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The gene for histone RNA hairpin binding protein is located on human chromosome 4 and encodes a novel type of RNA binding protein

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The hairpin structure at the 3′ end of animal histone mRNAs controls histone RNA 3′ processing, nucleo-cytoplasmic transport, translation and stability of histone mRNA. Functionally overlapping, if not identical, proteins binding to the histone RNA hairpin have been identified in nuclear and polysomal extracts. Our own results indicated that these hairpin binding proteins (HBPs) bind their target RNA as monomers and that the resulting ribonucleoprotein complexes are extremely stable. These features prompted us to select for HBP-encoding human cDNAs by RNA-mediated three-hybrid selection in *Saccharomyces cerevesiae*. Whole cell extract from one selected clone contained a Gal4 fusion protein that interacted with histone hairpin RNA in a sequence- and structure-specific manner similar to a fraction enriched for bovine HBP, indicating that the cDNA encoded HBP. DNA sequence analysis revealed that the coding sequence did not contain any known RNA binding motifs. The HBP gene is composed of eight exons covering 19.5 kb on the short arm of chromosome 4. Translation of the HBP open reading frame *in vitro* produced a 43 kDa protein with RNA binding specificity identical to murine or bovine HBP. In addition, recombinant HBP expressed in *S.cerevisiae* was functional in histone pre-mRNA processing, confirming that we have indeed identified the human HBP gene.

Keywords: histone gene expression/pre-mRNA processing/RNA 3′ processing/RNA–protein interaction/yeast three-hybrid system

Introduction

The replication-dependent animal histone genes (~50–70 genes in mammals) are transcribed by RNA polymerase II but otherwise follow an expression pathway that is distinct from that of all other protein-coding genes (reviewed in Marzluff and Pandey, 1988; Schümperli, 1988; Osley, 1991). The primary transcripts do not contain introns and are cleaved at their 3′ end in a reaction that is specific for the histone gene family and distinct from cleavage/polyadenylation, resulting in mRNAs without a poly(A) tail. This endonucleolytic cleavage is controlled by two RNA sequence elements: (i) a purine-rich spacer element that serves as an anchoring site for the essential U7 snRNP (Galli *et al.*, 1983; Schaufele *et al.*, 1986; Bond *et al.*, 1991) and (ii) a highly conserved 26 bp sequence encompassing a 6 bp stem–four base loop structure that is important but not essential for maximal processing efficiency (Mowry *et al.*, 1989; Vasserot *et al.*, 1989; Streit *et al.*, 1993). In addition, this hairpin structure, remaining at the 3′ end of the mature histone mRNAs, is involved in further aspects of histone RNA metabolism (reviewed in Marzluff, 1992). It is required for nucleo-cytoplasmic transport (Eckner *et al.*, 1991; Sun *et al.*, 1992), translation (Sun *et al.*, 1992) and stability of histone mRNA (Pandey and Marzluff, 1987). Moreover, it is a key target for a regulated destabilization of histone mRNA occurring upon interruption of cellular DNA replication (Pandey and Marzluff, 1987).

Proteins binding to the histone RNA hairpin have been identified in nuclear (Mowry and Steitz, 1987; Vasserot *et al.*, 1989; Pandey *et al.*, 1991; Melin *et al.*, 1992) and polyribosomal extracts (Pandey *et al.*, 1991) and termed hairpin binding (processing) factor (HBF) or stem–loop binding protein (SLBP), respectively. In both cases, a 40–45 kDa protein was cross-linked to hairpin RNA by UV irradiation (Pandey *et al.*, 1991), and recent experiments indicate that the polysomal protein can complement HBF-depleted nuclear extracts in histone RNA 3′ processing, suggesting that the two proteins are identical or share at least one common polypeptide (Dominski *et al.*, 1995).

Attempts at purification have provided enriched fractions which were useful for further biochemical analysis (Dominski *et al.*, 1995; Hanson *et al.*, 1996; A.Schaller, F.Martin and B.Müller, in preparation) but have not yet yielded the protein(s) in sufficient quantity for direct amino acid sequence determination. Using the newly developed *Saccharomyces cerevisiae* three-hybrid system for selection/screening of RNA binding proteins (Sengupta *et al.*, 1996), we have now isolated a human cDNA clone for a protein that binds specifically to the histone RNA hairpin. We demonstrate that this protein participates in histone pre-mRNA 3′ end processing. To indicate that we have cloned the RNA binding component of HBF/SLBP, we will henceforth refer to this protein/gene as HBP for (histone) hairpin binding protein. We also describe the cDNA and genomic structure as well as the expression pattern in different human tissues. Except for a putative *Caenorhabditis elegans* homologue, HBP has no significant homology to any other proteins or motifs in the SWISSPROT and PROSITE databases, and may thus represent a new type of RNA binding protein.

Results

Cloning of HBP cDNA

The yeast three-hybrid system (Sengupta *et al.*, 1996) has been derived from the two-hybrid system commonly

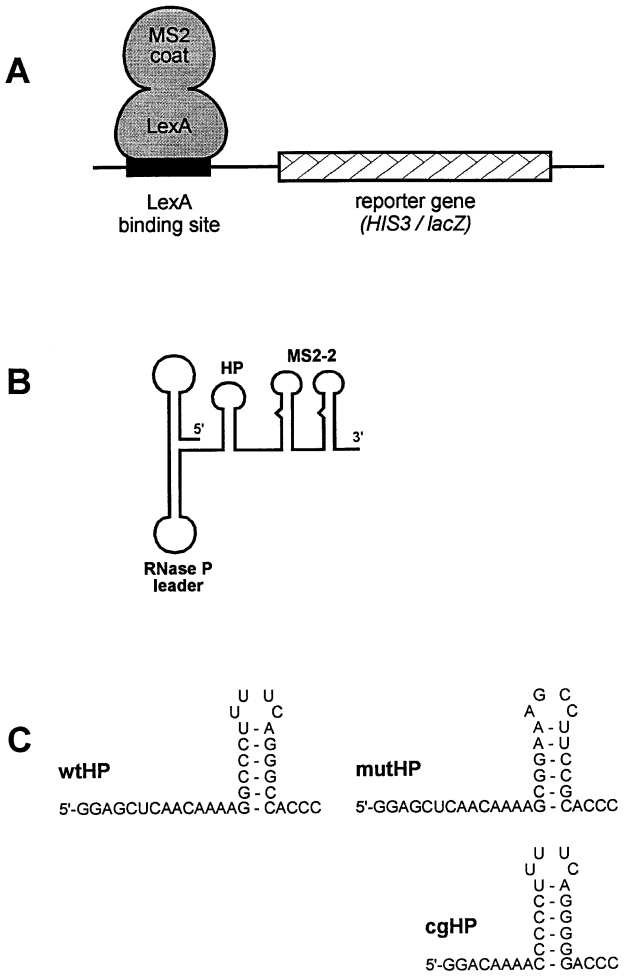

Fig. 1. Introduction to assay systems. (**A**) The yeast three-hybrid system was developed by M.Wickens, S.Fields and co-workers (Sengupta *et al.*, 1996) and depends on the activation of reporter genes (*HIS3* and *lacZ*) by an RNA-mediated interaction between the LexA–MS2 coat fusion protein and the Gal4 cDNA fusion protein. The structure of the RNA molecules used is as shown in (B). Reporter genes as well as the gene for the LexA–MS2 coat fusion protein are integrated in the genome of *S.cerevisiae* strain L40-coat. (**B**) Structure of RNA sequences used in the three-hybrid selection. RNAs containing either wild-type histone hairpin sequences or mutant hairpin sequences (marked HP) 3′ of the RNase P leader sequence and 5′ of two serial MS2 RNA elements were encoded by plasmids pIII/wtHP-MS2 and pIII/mutHP-MS2. The hairpin sequences are identical to wtHP and mutHP RNAs shown in (C). (**C**) RNA molecules used to detect the HBP by EMSA.

