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A synthetic histone pre-mRNA–U7 small nuclear RNA chimera undergoing *cis* cleavage in the cytoplasm of *Xenopus* oocytes

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ABSTRACT

The 3′ processing of histone pre-mRNAs is a nuclear event in which the U7 small nuclear ribonucleoprotein (snRNP) participates as an essential *trans*-acting factor. We have constructed a chimeric histone–U7 RNA that when injected into the cytoplasm of *Xenopus laevis* oocytes assembles into a snRNP-like particle and becomes cleaved at the correct site(s). RNP assembly is a prerequisite for cleavage, but, since neither the RNA nor the RNP appreciably enter the nucleus, cleavage occurs mostly, if not exclusively, in the cytoplasm. Consistent with this, cleavage also occurs in enucleated oocytes or in oocytes which have been depleted of U7 snRNPs. Thus all necessary components for cleavage must be present in the oocyte cytoplasm. The novel cleavage occurs *in cis*, involving only a single molecule of chimeric RNA with its associated proteins. This reaction is equally dependent upon base pairing interactions between histone spacer sequences and the 5′-end of the U7 moiety as the natural *in trans* reaction. These results imply that U7 is the only snRNP required for histone RNA processing. Moreover, the chimeric RNA is expected to be useful for further studies of the cleavage and assembly mechanisms of U7 snRNP.

INTRODUCTION

The endonucleolytic removal of spacer sequences from the 3′-ends of animal histone pre-mRNAs of the prevalent replication-dependent type is unique in several respects (reviewed in 1). Since these histone pre-mRNAs are devoid of introns, this is the only RNA processing step they undergo. This reaction is also exclusively used to cleave histone RNAs. Finally, histone genes are the only family of protein coding genes known to have evolved such a specialized processing pathway. The reaction is subject to regulation during the cell cycle or in response to variations in cell proliferation, thus contributing to the regulation of histone mRNA abundance under these conditions (reviewed in 2,3).

The cleavage reaction naturally occurs in the nucleoplasm and requires U7, one of the minor snRNPs (reviewed in 1). The 5′-end of U7 snRNA is complementary to a conserved spacer element located in the histone pre-mRNA, a few nucleotides downstream of the cleavage site. Base pairing between these complementary sequences is essential and serves to tether the U7 snRNP to the processing site (4–7). A hairpin binding factor (HBF), specific for a conserved stem–loop element located just upstream of the cleavage site in the histone pre-mRNA (8), and a heat-labile factor (HLF; 9) have been identified as additional *trans*-acting components. Whereas HLF is essential, HBF only enhances the processing reaction *in vitro* (8,10), most likely by stabilizing the interaction between histone pre-mRNA and the U7 snRNP (11,12). It cannot be excluded, however, that additional proteins or snRNPs participate in the reaction.

Analysis of the cleavage mechanism has been complicated by the low abundance of the *trans*-acting factors and by the fact that the contribution of one of them, HBF, is highly variable depending upon the particular histone pre-mRNA and type of nuclear extract used (11). Therefore, to study the mechanism more specifically it would be desirable to simplify the reaction and, in particular, to minimize the kinetic contributions of the assembly and disassembly of the processing complex. We have approached this goal by combining histone pre-mRNA and U7 RNA sequences into a single continuous molecule and by identifying conditions under which this RNA undergoes a single round of cleavage *in cis*.

That such conversions from a *trans* to a *cis* situation are possible was first suggested by the fact that the reverse, i.e. a conversion from *cis* to *trans*, could be achieved for other RNA processing reactions. Most catalytic RNAs, such as group I or group II introns and hammerhead and hairpin ribozymes (reviewed in 13), are *cis*-acting entities, i.e. 'enzyme' and 'substrate' are both contained in a single molecule. In several cases, however, such RNAs have been separated into two parts to create true *trans*-acting catalysts, termed 'ribozymes' (14–16). Moreover, the spliceosomal snRNPs U1, U2, U4/U6 and U5 are

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thought to have evolved naturally from *cis*-acting sequences originally present in group II self-splicing introns.

Inversely, it has been shown that nematode or trypanosome spliced leader RNAs, which are normally spliced *in trans* onto the bodies of various mRNAs, when joined to an adenovirus intron and 3′ exon will function in *cis* splicing in a mammalian extract (17). This novel splicing reaction is independent of U1 snRNPs, suggesting that the spliced leader sequences serve both as the 5′ exon and as the functional equivalent of U1 snRNA. A similar *trans* to *cis* conversion has recently been achieved for a protein-independent *in vitro* cleavage reaction, i.e. by covalently joining the catalytic RNA moiety of RNAse P to its substrate, pre-tRNA (18).

The chimeric histone pre-mRNA–U7 RNA designed in this paper is efficiently cleaved at the expected processing site when injected into the cytoplasm of *Xenopus* oocytes. This cleavage depends on correct assembly of the RNA with snRNP structural proteins and occurs predominantly, if not exclusively, *in cis* within the cytoplasmic compartment. These results imply that all the proteins required for cleavage are stored in the oocyte cytoplasm and that U7 is the only snRNP required for histone RNA processing.

MATERIALS AND METHODS

Construction of plasmids

The template for *in vitro* transcription of mouse U7 RNA (OT7U7) and mutants thereof have been described (19). Before *in vitro* transcription with T7 RNA polymerase these constructs were linearized with *Pst*I and the resulting ends made blunt with T4 DNA polymerase (Boehringer). This resulted in two additional G reidues at the 5′-end and an additional C residue at the 3′-end of transcribed RNAs, as compared with natural mouse U7 RNA. The template for short histone pre-mRNA from the 3′-end of the mouse histone *H4-12* gene (12/12 RNA; 6,11) was linearized with *Hin*dIII and transcribed with SP6 RNA polymerase. The template used for synthesis of chimeric 12/12–U7 RNA was constructed as follows. A 143 nucleotide (nt) *Hae*III fragment from OT7U7 containing the mouse U7 sequence preceded by 28 bp of OT7 vector and followed by part of the OT7 polylinker was cloned downstream of the histone pre-mRNA sequences into the filled-in *Hin*dIII site of construct 12/12. The plasmid was linearized for transcription by SP6 RNA polymerase as described for OT7U7 above. Combinations of various mutants of the two starting plasmids were constructed by the same strategy. The sequence of each construct was confirmed by dideoxy sequencing (20).

