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Biochemical demonstration of complex formation of histone pre-mRNA with U7 small nuclear ribonucleoprotein and hairpin binding factors

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Histone RNA 3′ end formation occurs through a specific cleavage reaction that requires, among other things, base-pairing interactions between a conserved spacer element in the pre-mRNA and the minor U7 snRNA present as U7 snRNP. An oligonucleotide complementary to the first 16 nucleotides of U7 RNA can be used to characterize U7 snRNPs from nuclear extracts by native gel electrophoresis. Using similar native gel technniques, we present direct biochemical evidence for a stable association between histone pre-mRNA and U7 snRNPs. Other complexes formed in the nuclear extract are dependent on the 5′ cap structure and on the conserved hairpin element of histone pre-mRNA, respectively. However, in contrast to the U7-specific complex, their formation is not required for processing. Comparison of several authentic and mutant histone pre-mRNAs with different spacer sequences demonstrates that the formation and stability of the U7-specific complex closely follows the predicted stability of the potential RNA-RNA hybrid. However, this does not exclude a stabilization of the complex by U7 snRNP structural proteins.

Key words: hairpin binding factors/histone genes/native gel electrophoresis/RNA 3′ end processing/U7 snRNA/U7 snRNP

Introduction

Native gel electrophoresis techniques have become established as invaluable tools for the analysis of nucleic acid-protein interactions. They were first developed for the study of sequence-specific DNA binding proteins (Gardner and Revzin, 1981; Fried and Crothers, 1981) but were soon adapted to the study of RNA-protein interactions. In particular, the analysis of splicing complexes resolved by electrophoresis in native polyacrylamide gels has shown that the assembly of the spliceosome is based on sequential, multiple interactions of snRNP and non-snRNP factors with the pre-mRNA (reviewed in Konarska, 1989). Similar methods have been used to demonstrate binding of a cellular factor to an iron responsive element in ferritin (Leibold and Munro, 1988) and transferrin receptor (Müllner *et al.*, 1989) mRNAs. More recently, it was shown that the stability of a specific ternary complex formed between pre-mRNA and two polyadenylation factors reflects the processing efficiency of poly(A) sites (Weiss *et al.*, 1991).

The 3′ ends of animal histone mRNAs are formed by a specific RNA processing reaction distinct from polyadenylation (reviewed in Birnstiel *et al.*, 1985). The signal for this reaction resides in a hairpin loop structure immediately preceding the cleavage site and a purine-rich spacer element located a few nucleotides (nt) further downstream. By complementation of a processing-deficient sea urchin H3 gene in microinjected *Xenopus laevis* oocytes, the U7 small nuclear ribonucleoprotein (snRNP) was identified as a *trans*-acting factor involved in histone RNA 3′ processing (Strub *et al.*, 1984; Strub and Birnstiel, 1986). The U7 snRNP is one of the minor snRNPs (Birnstiel and Schaufele, 1988) and complementation is dependent upon base pairing between the 5′ end of U7 RNA and the spacer element of histone pre-mRNA (Schaufele *et al.*, 1986; Bond *et al.*, 1991). Additional *trans*-acting factors comprise a hairpin binding factor (HBF) and a heat labile factor (HLF). Binding of a factor to the hairpin loop element was demonstrated by RNase protection experiments (Mowry and Steitz, 1987a). Moreover, mutations of the hairpin as well as competition experiments suggested that HBF, although not absolutely required, greatly increases the efficiency of processing *in vitro* (Cotten *et al.*, 1988; Mowry *et al.*, 1989; Vasserot *et al.*, 1989). In contrast, HLF is absolutely indispensable (Gick *et al.*, 1987) but its precise function or possible interaction with any specific part of the pre-mRNA is unknown.

We have studied the interactions of specific histone pre-mRNA sequences with factors present in a nuclear processing extract by native gel electrophoresis. Here, we present the characterization of a stable complex between histone pre-mRNA and the U7 snRNP. We show that the ability of transcripts to be processed largely reflects the stability of this U7-specific complex. Separate complexes appear to be due to interactions of factors with the 7-methyl guanosine cap and hairpin element of the pre-mRNA, respectively, but their formation is neither absolutely required nor plays a significant role in controlling the efficiency of histone RNA 3′ processing.

Results

Characterization of U7 snRNPs by native gel electrophoresis

To characterize U7 snRNPs in their native state, we developed a complementary oligonucleotide decoration (COD) assay: an oligodeoxynucleotide complementary to the first 16 nt of U7 RNA (oligo cA) base-pairs with U7 RNA stably enough to inhibit histone RNA processing by competi-

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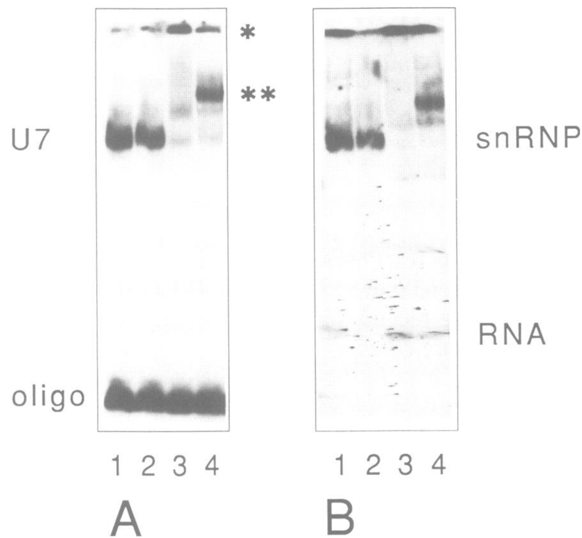