used for studying protein–protein interactions (Fields and Song, 1989; Durfee *et al.*, 1993). It allows for a selection for specific RNA binding proteins using the following features: two reporter genes (*HIS3* and *lacZ*, both preceded by a DNA binding site for LexA protein) and a fusion between *lexA* and the gene for phage MS2 coat protein are all stably integrated into the genome of *S.cerevisiae* strain L40-coat. Thus the LexA–MS2 coat fusion protein can bind to the LexA sites but this does not activate the reporter genes because of lack of an activation domain able to attract and stimulate the transcription machinery (Figure 1A). The second hybrid is an RNA, transcribed *in vivo* from a plasmid, that contains two binding sites for MS2 coat protein and a site for the RNA binding protein of interest (Figure 1B). The third hybrid should be a fusion protein between the corresponding RNA binding protein and the Gal4 activation domain. This can be provided on an appropriate plasmid expression vector or selected from a cDNA library cloned in such a vector.

To perform the three-hybrid selection, we introduced the wtHP sequence into the pIII/MS2-2 plasmid to yield plasmid pIII/wtHP-MS2 encoding a hybrid RNA containing the wild-type histone hairpin structure (Figure 1B and C). After this plasmid was introduced into *S.cerevisiae* strain L40-coat by *URA3* selection, we transformed the resulting strain with a Gal4 activation domain-tagged cDNA library from human lymphocytes (Durfee *et al.*, 1993). Titration on plates lacking uracil and leucine showed that the library was producing 300 000 original transformants. The culture was plated out on artificial medium lacking uracil, leucine and histidine to select for the presence of the plasmids and activation of the *HIS3* reporter gene. After 5 days of selection, four His^+ *S.cerevisiae* colonies were observed.

The four colonies (clones 1–4) were tested on URA-LEU-HIS selective media containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase, to visualize expression of the *lacZ* reporter gene. All four grew to blue colonies, indicating that the *lacZ* gene was also activated (Table I). We then isolated derivatives which had lost either the *URA3*-containing pIII/wtHP-MS2 plasmid or the *LEU2*-containing cDNA plasmid. For all clones, the His^+ and $lacZ^+$ phenotypes were lost with the Gal4 cDNA expression plasmid. However, only clone 2 also lost both phenotypes when the pIII/wtHP-MS2 plasmid was removed. The proteins encoded by the other three clones (1, 3 and 4) which retained both phenotypes therefore appeared to activate the reporter genes independently of the hybrid histone hairpin RNA, e.g. by binding either to the promoter DNA or to the LexA–MS2 coat fusion protein.

Three of the four cDNA plasmids (clones 1, 2 and 4) were recovered, amplified in *Escherichia coli* and then re-transformed into a series of *S.cerevisiae* L40-coat strains containing either no or different variants of the pIII RNA plasmid (Table I). In plasmid pIII/mutHP-MS2, the wtHP sequence was replaced by the mutant mutHP sequence (RNA structure and sequences are shown in Figure 1B and C) and plasmid pIII/IRE-MS2 contained an iron response element instead of the wtHP sequence (Sengupta *et al.*, 1996). Clones 1 and 4 produced His^+ and $lacZ^+$ phenotypes irrespective of whether the cell contained no pIII plasmid or plasmids pIII/IRE-MS2, pIII/wtHP-MS2 or pIII/mutHP-MS2, confirming that they activated both reporter genes by an RNA-independent mechanism. However, clone 2, the candidate for HBP cDNA, produced His^+ and $lacZ^+$ phenotypes only in the *S.cerevisiae* strain harbouring the pIII/wtHP-MS2 plasmid.

We then tested whether extracts prepared from the original *S.cerevisiae* transformants produce an RNA binding activity with the same characteristics as mammalian HBP. After incubation with radiolabelled 34 nucleotide wtHP RNA (Figure 1C) and native gel electrophoresis (electrophoretic mobility shift assay, EMSA), only clone 2 produced a ribonucleoprotein (RNP) complex with a strongly reduced mobility (Figure 2A, lane 4), whereas clones 1, 3 and 4 only produced faint, faster migrating and presumably unspecific complexes (lanes 3, 5 and 6).

Table I. His^+ and $lacZ^+$ phenotypes of clone 2 are wtHP RNA-mediated and dependent on cDNA

Clone Phenotype	1 His/lacZ	2 His/lacZ	3 His/lacZ	4 His/lacZ
Original transformants	+/+	+/+	+/+	+/+
after loss of pIII/wtHP-MS2[a]	+/+	−/−	+/+	+/+
after loss of pACT-cDNA	−/−	−/−	−/−	−/−
pACT-cDNA plasmids retransformed into yeast L40-coat:				
without pIII plasmid	+/+	−/−	n.d.	+/+
with pIII/IRE-MS2 plasmid[b]	+/+	−/−	n.d.	+/+
with pIII/wtHP-MS2	+/+	+/+	n.d.	+/+
with pIII/mutHP-MS2[c]	+/+	−/−	n.d.	+/+

[a]For the structure of pIII/wtHP-MS2 RNA, see Figure 1B and C.
[b]Containing iron response element RNA instead of wtHP RNA.
[c]Containing mutHP RNA (Figure 1C) instead of wtHP RNA.

The complex produced by *S.cerevisiae* clone 2 had a slower mobility than the murine HBP complex (compare lanes 2 and 4), consistent with the fact that the fusion protein additionally contained the Gal4 activation domain. We further compared the RNA binding specificity of the Gal4–HBP fusion protein with a highly enriched HBP fraction prepared from calf thymus using ion exchange chromatography (CT fraction V; A.Schaller, F.Martin and B.Müller, in preparation). Binding reactions were performed in the absence or presence of varying amounts of unlabelled wtHP, mutHP or cgHP competitor RNAs (sequences are shown in Figure 1C). Whereas mutHP contained a completely unrelated 6 bp stem–four base loop structure, in cgHP, only the two lowest base pairs of the stem were inverted from G–C to C–G. This mutant was reported to have ~3% of the wild-type affinity for HBP (Pandey *et al.*, 1994; Williams and Marzluff, 1995). The HBP-specific complexes produced by CT fraction V and by the extract from *S.cerevisiae* clone 2 were both partly competed by a 10-fold excess (Figure 2B, lanes 2 and 9, respectively) and inhibited by a 100-fold excess of wtHP RNA (lanes 3 and 10). In contrast, neither of the two mutant RNAs were able to inhibit the formation of HBP-specific complexes at these concentrations (lanes 4–7 and 11–14). The same RNA binding activity was also detected after re-transformation of the cDNA plasmid from clone 2 into *S.cerevisiae* strain L40-coat (data not shown), indicating that clone 2 cDNA encodes a protein with RNA binding specificity identical to mammalian HBP.

