Evolutionary conserved multiprotein complexes interact with the 3' untranslated region of histone transcripts

Richard Eckner* and Max L. Birnstiel

Research Institute of Molecular Pathology, Dr Bohr-Gasse 7, A-1030 Vienna, Austria

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ABSTRACT

The replication dependent histone transcripts terminate with a highly conserved stem-loop structure. This feature distinguishes them from most other eukaryotic mRNAs which end with a poly(A) tail. The 3' terminus of histone mRNA is a main determinant for rapid turnover of these transcripts. In this study, we report the identification of two cytoplasmic protein complexes that interact in a sequence specific fashion with 3' terminal sequences of a mouse histone H4 and a human histone H2A mRNA. The binding activities are conserved from frog to man. At least a fraction of one of the protein complexes appears to be specifically associated with polysomes. The evidence for an involvement of the observed protein complexes in turnover of histone transcripts is discussed.

INTRODUCTION

Different messenger RNAs display a wide variety in their half-lives. Stable transcripts as for example β -globin mRNA exhibit a half-life of more than 24 hours (1), whereas unstable messengers of immediate early genes like c-fos show a half-life of only 20 minutes (2, 3). The stability of a mRNA can be regulated by extracellular signals or during the cell cycle. Transferrin receptor mRNA is selectively stabilized under conditions of iron depletion (4, 5) and apolipoprotein II or vitellogenin transcripts are destabilized by estrogen withdrawal in chicken oviduct and liver (6–8). The transcripts of the replication dependent histone genes exhibit in G1 and S phase of the cell cycle a half-life of 45–60 minutes (9–11), which decreases to about 15 minutes at the end of S phase and in G2 phase (11).

For several unstable transcripts specific cis-acting elements have been identified that are required for their instability and that interact with trans-acting RNA binding proteins. AU-rich sequences found in the 3' untranslated region of certain labile transcripts have been identified as important determinants for rapid turnover (12). Although several RNA binding factors recognizing AU-rich domains have been described (13–16), it is presently not known whether binding of these proteins is required for stabilisation or destabilisation of such transcripts, or for translational control mechanisms also operating through this motif (17). Iron starvation induces the binding of a cytosolic

protein to the 3' untranslated region of the transferrin receptor mRNA and prolongs the half life of this transcript (18–20). In the majority of cases investigated to date, the transcripts are stabilised by drugs (e.g. cycloheximide) that prevent translational elongation, suggesting that continued translation is required for faithful RNA degradation. Based on this finding and on mutational analysis of protein coding regions, it has been proposed that a ribosome associated nuclease, for whose activation ongoing translation is one precondition, participates in the degradation process (21, 22).

The transcripts of the replication dependent histone genes end with a highly conserved stem-loop structure (reviewed in 23, 24). This structure has to be positioned at the mRNA 3' end in order to render the stability of a transcript sensitive to inhibition of DNA synthesis (25, 26). Cleavage within the terminal hairpin structure by an exonuclease seems to be the first event in histone mRNA degradation (27, 28). However, in vitro this exonuclease did not exhibit sequence specificity and degraded also transcripts unrelated to histone mRNA as c-myc RNA or an antisense transcript of a histone gene (29). Thus, it is not clear how the exonuclease recognizes the histone hairpin structure. Does the nuclease itself have an intrinsic affinity for this sequence element, or is the specificity of exonuclease action mediated by sequence specific RNA binding proteins directing the nuclease to its stem-loop target on histone mRNA?

A different set of experiments has revealed that histone RNAs carrying sequence extensions past the hairpin structure were substrates for an exonuclease removing the nucleotides downstream of the hairpin. This exonucleolytic activity has been detected in *Xenopus* oocytes (30) and in mammalian cells (31). It remains to be determined whether the trimming exonuclease and the above mentioned exonuclease cleaving within the hairpin are related with each other.

In this study we have examined whether cellular proteins can interact with a series of histone transcripts. Using gel retardation and crosslinking assays, we find that protein complexes present in cytoplasmic extracts bind in a sequence specific manner to the conserved 3' end of histone mRNA. The protein complexes appear to be evolutionary highly conserved because they can be detected in many species ranging from frog to man. We investigated whether protein binding correlates with the occurrence of the resection activity for a given transcript. Our

* Present address: Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

results suggest that the detected polypeptides are required for an efficient trimming reaction. We discuss the evidence for an involvement of the binding activities in the control of histone RNA stability and translation.

