 65-nt H2A-614 RNA that has both the stem-loop as well as the purine-rich HDE sequence and compared it to that of the 28-nt stem-loop that has previously been characterized by NMR Spectroscopy(6). Computational predictions using UNAFold(32) suggest two possible structures may exist for this region (Fig 1, A2 and A3). The 1D imino proton NMR spectrum (Fig. 1B) of the stem-loop RNA SL₂₈-UA (Fig. 1A1) shows five strong resonances in the Watson-Crick (W-C) base-paired region (12–15 p.p.m) corresponding to the stem of the hairpin as well as a weak U6 NH resonance for the closing U6-A11 base-pair at 13.8 p.p.m (Fig. 1A1, Fig. 1C) indicating that the imino proton of U6 is more prone to rapid exchange with bulk solvent. Intriguingly, at 25°C, the spectrum of the 65-nt SL+HDE RNA is almost identical to that of SL₂₈-UA with only one additional resonance at 12.5 p.p.m. (Fig. 1B) which, based on chemical shift, is likely to be a G-C base-pair. However, when we performed a temperature titration and lowered the temperature to 5°C (Fig. 1C), at least three additional resonances appear in the W-C base-paired region of the SL+HDE RNA spectrum. Therefore at least six additional nucleotides are capable of being base-paired in the absence of bound proteins or U7 snRNA, although the pairs may be solvent exposed or have lower stability than the stem-loop. These additional resonances do not appear in the spectrum of the SL₂₈-UA RNA at low temperature (Fig. 1C). Titration of 20-fold molar excesss of Mg²⁺ into the (SL+HDE) RNA sample did not result in the appearance of new resonances or cause spectral perturbations in the 1D imino spectra indicating that Mg²⁺ did not induce structure in this region of the RNA. We conclude that the 65-nt SL+HDE H2A-614 mRNA has residual structure that extends beyond the stem-loop, but the stem-loop sequence is the only stable element of secondary structure. It is not clear whether histone mRNA specific processing factors could stabilize the nascent structure in the downstream sequence of the pre-mRNA, and this is an avenue for future studies.

Structure of the SLBP RBD and recognition of the histone mRNA

Until now, the 105 amino acid SLBP RPD has been refractory to detailed structure calculation by NMR or crystallography. Computational approaches using secondary structure prediction algorithms and ab initio modeling suggest that the SLBP RPD has an all-helical RNA binding domain with either three or four helices (Fig. 2A). This is consistent with the helical nature of the RPD previously characterized by NMR and circular dichroism(19). All the SLBP RPD constructs we previously tested were not stably folded in solution. However, a peptide that corresponds to the first thirty residues of the human SLBP RPD (residues Glu129-Val158) and has many of the sequence determinants for RNA binding(18) was amenable to structure calculation by NMR. The solution NMR structure of residues Glu129-Val158 shows that, this region folds into two helices separated by a short loop in solution (Fig. 2C and D, Supplementary Fig 2). Helix-1 extends from residues Glu132-Ile142 and helix-2 from residues Thr147-Val158. The residues Arg137, Lys140, Tyr144, Asn147, Tyr151 and Tyr154 lie on one face of helix-1 forming an extensive basic and hydrophobic interface that likely contacts the RNA (Fig. 2D). Residues important for RNA processing (Asp152, Arg153, Ile155, and Lys156)(18) are oriented on the opposing face in helix-2. The structure statistics are summarized in Table 1.

To determine whether this 30 residue peptide could form a complex with histone mRNA stem-loop, we performed SPR studies in which biotinylated stem-loop RNA was loaded onto a streptavidin sensor chip and the change in response was measured in the presence of increasing concentrations of HTH peptide (Supplementary Fig. 1D). However, no measurable interaction was observed between the HTH peptide and the stem-loop RNA.

This result was confirmed by gel retardation experiments (data not shown). The K_D of histone mRNA hairpin for this peptide is therefore greater than the upper limit for SPR (10^{-4} M). We concluded that either the helices are not oriented favorably for RNA recognition when removed out of the context of the intact domain or formation of a stable and specific SLBP-RNA complex requires a second binding site C-terminal to the 30-residue RPD peptide.

NMR is uniquely suited to test molecular interactions over a wide range of affinities. Therefore we titrated the wild-type SLBP HTH peptide into different histone stem-loop RNAs and monitored spectral changes in the 1D imino proton NMR spectra in response to increasing concentration of peptide added (Fig. 3A, 3C). The titration was performed with both a 1-1 echo or an SS-pulse for water suppression to rule out artifactual changes due to the mode of solvent suppression or baseline correction. When the 30-residue peptide is titrated into either SL₂₈-CG or SL₂₈-UA (Fig. 3), the only spectral change observed in the 1D imino ¹H spectrum is an increase in the intensity of the imino protons of the loop uridines (U7, U8, U9) that lie in the unpaired region of the spectrum. These resonances are very broad at pH 6.8 and 25°C but become more protected from exchange in the presence of the peptide. To determine whether these changes were due to small differences in the phosphate buffer of the titrated solution, we repeated a similar titration with phosphate buffer alone and we did not observe any change in the intensity of the unpaired uridines (Fig. 3C). We also titrated the 30 residue peptide into a mutant form of the stem-loop, LSL₂₈, where the loop uridines at positions U7 and U9 (Fig. 3C) were replaced with adenosines. Titration of the peptide into the LSL₂₈ RNA mutant resulted in a small increase in the intensity of the U8 resonance but no other perturbation in the 1D imino proton spectrum was observed. Finally, to determine whether the effects were due to the non-specific interaction of a basic peptide with the RNA, we titrated a mutant 30mer RNA in which key arginines and tyrosine were replaced by lysines and alanines respectively. This peptide was not expected to bind the RNA and no changes in the 1D imino spectrum were observed (Fig. 3B).

We also compared 2D NOESY data for SL₂₈-UA in the absence and presence of a six-fold molar excess 30-mer peptide (Fig. 4). 2D NOESY spectra were used to assign the imino protons of the stem. For the free SL₂₈-UA five imino protons show clear sequential NH-NH NOEs in the W-C base-pair region, whereas the U6 NH is broad and observed only at 5°C. Notably, a strong NOE is observed between the G1 NH and the G2 NH in the free RNA which lie at the base of the stem. However, addition of peptide results in the loss of this sequential G1 NH-G2 NH NOE (Fig. 4A). The cross-strand NOE from the G1 imino to the C16 H41 amino proton was also not observed in the presence of peptide. The loss of these NOEs suggests that the first GC base-pair is either destabilized or is more exposed to solvent in the presence of peptide. Further evidence for destabilization of the first base-pair comes from the observation that two sets of resonances are found for the G1 NH in the presence of the peptide (G1 and G1'), both of which are shifted by 0.24 and 0.11 ppm downfield compared to the single resonance observed for the G1 NH in the free RNA (Fig. 4E). We also monitored NOE changes in the peptide in response to the addition of histone mRNA (Supplementary Fig. 2 and 7). A number of peptide resonances were shifted or exchange-broadened in the presence of RNA. The broadening of the peptide resonances prevents detailed characterization of the peptide in the complex by NMR. Notably, chemical shift perturbations are observed for all three Tyr Hδ and Hε ring protons of the HTH (Supplementary Fig. 2). Two tyrosines (Tyr151 and Tyr154) have previously been shown to be important for RNA recognition(18). Based upon the spectral changes observed in 1D and 2D NMR spectra, the data are consistent with the U7, U8, and U9 uridines in the RNA tetraloop possibly participating in stacking interactions with Tyr151 and Tyr154 in the

SLBP HTH. We have shown that interaction of the SLBP peptide with the SL₂₈ RNA destabilizes the first GC base-pair in the stem.

Evidence for a second binding site C-terminal to the HTH peptide comes from fluorescence experiments. The interaction of baculovirus-expressed SLBP RPD with histone mRNA was followed by monitoring tryptophan fluorescence quenching as a function of RNA concentration (Fig. 5). When histone mRNA stem-loop (SL₂₈) is added to a solution of phosphorylated SLBP the fluorescence data show slow biphasic kinetics (Fig. 5A). Initially, a rapid decrease in fluorescence intensity is observed at a red-shifted λ_{max} of 385 nm followed by a slow time-dependent and concentration-independent quench phase resulting in a 25% decrease in fluorescence intensity at 385 nm over the course of 5 days and an increase in fluorescence intensity at 442 nm (Fig. 5B). The increase in fluorescence intensity at 442 nm results from FRET due to electron transfer from the indole ring of one or more tryptophans in the C-terminus of the SLBP RPD to a purine or pyrimidine base in the RNA. This emission λ_{max} is close to that reported for cytidine (λ_{max} 450 nm) upon excitation of tryptophan at pH 7 and 77 K(33). The second and third base-pairs (G-C and C-G) from the base of the histone mRNA stem are important for RNA binding and it is likely that either one or both cytosines make direct contacts via stacking interactions with one or more tryptophans in the C-terminus of the SLBP RPD. The time-dependent and concentration-independent change in fluorescence during the slow phase is not observed in the presence of a non-cognate 30 nucleotide RNA sequence such as the histone-downstream element (HDE30) (Fig. 5C) or when dephosphorylated SLBP is incubated with the stem-loop RNA (Fig. 5D). Therefore there is a slow step in the reaction mechanism for phosphorylated SLBP that reaches equilibrium over the course of days. As shown below, the SLBP RPD undergoes prolyl isomerization, the time scale of which ranges between 10–1000 s⁻¹ due to a large energy barrier of 15–20 kcal mol⁻¹(34). The slow equilibration phase of the phosphorylated SLBP-RNA complex is a direct consequence of proline isomerization in the absence of a peptidyl-prolyl isomerase.