The sequence of HBP

The cDNA insert from clone 2 was subcloned into a pBluescript vector and its nucleotide sequence determined (EMBL nucleotide sequence database accession No. Z71188). Inspection of the sequence revealed a cDNA insert of 1716 bp with an additional tail of 22 A residues. The poly(A) tail is preceded by a perfect AATAAA sequence ending 17 nucleotides before the poly(A) addition site. The cDNA insert contains an open reading frame (ORF) beginning at the very 5′ end (reading frame 3) and ending with a TAA stop codon at position 915. The first ATG at position 105 (Figure 3A) is in a favourable sequence context for an initiation codon (Kozak, 1986). The ORF, from this position onwards, encodes a protein of 270 amino acids with a calculated M_r of 31 186 Da. This is lower than the 40–45 kDa estimated by SDS–PAGE of the murine protein labelled by UV cross-linking to radiolabelled RNA (Pandey *et al.*, 1991; A.Schaller and B.Müller, unpublished observation). However, we have expressed an HBP cDNA fragment starting from the ATG at position 105 in *S.cerevisiae* using a galactose-inducible expression system (see Materials and methods). The molecular mass of this protein determined by UV cross-linking was ~41 kDa (data not shown). In addition, we have translated the cDNA in a wheat germ extract (the AUG at position 105 being the first possible initiation codon), yielding a protein with an apparent M_r of 43 kDa (see below). These observations strongly suggest that we have isolated a virtually complete cDNA and that translation starts at the AUG at position 105.

Nucleotide sequence comparisons revealed that the genomic region of the HBP gene had been sequenced previously (McCombie *et al.*, 1992). In this report, three human cosmids, located within what was then the candidate region for the Huntington's disease gene (chromosome 4p16.3), were sequenced, yielding a contig of 58 864 bp. Based on the location of CpG-rich islands and ORFs, three genes were predicted to lie in this region, one of which was characterized further by cDNA cloning and named *hda1-1*. The 5′ end of the HBP cDNA is located in the next CpG island downstream of the *hda1-1* gene. If the long ORF of HBP cDNA is prolonged into the 5′-flanking genomic region, it meets a stop codon 19–17 nucleotides upstream of the cDNA's 5′ end (Figure 3A). These 19 bp contain neither an additional in-frame ATG nor a sequence resembling a 3′ splice site. Therefore, the first ATG and hence the correct initiation codon is the one at position 105. Furthermore, 55–50 nucleotides upstream from the cDNA's 5′ end is a TATA box-like sequence, CATAAA, flanked by two recognition sites for transcription factor SP1.

By comparison with the genomic sequence, the HBP gene comprises eight exons of ⩾163, 117, 105, 60, 138, 150, 67 and 916 bp, interrupted by introns of 139, 8133, 3524, 309, 3206, 1361 and 1060 bp, respectively. This adds up to ⩾19 448 bp for the entire gene.

Comparison of the amino acid sequence predicted by the ORF from position 105–915 with the SWISSPROT library revealed only one related protein, a putative 41.5 kDa protein predicted from the *C.elegans* genome project (SWISSPROT databank accession No. Q09599), but no known protein with significant homologies. This protein displayed a significant degree of sequence

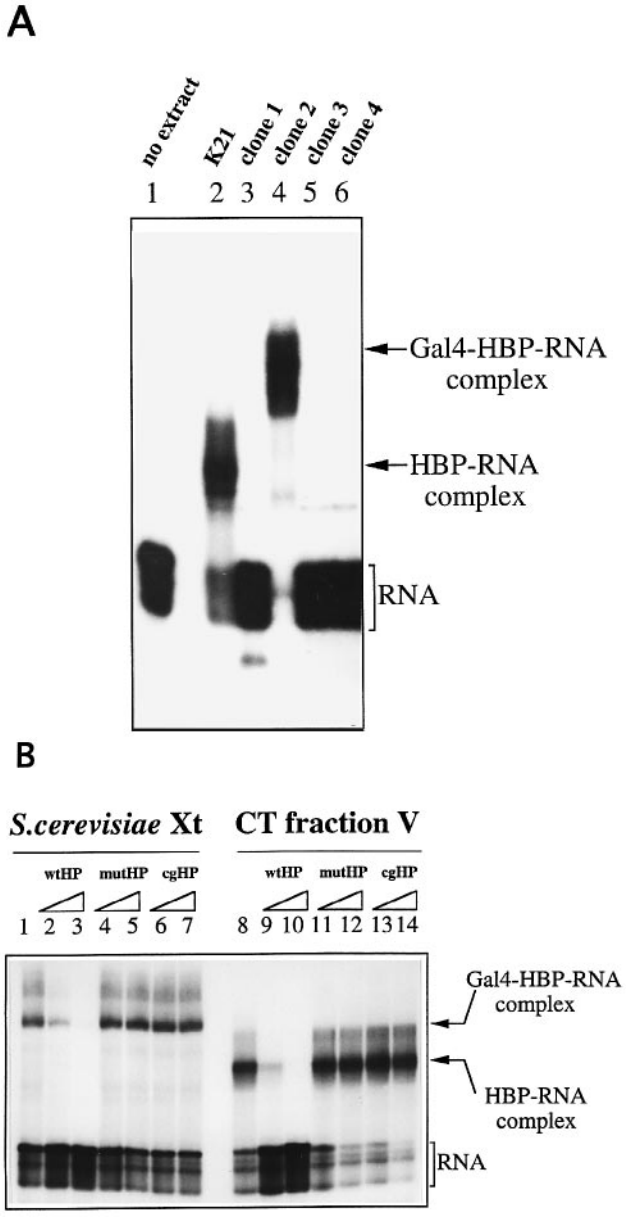

Fig. 2. cDNA from clone 2 encodes a Gal4–HBP fusion protein. (**A**) Extracts prepared from the four selected transformants were tested for the presence of HBP. Lane 1, ^{32}P-labelled wtHP RNA. Lane 2, 12 µl of mouse K21 cell nuclear extract (6 mg/ml) was mixed with ^{32}P-labelled wtHP RNA and incubated in 20 µl as described in Materials and methods. Lanes 3–6, as lane 2, but the nuclear extract was replaced by extracts prepared from the transformants 1–4 (3, 1.6, 1.7 and 2.2 mg/ml protein, respectively). Reaction products were analysed by EMSA as described in Materials and methods. (**B**) Comparison of RNA binding specificity of HBP encoded by clone 2 and of HBP enriched from calf thymus. Lanes 1–7, 25 fmol of ^{32}P-labelled wtHP RNA were incubated with 5 µl of extract prepared from clone 2 in a final volume of 10 µl as described in Materials and methods. Lanes 8–14, as lanes 1–7, but yeast extract was replaced by calf thymus fraction V (40 µg/ml). Competitor RNAs were included as follows: lanes 2 and 9, 250 fmol of wtHP RNA; lanes 3 and 10, 2.5 pmol of wtHP RNA; lanes 4 and 11, 250 fmol of mutHP RNA; lanes 5 and 12, 2.5 pmol of mutHP RNA; lanes 6 and 13, 250 fmol of cgHP RNA; lanes 7 and 14, 2.5 pmol of cgHP RNA.