In vitro transcription

All *in vitro* transcripts used for microinjections were synthesized with a monomethyl cap structure as follows. Linearized template (1 μg) was incubated in 10 μl 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 13 mM DTT, 2 mM spermidine, 0.1 mg/ml bovine serum albumin, 0.5 mM ^{m7}G(5′)ppp(5′)G (Boehringer), 0.05 mM GTP, 0.5 mM each of ATP, UTP, CTP, 25 μCi [α-^{32}P]GTP (800 Ci/mmol; New England Nuclear), 40 U RNAsin (Promega) and 5 U SP6 (Boehringer) or T7 RNA polymerase (Boehringer or a gift of B. Müller) for 1 h at 37°C. All labelled transcripts were purified by electrophoresis through a denaturing gel, followed by phenol extraction and ethanol precipitation.

In vitro processing

The preparation of mouse K21 nuclear extracts (a gift of R.Mital; 21,22) and conditions for *in vitro* processing of radioactively labelled histone pre-mRNAs (11) have been described.

Oocyte microinjection

Xenopus oocyte injections were as described (19,23), with 10–20 oocytes injected per sample. In some experiments endogenous *Xenopus laevis* U7 RNA was inactivated by injecting 5 ng oligonucleotide cA in 20 nl 88 mM NaCl, 10 mM Tris–HCl, pH 7.5, into each oocyte nucleus. Oligonucleotide cA is complementary to nt 1–16 of *Xenopus* and mouse U7 RNA (24,25). Injected oocytes were incubated for 5 h at 18°C before *in vitro* transcribed, radiolabelled RNAs (~1.5 fmol) were injected into the cytoplasm in 20 nl water. Enucleated oocytes were prepared by squeezing the nucleus through a hole punched into the cell membrane. Such oocytes were incubated at 18°C until healed and injected as above.

RNA analysis from injected oocytes

Total oocyte RNA was prepared as described (23). Oocyte nuclear RNA was extracted with phenol/chloroform from manually dissected nuclei. Processing was analysed by denaturing gel electrophoresis on 8% polyacrylamide–8.3 M urea gels. Endogenous *Xenopus* U7 RNA was analysed by primer extension with the primer oligonucleotide cBX (TGCTAGACAAAATAG-TA), complementary to nt 18–33 of *Xenopus* U7 RNA (23,24) (underlined nucleotides differ from the mouse U7 sequence). Input 12/12–U7 RNA and its 3′ product formed in oocytes were gel purified and their 5′-ends were mapped by primer extension with oligonucleotide cB (TGCTAGACAAATTCTA), complementary to nt 18–33 of mouse U7 RNA (25). For comparison the 12/12–U7 template plasmid was sequenced using the same primer.

Immunoprecipitation of snRNPs assembled in oocytes

Oocytes were injected with U7 Sm WT RNA and 12/12–U7 RNA and incubated overnight. Total extracts were made by homogenizing the oocytes in 130 mM NaCl, 34 mM Na phosphate, pH 7.4 (20 μl/oocyte). The homogenate was centrifuged for 4 min at 4°C and the clear supernatant used immediately for immunoprecipitation. Preparations of 25 μg purified monoclonal anti-Sm antibody Y-12 (26) or monoclonal antibody D5 (negative control, directed against strand exchange protein 1 of *Saccharomyces cerevesiae*; a gift of W.-D.Heyer) were coupled to 50 μl protein A–Sepharose (Pharmacia) and the beads washed with IPP150 (150 mM NaCl, 10 mM Tris–HCl, pH 8, 0.05% NP-40). Aliquots of 200 μl total oocyte extract were added and after 2 h incubation the beads were washed three times with IPP150. The RNA in the pellet and supernatant fractions was recovered by phenol/chloroform extraction and ethanol precipitation and analysed on a 6% denaturing polyacrylamide gel.

RESULTS

Structure and nomenclature of RNA substrates

We designed a template for the production of chimeric RNA in which a short sequence from a mouse histone pre-mRNA was placed 5′ of the mouse U7 RNA sequence. Practically this was

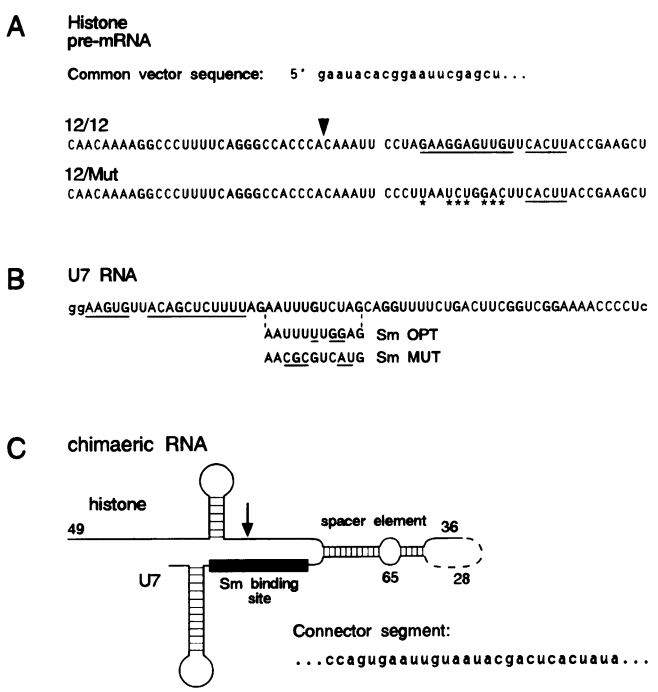

Figure 1. Structures of RNAs. (**A**) Histone pre-mRNAs of 85 nt (6,11). The prototype 12/12 RNA contains 20 nt of vector sequence (lower case) followed by the hairpin–loop and spacer elements of the mouse histone *H4-12* gene (upper case, complementarities to the 5′-end of U7 RNA underlined). The arrowhead indicates the major processing site used *in vitro*. Seven base changes in the spacer element of 12/Mut RNA are indicated by asterisks. (**B**) Mouse U7 RNAs of 65 nt (19). Complementarities to the spacer element of histone pre-mRNA are underlined. For technical reasons the synthetic RNA is longer than naturally occurring mouse U7 RNA (25) by two G residues at the 5′-end and one C residue at the 3′-end respectively (lower case). Shown below are the sequences of two mutants in the Sm binding site (23; altered nucleotides are underlined). (**C**) Schematic drawing of 178 nt chimeric 12/12–U7 RNA. The sequence exactly corresponds to the two RNA sequences shown in (A) and (B) (solid lines) joined in 5′→3′ orientation via the 28 nt connector segment (interrupted line, sequence shown below). Numbers indicate the lengths of relevant segments in nucleotides.

achieved by recombining fragments of transcription templates used previously for the separate synthesis of each individual RNA.

