Characterization of the Calf Thymus Hairpin-binding Factor Involved in Histone Pre-mRNA 3’ End Processing*

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Using ion exchange chromatography we have enriched the RNA hairpin-binding factor involved in histone pre-mRNA processing from calf thymus whole cell extract. We demonstrate that the interaction of the factor with its target RNA sequence, the hairpin structure located at the 3’ end of mature histone mRNA, is sequence-specific and highly salt-resistant. We have developed a simple in vitro system which allows detection of activities stimulating histone pre-mRNA 3’ end processing, based on mouse cell nuclear extract fractionated by Mono Q column chromatography. Using this system, we show that the bovine hairpin-binding factor participates in histone pre-mRNA 3’ end processing in vitro. We have further purified the hairpin-binding factor in form of a RNA-protein complex by RNA-mediated elution from phosphocellulose. This led to a fraction highly enriched for 2 proteins of 40 and 43 kDa.

In higher eukaryotes, replication-dependent histone genes are expressed during S phase, in parallel to DNA synthesis (for review, see Refs. 1 and 2). This is essential for the conservation of chromatin structure during the cell cycle. Regulation of gene expression occurs at transcriptional and post-transcriptional levels, and, at the post-transcriptional level, involves pre-mRNA 3’ end processing and histone mRNA stability (3). Unlike the majority of mRNAs, these histone mRNAs do not end in a poly(A) tail, but terminate a few nucleotides after a hairpin structure that is highly conserved between animal histone genes (1, 2). This 3’ end is formed by a cell cycle-regulated endonucleolytic cleavage between the hairpin structure and a purine-rich spacer element 3’ of the cleavage site (4, 5). These cis-acting sequences required for the cleavage reaction are well defined and three trans-acting factors have been identified, the U7 snRNP1 (6), the hairpin-binding factor (HBF) (7), and the heat-labile activity (8). However, of these factors only the U7 snRNP, which interacts with the spacer element (9–12), has been further characterized. The U7 snRNA sequence from several organisms has been determined (11, 13, 14), and the protein composition of the U7 snRNP was analyzed (15).

To better understand the mechanism and the regulation of the processing reaction, we intend to purify and characterize the other trans-acting factors involved in this reaction, with the ultimate goal to establish an in vitro processing system composed of purified components. Here, we describe the characterization and purification of the bovine HBF involved in histone RNA processing. Evidence for the role of this factor in histone gene expression is mostly indirect and was obtained by introducing mutations in the RNA hairpin, which were designed to change the RNA sequence but not the RNA structure. These mutations reduced RNA processing, nuclear export, and cytoplasmic regulation (16–18). The binding of the HBF to mutant RNA hairpin structures paralleled the effect of these mutations on gene expression (16, 17), suggesting a direct involvement of the HBF in all of these steps. Factors binding to the hairpin are present in nuclear as well as in cytoplasmic fractions of mouse cell extracts (19, 20) and the cytoplasmic fraction is able to participate in in vitro histone RNA processing, indicating that the nuclear and cytoplasmic factors are related (21). The cytoplasmic factor has been partially purified (22) and, using NorthWestern blotting, a 45-kDa protein has been identified as the RNA-binding protein.

In this manuscript, we describe the enrichment of the HBF from calf thymus extract by phosphocellulose, Affi-Gel blue, hydroxyapatite, and Mono Q column chromatography. Using a new in vitro complementation assay for components of the histone pre-mRNA processing machinery, we demonstrate that this enriched fraction is able to participate in histone pre-mRNA processing. We have further purified the HBF from the enriched fraction by RNA-mediated elution from a phosphocellulose column to a fraction enriched for two main proteins of 40 and 43 kDa.

MATERIALS AND METHODS

Buffers—Buffer A contained, if not otherwise indicated, 20 mM Hapes-KOH (pH 7.9), 100 mM KCl, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM pepstatin A, 2 μM leupeptin, and 0.1 mM benzamidine. Buffer B was 20 mM Hapes-KOH (pH 7.9), 50 mM KCl, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 1 μM pepstatin A, 2 μM leupeptin, and 0.1 mM benzamidine. Buffer C was 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 10% glycerol, 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF.