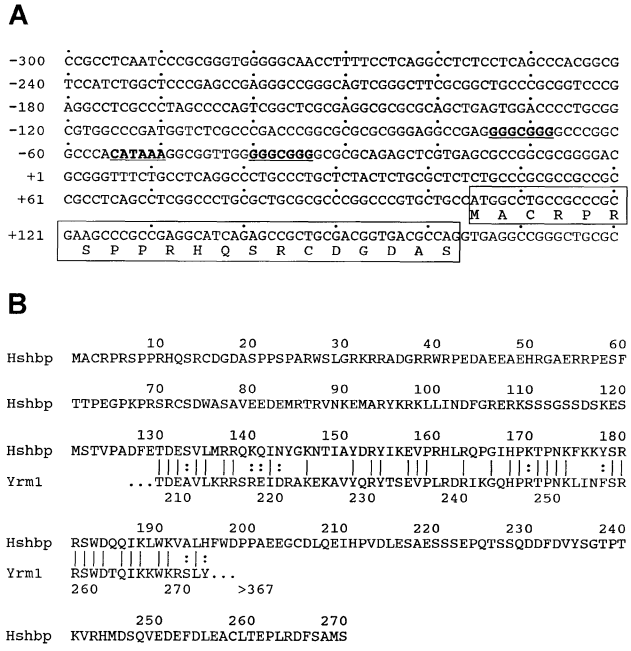

Fig. 3. (**A**) Promotor region and first exon of the HBP gene. The sequence of the HBP cDNA was deposited in the EMBL nucleotide sequence database (accession No. Z71188). Genomic DNA sequences are from McCombie and colleagues (McCombie *et al.* 1992; DDBJ/EMBL/GenBank nucleotide sequence database accession No. M63480). The TATA box-like sequence and possible SP1 recognition sites are underlined. The translated part of exon 1 is boxed; +1 marks the beginning of the cDNA. (**B**) Deduced amino acid sequence alignment between human HBP and the putative *C.elegans* histone HBP Yrm1 (SWISSPROT database accession No. Q09599) as determined using the BlastP program (Altschul *et al.*, 1990). Only the Yrm1 region with highest homology to HBP is shown. Identical amino acids (1, 53%) and conserved amino acids (:, 12 %) are indicated.

similarity to residues 33–195 of human HBP, but the highest conservation was observed for residues 130–195 (Figure 3B). Cloning and *in vitro* translation of the corresponding *C.elegans* cDNA produced a protein of apparent M_r ~58 kDa which bound specifically to RNA containing a *C.elegans* histone hairpin (data not shown; a detailed comparison of the human and *C.elegans* proteins will be presented elsewhere). Further analysis of the PROSITE library revealed no known motifs besides a potential nuclear localization signal RKRR (amino acids 31–34) and a number of putative phosphorylation sites.

The HBP gene product binds histone RNA hairpin structures

To exclude that the Gal4 activation domain was involved in RNA binding, the HBP cDNA by itself was transcribed and translated *in vitro* in wheat germ extract. The product was radiolabelled by the inclusion of [^{35}S]methionine and analysed by SDS–PAGE. Figure 4A shows that the main translation product, a 43 kDa protein, and some minor smaller peptides were formed only when the cDNA was in the sense orientation (lane 2). Reactions with the 'antisense' cDNA did not lead to any protein synthesis (lane 1).

To confirm that the 43 kDa protein contained the RNA binding activity, the translation mixtures were tested directly in an RNA binding assay. Binding reactions with the extract containing the 43 kDa protein and wtHP RNA formed an RNP complex which co-migrated with the one formed by the K21 nuclear extract (Figure 4B lanes 3 and 1, respectively). An additional, faster migrating complex, presumably originating from one of the minor translation

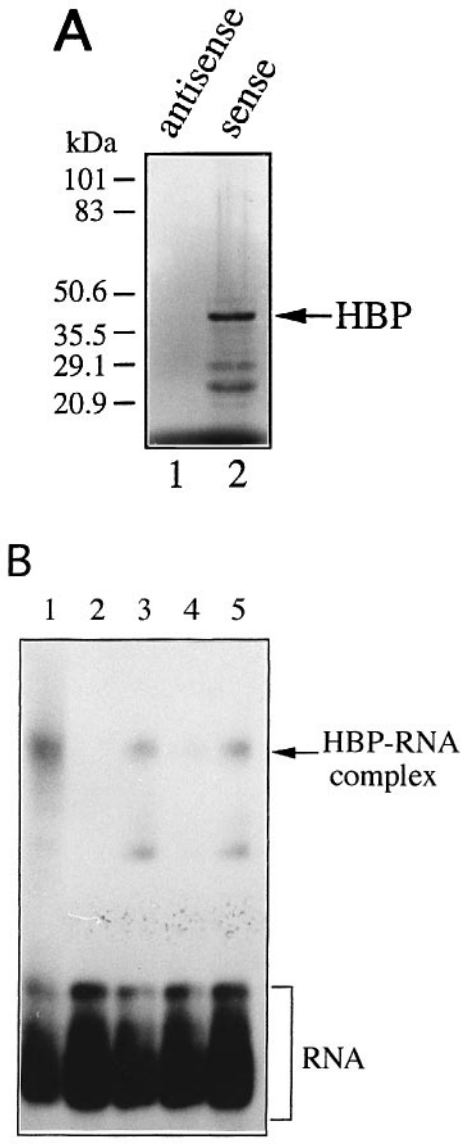

Fig. 4. Translation of HBP cDNA *in vitro*. (**A**) Analysis of translation products. pBluescript-HBP (antisense cDNA orientation) or pBluescript-rHBP DNA (sense cDNA orientation) were transcribed and translated in wheat germ extract as described in Materials and methods. Five µl of each reaction were precipitated by addition of trichloroacetic acid, analysed by SDS–10% polyacrylamide gel electrophoresis and visualized by autoradiography. The positions of the pre-stained marker proteins are indicated. (**B**) HBP translated *in vitro* binds histone hairpin RNA. Ten µl of K21 nuclear extract (lane 1) or 10 µl of translation reaction mixtures containing pBluescript-HBP (antisense cDNA orientation; lane 2) or pBluescript-rHBP DNA (sense cDNA orientation; lanes 3–5) were mixed with radiolabelled wtHP RNA and the reaction products analysed by EMSA as described in Materials and methods. The reactions in lane 4 and 5 were supplemented with 2.5 pmol of either wtHP or mutHP competitor RNA.

products, was also formed in these reactions. Both complexes were absent in incubations with the translation reaction of 'antisense' cDNA (lane 2) and were sensitive to the presence of wtHP but not mutHP competitor RNA (lanes 4 and 5, respectively). This confirmed that the cDNA-encoded protein has the binding specificity expected of mammalian HBP as well as the correct electrophoretic mobility both on SDS–polyacrylamide and native gels.

The HBP participates in histone pre-mRNA processing

To test whether the recombinant HBP was functional in histone pre-mRNA 3′ end processing, we used two different sources of K21 mouse cell nuclear extract deficient in HBP. Fractionation of K21 extract by MonoQ column chromatography separated hairpin binding factor (HBF)/HBP from U7 snRNP (Vasserot *et al.*, 1989), leading to side fractions with low processing activity. In our preparation, fraction 20 was enriched for HBF but lacked U7 snRNP, while fraction 24 was enriched for U7 snRNP and contained little HBF (A.Schaller, F.Martin and B.Müller, in preparation). Incubation of a histone H4 RNA 3′ end fragment encompassing the processing site (Vasserot *et al.*, 1989) with fraction 24 led to 1.7% of the RNA being processed (Figure 5A, lanes 2, 5 and 10; quantitation of two separate experiments is shown in Figure 5B), while ~58% was processed in a reaction with unfractionated nuclear extract from K21 cells (lane 1). In incubations with fraction 20, radiolabel at the position of the processing product was at background level (0.7%; lane 3). Mixing fraction 20 with fraction 24 increased cleavage of the histone RNA ~2.6-fold (lane 4), illustrating that the addition of HBF stimulated the activity of U7 snRNPs in this *in vitro* assay. However, the level of processing did not reach the level obtained with unfractionated K21 extract, indicating that, in addition to HBF, other factor(s) may be missing.