Fig. 1. Native gel analysis of U7 snRNPs. (**A**) Complementary oligonucleotide decoration (COD) assay. Nuclear extract from K21 cells was incubated in the presence of 5′ end-labelled oligo cA complementary to the first 16 nt of U7 snRNA (Soldati and Schümperli, 1988) and the samples were analysed by native gel electrophoresis. Oligo, position of free oligo cA; U7, decorated U7 snRNPs; * and **, antibody-induced shifts of U7 complex caused by anti-Sm and anti-3mG antibodies, respectively. (**B**) Blot hybridization analysis. Reaction mixtures identical to those in (A), except that oligo cA was omitted, were run on the same gel. The gel was blotted onto a filter and U7 RNA was detected by hybridization. In addition to U7 snRNPs (snRNP), the extract contains small amounts of free U7 snRNA (RNA). Lanes 1, no antibody; 2, non-immune control; 3, anti-SM antibody; 4, anti-3mG antibody.

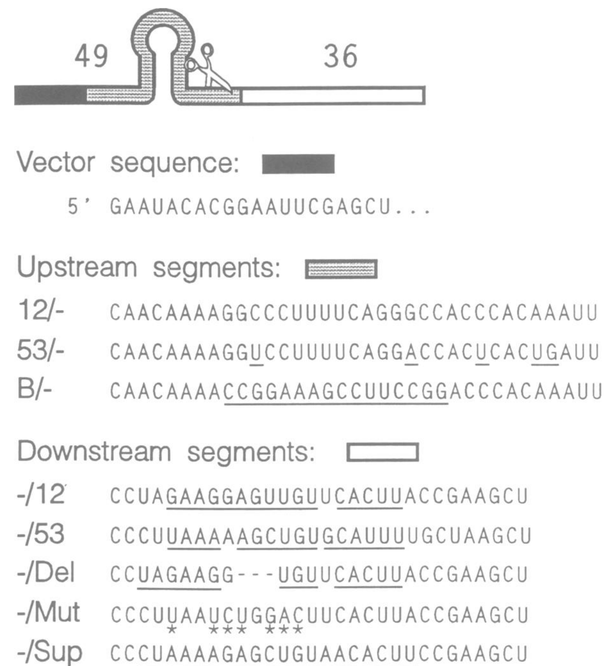

Fig. 2. Structure of pre-mRNAs used. Synthetic oligodeoxynucleotides corresponding to the upstream (stippled bar) and downstream (white bar) parts of the histone RNA processing signal were cloned into pSP65. Black bar, vector sequences at the 5′ end of resulting pre-mRNAs. Cleavage (scissors) results in 49 nt upstream and 36 nt downstream fragments. 12 and 53, sequences from the H4-12 and H4-53 genes, respectively (Meier *et al.*, 1989). Other sequences contain deliberately introduced mutations. For upstream segments, differences from H4-12 are underlined. For downstream segments, complementarities to mouse U7 RNA (Soldati and Schümperli, 1988) are underlined. Asterisks indicate seven point mutations in the Mut downstream segment which should prevent any base pairing with U7 snRNA.

tion (Soldati and Schümperli, 1988). Figure 1A shows that this interaction is also strong enough to survive non-denaturing gel electrophoresis conditions, such that radioactively labelled oligo cA can be used to reveal the position of U7 snRNP complexes on a composite agarose–polyacrylamide gel. After incubation in nuclear extract from K21 mouse mastocytoma cells, oligo cA yields a single retarded band in addition to the one produced by the free oligo (lane 1). A band of similar electrophoretic mobility is also observed after incubation of oligo cA in nuclear extracts from C127 mouse fibroblasts, human HeLa cells or calf thymus (D.Soldati and U.Albrecht, unpublished results). This band can be strongly competed by a 10- to 200-fold excess of unlabelled oligo cA or by a preparation of small nuclear RNAs, but neither by an unrelated oligo (in 10 000-fold excess) nor by tRNAs (data not shown).

Several control experiments demonstrate that the observed band corresponds to U7 snRNP complexes: if the extract is pretreated with micrococcal nuclease or by oligonucleotide-targeted digestion of U7 snRNA (Soldati and Schümperli, 1988), the complex can no longer be formed (data not shown). When the reaction mixture is incubated with anti-Sm antibodies (reacting with common proteins of U snRNPs), most of the complex becomes retarded or retained in the slot of the gel (Figure 1A, lane 3). Antibodies directed against the 2,2,7-trimethyl guanosine (3mG) cap structure of U snRNAs cause the complex to be shifted to a discrete position further up the gel (lane 4), whereas control antibodies have no such effect (lane 2). Furthermore, oligo cA was unable to form the complex when the nuclear extract had previously been depleted of snRNPs by immunoprecipitation with anti-Sm antibodies (data not shown).

Figure 1B shows another part of the same gel onto which reactions had been loaded that were identical except that they were carried out in the absence of oligo cA. This part of the gel was blotted onto a nylon filter and hybridized with a mixture of labelled oligos complementary to the first 49 nt of U7 RNA. As shown, U7 RNA is contained in a major complex that co-migrates with the one detected by the COD assay (lane 1). In addition, the extract contains very small amounts of U7 RNA of faster electrophoretic mobility which is not detected in the COD assay. Control experiments (not shown) indicated that this band is due to free U7 snRNA; a corresponding band is not visible in Figure 1A, but was sometimes observed also in COD assays (e.g. Figure 6). Most importantly, however, the major U7 snRNP complex detected by hybridization shows the same response to the different antibodies as the one detected by COD (lanes 2–4). Thus, the COD assay can be used to detect U7 snRNP complexes and to document their electrophoretic mobility on a non-denaturing gel.