Preparation of Pre-mRNAs and Nucleic Acids—Substrate RNAs with wild-type histone stem loop sequence derived from the mouse histone H4-12 gene (23) (wtHP; 5’-GGAGCUCAACAAAAGGCCCUUUUCAG-GGCCACCC) or mutant stem-loop sequence (mutHP; 5’-GGAGCUCAA-CAAAACCGAAGCCCUUCGCGACCC), and mutant stem sequence (eighthHP; 5’-GGACAAAAACCCCUUUCAGGGGGGACCC) were prepared in vitro by T7 RNA polymerase-mediated transcription and purified by denaturing polyacrylamide gel electrophoresis. Templates for transcription were either plasmids derived from pSP65 linearized with Smal (24) or partially double-stranded oligonucleotides (25).

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or [$\alpha$-32P]GTP incorporated into the 32P-labeled RNA.

**Purification of the Hairpin-binding Factor**—Unless indicated otherwise, all manipulations were performed on ice or at 4–6 °C. Fractions were frozen in liquid nitrogen, stored at -80 °C, and thawed quickly by incubation in H2O at room temperature. For the purification summarized in Table I, 1, 2 × 150 g of frozen calf thymus (Schlachthof, Bern) were partially thawed by incubation in 300 ml of buffer A containing 5 mM EDTA and 1 mM DTT each and homogenized separately in a Waring blender for 3 × 15 s and then centrifuged for 1 h at 25,000 × g. The supernatants (600 ml) (fraction I) was first filtrated through a cheese cloth and then directly applied to a 150-ml P11 phosphocellulose column (Whatmann) equilibrated with buffer A. The column was washed with 2 column volumes of buffer A and eluted with an 800-ml gradient from 100 to 1000 mM KCl in buffer A. The activity eluted between 200 and 400 mM KCl (fraction II). The pooled fractions were applied directly onto a 50-ml Affi-Gel blue column (Bio-Rad) equilibrated with buffer A containing 300 mM KCl. The column was washed with 2 column volumes of buffer A supplemented with 300 mM KCl and eluted with a 400-ml gradient from 300 to 1000 mM KCl in buffer A. The activity eluted in a broad peak between 750 and 1000 mM KCl (fraction III). The pooled fractions were adjusted to 3 mM CaCl2 and directly applied to a 25-ml hydroxyapatite column (Bio-Rad) equilibrated with buffer B. The column was washed with 2 volumes of buffer B and eluted with a 160-ml gradient from 0 to 400 mM potassium phosphate in buffer B. The column was washed with 2 column volumes of buffer B and eluted with a 510 mM potassium phosphate (fraction IV). The active fractions were pooled, dialyzed against buffer A, and applied onto a 1-ml Mono Q column (Pharmacia Biotech Inc.). The column was washed with 2 volumes of buffer A and eluted with a 12-ml gradient from 100 to 500 mM KCl in buffer A. The activity eluted as a broad peak between 180 and 320 mM KCl (fraction V). The pooled fractions were dialyzed against buffer A and applied onto a 5-ml phosphocellulose column in buffer A. The column was washed with 12.5 ml of buffer A collected in ~1.5-ml fractions. Proteins were eluted with 1 n mole of weakly radiolabeled wtHP RNA (5 × 107 dpm/nmol), applied in 300 µl of buffer A, and collected in 300-µl fractions (fraction VI). During the purification procedure, RNA-protein complexes with faster mobility were observed by mobility-shift assay. Fractions containing disproportional amounts (>30%) of this activity were discarded.

Protein concentrations were determined by the Bradford method (26) using bovine serum albumin as a reference. To determine the amount of HBF present in the different fractions, binding reactions were performed with 2.5 nm wtHP RNA as described below and different amounts of proteins and the amount of RNA bound per mg of protein was calculated. For the affinity elution, the amount of HBF was assumed to correspond to the amount of RNA complexed, as detected by mobility-shift assay. For electrophoresis on a SDS-10% polyacrylamide gel, the fractions were precipitated with trichloroacetic acid (15%). Proteins were visualized by staining with Coomassie Brilliant Blue followed by silver staining. Protein markers were from Bio-Rad.

