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3′ End processing of mouse histone pre-mRNA: evidence for additional base-pairing between U7 snRNA and pre-mRNA

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ABSTRACT

We have analysed the extent of base-pairing interactions between spacer sequences of histone pre-mRNA and U7 snRNA present in the *trans*-acting U7 snRNP and their importance for histone RNA 3′ end processing *in vitro*. For the efficiently processed mouse *H4-12* gene, a computer analysis revealed that additional base pairs could be formed with U7 RNA outside of the previously recognised spacer element (stem II). One complementarity (stem III) is located more 3′ and involves nucleotides from the very 5′ end of U7 RNA. The other, more 5′ located complementarity (stem I) involves nucleotides of the Sm binding site of U7 RNA, a part known to interact with snRNP structural proteins. These potential stem structures are separated from each other by short internal loops of unpaired nucleotides. Mutational analyses of the pre-mRNA indicate that stems II and III are equally important for interaction with the U7 snRNP and for processing, whereas mutations in stem I have moderate effects on processing efficicency, but do not impair complex formation with the U7 snRNP. Thus nucleotides near the processing site may be important for processing, but do not contribute to the assembly of an active complex by forming a stem I structure. The importance of stem III was confirmed by the ability of a complementary mutation in U7 RNA to suppress a stem III mutation in a complementation assay using *Xenopus* laevis oocytes. The main role of the factor(s) binding to the upstream hairpin loop is to stabilise the U7 – pre-mRNA complex. This was shown by either stabilising (by mutation) or destabilising (by increased temperature) the U7 – pre-mRNA base-pairing under conditions where hairpin factor binding was either allowed or prevented (by mutation or competition). The hairpin dependance of processing was found to be inversely related to the strength of the U7 – pre-mRNA interaction.

INTRODUCTION

Compared to the complex base-pairing interactions involved in the splicing of nuclear pre-mRNAs and self-splicing introns (reviewed in 1), a relatively more simple situation is found in 3′ processing of animal histone pre-mRNAs—the process leading to the formation of mature histone mRNA. Only two RNA moieties, the pre-mRNA substrate and the minor U7 snRNA, together with their associated proteins, are involved in this reaction (reviewed in 2). Genetic (3, 4) and biochemical evidence (5, 6) indicate that the U7 snRNP acts through base-pairing between the 5′ end of U7 RNA and a conserved spacer element downstream of the cleavage site of histone pre-mRNA. Stable intramolecular stem–loop structures form in the histone pre-mRNA immediately upstream of the cleavage site, and at the 3′ end of U7 RNA. Therefore, the overall secondary structure can be described as a RNA junction consisting of three base-paired stems with a variable number of unpaired nucleotides. The U7–histone spacer interaction, as it has been recognised so far, consists of six perfect base pairs in sea urchins, where the spacer element is absolutely conserved in sequence (7). In vertebrates, the traditionally recognised spacer element (stem II in Fig. 1) is more degenerate with the general consensus sequence $^{A}/_{G}AA^{A}/_{G}GAG^{C}/_{U}UG^{U}/_{G}$ (8) and its distance from the processing site is somewhat variable. This led to speculations that the spacer interaction may be less important in vertebrates than in sea urchins (an idea which is not borne out by experiments; 4, 9) or that the degeneracy of the vertebrate spacer element may be compensated for by its increased length.

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An apparent difference between histone RNA processing and the various splicing reactions is that, in splicing, base-pairing interactions are formed with nucleotides immediately surrounding the reactive phosphodiester bonds (reviewed in 1). Thus, it appears that the precise recognition of the reacting nucleotides, and hence at least part of the catalytic activity, are carried out by RNA moieties. In the current view of the histone–U7 RNA interaction, U7 RNA serves to anchor the processing complex and thereby loosely determine the processing site (6), but is unlikely to define that site precisely or to participate directly in catalysis.

During a mutational study of histone RNA processing in mammalian cell extracts (10), we noticed that our most efficiently processed histone pre-mRNA, originating from the mouse *H4-12* gene (11), could theoretically form additional base-paired structures with U7 RNA (Fig. 1). One of these (stem III) is located 3′ of the 'traditional' spacer element (stem II), is separated from the latter by an internal loop of unpaired nucleotides ('bubble' 2) and involves nucleotides from the very 5′ end of U7 RNA. A similar 3–5 bp complementarity can be found in most if not all mammalian histone genes (A.Furger, unpublished results). The other possible interaction (stem I) is also separated from stem II by an internal loop ('bubble' 1) and would involve nucleotides of the Sm binding site of U7 RNA, i.e. nucleotides interacting with snRNP structural proteins (12; B.Stefanovic, W.Hackl, R.Lührmann and D.Schümperli, submitted for publication). An attractive feature of this potential stem I is that it would include base-pairing interactions at or very close to the processing site. However, comparative sequence analyses show that the potential to form stem I is modest (often only 2–3 bp) for most other mammalian histone pre-mRNAs, which may question its biological significance (A.Furger, unpublished). In this work, we have investigated the functional importance of these potential RNA structures. We find that stem III is equally important as the previously recognised stem II for assembly of an active processing complex, whereas nucleotides near the processing site may be important for processing but do not appear to contribute to the assembly of an active complex by RNA–RNA base-pairing.

MATERIALS AND METHODS

Construction of plasmids and site-directed mutagenesis

The plasmids 12/12, 12/Sup, B/12 and B/Sup containing short 3′ segments from the mouse *H4-12* gene (11) downstream of the SP6 promoter have been described (5, 10). Mutant 12/st1.8 was constructed similarly by cloning double-stranded oligonucleotides into plasmid pSP65. Further mutants were made by site-directed mutagenesis (13, 14) after subcloning the histone segment from construct 12/12 into M13mp19.