Native gel analysis of histone pre-mRNAs

When we analysed capped, radioactively labelled histone pre-mRNAs in the same native gel system, the resulting pattern was much more complex. For this analysis, we used a short pre-mRNA from the H4-12 gene (Meier *et al.*, 1989) containing 49 nt preceding and 36 nt following the processing site, respectively (12/12; Figure 2). Qualitatively similar results were also obtained with transcripts from other

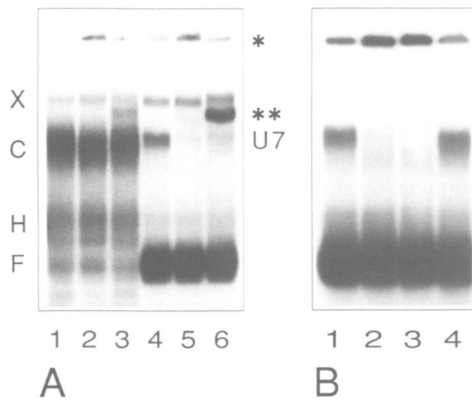

Fig. 3. Native gel analysis of histone pre-mRNA. Radiolabelled 12/12 RNA was incubated in nuclear extract and subjected to native gel electrophoresis. (**A**) Antibody reactivity of complexes formed in native (lanes 1–3) and heat inactivated (50°C) extract (lanes 4–6). Lanes 1 and 4, no antibody; 2 and 5, anti-Sm (Y12) antibody; 3 and 6, anti-3mG antibody. F, free 12/12 RNA; H, C and X, complexes formed in native extract (see text); U7, U7-specific complex; * and **, antibody induced shifts of U7 complex caused by anti-Sm and anti-3mG antibodies, respectively. (**B**) Oligonucleotide-targeted RNase H inactivation of U7 snRNPs. The 5′ end of U7 snRNA was destroyed by endogenous RNase H in the presence of 2 (lane 2) or 10 ng oligo cA (lane 3) per 10 μl extract. The extract was then heat-inactivated (50°C) prior to incubation with radiolabelled 12/12 RNA and native gel analysis. Lanes 1, no oligo; 4, 10 ng of unspecific oligo (complementary to the 5′ end of U1 RNA) per 10 μl extract.

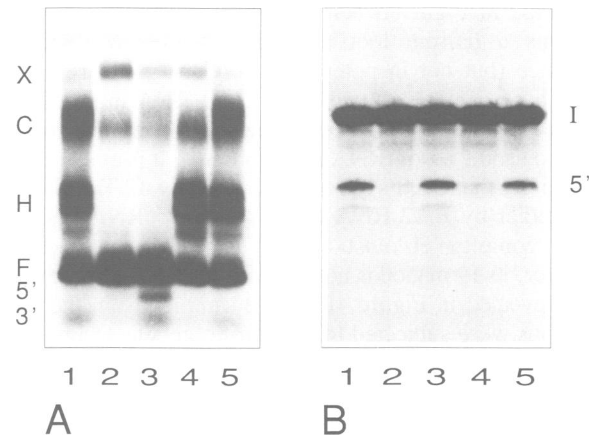

Fig. 4. Competition experiments. Radiolabelled 12/12 RNA was incubated in nuclear extract in the presence of various competitor RNAs and subjected to native (**A**) or denaturing gel electrophoresis (**B**). Complexes are labelled as in Figure 3. 5′, RNA 5′ cleavage product; 3′ RNA 3′ cleavage product; I, unprocessed input RNA. Lanes 1, no competitor RNA; 2, 12/12 competitor (100-fold excess); 3, 12/Del competitor; 4, B/12 competitor; 5, *E.coli* tRNA (5 μg) competitor.

histone genes (data not shown). Of eight histone genes analysed, H4-12 is the most efficiently processed *in vitro* (Streit, 1990). After native gel electrophoresis, the autoradiograph reveals a complex pattern which is caused by interactions of the pre-mRNA with different factors present in the nuclear extract (Figure 3A, lane 1). In addition to free 12/12 RNA (band F), three major groups of complexes are obained: complex H consists of two or three bands and is due to hairpin interactions; complex C, possibly consisting of two major bands, migrates roughly in the position of U7 snRNPs as defined by the COD assay (not shown), but is mainly due to interactions with cap-binding proteins; finally, there is a slowly migrating band, X, whose identity could not be unambiguously determined, but which is unrelated to the processing reaction (see below for data supporting these interpretations). In addition, we can positively state that a small fraction of the pre-mRNA must interact either directly or indirectly with a snRNP component, because after incubation with anti-Sm (lane 2) or anti-3mG antibodies (lane 3), some radioactive complex is shifted to similar positions as observed for U7 snRNPs in Figure 1. However, it is not possible to determine from where on the gel this complex originates, i.e. to detect its original position in the sample not treated with antibody.

