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# Differences and similarities between *Drosophila* and mammalian 3' end processing of histone pre-mRNAs

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## ABSTRACT

We used nuclear extracts from *Drosophila* Kc cells to characterize 3' end processing of *Drosophila* histone pre-mRNAs. *Drosophila* SLBP plays a critical role in recruiting the U7 snRNP to the pre-mRNA and is essential for processing all five *Drosophila* histone pre-mRNAs. The *Drosophila* processing machinery strongly prefers cleavage after a fourth nucleotide following the stem-loop and favors an adenosine over pyrimidines in this position. Increasing the distance between the stem-loop and the HDE does not result in a corresponding shift of the cleavage site, suggesting that in *Drosophila* processing the U7 snRNP does not function as a molecular ruler. Instead, SLBP directs the cleavage site close to the stem-loop. The upstream cleavage product generated in *Drosophila* nuclear extracts contains a 3' OH, and the downstream cleavage product is degraded by a nuclease dependent on the U7 snRNP, suggesting that the cleavage factor has been conserved between *Drosophila* and mammalian processing. A 2'-O-methyl oligonucleotide complementary to the first 17 nt of the *Drosophila* U7 snRNA was not able to deplete the U7 snRNP from *Drosophila* nuclear extracts, suggesting that the 5' end of the *Drosophila* U7 snRNA is inaccessible. This oligonucleotide selectively inhibited processing of only two *Drosophila* pre-mRNAs and had no effect on processing of the other three pre-mRNAs. Together, these studies demonstrate that although *Drosophila* and mammalian histone pre-mRNA processing share common features, there are also significant differences, likely reflecting divergence in the mechanism of 3' end processing between vertebrates and invertebrates.

**Keywords:** histone pre-mRNAs; 3' end processing; *Drosophila*; SLBP; U7 snRNP

## INTRODUCTION

Metazoan replication-dependent histone pre-mRNAs do not contain introns, and the only processing reaction necessary to generate mature histone mRNAs is a single endonucleolytic cleavage of the mRNA precursors (pre-mRNAs) to form the 3' end. Studies on 3' end processing were initially carried out in *Xenopus* oocytes using synthetic pre-mRNAs (Krieg and Melton 1984) and sea urchin histone genes (Birnstiel and Schaufele 1988) and later were facilitated by the development of an in vitro system based on nuclear extracts from mammalian cells (Gick et al. 1986; Mowry and Steitz 1987a). Replication-dependent histone pre-mRNAs contain two *cis* elements required for 3' end processing: a highly conserved stem-loop structure consisting of a 6-bp stem and a 4-nt loop and a less conserved

histone downstream element (HDE) located ~15 nt 3' of the stem-loop (Birnstiel and Schaufele 1988; Mowry et al. 1989). Mammalian histone pre-mRNAs are cleaved between the two elements, 5 nt downstream of the stem-loop. The stem-loop is recognized by the stem-loop binding protein (SLBP) (Wang et al. 1996), also referred to as the hairpin binding protein (HBP) (Martin et al. 1997). The HDE interacts with the U7 snRNP, which contains an ~60-nt U7 snRNA (Galli et al. 1983; Mowry and Steitz 1987b), and this interaction is primarily mediated by base-pairing between the HDE and the 5' end of U7 snRNA (Schaufele et al. 1986; Bond et al. 1991). In vitro studies in mammalian nuclear extracts suggest that SLBP stabilizes binding of the U7 snRNP to the pre-mRNA (Dominski et al. 1999) and is essential in processing of only those pre-mRNAs that do not form sufficiently stable duplexes with the U7 snRNA (Streit et al. 1993; Spycher et al. 1994). This role of SLBP in mammalian processing is most likely mediated by ZFP100, a 100-kDa zinc finger protein associated with the U7 snRNP and interacting with the SLBP/stem-loop complex (Dominski et al. 2002a). In addition to bridging the two factors bound to their respective sequence elements, ZFP100 may

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also play other roles in 3' end processing, possibly including the recruitment of the cleavage factor.

Purification of the U7 snRNP from mammalian cells resulted in identification of two novel Sm-like proteins: Lsm10 (Pillai et al. 2001) and Lsm11 (Pillai et al. 2003), which replace the D1 and D2 Sm proteins present in the spliceosomal snRNPs. Lsm11 interacts *in vitro* with ZFP100 (Azzouz et al. 2005) and plays a key role in recognizing the unique sequence of the Sm binding site in U7 snRNA (Grimm et al. 1993; Pillai et al. 2003). Orthologs of Lsm10 and Lsm11 are also found in the *Drosophila* U7 snRNP, demonstrating that the unique structure of the U7 snRNP in vertebrates and invertebrates is conserved (Azzouz and Schumperli 2003). A counterpart of ZFP100 has not been yet identified in the *Drosophila* genome, suggesting that ZFP100 is either weakly conserved between vertebrates and invertebrates or processing of histone pre-mRNAs in *Drosophila* does not require this protein.