Effect of threonine phosphorylation at Thr171/Thr230 on the kinetics of RNA recognition

When phosphorylated at Thr171, that lies C-terminal to the HTH, human and *Xenopus* SLBPs have been reported to form very stable and specific complexes with the histone stem-loop(20). Using nitrocellulose filter binding experiments, we previously showed that dephosphorylated SLBP exhibits 6–8 fold lower affinity for histone mRNA(22). Since filter binding experiments do not provide insight into the rates of RNA association and dissociation, we used surface plasmon resonance (SPR) to compare the on- and off- rates of phosphorylated and dephosphorylated full length dSLBP and the minimal dSLBP RBD (residues 189–259) for the histone mRNA hairpin as well as the 65-nt pre-mRNA (SL₂₈ + HDE) we had characterized by NMR. When expressed in baculovirus the full length dSLBP is a heterogeneous mixture of multiple phosphorylated species but with only a single phosphorylation site in the RBD(22). Consistent with this, the dSLBP RBD produced in baculovirus is a homogeneous protein that is phosphorylated only at Thr230 (Fig. 6A). The relative amounts of phosphorylated: dephosphorylated RBD after purification is 100:2 by mass spectrometry (Fig. 6A). We attribute this high level of phosphorylation not to the unknown kinase that carries out the reaction in the cell, but rather to the instability of the dephosphorylated form when over-expressed in either baculovirus or bacteria (Zhang & Thapar, unpublished observations). We were able to completely dephosphorylate the full length dSLBP and dSLBP RBD proteins by incubating with calf intestinal phosphatase for 12 hrs at 37°C. Complete dephosphorylation was determined by a mobility shift on SDS-PAGE (Fig. 6B) as well as a difference in the relative mobilities of the phosphorylated and dephosphorylated proteins in EMSA assays (Fig. 6C).

We used the phosphorylated and dephosphorylated full length and RBD proteins to measure their respective interactions with the histone mRNA stem-loop and the pre-mRNA we have characterized in this study. The SPR derived binding constants (Fig. 6, Supplementary Fig 5 and Table 2) reveal that the baculovirus-expressed phosphorylated full length SLBP has a slow association rate for the histone mRNA stem-loop ($k_{on} = 1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) compared to the diffusion-limited rate of $1.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and a slow dissociation rate ($k_{off} = 5.7 \times 10^{-4} \text{ min}^{-1}$) yielding a binding constant (K_D) of $47.5 \pm 17 \text{ nM}$. Our K_D values reported by SPR measurements are higher than those previously reported by filter binding ($K_D \sim 0.8\text{--}1.5 \text{ nM}$)(20) and could reflect possible differences in solution conditions, data fitting, and the methods used.

The first observation from the SPR studies is that the phosphorylated full length dSLBP protein binds more favorably to the pre-mRNA such that the K_D increased by ~15 fold corresponding to a $\Delta\Delta G$ of $-1.6 \text{ kcal mol}^{-1}$. The dephosphorylated full length dSLBP protein also had higher affinity towards the pre-mRNA (K_D (rel) 5.6, $\Delta\Delta G -1.0 \text{ kcal mol}^{-1}$). The increased affinity for the pre-mRNA (defined here as the SL28 + HDE sequence) suggests that either direct contacts are made by SLBP to regions outside the stem-loop in the 65 nt RNA used or that the secondary structure of the stem-loop is slightly different in the context of the 65 nt sequence.

Second, the minimal dSLBP RBD (residues 189–259) is not as effective in its interaction with either the stem-loop or the pre-mRNA as compared to the full length dSLBP protein. All K_D values obtained were 20–200 fold higher for the dSLBP RBD compared to full length dSLBP. The phosphorylated RBD also shows a much smaller difference in relative binding to the stem-loop vs the pre-mRNA (K_D (rel) 1.65, $\Delta\Delta G -0.3 \text{ kcal mol}^{-1}$). Since the longer dSLBP RPD (residues 189–276) does bind histone mRNA as effectively as the full length protein(22), we interpret this to mean that the additional C-terminal residues and/or phosphorylation of the four serines in the C-terminus of the dSLBP RPD (between residues 259–276) (25) stabilizes the entire domain or are involved in direct contacts with the RNA.

Next, we determined the effect of dephosphorylating SLBP at Thr230 on histone mRNA recognition. The more basic dephosphorylated full length SLBP has a ten-fold faster on-rate towards the stem-loop but also a 100 times faster off-rate of $7.44 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ yielding a K_D of $528.6 \pm 73 \text{ nM}$. This corresponds to a ~11 fold decrease in binding affinity for dephosphorylated full length dSLBP which is very similar to what we previously reported using filter binding measurements(22). A ~30 fold difference in affinity is observed for the phosphorylated and dephosphorylated dSLBP proteins towards the pre-mRNA sequence. We were not able to measure any reliable values for the interaction of the dephosphorylated RBD with stem-loop RNA and the sensorgrams were more consistent with a heterogenous analyte being present indicating that truncation of the C-terminus and dephosphorylation of the RBD severely destabilize the protein (Supplementary Fig. 5).

Measurements of on- and off-rates may also be performed using EMSA or filter binding. Therefore to test the validity of the SPR data, the off-rate was measured in competition experiments using EMSA (Fig 6E). In these experiments a preformed SLBP-RNA complex was incubated with a 100-fold excess of cold competitor RNA at 37°C and the dissociation of the radiolabeled RNA was monitored over the course of 24–36 hrs. As is clearly shown in Fig 6E, both phosphorylated full length *Drosophila* SLBP and the phosphorylated human SLBP RPD have a slow off-rate and the complex does not dissociate over 24 hrs. On the other hand, mutation of the threonine to alanine in *Drosophila* SLBP or dephosphorylation of baculovirus-expressed human SLBP RPD results in a complex in which the labeled RNA rapidly dissociates by the addition of cold competitor RNA. Therefore, the dephosphorylated

form is characterized by rapid dissociation indicative of an unstable complex. Thus, phosphorylation at Thr171 is a key determinant in the formation of a stable complex.

The SLBP RPD undergoes prolyl isomerization about the Thr171/230-Pro172/231 peptide bond

When expressed in baculovirus, the human SLBP RPD is also phosphorylated at only one site (Thr171), as is confirmed by mass spectrometry (Fig. 7C). However, two ^{31}P NMR resonances are observed at pH 8.4 for the single phosphate (Fig. 7A) at 3.00 p.p.m and 4.19 p.p.m, both of which lie within the range of that expected for O-phosphothreonine (3–5 p.p.m). To determine the ionization state of the phosphoryl groups, the ^{31}P chemical shifts were followed as a function of pH and the response of this phosphate to varying pH (4.33–9.09) was monitored by ^{31}P NMR (Fig. 7B). Similar to inorganic phosphate ($\text{pK}_\alpha 7.0 \pm 0.01$) and O-phosphothreonine standard ($\text{pK}_\alpha 6.3 \pm 0.02$), the ^{31}P resonances for the free protein go through a single ionization event corresponding to an apparent pK_α of 6.9. Therefore, in the absence of RNA, and at neutral pH, the phosphothreonine in free SLBP favors a dianionic form that is dominant in all the observed conformational states. In addition, the NMR line widths for the ^{31}P resonances (3–5 p.p.m) are very broad between pH 5.5–8.0 and a number of conformational sub-states are observed for the phosphates in the free protein at neutral pH (Fig. 7B). This suggests that the ^{31}P resonance is influenced by interactions in its local environment in addition to deprotonation of the orthophosphate. It is also consistent with our previous observations(19) using NMR that the SLBP RPD is not stably folded in the absence of RNA and undergoes conformational exchange between multiple states at physiological pH. A likely explanation for the observed conformational heterogeneity could be the presence of two prolines near the phosphothreonine in the sequence HPKTPNK that are potentially capable of generating four cis- and trans- proline conformers. In addition, the deprotonation of the phosphate may be coupled to the protonation state of His168.