A modification that greatly simplified the picture was to use heat inactivated, rather than native nuclear extract (lanes 4–6). It was previously shown that short incubation of nuclear extract at 50°C results in the inactivation of HLF and hence in a complete loss of processing activity, but that U7 snRNPs are resistant to temperatures up to 60°C (Gick *et al.*, 1987). Incidentally, heat treatment at 50°C also prevents the formation of some of the other complexes formed with 12/12 RNA. In fact, complexes H and C both disappear, whereas band F (free RNA) becomes more intense and band X is not significantly affected by the heat treatment. In place of band C, a fainter complex is now visible (lane 4) which can be completely shifted by incubation with anti-Sm (lane 5) or anti-3mG antibodies (lane 6). Figure 3B shows that this complex is U7-specific. Nuclear extract (5 μl) was pre-treated with either 1 or 5 ng of oligo cA (lanes 2 and 3) or with 5 ng of an unrelated oligo (complementary to the 5′ end of U1 RNA; lane 4) in the presence of 2.7 mM $MgCl_2$. This results in the targeted hydrolysis of the U7 RNA 5′ end by RNase H present in the extract (Soldati and Schümperli, 1988). Competitive (i.e. RNase H independent) inhibition requires amounts of oligo cA in excess of 10 ng (unpublished observations). The extracts were subsequently incubated at 50°C and used for native gel analysis to reveal the effects of RNase H digestion. As shown in Figure 3B, this oligonucleotide-targeted RNase H digestion of U7 RNA resulted in a complete loss of the appropriate complex. Band X, whose intensity varied between different extract preparations, was not observed in this particular experiment.

These experiments provide a direct biochemical demonstration of complex formation between histone pre-mRNA and U7 snRNPs. It should be noted that the intensity of this complex as revealed in the anti-3mG treated samples (Figure 3A, lanes 3 and 6) is stronger in heat treated than in native nuclear extract (see Discussion).

Complex H is due to hairpin interactions and its formation is not required for efficient RNA 3′ processing

The nature of interactions leading to the formation of band(s) H was revealed by competition experiments. Complex H was efficiently competed by a 100-fold excess of unlabelled 12/12 transcripts (Figure 4A, lane 2). In contrast, neither complex C nor X could be fully competed; in fact, band X became more intense in the presence of competing 12/12 RNA, albeit not always as much as in this particular experiment. Using two mutant RNAs as competitors (for structures see Figure 2) revealed that complex H is due to interactions of factors present in the extract with the hairpin loop element. One of these mutants, 12/Del, has a 3 nt deletion in the spacer element and is severely deficient in RNA 3′ processing as well as in the formation of the U7-specific complex (data

presented in Figure 6 below). The second mutant, B/12, contains a hairpin loop element completely altered in sequence (but not in potential secondary structure); B/12 RNA has a similar efficiency to 12/12 RNA in processing and in forming the U7 specific complex. As negative control, we also used 5 μg of tRNA as competitor. It is evident from Figure 4A that band(s) H are competed by 12/Del (lane 3) but neither by B/12 RNA (lane 4) nor by tRNA (lane 5). Thus, complex H must be due to hairpin interactions; however, its formation is not required for RNA 3′ processing as is revealed in Figure 4B where samples from the same reactions were subjected to denaturing gel electrophoresis. Processing is severely inhibited by 12/12 (lane 2) and B/12 (lane 4), i.e. the two competitor RNAs carrying an intact spacer element. However, competition of virtually all detectable hairpin binding activity by 12/Del RNA (lane 3) does not significantly reduce processing efficiency. It should also be noted that for the three samples with efficient processing, the cut-off spacer fragments migrate as free RNA on the non-denaturing gel (Figure 4A, band 3′) whereas the 5′ RNA fragment is released only in lane 3, where hairpin binding interactions have been competed. This suggests that hairpin binding factor(s) may primarily function in binding to the mature histone RNA (see Discussion).

Complex C is due to cap binding proteins

Although the experiment shown in Figure 3 proves that histone pre-mRNA interacts with the U7 snRNP, this complex could not be demonstrated directly in native extracts, probably due to its masking by large amounts of complex C. As shown in Figure 4, complex C was only poorly competed by an excess of unlabelled 12/12 RNA; because the main difference between the labelled and unlabelled 12/12 RNA was the presence of 5′ cap in the former, we suspected band C to be produced by cap binding proteins. However, band C could not be competed by an excess of ^{7m}GpppG either alone or in combination with unlabelled 12/12 RNA (data not shown). We therefore incubated labelled capped and uncapped 12/12 RNA in nuclear extracts which had been pre-incubated at various temperatures. The samples were again subjected to both native (Figure 5) and denaturing gel electrophoresis (data not shown). Histone RNA 3′ processing *in vitro* does not require the presence of a 5′ cap structure on the pre-mRNA, but uncapped RNAs may be unstable in some extract preparations. Thus, both the capped and uncapped 12/12 RNA were efficiently processed in native extract, but processing of both RNAs was slightly reduced by pre-treatment of the extract at 40°C and fully inactivated at and above 45°C (data not shown).

Upon native gel electrophoresis, uncapped 12/12 RNA does form complexes H and X, whereas complex C is missing (Figure 5B). In agreement with the above however, complex C is formed with the capped RNA (Figure 5A). Complexes C and H disappear between 45–50°C, whereas complex X is only eliminated at ~55°C. In addition, a new band (Y) is formed at 50 and 55°C which seems to be specific for the uncapped RNA and whose identity is, so far, unclear. Most importantly, however, the U7-specific complex can now be observed in Figure 5B in the absence of complex C. The electrophoretic mobility of this complex undergoes a characteristic change at higher temperatures which was also observed using the COD assay (data not shown). As already shown in Figure 3A, considerably more U7 specific complex is formed in processing deficient than in active extracts. This strongly suggests that the association of pre-mRNA with U7 snRNPs in active extracts is transient and that the complex dissociates when processing has occurred. In keeping with this idea, the intensity of U7 specific complex obtained in native versus heat treated extract was comparable for oligo cA, which binds to the U7 snRNP but does not get processed (data not shown).

