Assembly of U7 Small Nuclear Ribonucleoprotein Particle and Histone RNA 3′ Processing in Xenopus Egg Extracts*

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In animals, replication-dependent histone genes are expressed in dividing somatic cells during S phase to maintain chromatin condensation. Histone mRNA 3′-end formation is an essential regulatory step producing an mRNA with a hairpin structure at the 3′-end. This requires the interaction of the U7 small nuclear ribonucleoprotein particle (snRNP) with a purine-rich spacer element and of the hairpin-binding protein with the hairpin element, respectively, in the 3′-untranslated region of histone RNA. Here, we demonstrate that bona fide histone RNA 3′ processing takes place in Xenopus egg extracts in a reaction dependent on the addition of synthetic U7 RNA that is assembled into a ribonucleoprotein particle by protein components available in the extract. In addition to reconstituted U7 snRNP, Xenopus hairpin-binding protein SLBP1 is necessary for efficient processing. Histone RNA 3′ processing is not affected by addition of non-destructible cyclin B, which drives the egg extract into M phase, but SLBP1 is phosphorylated in this extract. SPII, the Xenopus homologue of human p80-coilin found in coiled bodies, is associated with U7 snRNPs. However, this does not depend on the U7 RNA being able to process histone RNA and also occurs with U1 snRNPs; therefore, association of SPII cannot be considered as a hallmark of a functional U7 snRNP.

The formation of histone mRNA 3′-ends is essential for the expression of replication-dependent histone genes and is restricted to the S phase in cultured cell lines (1–5). Formation of the 3′-end occurs by an endonucleolytic cleavage between a conserved RNA hairpin structure 5′ of the cleavage site and a purine-rich spacer element 3′ of the cleavage site (6). The analysis of the molecular mechanism of histone mRNA 3′ processing has been facilitated by the development of nuclear extracts that promote the cleavage of synthetic histone RNA in vitro. Three trans-acting factors are involved in this RNA processing reaction: the U7 small nuclear ribonucleoprotein particle (snRNP) (7–9), a heat-labile factor (10), and the hairpin-binding protein (HBP) or stem-loop binding protein (SLBP) interacting with histone hairpin RNA (11–13). The RNA moiety in the U7 snRNP, U7 RNA, is required for histone RNA 3′ processing (7, 9, 14–16) and base pairs with the purine-rich spacer element (8, 17). The protein components of the U7 snRNP are Sm proteins, which are common to all nucleoplasmic snRNPs (18), and other, as yet uncharacterized, U7-specific proteins (19, 20). The heat-labile factor, which is present in nuclear extract and inactivated by incubation at 50 °C (10), was implicated in the cell cycle regulation of RNA 3′ processing (21). HBP was first identified as a factor binding to the histone hairpin structure (11, 22), an element required for maximal processing efficiency in vitro (11, 23–25); in vivo, the hairpin element is most likely essential (26). In processing reactions in vitro, HBP bound to the hairpin RNA facilitates the formation of the 3′ processing complex composed of histone RNA, U7 snRNP, HBP, and presumably still other factors (12, 13, 25, 27–29). Interestingly, Xenopus oocytes contain two hairpin-binding proteins: SLBP1 involved in histone RNA 3′ processing and SLBP2, which is thought to be involved in the translational silencing of histone mRNA in oocytes (28).

In isolated Xenopus oocytes, histone RNA substrates are processed either by endogenous U7 snRNPs or, when these are inactivated using antisense oligonucleotides, in a reaction requiring synthetic U7 RNA injected into the nucleus or cytoplasm (15, 30, 31). This complementation is dependent on the Sm site sequence required for association of Sm protein with U small nuclear RNAs (snRNAs). Interestingly, U7 Sm OPT RNA with a U2 Sm site, as well as U7 Sm MUT RNA with a destroyed Sm site (Fig. 1A), are not able to process histone RNA (20). UV cross-linking studies have demonstrated different associations of proteins with U7 and U7 Sm OPT RNA, indicating that (i) Sm protein assembly is required, but not sufficient for processing, and that (ii) the U7 Sm site is necessary for assembly of U7-specific proteins (20). Within the nucleus, Sm proteins and U7 RNA are concentrated in C-type snurposomes or coiled bodies (32). Interestingly, Xenopus SLBP1 is also localized in coiled bodies (33), suggesting a common compartmentalization of histone RNA 3′ processing factors. Thus, Xenopus oocytes contain all of the components of the histone RNA-processing machinery.

Materials required for organelle biogenesis and cell cycle

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The abbreviations used are: snRNP, small nuclear ribonucleoprotein; EMSA, electrophoretic mobility shift assay; HBP, histone hairpin-binding protein; hHBP, human HBP; ME, mitotic extract; NRE, nuclear reconstitution extract; PAGE, polyacrylamide gel electrophoresis; SLBP, stem-loop binding protein; wHbps, wild-type histone hairpin RNA; snRNA, small nuclear ribonucleic acid; snoRNA, small nucleolar ribonucleic acid.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z71188 and U75681 (hairpin-binding protein sequences).