To further characterize the properties of this region we synthesized 20-residue unphosphorylated and Thr171/Thr230 phosphorylated peptides. The (^1H , ^{15}N) HSQC spectrum of the unphosphorylated 20-residue peptide corresponding to the sequence that follows the HTH (residues His161–Arg180 in human SLBP) and comprises the site of phosphorylation shows two sets of cross-peaks for 16 out of 18 (20 residues – 2 Pro) expected amide resonances (Supplementary Fig. 3) indicating that the unphosphorylated peptide interconverts slowly between two distinct conformations on the NMR timescale. When the (^1H , ^{15}N)-HSQC spectrum of the WT unphosphorylated peptide is compared to that of a peptide where two of the prolines (Pro169 and Pro172) are replaced by alanines in Pro169A-Pro172A only a single set of cross-peaks was observed for backbone NH and side-chain NH_2 resonances suggesting that the observed conformational heterogeneity in the peptide is most likely due to proline isomerizationin the HPKTPNK sequence.

The 20-residue peptides have three prolines, two of which lie in close proximity to the phosphothreonine in the sequence HPKTPNK. Assuming that the N-terminal proline (Pro165) does not substantially contribute to the backbone dynamics of the region in the peptides, there are four possible conformational states (trans/trans, trans/cis, cis/trans, cis/cis) for the backbone around HPKTPNK. To determine what the major and minor conformational states were, we assigned the proton NMR resonances of the 20 residue unphosphorylated and threonine phosphorylated peptides that corresponds to the region C-terminal to the site of threonine phosphorylation (residues H161–R180) and analyzed the 2D NOESY spectra (Fig. 7D, 7E). Analysis of NOESY spectra of the phosphorylated peptide (Fig. 7E) shows that the major conformation at pH 4 is the P169 trans/P172 trans conformer, and a minor fraction of the peptide adopts a P169 trans/P172 cis conformation. The major conformer shows NOEs between the phosphoThr171 H_α proton and the Pro172 $\text{H}_\delta 1$ and

H_δ2 protons that are diagnostic of a trans-Pro conformation. In addition, a weak NOE is observed between the phosphoThr171 H_α and the Pro172 H_α that is indicative of a cis-Pro conformer (Fig. 7E). Based upon NOE intensities as well as peak intensities for the observed conformers in TOCSY spectra, the population of the cis-Pro conformation in the peptides is expected to be ~15%. Therefore the peptides provide unequivocal evidence that the sequence around the phosphorylated threonine samples a number of conformational states which are at least in part attributed to cis-trans isomerization of one or more prolines.

In order to address whether the observed conformational heterogeneity of the dSLBP RPD was due to isomerization about the Thr230-Pro231 bond, we mutated Pro231 to glycine (Fig. 8). Our rationale was that introduction of a glycine at this position should result in increased backbone dynamics allowing greater sampling of conformational states that may be conducive to RNA binding. As shown in Fig. 8B, & 8C, the (¹H, ¹⁵N) HSQC spectrum of the P231G mutant of the dSLBP RPD shows a single conformation in solution as compared to the (¹H, ¹⁵N) HSQC spectrum of the wild type dSLBP RPD (Fig. 8D). We used an NMR chemical shift perturbation assay to test whether the dSLBP P231G mutant binds histone mRNA. When unlabeled SL₂₈-CG RNA is titrated into a solution of uniformly ¹⁵N-labeled P231G dSLBP RPD, the backbone amides of about 21 residues are significantly perturbed in (¹H, ¹⁵N) HSQC spectra indicating that these SLBP amide resonances experience a change in their chemical and electronic environment in the presence of RNA. In addition, the dSLBP P231G protein formed a stoichiometric complex with equimolar amounts of histone stem-loop RNA (Fig. 8C). The complex is in the slow chemical exchange regime on the NMR timescale, which is suggestive of a high affinity complex. No evidence of a second conformation is observed in our studies either with or without the histone mRNA stem-loop. However, P231G dSLBP RPD does not bind the histone stem-loop RNA with high affinity in an EMSA assay (Fig. 8A) as compared to the baculovirus expressed phosphorylated RBD. Since EMSA assays only detect complexes that are highly stable during electrophoresis, the assay underestimates binding affinities if the complex dissociates in the gel due to a faster off-rate, or if the amount of protein used is even 10-fold lower than the K_D for the interaction(35). The data are consistent with our observation that both phosphorylation and the correct proline conformer are key determinants of stability for the SLBP-histone mRNA complex.

We conclude that the SLBP RPD undergoes cis-trans proline isomerization about the Thr230-Pro231 bond and the backbone dynamics of this “hinge” region appears to be critical for histone mRNA recognition.

Contribution of His168 to observed conformational heterogeneity

His168/His227 is invariant in all SLBPs(36) and His-Pro sequences are also known to act as chemical catalysts, influencing proline isomerization rates in standard peptides(37). Mutation of H168 to phenylalanine has previously been shown to abrogate binding to histone mRNA stem-loop in EMSA assays(18). To determine whether H168/H227 affects the conformational dynamics of the region around the phosphothreonine, we measured the pKa values of His168 and His161 in the 20-mer phosphopeptides by following the chemical shift of the ¹H_e signal as a function of pH (Supplementary Fig. 6). The phosphorylated WT peptide shows a depressed pKa of 5.6 for the side-chain of His168 whereas His161 has a pKa value of 6.4 in the range expected for a histidine. A depressed pKa for a histidine usually results from either a H-bonding interaction or close proximity to a basic residue. Two lysines, Lys170 and Lys174 are present in this sequence and either one could act as a base. Next we determined the tautomeric states of the two histidines in the phosphopeptides at pH 6.5 and 25°C. The observed chemical shifts and intensities of the Nε2-Hε2, Nε2-Hδ2, and Nδ1-Hε1 crosspeaks are in agreement with a neutral tautomeric state where only the His168 Nε1 is protonated. On the other hand, His161 exists in a charged state where both

side-chain nitrogens are protonated. To see whether replacement of His168 by Ala altered the ionization of the phosphoryl group, we performed ^{31}P titrations as a function of pH (Fig. 9A, B, C). Two ^{31}P resonances are observed in the WT_{20mer} and MUT_{20mer} peptides as is observed in the context of the SLBP RPD, although the major conformer predominates. Replacement of His168 with Ala showed no change in either the relative populations of the major and minor components or the pKa of ionization of the phosphate. It is conceivable that His168 may modulate the rates of cis-trans proline isomerization in the HPKTPNK sequence and thereby play an indirect role in RNA recognition. Alternatively it may directly interact with histone mRNA. However, does not appear to be a major contributor to the observed conformational heterogeneity of the SLBP RPD.

DISCUSSION

The data presented in this paper provides new insights into the mechanism of SLBP-histone mRNA recognition and the structural role of posttranslational modifications in RNA-protein interactions. We show that the SLBP RPD is α -helical and appears to recognize at least two distinct sites on the histone stem-loop using two distinct binding interfaces in its RPD (Fig. 10). The first binding site on the SLBP RPD comprises residues Glu129-Val158 (human SLBP numbering) which forms a highly basic interface presented by helix-1/loop-1. The second binding site on the hSLBP RPD appears to lie between Arg180-Pro200 that is C-terminal to the site of threonine phosphorylation. Critical determinants in the RNA stem-loop for SLBP binding have previously been shown to be the second GC base-pair in the stem, uridines 7 and 9 in the loop, and the adenosines at -2 and 3 before the stem(20), suggesting that there are multiple contacts between SLBP and the RNA target. The protection of loop uridine imino protons (U7, U8 and U9) from exchange with solvent in the presence of the HTH peptide that we observed in this study is consistent with this region forming a binding interface for SLBP, possibly via stacking interactions with conserved tyrosines in the SLBP HTH. This interaction also destabilizes the first G-C base pair, “opening up” the base of the A-form stem. This may allow access of the SLBP RPD to the second GC base-pair that is critical for sequence-specific recognition of the RNA. In addition, the observation of FRET at 442 nm when the phosphorylated SLBP RPD is titrated into stem-loop RNA suggests that one or more of the conserved tryptophans toward the C-terminus of SLBP may be oriented toward the base of the RNA stem-loop and stack with either one or both G-C base-pairs at the base of the stem-loop and/or with the adenosines 5' of the stemloop. Our data suggests that the HPKTPNK sequence is a critical “hinge” region. The role of phosphorylation and prolyl isomerization in SLBP may be to orient the C-terminus of the RPD toward the base of the RNA stem and the single-stranded flanking regions in an “active” conformation. The existence of two binding sites on SLBP for double-stranded and single-stranded RNA interacting regions indicates that a unique mode of RNA recognition is employed in contrast to other modular RNA binding domains and could explain how high affinity and specificity is achieved by SLBP (Fig. 6). A more complete understanding of how SLBP contacts histone mRNA awaits NMR and X-ray crystallographic structures of the complex.