Using this complementation assay, we detected a stimulation of processing of fraction 24 by wheat germ extract containing recombinant HBP (data not shown) and by extract prepared from a *S.cerevisiae* strain expressing recombinant HBP from the ATG at position 105 (see Materials and methods). Addition of extract prepared from *S.cerevisiae* expressing HBP stimulated processing 2.6-fold (Figure 5A, lane 7), whereas no significant increase was observed with control yeast extract (lane 12), indicating that the presence of recombinant HBP in the extract contributed to processing. This was confirmed by the observation that inclusion of wtHP competitor RNA (lane 8), but not mutHP competitor RNA (lane 9), reduced processing to the level of processing obtained with fraction 24 alone. In contrast, product formation in the presence of control extract was not affected by the addition of either kind of competitor RNA (lanes 13 and 14). As expected, neither of the two yeast extracts showed any processing activity on its own (lanes 6 and 11).

In another series of experiments, we depleted K21 extract of HBP using biotinylated wtHP RNA and streptavidin–agarose (A.Schaller and B.Müller, unpublished results). This led to an ~6-fold reduction in processing activity (Figure 6A, lanes 1 and 2; quantitated in Figure 6B), and mixing of HBP-depleted with untreated extract showed that the depleted extract did not inhibit processing (lane 3). A 3.7-fold stimulation of processing could be achieved using the HBF-containing MonoQ fraction 20 (lane 14) already used in the above experiment. A 3-fold stimulation was obtained with an enriched preparation of bovine HBP (lane 10) and, more importantly, a 2-fold stimulation was also achieved with an identically fractionated preparation of recombinant HBP from cDNA-expressing *S.cerevesiae* strain BJ5465/pFMM5 (lane 6). Very similar effects were obtained in

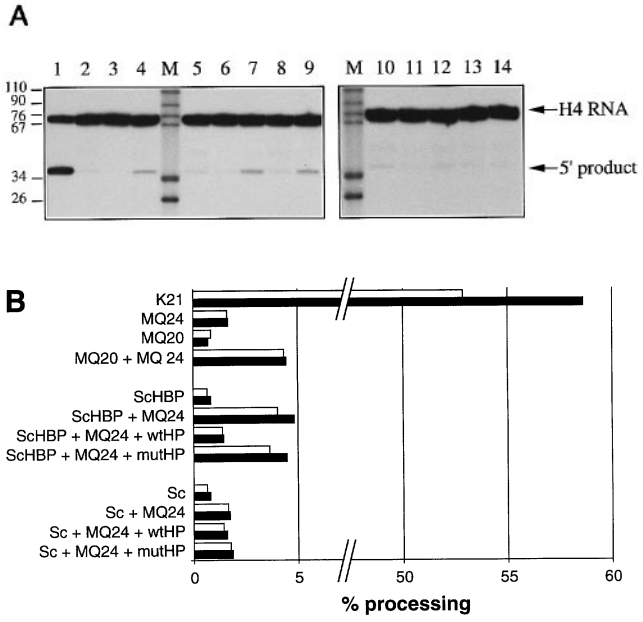

Fig. 5. Complementation of histone RNA 3′ processing using nuclear extract depleted of HBP by MonoQ column chromatography and *S.cerevisiae* extract containing recombinant HBP. (**A**) Processing reactions with a ^{32}P-labelled 69 nucleotide H4 RNA fragment contained mouse K21 cell nuclear extract fractionated by MonoQ column chromatography and were performed and analysed by denaturing gel electrophoresis as described in Materials and methods. Reactions contained 5 µl of K21 nuclear extract (5 mg/ml) (U7 snRNP and HBP; lane 1), 2.5 µl of MonoQ fraction 24 (1 mg/ml) (enriched for U7 snRNP; lanes 2, 5 and 10), 2.5 µl of MonoQ fraction 20 (0.8 mg/ml) (enriched for HBP/HBF; lane 3) or 2.5 µl of fraction 20 mixed with 2.5 µl of fraction 24 (lane 4). Reactions in lanes 7–9 contained 2.5 µl of MonoQ fraction 24 and 2.5 µl of BJ5465/pFMM5 extract (4 mg/ml) containing HBP. Reactions in lanes 12–14 contained 2.5 µl of MonoQ fraction 24 and 2.5 µl of BJ5465 extract (4 mg/ml). Lanes 8 and 13 additionally contained 2.5 pmol of unlabelled wtHP competitor RNA. Lanes 9 and 14 aditionally contained 2.5 pmol of unlabelled mutHP competitor RNA. The reaction in lane 6 was with 2.5 µl of BJ5465/pFMM5 extract only and the reaction in lane 11 with 2.5 µl of BJ5465 extract only. Molecular weight marker M is pBR322 DNA cleaved with *Hpa*II. (**B**) Quantitation of two independent experiments using a phosphorimager as described in Materials and methods. Processing reactions contained as indicated: K21, K21 nuclear extract; MQ20, MonoQ fraction 20; MQ24, MonoQ fraction 24; ScHBP, BJ5465/pFMM5 extract; Sc, BJ5465 extract; wtHP, wtHP competitor RNA; mutHP, mutHP competitor RNA. Results from the experiment in (A) are shown as black bars. For simplicity, only the mean (1.7%) of the three incubations with MonoQ fraction 24 in (A), lanes 2, 5 and 10 is shown (lane 2, 1.7%; lane 5, 1.8%; lane 10, 1.6%).

three separate experiments (Figure 6B) and in additional experiments which are not shown. With all three sources of HBP, this stimulation was prevented by inclusion of wtHP (Figure 6A, lanes 7, 11 and 15), but not mutHP competitor RNA (lanes 8, 12 and 16). These experiments demonstrate that recombinant HBP produced in *S.cerevisiae* is functional in histone pre-mRNA processing.

Expression of HBP in human tissues

A Northern blot of poly(A)$^+$ RNA from different human tissues was probed with an HBP-specific DNA probe and a ~2 kb mRNA was detected, consistent with the length of the cDNA (Figure 7). This RNA was detected in all tissues tested, and the differences in RNA levels were suggestive of slight tissue-specific variations in expression.

