Critical residues for RNA discrimination of the histone hairpin binding protein (HBP) investigated by the yeast three-hybrid system

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Abstract The histone hairpin binding protein (HBP, also called SLBP, which stands for stem-loop binding protein) binds specifically to a highly conserved hairpin structure located in the 3′ UTR of the cell-cycle-dependent histone mRNAs. HBP consists of a minimal central RNA binding domain (RBD) flanked by an N- and C-terminal domain. The yeast three-hybrid system has been used to investigate the critical residues of the human HBP involved in the binding of its target hairpin structure. By means of negative selections followed by positive selections, we isolated mutant HBP species. Our results indicate tight relationships between the RBD and the N- and C-terminal domains.

Key words: HBP; SLBP; 3′ UTR; Cell-cycle-dependent histones; RNA binding domain

1. Introduction

Replication-dependent histone genes in metazoans are unique in the eukaryotic kingdom in that they do not contain any intron [1,2]. In addition, the corresponding mRNAs are not polyadenylated at their 3′ end but are synthesized as longer mRNA precursors, which undergo processing by means of an endonucleolytic cleavage at their 3′ ends [3]. This processing occurs between two *cis*-acting elements; a highly conserved hairpin structure located five nucleotides upstream of the maturation site and a purine-rich sequence referred to as the spacer element (or HDE for histone distal element) downstream of the maturation site [4]. The cleavage also needs *trans*-acting factors. These are the U7 snRNP containing the U7 snRNA that anchors to the histone mRNA precursors by annealing to the spacer element, a poorly characterized heat sensitive factor called the heat labile factor (HLF) and the hairpin binding protein (HBP, also called SLBP, which stands for stem-loop binding protein) which binds specifically to the hairpin structure in the 3′ UTR of the histone mRNA. Finally, ZFP100 is a protein rich in zinc-finger motifs required for 3′ end processing and which interacts with the complex HBP–RNA hairpin [5]. After processing, HBP remains bound to the hairpin structure [6]. Besides its role in the 3′ end processing of histone mRNA, HBP has been shown to be required in several other steps of histone mRNA biogenesis. HBP is believed to be involved in nuclear export of mature histone mRNA [7,8], and has been demonstrated to be essential for efficient translation of histone mRNA by interacting with translation initiation factors eIF3 and eIF4G [9–11]. In addition, HBP is required for stability of the histone mRNA [8,12]. The genes encoding HBPs from various organisms have been characterized [13–15], and the homology between these genes is rather high, especially in a ~70 amino acid long minimal central RNA binding domain (RBD). HBP binds to its target RNA with a high affinity ($K_d = 0.85$ nM) and is able to discriminate the histone hairpin with high specificity [16]. In addition, this RBD domain has no significant homology with any previously described RNA binding motif. Therefore, HBP probably represents a new type of RBD. To further characterize this new type of RBD, we developed a three-hybrid strategy based on selection of loss-of-binding mutants of HBP as a first step and then, selection of compensating mutations restoring the binding to the histone hairpin target in a second step. We were able to select five point mutations abolishing partially or totally the binding to the histone hairpin. All these mutations mapped in the central RBD. Starting from these mutated HBPs, we selected intragenic compensating mutations restoring the binding to the histone hairpin. In contrast to the first mutants, most of these mutations were located outside of the RBD strongly suggesting that the N-terminal and the C-terminal domains modulate the conformation of the RNA binding site in order to accommodate the histone hairpin.

2. Materials and methods

2.1. Yeast strain and plasmid constructs

The three-hybrid system procedures were carried out in the *Saccharomyces cerevisiae* strain L40-coat [17]. Plasmid pIII/MS2.2 was used for expression of the hybrid RNA molecules in the yeast three-hybrid system [17]. The wild-type and mutant histone hairpins were cloned into the *Sma*I site of pIII/MS2.2. They are designated pIII/wtHP/MS2, pIII/G5U-U13A/MS2, pIII/cgHP/MS2 and pIII/mutHP/MS2 [18]. Wild-type and mutant HBPs were expressed as HBP-Gal4 activation domain (AD) fusion protein by the pAct-HBP [14].