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progression are stockpiled in Xenopus eggs for use during early development. In addition, the egg is arrested in metaphase and thus many components are stored in a mitotic, disassembled form. Extracts derived from eggs can be made to spontaneously enter interphase, and such extracts are capable of reconstituting nuclei that are structurally and functionally similar to somatic cell nuclei. Such reconstituted nuclei contain coiled body-like structures (34) and undergo semiconservative DNA replication (35). Alternatively, extracts can be made that are arrested in mitosis, either as a consequence of the stabilization of endogenous cyclin B/cdc2 kinase, or by the re-introduction into interphase extracts of indestructible recombinant cyclin B (35). Thus, extracts derived from Xenopus eggs provide a unique opportunity for in vitro analysis of the biochemistry and cell biology of nuclear assembly and function, and also of cell cycle progression (35). This encouraged us to test whether such extracts can be used as a source of factors for histone RNA 3' processing.

Here we demonstrate for the first time de novo assembly of U7 snRNP from synthetic RNA and proteins derived from Xenopus egg extract. We show that histone pre-mRNA processing can be reconstituted in this cell free system and that assembly and operation of processing complexes does not require the prior assembly of an interphase nucleus. As expected, we find endogenous U7 RNA in extracts, but surprisingly, we show that histone RNA processing in this system is dependent on de novo U7 snRNP assembly from exogenously added U7 RNA. We show that both the histone hairpin and SLBP1 have important functions in processing. SLBP1 is a mitotic phosphoprotein, phosphorylated in vitro by the mitotic kinase cyclin B/cdc2; however SLBP processing function is not dependent on phosphorylation status. Finally we show that SPH-1, the Xenopus homologue of human p80-coilin, associates with snRNAs with a functional Sm site but is not a hallmark of a functional U7 snRNP.

**EXPERIMENTAL PROCEDURES**

**Preparation of Plasmids and Nucleic Acids**—RNAs were prepared in vitro by transcribing U7 RNA polymerase with linearized plasmids or partially single-stranded oligonucleotides and purified by denaturing polyacrylamide electrophoresis. Plasmids encoding U7 RNAs (20) and the histone RNA fragment derived from the H4-12 gene (36) were described earlier (12/12 RNA (25, 27)). Plasmid pT7-U1 encoding Xenopus U1 RNA was obtained from I. Mattaj (37) and was cleaved with BamHI prior to RNA synthesis with T7 RNA polymerase. The short histone hairpin RNA wHsR RNA (5'-GGACAAAAGGCCCU-3') encoding a cytosolic S200 fraction and a membrane fraction as described (35). For histone RNA 3' processing and detection of U7 snRNP and Xenopus HBP, substrate RNAs were added to nuclear reconstitution extract (NRE). Briefly, NRE was composed of 1 volume of S200 fraction, 1/10 volume membrane fraction, an ATP-regenerating system (20 mM phosphocreatine, 2 mM ATP, 0.5 mg/ml phosphocreatine kinase), and up to 5000 Xenopus sperm chromatin per microliter (35). Where indicated, pre-mRNA fragments were supplied to prevent premature nuclear assembly (ME), or with EDTA, or microcin (CalBiochem). Unless stated otherwise, these mixtures were then incubated for 90–120 min prior to any subsequent reactions. Alternatively, S200 fraction supplemented with ATP-regenerating system was used instead of NRE.

**Immuno depletion of Xenopus Egg Extracts and Immunoprecipitations**—Preimmune serum antibodies and anti-SLBP1 antibodies were purchased from Santa Cruz (Santa Cruz Biotechnologies) using dimethyl pimelimidate (45). For immunodepletion, 45–50 μl of NRE were mixed with 100 μl of protein G-Sepharose-coupled antibodies washed five times with 10 mM Hepes KOH, pH 7.7, 50 mM KC1 and mixed on a wheel for 80–100 min at room temperature. The extract was then cleared by two subsequent spins (15 s at 12,000 × g). For immunoprecipitations, protein G-Sepharose beads with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl (ST) were incubated with monoclonal Y12 and H1 antibodies for 1 h at room temperature. Subsequently, unbound antibody was removed by four washes with ST. 30–90 fmol of 32P-labeled U7 or U1 RNA were incubated in 30–100 μl of S200 fraction supplemented with an ATP-regenerating system and 1 unit/μl RNasin (Promega) for 1 h at room temperature. Subsequently, reaction mixtures were diluted by addition of 1 volume of ST and then mixed with the antibodies coupled to protein G-Sepharose. After 1 h of incubation at 4 °C, the beads were concentrated by centrifugation and the supernatants were removed. The beads were then washed three times with ST, and subsequently, the amounts of 32P in pellet and bead supernatant were measured by liquid scintillation counting.

**Protein Modification in Xenopus Egg Extracts**—For the detection of
SLBP1 modification, normally a 1/15 to 1/10 volume in vitro transcription/translation mixture was added to the egg extract. After incubations at room temperature, reaction mixtures were supplemented with at least 2 volumes of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and analyzed by 12% SDS-PAGE. Detection of protein was achieved using the PhosphorImager.