The original histone pre-mRNA template generates a transcript consisting of 20 nt of vector sequence followed by 65 nt of histone pre-mRNA sequence (11; Fig. 1A). Two portions of this RNA, the hairpin (binding to the auxiliary processing factor HBF) and the spacer element (involved in base pairing with the 5′-end of U7 RNA), are relevant for histone RNA 3′ processing *in vitro* and *in vivo*. The prototype RNA has therefore been called 12/12, to indicate that both of these elements are derived from the *H4* gene on clone 12 (27). In the present paper we also make use of the derivative 12/Mut (where 7 nt of the spacer element have been altered to prevent formation of the longer of two adjacent intermolecular stems with U7 RNA; 11).

U7 RNA transcribed *in vitro* from its respective original template consists of the complete mouse U7 RNA sequence of 62 nt preceded by two G residues and followed by one C residue (Fig. 1B). This RNA (called U7 Sm WT) assembles with snRNP structural proteins and is imported into the nucleus to form a particle which is functional in histone RNA 3′ processing when injected into *Xenopus* oocytes (19). Two mutations of the Sm binding site AAUUUGUCUAG are relevant for the present paper, i.e. U7 Sm OPT (AAUUUUUGGAG, more efficient in assembly and nuclear import, but non-functional for histone RNA processing) and U7 Sm MUT (AACGCGUCAUG, lacking assembly and nuclear import; 19,23).

The prototype chimeric RNA is called 12/12–U7 (the extension 'Sm WT' has been omitted for simplicity; Fig. 1C). The sequence exactly corresponds to the two sequences of 12/12 and U7 Sm WT RNAs separated by a 28 nt linker derived from the parental U7 plasmid. Chimeric RNAs containing mutant histone or U7 segments are named analogously (e.g. B/12–U7 or 12/12–U7 Sm MUT).

Chimeric 12/12–U7 RNA is processed predominantly in the cytoplasm of *Xenopus* oocytes

We injected capped 12/12–U7 RNA into the cytoplasm of *X.laevis* oocytes, where we expected it to become assembled into a snRNP particle due to specific RNA–protein interactions at the Sm binding site. Since the same RNA contains the processing substrate, cleavage products could be expected either due to assembly of a complete processing complex on the single RNP particle or, following nuclear import, after its association with resident *Xenopus* U7 snRNPs. To detect such cleavage products total RNA was extracted at various time points after injection and analysed on a denaturing polyacrylamide gel (Fig. 2A). Progressively with time, 12/12–U7 RNA is converted into two major products of ~115 and 50 nt respectively. Whereas the longer is heterogeneous in length, the shorter one consists of two and sometimes only one (Figs 2B, 5A and 6B) discrete band(s). The sizes of these products are compatible with correct cleavage at the histone processing site and, if this interpretation is correct, the upper and lower bands should represent the 3′ and 5′ cleavage products respectively. This notion is supported by the following experiments (data not shown): (i) when 3′-end-labelled 12/12–U7 RNA is used as substrate only the uncleaved RNA and the longer product of ~115 nt are detected; (ii) after incubation in the oocytes a small fraction of the uncleaved RNA and of the shorter 50 nt product, but none of the longer product, becomes immunoprecipitable with an antibody directed against the trimethylguanosine cap structure of snRNAs. Considering the ratio of G residues in these cleavage products (10:26), the 5′ product is less intense than expected, suggesting that the stabilities of the two product RNAs are different. Additional minor products of ~150 nt do not increase over time and are obtained in variable intensities in different experiments. Therefore, they most likely represent non-specific degradation products.

The early appearance of specific cleavage products after cytoplasmic injection of 12/12–U7 RNA is surprising. While the shorter 5′ product only becomes visible 1.5 h after injection on this particular autoradiogram (Fig. 2A), the longer 3′ fragment, due to its higher content of labelled nucleotides, can already be detected after 30 min. We have previously analysed assembly of the single *in vitro* transcribed U7 Sm WT RNA (Fig. 1B) after cytoplasmic injection into *Xenopus* oocytes (19). In these studies the RNA did not reach the nucleus until 3 h after injection, but cytoplasmic assembly with snRNP proteins could be detected by UV cross-linking after 15 min. Thus it seemed likely that the potential histone-specific cleavage of 12/12–U7 RNA occurs, at least partly, in the cytoplasmic compartment. We therefore determined the

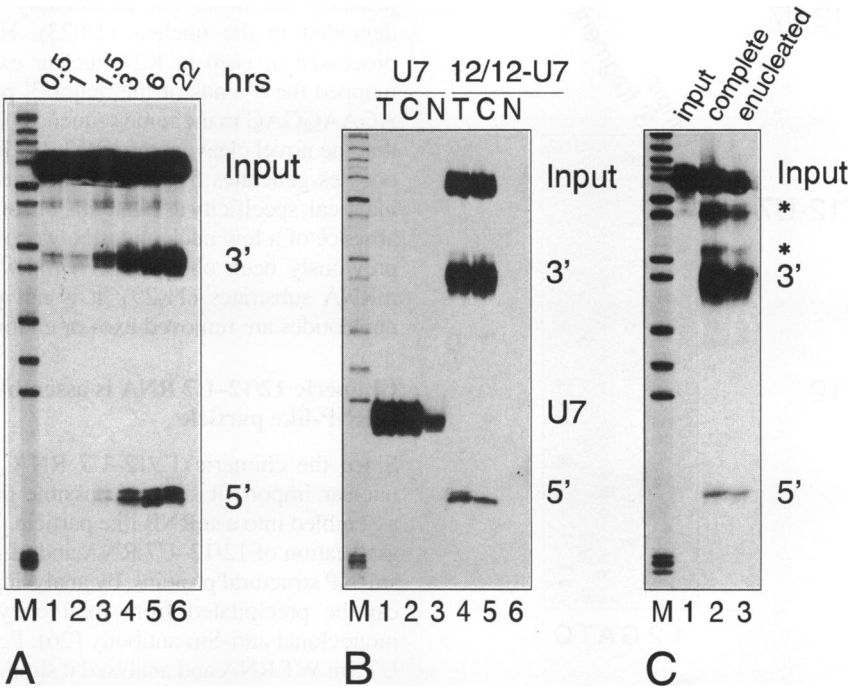

Figure 2. Chimeric 12/12–U7 RNA is predominantly cleaved in the cytoplasm of *Xenopus* oocytes. (**A**) *In vitro* transcribed, capped 12/12–U7 RNA was injected into the cytoplasm of *X.laevis* oocytes. At the times indicated RNA was isolated and analysed by denaturing polyacrylamide gel electrophoresis (3 oocyte equivalents/sample). The positions of uncleaved 12/12–U7 RNA of 178 nt (input), a 3′ fragment(s) of ~115 nt and a 5′ fragment(s) of ~50 nt are indicated on the right. A faint, presumably non-specific, product of ~150 nt is also visible. Lane M, size marker, *Hpa*II digest of pBR322 (bands between 217 and 34 nt are visible). (**B**) Nucleocytoplasmic distribution of U7 Sm WT RNA of 65 nt (lanes 1–3; Fig. 1B) and of chimeric 12/12–U7 RNA of 178 nt (lanes 4–6) after injection into the cytoplasm of *Xenopus* oocytes and overnight incubation (4 oocyte equivalents/sample). Lane T, total RNA; lane C, cytoplasmic RNA; lane N, nuclear RNA. (**C**) Cleavage of 12/12–U7 RNA in enucleated oocytes. *In vitro* transcribed, capped 12/12–U7 RNA (lane 1) was injected into the cytoplasm of complete (lane 2) or manually enucleated (lane 3) *X.laevis* oocytes. After overnight incubation RNA was isolated and analysed by denaturing polyacrylamide gel electrophoresis (4 oocyte equivalents/sample). Asterisks indicate presumably non-specific products of ~150 and 130 nt.