2.2. Negative and positive screening procedures

Loss-of-binding mutants of HBP were selected on the basis of the

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Abbreviations: RBD, RNA binding domain; HBP, hairpin binding protein; SLBP, stem-loop binding protein; HDE, histone distal element; ORF, open reading frame

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blue coloration of colonies on X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)-containing medium as previously described [18]. The plasmids encoding these mutants were randomized by hydroxylamine treatment and then introduced into L40-coat cells containing the plasmid pIII/wtHP/MS2. The positive screens for revertants were selected by transformants that could grow on a synthetic medium (YNB) lacking uracil, histidine and leucine. Ten mM 3-amino-1,2,4-triazole (3-AT) was included in the medium in order to identify reverting HBP able to bind with strong affinity to the wild-type hairpin structure (wtHP). Colonies appeared after 5–6 days and were further analyzed for *lacZ* expression by plating on YNB medium lacking uracil and leucine and supplemented with 80 μg/ml X-Gal. To confirm that changes in reporter gene activation were caused by mutations in the HBP open reading frame (ORF), the corresponding pAct-HBP were reintroduced into fresh yeast cells containing pIII/wtHP/MS2 and transformants were tested for activation of the *HIS3* and *lacZ* on appropriate selective media. The coding regions of the mutant proteins that still confirmed their phenotype were cloned into non-mutagenized pAct2 plasmids and tested for activation of both reporter genes as described above. The HBP cDNA insert of the positive clones was sequenced.

2.3. *Measurement of* HIS3 *activation in the yeast three-hybrid system*

In vivo, the gene expression level of *HIS3* directly reflects the strength of the RNA–protein interaction between the two hybrids MS2-HP and HBP-GAL4AD. To quantify the *HIS3* gene expression level, we monitored the growth of L40-coat cells expressing the hybrids on increasing concentrations of the *HIS3* gene product inhibitor 3-AT. The expression level is defined as the highest concentration of 3-AT that still allows growth. Four μl drops of yeast double transformants grown to $OD_{600} = 0.1$ were applied to plates prepared with YNB medium lacking uracil, leucine and histidine but supplemented with increasing 3-AT concentrations (0–225 mM). After three days of incubation at 30°C, levels of *HIS3* expression were defined by the highest 3-AT concentration allowing growth.

3. Results

The interactions between HBP and its target hairpin RNA can be monitored by the yeast three-hybrid method. The minimal 24 nucleotide RNA binding site has been cloned into the plasmid pIII/MS2.2, which allows the expression of a hybrid RNA molecule containing a MS2 binding site fused to histone RNA hairpins (Fig. 1A). A fusion protein consists of the *GAL4* activation domain fused to the human HBP. The interaction between the histone hairpin and HBP then triggers the expression of the reporters *HIS3* and *lacZ*. To better understand the molecular mechanism of this interaction we chose to use this method to isolate HBP mutants. Negative and positive selections were employed in order to identify critical residues involved in the RNA hairpin binding.

3.1. *Selection of loss-of-binding mutations and counterselection of suppressors that revert the negative effect*

From a randomly mutagenized library of pAct-HBP, we selected mutated HBP proteins that abolished or diminished binding to the wild-type histone hairpin. For this, we used the blue/white color phenotype on X-Gal plates. The yeast transformants were replica-plated on minimal selective media supplemented with X-Gal. Among a large majority of blue clones, the white ones were further analyzed. After phenotype confirmation, the HBP ORFs from the corresponding plasmids were sequenced. The most severe effects for binding to the RNA were observed for five single mutant proteins; G145R, P172S, P172L, R181H and D184N (Fig. 1B). Inter-