In Vitro Phosphorylation—Reactions were done in 50 mM Tris-HCl (pH 7.5), 10% glycerol, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM ATP 6 mM [γ-32P]ATP (3000 Ci/mmol), 0.14 mg/ml SLBP1 (or histone H1), and 0.5 μl of cyclin B/cdc2 kinase in a volume of 5 μl. Incubations were done at 30 °C

RESULTS

U7 Content of Xenopus Egg Extracts—Xenopus egg extracts are rich in components used for the first rapid cell divisions during embryonic development. Fractionation of Xenopus eggs produces a membrane fraction and a cytosolic S200 fraction (35). These can be mixed to form a nuclear reconstitution extract (NRE), which, in the presence of DNA or chromatin, is able to assemble nuclei that undergo semiconservative DNA replication in vitro (35). First, we tested whether endogenous U7 snRNPs, which are functional in oocytes (20), are able to process synthetic histone mRNA. Because we did not detect any processing (data not shown and see Figs. 4–6 below), we wondered whether this was due to the absence of the U7 snRNA from the Xenopus egg extract preparation used. We therefore prepared RNA from oocytes and from various stages of the Xenopus egg extract preparation and estimated the amount of U7 RNA present by detecting U7 RNA by primer extension from U7 RNA-specific primers. The efficiency of primer extension was then compared with primer extension in control reactions with known amounts of synthetic Xenopus U7 RNA. This comparison indicated that the amounts of U7 RNA in oocytes, eggs, and in NRE were very similar, corresponding to the proportion of primer extended with 0.3, 0.2, and 0.3 pmol of synthetic U7 RNA, respectively (data not shown). U7 was present in both the cytosolic S200 and in the membrane fraction used for reconstitution at a ratio of ~2:1 (data not shown).

No significant loss of U7 RNA occurred at the different stages of the preparation. This suggests that the absence of processing in our reactions was not caused by the absence of U7 RNA. Attempts to further investigate this lack of processing activity, which included testing whether the Xenopus U7 RNA 5′-end was accessible for hybridization to oligonucleotides, were not conclusive. We therefore decided to test whether addition of synthetic U7 snRNA would restore processing activity in these extracts.

Assembly of U7 snRNPs with Synthetic U7 RNA in Egg Extracts—To test whether these extracts contain the factors required for assembly of U7 snRNPs, we added three different labeled synthetic U7 RNAs into NRE. The three RNAs vary in the Sm site required for the binding of the snRNP Sm core proteins (illustrated in Fig. 1A). The complexes resulting from this incubation were analyzed by EMSA. Incubation of wild-type mouse U7 RNA in NRE led to the formation of a well-defined complex with low mobility (Fig. 2, lane 6, indicated snRNP). A distinct complex was formed with U7 Sm OPT RNA, which contains the Sm site of U2 RNA sequence. In contrast, the addition of U7 Sm MUT RNA, a molecule where the Sm site was abolished by mutagenesis, did not form a distinct low mobility complex (lane 4). The presence of a Sm sequence was essential for the formation of the slowly migrating complex. The complex formation was sequence-specific. Additional retardation of the complex with wild-type U7 RNA was observed in incubations containing the Y12 monoclonal antibody specific for Sm proteins (41) (lane 11) but not with an irrelevant control antibody (lane 12). Taken together, we conclude that incubation of U7 RNA in Xenopus egg extract leads to the assembly of a U7 snRNP particle.

Reassembled nuclei formed in Xenopus egg extract have been shown to contain coiled body-like structures (34). These structures contain small nuclear RNAs (snRNAs), spliceosomal snRNAs, and U7 snRNA and have been proposed to be sites of assembly of functional processing complexes (34, 47). Many subnuclear bodies undergo significant alterations as cells enter mitosis, and the timing and mechanism of relocation following the re-establishment of the nucleus in interphase are increasingly studied (48). We wished to determine whether postmitotic nuclear reassembly is a prerequisite for U7 snRNP assembly. In our first approach, U7 RNA was incubated with the S200 fraction only, thus omitting the membrane fraction and chromatin from the mixture. Under these conditions, events such as nuclear envelope assembly and DNA synthesis cannot take place (35). U7 snRNPs were formed with the same sequence specificity in the cytosolic S200 fraction as in NRE (lanes 16–18). This indicates that nuclear reassembly is not required for U7 snRNP assembly. In our second approach we examined U7 snRNP assembly in mitotic extract (ME) generated by addition of recombinant indestructible cyclin B into NRE. This leads to chromosome condensation, depolymerization of the nuclear lamina, and dispersal of the nuclear envelope (35). Addition of synthetic U7 RNAs into ME still led to snRNP assembly (lanes 13–15), indicating that mitotic extracts
do not bring about changes in U7 snRNP assembly that can be detected in our assay. Taken together, these results indicate that U7 snRNP assembly is not dependent on the prior assembly of a nucleus and appears unaffected by the cell cycle status in this system.

Assembled U7 snRNPs Are Functional—To test whether the assembled U7 snRNPs were functional in histone RNA 3’ processing, we included the short 86-nucleotide synthetic 12/12 RNA fragment derived from the mouse histone H4-12 gene (36) in the NRE. This RNA molecule encompasses all the sequence elements required for 3’ processing (illustrated in Fig. 1B). In previous experiments, it was processed in mouse nuclear extract (25, 27, 43) and upon injection into Xenopus oocytes (20). Inclusion of 32P-labeled synthetic mouse U7 RNA together with 32P-labeled 12/12 RNA into the reaction led to the formation of a faster migrating RNA species (Fig. 3B, compare lanes 1 and 3). These molecules comigrated with the 5’ product of a histone processing reaction in K21 cell nuclear extract (compare lanes 1 and 3). They were absent in a reaction containing only 32P-labeled U7 RNA (lane 6) but were formed in a reaction containing 32P-labeled histone RNA and non-labeled U7 RNA (lane 7), indicating that they derived from the histone RNA fragment. Furthermore, these products were not formed when wild-type U7 RNA was replaced by U7 Sm MUT RNA or by U7 Sm OPT RNA (lanes 4 and 5) (20). All these observations indicate that this RNA species is the product of a bona fide histone RNA 3’ processing reaction.

