Both Conserved Signals on Mammalian Histone Pre-mRNAs Associate with Small Nuclear Ribonucleoproteins during 3' End Formation In Vitro

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Pre-mRNA substrates containing sequences from human and mouse histone genes are accurately processed in a HeLa cell nuclear extract to generate mature 3' termini. When in vitro processing reactions containing either human histone H3 or mouse histone H3 transcripts are treated with RNase T₁ and probed with antibodies specific for the Sm protein determinants or for the trimethylguanosine cap structure unique to the U RNAs present in small nuclear ribonucleoproteins, RNA fragments that encompass the site of 3' end formation on the pre-mRNA transcript are selectively recovered. Several different interactions are detected: at time zero, the protected region contains the upstream conserved hairpin loop structure; at later times during the reaction, protection extends beyond the site of 3' end formation to include the downstream conserved sequence element and the 5' cap of the transcript is bound as well. Possible interactions between Sm small nuclear ribonucleoproteins and these conserved sequence elements in histone pre-mRNAs are discussed.

Small nuclear ribonucleoprotein particles (snRNPs) are highly conserved RNA-protein complexes found in the nuclei of eucaryotic cells (reviewed in references 11 and 41). snRNPs containing U RNAs—U1, U2, U4, U5, and U6 (from vertebrate cells) and U7 (from the sea urchin)—can be immunoprecipitated with antisera of the Sm specificity obtained from patients with autoimmune disease (30, 42). The antigenic determinants recognized by anti-Sm antibodies reside on several proteins common to the different particles (38); the U RNA moieties of snRNPs are specifically bound by anti-trimethylguanosine (m₃G) antibodies, which target the unusual 5' cap structure of small nuclear RNAs (10, 13).

Recent studies have assigned roles for a number of the Sm snRNPs in RNA processing. The most abundant particles, containing U1, U2, U5 (reviewed in reference 37), or U4 and U6 RNAs (2, 9), are all implicated in pre-mRNA splicing. Direct interactions with the pre-mRNA have been suggested for several of these: the U1 snRNP binds the 5' splice site (8, 35, 48), a protein probably associated with the U5 snRNP recognizes the 3' splice site (13, 20), and the U2 snRNP interacts with the branch point in the intron (8, 14).

An as yet unidentified Sm snRNP has also been implicated in polyadenylation (33), a 3' processing event involving cleavage of a longer precursor RNA (34, 36; for a review, see reference 7). This snRNP interacts, either directly or via some other component, with the highly conserved AAUAAA signal located upstream from the cut site (24).

The 3' ends of histone mRNAs are likewise generated by RNA processing (6, 7, 28). Two conserved sequence elements flank the site of 3' end formation in histone pre-mRNAs and have been shown to be required for maturation of sea urchin histone transcripts in the *Xenopus* oocyte (4-6, 18). The upstream element is a hairpin loop structure which is highly conserved among the nonpolyadenylated histone mRNAs of all species analyzed (reviewed in references 26 and 46). The consensus among vertebrates for this element is GGC₃CTTTTCAG₃GCC (46). The downstream element is

absolutely conserved among sea urchin histone transcripts and consists of a CAAGAAAGA sequence lying 13 or 14 nucleotides 3' to the mature 3' end (12). In the vertebrate histone genes that have been analyzed, however, the downstream element is not highly conserved (7, 46). The consensus G₃AAAGA has been suggested (7), but the fit of any individual histone gene to this consensus is rather loose.

The U7 snRNP, a low-abundance Sm particle in the sea urchin, has been shown to be required for the correct 3' end processing of sea urchin histone H3 mRNA in the *Xenopus* oocyte (17). The 60-nucleotide-long U7 RNA has been proposed to interact with both of the conserved sequence elements in the pre-mRNA through base pairing interactions (43). A recent experiment, in which a processing-defective mutation in the CAAGAAAGA box of sea urchin H3 was suppressed by compensating base changes in the U7 RNA (40), provides strong support for such an interaction with the downstream element.

By analogy with the sea urchin system, a low-abundance Sm snRNP may participate in the 3' end formation of mammalian histone pre-mRNAs (21). As a first step toward identifying this particle and delineating its role in RNA processing, we have developed an in vitro system that accurately matures the 3' ends of model histone transcripts. Using this extract as a source of snRNPs, we have performed binding and RNase protection experiments to identify regions on the pre-mRNA substrate which are specifically recognized by Sm snRNP particles. Two distinct interactions involving the region around the processing site have been detected. Early in the reaction, protected fragments containing the upstream element are selectively recovered by using snRNP-specific antibodies. At later times, the interaction extends to include conserved sequences downstream of the site of 3' end formation.