**Gel Filtration Chromatography**—A Superdex 75 gel filtration column (Pharmacia) was equilibrated with 300 mM KCl in buffer A. 50 µl of fraction V were applied onto the column, and 50-µl fractions were collected and assayed for the presence of HBF. The relative amount of HBF in the various fractions was determined by calculating the ratio of HBF-RNA complexes divided by the amount of free RNA. Marker proteins (Pharmacia) phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were run in parallel, detected by UV absorption at 280 nm, and identified by SDS-10% polyacrylamide gel electrophoresis.

**Binding Assays**—Unless indicated otherwise, 2.5 nm 32P-labeled RNA were incubated with protein (usually 50% of reaction volume) in the presence of 10 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT, and 1 mg/ml yeast tRNA in 10–20 µl for 20 min on ice. Subsequently, the reaction products were analyzed directly by electrophoretic mobility-shift assay (EMSA). 5% polyacrylamide gel electrophoresis was done at 4–6 °C with 10 V/cm, using 50 mM Tris, 50 mM glycine as buffer system. Reaction products were visualized by autoradiography or analyzed by a Molecular Dynamics PhosphorImager.

Alternatively, RNA and proteins were cross-linked by UV light and the reaction products analyzed by gel electrophoresis. Uniformly labeled RNA prepared with [$\alpha$-32P]UTP and, if indicated, mixed with competitor RNA, were incubated with proteins as above and, after 20 min on ice, irradiated with 840–960 mJ of 260-nm UV light in a UV Stratagene (Stratagene) and analyzed by SDS-10% polyacrylamide gel electrophoresis. The photoadducts were detected by autoradiography or using a PhosphorImager. Prestained protein standards were from Bio-Rad.

**Complementation Assay**—Mouse K21 cell nuclear extract was prepared as described, except that 1 mM PMSF was included in the hypotonic buffer (27). 5 ml of extract (40 mg of protein) was applied onto a 1-ml Mono Q column (Pharmacia) equilibrated with buffer C. The column was washed with 2 column volumes of buffer C and eluted with a 12-ml 100–500 mM KCl gradient in buffer C. HBF and U7 snRNP eluted in two overlapping, but separate, peaks. HBF eluted in fractions 18–24, at 200–340 mM KCl, and the peak was in fractions 19–22, between 220 and 300 mM KCl. U7 snRNP started to elute in fraction 22, at 300 mM KCl, and most eluted between 320 and 340 mM KCl in fractions 23 and 24. All fractions were tested for histone pre-mRNA 3′ end processing, and the peak of processing activity was in fractions 22 and 23, indicating that a factor other then U7 snRNP is limiting in fraction 24. Therefore, fraction 24 was used to test for stimulation of processing by HBF. Processing reactions were essentially as described (28) and contained 20 mM EDTA, 0.3 mg/ml tRNA, 1 unit/µl RNasin (Promega), 2.5 mM histone H4wt pre-mRNA 3′ end fragment labeled with [$\alpha$-32P]GTP, the indicated amounts of the different fractions and, where necessary, buffer A, 200 mM KCl up to 50% of the reaction volume, in 10 µl. The reaction products were analyzed by 7% urea, 10% polyacrylamide gel electrophoresis. Visualization and quantitation of results was as described above. Processing efficiency (percentage of RNA processed) was calculated from the fraction of RNA present as 5′ cleavage product, taking into account the proportion of radiolabeled nucleotide in this fragment.