Because U7 snRNPs were also formed in incubations with the S200 fraction only (Fig. 2, lane 18), we wished to establish whether the S200 fraction alone would support histone RNA 3’ processing. Fig. 3B demonstrates the appearance of a similar product under these conditions as in a processing reaction with NRE (compare lanes 3 and 5), indicating that this fraction contains all the factors necessary for processing.

As described above, the presence of U7 RNA in NRE led us to expect that endogenous U7 snRNPs would participate in processing as occurs with nuclear extracts of somatic cells. We wished to exclude the possibility that processing observed following the addition of exogenous U7 RNA was not the consequence of spurious activation of the endogenous molecules and to confirm that it was the consequence of de novo assembly of functional U7 snRNPs. To do this, we utilized the observation that histone pre-mRNA processing is dependent on base pairing between U7 RNA and the spacer element of histone pre-mRNA (17, 49). We utilized all combinations of wild-type and mutant histone RNAs with wild-type and mutant U7 RNAs for processing reactions. In the mutant histone 12/st3.5 RNA, the sequence 10–14 nucleotides away from the 3’-end was changed from CACUU to GGGAA, thus changing a 5-base region complementary to mouse U7 RNA (43) (Fig. 4A). To restore histone RNA-U7 RNA base pairing potential in this region, U7 RNA was changed at the 5’-end to generate the U7snp3.5 mutant RNA. In incubations with 12/12 RNA, only reactions containing wild-type U7, but not the mutant U7snp3.5 RNA, led to the formation of detectable amounts of processing products (Fig. 4B, compare lanes 3 and 4). On the other hand, in incubations with mutant 12/st3.5 histone RNA, only reactions with mutant, but not wild-type U7 RNA, produced detectable amounts of product (lanes 7 and 6, respectively). These results clearly show that newly assembled U7 snRNPs are directly participating in the processing reaction.

We have also tested other histone RNA substrates such as a H4-wt RNA fragment (11) and a fragment from the 3’-end of the mouse H2A.614 gene (50) used for in vitro processing in nuclear extracts prepared from mammalian cells. Although we were able to observe processing, background cleavage in the absence of U7 RNA was for unknown reasons higher with these RNAs, making an interpretation more difficult. We therefore decided to use the 12/12 RNA substrate for our RNA 3’ processing reactions. We also noticed that nuclease activities in the egg extracts varied from batch to batch; however, the use of good quality eggs and quick processing of the extract kept these activities low in general.

Efficient Histone RNA 3’ Processing Is Dependent on the Hairpin Structure and Hairpin-binding Proteins—To determine whether the conserved histone hairpin element is important for histone RNA 3’ processing in Xenopus egg extracts, we compared processing of wild-type 12/12 RNA to processing of B/12 RNA carrying a mutant hairpin element (Fig. 5A). As in previous experiments (25), both RNA molecules were processed...
in K21 mouse cell nuclear extract, but processing with the mutant RNA was significantly less efficient (Fig. 5B, compare lanes 2 and 4; 18% of RNA processed instead of 34%). In fact, in four experiments processing efficiency of B/12 RNA in K21 nuclear extract was at 45–59% of processing of 12/12 RNA, lower than reported earlier (25), reflecting probably batch-to-batch variations of nuclear extract preparations and emphasizing the importance of the hairpin element for processing. In
NRE, product formation with the B/12 substrate was about three times lower than product formation with the 12/12 substrate (lanes 6 and 8, 3.8% of RNA processed instead of 10.9%). This indicates that in NRE, similar to nuclear extract, the hairpin RNA sequence is important, but not essential for processing histone RNA.

To determine whether the hairpin is a binding site for factors, we performed binding experiments with various short hairpin RNAs and detected a factor binding, specifically, the wild-type histone hairpin RNA (Fig. 5C) but not the mutant hairpin (data not shown). To determine whether this factor was the Xenopus SLBP1 (12), we raised antibodies against this protein and used the antibodies to deplete NRE of SLBP1. Fig. 5C shows that the formation of a major RNA-protein complex detected in NRE and also in mock-depleted NRE was prevented when SLBP1 was depleted from the extract (lanes 2, 3, and 4, respectively). This indicates that this complex was formed by SLBP1 and that extract could be effectively depleted of SLBP1. The SLBP1-depleted extract was then used for processing reactions. SLBP1 depletion essentially abolished processing (Fig. 5D, compare lanes 1 and 2), and this was rescued by the addition of either recombinant Xenopus SLBP1 (lane 3) or recombinant human HBP (lane 4), at least in part. These results strongly suggest that removal of SLBP results in the loss of an essential function in the processing reaction and rescue by recombinant protein indicates that depletion of SLBP1 does not remove any other essential factor required for processing. We thus conclude that SLBP1 participates in histone mRNA 3'-end formation and is a true homologue of hHBP.

SLBP1 Modification and Histone RNA Processing in Mitotic Extracts—During mitosis, the nucleus and many of the sub-compartments contained within, including the nucleolus and coiled bodies, undergo disassembly or dramatic rearrangement (51). In addition, many fundamental cellular processes are inhibited in M phase. Entry into mitosis is driven by the highly conserved mitotic protein kinase cyclin B/cdc2, and subsequently, the cyclin subunit undergoes proteolytic degradation for the cell to exit M phase. In Chinese hamster ovary cells that have been synchronized by mitotic shake-off, histone mRNA levels in G1 are dramatically reduced compared with cells in S phase, and this change is due in part to significant up-regulation of processing at the G1/S boundary (52). At the end of S phase, the decrease in histone RNA levels is principally due to decreased stability of the mRNA, raising the question of when in the cell cycle the processing machinery is down-regulated and whether processing components per se undergo cell cycle-regulated control.