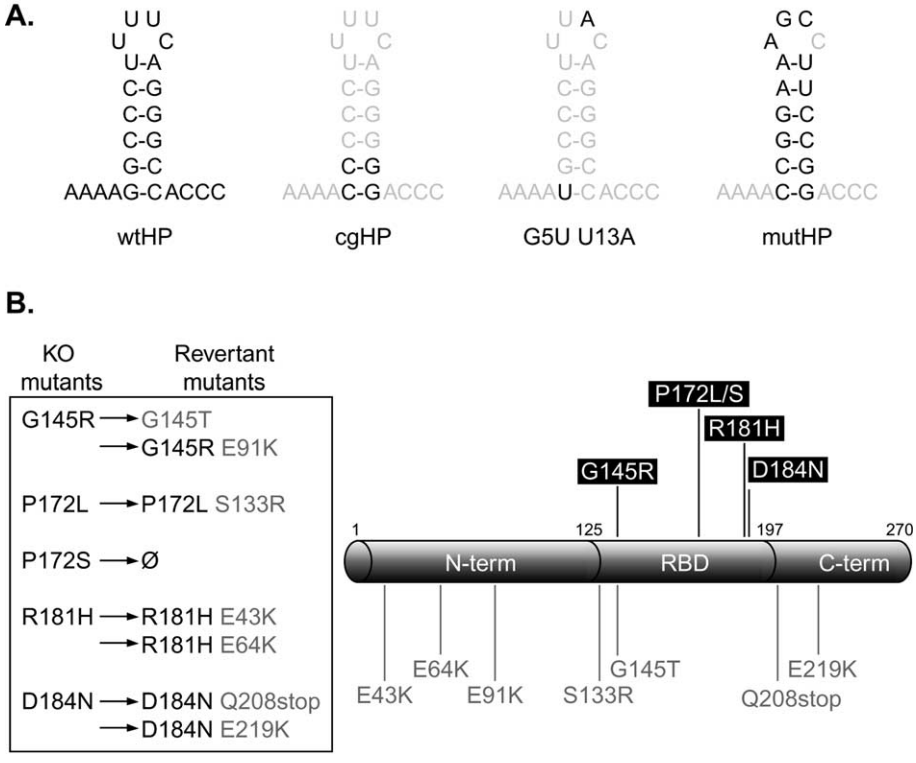

Fig. 1. A: Representation of the histone wild-type hairpin RNA target and mutated hairpins used in this work. The nucleotides that are identical in the three mutant hairpins compared to the wild-type hairpin are shown in gray and the mutations in black. B: Schematic representation showing the mutated residues selected by the yeast three-hybrid system. Human HBP is depicted as a cylinder on the right side. The protein consists of a central minimal RBD and N- and C-terminal domains. The selected loss-of-binding mutations are shown in black squares above the cylinder and the compensating mutations are shown in gray under the cylinder. On the left side, all the single and double mutants are listed.

estingly, all these mutations are mapped in the minimal RBD [13]. As these mutants were in vivo produced and were tested as fusion proteins with the Gal4 activation domain we separated the HBP part from the Gal4 domain in order to confirm that the observed effects were not caused by the fusion. For this, we subcloned the corresponding mutated ORFs into pFastBac plasmid for baculovirus expression. Then, the recombinant purified HBPs were tested for binding to the wild-type histone hairpin by electrophoretic mobility shift assay. These experiments confirmed that the selected single mutants are not able to bind to the wild-type histone hairpin (data not shown).

These single mutants of HBP were then used as templates to select intragenic suppressive mutations that restore the binding to the wild-type histone hairpin. The previously mutated pAct-HBP vectors were randomized with hydroxylamine and screened for binding to the wild-type hairpin. For G145R, two suppressors were selected, a double mutant G145R/E91K and a reversion of G145R to G145T (Fig. 1B). Concerning position P172, we could only select suppressive mutations from P172L (P172L/S133R) but not from P172S. Finally we selected two suppressive mutations from R181H and two from D184N (Fig. 1B). Among the suppressive mutations, four substitutions from glutamate to lysine residues were selected at positions 43, 64, 91 and 219. In addition, one mutation introduced a non-sense codon (Q208stop) leading to a C-terminal-truncated protein. Interestingly, all these suppressive mutations are located outside of the RBD except mutation S133R, which suppresses the P172L mutation. Compensating mutations selected from D184N mutant led to selection of two mutations in the C-terminal domain whereas all the remaining compensating mutations are located in the N-terminal domain of HBP.