To avoid repeated cloning between the M13 phage and pSP65 plasmid systems, we constructed further mutants in a vector derived from phagemid OT7, a pBlScr(M13+) derivative (a gift of K.Kälin, Institute for Molecular Biology I, University of Zürich). To ensure that the SP6- and T7-derived RNAs are identical in sequence, we introduced an adaptor oligonucleotide containing the sequence from the SP6 promoter to the *Eco*RI site of pSP65 (GAATACACGGAATTC; first nt of SP6/T7 transcripts and *Eco*RI site underlined) between the blunt-ended *Nsi*I site (at the T7 promoter) and the *Eco*RI site of OT7, resulting in plasmid OT7.6. *Eco*RI/*Hin*dIII fragments containing the histone 3′ ends from 12/12 and 12/st1.8 were then subcloned into OT7.6. The resulting plasmids were used for site-directed mutagenesis, as modified for phagemid vectors (13, 14). The sequence of each mutant was confirmed by dideoxy sequencing (15). The structures of these constructs and a description of the nomenclature are presented in Fig. 1.

Plasmid OT7-U7sup3.5 was constructed by site-directed mutagenesis of OT7-U7 (B.Stefanovic, W.Hackl, R.Lührmann and D.Schümperli, submitted for publication). When transcribed as described below, this plasmid yields mouse U7 RNA with the 5′-terminal sequence GGUUCCCUUACA... (nucleotides differing from wild-type sequence underlined) and containing an extra C at the 3′ end. The two Gs at the 5′ end should ensure efficient *in vitro* transcription. In construct HP-12, an oligonucleotide encompassing the T7 promoter and the hairpin of the *H4-12* gene was inserted into the *Sma*I site of pSP65 (A.Schaller, unpublished results).

In vitro transcription

All templates were linearised with *Hin*dIII [except for HP-12 (*Sma*I) and OT7-U7sup3.5 (*Pst*I, followed by removal of 3′ overhangs with T4 DNA polymerase)], precipitated with ethanol, washed with 80% ethanol and resuspended in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, to a final concentration of 1 μg/μl. Transcription reactions were carried out as described (10) with some modifications. The reaction mix (10 μl) contained 1 μg linearized template, 0.05 mM rGTP, 0.5 mM each of rATP, rCTP and rUTP, 0.1 μg BSA, 3 mM DTT, 1× SP6 buffer (Boehringer Mannheim), 40 U RNasin (Promega), 25 μCi [α-^{32}P] GTP (800 Ci/mmol; New England Nuclear; NEG-006X) and 5 U SP6 or T7 RNA polymerase (Promega, Boehringer Mannheim). For RNAs used in oocyte injections, 0.5 mM ^{m7}G(5′)ppp(5′)G (Boehringer Mannheim) was included. Reactions were carried out for 1 h at 40°C (SP6) or 37°C (T7). The RNA was purified on 6% polyacrylamide–8.3 M urea gels and eluted as described (10).

In vitro processing and complex formation in nuclear extract of mouse K21 cells

Mouse K21 nuclear extracts (a gift of R.Mital) were prepared as described (16, 17). 3 μl yeast RNA (1 mg/ml), 2 μl EDTA (100 mM) and 5 μl nuclear extract were preincubated for 10 min on ice. 20 000 c.p.m. (~0.03 pmol) of histone pre-mRNA was added and processing was allowed to proceed for 2 h at 30°C. The RNA was extracted once with phenol, once with chloroform, precipitated with ethanol and analysed on 6 or 8% polyacrylamide–8.3 M urea gels. In some experiments, unlabelled competitor RNAs were added to the reaction mixture as described (10). The signals were quantitated by densitometry of autoradiograms using a GS300 Scanning Densitometer (Hofer Scientific Instruments; Figs 1, 2, and 3A) or with a PhosphorImager (Molecular Dynamics; Fig. 6).

Nuclear extract was heat-inactivated by incubation for 15 min at 50°C (18). RNAse H inactivations were performed with oligo-cA complementary to nucleotides 1–16 of U7 RNA (17, 19) or with oligo-U1a complementary to nucleotides 10–25 of U1 RNA (negative control). 15 μl extract, 1 μl 50 mM $MgCl_2$ and 75 ng of oligonucleotide were incubated for 15 min at 15°C. The reaction was stopped by adding 1 μl 400 mM EDTA.

Complex formation between histone pre-mRNA and the U7 snRNP was analysed in heat-inactivated extract as described (5). Antibody shifts of the U7–histone pre-mRNA complexes were induced with monoclonal anti-Sm antibody Y-12 (20; purified

by I.Haussmann). D5 Ig, a mouse IgG_1 antibody against the *S.cerevisiae* SEP I protein (a gift from W.-D.Heyer), was used as a negative control. 5 μg of antibody was added to the reactions after processing. After incubation for 30 min on ice, the samples were treated with heparin and loaded on a composite gel (5).

Oocyte microinjection

Xenopus oocyte injections (10–20 oocytes per sample) were done as described (12). *In vitro*-made and radiolabelled U7 RNAs (about 1.5 fmol) were injected into the ooplasm in 20 nl of water and the oocytes were incubated for 16–18 h at 18°C, then 7.5 fmol of *in vitro*-made histone pre-mRNA (5000 c.p.m.) was injected into the nucleus in 20 nl of water. After 2 h, total oocyte RNA was extracted (12) and analysed.