nucleocytoplasmic distribution of 12/12–U7 RNA (Fig. 2B). After cytoplasmic injection and overnight incubation virtually all RNA, both cleaved and uncleaved, is present in the cytoplasmic fraction (lane 5). Even after different times of incubation (corresponding to those used in Fig. 2A), 12/12–U7 RNA does not accumulate in the oocyte nucleus (data not shown). In comparison, ~10–20% of injected single U7 Sm WT RNA accumulates in the nucleus after overnight incubation (lanes 1–3). We therefore conclude that nuclear import of 12/12–U7 RNA, either as naked RNA or as RNP, is very inefficient and that the observed cleavage must occur predominantly or exclusively in the cytoplasmic compartment. This was directly tested by injecting 12/12–U7 RNA into enucleated oocytes (Fig. 2C, lane 3). Although nuclear import is no longer possible, the specific cleavage products are still formed, thus proving that cleavage of chimeric 12/12–U7 RNA can occur in the oocyte cytoplasm. Moreover, the efficiency in enucleated oocytes is very similar to that of native oocytes (lane 2), again suggesting that very little, if any, cleavage normally occurs in the nucleus.

We next analysed whether the cleavage fragments of 12/12–U7 RNA produced in oocytes terminate at the correct positions. Since the 5′-end of 12/12–U7 RNA is identical to that of the single histone pre-mRNA 12/12 (Fig. 1), the 5′ cleavage products should be directly comparable. The partial trimethylation of the cap of 12/12–U7 RNA should not significantly alter the mobility for the following reasons: the two additional methyl groups represent <10% of the mass of a nucleotide and do not alter the charge of the RNA; moreover, in immunoprecipitations of RNAs with anti-3mG antibody we have not observed a systematic difference in electrophoretic mobility between RNAs in the pellet and supernatant fractions (unpublished results). Therefore, m7G-capped 12/12 RNA was either injected into oocyte nuclei (where processing depends upon resident *Xenopus* U7 snRNPs) or incubated in mouse nuclear extract (where processing depends upon authentic mouse U7 snRNPs) and the products were analysed by electrophoresis on a denaturing polyacrylamide gel alongside the products obtained after injection of the chimeric 12/12–U7 RNA into the oocyte cytoplasm (Fig. 3A). Both the single 12/12 (lane 1) and chimeric 12/12–U7 RNAs (lane 3) when processed in oocytes produce two 5′ fragments which appear to differ by 1 nt. The longer of these co-migrates with the major 5′ product obtained from processing of 12/12 RNA *in vitro* (lane 2). The latter has been shown to end after the nucleotides ACCCA, immediately following the histone hairpin (7,28) and is 50 nt long (49 plus the cap). It is not known whether both 5′ fragments observed in the oocyte are direct products of the initial cleavage reaction. More likely the smaller one (which is not observed in all experiments) results from exonucleolytic nibbling once primary cleavage has occurred. Most importantly, however, both the U7 snRNP-mediated cleavage of 12/12 RNA in oocyte nuclei and the novel cleavage of 12/12–U7 RNA appear to generate identical 5′ products.

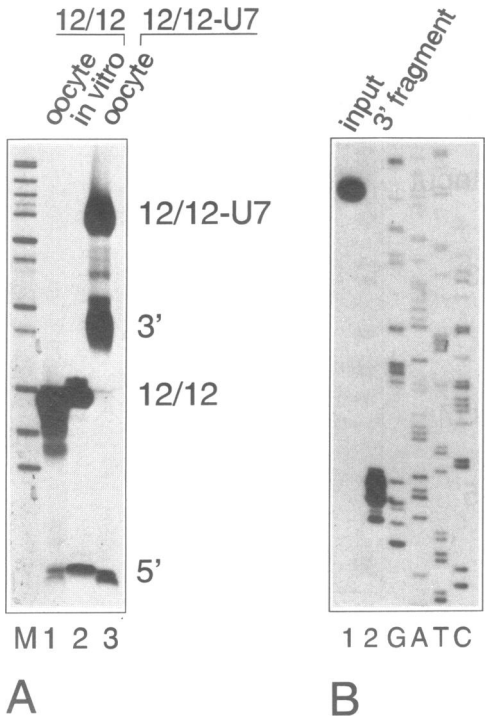

Figure 3. Mapping of cleavage fragments of the chimeric RNA. (**A**) Mapping of 5′ fragments. Capped histone pre-mRNA 12/12 of 85 nt (Fig. 1A) was either injected into the nucleus of *Xenopus* oocytes (lane 1) or incubated for 2 h in nuclear extract from mouse mastocytoma cells (lane 2). Capped chimeric 12/12–U7 RNA was injected into the oocyte cytoplasm (lane 3). Microinjected oocytes (lanes 1 and 3) were incubated overnight. All RNAs were re-isolated and analysed on a denaturing polyacrylamide gel (4 oocyte equivalents/sample). The positions of the various RNAs and of their fragments are indicated on the right. Lane M, size marker, *Hpa*II digest of pBR322 (bands between 242 and 67 nt are visible). (**B**) Mapping of 3′ fragments. Uncleaved input 12/12–U7 RNA (lane 1) and 3′ fragments obtained after injection into the cytoplasm of 100 *Xenopus* oocytes (lane 2) were gel purified and subjected to reverse transcription using an oligonucleotide primer complementary to nt 18–33 of mouse U7 RNA (24). Reaction products were analysed on a denaturing polyacrylamide gel alongside sequencing reactions of the plasmid template using the same primer (lanes G, A, T and C). To reveal the sequence of the RNA strand all lanes are labelled with the complementary nucleotide, i.e. lane G is the product of a dideoxy-C reaction, lane A that of a dideoxy-T reaction, etc.