MATERIALS AND METHODS

Preparation of model histone pre-mRNA substrates. Plasmid pSPmH33' (provided by D. Black) was constructed by standard cloning methods (31) with a 217-base-pair (bp)

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*Pst*I-*Dde*I fragment from pH3614 (a gift of W. Marzluff [44]) which contains 134 bp of the coding sequence for mouse histone H3 and 83 bp of 3' noncoding sequences. The *Dde*I site was filled in with the Klenow fragment of DNA polymerase I (provided by C. Joyce), and the resulting DNA fragment was ligated into pSP64 (32) which had been cleaved with *Pst*I and *Sma*I. Cleavage of pSPmH33' with *Eco*RI followed by transcription with SP6 RNA polymerase (32) generates a 249-nucleotide runoff transcript with the site of 3' end formation 200 nucleotides from the 5' end. Plasmid pSPmH33' was constructed with a 271-bp *Sac*I-*Ava*II fragment of pHh5B (a gift of N. Heintz [47]) which includes 149 bp of coding sequence for human histone H3 and 122 bp of 3' noncoding sequences. The blunt ended *Sac*I-*Ava*II fragment was ligated into pSP65 (32) which had been cut with *Sac*I and *Sma*I. Cleavage of pSPmH33' with *Bst*II (an isoschizomer of *Bam*HI, kindly provided by C. Joyce) followed by transcription with SP6 RNA polymerase generates a 293-nucleotide runoff transcript with the site of 3'-end formation 222 nucleotides from the 5' end. Similar constructions were made using sequences from human H2A (130 bp of coding sequences, 110 bp of 3' noncoding sequences), H2B (145 bp of coding sequences, 310 bp of 3' noncoding sequences), and H4 (200 bp of coding sequences, 250 bp of 3' noncoding sequences) genes (all provided by N. Heintz).

Precursors for the in vitro processing reactions were transcribed with SP6 RNA polymerase as described in Black et al. (8), except that 25 μ Ci of [α -³²P]GTP (410 Ci/mmol; Amersham Corp.) was used in a reaction volume of 25 μ l. The RNA transcripts were isolated after electrophoresis on a 4% polyacrylamide-8 M urea gel.

The substrates used in RNase T₁ protection and immunoprecipitation experiments were labeled to a high specific activity at G residues and to a lower specific activity at C and U residues. The transcription reactions contained 40 mM Tris hydrochloride (pH 7.5), 6 mM MgCl₂, 4 mM spermidine, 16 mM dithiothreitol, 0.4 mM G(5')ppp (5')G, 0.25 mM ATP, 10 μ M each UTP and CTP, 40 μ Ci each of [α -³²P]CTP and [α -³²P]UTP (410 Ci/mmol), 125 μ Ci of [α -³²P]GTP (410 Ci/mmol), 30 U of SP6 RNA polymerase (Boehringer Mannheim Biochemicals), and 2.5 μ g of template DNA in 25 μ l. After incubation for 1 h at 40°C, RNA transcripts were gel purified as described above.

Nuclear extract and processing reactions. The nuclear extract was prepared by the method of Dignam et al. (15), incorporating modifications of Heintz and Roeder (25). The processing reactions contained 20% (vol/vol) nuclear extract in buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride); 40 mM KCl (to give a final concentration of 60 mM); 0.2 mM each ATP, UTP, CTP, and GTP; 5 mM creatine phosphate; and 40,000 cpm (Cerenkov) of precursor RNA in a final volume of 10 μ l. The reactions were stopped after incubation at 30°C by the addition of 10 μ l of proteinase K solution (2 mg of proteinase K per ml, 1 mg of yeast tRNA per ml, 1% [wt/vol] sodium dodecyl sulfate, and 10 mM EDTA) and 15 min of incubation at 65°C. The samples were extracted with phenol-chloroform-isoamyl alcohol (50:49:1), ethanol precipitated, and resolved on 6% polyacrylamide (19:1 ratio of acrylamide to bisacrylamide)-8 M urea gels in TBE buffer (90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA).

S1 nuclease mapping. To prepare a probe for mapping the 3' end of mouse histone H3 RNA, the *Stu*I site of pSPmH33' was labeled at its 3' end by incubating *Stu*I-digested plasmid

in the presence of 200 μ M dCTP and T4 DNA polymerase (New England Biolabs) followed by the addition of 200 μ M dTTP and 50 μ Ci each of [α -³²P]dATP and [α -³²P]dGTP (3,000 Ci/mmol; Amersham). The plasmid was then cut with *Eco*RI, and the 213-nucleotide probe strand was isolated from a 6% polyacrylamide-8 M urea gel in 0.5 $\times$ TBE buffer.

RNA samples were prepared from processing reactions as described above. Gel-purified precursor and product RNA were prepared by eluting slices of a 6% polyacrylamide gel for 16 h at 25°C in 0.5 M sodium acetate-10 mM Tris hydrochloride (pH 7.5)-10 mM EDTA-0.1% (wt/vol) sodium dodecyl sulfate. Cytoplasmic RNA was prepared from the postnuclear supernatant of mouse L cells lysed with Nonidet P-40 (3).

The hybridization reactions and S1 digestions were done as described by Berk and Sharp (3) and modified by Weaver and Weissmann (45). Hybridization was for 3 h at 25°C, and the S1 digestions were done with 200 U of S1 nuclease (PL Biochemicals) for 30 min at 25°C. The S1-resistant DNA was analyzed on 6% polyacrylamide-8 M urea gels in TBE buffer.