RESULTS

Experimental strategy and nomenclature of mutants

To determine if the additional complementarities detected between the 3′ spacer of the mouse *H4-12* gene and mouse U7 RNA (Fig. 1, stems I and III) play a similiar role as the one in the traditional spacer element (stem II), we introduced small mutations that should decrease the complementarity in each potential stem. We further asked if the effect of these mutations could be compensated for by allowing base-pairing in each of the internal loops ('bubbles' 1 and 2). In the nomenclature used to describe the mutants, the altered stems, the number of mutations in each stem and eliminated 'bubbles' are indicated. For example 12/st1.2-b1 means that starting from the original RNA, 12/12, two nucleotides have been changed in stem I and that the nucleotides in 'bubble' 1 have been altered to allow base-pairing with U7 snRNA.

In vitro-synthesised RNAs from these constructs were incubated in nuclear extract from K21 mouse mastocytoma cells. Processing was assessed after 2 h of incubation by electrophoresis on a denaturing polyacrylamide gel (10). For complex formation experiments, the same transcripts were incubated for 30 min in heat-inactivated extract and then analysed on a non-denaturing polyacrylamide–agarose gel (5). The rationale for using heat-inactivated extract (where processing is abolished due to the inactivation of heat-labile processing factor; 18) rather than native extract is that, in heat-treated extract, no cleavage of substrate or dissociation of products can occur; thus the amount of complex formed is only a function of the affinity between pre-mRNA and the U7 snRNP. Although the binding behaviour of the U7 snRNPs may be altered by this treatment, it has been shown that heat-treated U7 snRNPs are still functionally active in complementation assays (18, 21) and that the binding between U7 snRNPs and pre-mRNA closely follows the predicted base-pairing stabilities of potential RNA–RNA hybrids (5).

Lack of evidence for a role of potential stem I

To test the importance of base-pairing in potential stem I, we interrupted 2 or all 8 of its base pairs. Compared to wild-type 12/12 RNA (Fig. 2A, lane 1), the processing efficiency of 12/st1.2 (lane 2) is only reduced by 15% (for quantitation, see Fig. 1). Interrupting the whole potential stem I (12/st1.8, lane 4) reduces processing efficiency by 43%. In addition to the changes in stem I, mutant 12/st1.8 has an additional alteration in 'bubble' 1 (U→C), but this does not affect the potential base-pairing with U7 RNA.

We then determined if the disruption of a potential hybrid could be compensated for by creating a base-pairing potential across

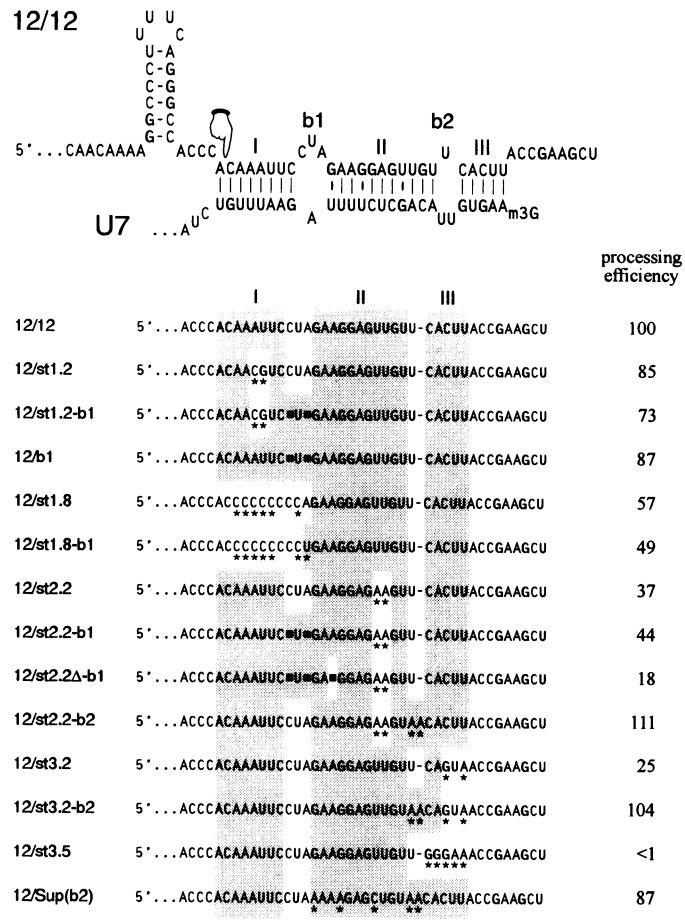

Figure 1. (**Top**) Schematic representation of potential hybrid between 12/12 histone pre-mRNA originating from the mouse *H4-12* gene (11) and U7 RNA (19). Three regions of base complementarity (potential stems I, II and III) are interrupted by two internal loops ('bubbles' b1 and b2). The major processing site is indicated by a pointing finger. (**Bottom**) Structures of mutant histone pre-mRNAs used in this work. All mutant RNAs are derivatives of 12/12 RNA. The 5′ parts of the RNAs, including the hairpin–loop structure and sequences at the processing site are identical. In the nomenclature used, st1, st2 and st3 indicate which stem structure was altered; the number after the period indicates how many potential base pairs of the stem are prevented by the mutation; b1 and b2 indicate when base complementarity across one of the internal loops was created. 12/st2.2Δ-b1 contains a single nucleotide deletion and was isolated in the screen for 12/st2.2-b1; it is probably the result of an error in oligonucleotide synthesis. Filled squares indicate deleted nucleotides, asterisks mark mutated positions. The shaded areas indicate sequence complementarity to mouse U7 RNA. (**Right**) Processing efficiencies determined from gels shown in Figs 2 and 4A and from repeat experiments. Autoradiograms were scanned densitometrically and molar percentages of processed RNA generated during the 2 h incubation calculated. Processing efficiencies are expressed in percent of 12/12 RNA, the control used in each experiment, whose absolute efficiency was 50.9 ± 6.6% (n = 8). Each value represents the average of 2–3 independent determinations, except for 12/st2.2Δ-b1 RNA which was analysed only once.