To map the 3′ products we re-extracted 12/12–U7 RNA from injected oocytes and gel purified the 3′ products. These were subjected to reverse transcription with an oligonucleotide primer complementary to nt 18–33 of mouse U7 RNA (25). As controls the input 12/12–U7 RNA was mapped by the same procedure and both samples were subjected to electrophoresis alongside a set of sequencing reactions of the template plasmid using the same primer (Fig. 3B, lanes G, A, T and C). Thus the positions of the extension products with respect to the sequencing ladder directly reveal the 5′-ends of the corresponding RNA molecules. The extension product of input 12/12–U7 RNA (lane 1) is present as a single band co-migrating with the initiating G of the pSP65 promoter. For the 3′ product (lane 2) a heterogeneous array of extension products are formed which map to the nucleotide sequence AGAAGGAG (end-points of strongest extension products underlined). This sequence corresponds to the purine-rich part of the histone spacer element. If 12/12 RNA is processed by endogenous U7 snRNPs in the *Xenopus* oocyte nucleus no 3′ products are observed, presumably because they are rapidly degraded in the nucleus (19,23). However, for 12/12 RNA processed *in vitro* in K21 nuclear extract we have previously mapped the 5′-ends of the major 3′ products to the nucleotides AGAAGGAG in the same sequence (11). We therefore conclude that the novel cleavage of 12/12–U7 RNA observed in *Xenopus* oocytes generates 5′ and 3′ fragments with very similar, if not identical, specificity as *bona fide* histone RNA 3′ processing. The absence of a few nucleotides between the 5′ and 3′ products has previously been observed *in vitro* with different histone pre-mRNA substrates (11,29); it is not yet known whether these nucleotides are removed exo- or endonucleolytically.

Chimeric 12/12–U7 RNA is assembled into a snRNP-like particle

Since the chimeric 12/12–U7 RNA is extremely deficient in nuclear import, it seemed possible that it is also inefficiently assembled into a snRNP-like particle. We therefore analysed the association of 12/12–U7 RNA and of its cleavage products with snRNP structural proteins, by analysing the fraction of RNA that can be precipitated from total oocyte extracts by the Y-12 monoclonal anti-Sm antibody (26). For comparison we injected U7 Sm WT RNA and analysed it similarly. The short U7 Sm WT RNA is specifically precipitated by Y-12 antibody (Fig. 4, lane 1), whereas only trace amounts can be precipitated with a control monoclonal antibody (lane 2). Nevertheless, a large proportion of the injected RNA remains in the supernatant (lanes 5 and 6). For the uncleaved chimeric 12/12–U7 RNA the efficiency of immunoprecipitation by the Y-12 antibody (lanes 3, pellet, and 7, supernatant) is comparable with that of U7 Sm WT RNA, but, in contrast to the latter, a significant amount is also found in the pellet fraction with the control antibody (lane 4). Nevertheless, the proportion precipitated by the Y-12 antibody is substantially higher than that obtained with the control antibody. The 3′ fragment, which carries the Sm binding site, is almost quantitatively precipitated by Y-12, but not at all by the control antibody. Finally, the 5′ cleavage product is very much under-represented in the Y-12 pellet fraction. Since this 5′ fragment is not associated with a Sm binding site, the low amounts present in the pellet could reflect incomplete dissociation of the processing complex after RNA cleavage or cleavage continuing during the immunoprecipitation procedure. Similar immunoprecipitation experiments carried out with antibodies directed against the trimethylguanosine cap structure of U7 snRNAs (data not shown) demonstrate that chimeric 12/12–U7 RNA undergoes cap hypermethylation. However, the efficiency of this reaction seems to be lower than for the short U7 Sm WT RNA.

We conclude from these experiments that 12/12–U7 RNA does assemble with snRNP structural proteins present in the oocyte cytoplasm. However, it is premature to say whether the extreme deficiency in nuclear import is due to reduced cap hypermethylation, increased RNP size or other reasons.

Primary sequence requirements for cleavage of 12/12–U7 RNA

We next analysed the sequence requirements for cleavage of the chimeric histone–U7 RNA. Two alterations, Sm MUT and Sm OPT, were introduced at the Sm binding site (AAUUUGUCUAG) of the U7 moiety. We have previously shown that U7 Sm MUT RNA (AACGCGUCAUG, mutated nucleotides underlined)

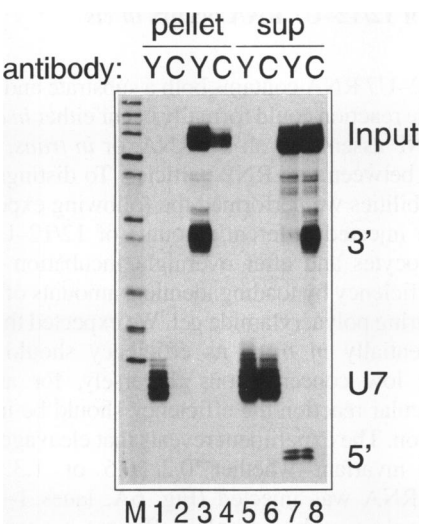

Figure 4. Association of injected RNAs with snRNP structural proteins. *In vitro* transcribed, capped U7 Sm WT RNA (lanes 1, 2, 5 and 6; Fig. 1B) or chimeric 12/12–U7 RNA (lanes 3, 4, 7 and 8) were injected into the cytoplasm of *X.laevis* oocytes. After overnight incubation total oocyte extracts were prepared and subjected to immunoprecipitation with either monoclonal anti-Sm antibody Y-12 (lanes Y; 26) or the unrelated monoclonal antibody D5 (lanes C; see Materials and Methods). RNAs from pellet and supernatant fractions were extracted and analysed by denaturing polyacrylamide gel electrophoresis (5 oocyte equivalents/sample). The positions of the various RNAs and of their fragments are indicated on the right. Lane M, size marker, *Hpa*II digest of pBR322 (bands between 217 and 67 nt are visible).