What are the possible roles of phosphorylation and prolyl isomerization of the SLBP RPD? From a functional perspective, threonine phosphorylation and prolyl isomerization of the SLBP RPD may allow the SLBP RNA binding domain to adopt a unique structure that is recognized by another protein in the processing complex. Consistent with this, mutation of the threonine to alanine in *Drosophila* SLBP (T230A SLBP) results in misprocessing of the histone pre-mRNA *in vitro* and *in vivo*(23). A full length T230A mutant *Drosophila* SLBP (equivalent of T171A in human SLBP) is also unable to restore viability to *Drosophila* SLBP null mutants indicating that phosphorylation at T230 is critical for the function of *Drosophila* SLBP in the cell. In unpublished studies from our laboratory (Krishnan, N and

Thapar, R, in preparation) we find that the phosphorylated TPNK sequence in the SLBP RPD is a substrate for the prolyl isomerase Pin1 *in vivo* and *in vitro*. Pin1 regulates cis-trans prolyl isomerization about the Thr171-Pro172 sequence dissociating SLBP from the histone mRNA complex *in vitro* and regulating the SLBP ubiquitination and histone mRNA decay *in vivo*.

From a structural perspective our studies highlight the importance of dynamics in RNA protein interactions. We have shown in this study that phosphorylation at this conserved threonine is a key determinant for the stability of the SLBP-RNA complex and that the SLBP RPD exists in multiple folded conformations that are attributed to proline isomerization. Threonine phosphorylation occurs in a highly basic region of the RBD and both positions (N-1) and (N+4) from the Thr are conserved Arg/Lys residues in all SLBP proteins. Therefore it is likely that the interaction of the phosphate with Lys and Arg residues in close proximity stabilizes the domain. Phosphorylation may also alter the conformational equilibrium between multiple states, thereby affecting conformational capture by the RNA. In such a model, the RNA binding domain would interconvert between multiple conformations, only one of which binds RNA with high-affinity. Quantitative studies in the literature on model peptides have shown that the introduction of a phosphate on a Ser/Thr N-terminal to a proline increases the cis-Pro content in peptides by changing the thermodynamic equilibrium for prolyl bond isomerization(38). In the absence of a PPIase, the cis to trans isomerization rate is 8-fold slower for a Thr/Ser phosphorylated peptide as compared to an unphosphorylated peptide. Intriguingly, we observe a similar 6–15 fold stabilizing effect of phosphorylation on the affinity for the stem-loop RNA, by filter binding experiments as well as SPR. The P231G SLBP mutation may alter conformational sampling so as to favor the binding competent conformation in SLBP. Therefore phosphorylation and prolyl isomerization of the SLBP RPD likely plays both structural and regulatory roles in histone pre-mRNA processing and histone mRNA metabolism.

Finally, we provide NMR evidence that the SL+HDE region of the pre-mRNA has secondary structure beyond the stem-loop. An important question in the field of histone pre-mRNA processing is how the two cis-elements, namely the stem-loop and the HDE cooperate to stimulate histone 3' end formation. We suggest that the two essential cis elements, the stemloop and the HDE, may be involved in a higher order RNA structure that is necessary for specific recognition by the multifactor protein complex that catalyzes the cleavage reaction. Ongoing studies will test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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(accession code 2kjm) and the assignments for the 30-mer and 20-mer RPD peptides deposited in the BioMagResBank (deposition numbers 16274 and 16323).

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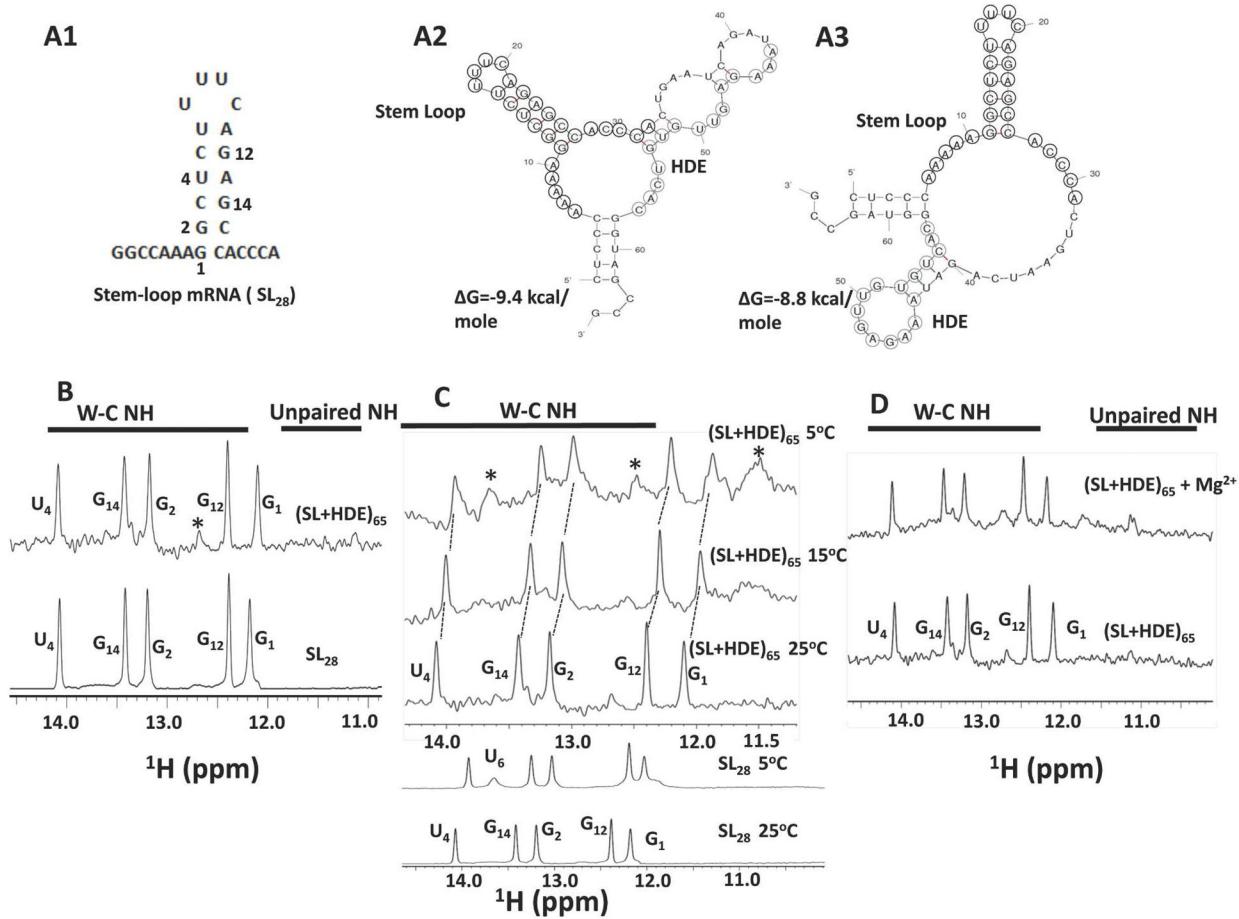


Figure 1. Structural determinants in the histone 3'UTR of the mouse H2A-614 RNA

Secondary structure of the histone stem-loop (SL28) mRNA the structure of which has previously been confirmed by NMR (A1), and secondary structure predictions (A2 and A3) of the 65-mer (SL + HDE) corresponding to the histone 3'UTR of the mouse H2A-614 histone gene by UNAFold. The sequence corresponding to the stem-loop and the HDE have been highlighted in circles. (B) Comparison of one-dimensional imino proton NMR spectra of 28-nt stem-loop SL28 (shown in A1) and the 65-mer (SL+HDE) sequence collected at 600 MHz, pH 6.8, and 25°C. (C) Comparison of one-dimensional imino proton NMR spectra of SL28 and (SL+HDE) collected at different temperatures at 600 MHz and pH 6.8. (D) Comparison of one-dimensional imino proton NMR spectra of (SL+HDE) in the presence of Mg²⁺. Assignments are given for the bottom spectra and the numbering corresponds to that shown for the stem-loop sequence in A1. Asterisks show new resonances.