Xenopus egg extracts retain the core cell cycle components necessary to recapitulate in vitro a simplified cell cycle that operates in vivo during the early embryonic cell cycles, oscillating between S phase and mitosis (35). This system is thus biochemically tractable and amenable for determining whether specific cellular elements are subjected to cell cycle-dependent translational or post-translational control. Even cell cycle-regulated events such as mitotic repression of RNA polymerase III transcription or replication checkpoint control, which do not ordinarily operate in early embryonic cell cycles, can be reconstituted in appropriate circumstances (38, 51). Because depletion of SLBP1 from extracts abolished processing (Fig. 5D), we wished to determine whether SLBP1 might be a target for mitotic regulation. As for the experiment described in Fig. 2, lanes 13–15, we prepared ME by inclusion of indestructible cyclin B into the extract. First, we established whether SLBP1 was affected by this treatment. The comparison of SLBP1 in NRE and ME by Western blotting revealed that the mobility of SLBP1 was reduced in ME (Fig. 6A). To further investigate this change in Xenopus SLBP1 mobility, we prepared 35S-labeled Xenopus SLBP1 in vitro and added this protein into NRE. 35S-SLBP1 migrated as a single polypeptide during SDS-PAGE (Fig. 6B, lane 1), and incubation in NRE did not affect this mobility (lanes 2 and 5). However, in ME as well as in extracts supplemented with the protein phosphatase inhibitor microcystin, the protein quantitatively underwent band shifts characteristic of phosphorylation (lanes 3 and 6, and lane 4, respectively). This change in mobility suggests that SLBP1 may be phosphorylated in mitotic extracts, and may also be a target for phosphorylation in NRE by a kinase other then cyclin B/cdc2. This latter phosphorylation, however, appears nor-

![Fig. 5. Histone RNA 3' processing in Xenopus egg extracts is stimulated by the presence of a wild-type histone hairpin RNA sequence and by hairpin-binding proteins. A, wild-type (12/12) and mutant (B/12) hairpin RNA sequences inserted between A-28 and A-45 of the histone RNA shown in Fig. 1B. B, 12 μl of NRE was supplemented with either 1.2 μl of H2O or 1.2 μl of 200 nM U7 RNA and incubated at room temperature for 2 h. Subsequently, 5-μl aliquots of each mixture were used in processing reactions with either 32P-labeled 12/12 RNA (lanes 7 and 8) or 32P-labeled B/12 RNA (lanes 5 and 6). The reactions were performed and analyzed as described under “Experimental Procedures” and visualized using a Molecular Dynamics PhosphorImager. Processing reactions with R21 nuclear extract (lane 1–4) were done as described under “Experimental Procedures.” C, NRE was treated with either preimmune serum or anti-SLBP1 serum coupled to protein G-Sepharose as described. Aliquots (3 μl) of NRE (lane 2) NRE treated with preimmune serum (mock-depleted NRE, lane 3) or NRE treated with the antisemur (△SLBP1 NRE, lane 4) were mixed with 32P-labeled wHPrs RNA as described, and complexes were analyzed by EMSA. D, for processing reactions, aliquots (6 μl) of NRE or of SLBP1-depleted NRE were supplemented with U7 RNA and for reactions shown in lanes 3 and 4, with 1 μl of SLBP1 or hHBP (0.2 mg/ml). EDTA, rRNA, and finally 32P-labeled 12/12 histone RNA were added subsequently as described. After 3-h incubation, the reactions were stopped and the products analyzed as described above.](https://example.com/figure5.png)
mally to be subject to immediate dephosphorylation by endogenous protein phosphatase(s).

To confirm that the bandshift in ME was due to phosphorylation, labeled protein was incubated for 2 h in ME. Subsequently, the reaction mixture was split into three aliquots that were treated with either EDTA (acting as kinase inhibitor, thus allowing endogenous phosphoxygenase 1 and 2A to dephosphorylate proteins); EDTA and additional exogenous protein phosphatase 2A (PP2A); or with EDTA, PP2A, and the protein phosphatase inhibitor microcystin (lanes 7–9) for 90 min. Incubations in EDTA (lane 7) partially reversed the bandshift induced in mitotic extract and the presence of additional PP2A (lane 8) reduced most of SLBP1 to a mobility corresponding to that observed in NRE. These results indicate that most of the cyclin B/cdc2-induced modification was caused by threonine/serine phosphorylation. Finally, addition of microcystin inhibited PP2A action (lane 9), confirming that the difference of mobility observed between lanes 7 and 8 was caused by dephosphorylation by PP2A. This indicates that SLBP1 is a mitotic phosphoprotein. SLBP1 has two consensus cdk phosphorylation sites (Thr-169 and Thr-228) as well as a non-consensus site (Thr-60), and it is possible that in the extract, SLBP1 is a direct target of this kinase. To test this in vitro, recombinant SLBP1 was incubated with recombinant cyclin B/cdc2 kinase in the presence of [γ-32P]ATP (Fig. 6C). Transfer of the radiolabeled phosphate group by the kinase was then detected by gel electrophoresis followed by autoradiography. This experiment demonstrates that SLBP1 is a substrate for recombinant cyclin B/cdc2 kinase and may well be directly phosphorylated by this kinase in mitosis.