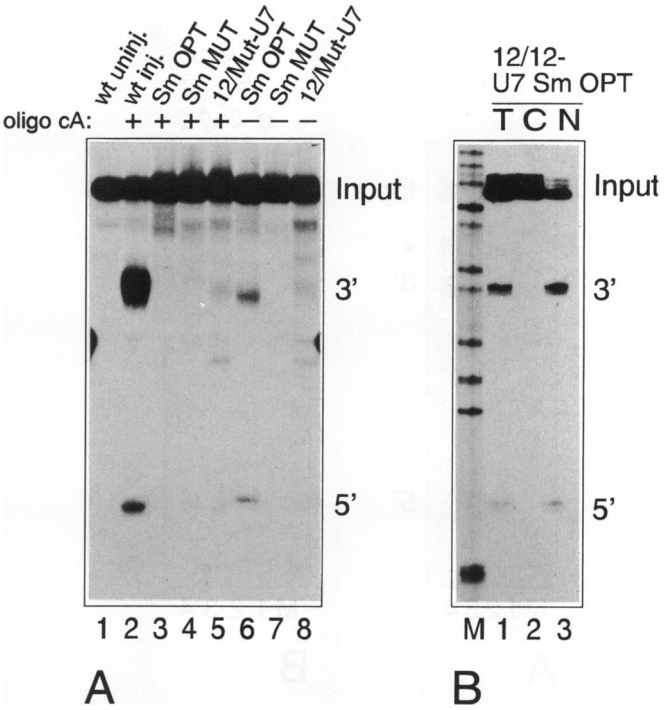

Figure 5. Analysis of mutant derivatives of the chimeric histone–U7 RNA. (**A**) Cleavage analysis of mutations in the histone spacer element and the U7 Sm binding site. In lanes 2–5 *X.laevis* oocytes were depleted of endogenous U7 snRNPs by intranuclear injection of oligonucleotide cA complementary to nt 1–16 of *Xenopus* (or mouse) U7 RNA (23,24). A delay of 5 h elapsed between this injection and subsequent cytoplasmic injection of *in vitro* transcribed, capped chimeric RNAs. Three oocyte equivalents were loaded per sample. The positions of uncleaved RNAs (input) and of the 3′ and 5′ fragments are indicated on the right. Lane 1, uninjected 12/12–U7 RNA. The injected RNAs were: 12/12–U7 (lane 2); 12/12–U7 Sm OPT (lanes 3 and 6); 12/12–U7 Sm MUT (lanes 4 and 7); 12/Mut-U7 (lanes 5 and 8). The sequences of the RNAs are shown in Figure 1. (**B**) Nucleocytoplasmic distribution of 12/12–U7 Sm OPT RNA after injection into the cytoplasm of *Xenopus* oocytes and overnight incubation (4 oocyte equivalents/sample). Lane T, total RNA; lane C, cytoplasmic RNA; lane N, nuclear RNA.

is unable to interact with snRNP proteins, to accumulate in the nucleus and to function in histone RNA processing. In contrast, U7 Sm OPT RNA (AAUUUUUGGAG), designed to resemble the consensus Sm binding sequence derived from spliceosomal U snRNAs, shows enhanced assembly with snRNP proteins and nuclear import, but the resulting particle is non-functional in histone RNA processing, presumably because it fails to incorporate one or more of the U7-specific proteins (19,23). When injected into the oocyte cytoplasm chimeric 12/12–U7 Sm OPT RNA produces low, but significant, amounts of cleavage products (Fig. 5A, lane 6). Because of the enhanced assembly and nuclear accumulation previously observed with U7 Sm OPT RNA, it seemed possible that this cleavage occurs in the nucleus and involves *Xenopus* U7 snRNPs acting *in trans*. Indeed, when the nucleocytoplasmic distribution of 12/12–U7 Sm OPT RNA was analysed ~20% of the RNA was found in the nucleus after overnight incubation (Fig. 5B, lanes 1–3). Interestingly, both cleaved and uncleaved RNAs were present in the nucleus, whereas the cytoplasmic fraction contained exclusively uncleaved molecules. This strongly suggests that the low amount of cleavage of 12/12–U7 Sm OPT RNA occurs in the nucleus and not in the cytoplasm, as is the case for wild-type 12/12–U7 RNA (see above). We therefore re-analysed 12/12–U7 Sm OPT RNA in oocytes in which the resident U7 RNA had previously been degraded by endogenous RNAse H targetted to nt 1–16 by nuclear injection of an antisense oligonucleotide (23). The two injections were separated by an interval of 5 h to ensure that the targeting oligonucleotide had itself been degraded. Under these conditions no cleavage products were observed (Fig. 5A, lane 3), thus proving that cleavage of 12/12–U7 Sm OPT RNA indeed depends on endogenous U7 snRNPs. In contrast, cleavage of the wild-type 12/12–U7 RNA (which, as shown above, occurs predominantly, if not exclusively, in the cytoplasm) is efficient even in U7-depleted oocytes (lane 2).

Interestingly, the products formed in a U7-dependent fashion from 12/12–U7 Sm OPT RNA have a different electrophoretic mobility than the products of wild-type 12/12–U7 RNA; the 3′ product(s) appears smaller and the 5′ product(s) larger respectively. Considering that the 5′ products of 12/12 RNA (nuclear, U7-dependent cleavage) and of wild-type 12/12–U7 RNA (cytoplasmic cleavage) migrate identically (Fig. 3A), this result may indicate that the specificity of cleavage mediated by the endogenous U7 snRNPs can be modified by the presence of the extra U7 sequences complexed with RNP proteins at the 3′-end of 12/12–U7 Sm OPT RNA. However, the important result remains that 12/12–U7 Sm OPT RNA is not cleaved in oocytes devoid of U7 RNA, whereas 12/12–U7 RNA is still cleaved efficiently.

For the other mutant, 12/12–U7 Sm MUT, no cleavage was observed, irrespective of whether the endogenous U7 snRNA was intact (lane 7) or degraded (lane 4). It is therefore evident that cleavage of 12/12–U7 RNA is dependent upon the correct

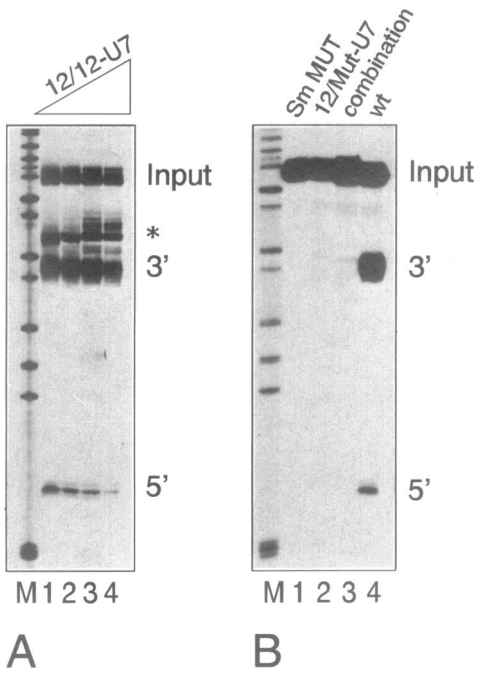

Figure 6. Cleavage of chimeric RNA occurs *in cis*. (**A**) Substrate titration. Either 0.2 (lane 1), 0.6 (lane 2), 1.3 (lane 3) or 3.2 fmol/oocyte (lane 4) *in vitro* transcribed, capped 12/12–U7 RNA were injected into the oocyte cytoplasm. After overnight incubation RNA was isolated and identical amounts of radioactive RNA (corresponding to 6.6, 2, 0.9 and 0.35 oocyte equivalents respectively) were analysed by denaturing polyacrylamide gel electrophoresis. The positions of uncleaved 12/12–U7 RNA (input) and of its 3′ and 5′ fragments are indicated on the right. The asterisk indicates a presumably non-specific product of ~135 nt. Lane M, size marker, *Hpa*II digest of pBR322 (bands between 238 and 34 nt are visible). (**B**) Absence of *trans*-complementation. Either 12/12–U7 Sm MUT (lane 1, 0.8 fmol/oocyte), 12/Mut-U7 (lane 2, 0.8 fmol) or 12/12–U7 RNA (lane 4, 1.6 fmol) were injected into the oocyte cytoplasm. In lane 3 a combination of 0.8 fmol/oocyte of each of the two mutant RNAs shown in lanes 1 and 2 was injected. After overnight incubation RNA was isolated and analysed by denaturing polyacrylamide gel electrophoresis (3 oocyte equivalents/sample) The positions of the RNAs (input) and of their 3′ and 5′ fragments are indicated on the right.