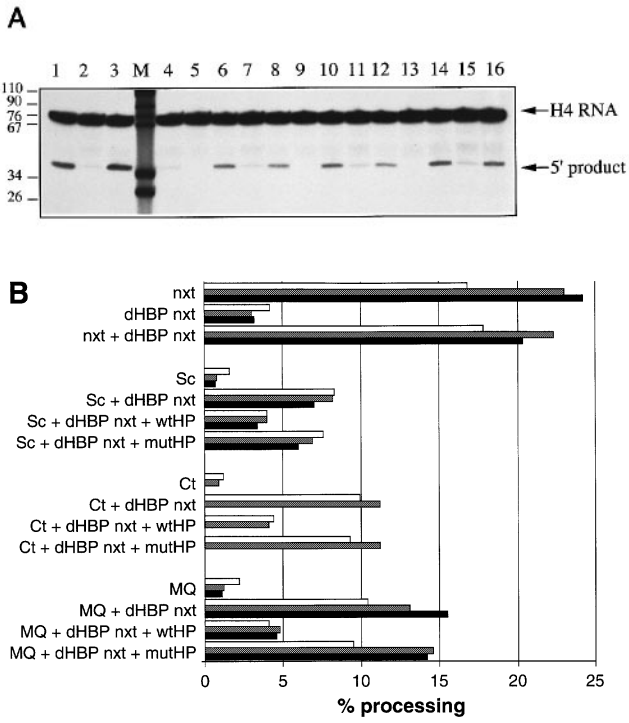

Fig. 6. Complementation of histone RNA 3′ processing in nuclear extract depleted of HBP using biotinylated histone hairpin RNA with enriched recombinant HBP. (**A**) Processing reactions were performed and analysed by denaturing gel electrophoresis as described in Materials and methods. Reactions contained 2.5 µl of K21 nuclear extract (3.1 mg/ml) (lane 1) or 2.5 µl of nuclear extract depleted of HBP with biotinylated histone hairpin RNA as described in Materials and methods (1.8 mg/ml) (lanes 2–4, 6–8, 10–12 and 14–16). Incubations of reactions in lanes 2 and 4 were without further addition. The reaction in lane 3 was supplemented with 2.5 µl of K21 nuclear extract. Reactions in lanes 6–8 were supplemented with 4.3 µl of recombinant human HBP enriched from BJ5465/pFMM5 (0.25 mg/ml) by ion exchange chromatography as described in Materials and methods. Reactions in lanes 10–12 were supplemented with 4.3 µl of HBP enriched from calf thymus (1.1 mg/ml) as described in Materials and methods. Reactions in lanes 14–16 were supplemented with 4.3 µl of K21 MonoQ fraction 20 used in Figure 5. In addition, reactions in lanes 7, 11 and 15 were supplemented with 2.5 pmol of unlabelled wtHP competitor RNA and reactions in lanes 8, 12 and 16 with 2.5 pmol of unlabelled mutHP competitor RNA. In lanes 5, 9 and 13, 4.3 µl of the respective BJ5465/pFMM5, calf thymus and K21 MonoQ fractions were tested on their own. Molecular weight marker M is pBR322 DNA cleaved with *Hpa*II. (**B**) Reactions of three independent experiments performed as described above were quantitated using a phosphorimager and the percentage of processed H4 RNA molecules is shown. Processing reactions contained as indicated: nxt, K21 nuclear extract; dHBP nxt, HBP-depleted nuclear extract; Sc, recombinant HBP enriched from BJ5465/pFMM5; Ct, HBP enriched from calf thymus; MQ, MonoQ fraction 20; wtHP, wtHP competitor RNA; mutHP, mutHP competitor RNA. Results from the experiment in (A) are shown as white bars. For simplicity, only the mean (4.2%) of the two incubations with HBP-depleted nuclear extract in (A), lanes 2 and 4 is shown (lane 2, 4.1%; lane 4, 4.3%).

Discussion

Here we describe the isolation and characterization of a cDNA encoding the human HBP, a protein that is involved in multiple steps of histone gene expression. The cDNA was isolated using the recently developed *S.cerevisiae* three-hybrid system (Sengupta *et al.*, 1996). In our screen, we obtained four *S.cerevisiae* transformants which activated both reporter genes, but only in one case, clone 2, was this activation dependent on the presence of

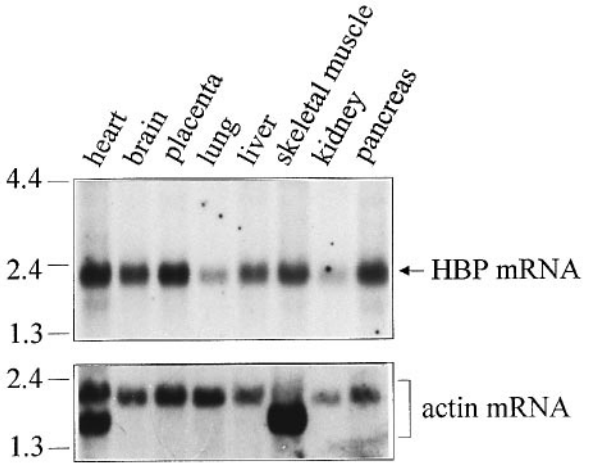

Fig. 7. Expression of HBP in different human tissues. Top: a Northern blot displaying poly(A)$^+$ RNA from the indicated human tissues was probed with an HBP DNA probe as described in Materials and methods. Bottom: the same blot was stripped and tested for the presence of human β-actin mRNA. This probe detects β-actin mRNA (2 kb) as well as α- or γ-actin mRNA (1.6–1.8 kb) in heart and skeletal muscle poly(A)$^+$ RNA. The positions of the size markers (kb) are indicated on the left.

both the cDNA and the RNA containing the correct histone hairpin sequence (Table I).

Further indications that clone 2 encoded human HBP came from functional assays. The expression level of the fusion protein between the Gal4 transactivation domain and HBP was sufficient to allow detection of RNP complexes by EMSA (Figure 2A). In competition experiments, the complex formed between Gal4–HBP and wtHP RNA was not competed by mutant hairpin RNA structures (Figure 2B), indicating that the binding had the same specificity for wild-type histone HP sequences as mammalian HBP.

Translation of the HBP cDNA without the Gal4 activation domain *in vitro* produced a 43 kDa protein which was able to bind to histone hairpin RNA (Figure 4). Initial experiments using this preparation of HBP indicated that the HBP was able to participate in histone pre-mRNA *in vitro* processing. This was confirmed with extract prepared from *S.cerevisiae* expressing HBP. This extract was used to complement a fraction rich in U7 snRNP but containing little HBP produced by fractionation of mouse K21 nuclear extract by MonoQ column chromatography (Figure 5). Similarly, an enriched preparation of *S.cerevisiae*-derived recombinant HBP stimulated processing of an HBP-depleted K21 extract (Figure 6). In both cases, the 2- to 3-fold stimulation of processing was hairpin RNA dependent, demonstrating that the recombinant HBP participates in processing by interaction with the hairpin structure.

The sequence of the cDNA revealed an ORF of 270 amino acids, coding for a protein with a mass of slightly over 31 kDa (Figure 3B). This was in contradiction to the M_r of ~44 kDa calculated from the migration in SDS–PAGE of the mouse HBP cross-linked to wtHP RNA (Pandey *et al.*, 1991) and of extensively purified bovine HBP (A.Schaller, F.Martin and B.Müller, in preparation). However, translation of the cDNA in *S.cerevisiae* or *in vitro* (Figure 4A) produced either a 41 or a 43 kDa protein able to bind specifically to wtHP RNA, in agreement with these earlier observations. A similar size discrepancy between the mobility in SDS–polyacrylamide gels (~58 kDa) and the predicted M_r (41.5 kDa) was also observed for the putative HBP homologue from *C.elegans*.