We tested different SLBP1 functions for possible effects of phosphorylation. Phosphorylation in mitotic egg extract or by cyclin B/cdc2 kinase does not abolish hairpin RNA binding (data not shown). We then wished to establish whether SLBP1 phosphorylation affects histone mRNA 3′ processing. We tested processing in ME, and, as illustrated in Fig. 6D, this did not prevent 3′-end formation (compare lanes 1 and 3) and there was no significant difference between time courses of processing in ME versus NRE (Fig. 6E). In summary, in these and other experiments, we were not able to detect any significant difference in processing between mitotic and S phase extracts.

Interaction of the Xenopus p80-coilin Homologue SPH1 with U7 snRNA—In Xenopus oocytes, U7 snRNA was found to be localized in coiled bodies, which are also referred to as C snurposomes or Cajal bodies in the oocyte nucleus (32, 53, 54). In oocytes, U7 snRNP can associate with Xenopus SPH1 protein, the homologue of human p80-coilin characteristic for coiled bodies (42, 55). To determine whether this association is a hallmark of a U7 snRNP functional in histone RNA 3′-end processing, we tested whether SPH1 was interacting with U7 RNA in the S200 fraction, our minimal system allowing for U7 snRNP assembly (Fig. 2) and histone mRNA 3′ processing (Fig. 3). Radiolabeled U7 RNA was incubated in the S200 fraction and then mixed with either the monoclonal anti-Sm antibody Y12 or the monoclonal antibody H1 recognizing SPH1 (42) bound to protein G-Sepharose beads. The fraction of U7 snRNA
reflects a model substrate. Four distinct lines of evidence presented in Figs. 3 and 4 indicate that the observed modification of the model substrate processing reaction: First, the processed RNA is clearly functional in oocytes that support processing of these RNAs, precipitations with Y12 antibodies were more efficient than precipitations with H1 antibodies. In these reactions, precipitations with U7 Sm OPT RNA were slightly more efficient than precipitations with U7 RNA, and, for each of these RNAs, precipitations with Y12 antibodies were more efficient than precipitations with H1 antibodies. Similar results were obtained in additional experiments where the integrity of the RNA isolated from supernatants and pellet fractions was confirmed by denaturing gel electrophoresis (data not shown). We conclude from these experiments that the Sm sequence is important for the interaction with Sm proteins and SPH-1.

An interesting further question is then whether the association of SPH-1 is specific for U7 RNA. To test this we produced synthetic U1 RNA and added this RNA into Xenopus egg extracts. A second set of experiments was then performed with Y12 and H1 antibodies as above. The data summarized in Table I demonstrate that, similar to U7 RNA, U1 RNA is precipitated by Y12 and H1 antibodies, indicating that it becomes associated with Sm proteins and also with SPH-1. These results indicate that, in these extracts, SPH-1 does not serve as a hallmark for a functional U7 snRNP but appears to associate with RNA-protein complexes containing Sm proteins.

**DISCUSSION**

In this paper we report the characterization of a novel in vitro system for the analysis of histone mRNA 3' processing. We have utilized extracts derived from Xenopus eggs as a source of trans-acting factors required for the processing reaction. Four distinct lines of evidence presented in Figs. 3 and 4 indicate that the observed modification of the model substrate reflects a bona fide processing reaction: First, the processed reaction product comigrated with the product obtained from previously characterized K21 cell nuclear extracts (25, 43). Second, processing was dependent on the addition of U7 RNA.

The newly assembled snRNPs participate directly in the processing reaction. We conclude that xenopus egg extracts support bona fide U7-dependent snRNP assembly and histone 3' processing.

Previous work involving microinjection in Xenopus oocytes indicated that oocytes support the efficient processing of histone mRNA in a reaction dependent either on endogenous U7 RNA or, after its destruction, on the injection of an appropriate synthetic U7 RNA (20, 31). Such processing presumably reflects the requirement of the oocyte to stockpile histone mRNA and protein needed to provide for the histones necessary for the condensation of the exponentially increasing amount of chromatin synthesized in the early preblastula cell cycles, during which all transcription is inhibited (56–58). The detection of processed products in extracts derived from metaphase-arrested eggs strongly suggests that the protein components involved in histone 3' processing are retained following oocyte maturation and fertilization. They may be stored as a relatively abundant maternal pool to be used at the onset of zygotik histone gene expression, and their assembly is not dependent on the prior assembly of an interphase nucleus.

In contrast to the retention of functional protein components, we were surprised to find that reconstitution of processing in egg extracts required the addition of U7 RNA. Xenopus U7 RNA is clearly functional in oocytes that support processing of sea urchin, Xenopus, and mammalian histone pre-mRNA, including the 12/12 RNA used here, in the absence of any exogenous U7 RNA (20, 31). In addition, U7 RNA has been shown, by hybridization with labeled complementary probes, to be enriched in coiled bodies in the oocyte nucleus as well as in similar structures in nuclei reconstituted in cell-free extracts (32, 34). The function(s) of these subnuclear structures, which have been proposed to disassemble in M phase and reassemble in interphase, remain(s) elusive, and suggestions range from storage sites to essential processing locations for elements involved in RNA processing (discussed in Ref. 34). Our observation that processing occurs in both interphase S200 and mitotic extract, both unable to form reconstituted nuclei (for different reasons), indicates that nuclear reassembly is not a prerequisite for the assembly of functional processing complexes. This is in sharp contrast to the assembly of functional RNA replication complexes in the extract, which are absolutely dependent on nuclear formation (35). In addition, our results suggest that the in vitro reassembly of coiled bodies is not an essential step for the formation of functional processing complexes.