'bubble' 1. Processing efficiency of 12/st1.2-b1 (lane 3) is reduced by 14% compared to 12/st1.2. An identical reduction of processing efficiency can be measured in the case of 12/st1.8-b1 (lane 5) compared to 12/st1.8. In the context of wild-type stem I sequences, the same 'bubble' 1 mutation (12/b1) reduces processing efficiency by 13% (Fig. 2B, lane 5; but see also below and Fig. 6). It therefore appears that an additional base-pairing potential in 'bubble' 1 not only does not compensate

for the effect of destabilising mutations in potential stem I, but may even affect processing negatively.

To determine if the effect of mutating stem I sequences is due to inefficient base-pairing with U7 RNA, we analysed the intensity of U7–pre-mRNA hybrids in gel retardation experiments (Fig. 3A and B). On the autoradiogram of a typical non-denaturing gel, several bands are visible. One is formed by free histone pre-mRNAs (F), two are due to unknown complexes (Y and X) and one represents the complex formed with the U7 snRNP (U7; 5). Compared to 12/12 RNA (Fig. 3A, lane 1), the intensity of U7 complex formed with 12/st1.8 (lane 4) and 12/st1.8-b1 (lane 6), if anything, is slightly increased. Therefore the reduction in processing efficiency observed for these two mutants cannot be due to a reduced stability of the U7 interaction. The specificity of the analysed complex is illustrated by the fact that it can be super-shifted to the pocket of the gel by preincubation with the monoclonal anti-Sm antibody Y-12 (20; lanes 2, 5 and 7) and that it is not formed if the U7 RNA present in the extract has been destroyed with the help of a complementary oligonucleotide and RNAse H (data not shown). The control antibody D5 Ig did not alter the intensity or mobility of the bands formed with 12/12 RNA (lane 3). Of the other mutants, 12/st1.2 (Fig. 3B, lane 12) shows a slight reduction of complex intensity compared to 12/12 RNA (lane 1). Mutants 12/b1 and 12/st1.2-b1 (lanes 10 and 14) produce a more intense U7-specific complex than 12/12 RNA.

These data indicate that the rather modest effects of mutations in potential stem I are not due to a decrease in binding to the U7 snRNP. An increase in base-pairing in 'bubble' 1 may increase U7 binding, as revealed in the complex formation experiments, but has either no effect or a slight inhibitory effect on processing.

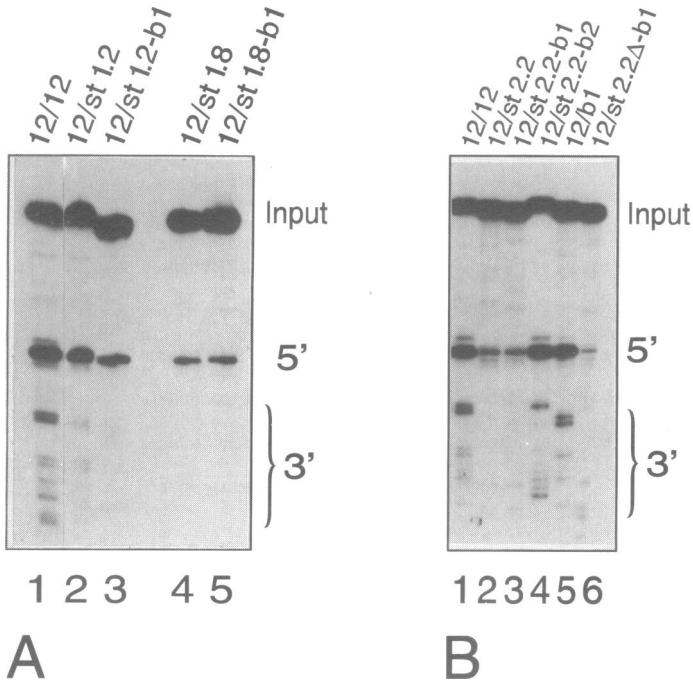

Figure 2. *In vitro* processing by mutants in the stem I/stem II region. (**A**) Mutations in stem I and 'bubble' 1. (**B**) Mutations in stem II and 'bubbles' 1 or 2. The names of the constructs used to generate universally labelled pre-mRNAs are indicated above each lane. For nomenclature see Fig. 1. The positions of unprocessed input RNAs and of processed 5′ and 3′ fragments are indicated.

The importance of the classical spacer element for 3′ end processing

We then tested the effects of a 2 base mutation in stem II (the classical spacer element) and analysed if this mutation (12/st2.2) could be compensated for by extending the hybrid through the neighboring 'bubbles' either in the 5′ (12/st2.2-b1) or in the 3′ direction (12/st2.2-b2). The processing efficiency of 12/st2.2 (Fig. 2B, lane 2) is reduced by 63% compared to 12/12 and cannot be compensated for by 12/st2.2-b1 (lane 3; 56% reduction from 12/12). Mutant 12/st2.2Δ-b1 (lane 6), a construct with a single nucleotide deletion in the spacer in additon to the changes as in 12/st2.2-b1, has the lowest processing efficiency (82%