sequence of the Sm binding site (see Discussion). We further analysed 12/Mut–U7 RNA, a derivative in which the histone spacer sequence has been altered from GAAGGAGUUGU to UAAUCUGGACU. This mutation prevents formation of the longer of two base paired stems between the spacer element and U7 RNA (Fig. 1A and C). Cleavage of the chimeric 12/Mut–U7 RNA is drastically reduced compared with 12/12–U7 RNA, both in native oocytes (Fig. 5A, lane 8) and in oocytes depleted of U7 snRNA (lane 5). Although this reduction may be slightly less dramatic than was previously observed when 12/Mut RNA was analysed in a K21 nuclear extract (0.8% of 12/12 RNA; 11), this result nevertheless indicates that cleavage of the chimeric RNA strongly depends upon base pairing interactions between the histone spacer and U7 moieties. In the RNA samples corresponding to lanes 2–5 (U7-depleted oocytes) *Xenopus* U7 RNA was undetectable by primer extension, whereas it was readily detected in an RNA sample from oocytes which had not been injected with the antisense oligonucleotide (data not shown).

Cleavage of 12/12–U7 RNA occurs *in cis*

Since 12/12–U7 RNA contains both a substrate and a U7 moiety, the cleavage reaction could formally occur either *in cis*, involving a single RNP assembled on the RNA, or *in trans*, involving an interaction between two RNP particles. To distinguish between these possibilities we performed the following experiments.

First, we injected different amounts of 12/12–U7 RNA into *Xenopus* oocytes and after overnight incubation analysed the cleavage efficiency by loading identical amounts of radioactivity on a denaturing polyacrylamide gel. We expected that if cleavage occurs essentially *in trans* its efficiency should be strongly reduced at low concentrations. Inversely, for an essentially monomolecular reaction the efficiency should be invariant with concentration. The experiment reveals that cleavage efficiency is practically invariant whether 0.2, 0.6 or 1.3 fmol/oocyte 12/12–U7 RNA was injected (Fig. 6A, lanes 1–3). With 3.2 fmol/oocyte cleavage becomes less efficient, presumably because of titration of some limiting factor(s) for assembly and/or cleavage (lane 4). Essentially identical results were obtained in a separate experiment in which the amount of 12/12–U7 RNA was varied between 0.05 and 6.4 fmol/oocyte and the incubation time was reduced from 18 to 4.5 h (data not shown).

Secondly, we injected U7-depleted oocytes with 0.8 fmol of either 12/12–U7 Sm MUT or 12/Mut-U7 RNA. In agreement with the results of Figure 5A, 12/12–U7 Sm MUT RNA is not cleaved at all (Fig. 6B, lane 1) and 12/Mut-U7 RNA is cleaved very inefficiently (lane 2, only low amounts of 3′ product are visible). In addition, we co-injected oocytes with a combination of 0.8 fmol of each of the mutant RNAs. More efficient cleavage than that observed for each mutant alone would only be possible if the RNA molecules reacted *in trans*, i.e. if cleavage of 12/12–U7 Sm MUT RNA (which has an intact histone pre-mRNA moiety) was mediated by 12/Mut–U7 RNA (whose U7 moiety has a functional Sm binding site). Thus if cleavage occurred exclusively *in trans*, the combination of the two mutants should be processed with ~50% apparent efficiency compared with 12/12–U7 RNA. Inversely, if cleavage occurred exclusively *in cis* the combination of mutant RNAs should not be processed more efficiently than either mutant alone. Upon co-injection (Fig. 6B, lane 3) the same low amount of 3′ cleavage product is observed as with 12/Mut–U7 RNA alone (lane 2). In contrast, cleavage of wild-type 12/12–U7 RNA is still efficient when 1.6 fmol/oocyte RNA was injected (lane 4). This indicates that cleavage of 12/12–U7 RNA is a *cis*- rather than a *trans*-mediated event.

A third experiment confirmed that interactions *in trans* between two molecules of chimeric RNA are inefficient compared with the predominant *cis* cleavage. Unlabelled 12/12–U7 Sm MUT RNA (which neither associates with snRNP proteins nor is cleaved in the oocytes, but which remains in the cytoplasmic compartment) was co-injected in a 10- or 100-fold excess over the labelled wild-type 12/12–U7 RNA. The rationale was that if associations *in trans* between the two RNAs occur this should be reflected in a competitive inhibition of cleavage of the labelled 12/12–U7 RNA. However, with the competitor RNA being present at 10-fold excess no change in processing was observed and at 100-fold excess a slight reduction in the reaction rate, but no change in the final yield of products, was observed (data not shown). Therefore, even in the presence of an excess of non-cleavable competitor RNA the *cis* interaction (cleavage of

12/12–U7 RNA) prevails over a possible interaction *in trans* (competitive inhibition).

DISCUSSION

Cleavage of chimeric histone–U7 RNA occurs in the ooplasm and is independent of preformed, endogenous U7 snRNPs

By combining the sequences of a mouse histone pre-mRNA and of mouse U7 snRNA into a single chimeric molecule we have created a biologically functional RNA which upon injection into the cytoplasm of *Xenopus laevis* oocytes assembles into a RNP particle using snRNP structural proteins stored in the ooplasm (Fig. 4). In the oocyte the cap structure present in the chimeric RNA becomes at least partly hypermethylated (data not shown) and, most importantly, the RNA is cleaved at positions corresponding to the normal cleavage sites of histone pre-mRNA (Fig. 3).