Formation of the U7 specific complex with different natural and mutant pre-mRNAs

The above experiments suggest that binding assays performed with either capped or uncapped transcripts in extracts pre-treated at 50°C can be used to compare the binding of different pre-mRNAs to the U7 snRNP. How the interpretation of such experiments is affected by the observed mobility differences of U7 complexes (Figure 5) will be discussed below (Discussion). The different pre-mRNAs used for this type of analysis are shown in Figure 2. We have found that the processing efficiencies *in vitro* of two mouse H4 genes, H4-12 and H4-53, differ by ~5-fold (Streit, 1990). Using appropriate oligonucleotides, we constructed SP6 templates for the synthesis of short RNAs containing the hairpin and spacer elements of these genes in all four combinations. Using the resulting 12/12, 53/53, 12/53 and 53/12 RNAs, we could show that the difference in processing efficiency between the two genes is entirely due to the spacer element, i.e. the two constructs with the H4-12 spacer are efficiently processed, those with the H4-53 spacer are not (Streit, 1990). An obvious difference between the two spacer sequences is the presence in H4-53 of an internal A residue that is unable to pair with a C in a potential hybrid with U7 RNA (Figure 2). While screening these clones, we additionally isolated a template which is identical to the 12/12 construct, except for a 3 nt deletion in the spacer element (12/Del). Additional variations of the 12/12 construct were created using appropriate oligonucleotides: 12/Mut has 7 nt of the spacer substituted which should completely prevent base pairing with U7 RNA; 12/Sup contains a perfect complement to the first 20 nt of U7 RNA; B/12 contains a highly divergent hairpin. 12/Del and B/12

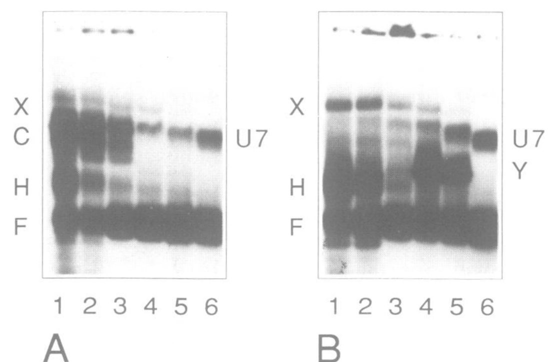

Fig. 5. Heat sensitivity of complexes. Extracts were incubated for 15 min at various temperatures prior to complex formation and native gel analysis with either capped (**A**) or uncapped (**B**) radiolabelled 12/12 RNA. Complexes are labelled as in Figure 3. Y, unidentified complex formed with uncapped RNA in extracts pre-incubated at 50 or 55°C. Lanes 1, no heat treatment; 2, 40°C; 3, 45°C; 4, 50°C; 5, 55°C; 6, 60°C.

were already introduced in the competition experiment of Figure 4. 12/Del and 12/Mut are both extremely deficient in processing, whereas B/12 and 12/Sup are processed with efficiencies similar to 12/12 RNA. The abilities of the different transcripts to be processed are listed in Figure 6, but will be fully documented in a forthcoming paper, along with further data on histone RNA processing (A.Streit, D.Soldati, T.Wittop Koning, L.Helin, D.Albrecht and D.Schümperli, manuscript in preparation).

When these templates were tested for the formation of a U7-specific complex in extracts pre-treated at 50°C (Figure 6), we found that the ability to form the complex closely followed the competence in processing. 53/53, 12/53, 12/Del and 12/Mut RNAs did not form any detectable complex, whereas complex formation was observed with 12/12, 53/12, 12/Sup and B/12 RNAs. It is also evident from Figure 6 that formation of the slowly migrating complex X reflects a property of the spacer part of the RNA substrate, yet its formation does not go along with the competence in processing. Complex X is formed by all RNAs that carry either the −/12, −/Del, or −/Mut spacer fragment, but not by RNAs carrying the −/53 or −/Sup spacer. In four separate experiments, we counted the radioactivity contained in the region of the U7 specific complex (without background subtraction). This analysis clearly indicates that 12/Sup RNA binds to the U7 particle more efficiently than any of the other RNAs, as would be expected for a complex formed by RNA−RNA base pairing (Figure 6).

Discussion

An 85 nt histone pre-mRNA interacts with several factors from the nuclear extract that are unrelated to RNA 3′ processing

Besides the expected interaction with U7 snRNPs, our native gel analysis has revealed at least three types of interactions of the 85 nt 12/12 RNA with factors from K21 nuclear processing extracts:

Complex X. Among all observed complexes, this is the most difficult to interpret. It is relatively heat stable (destroyed at 55°C) and it displays a certain sequence specificity which apparently resides in the downstream portion of the pre-mRNA, but its formation by different pre-mRNAs clearly does not follow their competence for processing. The surprising finding that it cannot be competed by unlabelled pre-mRNA (Figure 4A) seems to suggest that it may be dependent on total nucleic acid concentrations. Preliminary experiments indicate that it is not formed in MN treated extracts. However, incubation of various concentrations of 12/12 RNA in the absence of extract did not lead to formation of the complex (data not shown).