**RESULTS**

To better understand the mechanism of histone pre-mRNA 3′ end processing, we have initially characterized the interaction of the hairpin structure at the mRNA 3′ end with HBF using mouse cell nuclear extract as the HBF source. This interaction was not sensitive to mild heat treatment and not dependent on the presence of a functional U7 snRNP, but was sensitive to proteinase K (data not shown), confirming earlier results (7). Subsequently, we attempted to purify the HBF from mouse cell nuclear extract. Unfortunately, the amount of factor in nuclear extracts, estimated by EMSA using RNA containing a histone hairpin sequence as substrate (see below), was low (~8000 molecules/nucleus). However, we detected HBF in calf thymus, a more abundant source of material, and enriched HBF from calf thymus whole cell extract using phosphocellulose, Affi-Gel blue, hydroxyapatite, Mono Q column chromatography, followed by RNA-mediated elution from phosphocellulose.

### Table I

**Purification of the hairpin-binding factor from calf thymus**

| Fraction                  | Protein | Hairpin-binding factor | Concentration | Purification factor | Recovery |
|---------------------------|---------|------------------------|---------------|---------------------|----------|
| I. Calf thymus extract    | 9572    | 1890                   | 0.2           | 1                   | 100.0    |
| II. Phosphocellulose pool | 384     | 230                    | 0.7           | 3.5                 | 11.6     |
| III. Affi-Gel blue pool   | 20.9    | 153                    | 7.3           | 36.5                | 7.7      |
| IV. Hydroxyapatite pool   | 6.2     | 75                     | 12.1          | 60.5                | 3.8      |
| V. Mono Q pool            | 1.0     | 23                     | 23.0          | 115.0               | 12.2     |
| VI. Phosphocellulose-RNA affinity elution | 20     |                        |               |                     | 1.0      |
were performed with affinity elution from phosphocellulose (wtHP). B, binding reactions were performed with 5 μl of fraction V in 10 μl with the indicated 32P-labeled RNAs, and products were analyzed by EMSA as described under “Materials and Methods.” Lanes 1–3, reactions in the absence of fraction V. Lanes 4–12, reactions in the presence of fraction V. In reactions in lanes 7–12, either 250 fmol or 2.5 pmol of the indicated competitor RNA were included prior to addition of protein. C, binding reactions with fraction V were performed in 20 μl as described under “Materials and Methods” and contained 150 fmol 32P-labeled wtHP RNA (lane 1), 150 fmol of 32P-labeled wtHP RNA mixed with 50 pmol of mutHP competitor RNA (lane 2), 150 fmol of 32P-labeled wtHP RNA mixed with 50 pmol of wtHP competitor RNA (lane 3). Cross-linking with UV light, analysis by 10% SDS-polyacrylamide gel electrophoresis, and visualization by autoradiography were as described under “Materials and Methods.” The major specific UV cross-links are indicated by a bracket.

To test for sequence-specific binding, we prepared the two mutant RNA molecules mutHP RNA and cgHP RNA shown in Fig. 1A. In mutHP RNA, the hairpin sequence was changed in the stem and the loop, but the flanking sequences were unchanged. In vitro processing experiments have shown that this mutant hairpin structure does not interact with HBF (29). In cgHP RNA, the sequence was changed to form a stem composed of five C-G base pairs and an U-A base pair, and the 5′-flanking sequence was shortened by 6 nucleotides. When we compared the binding of the HBF to the wtHP RNA with the binding to the two mutant RNAs, we found that a complex was formed only with wtHP RNA (Fig. 1B, lanes 4–6). In addition, only wtHP competitor RNA could efficiently compete for the factor (lanes 7 and 8), indicating that the binding to RNA is sequence- and structure-specific. To rule out that, using this assay, we detected La protein, a ubiquitous RNA-binding protein with a preference for U-tracks, we performed competition experiments with an artificial tRNA precursor ending in UUUUOH (30). Inclusion of a 100-fold excess of this RNA, which is bound efficiently by La protein, did not influence the binding of the HBF to wtHP RNA (data not shown), thus excluding La protein as a candidate. To estimate the molecular mass of the HBF, wtHP RNA prepared with [32P]UTP of high specific activity was incubated with fraction V, either in the presence or absence of excess wtHP or mutHP competitor RNA. Proteins and RNA were then covalently linked by irradiation with 260-nm UV light and analyzed subsequently by SDS-polyacrylamide gel electrophoresis. Fig. 1C shows that fraction V contains two main double bands of ~48–50 and ~55–57 kDa (lane 1), which are sensitive to competition with wtHP, but not with mutHP RNA (lanes 3 and 2, respectively), indicating that these double bands are formed by a sequence-specific interaction, as are the HBF-RNA complexes in Fig. 1B. Incubation of these samples with RNase decreased the molecular mass of these complexes to between ~40 and 50 kDa, giving a more accurate mass estimate for the protein component (data not shown).