MATERIALS and METHODS

Plasmid constructs and in vitro transcription

Construction of the plasmids coding for the wt, mut or long pal full length histone H4 RNAs has been described previously (31). For transfection into tissue culture cells, constructs in plasmid pSP65CMV (31) were utilised, and for in vitro transcription, constructs cloned into pSPT19 (Pharmacia) were used. The plasmid coding for the wt pal-ClaI RNA was generated by replacing the mutated palindrome of the mut rib (31) construct (cloned into pSPT19) with an oligonucleotide containing a wildtype palindrome. The sequence of this oligo is: (ClaI) 5'-CG-ATCATCCCTAACGGCCCTTTTAGGG-CCAACCGTCA-3' (MluI). Expression of the wt pal-ClaI transcripts in tissue culture cells was made possible by subcloning the complete histone H4 coding region containing the wt-pal-ClaI 3' end as a BamHI fragment into pSP65CMV. To generate the templates for the 3' end transcripts, EcoRI-BamHI fragments encompassing 115 bp of histone H4 specific sequences were transferred from the full length histone gene constructs and were inserted downstream of the T7 promoter of pSPT19. Each of the above templates was cut with MluI, so that run-off transcripts with a 12 nucleotide extension were formed by in vitro transcription with T7 RNA polymerase. For nuclease Bal 31 deletions, the plasmid pSP65-H4-119/70 (32) was cleaved with SacI and incubated with Bal 31 for various timepoints under standard conditions (33). Fragments with progressive deletions of sequences upstream of the stem-loop structure were recovered by digestion with BamHI and subsequently cloned into SmaI-BamHI cut plasmid pSP65. The deletion endpoints were determined by sequencing with Sequenase according to the supplier's protocol (USB).

In order to obtain a template coding for the 3' end of a human H2A gene (34), a 185 bp HindII-PstI fragment spanning the conserved hairpin structure was inserted into pSP65 between the SmaI and PstI sites. This H2A fragment includes H2A specific sequences 55 bp upstream of the hairpin and 114 bp downstream of this secondary structure. To synthesize a run-off transcript, the above plasmid was linearized with PstI.

In vitro transcription reactions were performed with T7- or SP6 RNA-polymerase in the buffer recommended by the supplier (Boehringer Mannheim). The labelled nucleotide was [α -³²P] GTP, except for the crosslinking experiment for which [α -³²P] UTP was used. Cold competitor transcripts were generated by replacing the labelled GTP with 500M cold GTP. All transcripts were purified over 10% denaturing polyacrylamide gels containing 8M urea.

Transfections and S1 mappings

Transcription of the histone gene variants used in Fig. 1C was driven by the CMV promoter/enhancer. Transfections into COS1 cells and RNA isolations were done as described (31). For each of the four constructs an individual S1 probe was labelled at the EcoRI site present in the histone H4 coding region. The S1 probes were the same as described earlier (31). S1 hybridisation reactions were carried out at 48°C for at least 15 hours. Digestion with nuclease S1 and gel electrophoresis were performed according to standard procedures (32).

Gel retardation assays and crosslinking

For gel retardation assays, 10,000 cpm of the respective transcripts were incubated with 6–8 μ g proteins from cytoplasmic extracts at 30°C for 10 minutes. Subsequently, heparin was added to 5 μ g/ μ l final concentration and after another 5 min. unbound transcripts were digested with 200 units of RNase T1 (Boehringer Mannheim) for 10 min. at 30°C. The composition of the binding buffer was 80mM KCl, 25mM Tris pH 7.9, 1.5mM EDTA and 5% glycerol. The final volume of the reaction was 20 μ l. 4% native polyacrylamide gels (40 : 1 ratio acrylamide to methylenebisacrylamide) were run in a buffer containing 25mM Tris, 190mM glycine and 1mM EDTA. For the in situ crosslinking experiment, binding reaction were set up with 25 μ g proteins in a 30 μ l volume as described above. After electrophoresis, the position of the retarded complexes was visualised by autoradiography. Crosslinking was performed for 10 min. with a 254nm UV lamp (model UVGL-25, UVP) at 4cm distance. The complexes were cut out, soaked for one hour in SDS-sample buffer and the crosslinked proteins were resolved on an SDS/8% PAGE. For the crosslinking in solution, 20 μ l binding reactions were put on ice and irradiated for 5min. with the same UV lamp as above. The reactions were stopped by the addition of SDS sample buffer and the crosslinked proteins were analyzed on SDS/8% PAGE.