Complex C. Interactions of the capped 5′ ends of histone pre-mRNAs with factors from nuclear processing extracts have been demonstrated by Mowry and Steitz (1987a). In their experiments, the 5′ end was shown to become protected from RNase T1 attack and the protected fragment was immunoprecipitable by anti-Sm antibodies. Our experiments suggest that complex C (possibly multiple bands) is due to cap interactions. In our hands, however, complex C does not appear to interact directly with anti-Sm antibodies. It also follows from our experiments that cap interactions of the pre-mRNA *in vitro* are required neither for interaction with U7 snRNPs nor for RNA 3′ processing.

Complex H. Interactions of factors from nuclear extracts with the conserved histone hairpin sequence were first demonstrated using the same RNase T1 protection/anti-Sm immunoprecipitation approach which also revealed cap interactions (Mowry and Steitz, 1987a). Later it was shown that mutations in the hairpin, although not completely preventing histone RNA processing *in vitro*, caused a severe reduction in its efficiency (Cotten *et al.*, 1988; Mowry *et al.*, 1989; Vasserot *et al.*, 1989). Furthermore, competition experiments indicated that the hairpin interacts with a titratable factor that enhances processing efficiency (Vasserot *et al.*, 1989). Our results provide direct biochemical evidence for an interaction of hairpin sequences with components of a nuclear processing extract. Binding of the factor(s) is clearly sequence- and not just structure-specific,

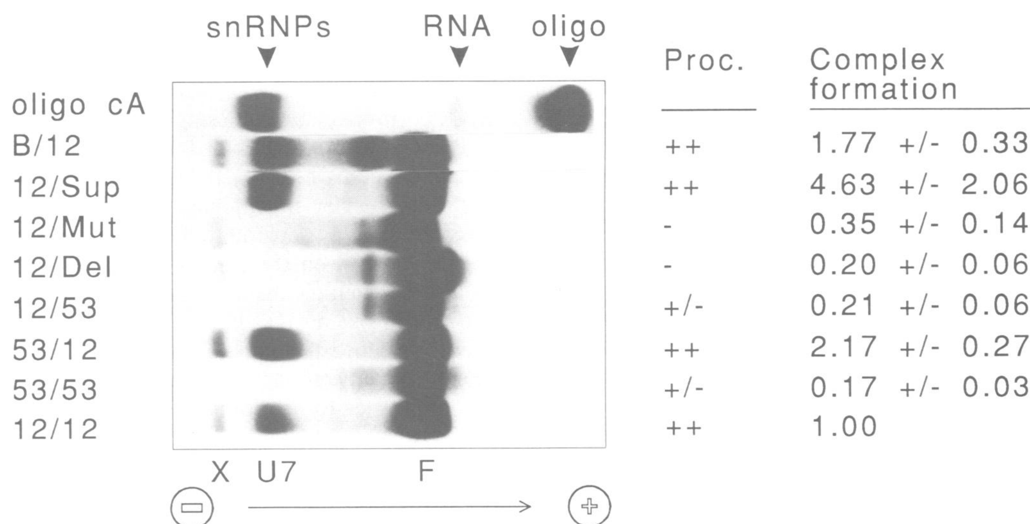

Fig. 6. Complex formation of different histone pre-mRNAs in heat-inactivated (50°C) extracts. Details of the constructions are given in Figure 2. The competence of the various pre-mRNAs in RNA processing is indicated. ++, ~20% of substrate processed in 2 h incubation; +/−, product bands barely visible on long autoradiographic exposures; −, no detectable processing. The amounts of U7-specific complex formed were determined in four separate experiments and are expressed relative to the value of 12/12 RNA (Cerenkov counting of bands without background subtraction; Mean ± standard deviation).

as indicated by the failure of B/12 RNA to compete. Using the methods presented in this paper, it will now be possible to study in more detail the specific requirements for this interaction. So far, we have no indication for a direct interaction of the factor(s) causing complex H with anti-Sm antibodies. Moreover, competition with a 100-fold excess of unlabelled competitor RNA, which virtually eliminates formation of complex H, does not significantly affect histone RNA processing. This very strongly suggests that formation of complex H neither is required for nor plays a major role in histone RNA 3′ processing. A formal but not very probable possibility is that our processing extract lacks the hairpin binding factor(s) described in the work of the other groups. Alternatively, the interaction of 12/12 RNA with the U7 snRNP by itself could be optimal for processing so that an ancillary function of hairpin binding factor(s) (important for other genes) might be dispensable. A presentation and discussion of detailed processing experiments designed to analyse the relative contributions of hairpin and spacer sequences will form the subject of a forthcoming paper (Streit *et al.*, in preparation).

On gels with good resolution, complex H is resolved into two or possibly three different bands. It is therefore possible that the hairpin is recognized by multiple factors. In agreement with this, hairpin binding factors of different molecular weight have been found in nuclear and cytoplasmic extracts (W.F.Marzluff, personal communication). It is evident from our experiments that the factors causing the formation of complex H can bind to both unprocessed and processed RNA (competition with the unprocessable 12/Del RNA releases the processed 5′ fragment of 12/12 RNA from the complex). However, the binding affinities for processed and unprocessed RNAs cannot be the same. It is therefore quite possible that hairpin binding factors *in vivo* bind preferentially to mature histone mRNA and play a role in more downstream functions of RNA metabolism such as nucleocytoplasmic transport and regulation of histone mRNA stability.