To determine whether HBF in fraction V is involved in histone pre-mRNA processing, we tested fraction V in a simple in vitro RNA processing system that allows us to identify components of the 3′ end processing reaction. To prepare this system, histone pre-mRNA processing proficient mouse cell nuclear extract was fractionated by Mono Q column chromatography, leading to a separation of HBF from U7 snRNP. We then tested whether the mouse HBF was part of the histone pre-mRNA processing machinery. Fig. 2 shows that Mono Q fraction 20, containing the mouse HBF, did not cleave a short RNA fragment containing all the sequence elements required for histone pre-mRNA processing (lane 3) (7), while processing using Mono Q fraction 24, containing U7 snRNP and little HBF, was very low (lane 4). However, mixing these two fractions stimulated RNA processing ~5-fold, to about 20% of processing observed with a peak fraction (compare lanes 2 and 5). When the mouse HBF-containing fraction 20 was replaced by the calf thymus fraction V, which did not process RNA by itself (lane 6), proc-
Materials and Methods.

fractionated by Mono Q column chromatography as described under "Materials and Methods." Processing reactions were done as described under "Materials and Methods." in 10 µl and contained: 5 µl of mouse Mono Q fraction 22 showing the peak of processing activity (lane 2), 2.5 µl of mouse Mono Q fraction 20 showing processing with HBF, but without U7 snRNP (lane 3), 2.5 µl of mouse Mono Q fraction 24 showing processing with U7 snRNP and limiting HBF (lane 4), complementation with 2.5 µl of Mono Q fraction 20 and 2.5 µl of Mono Q fraction 24 (lane 5), processing with 2.5 µl of fraction V (lane 6), processing with 2.5 µl of Mono Q fraction 24 and 2.5 µl of fraction V (lane 7), same as lane 6 but with 125 pmol of mutHP RNA (lane 8), same as lane 7 but with 125 pmol of wtHP RNA (lane 9). The percentage of RNA processed is indicated at the bottom of the figure. The marker in lane 1 is 32P-end-labeled pBR322 DNA cut with HpaII.

processing was stimulated ~5-fold (lane 7), similar to the reaction in the presence of the mouse Mono Q fraction 20. This stimulation was reduced to near background level by the inclusion of wtHP competitor RNA (lane 9), but not by mutHP competitor RNA (lane 8), indicating that the HBF in fraction V is involved in histone pre-mRNA processing.

To exclude the possibility that stimulation of histone pre-mRNA processing by a fraction enriched for HBF is coincidental, we tested whether HBF co-eluted with the processing-stimulating activity from hydroxyapatite and Mono Q columns. HBF eluted from the Mono Q column in fractions 6–10 (Fig. 3A, lanes 3–7), with the main peak in fractions 7–9. In parallel, we tested these fractions for stimulation of histone pre-mRNA processing, using the assay described above. About 3-fold stimulation of processing was observed in reactions with the peak fraction 7–9 (Fig. 3B, lanes 11, 13, and 15), ~2-fold stimulation with fraction 6 (lane 9), and ~1.4-fold stimulation with fraction 10 (lane 17), in parallel to the amount of HBF present in these fractions. This demonstrates that HBF and processing-stimulating activity co-elute form the Mono Q column. Co-elution of these activities was also observed in fractions from the hydroxyapatite column (in fractions 2–7, 6.3, 46.3, 67.1, 44.2, 24.1, and 11.9% wtHP RNA was bound by HBF, and stimulation of processing with these fractions was 1-, 2-, 2.2-, 1.3-, 1-, and 0.9-fold, respectively). These results confirm that HBF enriched in fraction V is involved in histone pre-mRNA processing.