We found endogenous U7 levels in extracts to be very similar to those observed in oocytes, excluding the possibility that U7 RNA is specifically lost during oocyte maturation. The inability of endogenous U7 to support a significant degree of mRNA processing in this study may reflect a functional inactivation of U7 RNA during early development. Interestingly, both sense and antisense probes to U7 RNA have been reported to localize to the same coiled body-like structures in nuclear reconstitution extracts but not in oocytes (34), raising the intriguing possibility that, during Xenopus oocyte maturation, U7 snRNPs may be sequestered by a complementary nucleic acid. Alternatively, U7 RNA may be limiting in G2-arrested oocytes, and the disassembly of the oocyte germinal vesicle, which occurs on maturation (35), may result in the dilution of essential factors throughout the cytoplasm such that in vitro processing cannot be detected by current assays unless exogenous U7 is added. Whatever the reason for the lack of detectable processing by the endogenous U7 RNA, Xenopus egg extracts have provided us with the first cell-free system allowing the direct analysis of the interactions between the two RNAs involved in the processing reaction. Processing in this system was in gen-

**TABLE I**

| Association of SPH-1, the Xenopus p80-collin homologue, with snRNAs in Xenopus egg extract |
|---------------------------------------------------------------|
| The indicated RNAs were incubated in S200 fraction and subsequently precipitated with either anti-Sm (Y12) or anti-SPH-1 (H1) antibodies as described under “Experimental Procedures.” |

| RNA/antibody | U7wt | U1 | U7wt/Y12 | U7 Sm OPT/Y12 | U7 Sm MUT/Y12 | U7wt/H1 | U7 Sm OPT/H1 | U7 Sm MUT/H1 | U1/Y12 | U1/H1 |
|--------------|------|----|----------|---------------|---------------|--------|-------------|-------------|--------|------|
| 1            | 2.0  | 2.2| 1.7      | 4.2           | 5.3           | 5.1    | 5.6         | 11.3        | 6.4    | 5.4  |
| 2            | 21.3 | 27.5| 16       | 31.3          | 21.3          | 11.3  | 11.4        | 17.1        | 16.1   | 15.2 |
| 3            | 24.4 | 33.7| 16       | 31.3          | 21.3          | 4.2    | 11.3        | 17.1        | 16.1   | 15.2 |
| 4            | 1.6  | 2.1| 1.7      | 6.4           | 5.4           | 1.8    | 3.8         | 4.3         | 11.5   | 17.1 |
| 5            | 8.2  | 10.1| 4.2      | 16.7          | 15.2          | 4.2    | 11.3        | 17.1        | 16.1   | 15.2 |
| 6            | 2    | 2.3| 1.8      | 3.8           | 4.3           | 4.5    | 14.5        | 12          | 4.5    | 12   |

a The results of six independent experiments are shown.
eral less efficient than that observed in somatic cell nuclear extracts, although it is clear that products accumulated linearly as a function of time (Fig. 6E). The difference in efficiency is likely due to the fact that, in the system described here, total cytoplasm, and not simply nuclear extract, is the starting source for the protein factors involved.

In addition to NRE composed of membrane and cytosolic fractions, U7 snRNPs were also formed in incubations with only the cytosolic S200 fraction (Fig. 2, lane 18). Thus, we wished to establish whether the S200 fraction alone would support histone RNA 3′ processing. Fig. 3B demonstrates the appearance of a similar product under these conditions as in a processing reaction with NRE (compare lanes 3 and 5), indicating that this fraction contains all the factors necessary for processing.

A system allowing the direct analysis of the interactions between the two RNAs involved in the processing reaction is of particular interest. Despite reports of the recovery of polypeptides specifically associated with U7 RNAs (19), their molecular identity remains unknown, and to date a system for the recovery and molecular characterization of functional U7 snRNPs has been lacking. Interestingly, snRNP assembly with U7 snRNA is not sufficient for processing, as is demonstrated by the example of U7 Sm OPT RNA, which has an Sm site similar to U2 snRNA and assembles into a non-functional snRNP (Figs. 2 and 3). This is in agreement with earlier observations, where U7 Sm OPT was found to associate with Sm proteins in Xenopus oocytes but was not able to process histone RNA, and probably reflects a requirement for further U7-specific proteins (20).

When we were testing different substrates for processing, we found that processing efficiency decreased when we exchanged the hairpin in the 12/12 histone RNA with a hairpin that was not bound by hairpin-binding proteins (Fig. 5 and data not shown). This was not surprising, because similar observations were made with this substrate in other cell free systems (25). *Xenopus SLBP1* is the major activity binding to hairpin RNA in these extracts. Immunoprecipitation of SLBP1, however, reduced RNA processing activity to background levels. This was reversed by the re-addition of either SLBP1 or human HBP. Thus the presence of a mutant hairpin, which cannot bind SLBP1, results in suboptimal processing, whereas removal of SLBP1 makes processing undetectable. Taken together, these results suggest that SLBP1-depleted extracts lack an essential function in processing that is independent of SLBP1 hairpin binding function. Recovery of processing was achieved by the re-addition of either SLBP1 or human HBP. This strongly suggests that no other essential protein was removed in the immunoprecipitation. The reason for only partial reversal is unknown. Protein is in excess over histone RNA in these reactions, but it is possible that recombinant protein lacks full functionality. Alternatively, it is possible that we have removed a stimulatory factor, and the analysis of the immunoprecipitate will be of great interest. A role for SLBP1 in histone RNA processing was also observed by Marzluff and coworkers in *Xenopus* oocytes (28). In some extract preparations, traces of a second hairpin-RNA binding activity, probably the *Xenopus* SLBP2 (28) involved in silencing of maternal histone mRNA in oocytes, were observed.