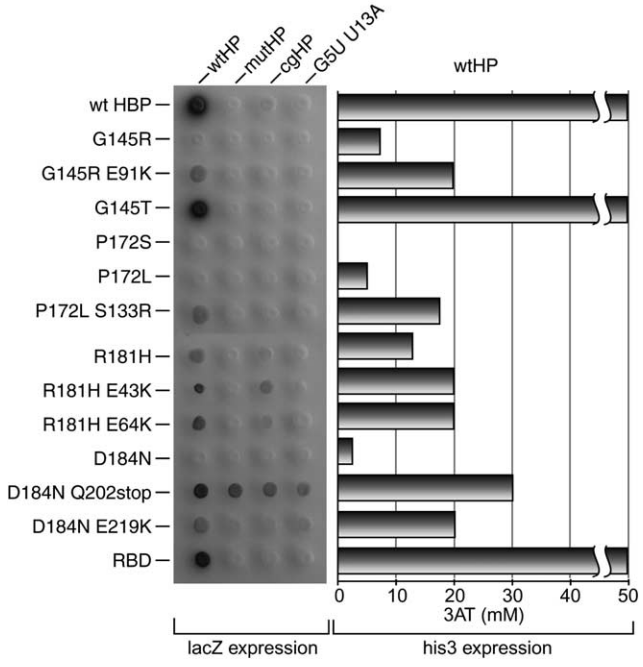

Fig. 2. Measurements of *lacZ* expression by drop test on X-Gal containing medium of the HBP variants and the four hairpin RNAs on the left. The histogram on the right represents the higher 3-AT concentration, which still allows growth of yeast expressing the wild-type hairpin RNA.

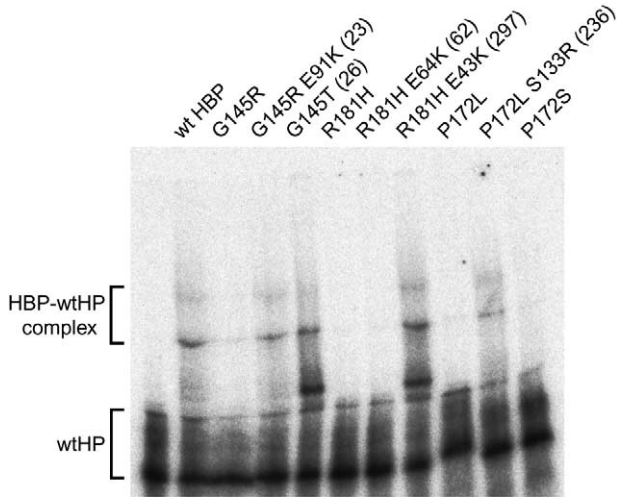

Fig. 3. Electromobility shift assays of internally radio labelled wtHP RNA incubated with yeast extracts expressing the wild-type and mutant HBP fusion proteins used in the yeast three-hybrid system.

3.2. Analysis of the binding properties of the mutants

To determine the affinity between the mutant HBP and the hairpin RNA, we performed drop tests experiments on media containing increasing concentrations of the drug 3-AT. This allowed the determination of the expression level of *HIS3* that reflects the binding strength of the HBP protein–histone hairpin complex in the three-hybrid system [17]. The starting loss-of-binding mutants G145R, P172S, P172L, R181H and D184N were able to promote growth up to 10 mM 3-AT (Fig. 2). The most drastic mutation was P172S, which did not promote growth even without 3-AT in the medium. As expected, introduction of the suppressive mutations led to a higher affinity for the wild-type hairpin, as can be deduced from the increased resistance to 3-AT. Mutant G145T and double mutant D184N/Q208stop turned out to be the most efficient in restoring binding to the histone hairpin since they promoted growth of yeast cells on media containing more than 100 mM and 30 mM 3-AT, respectively. The other mutations improved the binding to the wild-type hairpin and promoted growth on media containing from 17.5 to 20 mM 3-AT. By electromobility shift assays of radioactively labelled wtHP RNA with yeast extracts, we confirmed that the loss-of-binding single mutants did not bind to the hairpin whereas the compensating mutations restore efficiently the binding (Fig. 3). However the band shift was rather faint in the case of the mutants R181H E64K.

3.3. Some suppressors display relaxed RNA binding properties

After determination of the tightness of the complex with the wild-type hairpin, we assayed the binding properties for different RNA targets shown in Fig. 1A. The growth limiting concentrations of 3-AT were measured for the four RNA baits and listed in Table 1. The wild-type HBP bound specifically to the wtHP with high affinity (225 mM 3-AT) and very slightly to cgHP and G5U/U13A (5 mM 3-AT). However it did not bind at all to the negative control mutHP, which consists of a mutated six base pair stem and four member-nucleotide loop. All the tested HBP mutants bind preferentially to the wtHP meaning that they keep selectivity for the cognate RNA target. As expected, the loss-of-binding mutants show very weak