The polypeptides detected by UV cross-linking to wtHP RNA in fraction V (Fig. 1C) have similar sequence requirements for hairpin RNA binding as the HBF detected by EMSA (Fig. 1B), indicating that they are part of the HBF. To test whether these polypeptides co-elute with HBF from the Mono Q column, we mixed Mono Q fractions 4–12 with 32P-labeled wtHP RNA and cross-linked RNA-protein interactions with UV light. The cross-links were visualized by autoradiography. The major HBF-specific UV cross-links are indicated by a bracket.
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During the purification of HBF from calf thymus extract as well as from HeLa and K21 cell nuclear extracts (data not shown), suggesting that HBF might be easily modified.

To better characterize the conditions for HBF-wtHP complex formation, salt concentrations were varied in binding reactions with fraction V. Complex formation was stimulated by increasing the KCl concentration from 100 to 400 mM and not inhibited by the presence of up to 1 mM KCl (Table II). Binding at still higher salt concentrations was observed but not quantitated, since the migration during electrophoresis was affected (data not shown). Our standard binding assay is performed in the presence of EDTA; however, inclusion of MgCl2 and ATP had no influence on complex formation (Table II).

To further purify the HBF from fraction V, we exploited the high affinity of the factor for wtHP RNA and made use of the observation that the HBF, but not HBF-RNA complexes, binds to phosphocellulose at 100 mM KCl. We applied an aliquot of fraction V onto a small phosphocellulose column and washed the column with buffer containing 100 mM KCl. Fig. 5A shows that using a normal binding assay followed by EMSA, we did not detect HBF in the wash fractions (lanes 2–11). HBF was then eluted with wtHP RNA radiolabeled with high specific activity UTP, and analyzed directly by EMSA (lanes 13–21). The amount of HBF-RNA complexes in each fraction paralleled the amount of free radiolabeled RNA, and complexes were present mainly in fractions 12 and 13 (lanes 14 and 15). Comparison with the HBF in fraction V (lane 1), detected using a standard binding assay, showed no significant differences in appearance of the HBF. To visualize the wtHP RNA-binding proteins in the peak fractions 11–14 (Fig. 5A, lanes 13–16), samples of these fractions were cross-linked with UV light immediately after 32P-labeled RNA-mediated elution from the phosphocellulose column and analyzed by SDS-polyacrylamide gel electrophoresis. The pattern of RNA-protein complexes in these fractions is best visible in the cross-linked fraction 12 and consists of 3–4 RNA-protein complexes between ~48 and 57 kDa (Fig. 5B, lane 2), very similar to the cross-links with Mono Q fractions and fraction V (Figs. 1C and 3C). This indicates that the proteins eluted with wtHP RNA are (components of) the HBF. The small differences revealed by closer inspection are probably caused by a weak Rnase activity during RNA-mediated elution from phosphocellulose, as indicated by the appearance of shorter RNA fragments in Fig. 5A (lanes 14–17).

Fractions 12 and 13 were pooled, precipitated with 15% trichloroacetic acid, and analyzed by SDS-polyacrylamide gel electrophoresis. Extensive staining with silver revealed the presence of two polypeptides of apparent molecular mass of ~40 and ~43 kDa in these pooled fractions (data not shown), arguing again that the double bands produced by UV cross-linking were caused by two proteins. These polypeptides are better visible in fraction VI (Fig. 5C, lane 1) of a large-scale HBF preparation (summarized in Table I). As already mentioned above, Rnase A digestion reduced the apparent molecular mass of these RNA-protein complexes from ~48–57 to ~40–50 kDa. Assuming a minimal HBF binding site of ~22 nucleotides, and keeping in mind that the loop in the hairpin structure and the sequences immediately 5′ of the hairpin structure are accessible to Rnase A in complexes formed with crude preparations of a mouse factor binding to histone hairpin RNA (31), it is likely that RNA fragments from 5 to 22 nucleotides may remain cross-linked to HBF upon Rnase A digestion. This is in good agreement with the apparent molecular mass of 40 and 43 kDa for the proteins enriched in fraction VI.