RNase T₁ protection and immunoprecipitation analyses. Antibodies were prebound to protein A-Sepharose (Pharmacia Fine Chemicals) by mixing 20 μ l of of ascites fluid (Sm) or antiserum with 4 mg of protein A-Sepharose in 0.4 ml of NET-2 buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Nonidet P-40). Binding was for 1 h at 25°C followed by four washes with NET-2 buffer. The antibodies used in these experiments were Y12, an anti-Sm mouse monoclonal (29), normal human serum, and rabbit preimmune and anti-m₃G sera (13). The amount of Y12 used in protection experiments was titrated to ensure that it was adequate to bind all Sm snRNPs. Anti-m₃G serum was used in suboptimal amounts because of the limited availability and low titer of the antibody.

Processing reactions were as described above except that a final volume of 20 μ l was used, with 3×10^6 to 8×10^6 (Cerenkov) cpm of transcript in each reaction. After appropriate incubation at 30°C, 6.5 μ l of a 50-U/ μ l solution of T₁ RNase (Calbiochem) was added to the 20- μ l processing reaction and incubated for 30 min on ice. The entire reaction was then added to the prebound antibodies on protein A-Sepharose beads and incubated for 30 min on ice with gentle mixing. The samples were pelleted, washed four times with NET-2 buffer, extracted with phenol-chloroform-isoamyl alcohol, and ethanol precipitated. The RNA fragments were resolved on a 15% polyacrylamide-8 M urea gel in TBE buffer.

To analyze the labeled RNA fragments resolved in the 15% gel, fragments were cut from the gel and eluted in a buffer containing 0.5 M sodium acetate-10 mM Tris hydrochloride (pH 7.5)-10 mM EDTA-0.1% (wt/vol) sodium dodecyl sulfate and ethanol precipitated. Each fragment was analyzed by complete digestion with T₁ RNase at 3.5 U/ μ l. The resulting T₁ oligonucleotides were separated on a 20% polyacrylamide gel, cut and eluted from the gel, and subjected to secondary analysis with RNase A. RNase T₁ fingerprint analysis, as described by Barrell (1) was performed on some of the protected RNA fragments. The first dimension was electrophoresis on cellulose acetate strips (Schleicher & Schuell Co.) at pH 3.5 followed by chromatography on polyethyleneimine thin-layer plates (Brinkmann) as the second dimension. This was followed by RNase A secondary analysis of each oligonucleotide with electrophoresis on DEAE paper at pH 3.5.

FIG. 1. Time course of in vitro processing of the mouse histone H3 pre-mRNA. Precursor RNA was incubated at 30°C in the presence of nuclear extract as described in Materials and Methods. RNAs extracted from samples withdrawn at the times indicated (in minutes) were electrophoresed on a 6% polyacrylamide gel. The resulting autoradiogram is shown, indicating the positions of the 248-nucleotide precursor RNA and the product RNA of ~200 nucleotides at the right. The sizes (in nucleotides) of the DNA markers (lane M) are labeled at the left.

RESULTS

Histone pre-mRNA substrates are accurately processed in vitro. Model histone pre-mRNA substrates were generated by transcribing recombinant DNAs containing the 3' portions of several different mammalian histone genes, each cloned behind an SP6 promoter. When these ³²P-labeled substrates were incubated in a HeLa cell nuclear extract (15, 25), RNA species accumulated whose lengths corresponded to those expected if in vitro processing were generating the proper 3' end. The time course of the reaction for a mouse histone H3 substrate is shown in Fig. 1. The 248-nucleotide precursor (lane 1), upon incubation at 30°C in the nuclear extract, gave rise to a product of about 200 nucleotides with little or no lag (lanes 2 through 8). Little additional product was generated after 1 h (lanes 6 through 8). Substrates containing 3' end sequences from human histone genes H2A, H2B, H3 and H4 similarly yielded products of the expected size when incubated in the extract (data not shown).

Before initiating binding studies, the reaction conditions were varied to optimize product formation for the two substrates to be analyzed in detail: mouse histone H3 and human histone H3 (data not shown). We find that the appearance of product is dependent upon the presence of

nuclear extract, the optimum being 15 to 20% extract in the reaction. The activity is heat labile and sensitive to protease treatment. The salt optimum is rather broad for both substrates, peaking around 60 mM KCl, with 50% activity at 20 mM and 80% activity at 100 mM. Processing reactions were routinely performed with added nucleotides and creatine phosphate; however, reactions performed in their absence exhibited no apparent requirement for an energy source. As reported by Gick et al. (21), the 3' end processing of histone