Interactions of spacer sequences with the U7 snRNP follow the predicted stability of potential RNA–RNA hybrids

So far, U7 is the only snRNP that has been identified on the basis of its function. Sea urchin U7 snRNPs were found to complement defective 3′ end formation of a sea urchin H3 gene in the heterologous *Xenopus* oocyte system (Galli *et al.*, 1983; Strub *et al.*, 1984). Using a mutational approach, it was possible to show that this complementation requires the presence of complementary sequences in the pre-mRNA spacer and at the 5′ end of U7 RNA (Schaufele *et al.*, 1986). A similar approach has recently yielded the same result in a mammalian *in vitro* system (Bond *et al.*, 1991). Further evidence for base pairing interactions was obtained in RNase H inactivation studies (Mowry and Steitz, 1987b; Cotten *et al.*, 1988; Soldati and Schümperli, 1988). Evidence for an interaction of the spacer element with a snRNP component was also obtained by RNase T1 protection/anti-Sm immunoprecipitation (Mowry and Steitz, 1987a). In this paper, we have shown by a direct biochemical approach that histone spacer sequences interact with the U7 snRNP. To compare this interaction for different natural and mutant histone pre-mRNAs, we have used heat-inactivated extracts where processing and subsequent dissociation of the complex should be precluded. An important point in this respect is the small mobility difference of U7 complexes between native extracts and extracts incubated at 50°C which becomes more pronounced after pre-treatment at higher temperatures (Figure 5). Although we do not yet know the molecular basis for this difference, the U7 particles do remain reactive with anti-Sm antibodies up to 60°C (data not shown). Moreover, particles from extracts pre-treated at 50°C can fully complement the processing activity of a snRNP-depleted extract (Gick *et al.*, 1987). Bearing in mind that the binding properties of U7 particles in extracts treated at 50°C may not be identical to those of native U7 snRNPs, we find that this binding closely follows the predicted base-pairing stabilities of potential RNA–RNA hybrids. However, we still consider it possible or even likely that U7 snRNP proteins also contribute to the binding.

The decoration of U7 snRNPs by a COD assay may have several useful applications. It may be used to analyse structural changes in the U7 snRNP particles such as the temperature-induced mobility change seen in Figure 5 or the cell cycle-dependent masking of the U7 RNA 5′ end reported by Hoffman and Birnstiel (1990). In combination with treatment of nuclear extracts with various agents, it can be used to study the stability and possibly the composition of the U7 snRNP particle. The assay may also be adapted to *in situ* localization of U7 snRNPs. Finally, the stability of the interaction could be exploited in the purification of U7 snRNPs.

Using a processable pre-mRNA as the substrate, only very small amounts of the U7 specific complex are formed in native, fully active processing extracts, in contrast to the large amounts formed in heat inactivated extracts (Figure 5B). This suggests that the complex forms only very transiently during the processing reaction; this is in agreement with earlier findings that histone RNA processing *in vitro* begins very rapidly without any detectable time lag (Gick *et al.*, 1986). In contrast, pre-mRNA splicing requires the assembly of a large multimeric spliceosome and a considerable time lag is observed before any spliced products can be detected. Thus, it appears that the assembly of a functional histone RNA processing complex is a rapid and relatively simple matter. Indeed, we have so far failed to detect stable interactions of histone pre-mRNA with any other snRNP components which may suggest that histone RNA 3′ processing is a ‘single snRNP’ reaction.

These apparent kinetic differences of complex formation in active versus heat inactivated extracts may prove to be useful in further mutational analyses. In fact, it is now possible to assay separately the effects of any mutations in the pre-mRNA on U7 binding and on RNA processing. It would be extremely interesting to obtain mutations that affect processing but not U7 binding. Such mutant pre-mRNAs should produce the same amount of U7 specific complex in native and heat-inactivated extracts and might help define the target sites for additional processing factors such as the so far elusive heat-labile factor.

Materials and methods

Preparation of templates and in vitro transcriptions

Double stranded DNA oligonucleotides corresponding to the hairpin loop or spacer elements of the H4 genes on clones 12 and 53 (Meier *et al.*, 1989) and defined mutants thereof, were synthesized with appropriate 5′ and 3′ overhanging ends and clones in various combinations between the *Sac*I and *Hin*dIII sites of pSP65 (see Figure 2). The relevant nucleotide sequences of the resulting plasmids were determined by the dideoxy method (Sanger

et al., 1977). All templates were linearized for *in vitro* transcription with *Hin*dIII, successively extracted with phenol, phenol–chloroform (1:1) and chloroform and precipitated with ethanol.

1 μg linear template was incubated for 1 h at 40°C in the presence of 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 13 mM DTT, 2 mM spermidine, 0.1 mg/ml bovine serum albumin, 2.5 mM $m^7G(5')ppp(5')G$ (Boehringer Mannheim), 0.1 mM rGTP, 0.5 mM each of rATP, rCTP and rUTP, 25 μCi [α-^{32}P]GTP (800 Ci/mmol; New England Nuclear), 40 U RNAsin (Promega) and 5 U SP6 RNA polymerase (Boehringer Mannheim). Uncapped transcripts were synthesized by the same protocol except for the omission of $m^7G(5')ppp(5')G$.

The RNA was purified by electrophoresis on 42% urea, 5% polyacrylamide gels, excised and eluted in 300 μl of 0.3 M NaCl, 0.1% SDS, 0.1 M EDTA, 10 mM Tris–HCl, pH 7.5 for 2 h. The buffer was extracted twice with phenol and once with chloroform and the RNA was precipitated with ethanol.