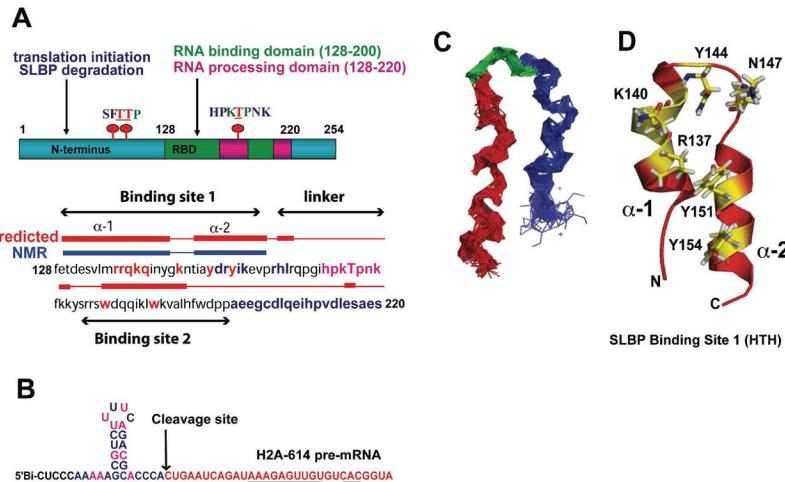


Figure 2. Solution NMR Structure of the 30-residue peptide fragment of the SLBP RPD

(A) The domain structure of human SLBP consists of an N-terminal domain important for translation initiation and SLBP degradation and a central domain that has the sequence determinants for RNA binding and processing. Residues previously shown to be important for RNA binding are in green and residues important for RNA processing but not RNA binding are shown in pink. The phosphorylated threonine in the sequence HPKTPNK is depicted. The two binding sites and the linker regions are shown. (B) The sequence of two cis-elements important for histone pre-mRNA processing in the H2A-614 3' untranslated region (UTR) are shown. The 26 nucleotide hairpin is conserved in all metazoans and binds SLBP. Nucleotides that are important for SLBP interaction are highlighted in pink. The HDE sequence that base-pairs with the U7 snRNP is underlined. In (C), superposition of the top 20 NMR conformers (out of 100 calculated) is depicted and in (D) a ribbon representation of the average solution structure of the first 30 residues of the hSLBP RPD is shown. Residues important for RNA binding from previous mutagenesis studies are depicted in stick representation.

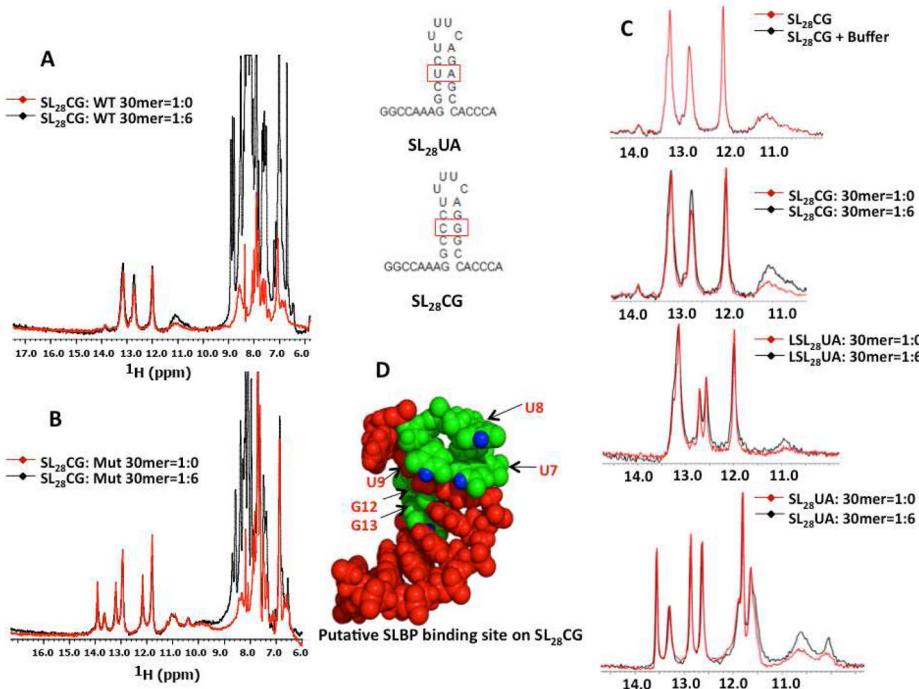


Figure 3. Interaction of the SLBP HTH motif with the histone mRNA hairpin monitored by 1D imino proton NMR spectra

(A) One-dimensional ¹H imino NMR spectrum (17–6.0 ppm) of the stem-loop RNA SL₂₈-CG in the presence of increasing concentration of HTH peptide collected at 1°C and 600 MHz are shown. The molar equivalents of SL₂₈ RNA: SLBP HTH peptide is 1:6. The samples were in phosphate buffer pH 6.8 as described in Methods. (B) One-dimensional ¹H imino NMR spectra of the stem-loop RNA SL₂₈-UA in the presence of increasing concentration of mutant HTH peptide (see methods) collected at 1°C and 600 MHz is shown. The molar equivalents of SL₂₈ RNA: SLBP HTH peptide is 1:5. The increased protection of unpaired imino protons is not due to baseline corrections. In (C), the imino region of the various 1D spectra are superimposed to highlight the small increase in intensity of the unpaired uridine iminos observed in the presence of the WT 30 mer peptide. No spectral change is observed with the buffer control. Both SL₂₈-CG and SL₂₈-UA show this effect. (D) The U7, U8, and U9 uridine bases are highlighted in green CPK and the imino protons that show protection from exchange in the presence of the HTH peptide are shown in blue. Small but significant changes in peak intensities were also observed for G12 and G13.

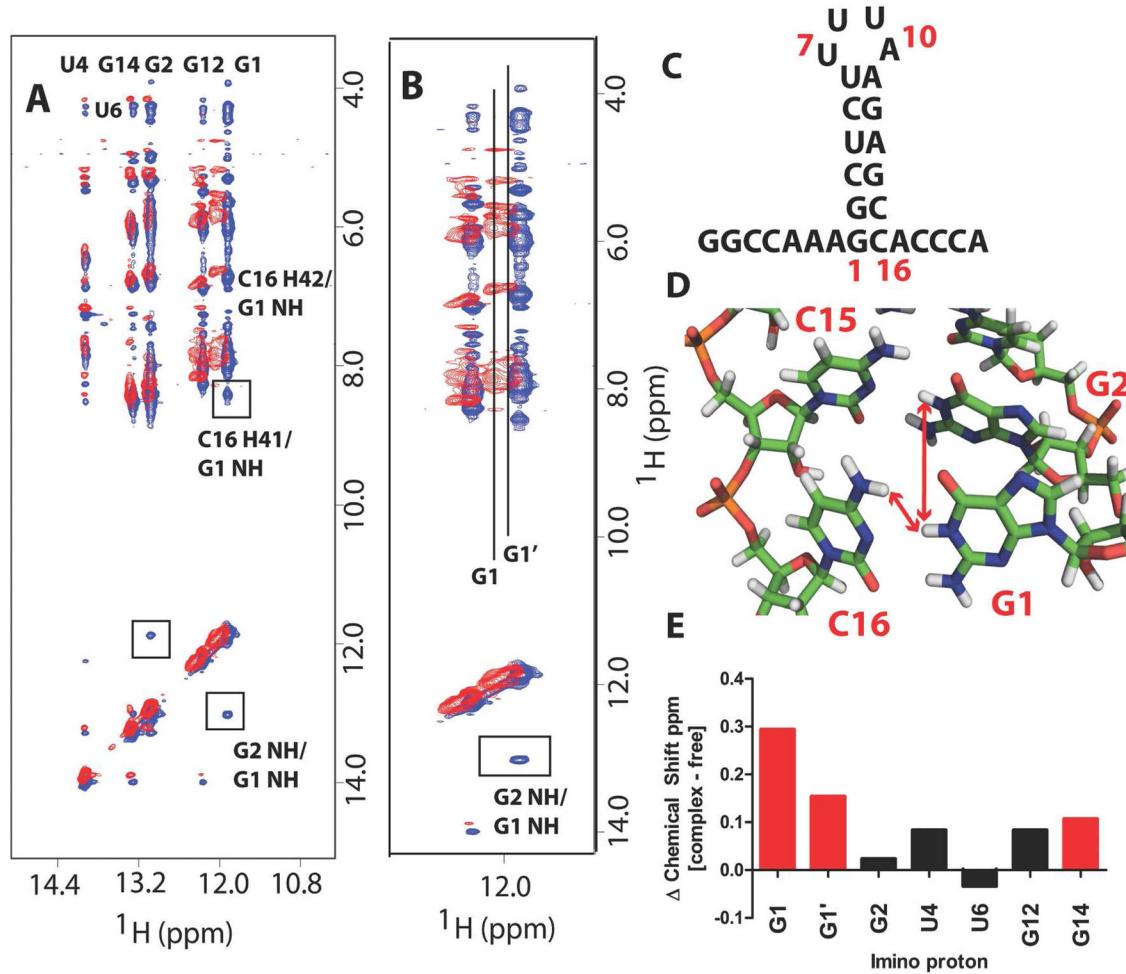


Figure 4. The first G-C base pair at the base of the stem is destabilized in the presence of the HTH peptide

(A) 2D (^1H , ^1H) NOESY data (125 ms) were collected at 900 MHz with cryoprobe on a 0.3 mM sample of SL28-UA at 1°C as described in Methods. The imino region of the free RNA is shown in blue and the same region in the presence of a six-fold excess of HTH peptide is shown in red. The most significant differences in the two spectra were the loss of the sequential G1-G2 imino-imino NOE that is characteristic of stem regions of RNA structure and the loss of the cross-strand NOE between the G1 NH and the C16 H4 amino proton. These NOEs are depicted on the structure of the SL28 RNA in (D). In (B) the expanded region of the NOESY spectrum is shown. In the presence of HTH peptide, two unique conformations for the G1 NH are observed (highlighted and labeled G1 and G1'), both with a different chemical shift compared to that of the single conformation observed in free RNA. The secondary structure and numbering of the stem-loop RNA used is shown in (C). The difference in ^1H chemical shift for the imino protons between the RNA complexed to the 30mer peptide and the free RNA is shown. In red bars are the imino protons for which the largest changes in chemical shift were observed. G1 and G1' represent the two different conformations of the first guanine base at the bottom of the stem.