FIG. 2. S1 nuclease analyses of mouse histone H3 transcripts. A single-stranded probe was prepared from a 213-bp *StuI-EcoRI* fragment of pSPmH33' labeled at the 3' end of the *StuI* site. The probe was hybridized with RNA samples prepared from in vitro processing reactions similar to that in Fig. 1 at 0 (lane 1), 30 (lane 2), and 60 min (lane 3); to gel-purified precursor (lane 6) and product (lane 7) RNA from a 60-min processing reaction; and to in vivo synthesized RNA isolated from the cytoplasm of mouse L cells (lanes 4 and 8). The hybrids were treated with S1 nuclease, and the protected DNA fragments were resolved on a 6% polyacrylamide gel. The positions of the DNA fragments generated by protection with the full-length precursor (213 bp), the double-stranded probe (209 bp), and RNAs with mature 3' termini (165 bp) are indicated in the center and in the diagram at the bottom. The sizes of the DNA markers (lane M) are labeled on the right in nucleotides, and a sequencing ladder of the probe DNA with cleavage at A's and G's is shown in lane 5. The ~115-nucleotide fragment generated with the L-cell RNA (lanes 4 and 8) would correspond to protection extending only to the termination codon and thus probably represents mRNA expressed from other H3 alleles.

substrates does not require added Mg^{2+} and will in fact proceed in 20 mM EDTA. In the absence of added Mg^{2+} , the efficiency of product formation depends on the gene used for substrate: 1 to 5% of the human H3 precursor is converted to product, whereas for the mouse H3 precursor the efficiency of product formation is 30 to 40% (Fig. 1). The efficient processing of the latter transcript is consistent with the observation that this mouse H3 gene is highly expressed in vivo (22).

To determine whether in vitro processing produces the correct 3' end on histone pre-mRNAs, we used S1 nuclease mapping to compare the termini generated in vitro in the absence of Mg^{2+} with those found on in vivo-synthesized histone mRNAs (Fig. 2). To map the 3' ends of the mouse H3 RNAs, a 213-bp *StuI-EcoRI* fragment from pSPmH33' (see Materials and Methods) was labeled at the 3' end of the *StuI* site with T4 DNA polymerase, and a single-stranded probe was prepared. Total RNA extracted at various times from the processing reaction, as well as gel-purified precursor RNA, gel-purified product RNA, and in vivo RNA rapidly isolated from the cytoplasm of mouse L cells were hybridized to the probe. The hybrids were treated with S1 nuclease, and the products were resolved on a 6% polyacrylamide

gel with an adjacent sequencing lane. As the in vitro reaction proceeded, increasing levels of a 165-nucleotide S1 nuclease-resistant fragment appeared in the analyses (Fig. 2, lanes 1 through 3). The gel-purified product RNA generated an identically sized S1-protected fragment (lane 7), as does the mouse L cell RNA (lanes 4 and 8). S1 nuclease analyses comparing the in vivo and in vitro products of the human H3 gene (data not shown) gave similar results to those presented for the mouse H3 substrate. Within the limits of resolution of the S1 mapping procedure (one or two nucleotides), these analyses establish that the 3' ends generated in vitro are indistinguishable from the 3' ends formed in vivo for the mouse and human H3 histone mRNAs.

RNA fragments containing the upstream conserved signals are precipitated by anti-Sm antibodies from a processing reaction at zero time. Purified ^{32}P -labeled SP6 transcripts of the mouse and human H3 histone genes were added to the nuclear extract under optimal processing conditions. Samples were removed from the mixture at various times during the reaction. RNase T_1 was added to degrade the transcript except where protected by bound factors. Subsequently, monoclonal anti-Sm antibodies bound to protein A (29) were used to select those RNA fragments bound and protected by

FIG. 3. RNA fragments containing sequences flanking the site of 3' end formation are precipitated by anti-Sm antibodies. (A) Samples were taken at 0 min (lanes 1 and 2) and 30 min (lanes 3 and 4) from a processing reaction containing human histone H3 precursor RNA labeled with $[\alpha\text{-}^{32}P]\text{GTP}$, $[\alpha\text{-}^{32}P]\text{CTP}$, and $[\alpha\text{-}^{32}P]\text{UTP}$ and treated with T_1 RNase. The T_1 -resistant RNA fragments were immunoprecipitated with anti-Sm antibodies (lanes 2 and 4) or nonimmune control serum (lanes 1 and 3) and resolved on a 15% polyacrylamide gel. The recovery of protected fragments in the anti-Sm precipitates (lanes 2 and 4) corresponds to 1.5% of the theoretical yield, but note that greater than saturating amounts of substrates were used in protection experiments to optimize fragment analysis. The lengths (in nucleotides) of the recovered RNA fragments were determined by comparison to RNA or DNA size markers and by oligonucleotide analysis to be as follows: a_h , 70; b_h , 51; b'_h , 47; c_h , 28; d_h , 25; e_h , 20; and f_h , 11. Abbreviations: n.i., nonimmune control serum; Sm, monoclonal anti-Sm antibodies (Y12). (B) The RNA fragments shown in panel A were eluted from the gel, digested to completion with RNase T_1 , and fractionated on a 20% polyacrylamide gel. The T_1 oligonucleotides contained in fragments a_h through f_h (lanes 1 through 7) are compared with those from a T_1 digest of the total human H3 transcript (lane M), whose sizes are indicated on the right. (C) Samples were taken at 0 min (lanes 1 and 2) and 30 min (lanes 3 and 4) from a processing reaction containing mouse histone H3 precursor labeled with $[\alpha\text{-}^{32}P]\text{GTP}$, $[\alpha\text{-}^{32}P]\text{CTP}$, and $[\alpha\text{-}^{32}P]\text{UTP}$ and treated with RNase T_1 . The T_1 -resistant RNA fragments were immunoprecipitated with anti-Sm antibodies (lanes 2 and 4) or nonimmune control serum (lanes 1 and 3) and electrophoresed on a 15% polyacrylamide gel. The recovery of protected fragments was 1.5% of the theoretical yield. The lengths (in nucleotides) of the recovered RNA fragments were determined as in panel A to be as follows: a_m , 53; b_m , 45; c_m , 23; d_m , 18; e_m , 14; and f_m , 11. (D) The RNA fragments shown in panel C were eluted from the gel, digested to completion with T_1 RNase, and electrophoresed on a 20% polyacrylamide gel (lanes 1 through 6). The T_1 oligonucleotides containing fragments a_m through f_m are compared with those in a T_1 digest of the entire mouse histone H3 transcript (lane M), whose sizes are indicated on the right.