Preparation of cytoplasmic extracts

Cytoplasmic extracts were prepared from mouse NIH 3T3 cells according to the procedure of Dignam et al. (35) or according to Müllner et al. (20). Both methods gave identical results. The latter method was used to prepare cytoplasmic extracts from rat, monkey and chicken cells. The chicken cells were a kind gift of Drs. C. Schröder and H. Beug. The *Xenopus* oocyte extract was a whole cell extract and was prepared as described (36).

Sucrose gradient centrifugation

For polysomes containing extracts, 7×10^7 cells were lysed exactly as described by Sive et al. (37) in a volume of 0.5ml extraction buffer. 140 μ l of this preparation was treated with 5mM EDTA (final conc.) for 5min. on ice to release the ribosomal subunits from messenger RNAs. This sample and 140 μ l of the untreated extract were loaded on linear 4ml 10–30% sucrose gradients and centrifuged at 4°C for 3 hours at 50,000 rpm in a Beckman SW60 rotor. An identical gradient loaded with total RNA from NIH 3T3 cells was run as reference gradient in parallel with the polysome gradients. Immediately after centrifugation, gradients were separated in 10 fractions, the pellets of the gradients were dissolved in 40 μ l of polysome extraction buffer. 10 μ l of each fraction and 5 μ l of the dissolved pellet, respectively, were used for gel retardation assays. In addition, one quarter of each fraction was analyzed for RNA composition on agarose gels.

RESULTS

Sequence specific binding of cytoplasmic factors to the 3' terminus of a histone H4 mRNA

Previous in vivo experiments have shown that transcripts of the replication dependent histone genes were subject to a resection activity which removed nucleotides extending past the conserved terminal stem-loop structure of this class of mRNAs (30, 31). This resection activity was strictly sequence specific since histone RNAs with altered hairpin structures and an insertion of a ClaI

Figure 1. Sequence specific binding of cytoplasmic proteins to a histone H4 transcript and its correlation with the activity of a trimming exonuclease. **A.** Schematic drawing of the four variants of the histone H4 RNA. The scheme shows only the 3' portion of the transcripts with the mutations in and upstream of the canonical hairpin structure. The wt-pal construct comprises the natural histone palindrome with 6 basepairs in the stem and 4 nucleotides in the loop, the mut-pal RNA preserves the secondary structure of the natural palindrome but contains a different primary sequence, and the long-pal transcript carries an elongated hairpin with 8 basepairs in the stem and 5 nucleotides in the loop. The wt-pal-ClaI construct differs from the wt-pal construct by the presence of a ClaI site 10 bp upstream of the wildtype palindrome. This ClaI site is also present in the mut and long-pal RNAs. The transcripts shown in this figure correspond to the 3' end RNAs used in Fig. 1B, lanes 4–8. These RNAs encompass 87 nucleotides of histone H4 specific sequences upstream of the stem-loop and expose 12 nucleotides downstream of it. **B.** RNA gel retardation assay using the four histone transcripts depicted under A. The 32 P-labelled RNAs were incubated with cytoplasmic extracts derived from mouse NIH3T3 cells. Subsequently, the reaction mixtures were treated with heparin and RNase T1. The digestion products can be seen near the bottom of the gels. The transcripts marked with 'full-length' (lanes 1–3) encompass the complete histone H4 coding region and carry the indicated version of the palindrome. The RNAs denoted with '3' end' (lanes 4–8) span only the 3' terminal 115 nucleotides of the histone H4 messenger. Lane 7 shows the RNase T1 digestion pattern of the wt-pal-ClaI transcript in the presence of extract, and lane 8 the pattern without extract. In lanes 9–12 the radiolabelled wt-pal 3' end transcript was utilised for competition studies; the cold competitor RNA was in lanes 10 and 11 the homologous wt-pal 3' end transcript and in lane 12 E. coli tRNA. The excess of competitor RNA over labelled transcript is indicated above the lanes. **C.** Nuclease S1 protection analysis of cytoplasmic (marked with C) and nuclear (N) RNA isolated from transiently transfected COS1 cells. The transfected constructs are driven by the CMV promoter/enhancer, comprise the complete histone coding region and the transcribed RNAs receive the 3' end by a cis-acting ribozyme (see 31 for details). Ribozyme cleavage results in a 3' terminus 7 nucleotides past the hairpin structure. The cytoplasmic form of the wt-pal and the wt-pal-ClaI transcript is subject to a resection activity removing 6–7 nucleotides beyond the hairpin. The length of the protected fragments is for the wt-pal construct 115 nt (untrimmed RNA) and ~109 nt (resected RNA), for the mut-pal construct 112 nt, for the long-pal construct 117 nt and for the wt-pal-ClaI construct 112 nt for the intact RNA and ~106 nt for the resected RNA.