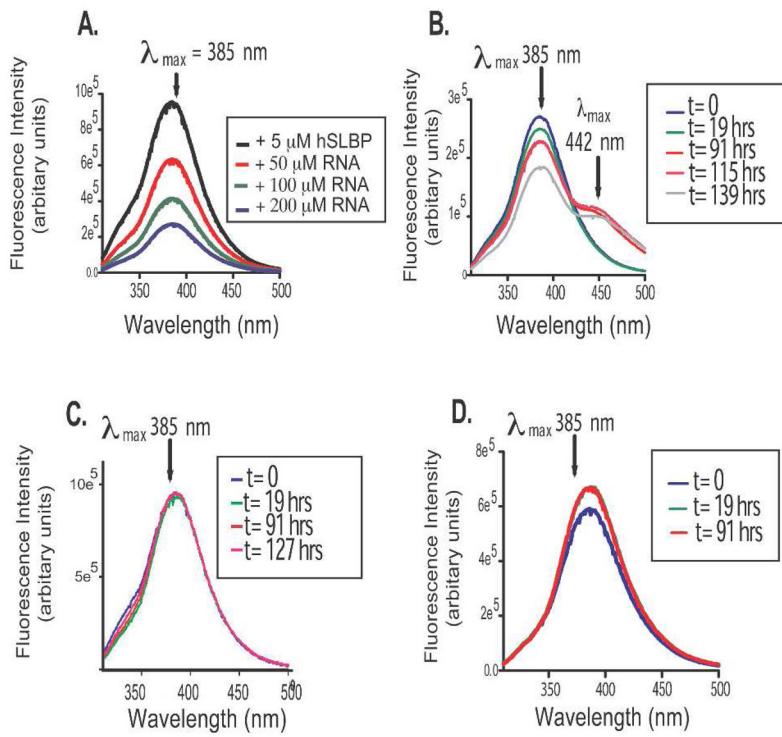


Figure 5. A second binding site for histone stem-loop RNA exists C-terminal to the HPKTPNK sequence

Fluorescence emission spectra obtained after fluorescence excitation of intrinsic tryptophans at 295 nm. (A) Fluorescence quenching observed as a function of increasing concentration of histone stem-loop RNA added; (B) time-dependent fluorescence quenching observed with 5 μM hSLBP in the presence of 200 μM stem-loop RNA at 385 nm and a corresponding increase in fluorescence intensity is observed at 442 nm resulting from Trp-Cytosine FRET; no time-dependent change in fluorescence is observed for (C) 5 μM hSLBP RPD in the presence of 200 μM HDE30 RNA; or (D) 5 μM dephosphorylated SLBP RPD in the presence of histone stem-loop RNA.

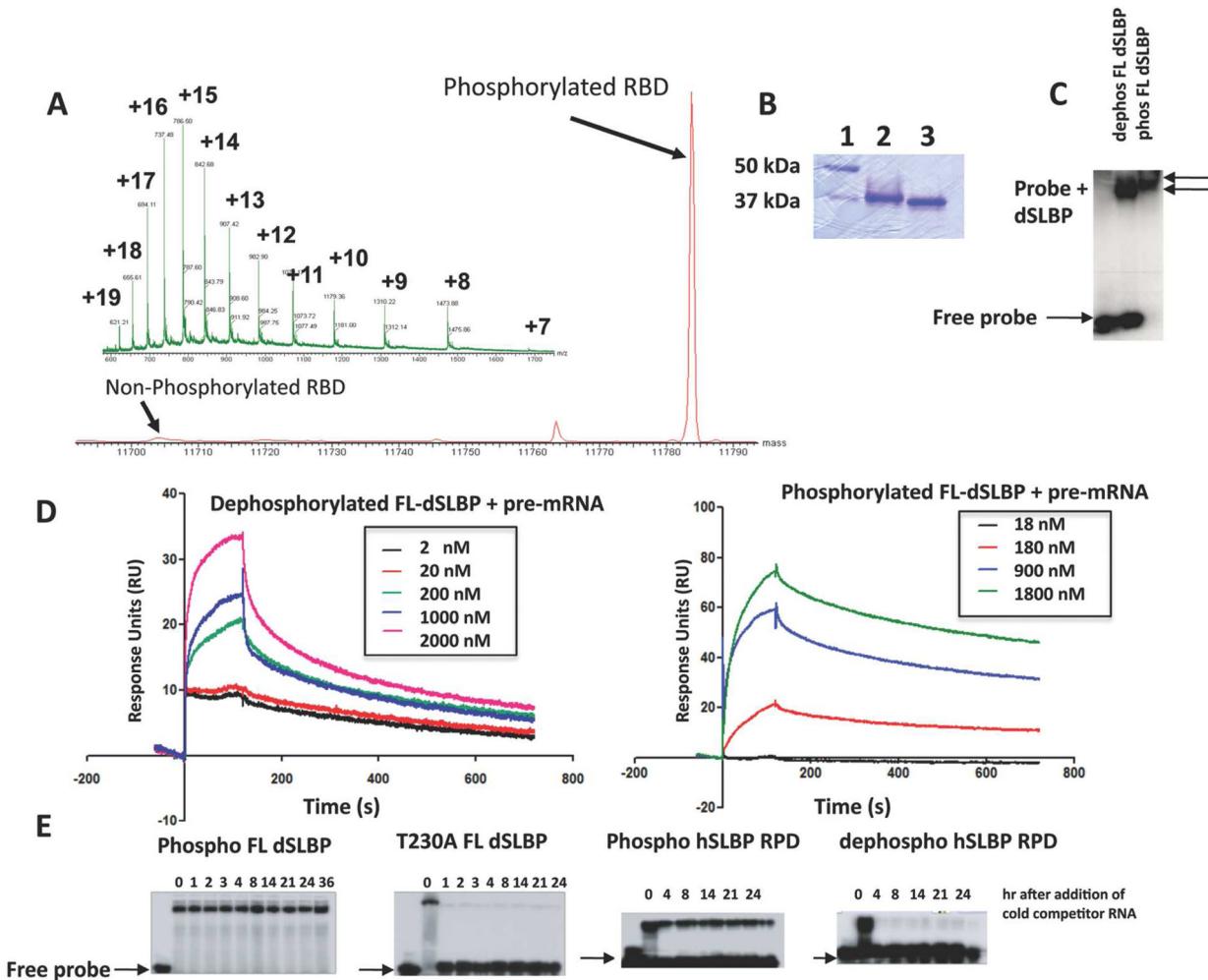


Figure 6. Effect of SLBP RPD phosphorylation on kinetics of association with histone mRNA

In (A), the electrospray mass spectrum (ES-MS) of the dSLBP RBD obtained on a Qtof-Micro mass analyzer is shown. The mass obtained was 11783.00 Da corresponding to a single phosphate at Thr230, cleavage of the N-terminal Met and acetylation of the N-terminal serine. Based on peak heights, the ratio of the phosphorylated: dephosphorylated RBD was 100:2. (B) SDS-PAGE on full length dSLBP proteins used for SPR. The molecular weight markers are in lane 1, the phosphorylated full length dSLBP is in lane 2, and the dephosphorylated full length dSLBP in lane 3. (C) EMSA on the phosphorylated and dephosphorylated full length dSLBP proteins shows a difference in mobility in a native gel. (D) Representative SPR sensograms for unphosphorylated and baculovirus-expressed phosphorylated *Drosophila* SLBP RPDs. The association phase was measured between time 0 and 120s and the dissociation phase was up to 600s. The baseline was re-established by regeneration of the chip with 3M NaCl. (E) Dissociation of *Drosophila* SLBP full length and the human RPD proteins measured using EMSA in the presence of 100-fold excess cold competitor RNA at 37°C. All proteins were expressed in baculovirus. The hSLBP RPD was dephosphorylated where indicated by calf intestinal phosphatase.