Using the human HBP cDNA sequence in a database search, we discovered that the genomic region had been sequenced previously within an anonymous stretch of DNA near the Huntington's disease locus on the short arm of chromosome 4 (location 4p16.3; McCombie *et al.*, 1992). The comparison between cDNA and genomic DNA revealed that the *hbp* gene covers 19.5 kb and contains eight exons. Most of the exons are relatively short (60–163 bp); only the 3′-terminal exon is 916 bp long and ends with a conventional polyadenylation signal. The region upstream of the cDNA 5′ end has many features of a promoter region, a TATA-like sequence (CATAAA) and two perfect recognition sites for the ubiquitous transcription factor SP1 (Figure 3A), as well as several other putative sites for additional transcription factors. The gene does not contain a histone-like hairpin sequence (something which might have been expected as an autoregulatory feature).

Consistent with the presence of SP1 sites in the promoter region and with the fundamental role of HBP, a ~2 kb transcript was detected in all human tissues tested (Figure 7). The size of the transcript corresponds reasonably well with the length of the cDNA (1716 bp), if one assumes a poly(A) tail length of ~100 nucleotides.

The HBP protein as predicted by the cDNA sequence (Figure 3B) has no motifs in common with any known RNA binding or other proteins in the databases. It appears, therefore, to represent a new type of RNA binding protein and it will be interesting to investigate how it interacts so strongly and specifically with its RNA target. The putative *C.elegans* homologue detected by screening of the SWISSPROT database and found by us to bind to *C.elegans* histone hairpin RNA shows a particularly high degree of sequence conservation in residues 130–195, making this an ideal candidate region for an RNA binding domain. Indeed, Marzluff and colleagues, who independently have cloned human HBP cDNA using a very similar yeast three-hybrid strategy, have delimited the RNA binding activity to a 75 amino acid region between residues 125 and 199 (Z.-F.Wang, M.L.Whitfield, T.C.Ingledue III and W.F.Marzluff, in preparation). Using the human and *C.elegans* sequences, the PHD protein folding program (EMBL Heidelberg) with high confidence predicts this region to contain two α-helices (residues 132–144 and 173–190). Interestingly, the latter of these α-helices in both species contains the sequence SRR, a putative phosphorylation site for protein kinase C.

Based on previous experiments, the HBP is expected to interact not only with its RNA target, but also with a wide variety of other cellular components. An interaction with the U7 snRNP during histone RNA 3′ processing is likely, in view of the fact that it appears to contribute to efficient processing by stabilizing the complex between histone pre-mRNA and the U7 snRNP (Streit *et al.*, 1993; Spycher *et al.*, 1994). Moreover, HBP is supposed to play an important role in targeting mature histone mRNA from the nucleus to the cytoplasm and to the translation

machinery (Eckner *et al.*, 1991; Sun *et al.*, 1992). Finally, HBP stabilizes histone mRNA and is critical for a regulated destabilization of histone mRNA when DNA synthesis ceases at the end of S phase or when cells are treated with inhibitors of DNA synthesis (Pandey and Marzluff, 1987). It is by this step that HBP presumably contributes to the cell cycle regulation of histone gene expression (reviewed in Schümperli, 1988; Marzluff, 1992). Given the possibility to produce recombinant HBP and antibodies directed against it, it will now be possible to study these processes in more detail.

Materials and methods

Strains

All plasmids used in this work were amplified in *E.coli* strain XL-1 blue. The three-hybrid system screening procedure was done in *S.cerevisiae* strain L40-coat [*MAT***a**, *ura3-52, leu2-3, 112, his3Δ200, trp1Δ1, ade2, LYS2::(lexAop)-HIS3, ura3::(LexAop)-lacZ, LexA-MS2 coat (TRP1)*] (Sengupta *et al.*, 1996). Strain BJ5465 (*MAT***a**, *ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS2, prb1Δ1.6R, can1, GAL*) (Jones, 1991) was used to express recombinant HBP.

Plasmids

The different hybrid RNAs used in this study were expressed from derivatives of plasmid pIII/RPRx426 which carries the *URA3* gene (Good and Engelke, 1994). Plasmids encoding hybrid RNAs with additional hairpin structures were constructed by insertion of double-stranded oligonucleotides into the *Sma*I site of pIII/MS2-2 (Sengupta *et al.*, 1996) between the RNase P leader sequence and the two binding sites for MS2 coat protein. Below, the sequence of one strand is shown for each inserted hairpin. For insertion of the wild-type histone sequence (wtHP), 5′-GGAGCTCAACAAAAGGCCCTTTTCAGGG-CCACCC was used; for insertion of a mutant hairpin sequence (mutHP) 5′-GGAGCTCAACAAAAGCGGAAAGCCTTCCGCACCC was used; and for the insertion of a hairpin sequence with a changed stem sequence (cgHP) 5′-GGAGCTCAACAAAACCCCCTTTTCAGGGGG-ACCC was used to produce the plasmids pIII/wtHP-MS2, pIII/mutHP-MS2 and pIII/cgHP-MS2, respectively. All constructs were verified by sequencing of the inserts using the dideoxy chain termination method (Sanger *et al.*, 1977). The structure of the RNA molecules is shown schematically in Figure 1B. Plasmid pIII/IRE-MS2 is described elsewhere (Sengupta *et al.*, 1996). Plasmids pSP65wtHP and pSP65mutHP contained the wtHP and mutHP sequences joined to the T7 RNA polymerase promotor inserted into the *Sma*I site and were used to prepare RNA for the detection of RNA–protein interactions using the *in vitro* assays described below. For galactose-induced expression in *S.cerevisiae* strain BJ5465, the *SEP*1 gene in the yeast shuttle vector pWDH129 (Holler *et al.*, 1995) was replaced by the 1603 bp *Nco*I–*Xho*I HBP cDNA fragment to produce plasmid pFMM5. In pFMM5, the ATG at position 105 in the cDNA sequence is immediately downstream of the strong *GAL1-10* promotor.

cDNA library

The human cDNA expression library was a gift from Robert Hindges. cDNA prepared from human Epstein–Barr virus-transformed peripheral lymphocytes was inserted into a *Xho*I site downstream of the *GAL4* transactivation domain in the plasmid pACT with the selectable marker *LEU2* (Durfee *et al.*, 1993).

Screening procedure

The human cDNA expression library was introduced in *S.cerevisiae* L40-coat cells carrying pIII/wtHP-MS2 as described (Gietz *et al.*, 1992). Transformants were grown on synthetic complete medium (YNB) lacking uracil, histidine and leucine for selection of the *URA3*, *HIS3* and *LEU2* marker genes. To favour the selection of high affinity RNA binding proteins, residual *HIS3*-independent growth was prevented by inclusion of 25 mM 3-aminotriazole in the medium. Growing colonies were analysed further for *lacZ* expression by plating on media supplemented with 80 mg/l of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase. Four blue transformants (clones 1–4) were selected and analysed further. The His^+ $lacZ^+$ phenotype was shown to be plasmid dependent by rescue of the pACT-cDNA plasmid as described (Hoffman and Winston, 1987), amplification in *E.coli* XL-1 blue and re-transformation into *S.cerevisiae* L40-coat, either in the absence of other plasmids or in the presence of the plasmids pIII/IRE-MS2, pIII/wtHP-MS2, pIII/mutHP-MS2 and pIII/cgHP-MS2.