sequence (see top of Fig. 1) did not serve as substrates for the putative trimming exonuclease (31). These observations pointed to the presence of a sequence specific cellular component, maybe the nuclease itself, recognizing only natural histone transcripts. In order to see whether we can detect cellular proteins interacting specifically with histone mRNAs, we performed gel retardation assays. We synthesized radiolabelled *in vitro* transcripts derived from the same three constructs previously employed for our *in vivo* study (31). The three RNAs differ in their stem-loop structures at the 3' end: the wt-pal transcript carries the natural histone hairpin structure, whereas the mut-pal and long-pal RNAs have altered stem-loop structures (see Fig. 1A). The three transcripts encompass the complete histone H4 coding region. They were incubated with cytoplasmic protein extracts from exponentially growing NIH 3T3 cells. To remove unspecific RNA-protein complexes, RNase T1 and heparin was added to the samples, and complex formation was monitored by electrophoresis on non-denaturing polyacrylamide gels (38). Only in the case of the wt-pal transcript, two RNase T1 resistant complexes could be observed (Fig. 1B, lanes 1–3). No retarded bands were seen when the extract was either treated with proteinase K or heated to 65°C for 10 min. prior to addition of the radiolabelled wt-pal RNA, indicating that proteinaceous factors caused the two retarded complexes (data not shown).

To determine whether sequences from the histone H4 coding were required for complex formation, transcripts spanning the terminal 115 nt of the 3' part of the three histone gene constructs were synthesized. These RNAs lack most of the coding region. In addition, we used a second transcript with a wildtype palindrome (named wt-pal-ClaI) that contained a ClaI site inserted at exactly the same position upstream of the stem-loop structure as in the mut-pal and long-pal transcripts. This RNA served as a control allowing to assess the effect of the inserted ClaI site alone on factor binding (i.e. separated from the effect of the altered stem-loop structures). Transcripts were incubated with cytoplasmic extracts as described above. Again, only the RNA carrying the natural histone 3' terminal sequences gave rise to two retarded bands (Fig. 1B, lanes 4–8), indicating that most of the histone H4 protein coding region is dispensible for complex formation. The insertion of a ClaI site was sufficient to abolish complex formation *in vitro*. This result therefore suggested that the mut-pal and long-pal RNAs were defective in factor binding merely due to the insertion of the ClaI site.

The formation of the upper as well as of the lower complex between the histone RNA fragments and cytoplasmic components could be specifically titrated out by an excess of unlabelled homologous competitor RNA (Fig. 1B, lanes 9–11). A 100-fold molar excess of cold transcript over labelled RNA completely

Figure 2. Sequence requirements for complex formation. **A.** Schematic representation of the progressively deleted DNA templates utilised for the generation of transcripts for gel retardation assays. The different plasmids are designated according to the number of histone H4 derived nucleotides being left intact upstream of the stem-loop structure. The top four templates were cut with either MboII (giving rise to a transcribed RNA with a 2 nucleotide extension beyond the palindrome) or with BamHI (leading to a 68 nucleotide overhang). The template at the bottom was cleaved with Sau 96I resulting in the production of a transcript containing only the first four nucleotides of the hairpin. **B.** Gel retardation analysis of the binding ability of the truncated RNAs. The enzyme used for cleaving the template is indicated. In this gel, a radioactive spot co-migrates with the specific complexes; this spot is also present in the control reaction (marked K, lane 7) displaying the RNase T1 digestion products of the +0 transcript in the absence of cytoplasmic extract. The spot therefore does not represent specific binding and is most likely a digestion product.

competed for factor binding. At the same time, a 1000-fold molar excess of unrelated competitor RNA (*E. coli* tRNA) did not eradicate complex formation (Fig1B, lane 12). Similarly, a bacterial RNA encompassing sequences from plasmid pSP64 could not compete (data not shown). Taken together, these experiments demonstrate that cytosolic factors interact in a specific manner with sequences near the 3' end of the histone H4 RNA.