**DISCUSSION**

In this manuscript, we demonstrate that an assay for factors involved in histone pre-mRNA processing can be used to deter-
mine whether enriched fractions, and probably also pure proteins, take part in histone pre-mRNA processing. Furthermore, we describe the purification of the HBF involved in this RNA processing reaction from calf thymus.

To separate two components of the processing machinery, HBF and U7 snRNP, processing-proficient mouse cell nuclear extract was fractionated by Mono Q column chromatography. The two activities overlapped only in a few fractions, and not surprisingly, the peak of histone RNA processing activity was found to be in these fractions. For the in vitro complementation assay, a side fraction enriched for HBF was mixed with a side fraction enriched for U7 snRNP, leading to processing at ~20% of the peak activity fraction. These side fractions were chosen as that neither U7 snRNP nor the HBF were limiting. Therefore, the low level of restoration suggests that another factor, perhaps the heat-labile activity, was limiting in this reaction.

This assay system was used to confirm that the bovine HBF enriched from calf thymus whole cell extract by ion exchange chromatography is able to participate in histone pre-mRNA processing. HBF, as detected by EMSA, co-eluted with an activity stimulating histone pre-mRNA processing from hydroxyapatite and Mono Q columns, indicating that they are identical. In addition, in reactions with fraction V of the purification, which was at least 115-fold enriched for HBF, stimulation of processing was prevented by the inclusion of wtHP, but not mutHP competitor RNA. Since HBF binds specifically and with high affinity to wtHP RNA, this is further proof that HBF is involved in histone pre-mRNA 3’ end processing. This assay system will be very useful during the further characterization of HBF and in addition promises to be an essential tool to study additional factors involved in processing, such as the heat-labile activity.

Attempts to further purify HBF from fraction V revealed a common problem in the purification of RNA-binding proteins, an extreme stability of RNA-protein complexes. This stability did not allow for an affinity purification step, using the dissociation of nucleic acid-protein interactions by high salt concentrations, a method commonly used for the dissociation of DNA-protein complexes. Instead, we used hairpin RNA to specifically elute the HBF from phosphocellulose at low salt. UV cross-linked RNA-protein complexes eluted from phosphocellulose had the same apparent molecular mass (48–57 kDa) as complexes formed with HBF in Mono Q fractions and fraction V, indicating that only minor changes, if at all, occurred during the last purification step. Analysis of proteins in fraction VI showed that two proteins of ~40 and ~43 kDa were enriched by the RNA-mediated elution. The presence of two enriched proteins agrees well with the observation that fraction V contained two hairpin RNA binding activities of apparent molecular masses of ~43 and ~53 kDa detected by gel filtration and that the HBF appears as double bands of ~48–50 and ~55–57
kDa in UV cross-linked samples. Our interpretation of these observations is that the HBF consists of two polypeptides with different molecular mass, interacting in a sequence-specific manner with histone hairpin RNA. Currently, we assume that the mass difference is due to different modifications of the same polypeptide, which might arise during cell cycle regulation, perhaps by phosphorylation, and perhaps reflect a regulatory step in the processing reaction. Alternatively, it is possible that the polypeptide itself is unstable and that truncated forms appear during the purification. However, we cannot presently exclude the possibility that the HBF consists of two different RNA-binding proteins.

The protein purification procedure described here consists of four standard ion exchange chromatography steps, followed by RNA-mediated elution from phosphocellulose. This procedure reproducibly leads to the enrichment of two polypeptides of ~40 and ~43 kDa. However, we observed that the appearance of HBF analyzed by EMSA was variable from purification to RNA-mediated elution from phosphocellulose. This procedure involved four standard ion exchange chromatography steps, followed by a final step in the processing reaction. We cannot presently exclude the possibility that the HBF consists of two different RNA-binding proteins.

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