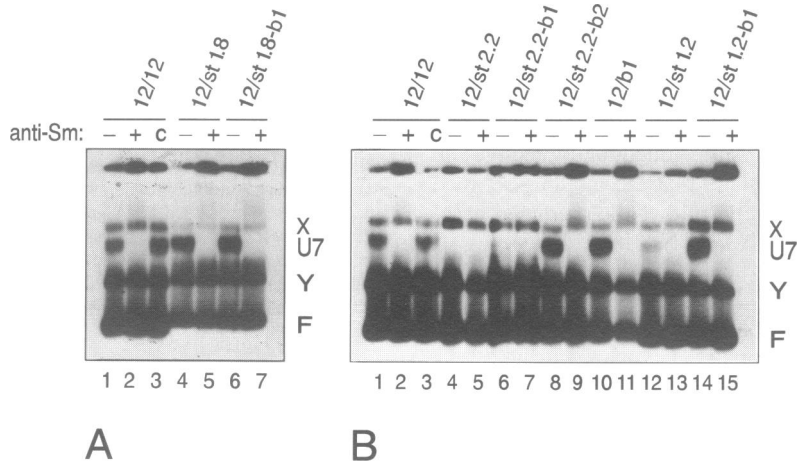

Figure 3. Native gel analysis of mutants in the stem I/stem II region. F, free RNA; U7, U7-specific complex; X and Y, unknown complexes (5). The names of the constructs used to generate universally labelled pre-mRNAs are indicated above the lanes. For nomenclature see Fig. 1. Incubations with monoclonal anti-Sm antibody Y-12 (+; 20) induce a shift of the U7-specific complex to the slot of the gel. Incubations without antibody (–) or with an unrelated antibody (c; see Materials and Methods) served as controls.

reduction from 12/12). However additonal base-pairing through 'bubble' 2 (12/st2.2-b2; lane 4) rescues the stem II down-mutation up to 111% of the original 12/12 RNA (lane 1). As already mentioned, 12/b1 (lane 5) is processed with slightly lower efficiency than 12/12 RNA. It must also be noted that most of these spacer mutations alter the pattern of 3′ products which are generated during the reaction.

For this set of mutants, the formation of a U7–pre-mRNA complex correlates reasonably well with the measured processing efficiencies and with the predicted hybrid stabilities (Fig. 3B). U7 complexes are not detectable for 12/st2.2 (lane 4) and at the most very weakly for 12/st2.2-b1 (lane 6). Mutant 12/st2.2-b2 (lane 8) produces an even more intensive U7 complex than the original 12/12 RNA (lane 1).

These results confirm the importance of base-pairing between the histone pre-mRNA and U7 RNA in the classical spacer element. Base-pairing can be extended through 'bubble' 2 to compensate for a reduction in processing efficiency caused by a two base mutation in stem II. Conversely, base-pairing in the region of 'bubble' 1 does not lead to better processing.

A novel base-pairing interaction in stem III

The potential hybrid in stem III has not been noticed previously and its role has therefore not been investigated. We made constructs containing 2 or 5 base mutations in stem III (12/st3.2 and 12/st3.5). Thus, in 12/st3.5, the potential to form stem III is completely abolished. In mutant 12/st3.2 (Fig. 4A, lane 3), processing efficiency is reduced by 75% and this effect is compensated for by allowing additional base-pairing in the region of 'bubble' 2 (12/st3.2-b2; lane 4; processing efficiency 104% of 12/12). Processing of 12/st3.5 RNA (lane 5) is completely abolished. Therefore stem III seems to be an important element in the 3′ end cleavage reaction. Construct 12/Sup(b2) (lane 2) is perfectly complementary to the first 20 nt of U7 RNA, i.e. throughout the entire stem II–'bubble' 2–stem III region. Thus it is not a pure 12/b2 construct in the sense that, of the original G–U base pairs in stem II, two have been replaced by A–U and one by G–C. In accord with previous results (10), 12/Sup(b2) is not processed more efficiently than 12/12 RNA (87% of 12/12 in these experiments; see also Fig. 6) and two different 5′ fragments of equal intensity are formed, whereas, for 12/12 RNA the shorter product is formed preferentially.

Complex formation experiments indicate that the effect of mutations in stem III are due to differences in binding of the U7 snRNP. Mutants 12/st3.2 (Fig. 4B, lane 4) and 12/st3.5 (lane 8) both form no detectable U7 complex. Mutant 12/st3.2-b2 (lane 6) forms a slightly stronger complex than 12/12 RNA (lane 1). As might be expected, 12/Sup(b2) RNA produces the most intensive band (lane 10).

Interestingly, complex X disappears in all mutations in which stem III has been changed. Therefore this experiment provides first evidence as to the possible specificity of this complex. An additional band, migrating slightly slower than complex Y is formed with mutants 12/st3.2 and 12/st3.2-b2 (lanes 4–7). This has not been further characterised. Most importantly, however, these experiments demonstrate that a base-pairing interaction in the region of stem III is crucial for histone RNA 3′ processing.

Suppression of a stem III mutation by a complementary change in U7 RNA

As final proof that the novel stem III is indeed formed and contributes to efficient histone RNA processing we wanted to

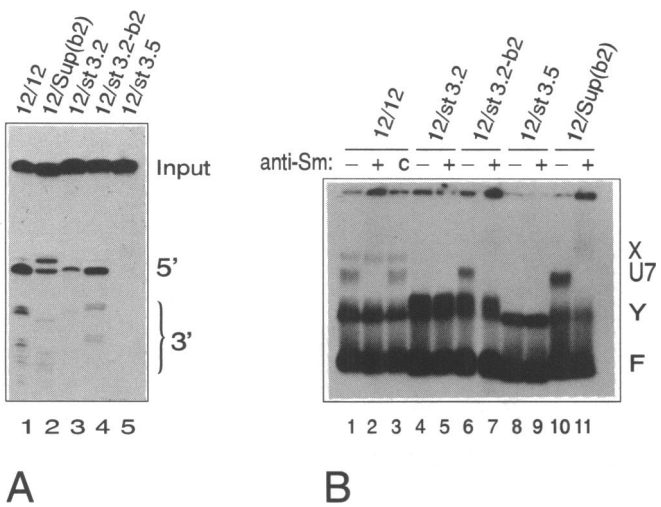

Figure 4. (**A**) *In vitro* processing and (**B**) native gel analysis of mutants in the stem III region. The names of the constructs used to generate universally labelled pre-mRNAs are indicated above each lane. For nomenclature see Fig. 1. (**A**) The positions of unprocessed input RNAs and of processed 5′ and 3′ fragments are indicated. (B) F, free RNA; U7, U7-specific complex; X and Y, unknown complexes (5). Incubations with monoclonal anti-Sm antibody Y-12 (+; 20) induce a shift of the U7-specific complex to the slot of the gel. Incubations without antibody (–) or with an unrelated antibody (c; see Materials and Methods) served as controls.