We next analyzed the ability of the four histone RNA versions to serve as substrates for the trimming exonuclease *in vivo*. To this end, plasmids carrying the four constructs, each of them driven by the CMV promoter/enhancer, were transfected into COS1 cells. The plasmids code for transcripts comprising the complete histone H4 coding region. They received a 3' terminus 7 nucleotides past the palindrome by the cleavage activity of a cis-acting ribozyme (as described in 31). Nuclear and cytoplasmic RNA was isolated and subject to S1 mapping analysis. Consistent with previous results (31), the 7 nucleotide extension beyond the stem-loop of the cytoplasmic form of the wt-pal transcript was very efficiently resected, while the nuclear species of this RNA was still largely intact (Fig. 1C, lanes 1,2). Both the mut-pal and long-pal transcripts were unresected in both compartments and thus were no targets for the trimming exonuclease (lanes 3–6). In the case of the wt-pal-ClaI RNA, exonuclease action was clearly impaired as is evident by the substantial amount of untrimmed transcript in the cytoplasm; less than 50% of the cytoplasmic form underwent resection (compare in lane 7 the signal above the 110 bp marker band with the signal below this marker band). The behavior of the wt-pal-ClaI transcript suggests that the trimming exonuclease can resect histone transcripts that fail to interact with the above detected binding proteins, provided

a natural histone hairpin is present. However, our *in vivo* experiments also indicate that binding of the cytoplasmic factors clearly enhances the efficiency of the resection reaction (see Discussion).

Sequences upstream of the hairpin structure are essential for factor binding

To determine more precisely the sequence requirements for binding of the 2 factors, we made a series of progressive Bal31 deletions of a DNA template used in an earlier study for the generation of transcripts for *in vitro* 3' end processing reactions (32). This DNA template closely resembles the template used for generating the wt-pal 3' end transcript of Fig. 1. The deletions removed increasing parts of the sequence upstream of the wildtype stem-loop structure (see Fig. 2A). The constructs are designated according to the number of nucleotides derived from the histone H4 gene that are left 5' (upstream) of the palindrome. Each of the four DNA templates was cut with either one of two restriction enzymes: cleavage with BamHI resulted in the production of an RNA with a 68 nucleotide extension after the palindrome, whereas cutting with MboII allowed to generate a transcript exposing only 2 nucleotides beyond the stem-loop structure (Fig. 2A). Immediately after 3' end formation, the histone H4 transcript possesses a 5 nucleotide extension past the stem-loop (32). In addition, the +34 template was also cleaved with Sau96I; this enzyme cuts within the hairpin coding sequence. The run-off transcript produced from this template contains only the first four nucleotides of the palindromic sequence and lacks therefore this secondary structure (Fig. 2A, bottom).

No differences in the ability to form upper or lower complexes were seen between transcripts having either a 68 or a 2 nucleotide extension after the palindrome (Fig. 2B, compare lanes 1,3 and 5,6). From the RNAs transcribed from the BamHI or MboII cut templates only the +0 RNA could no longer interact with the cytoplasmic factors (Fig. 2B, lane 4), the other three transcripts were readily capable to undergo complex formation. Thus, the 34 nucleotides upstream of the hairpin structure contain information essential for factor binding, removal of this sequence prevents the formation of RNA-protein complexes. Significantly, the RNA lacking the intact stem-loop (+34 Sau96I) was able to form both complexes, albeit it was slightly impaired in forming the lower complex (compare lanes 8 and 9). In comparison to the +72 RNA, both complexes formed by the +34 Sau96I RNA exhibited a lower electrophoretic mobility. The fact that factor binding to this transcript could still be competed by the presence of an excess of unlabelled +72 competitor RNA (data not shown) indicated that the same proteins must interact with the two transcripts. We suspect that the lack of the hairpin structure causes the altered mobility of the +34 Sau96I RNA–protein complexes. Owing to its double stranded structure and the absence of G residues in the loop, the hairpin is protected against RNase T1 digestion. Hence, the stem-loop is still present in the shifted complexes containing RNAs derived from the BamHI or MboII cleaved templates. The greater negative net charge contributed by the phosphate groups of the stem-loop element most likely increases the mobility of the hairpin-containing RNAs in comparison to the hairpin-less RNA. Alternatively, the lack of the palindrome may induce an altered sterical conformation of the bound proteins, and this may cause the lower mobility.

We conclude that the two cytoplasmic factors mainly interact with sequences immediately upstream of the palindrome. The stem-loop structure does not appear to be required for binding

of the proteins and does not interact with soluble cytoplasmic proteins by itself. Consistent with this interpretation is the finding that the +0 RNA, retaining no sequences upstream of the stem-loop, does not give rise to retarded complexes. In retrospect, the results of this section also suggest an explanation as to why the wt-pal-ClaI transcript is defective for protein binding. The inserted ClaI site is located in the middle of the minimal binding site defined here and thus seems to disrupt the binding region.