Sm snRNPs. The fragments were separated on a 15% polyacrylamide gel (Fig. 3A and C), eluted, and subjected to complete digestion with RNase T₁. The resulting T₁ oligonucleotides were fractionated on a 20% polyacrylamide gel adjacent to total digests of the two transcripts (Fig. 3B and D). Bands were further analyzed by digestion with RNase A followed by electrophoresis on DEAE paper to determine their identity.

The protected RNA fragments recovered with anti-Sm antibodies from the human histone H3 substrate at time zero (no incubation at 30°C) are shown in Fig. 3A (lane 2) compared with a nonimmune control serum (lane 1). The major fragment b_h is composed of three large T₁ oligonucleotides, 19, 15, and 10 nucleotides long (Fig. 3B, lane 3). b_h was analyzed further by RNase T₁ fingerprint analysis. The fingerprint (Fig. 4) shows four oligonucleotides, the 19-mer CCACCCACUUAUCCAACG, the 15-mer UCUUAAAA CCCAAAG, the 10-mer CUCUUUUCAG, and an AG dinucleotide. Together, these oligonucleotides define a region of the human histone H3 RNA sequence which contains the entire conserved hairpin loop structure and extends just beyond the site of 3' end formation (Fig. 5A).

An SP6 transcript of the mouse histone H3 gene was used in similar experiments. The RNA fragments immunoprecipitated by the monoclonal anti-Sm antibody at time zero in the processing reaction are shown in Fig. 3C (lane 2) compared with a nonimmune control (lane 1). The two major fragments obtained, b_m and c_m, both contain a 23-nucleotide T₁ oligonucleotide (Fig. 3D, lanes 2 and 3). Fragment b_m

contains in addition a 10-mer and a 9-mer (Fig. 3D, lane 2). RNase T₁ fingerprint analysis performed on fragment b_m (Fig. 4) reveals the presence of four oligonucleotides: an AG dinucleotide, the 9-mer CCACCACAG, the 10-mer CUCUUUUCAG, and the 23-mer UCUCCAUCCAU CCCACAAAG. These sequences define a region on the mouse histone H3 pre-mRNA which includes the hairpin loop structure and the site of 3' end formation (Fig. 5B).

Protection extends to include downstream sequences at later times during in vitro processing of histone pre-mRNAs. In vitro processing reactions containing SP6 transcripts of either human or mouse H3 sequences were also probed at later times for interactions between Sm snRNPs and the precursor RNA. The RNA fragments immunoprecipitated from the human H3 substrate after 30 min of incubation at 30°C (Fig. 3A, lane 4) include b_h, which was the major band obtained at time zero, but the overall pattern is much more complex. Of the additional RNA fragments precipitated at later time, a_h and f_h (Fig. 3A, lane 4) are most prominent. Fragment a_h contains the same T₁ oligonucleotides as does fragment b_h (the 19-mer, 15-mer, and 10-mer) but has an additional oligomer of 11 nucleotides and several of less than 6 nucleotides (Fig. 3B, lanes 1 and 3). Comparison of the fingerprints of b_h and a_h (Fig. 4) verified that they contain certain oligonucleotides in common: the 19-mer CCAC CCACUUAUCCAACG, the 15-mer UCUUAAAAACCC AAAG, the 10-mer CUCUUUUCAG, and an AG dinucleotide. The additional a_h oligonucleotides (Fig. 4) are AUAUUUUUUUG (which is seen clearly in the limit T₁

FIG. 4. Fingerprint analyses of the major protected RNA fragments. Fingerprint analyses were performed as described in Materials and Methods for the large protected fragments a_h and b_h from the human and a_m and b_m from the mouse transcripts labeled with [α -³²P]GTP, [α -³²P]CTP, and [α -³²P]UTP. The fingerprint of a_h was not run as far as b_h in the first dimension to retain the rapidly migrating 11-mer AUAUUUUUUUG. An arrow shows the position of the 23-mer in the fingerprint of a_m, which is under-represented due to poor transfer of larger oligonucleotides. The oligonucleotide CUCUUUUCAG in the fingerprints of both b_h and b_m splits into two spots which are identical by RNase A secondary analysis.