Sequencing of the hbp gene

The cDNA contained in the pACT plasmid of clone 2 was excised using *Bgl*II and subcloned into the *Bam*HI site of pBluescript KS(–) (Stratagene) to produce pBluescript-HBP or pBluescript-rHBP. The sequences of both strands were determined using an automatic DNA sequencer (Applied Biosystems). Using the GCG program (Genetics Computer Group, Madison), 100% homology to three cosmids containing contiguous human DNA (McCombie *et al.*, 1992) was detected.

Preparation of extracts

Nuclear extract was prepared from mouse mastocytoma K21 cells as described (Stauber *et al.*, 1990). To prepare *S.cerevisiae* whole cell extracts, 10 ml of YNB medium lacking uracil and leucine were inoculated with a single colony and the culture was grown at 30°C for 16 h. Cells were then harvested by centrifugation and the pellet washed in 10 ml of extraction buffer [20 mM Tris–HCl (pH 7.5), 10% glycerol 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C. Subsequently, the cells were collected again by centrifugation and resuspended in 250 µl of extraction buffer at 4°C. The cells were broken by vortexing in the presence of an equal volume of glass beads six times for 30 s at 4°C. Debris was removed by centrifugation and the extract stored at –80°C. For galactose induction of HBP, *S.cerevisiae* BJ5465/pFMM5 and BJ5465 were grown and induced with 2% galactose essentially as described (Johnson and Kolodner, 1991). Cells were harvested by centrifugation, washed in extraction buffer and then lysed as described above. Protein concentrations were determined by the Bradford assay, using bovine serum albumin as reference (Bradford, 1976).

Enrichment of HBP

Bovine HBP was partially purified from calf thymus whole cell extract by phosphocellulose, Affigel-Blue, hydroxyapatite and MonoQ column chromatography (fraction V). The detailed procedure will be described elsewhere (A.Schaller, F.Martin and B.Müller, in preparation). Recombinant HBP was induced in a 1 l culture of BJ5465/pFMM5 as described above. After 6 h induction, cells were harvested and washed in buffer A [20 mM HEPES–KOH (pH 7.9), 10% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 µM leupeptin, 1 µM pepstatin A, 1 mM benzamidine] and resuspended in buffer A to a final volume of 5 ml. Cells were then broken by vortexing at 4°C six times for 30 s in the presence of an equal volume of glass beads and the debris removed by centrifugation. The lysate was recovered and applied immediately onto a 20 ml P11 phosphocellulose column (Whatmannn) equilibrated with buffer A. Subsequent purification steps were as described for bovine HBP (A.Schaller, F.Martin and B.Müller, in preparation). Both *S.cerevisiae* and calf thymus MonoQ fractions (fraction V) used for the experiments described in Figure 6 were dialysed extensively against buffer A.

Assays to detect RNA–protein interactions in vitro

Uniformly ^{32}P-labelled RNA was produced by T7 RNA polymerase transcription of plasmids pSP65wtHP and pSP65mutHP linearized with *Sma*I and purified by denaturing polyacrylamide gel electrophoresis. cgHP RNA was transcribed directly from oligonucleotides by T7 RNA polymerase (Milligan *et al.*, 1987) and purified as described above. To detect RNA–protein interactions by EMSA, protein was mixed with 50 fmol of uniformly ^{32}P-labelled wtHP RNA and 20 µg of yeast tRNA and incubated for 20 min in 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 10% glycerol in 20 µl at 0°C. Reaction products were analysed by electrophoresis on native 5% polyacrylamide gels using 50 mM Tris–50 mM glycine as buffer system and visualized by autoradiography. In some experiments, unlabelled competitor RNAs were included as indicated. RNA molecules used for EMSA are shown in Figure 1C.

Preparation of K21 mouse cell nuclear extract depleted of HBP

Depletion by MonoQ column chromatography. K21 cell nuclear extract was prepared as described above and the components of the histone 3′ processing reaction fractionated similarly to earlier procedures (Vasserot *et al.*, 1989; A.Schaller, F.Martin and B.Müller, in preparation). Briefly, 5 ml of extract (8 mg/ml protein) were applied onto a 1 ml MonoQ

column (Pharmacia) in 20 mM Tris–HCl (pH 7.9), 100 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF (buffer C) and eluted with a 12 ml 100–500 mM KCl gradient in buffer C. All fractions (500 µl) were tested for histone mRNA processing, the presence of HBF/HBP (by EMSA) and U7 snRNP (by primer extension). The peak of histone processing activity was in fractions 22 and 23 (300–320 mM KCl); however, the peak of U7 snRNP was in fractions 23 and 24 and the peak of HBF in fractions 19–22. In Figure 5, U7 snRNP-containing fraction 24 was used to test for stimulation of processing by HBP.

Depletion using biotinylated histone hairpin RNA and streptavidin. Biotinylated RNA [5′ biotin-(2′-*O*-Me)ACAAAAGGCCCUUUUCA-GGGCCACCCA(2′-*O*-Me)C(2′-*O*-Me)A] was mixed with K21 nuclear extract and the HBP–RNA complex removed upon incubation with streptavidin–agarose as described (Dominski *et al.*, 1995).

Complementation of histone mRNA 3′ processing in vitro

Processing reactions (10 µl) were performed essentially as described (Spycher *et al.*, 1994) and contained 20 mM EDTA, 0.3 mg/ml tRNA, 800 U/ml RNasin (Promega), the indicated amounts of competitor RNA, 2.5 nM histone ^{32}P-labelled H4wt RNA fragment, the indicated amounts of the different fractions [if necessary made up to equal volume by inclusion of 20 mM HEPES–KOH (pH 7.9), 20% glycerol, 100 mM KCl, 0.5 mM DTT and 1 mM PMSF]. The reaction products were analysed by electrophoresis on 7 M urea–10% polyacrylamide gels, visualized by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics).

Translation of HBP cDNA in vitro

Plasmids pBluescript-HBP (antisense cDNA orientation) or pBluescript-rHBP (sense cDNA orientation) were cleaved with *Sma*I and transcribed and translated in a coupled transcription–translation wheat germ extract system as described by the manufacturer (Promega). Translation products were radiolabelled by the inclusion of [^{35}S]methionine, precipitated by the addition of trichloroacetic acid (final concentration 15%), analysed by SDS–10% polyacrylamide gel electrophoresis and visualized by autoradiography.

Northern blot analysis

A 900 bp *Hin*dIII fragment containing the HBP ORF lacking the 14 C-terminal amino acids was excised from pBluescript-HBP, labelled with digoxigenin according to the manufacturer's instructions (Boehringer) and used to probe a Northern blot with 2 µg of poly(A)$^+$ RNA from different human tissues (Clontech). Pre-hybridization was for 3 h at 42°C in 5× SSPE, 1% Denhardt's solution, 100 µg/ml calf thymus DNA, 100 µg/ml herring sperm DNA, 0.5% SDS, 50% formamide and 1% blocking solution (Boehringer). Hybridization was for 18 h at 42°C in 5× SSPE, 1% Denhardt's solution, 0.5% SDS, 50% formamide and 10 ng/ml digoxigenin-labelled DNA fragment. The blot was washed once with 2× SSC, 0.05% SDS at room temperature and once with 0.1× SSC, 0.1% SDS at 50°C and the hybrids were detected by chemiluminescence according to the manufacturer's instructions (Boehringer). After exposure, the membrane was stripped and reprobed with a human β-actin probe labelled with digoxigenin as above.

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