Two multiprotein complexes recognize the wildtype histone H4 mRNA

We next performed UV crosslinking experiments to obtain information about the number and the size of the proteins binding to the 3' terminus of histone RNAs. Labelled wt-pal and mut-pal transcripts, covering the 3' end of the mouse H4 gene, were incubated with extracts (as described for fig. 1) and then irradiated with short wave UV light (crosslinking in solution). RNA-protein adducts were then resolved by SDS-gel electrophoresis. Alternatively, transcripts and bound proteins were crosslinked after separation of the specific complexes in a retardation gel (crosslinking in situ). In this case, the specific complexes were cut out as slices after UV-irradiation, soaked in SDS and finally also loaded on a protein gel.

Figure 3 shows an autoradiogram of such a denaturing protein gel displaying crosslinked proteins. With the crosslinking in solution approach, 9 proteins were labelled when the wt-pal transcript was used, but none when the mut-pal RNA was employed (compare lanes 3,4 with lane 2, Fig. 3). This result demonstrates that the used crosslinking procedure is specific and detects only proteins interacting with the natural 3' end of histone mRNA. The protein pattern was the same no matter whether the RNase T1 digestion was carried out before or after UV crosslinking (lanes 3 and 4). However, the crosslinked proteins lost most of their label by RNase A digestion conducted after UV irradiation (lane 5). This result indicates that crosslinking does not occur at uracil residues (the labelled nucleotide is UTP in these experiments), but rather at one of the other 3 bases. We also never observed retarded complexes in mobility shift experiments using RNase A instead of RNase T1. For the in situ crosslinking experiment, the upper and the lower complex could be analyzed in separate lanes. The lower complex contains 7 different protein species detectable by crosslinking, while the upper complex contains 5 proteins (lanes 6 and 7). Interestingly, the protein composition of the upper and lower complex seems to be quite different. Most of the proteins labelled by the in solution crosslinking approach can also be found in either the upper or lower complex of the in situ crosslinking approach. As the employed UV-crosslinking procedure is a label transfer reaction during which the bound proteins acquire a radioactive label by the formation of a UV-light induced covalent linkage between the protein and the labelled, RNase T1 resistant RNA fragment, the effective molecular weight of the crosslinked proteins is overestimated. The length of the RNase T1 resistant fragment is 58 nucleotides and therefore contribute about 19 kD to the molecular weight of the crosslinked proteins.

The binding activities are phylogenetically conserved

The trimming exonuclease acting on the 3' end of histone transcripts has been detected not only in mammalian cells (31) but also in *Xenopus* oocytes (30). These observations argue for an evolutionary conservation of the exonuclease. To examine whether the components binding to the 3' end of histone

Figure 3. Protein gel displaying the UV crosslinked proteins present in the two binding complexes. Bound proteins were crosslinked either by irradiating a normal binding reaction set up in a test tube (lanes marked with 'in solution') or by irradiating protein-RNA complexes resolved on a gel (lanes labelled with 'in situ'). In this experiment, the 3' end transcripts described in Fig. 1 were used. In lane 1 the wt-pal RNA alone without extract was treated with UV-light and digested with RNase T1. The blob of radioactivity at the bottom of this lane represents RNA digestion products. Lane 2: mut pal transcript irradiated in the presence of extract; this lane shows that no proteins can be crosslinked to the mut-pal RNA. Lanes 3-5: wt pal transcripts irradiated with UV-light in the presence of extract; in lane 3, RNase T1 digestion was performed before treatment with UV-light and in lane 4 after this step; in lane 5, the binding reaction was treated first with RNase T1 followed by digestion with RNase A and UV irradiation. Lane 6: proteins present in the lower gel shift complex. Lane 7: proteins present in the upper gel shift complex. The labelled protein species are marked by dots on the right hand side of the lanes.

transcripts are similarly conserved, we prepared extracts from *Xenopus* oocytes and cultured cells derived from five different species. Fig 4A shows that the extracts of all six species do indeed contain such a binding activity. A longer exposure of the autoradiograph revealed that also the *Xenopus* oocyte extract gives rise not only to an upper complex but also to a lower one. The mobility of the two complexes differed somewhat between the various species suggesting that they might possess complexes of slightly different total molecular weight. This experiment demonstrates that the proteins recognizing the histone RNA 3' end are similarly conserved as the trimming exonuclease.