Human H3

Mouse H3

FIG. 5. Positions of the protected regions in each transcript. The nucleotide sequences of the human histone H3 transcript (A) and of the mouse H3 transcript (B) were derived from DNA sequences (44, 47). The thickness of the lines indicate the relative yields of the immunoprecipitated fragments. The gray lines correspond to fragments detected exclusively at late times, and the black lines correspond to fragments obtained at both early and late times during the reaction. Sequences in the stem of the hairpin-loop elements are designated by opposing arrows, the A + G-rich boxes are shaded, and the nucleotides at the mature 3' ends are indicated. Fragments a_h through f_h from the human transcript are labeled a through f in panel A; fragments a_m through f_m from the mouse transcript are labeled a through f in panel B.

digestion; Fig. 3B, lane 1), AAAG, UAG, CUG, and UG. These sequences correspond to an extension downstream of the protected region defined by fragment b_h to include the second block of conserved sequence, the "GAAAG" box, and sequences just beyond (Fig. 5A).

The other major RNA fragment immunoprecipitated from the human H3 substrate at later time is f_h (Fig. 3A, lane 4). This product migrates between marker oligonucleotides 10 and 11 nucleotides long and yields an 8-mer upon subsequent T_1 digestion (Fig. 3B, lane 7). Analysis by RNase A digestion of the eluted 8-mer indicates that it corresponds to the first oligonucleotide at the 5' end of the transcript AAUACAG. We deduce that fragment f_h is the capped oligonucleotide GpppGAUACAG.

Several minor fragments, b'_h, c_h, d_h, and e_h (Fig. 3A, lane 4), are reproducibly immunoprecipitated from the human histone H3 substrate after 30 min of incubation in the *in vitro* system. Their T₁ RNase digests (Fig. 3B; lanes 2, 4, 5, and 6) show b'_h, c_h, and e_h as unique fragments, whereas d_h is a mixture. The identities of these minor fragments (Fig. 5A) further confirm that at later times in the processing reaction both sequence elements flanking the site of 3' end formation, as well as the 5' end of the transcript, are associated with a component containing Sm determinant(s).

The mouse H3 substrate also shows a more complex pattern of anti-Sm immunoprecipitable RNA fragments after 30 min of incubation in the nuclear extract (Fig. 3C, lane 4). All fragments were analyzed as described above for those from the human H3 substrate (Fig. 3D and 4). Fragments b_m and c_m are identical to those seen at time zero, whereas a_m , d_m , e_m , and f_m appear exclusively at the later time. The T_1 oligonucleotide composition of the largest protected fragment a_m (Fig. 3D, lane 1; Fig. 4) demonstrates that it contains all of the oligonucleotides of b_m , plus an oligomer of 7 nucleotides (CUUCCCG) and possibly an additional G residue. Fragment a_m therefore represents an extension of fragment b_m either seven or eight nucleotides in the 3' direction (Fig. 5B), so that the protected region includes the beginning of the A+G-rich box. However, the entirety of the A+G-rich box is not protected in the mouse H3 transcript, since the oligonucleotide AAAG is not present in the fingerprint of fragment a_m (Fig. 4).

The remaining RNA fragments, d_m , e_m , and f_m (Fig. 3C, lane 4), which are precipitated at 30 min, map elsewhere on the mouse H3 sequence (Fig. 5B). Fragment d_m contains oligonucleotides 11, 4, and 2 nucleotides long (Fig. 3D, lane 4) and probably corresponds to a region which begins 50 nucleotides from the 5' end of the transcript. Fragment e_m

FIG. 6. Protected RNA fragments are precipitated with anti- m_3G antibodies. (A) RNase T_1 -resistant fragments from an in vitro processing reaction containing ^{32}P -labeled human histone H3 precursor RNA after 0 min (lane 2) and 30 min (lanes 1 and 3) of reaction were precipitated with anti- m_3G antibodies (lanes 2 and 3) or preimmune rabbit serum (lane 1). The RNA fragments recovered by the anti- m_3G serum were resolved on a 15% polyacrylamide gel and are labeled on the right as follows: (sizes in nucleotides) a_h , 70; b_h , 51; and f_h , 11. Their lengths were determined using DNA and RNA size markers; these fragments comigrated with fragments a_h , b_h , and f_h precipitated with anti-Sm antibodies (Fig. 3A). Densitometry of a less-exposed autoradiogram showed that the amount of fragment b_h present in the preimmune precipitate (lane 1) was 3.7% of that obtained by precipitation with the anti- m_3G serum (lane 3). Abbreviations: p.i., preimmune rabbit serum; α - m_3G , anti- m_3G antiserum. (B) RNase T_1 -resistant fragments from a processing reaction containing ^{32}P -labeled mouse H3 precursor RNA after 0 min (lane 2) and 30 min (lanes 1 and 3) of incubation at 30°C were precipitated using anti- m_3G antibodies (lanes 2 and 3) or preimmune rabbit serum (lane 1). Labeled on the right are the following immunoprecipitated RNA fragments (sizes in nucleotides): a_m , 53; b_m , 45; c_m , 23; e_m , 14; and f_m , 11. Their lengths were assigned by comparison with RNA and DNA size markers; fragments a_m through f_m comigrated on a 15% polyacrylamide gel with fragments a_m through f_m obtained with anti-Sm antibodies (Fig. 3C). The amount of fragment b_m present in the preimmune precipitate (lane 1) was determined to be 3.6% of that obtained in the anti- m_3G immunoprecipitate (lane 3) by densitometry of a less-exposed autoradiogram. (C) The T_1 -resistant fragments were eluted from the 15% gel (panels A and B) and digested with RNase T_1 , and the products were resolved on a 20% polyacrylamide gel. Analysis of fragment e_m was not included due to low yield. Labeled on the right are lengths of marker oligonucleotides obtained by T_1 digestion of the mouse and human H3 transcripts.