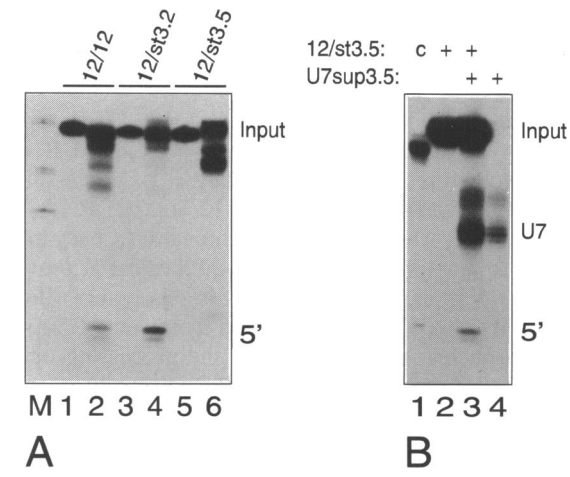

Figure 5. Suppression of a stem III muation by complementary base changes in U7 RNA. (**A**) Processing of stem III mutations in *Xenopus laevis* oocytes. Histone pre-mRNAs 12/12 (lanes 1, 2), 12/st3.2 (lanes 3, 4) and 12/st3.5 (lanes 5, 6) were injected into oocyte nuclei. After 2 h, the RNA was extracted and analysed on denaturing polyacrylamide gels. Odd numbered lanes, uninjected controls; even numbered lanes, injected RNAs; lane M, pBR322 marker plasmid cut with *Hpa*II (fragments of 90, 76 and 67 nucleotides are visible). The positions of unprocessed input RNAs and of processed 5′ fragments are indicated. Some unspecific degradation products are visible in lane 6. (**B**) Suppression by complementary U7 mutant. *In vitro*-transcribed U7sup3.5 RNA (U7) was injected into the cytoplasm (lanes 3, 4) and the oocytes incubated overnight to allow for snRNP assembly. The oocytes were then injected intranuclearly with 12/12 (lane 1, c; less material loaded) or 12/st3.5 RNA (lanes 2, 3) and analysed as in (A). The U7sup3.5 transcript shows some size heterogeneity; the shorter, more intense band has the correct size.

rescue a stem III mutation by a compensatory base change in U7 RNA. Such experiments have previously been described for sea urchin genes tested in *Xenopus laevis* oocytes (3) and for mammalian genes tested in HeLa cells (4). We have recently established a similar complementation assay for mouse genes using *Xenopus* oocytes (12; B.Stefanovic, W.Hackl, R.Lührmann and D.Schümperli, submitted for publication). We therefore injected 12/12, 12/st3.2 and 12/st3.5 RNAs into the nuclei of oocytes and tested how efficiently they could be handled by the *Xenopus* processing machinery. In contrast to our findings in the mammalian *in vitro* system, 12/st3.2 (Fig. 5A, lane 4) was processed at least as well as 12/12 RNA (lane 1). For 12/st3.5 RNA, some unspecific degradation products were formed (lane 6), but neither in this nor in a separate, cleaner experiment (Fig. 5B, lane 2) could any mature processing product be detected.

We therefore mutated a template for mouse U7 RNA to make it complementary to the 12/st3.5 mutation. The T7 transcript from this template (U7sup3.5 RNA) is equivalent to U7 RNA but has the first 5 nucleotides altered to allow base-pairing with 12/st3.5 RNA. In addition, the RNA has two extra Gs at the 5′ end (to allow efficient *in vitro* transcription) and an extra C at the 3′ end. The corresponding wild-type U7 RNA forms functional U7 snRNPs when injected into the cytoplasm of *Xenopus* oocytes (B.Stefanovic, W.Hackl, R.Lührmann and D.Schümperli, submitted for publication). Oocytes were injected with U7sup3.5 RNA, incubated overnight to allow for snRNP assembly and then injected intranuclearly with 12/st3.5 RNA. The resulting oocytes therefore contain two labelled RNAs. Most importantly, a product corresponding to processed 12/st3.5 RNA is formed in the oocytes having received both injections (Fig. 5B, lane 3), but not in the oocytes having received a single injection of either 12/st3.5 (lane 2) or U7sup3.5 RNA (lane 4).

Effects of temperature and hairpin factor binding on U7–pre-mRNA interactions

To further define the importance of the histone–U7 base-pairing interactions, we studied the processing of selected RNAs at elevated temperatures. Quantitative analyses of processing gels with a PhosphorImager showed that 12/Sup(b2) RNA, when incubated at 30°C, was processed with similar efficiency to 12/12 RNA (Fig. 6), in agreement with results presented previously (10; Fig. 4). A slight (20%) reduction in processing efficiency was measurable for 12/b1 RNA. When these same RNAs were incubated in nuclear extract at 40°C, the processing of 12/12 and 12/b1 RNAs was significantly reduced, but processing of 12/Sup(b2) RNA was even increased. This is in keeping with the notion that base-pairing through 'bubble' 2 contributes to the stability of the U7–pre-mRNA complex. However since the reduction in processing was more pronounced for 12/12 than for 12/b1 RNA, it appears that potential base-pairing through 'bubble' 1 (which is neutral or slightly inhibitory at 30°C) may contribute to more efficient processing at higher temperatures.