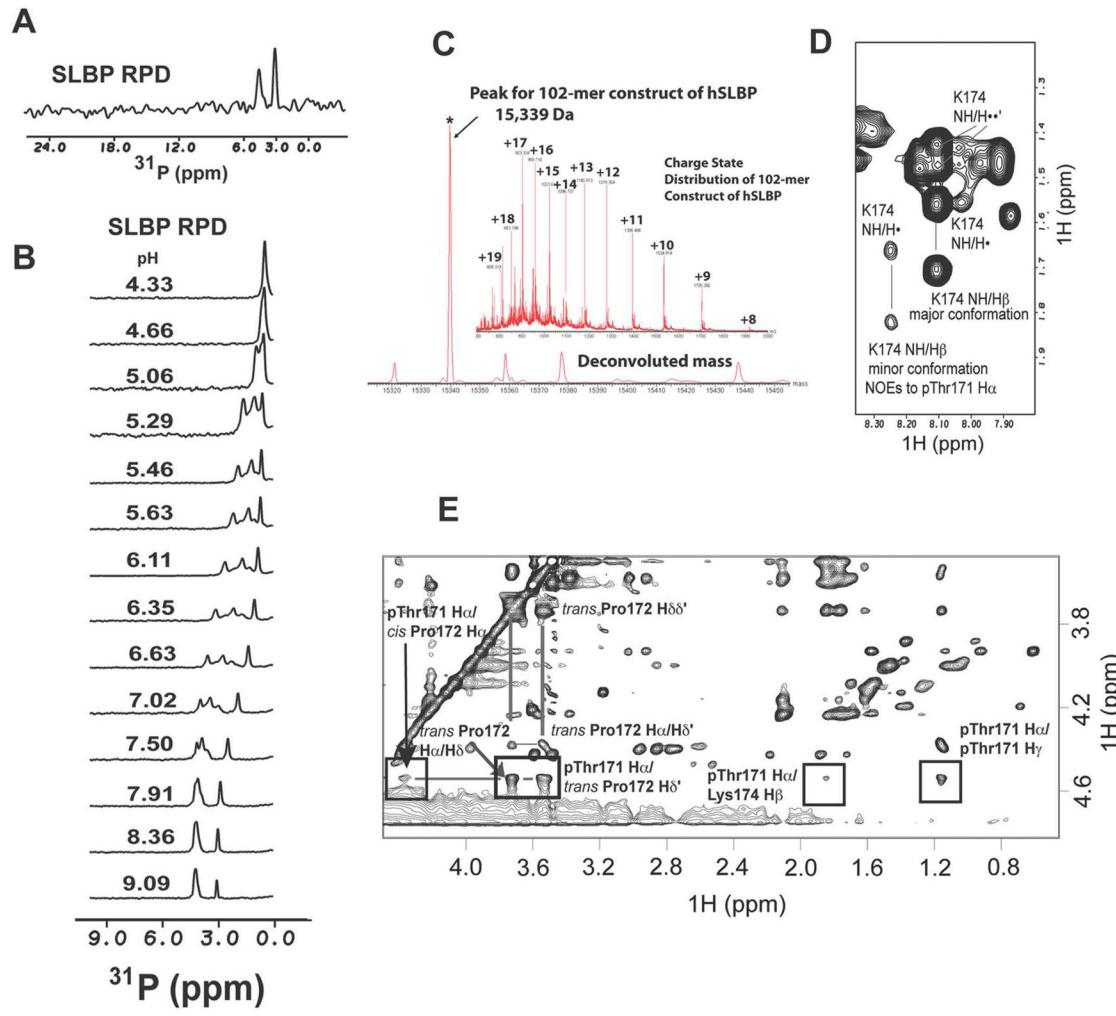


Figure 7. The hSLBP RPD shows evidence for multiple conformations that are attributed to prolyl isomerization

(A) ^{31}P NMR spectrum of human SLBP RPD collected at 500 MHz, pH 8.1, and 25°C. Two ^{31}P resonances are observed for the RPD that is phosphorylated only on a single threonine in the HPKTPNK sequence. (B) pH titration of the SLBP RPD monitored by ^{31}P NMR. In (C), the ES-MS mass spectrum used for ^{31}P NMR of the SLBP RPD is shown. The protein exists as a single species that is phosphorylated only at T171. In (D) NH-aliphatic NOEs for the major and minor conformations of Lys174 are shown in the spectrum of the 20-mer unphosphorylated peptide, providing additional evidence that the peptides exist as two conformations that are in slow exchange on the NMR time scale. In panel (E), a portion of the NOESY spectrum of the WT phosphopeptide (pepWT) is shown. The pattern of NOEs provide clear evidence for proline cis-trans isomerization about the phosphoThr171-Pro172 bond. The NOEs originating from the H α chemical shift of phosphoThr171 are highlighted. As discussed in the text, a weak NOE is observed between the phosphoThr171 H α and the Pro172 H α that is diagnostic of a cis-Pro conformation. Medium to strong NOEs are observed between the phosphoThr171 H α and the Pro172 H δ that are diagnostic of a trans-Pro bond.

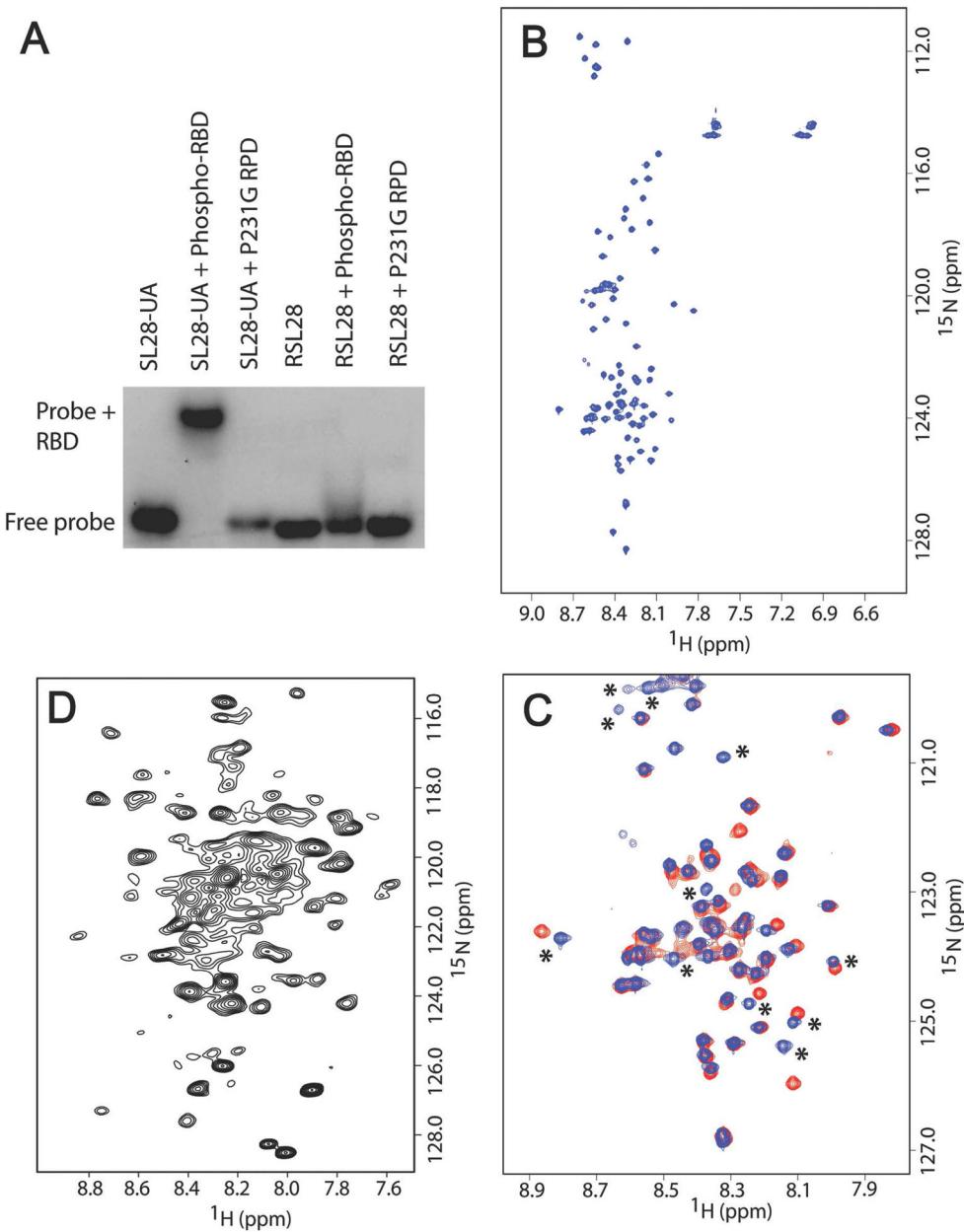


Figure 8. The P231G dSLBP mutant forms a single conformer that binds SL28 histone mRNA

(A) $5'$ ^{32}P labeled SL₂₈-CG RNA was used to compare the affinity of the baculovirus expressed phosphorylated dSLBP RBD to the bacterially expressed non-phosphorylated P231G mutant SLBP in EMSA assays. The baculovirus expressed dSLBP RBD forms a specific and high affinity complex with SL₂₈-CG RNA but not with the RSL₂₈-CG mutant RNA. In contrast, the P231G dSLBP RBD does not shift the probe, but a decrease in the relative amount of free probe is observed, indicating that the complex likely dissociates throughout the gel due to weak binding. No binding was observed for the RSL₂₈-CG mutant. (B) (^1H , ^{15}N) HSQC spectrum of uniformly ^{15}N -labeled P231G SLBP RPD shows a single conformation for all backbone amide resonances. The spectrum was collected on an 80 μM sample at 900 MHz with cryoprobe at 25°C. (C) Portion of the (^1H , ^{15}N) HSQC spectrum of uniformly ^{15}N -labeled P231G SLBP RPD collected in the absence (in blue) and presence (in red) of SL₂₈-CG RNA. The difference spectrum is shown in black. Asterisks indicate residues that show significant changes in chemical shifts upon binding.

red) of equimolar amount of unlabeled SL₂₈-CG RNA. (D) Portion of the (1H, 15N) HSQC spectrum of the wild type 15N-labeled dSLBP RPD collected at 700 MHz with cryoprobe at 25°C is shown for comparison.