initially migrates as a 14-mer in a 15% polyacrylamide gel (Fig. 3C, lane 4) and is reduced to an 8-mer plus a 4-mer upon redigestion with RNase T_1 (Fig. 3D, lane 5). Secondary analysis of the 8-mer showed it to be AAUACAAG, the first T_1 oligonucleotide after the cap structure. Thus, e_m corresponds to protection of the capped end of the transcript including the first two T_1 oligonucleotides (Fig. 5B). Similarly, f_m represents protection at the 5' end, but in this case only the first oligonucleotide, the 8-mer (Fig. 3D, lane 6), is present in the complete T_1 digest of f_m , which originally migrated between the markers for 10 and 11 nucleotides. Anti-Sm selection of fragments derived from the capped 5' end of the transcript is thus seen with both the mouse and the human H3 transcripts at later times in the reaction (Fig. 5).

Protected fragments are recovered with anti- m_3G antibodies. To establish whether the extract component(s) responsible for protection contain RNA as well as Sm protein determinants, we probed processing reactions with an antiserum directed against the unique m_3G cap structure of the U RNAs, U1, U2, U3, U4, U5, and U7 (U6 does not contain an m_3G cap) (10, 13, 43). The RNA fragments protected from T_1 RNase digestion and immunoprecipitated by the m_3G antiserum from the human H3 transcript (Fig. 6A) and the mouse H3 transcript (Fig. 6B) give exactly the same pattern as those immunoprecipitated by anti-Sm antibodies (Figure 3A and C). Further analysis confirms the identity of the two sets of fragments (Fig. 6C). Although the amount of b_h and b_m selected by the preimmune serum appears high in this exposure of the gel (Fig. 6A and B; lanes 1), densitometry of a shorter exposure reveals that the yield is only about 4% in both cases relative to that obtained with the m_3G antiserum (lanes 2 and 3). Note that the lack of immunoprecipitation at time zero of fragments f_h and f_m indicates that the m_3G antiserum does not cross-react with the GpppG caps on the 5' ends of the two pre-mRNA substrates.

DISCUSSION

We have presented evidence for several distinct interactions involving mammalian histone pre-mRNAs and factors present in an in vitro histone 3' end processing system. When probed at time zero by T_1 RNase digestion and immunoprecipitation with anti-Sm or anti- m_3G antibodies, sequences containing the hairpin loop region of the pre-mRNA are recovered. At later times in the reaction, protection extends to include sequences downstream of the site of 3' end formation, and fragments from the 5' capped end of the transcript are also bound. We conclude that one or more Sm snRNPs are among the factor or factors responsible, because the protected histone pre-mRNA fragments can be selected by antibodies directed either against the Sm determinants on snRNP proteins or against the 5' cap structure of U RNAs.

The mammalian in vitro histone pre-mRNA processing system we have developed is similar to that recently reported by Gick et al. (21). They present evidence that the cleavage occurring in the presence of EDTA is endonucleolytic and requires Sm snRNPs. They further argue that an Mg^{2+} -dependent 3' cleavage reaction observed in their system and previously reported by Price and Parker (39) in a *Drosophila* cell extract, is due to a 3' to 5' exonuclease activity. Since it had been demonstrated by recovery of downstream cleavage products (21) that the 3' end processing proceeded via an endonucleolytic cleavage, we did not search extensively for such products in our in vitro system. Rather, we have ascertained the accuracy of 3' end forma-

tion in our system by comparing the 3' termini produced in vitro either in the absence of added Mg^{2+} or in 20 mM EDTA with the 3' ends on histone mRNAs recovered from the cytoplasm of cultured cells (Fig. 2). In the presence of ≥ 2.5 mM Mg^{2+} , we observe the accumulation of products two or three nucleotides shorter at the 3' end (data not shown). Moreover, we constructed a mutant human H3 substrate which lacked the entire conserved downstream element and found that the reaction in the presence of Mg^{2+} did not require this sequence, whereas the Mg^{2+} -independent reaction did (data not shown). These results are consistent with the proposal (21) that the Mg^{2+} -dependent reaction involves a different set of extract component(s) from those involved in the Mg^{2+} -independent reaction. Yet, we observe only minor differences in the immunoprecipitated fragments recovered with anti-snRNP antibodies from reactions containing or lacking Mg^{2+} (data not shown): the patterns are identical with and without Mg^{2+} at both 0 and 30 min in the case of the human H3 substrate; for the mouse H3 transcript, the patterns are again identical at 0 min, but at 30 min in the presence of Mg^{2+} the recovery of fragments extending into the region downstream of the cut site is lower and more variable. These observations suggest that the presence of Mg^{2+} does not abolish the authentic-snRNP-mediated 3' end processing reaction in the mammalian in vitro system, but rather allows it to be obscured by the Mg^{2+} -dependent reaction.