Also shown in Figure 6 are experiments carried out with two further mutants, B/12 and B/Sup(b2), which are identical to 12/12 and 12/Sup(b2) in the spacer region (10), but carry a mutated hairpin sequence that is unable to bind the nuclear hairpin binding factor (HBF). In the present experiments, when incubated at 30°C, processing of B/12 RNA is reduced by 37% with respect to 12/12 (reduction of 20% reported in ref. 10), whereas B/Sup(b2) RNA is processed with similar efficiency to 12/12. However, at 40°C, processing is virtually abolished for B/12 but is increased for B/Sup(b2) to a similar level as for 12/Sup(b2) RNA. Thus HBF interactions become more important when the spacer interaction with U7 RNA is weakened (by increased temperature) and become completely dispensable if the spacer interaction is strengthened [in B/Sup(b2)].

This is confirmed by an identical set of experiments carried out in the presence of a 200-fold excess of a short unlabelled RNA that contains only the histone stem–loop structure with 8 and 4 nucleotides of 5′ and 3′ flanking sequences, respectively (see Materials and Methods). At 30°C, this competitor RNA had only small effects on processing of 12/12 and 12/Sup(b2) RNAs (12–16% reduction). This is in keeping with our previous data indicating that processing of pre-mRNA from the *H4-12* gene in nuclear extract from K21 cells is largely independent of hairpin interactions under these conditions (10). However, in the case of 12/b1, processing efficiency was reduced by about 40%, indicating that this mutant has become more dependent on HBF interactions. At 40°C, however, processing of 12/12 and 12/b1 RNAs was equally strongly inhibited by the competitor RNA. The inefficient processing of B/12 and the efficient processing of 12/Sup(b2) and B/Sup(b2) RNAs remained unaffected. Very similar results to the ones shown in Fig. 6 were also obtained with incubations carried out at 43°C, although the overall processing efficiency was substantially lower, presumably because of irreversible damage to some of the processing components (data not shown). At 47°C, processing was almost completely abolished.

DISCUSSION

Extent and possible functions of base-pairing interactions between histone pre-mRNA spacer sequences and U7 RNA

In this work, we have defined the limits of the region in histone pre-mRNA which contributes to the formation of a binary complex by base-pairing with U7 RNA in a mammalian *in vitro* processing system. The contributing regions called stems II and III and 'bubble' 2 collectively extend over nucleotides 1–18 of

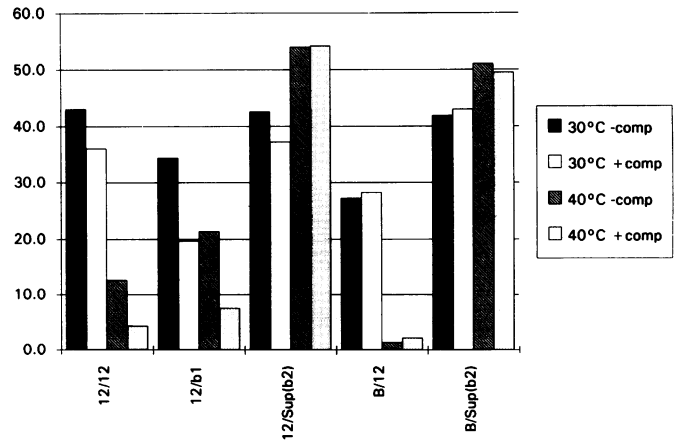

Figure 6. Effects of temperature and hairpin interactions on *in vitro* processing. Processing of the indicated RNAs was carried out at 30°C and 40°C and in the presence or absence of a 200-fold excess of unlabelled competitor RNA containing the hairpin element (see Materials and Methods). Processing was quantitated on a PhosphorImager and is expressed as molar percent of input RNA processed after a 2 h incubation. Each bar represents the average of three independent measurements.

mouse U7 RNA. We have found by primer extension analysis that human (and bovine) U7 RNAs are lacking the first nucleotide present in mouse U7 RNA (22); thus, for these species, the first 17 nucleotides of U7 RNA could interact with histone pre-mRNAs. It is possible that this region may extend 1–2 nucleotides further, although the results with mutants allowing base-pairing across 'bubble' 1, at least when tested at 30°C, seem to argue against this possibility. Although a potential for base-pairing across 'bubble' 1 increased formation of a U7-specific complex for 12/12 and 12/st1.2 (Fig.3), in none of the four different contexts did such base-pairing augment the efficiency of processing and in some cases processing was even inhibited (Fig. 2). However, since the mutation used to allow base-pairing across 'bubble' 1 involved deleting two nucleotides, this shortening could have neutralised a potential beneficial effect of the increased base-pairing. Consistent with this notion is the fact that processing of 12/b1 RNA is more dependent on HBF interactions than 12/12 RNA at 30°C, but is slightly more efficient at elevated temperatures (Fig. 6). Despite this remaining uncertainty about the region of 'bubble' 1, our present definition of the possible base-pairing region agrees well with the fact that the first 18–20 nucleotides of U7 RNA are susceptible to nucleases and can bind complementary oligonucleotides (19, 23, 24).