So far, our protein binding assays have relied on a single mouse histone H4 RNA. To extend our analysis to another histone transcript, we studied protein binding to a human histone H2A transcript (34). An RNA encompassing 55 nucleotides upstream of the palindrome of this gene gave rise to two predominant, retarded complexes (and some minor complexes displaying a lower mobility) upon incubation with cytoplasmic extract derived from mouse cells (Fig. 4B, lane 8). These complexes were not seen in a control sample in which extract was omitted (lane 7). To determine the relationship between the factors binding to the mouse H4 RNA and those interacting with the human histone H2A RNA, we tried to compete factor binding to the H2A transcript by the addition of an excess of unlabelled H4 transcript prior to extract addition. Only the upper of the two specific complexes could be competed for, indicating that the two investigated histone RNAs may share some but not all of the factors binding to them (lane 9). This competition was specific

Figure 4. Phylogenetic conservation of the two protein complexes and binding to the 3' terminus of a human histone H2A transcript. **A.** Cytoplasmic extracts prepared from mouse (NIH 3T3), human (HeLa), monkey (COS1), rat (208F) and primary chicken embryo fibroblast cells and a *Xenopus* oocyte whole cell extract were tested for their ability to form complexes with the 3' end wt-pal RNA derived from the mouse histone H4 gene. **B.** At least two protein complexes interact with the 3' end of a human histone H2A transcript. Lane 7: H2A transcript digested with RNase T1 in the absence of extract; lane 8: same as in lane 7 but with added extract; lane 9: shift pattern in the presence of a 100-fold excess of unlabelled H4 wt-pal 3' end transcript over labelled H2A RNA and lane 10: 1000-fold excess of tRNA over labelled H2A transcript.

because a 1000-fold molar excess of tRNA over radiolabelled H2A RNAs did not diminish the two complexes (lane 10). Furthermore, the proteins interacting with the histone H2A transcript exhibited the same phylogenetic conservation as the proteins binding to the histone H4 RNA; they could be detected from frog to man (data not shown).

A fraction of the lower complex is associated with polysomes

Finally, we analyzed whether the detected binding activities are associated with polysomes. This question was of interest, because it had been proposed that a polysome bound nuclease may be involved in histone mRNA turnover (28). Cytoplasmic extracts were prepared from NIH 3T3 cells and loaded on a 10–30% sucrose gradient to separate polysomes from ribosomal subunits and free mRNPs. Under the running conditions employed, intact polysomes are pelleted. Two sucrose gradients were run in parallel. For the first one, all manipulations were performed in the presence of magnesium ions to keep polysomes as intact as possible and for the second gradient, polysomes were dissociated by the addition of an excess of EDTA to the cytoplasmic extract prior to loading on a gradient containing also EDTA. Chelation of magnesium ions is known to dissociate the ribosomes into the 40S and 60S subunits, leading to the release of the messenger RNAs from the polyribosomes (39). After completion of the run, different fractions of the gradient were tested by gel shift assays for the presence of the protein complexes. To determine the position of the ribosomes or ribosomal subunits on the gradients, a quarter of each fraction was analyzed on agarose gels in respect to ribosomal RNA content.

Figure 5. Sedimentation behavior of the two protein complexes on sucrose gradients. Polysome preparations from NIH 3T3 cells were loaded on 10–30% sucrose gradients containing either Mg^{++} (5A) or EDTA (5B). Before loading, the material for the EDTA gradient was adjusted to 5mM EDTA. After the run, the gradients were separated into 10 fractions and the pelleted material. 1/40th of the 10 fractions and 1/5th of the pellet was assayed by gel retardation analysis for the presence of the two binding activities. The wt-pal 3' end transcript served as radiolabelled probe. Fractions containing the intact ribosomes, denoted with 80S in the case of the Mg^{++} gradient, or the 40S and 60S ribosomal subunits in the case of the EDTA gradient, are indicated below the autoradiograms.

In both gradients, the top fractions displayed essentially all binding activity for the upper complex, indicating that it is not associated with large particles (Fig. 5). By contrast, the migration of the lower complex was clearly different on the two gradients. Some of the lower complex was found to be pelleted in the case of the Mg^{++} -gradient (Fig. 5A, lane 11), whereas on the EDTA-gradient the position of majority of the lower complex was shifted towards the middle of the gradient (Fig. 5B, lanes 5 and 6), the pellet of this gradient contained only trace amounts of binding activity. Since 1/8th of the resuspended pellet but only 1/40th of the individual gradient fractions was tested for the presence of the RNA binding factors, the signal intensities of the RNA-protein complexes of fractions 1–10 of the two gradients have to be multiplied by a factor of five for a direct comparison with the pelleted material. Agarose gel electrophoresis showed that for the Mg^{++} -gradient, most of the 18S and 28S ribosomal RNAs were present in equal amounts in the pellet, while for the EDTA gradient, the peak of the 18S rRNA was in fractions 7, 8 and that for 28S rRNA was in fraction 9 and 10. From this experiment we conclude that at least a fraction of the lower complex can be associated with polysomes. As revealed by the EDTA-gradient, the lower complex does not

seem to possess an intrinsic affinity for ribosomal subunits, as it sediments above these particles, nor seem the ribosomal subunits themselves to be able of interacting directly with the 3' untranslated region of the histone H4 RNA, since they sediment below the peak of the binding activity.