Table 1
The results of drop test experiments for the mutant HBPs with the wild-type histone hairpin (wtHP) and three mutated hairpins (mutHP, cgHP and G5U/U13A)

	wtHP	mutHP	cgHP	G5U/U13A
wt HBP	225	0	2.5	7.5
RBD	150	0	2.5	2.5
G145R	7.5	0	0	0
G145R E91K	20	2.5	2.5	5
G145T	>100	0	0	0
P172S	0	0	0	0
P172L	5	0	0	0
P172L S133R	17.5	0	0	0
R181H	12.5	0	5	0
R181H E43K	20	0	15	0
R181H E64K	20	0	7.5	0
D184N	2.5	0	2.5	2.5
D184N Q208stop	30	12.5	12.5	12.5
D184N E219K	20	10	10	10

The values correspond to the highest 3-AT concentration (mM) that allows growth of the yeast L40-coat. They reflect the affinity of the RNA–HBP interaction measured in the three-hybrid system.

affinity to the wild-type and mutant hairpins. The suppressors of these HBP mutants recognize the mutHP, cgHP and G5U/U13A targets with rather low affinity, as does the wild-type HBP. However, examination of the binding strength ratio between the wtHP and the different mutated hairpins suggests that some loss-of-binding mutants and their derived suppressors exhibited a less stringent recognition. For instance, mutant D184N recognizes wtHP as well as cgHP and G5U/U13A. On the other hand, the G145T mutant, a true revertant of the loss-of-binding G145R mutant became more specific than the wild-type HBP as showed by its exclusive recognition of the native hairpin.

4. Discussion

Using the yeast three-hybrid system, we selected loss-of-binding mutations located in the RBD. The five selected loss-of-binding mutations concerned residues that are absolutely conserved in the HBP from various organisms except for G145, which is an alanine in *Caenorhabditis elegans* HBP (Fig. 4). The essential role of three of these residues was already shown. R181 was isolated as a Q and C mutant and D184 as an N mutant [18]. In HBP from *C. elegans* the equivalent-P172S mutation (P249S) was shown to be lethal for the worm development [19].

Wild-type HBP and its RBD alone bind the wtHP with high affinity and discriminate very efficiently for non-cognate hairpins like mutHP, cgHP and G5U/U13A (Fig. 1A). The stem loop mutHP that differs in its entire sequence is not recognized at all (Table 1). The partially mutated cgHP and G5U/U13A stem loops are poorly bound, showing the high selectivity of native HBP for the cognate hairpin. Analysis of the binding properties of the five selected loss-of-binding mutants revealed for two of them unexpected decreases of the recognition stringency. Mutants R181H and D184N displayed decreases of the binding strength for the wild-type hairpin but without significant decrease for the mutated hairpins (Table 1). These data suggest that R181 and D184 are proper elements of the binding site of wtHP that do not participate to