Native gel analysis and in vitro processing

Preincubation mixtures were prepared containing 5 μl nuclear extract from K21 mouse mastocytoma cells (Stauber *et al.*, 1990), 3 μg yeast RNA, 20 mM EDTA and cold competitor RNAs, formamide and/or antibodies as specified, in a total volume of 10–15 μl and were incubated for 10 min at room temperature. 60 fmol of radiolabelled transcript were added and the mixtures further incubated for 30 min at 30°C. Heparin was added to a final concentration of 5 mg/ml and incubation continued for 10 min at 4°C. This material was directly analysed on a composite agarose–polyacrylamide gel (see below). In some cases, one-third of the reactions was mixed with 10 μl of 80% formamide loading buffer and analysed on a denaturing 6% polyacrylamide–50% urea gel (Sambrook *et al.*, 1989). Rabbit polyclonal anti-3mG cap antibodies were obtained from R.Lührmann (University of Marburg) and cells producing monoclonal anti-Sm antibodies (Y12) were originally obtained from I.W.Mattaj (EMBL, Heidelberg).

For composite agarose–polyacrylamide gels. 0.66% (all final concentrations) low melting point agarose (BRL or SIGMA) in 0.3 × Tris–borate–EDTA buffer (Sambrook *et al.*, 1989) was boiled, 10% glycerol was added and the solution cooled down to 41°C; then, 3% acrylamide–bisacrylamide (79:1) was added. Polymerization was induced by adding 1 ml 10% ammonium persulphate and 40 μl TEMED per 100 ml. The gel was cast between sand-blasted glass plates (15 × 15 cm) equipped with 1.5 mm spacers. Electrophoresis was at 12 mA and 4°C for 4–6 h (until bromophenol blue, loaded in a separate lane, had migrated 6–8 cm).

Heat inactivation and oligonucleotide-targeted RNase H treatment of nuclear extract were performed as described (Stauber *et al.*, 1990).

COD assay and blot hybridization of native gels

Preincubation mixtures were modified to contain 5 μl nuclear extract, short unspecific oligodeoxynucleotides (in 100- to 500-fold molar excess over oligo cA), 10 mM EDTA, and antibodies as specified. After incubation for 10 min at room temperature, 15 fmol of 5′ end-labelled (Sambrook *et al.*, 1989) oligo cA (Soldati and Schümperli, 1988) were added and incubation continued for 30 min at 30°C. Heparin was added and native gel analysis was performed as described above.

For hybridization analysis, the gel was transferred to a Biodyne A membrane (Pall; 0.2 μm pore size), using a semi-dry electroblotting system and a protein transfer buffer (48 mM Tris, 39 mM glycine, 0.375% SDS, 20% methanol) for 2 h at room temperature. The membrane was UV irradiated for 10 min, baked and then hybridized with a mixture of three 5′ end-labelled oligodeoxynucleotides complementary to nt 1–16, 18–33 and 34–49 of U7 RNA, respectively (Soldati and Schümperli, 1988).

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References

Birnstiel,M.L., Busslinger,M. and Strub,K. (1985) *Cell*, **41**, 349–359.
Birnstiel,M.L. and Schaufele,F.J. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer, Berlin, pp. 155–182.
Bond,U.M., Yario,T.A. and Steitz,J.A. (1991) *Genes Dev.*, **5**, 1709–1722.
Cotten,M., Gick,O., Vasserot,A., Schaffner,G. and Birnstiel,M.L. (1988) *EMBO J.*, **7**, 801–808.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Galli,G., Hofstetter,H., Stunnenberg,H.G. and Birnstiel,M.L. (1983) *Cell*, **34**, 823–828.
Gardner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047–3060.
Gick,O., Krämer,A., Keller,W. and Birnstiel,M.L. (1986) *EMBO J.*, **5**, 1319–1326.
Gick,O., Krämer,A., Vasserot,A. and Birnstiel,M.L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8937–8940.
Hoffmann,I. and Birnstiel,M.L. (1990) *Nature*, **346**, 665–668.
Konarska,M.M. (1989) *Methods Enzymol.*, **180**, 442–453.
Leibold,E.A. and Munro,H.N. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2171–2175.
Meier,V.S., Böhni,R. and Schümperli,D. (1989) *Nucleic Acids Res.*, **17**, 795.
Mowry,K.L. and Steitz,J.A. (1987a) *Mol. Cell. Biol.*, **7**, 1663–1672.
Mowry,K.L. and Steitz,J.A. (1987b) *Science*, **238**, 1682–1687.
Mowry,K.L., Oh,R. and Steitz,J.A. (1989) *Mol. Cell. Biol.*, **9**, 3105–3108.
Müllner,E.W., Neupert,B. and Kühn,L.C. (1989) *Cell*, **58**, 373–382.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schaufele,F., Gilmartin,G.M., Bannwarth,W. and Birnstiel,M.L. (1986) *Nature*, **323**, 777–781.
Soldati,D. and Schümperli,D. (1988) *Mol. Cell. Biol.*, **8**, 1518–1524.
Stauber,C., Soldati,D., Lüscher,B. and Schümperli,D. (1990) *Methods Enzymol.*, **181**, 74–89.
Streit,A. (1990) University of Bern, diploma thesis.
Strub,K. and Birnstiel,M.L. (1986) *EMBO J.*, **5**, 1675–1682.
Strub,K., Galli,G., Busslinger,M. and Birnstiel,M.L. (1984) *EMBO J.*, **3**, 2801–2807.
Vasserot,A.P., Schaufele,F.J. and Birnstiel,M.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4345–4349.
Weiss,E.A., Gilmartin,G.M. and Nevins,J.R. (1991) *EMBO J.*, **10**, 215–219.

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