We recently reported that nuclear extracts from *Drosophila* S-2 and Kc cultured cells and embryos are capable of 3' end processing of presynthesized *Drosophila* histone pre-mRNAs (Dominski et al. 2002b) and identified the *Drosophila* U7 snRNA (Dominski et al. 2003b). Nuclear extracts from Kc cells are also capable of cotranscriptional processing of histone pre-mRNAs (Adamson and Price 2003). Unlike the auxiliary role played by SLBP in mammalian *in vitro* processing, *Drosophila* SLBP is indispensable for processing of all *Drosophila* histone pre-mRNAs (Dominski et al. 2002b). This observation suggests that *Drosophila* SLBP plays a much more important role in recruiting the U7 snRNP to the pre-mRNA than it does in the mammalian processing. Here we used the *in vitro* system based on *Drosophila* nuclear extracts to characterize 3' end processing of *Drosophila* histone pre-mRNAs and to define differences and similarities in processing between this model invertebrate processing system and processing in mammalian nuclear extracts.

## RESULTS

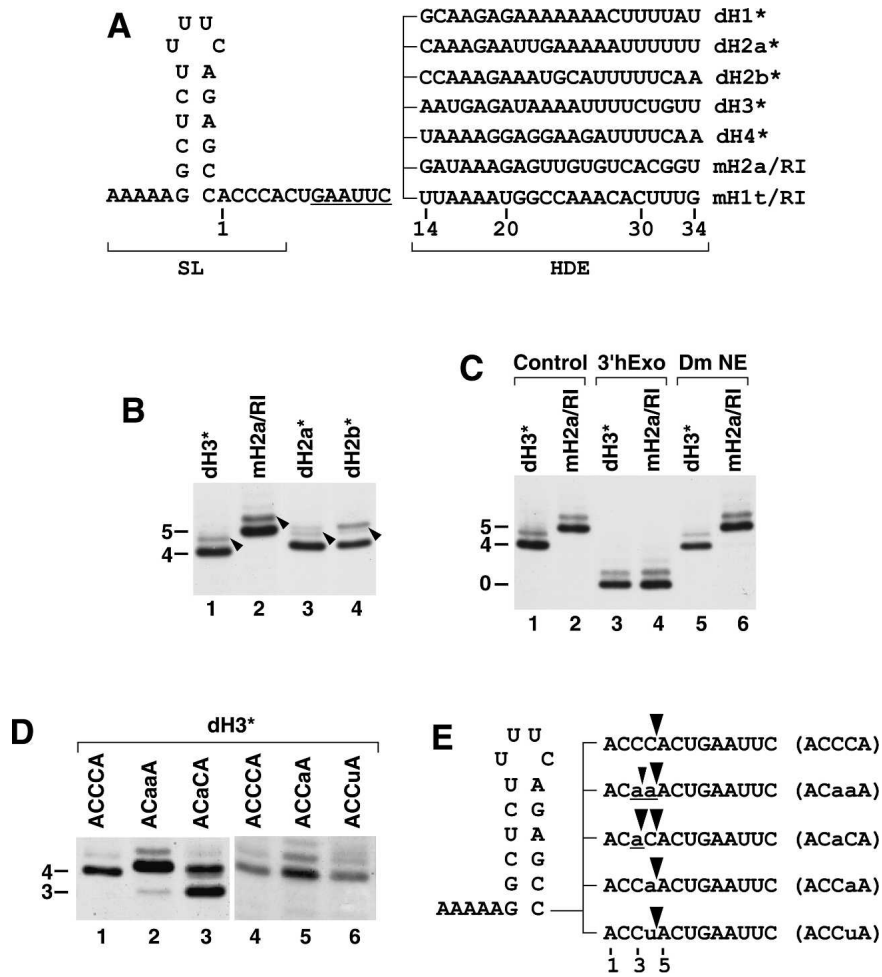
### Mapping the cleavage site in *Drosophila* 3' end processing