DISCUSSION

Knowledge about the nucleases cleaving messenger RNAs represents an important aspect in the understanding of mRNA decay (40). Starting from our previous observation of a sequence specific exonuclease removing nucleotides beyond the natural hairpin structure of histone transcripts (30, 31), we now tried to analyze the basis for this sequence specificity using a set of mutant histone RNAs. Gel retardation assays allowed to identify two evolutionary conserved protein complexes that mainly interact with sequences immediately upstream of the canonical stem-loop structure of a histone H4 transcript. The two cytoplasmic complexes appear to consist of several proteins and cannot bind to histone H4 mRNAs with altered 3' terminal sequences. Transient transfection experiments were employed to test whether a correlation exists between protein binding and exonuclease activity. The most informative transcript in this respect was the wt-pal-Clal RNA which exhibited no factor binding and showed a reduced level of trimming. A straightforward interpretation of this result is that factor binding is not essential for the trimming process. It seems, however, that binding of the detected proteins increases the efficiency with that the resection occurs because trimming was only maximally efficient in the case of the wt-pal RNA. In this sense, factor binding is required for reaching maximal levels of resection activity. Trimming is completely abolished, if, in addition to the Clal insertion, the sequence of the hairpin is changed. This finding defines the wildtype stem-loop as essential element for the resection reaction.

Although the histone RNA binding proteins are evolutionary conserved from frog to man, their recognition site on the RNA is clearly less well conserved. The sequence upstream of the used histone H4 stem-loop is characterized by two features. The 8 nucleotides preceding the hairpin are quite well conserved among vertebrate histone genes (41). A and C are the predominant bases found in this region. The second feature is a stretch of 14 consecutive C residues. This pyrimidine stretch is phylogenetically not conserved and is also not shared by the histone H2A RNA employed in this study. Perhaps this difference can account for the fact that only one of the two RNA-protein complexes formed over the H2A transcript could be competed by the H4 RNA. Based on this competition experiment, it appears that several distinct types of protein complexes, which however may share some of their subunits, interact with the 3' untranslated region of different histone transcripts. This situation is somewhat reminiscent of the diversity of subtype-specific transcription factors stimulating expression of the replication dependent histone genes during S phase (42, 43).

Given the only partial dependence of the trimming exonuclease on the binding activities, it is likely that the protein complexes participate in additional aspects of histone RNA metabolism. As our experiments did not directly address other possible functions, one has to speculate. An earlier report has shown that ribosomes continuing translation into the histone 3' untranslated region interfered with the down-regulation of this class of mRNAs following exposure of cells to DNA-synthesis inhibitors (44). In the light of our results, it is possible that the translating ribosomes

displaced the detected binding factors, and thereby may have prevented faithful histone RNA destabilisation. If this were true, the protein complexes would play an essential role in histone mRNA degradation. In this context it is also noteworthy that for instance the translation dependent resection of the poly(A) tail precedes the degradation of both c-myc (45) and c-fos (46) messengers. By analogy, the trimming reaction of the histone RNA 3' terminus might be the prelude to the actual degradation event. Finally, considering the role of the poly(A) binding protein in initiation of translation (47, 48) and in poly(A) tail shortening (47), it is tempting to speculate that one or both of the two factors binding to 3' terminal sequences of histone RNA could fulfill similar functions in the case of histone mRNAs. It should be possible to test this model in an in vitro translation system, taking advantage of the transcripts characterized here with regard to formation of RNA-protein complexes.

Very recently, Marzluff and coworkers have identified a polysome associated 50kD protein that binds directly to the histone hairpin structure (49). In agreement with our data showing that there is no soluble hairpin specific binding activity in cytoplasmic extracts (see e. g. wt-pal-Clal RNA in Fig. 1B or +0 RNA in Fig. 2B), the 50kD protein can only be detected after a high salt extraction of polysomes (49). Interestingly, this protein appears to interact preferentially with histone transcripts with few protruding nucleotides past the stem-loop. The 50kD protein shares this property with the trimming exonuclease which exhibits considerably more activity on histone RNA substrates with small nucleotide extensions beyond the stem-loop compared to RNA substrates with long extensions (30).

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