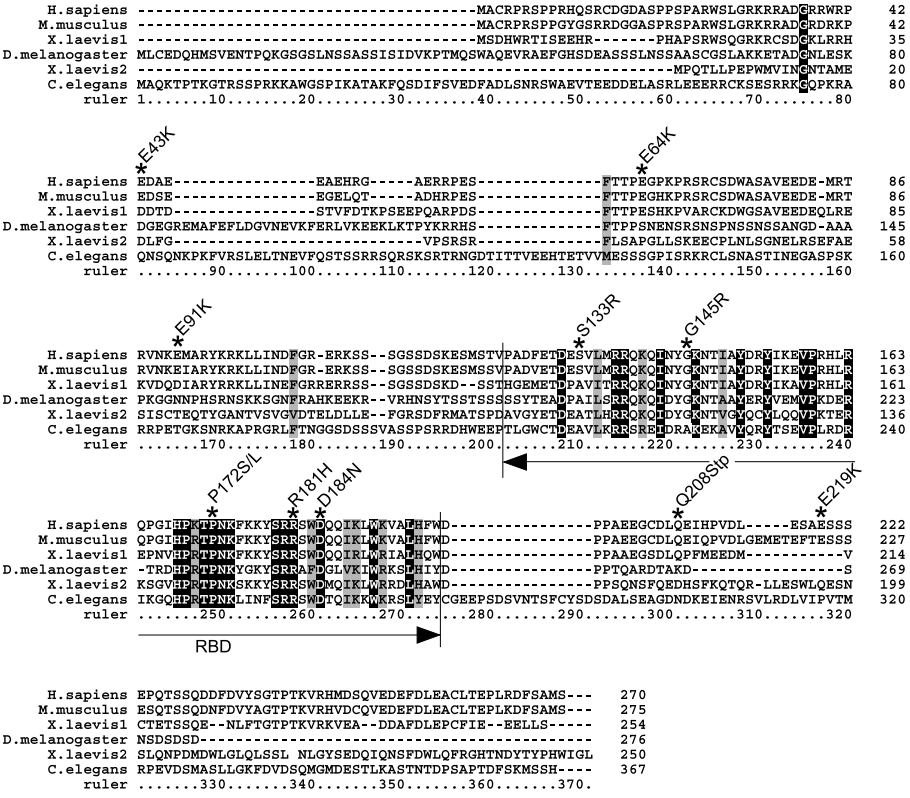

Fig. 4. Alignment of HBPs from *H. sapiens*, *M. musculus*, *X. laevis*, *D. melanogaster* and *C. elegans*. Positions of mutations selected by the three-hybrid system are shown above the alignment. Strictly conserved residues are boxed in black and the partially conserved residues are boxed in gray. The position of the central RBD is also indicated.

the binding of cgHP and G5U/U13A hairpins. It would also mean that these mutated stem loops bind HBP in a way at least partially distinct from the native hairpin. On the contrary, the other loss-of-binding mutations G145R, P172S and P172L exhibited global decreases of the binding strength for all the hairpins. Proline and glycine residues are known to constraint the protein structure. The selected mutations introduce residues exhibiting contrasting physicochemical properties with strong effects. This might reflect the essential structural role of G145 and P172.

Compensating mutations were selected from mutagenized loss-of-binding DNA libraries with the three-hybrid strategy. Six new mutations were selected with increased affinity for the histone hairpin. A seventh mutant contained a reversion of G145R in G145T and exhibited binding properties similar to native HBP. Therefore, it can be considered as a simple silent mutation of G145. Remarkably, all suppressive mutations except one mapped outside of the RBD from where all the loss-of-binding mutations are located. Additionally, most of the mutations change negatively charged residues into positively charged residues that could interact with the phosphate groups of the RNA hairpin structure. In all cases, the mutations exhibited an increased affinity for the histone hairpin, as measured by resistance to 3-AT. Nevertheless, this affinity never reaches the level of the native HBP even with the most efficient compensating substitutions (E43K, E64K, E91K, and E219K). But curiously, the best binding restoration was measured for a deletion mutant deprived of its 62 C-terminal residues (Q208stop) that reverted the loss-of-binding effect of D184N. Thus, partial removal of the C-terminal domain produced an effect comparable to those observed for the single mutations selected in the same domain. This also shows that the C-terminal domain plays a negative role in the recognition of the hairpins that are not correctly bound to the central RBD domain. It might control the initial binding to the RBD and helps to discriminate the non-cognate hairpins. Altogether, these data suggest that the C- and probably N-terminal parts of the protein play a role in the recognition process of the stem loop. The N- and C-terminal domains could establish specificity by steric hindrance or charge effect. Thus, changing a residue charge would transform a negative effect on non-cognate RNA into a positive effect by increasing its binding strength. In the same manner, removing the C-terminal domain that discriminates for the correct hairpin would increase the binding of non-cognate substrates. Interestingly, the mutations Q208stop and E219K have already been selected in a screen for HBP mutants that were able to recognize non-cognate hairpins [18]. The single mutants exhibited affinity constants for the mutated hairpins very similar to the double mutants selected here and an unchanged affinity for wtHP. In this work, the double mutants D184N/Q208stop and D184N/E219K exhibited a non-specific increase of binding efficiency for all hairpins, confirming that they induce a global expansion of the substrate recognition and a moderate increase of the binding energy. This emphasizes the central role played by the RBD in providing most of the binding energy of the hairpin and the discriminating role played by the N- and C-terminal domains.

Only one compensating mutation was selected inside of the RBD. The effect of the positive charge of S133R concerns specifically the binding of the cognate hairpin, which was increased whereas no binding was measurable for the non-cognate hairpins. This reinforces the presumption that the interactions with the RBD mainly contribute to the binding energy of the cognate hairpin and that the RBD probably adopts a rigid conformation highly adapted to the binding of wtHP.