Addition of non-destruclible cyclin B converts an interphase extract (NRE) into a mitotic extract (ME). Interestingly, SLBP1 was phosphorylated in ME and also directly by cyclin B/cdc2 protein kinase (Fig. 6). SLBP1 has three potential cdk phosphorylation sites, Thr-60, Thr-169, and Thr-228. Preliminary results indicate that, in ME and in incubations with cyclin B/cdc2 kinase, Thr-60 is the major phosphorylation site. This modification is clearly of interest. However, we did not detect any differences between histone RNA processing in NRE and ME, indicating that the SLBP1 modification had no effect on this reaction. Other possible functions, which will be addressed in future experiments, are for example in regulating translation or protein stability.

As mentioned above, the protein composition of the U7 snRNP is not well characterized. As a first step toward the molecular characterization of the U7 snRNP, we investigated the significance of the association of SPH-1 protein with U7 snRNPs in our system. In *Xenopus* oocytes, wild-type U7 RNA was found to be enriched in coiled bodies and associated with SPH-1 protein, the *Xenopus* homologue of p80-coilin associated with coiled bodies (32, 53, 55). In addition, injected U7 RNA has been shown to induce the formation of coiled body-like structures in oocytes (60), and endogenous U7 RNA was enriched in structures similar to coiled bodies in nuclei reconstituted in cell free extracts (34). These latter coiled body-like structures also contained spliceosomal snRNAs and snRNAs (34). The S200 fraction contains all the protein components for histone m RNA 3′ processing but, in the absence of chromatin and membrane fraction, does not form nuclear structures (35). We used immunoprecipitation with H1 and, as a control, Y12 antibodies to test for interactions of SPH-1 and Sm proteins with U RNAs upon incubation in the S200 fraction. Although the fraction of RNA precipitated with, e.g., U7 Sm OPT did vary between experiments, probably reflecting a batch-to-batch variation between extracts, the results obtained were consistent. They confirmed the interaction between Sm proteins and U7 and U7 Sm OPT (Fig. 2 and Ref. 20) and between U1 RNA and Sm proteins. In addition, we also detected interactions between U1, U7, and U7 Sm OPT and SPH-1. The data in Table I suggest that these interactions are weaker than with Sm proteins. This may be due to a less efficient precipitation, caused either by poorer assembly or poorer interaction between antibody and epitope in the immunoprecipitation. Our preferred interpretation of this observation is, however, that in egg extract, SPH-1 associates with U-RNA·Sm protein complexes but not with RNA directly. Thus immunoprecipitation would depend on Sm protein assembly and the interaction between SPH-1 and U RNA Sm protein complex. Both of these interactions may be inefficient in our system, thus leading to the poor but reproducible precipitation of U1, U7 Sm OPT, and U7 RNAs with H1 antibodies (Table I). This indicates that association of the *Xenopus* p80-coilin homologue with U7 snRNP is not a hallmark of a functional snRNP.

Gall and coworkers (32, 55) made apparently contradictory observations when the interaction between SPH-1 and U7 RNA was tested upon injection of U7 RNA into oocytes RNA. In these experiments, an interaction was observed between SPH-1 and U7 RNA or a U7 mutant similar to U7 Sm OPT but not with U1 RNA. It is likely that this is related to the different localization of spliceosomal U RNAs (as illustrated at the example of U2 RNA) and U7 RNA within subnuclear structures in oocytes and the colocalization of SPH-1 and U7 RNA (32, 47, 55). However, the same authors reported that in *Xenopus* egg extracts, endogenous spliceosomal snRNAs, snoRNAs, U7 RNA, and SPH-1 colocalize to the same structures, also referred to as coiled bodies (34). SPH-1 is essential for this localization of Sm proteins, and therefore presumably also for the localization of spliceosomal RNA and U7 RNA (61). Although no quantitative interaction between SPH-1 and endogenous spliceosomal snRNAs or U7 RNA was detected in these extracts (61), this sug-

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2 J. Link and B. Müller, unpublished observation.
gests that the Xenopus coilin homologue and Sm snRNPs must somehow interact for a correct localization. The results of our experiments with synthetic RNAs and S200 fraction (where nuclear assembly does not take place), indicate that a direct interaction between U RNAs and SPH-1 that is dependent on the association of Sm proteins with U RNA is possible. This interaction may be instrumental for the localization observed.

In conclusion, we have identified a system that allows histone RNA processing in a reaction dependent on the addition of U7 snRNA. Our observations indicate that the requirements for other factors are similar to processing in Xenopus oocytes or nuclear extract, the other commonly used in vitro systems to study histone RNA processing. We have also more closely investigated the association of one particular protein, the coilin homologue SPH-1, with U7 snRNPs and found that SPH1 associates with both functional and non-functional snRNPs.

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