Despite being assembled into a snRNP-like structure, neither the full-length chimeric 12/12–U7 RNA nor the 3′ cleavage product accumulate in the nucleus (Fig. 2B). It is possible that the considerable 5′ extension compared with U7 RNA of normal size and perhaps the additional RNA secondary structure interfere with nuclear import. As a consequence, the observed cleavage must occur mostly, if not exclusively, in the cytoplasm. Consistent with this, efficient cleavage occurs even when the chimeric RNA has been injected into enucleated oocytes (Fig. 2C). In contrast, single histone pre-mRNAs whose cleavage is dependent on *Xenopus* U7 snRNPs can only be processed in the oocyte nucleus and not in the cytoplasm (30). Presumably only the nucleus contains U7 snRNPs and/or other processing factors in sufficient quantity and in functional form. Cytoplasmic cleavage of the chimeric RNA is therefore most likely independent of *Xenopus* U7 snRNPs. Indeed, 12/12–U7 RNA is also efficiently cleaved in oocytes depleted of endogenous U7 snRNA by oligonucleotide-targeted RNAse H digestion (Fig. 5). It is not yet known whether this cytoplasmic, U7 snRNP-independent cleavage is unique to *Xenopus* oocytes or whether it is also present in other cell types.

The results obtained with two mutant versions of the Sm binding site indicate that for this cytoplasmic cleavage to occur the chimeric RNA must be assembled into a correct U7 snRNP-like structure (Fig. 5A). The Sm MUT mutation, which prevents snRNP assembly, (19,23) results in a complete loss of cleavage. The Sm OPT mutation, which allows more efficient assembly and nuclear import (Fig. 5B), leads to a reduced level of cleavage. However, this cleavage of 12/12–U7 Sm OPT RNA occurs in the nucleus (Fig. 5B) and is entirely dependent upon intact resident *Xenopus* U7 snRNPs (Fig. 5A). In other words, 12/12–U7 Sm OPT RNA (or its resulting RNP) is incompetent for the novel U7 snRNP-independent, cytoplasmic cleavage. This is in agreement with our previous results indicating that U7 Sm OPT RNA upon injection into *Xenopus* oocytes forms a particle that cannot complement histone RNA 3′ processing (23). Possible structural reasons for this deficiency have been identified (19): in comparison with wild-type U7 RNA, U7 Sm OPT RNA can no longer be cross-linked to a 40 kDa, apparently U7-specific protein; in addition, it is more efficiently associated with common snRNP proteins. Our present results not only confirm that a RNP containing the Sm OPT sequence does not participate in histone RNA processing, but conclusively prove that this RNP is deficient in the cleavage step itself. In particular, by studying the *cis* cleavage reaction occurring in the cytoplasmic compartment we can rule out that the deficiency of U7 Sm OPT snRNP observed previously might have been due to its sequestration at a special subnuclear site where it was physically unable to interact with histone pre-mRNA and to participate in 3′ processing.

Mechanistic implications for histone RNA 3′ processing

An important implication of our results is that histone RNA processing must be a single snRNP reaction. Although only the U7 snRNP has been proven to be required, the possible involvement of other snRNPs could previously not be excluded. The best way to clarify this point was to perform processing in an environment where U7 is the only snRNP present. This has now been achieved by 'exporting' the reaction to the cytoplasm.

Previous studies of histone RNA 3′ processing with the pre-mRNA and the U7 snRNP being present *in trans* consistently revealed an absolute requirement for base pairing interactions between the histone spacer element and the 5′-end of U7 RNA (4,5). However, it was conceivable that such a requirement for base pairing could be less stringent or even non-existant if the U7 snRNP and the histone pre-mRNA were covalently joined into a single molecular complex. Our experiments with 12/Mut–U7 RNA show that this is not the case and that base pairing interactions are equally important for *cis* cleavage of the chimeric RNA as for normal histone RNA processing *in trans*. Thus for cleavage to occur tethering between the histone pre-mRNA substrate and the U7 component must be more precise than is the case for 12/Mut–U7 RNA; so far it appears that this increased precision can only be guaranteed by RNA–RNA base pairing.

Furthermore, of the three *trans*-acting factors implicated in histone RNA processing U7 snRNP is the only one accounted for in the above-mentioned single molecular complex. The other two, HBF and HLF, should, in principle, be nuclear proteins. However, the oocyte, as a typical storage cell, may also contain non-snRNP factors destined to function in the nucleus within its cytoplasm. Alternatively, HBF, which is not absolutely required for processing *in vitro*, but rather appears to function as an auxiliary component stabilizing the interaction between histone pre-mRNA and the U7 snRNP (8,10–12), may not be required for this novel *cis* reaction. In addition, it is possible that HLF, although it was originally identified as a separate entity (9), may in fact be a component of the U7 snRNP. Using the chimeric histone–U7 RNA, it should be possible to test some of these hypotheses.

Potential of studying histone RNA processing as a *cis* cleavage reaction

In designing a chimeric histone–U7 RNA it was our aim to reduce the complexity of histone RNA 3′ processing to facilitate further studies of the cleavage mechanism. The fact that the reaction proceeds *in cis*, i.e. involves only interactions within a single molecule of chimeric RNA present as a RNP (Fig. 6), should considerably simplify such studies. First, only one round of cleavage is possible for every assembled chimeric RNP and, therefore, the kinetic contribution of product dissociation becomes irrelevant. Second, the association of the pre-mRNA and U7 RNA moieties by base pairing is strongly favoured by their physical proximity.

In contrast, an additional step, RNP assembly, is required if the chimeric RNA is injected into the oocyte cytoplasm. Thus the novel *cis* cleavage reaction consists of two distinct phases: (i) assembly of the chimeric RNA with structural proteins of the U7

snRNP and HLF (which, as discussed above, may or may not be a U7-specific protein); (ii) the cleavage step itself.

The chimeric histone–U7 RNA offers the potential to study both of these phases in more detail. On the one hand, it may be possible to re-isolate fully assembled, uncleaved chimeric RNPs from injected oocytes and to study their cleavage *in vitro*. That this may be feasible is suggested by the fact that significant amounts of uncleaved 12/12–U7 RNA can be immunoprecipitated with anti-Sm antibodies and that low amounts of the 5′ product (which does not contain a Sm binding site) are found in the pellet fraction (Fig. 4). On the other hand, it has so far not been possible to demonstrate assembly of functional U7 snRNPs *in vitro*. Only an association of U7 RNA with common snRNP proteins and the formation of a particle that reacts with anti-Sm antibodies has recently been achieved (31). One problem in such studies is the low abundance of U7-specific proteins in most sources of snRNP proteins. Thus even if a few molecules of U7 RNA should get assembled into functional particles, these would presumably have to compete for the histone pre-mRNA substrate with large excesses of incompletely assembled and free U7 RNA. The chimeric 12/12–U7 RNA appears as an ideal substrate for such studies, because, as soon as a molecule has assembled with all the necessary components, this should be scored by cleavage *in cis*. This reaction should be relatively resistant to competition by incompletely assembled or free RNA molecules.

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