The location of protected fragments relative to the conserved elements flanking the 3' processing site appears slightly different for the two substrates we have analyzed (Fig. 5). However, upon closer examination, it may be only the relative scarcity of G residues in this region of the transcripts that is responsible. For example, with both substrates the upstream hairpin loop and the cleavage site are included in the major bound fragments before the appearance of product (zero time). But since there are no G residues between the base of the hairpin and the mature 3' end in either substrate, it is impossible to say whether the site of 3' end formation is actually associated with factor(s) at this time. The fact that zero time protection includes part of the downstream element of the human H3 transcript may likewise be fortuitous, since in this transcript the first G beyond the cut site is in the GAAAG box. Similarly, upstream of the hairpin loop the boundary of protection can only be defined to lie somewhere between the base of the hairpin and the first upstream G residue. This corresponds to a 22-nucleotide region for the mouse H3 transcript and to a 14-nucleotide region for the human H3 transcript. Whatever the exact boundaries of the protected regions at the early time, it is clear that they encompass the conserved hairpin loop structure of both histone pre-mRNA transcripts.

At later times during the processing reaction, as product is being formed, the pattern of protection on both transcripts extends downstream in the direction of the second conserved element, which includes the GAAAG box. The extent of the downstream protection again appears different for the human and the mouse H3 substrates. For the human H3 transcript (Fig. 5A), bound fragments include the entire GAAAG box and sequences just beyond, whereas for the mouse H3 transcript, protection extends only to the beginning of the conserved downstream element stopping just short of the GAAAG box (Fig. 5B). Since this region of both transcripts does contain G residues, the difference is probably real. However, the implications of this difference for the processing mechanism are not clear. The two transcripts do differ markedly in their efficiency of product formation in

vitro; the human H3 substrate is inefficient, whereas the mouse H3 substrate is quite efficient (Fig. 1). A scenario could be envisioned where the inefficient processing of the human H3 transcript involves a more persistent association between factor(s) and the downstream sequences, but with the efficient processing of the mouse H3 transcript the complex would dissociate more easily, lowering recovery of the downstream sequences. It is interesting that we do not observe oligonucleotides possessing the mature 3' end sequence among protected fragments recovered at late times, consistent with the idea that complexes may dissociate once cleavage has occurred.

A second interaction with Sm snRNPs that is detected only at later times in the in vitro processing reaction is with the 5' GpppG capped end of the two histone pre-mRNA substrates. Similar protection of this region has been reported for polyadenylation and splicing substrates (14, 24). As is the case here (Fig. 3A and C; Fig. 6), incubation in an active processing extract was required to observe these interactions. We were unable to test whether the cap itself is essential for protection, because transcripts lacking a 5' cap moiety are unstable in our in vitro system (data not shown). It has previously been reported that with histone 3' processing (19), polyadenylation (23), and splicing (16, 27) the efficiency of the reaction is enhanced by the presence of a 5' cap on the substrate. Hence, it appears that the 5' cap is somehow involved in histone 3' end maturation. However, whether it interacts with the same complex that forms at the 3' end processing site or becomes involved in a distinct snRNP-containing complex requires further investigation.

We have demonstrated two different protection patterns involving the conserved sequences of histone pre-mRNAs at different times during 3' end processing in vitro. Our results argue strongly for the presence of Sm snRNPs in the assemblies responsible for both these patterns, but many possibilities can be envisioned. A snRNP (perhaps with accessory protein factors) could interact initially with the hairpin loop, and as the reaction proceeds a conformational change could allow interaction of the same snRNP with downstream sequences. Alternatively, after an initial interaction, other factors might be recruited to form a larger complex involving the downstream sequences. A third possibility is that two separate complexes (and perhaps separate snRNPs) assemble independently and then interact with one another.

A specific model has been proposed for sea urchin histone pre-mRNA processing in which the U7 RNA base pairs both with sequences in the hairpin loop structure and with the GAAAGA portion of the CAAGAAAGA box (43). Experimental support for such an interaction within the downstream element has been obtained by introducing compensating base changes into U7 RNA that suppress a processing defect in the sea urchin H3 gene caused by a double-base change (GAAAGA to GAAUCA) in the CAAGAAAGA box (40). Our data provide the first evidence for associations between mammalian Sm snRNPs and the two conserved sequence elements flanking the 3' cleavage site of mammalian histone pre-mRNAs. Whether base pairing occurs with either the hairpin loop region or the downstream A+G-rich sequence has not been addressed. The relative heterogeneity among vertebrates in the sequence of the downstream element (46) complicates any model for base pairing with a single U7 RNA. Only after the mammalian U7 snRNPs have been identified and characterized will their potential for interactions with the conserved elements and the cleavage sites on histone pre-mRNAs become clear.

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