The novel finding in this work is the recognition of stem III as an important contribution to the stability of the U7–pre-mRNA complex and to efficient processing. Mutations in stem III have equally strong effects on both parameters, as do mutations in the 'conventional' spacer element, stem II (Figs 2–4). Moreover, suppression of a stem III mutation by a complementary U7 mutation in *Xenopus* oocytes (Fig. 5) confirms that stem III interactions are important for efficient histone RNA processing. Although it has not been recognised previously, a hybrid of typically 3–5 bp corresponding to stem III can be formed with all mammalian histone pre-mRNAs whose sequence is known. Stem III considerably adds to the stability of the U7–pre-mRNA hybrid. As already mentioned in the Introduction, this may be partly due to the fact that the 'conventional' spacer element is less well conserved than in sea urchins. However, this degeneracy may be relatively unimportant functionally, if one accepts G–U wobbles as *bona fide* RNA base pairs. Rather, it is likely that the increased length of possible histone–U7 base-pairing in mammals is related to increased body temperature, since, at 40°C, a further increase in hybrid stability over that of 12/12 RNA can contribute to more efficient processing (Fig. 6). This idea is supported by the fact that the 12/st3.2 mutation is not inhibitory in *Xenopus* oocytes (incubated at 18°C), in contrast to its significant deficiency in the mammalian *in vitro* system.

It is also noteworthy that the novel stem III involves nucleotides at the very 5′ end of U7 RNA. Although only six base pairs can be formed in sea urchins, the base-pairing also extends to the 5′ end of U7 RNA (3, 7). Thus an involvement of these first nucleotides seems to be a conserved feature of the processing reaction.

Although the limits of the potential base-pairing region have now been defined, it is not yet clear whether all nucleotides or base pairs in this region play equivalent roles. In their mutational analysis, Bond *et al.* (4) obtained evidence that certain nucleotides or base pairs in the 'conventional' spacer element (stem II) may be more important than others, although the effects of these mutations on the potential stability of the entire hybrid were not examined systematically. However, the same study also revealed that certain mutations in the pre-mRNA may be less efficiently suppressed by complementary base changes in U7 RNA than others. This could mean that parts of the RNA–RNA hybrid, besides the stabilising function of their base pairs, may also have to be conserved in primary sequence and/or to be present in a particular three-dimensional conformation to allow optimal histone RNA processing. A possible reason for such primary sequence requirements is that the 3′ products generated during the processing reaction at least partly result from additional cleavages within the proximal portion of stem II and that these cleavages may have some sequence specificity (10, 25). Alternatively, purine–pyrimidine stretches such as are present in stem II may assume a special three-dimensional conformation and/or provide binding sites for small cofactors such as metal ions or nucleotides. However such an explanation seems less likely, considering the fact that histone RNA 3′ processing in nuclear extracts from mammalian cells is independent of ATP and metal ions (25).

What may be the role of the 'stem I' region?

Destabilising mutations in the putative stem I had relatively mild effects on processing efficiency and did not impair the ability of the pre-mRNA to form a U7-specific complex (Figs 2 and 3). This strongly argues against this region being involved in base pairing with U7 RNA. Nevertheless it is evident that certain mutations, such as 12/st1.8, do impair processing without affecting the formation of a U7–pre-mRNA complex. This is in agreement with a result of Georgiev and Birnstiel (26) who introduced an *Eco*RI site between the hairpin and spacer elements of a sea urchin H3 gene, which impaired but did not completely prevent U7 RNA-dependent 3′ processing. What further argues against an extensive base pairing in this region are the facts that the corresponding region of U7 RNA is involved in protein interactions (12, 27; B.Stefanovic, W.Hackl, R.Lührmann and D.Schümperli, manuscript in preparation) and that only very limited base complementarity to U7 RNA is found in this region in other vertebrate histone genes.

Nevertheless, it is clear that mutations in this region and perhaps at the processing site itself do affect processing efficiency. Thus nucleotides in this region may interact with other components of the processing apparatus, such as proteins of the U7 snRNP or the so far unidentified cleavage component. Even base-pairing contacts with U7 RNA cannot be ruled out, provided that they are very limited in extent and serve other purposes than to stabilise the U7–pre-mRNA complex, e.g. to specify the cleavage site. A systematic mutational analysis of this region might shed more light on this question.

A possible interplay between the hairpin binding factor and the U7 snRNP

The hairpin loop element of histone pre-mRNA is required for 3′ processing and production of mature histone mRNA *in vivo* (28–30). *In vitro*, the hairpin has been shown to interact with a processing factor (hairpin binding factor, HBF; 31, 32) which has somewhat variable effects on processing efficiency, but is not absolutely required for the reaction (10, 32, 33). Little is known so far about the exact role played by HBF in the processing reaction. One of our present experiments demonstrates that processing of pre-mRNA from the mouse *H4-12* gene, which, in K21 nuclear extract and at 30°C, is relatively independent of HBF interactions (10), becomes severely impaired at 40°C if these HBF interactions are prevented (Fig. 6). A less significant

reduction in processing is observed if the wild-type hairpin sequence is present and is allowed to interact with HBF. However, if the base-pairing potential of the pre-mRNA with U7 RNA is improved by the Sup(b2) mutation, processing is still efficient independently of HBF interactions even at 40°C. Thus, with a weak U7–pre-mRNA hybrid, HBF interactions are more important than if this hybrid is very stable. This is reminiscent of our previous experiments using a HeLa cell nuclear extract, where processing of 12/12 RNA was found to be HBF-dependent (10). There, the impairment of processing caused by preventing HBF interactions could also be counteracted by improving the base-pairing potential with U7 RNA.

Taken together, these results provide a strong argument for the notion that binding of HBF to the hairpin–loop element contributes to efficient processing by stabilising the interaction between the histone pre-mRNA and the U7 snRNP. If this is true, then HBF, besides binding to the hairpin–loop element, must also have some affinity for the U7 snRNP. Although non-denaturing gel analysis can be used to demonstrate interactions of histone pre-mRNA with either HBF or the U7 snRNP (5), we have never detected a complex containing all three components. However, the failure to detect this ternary complex may have trivial technical reasons and such an interaction should be re-investigated as other methods become available.

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