In conclusion, the yeast three-hybrid system is a very useful tool for the characterization of critical residues involved in RNA–protein interactions. We have shown that loss-of-binding mutations are exclusively found in the RBD domain whereas revertants of these mutants mapped to residues located in the N- and C-terminal parts. It was shown before that HBP mutants, which are able to recognize mutated hairpins contained mutations in the same N- and C-terminal parts [18]. In addition, the flanking regions of HBP play other functions in histone mRNA metabolism. It has been reported that the 113 C-terminal amino acids of *Xenopus laevis* SLBP1 are sufficient for localization of SLBP1 into the cajal bodies [20]. It has also been shown that a 20 amino acid motif in the C-terminal domain and a nine amino acid motif in the RBD are required for efficient processing of histone mRNA precursor and for recruitment of U7 snRNP [21] and ZFP100 [5]. The RBD and flanking sequences from the N- and C-terminal domains are also required for translation [10,11]. Finally, it has been demonstrated that several phosphorylation events occur in the C-terminal domain and modulate the binding to the target RNA [22]. All together, these data suggest that probably HBP is not subdivided in three independent domains since they appear tightly related to ensure its functions in the cell.

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References

[1] Müller, B. and Schümperli, D. (1997) Semin. Cell Dev. Biol. 8, 567–576.
[2] Marzluff, W.F., Gongidi, P., Woods, K.R., Jin, J. and Maltais, L.J. (2002) Genomics 80, 487–498.
[3] Dominski, Z. and Marzluff, W.F. (1999) Gene 239, 1–14.
[4] Marzluff, W.F. and Duronio, R.J. (2002) Curr. Opin. Cell Biol. 14, 692–699.
[5] Dominski, Z., Erkmann, J.A., Yang, X., Sanchez, R. and Marzluff, W.F. (2002) Genes Dev. 16, 58–71.
[6] Dominski, Z., Sumerel, J., Hanson, R.J. and Marzluff, W.F. (1995) RNA 1, 915–923.
[7] Eckner, R., Ellmeier, W. and Birnstiel, M.L. (1991) EMBO J. 10, 3513–3522.
[8] Williams, A.S., Ingledue, T.C., Kay III, B.K. and Marzluff, W.F. (1994) Nucleic Acids Res. 22, 4660–4666.
[9] Sun, J., Pilch, D.R. and Marzluff, W.F. (1992) Nucleic Acids Res. 20, 6057–6066.
[10] Sanchez, R. and Marzluff, W.F. (2002) Mol. Cell. Biol. 22, 7093–7104.
[11] Ling, J., Morley, S.J., Pain, V.M., Marzluff, W.F. and Gallie, D.R. (2002) Mol. Cell. Biol. 22, 7853–7867.
[12] Pandey, N.B. and Marzluff, W.F. (1987) Mol. Cell. Biol. 7, 4557–4559.
[13] Wang, Z.F., Whitfield, M.L., Ingledue, T.C., Dominski III, Z. and Marzluff, W.F. (1996) Genes Dev. 10, 3028–3040.
[14] Martin, F., Schaller, A., Eglite, S., Schümperli, D. and Müller, B. (1997) EMBO J. 16, 769–778.
[15] Sullivan, E. et al. (2001) Genes Dev. 15, 173–187.
[16] Battle, D.J. and Doudna, J.A. (2001) RNA 7, 123–132.
[17] SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S. and Wickens, M. (1996) Proc. Natl. Acad. Sci. USA 93, 8496–8501.

[18] Martin, F., Michel, F., Zenklusen, D., Müller, B. and Schümperli, D. (2000) Nucleic Acids Res. 28, 1594–1603.
[19] Kodama, Y., Rothman, J.H., Sugimoto, A. and Yamamoto, M. (2002) Development 129, 187–196.
[20] Abbott, J., Marzluff, W.F. and Gall, J.G. (1999) Mol. Biol. Cell 10, 487–499.
[21] Dominski, Z., Erkmann, J.A., Greenland, J.A. and Marzluff, W.F. (2001) Mol. Cell. Biol. 21, 2008–2017.
[22] Dominski, Z., Yang, X.C., Raska, C.S., Santiago, C., Borchers, C.H., Duronio, R.J. and Marzluff, W.F. (2002) Mol. Cell. Biol. 22, 6648–6660.