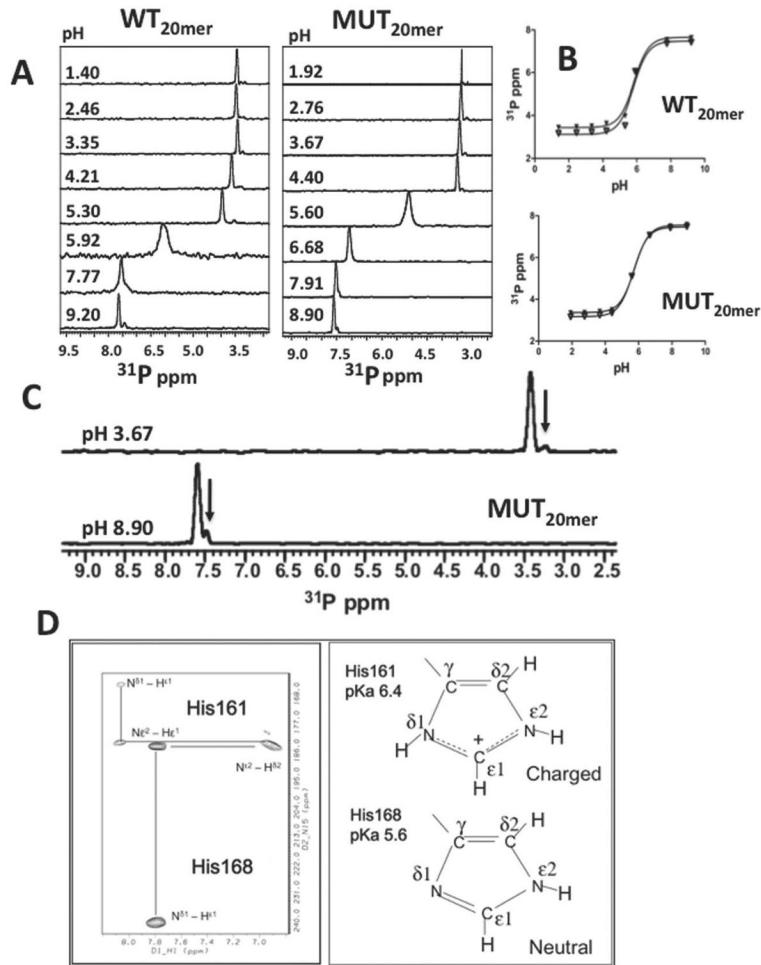


Figure 9. Role of His168 in the HPKTPNK sequence in the observed conformational heterogeneity of the SLBP RPD

(A) A 20 residue threonine phosphorylated peptide corresponding to the WT sequence and a mutant 20 residue peptide in which His168 was replaced by alanine were used. The sequences of the peptides are in Methods. The peptides were taken up in 20 mM Tris acetate buffer pH 6.8, 50 mM NaCl and 90% H_2O /10% D_2O . The dependence of the ^{31}P chemical shifts on pH was monitored. In (B) the change in chemical shift was plotted as a function of pH to obtain the pKa for the ionization of the phosphate. Closed triangles represent the major conformer and open triangles the minor conformer. In (C) an expanded portion of the ^{31}P NMR spectra for the mutant peptide is shown at two different pH values. Arrows point to the minor conformer in the peptide which was also observed in the WT peptide. (D) Portions of (^1H , ^{15}N) HMQC-J spectra of the WT phosphopeptide are shown on the left and the tautomeric states of the histidines is shown on the right. The spectra were collected using phosphopeptides at natural abundance at 700 MHz using a cryoprobe at 25°C and pH 6.5.

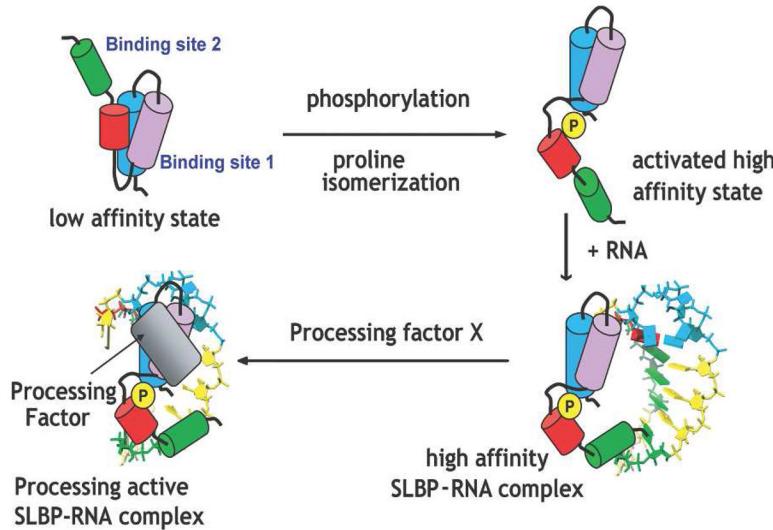


Figure 10. Our proposed model for how SLBP recognizes histone mRNA stem-loop and the role of phosphorylation and prolyl isomerization in RNA recognition

The SLBP RPD can exist in two unique conformational states of low and high activity i.e a major, low affinity state, and a scarcely populated, activated high affinity state. Phosphorylation populates the high-affinity conformation which is captured by the RNA. Once bound, SLBP forms a stable complex with the histone mRNA hairpin and may interact with another factor in the pre-mRNA processing reaction thereby participating in 3' end formation.

Table 1

NMR Structure Statistics for the SLBP RPD HTH peptide

NMR distance and dihedral constraints	
Distance constraints	
Total NOE	409
Intra-residue	166
Inter-residue	243
Sequential ($ i - j = 1$)	145
Medium-range ($ i - j < 4$)	77
Long-range ($ i - j > 5$)	21
Hydrogen bonds	0
Total dihedral angle restraints	
ϕ	18
Structure Statistics	
Ramachandran analysis (%)	
Residues in most favored regions	86.1
Residues in additional allowed regions	13.9
Deviations from idealized geometry	
Bond lengths (Å)	0.0048 ± 0.0002
Bond angles (°)	0.582 ± 0.017
Average pairwise r.m.s. deviation, 20 conformers (Å)	
Heavy	1.72 ± 0.21
Backbone	0.90 ± 0.27

Table 2

Rate constants derived from Surface Plasmon Resonance (SPR) for *Drosophila* SLBP full length and RBD (residues 189–259) towards Histone mRNA.
at 25°C

Proteins and RNA	k_{+1} ($M^{-1}s^{-1}$)	k_{-2} (s^{-1})	K_D (nM)	K_D (rel)*	$\Delta\Delta G$ (kcal mol $^{-1}$)
Phosphorylated SLBP					
Full length dSLBP- SL28 RNA	1.2E4	5.7E-4	47.5 ± 17.0	14.8	1.6
Full length dSLBP- pre-mRNA	4.1E4	1.3E-4	3.2 ± 2.9	-	
RBD (residues 189–259)-SL28 RNA	6.9E3	8.7E-2	1268 ± 195	396.2	3.5
RBD (residues 189–259)-pre-mRNA	2119	1.63E-3	766.8 ± 138	239.6	3.2
Dephosphorylated SLBP					
Full length dSLBP- SL28 RNA	1.4E5	7.4E-2	528.6 ± 73	165.2	3.0
Full length dSLBP- pre-mRNA	8522	8.05E-4	94.5 ± 18	29.5	2.0
RBD (residues 189–259)-SL28 RNA	Unstable#	Unstable#	-	-	-
RBD (residues 189–259)-pre-mRNA	307.7	1.79E-3	5827 ± 115	1821	4.4

* K_D values relative to the interaction of full length phosphorylated SLBP with the pre-mRNA sequence used

See Supplementary Fig. 5