To characterize 3' end processing of histone pre-mRNAs in *Drosophila* we used an *in vitro* system based on nuclear extracts from *Drosophila* Kc cells. A simplification of the *Drosophila* system is that there are only five different histone genes (each repeated multiple times) compared with the more than 60 nonallelic histone genes present in mammals (Marzluff et al. 2002). As substrates in most of these studies we used hybrid pre-mRNAs consisting of the stem-loop and cleavage site from the mouse H2a-614 pre-mRNA and the HDE from each of the five different *Drosophila* histone pre-mRNAs (Dominski et al. 2002b). The two elements were joined by introducing an EcoRI restriction site 8–13 nt

downstream of the stem-loop of the H2a-614 DNA clone, in a region that does not play an important role in 3' end processing (Fig. 1A). The H2a/RI pre-mRNA, a derivative of the H2a-614 pre-mRNA containing the EcoRI sequence, is processed in a mouse nuclear extract with the same efficiency as the parental pre-mRNA (Dominski et al. 1999).

All the five hybrid pre-mRNAs, designated dH1\*, dH2a\*, dH2b\*, dH3\*, and dH4\*, are processed equally well in *Drosophila* nuclear extracts (Dominski et al. 2002b). The mouse H2a-614 pre-mRNA, containing the original mouse-specific HDE, was also processed in the *Drosophila* nuclear extract, although with much lower efficiency (Dominski et al. 2002b). We previously showed using high-resolution electrophoresis capable of detecting single nucleotide differences in RNA length that the upstream cleavage product generated from the dH3\* pre-mRNA in a *Drosophila* nuclear extract migrated faster than the product of the mouse H2a pre-mRNA generated in a mouse nuclear extract. To establish whether generation of the faster migrating processing product is a general property of *Drosophila* nuclear extracts, we tested two other *Drosophila*-specific pre-mRNAs, dH2a\* and dH2b\*. These pre-mRNAs were incubated in a *Drosophila* nuclear extract and their cleavage products analyzed in a high-resolution gel next to products of the dH3\* pre-mRNA generated in the *Drosophila* nuclear extract and of the mouse H2a/RI pre-mRNA, generated in a mouse myeloma nuclear extract. Processing of dH2a\* and dH2b\* generated the upstream cleavage product that had the same increased mobility as the dH3\* product (Fig. 1B, lanes 1,3,4). Note that the minor band migrating more slowly than the major processing product in this and other lanes is the cleavage product of the longer substrate containing an additional nucleotide at the 5' end as a result of an alternative transcription start site. The other two *Drosophila*-specific pre-mRNAs, dH1\* and dH4\* pre-mRNAs, were also cleaved to an identical product migrating slightly faster than the upstream processing product generated in a mouse nuclear extract from the mouse H2a/RI pre-mRNA (not shown). The processing product of the mouse H2a/RI pre-mRNA generated in *Drosophila* nuclear extracts also had a higher mobility, demonstrating that this difference is due to a unique property of *Drosophila* extracts rather than processing signals in *Drosophila* histone pre-mRNAs (Dominski et al. 2002b).

The difference in migration could indicate that *Drosophila* nuclear extracts cleave pre-mRNAs 1 nt closer to the stem, thus producing slightly shorter mRNAs, or cleave at the same site as mammalian extracts but generate a 3' end containing a phosphate group, which would increase mobility of the RNA (Sollner-Webb et al. 2001). Alternatively, *Drosophila* nuclear extracts might contain a 3' exonucleolytic activity resistant to EDTA that shortens the upstream cleavage product. The upstream cleavage product generated in mammalian nuclear extracts ends with the ACCCA following the stem-loop and contains a 3' hydroxyl group



**FIGURE 1.** Mapping of the cleavage site in *Drosophila* 3' end processing. (A) Sequences of pre-mRNA substrates used in this study. The histone downstream element (HDE) specific to each pre-mRNA was fused to the stem-loop (SL) and the flanking sequences (including the cleavage site) from the mouse H2a-614 pre-mRNA using the EcoRI restriction site (underlined) generated between the two sequence elements. The nucleotides are numbered starting from the first residue after the stem-loop. (B) The upstream cleavage products of the dH3\*, dH2a\*, and dH2b\* pre-mRNAs generated in a *Drosophila* nuclear extract (lanes 1,3,4) were analyzed in a high-resolution polyacrylamide gel next to the upstream cleavage product of the mouse H2a/RI pre-mRNA generated in a mouse nuclear extract (lane 2). The unprocessed input pre-mRNAs are not shown. The minor bands (arrow heads) that migrate over the major cleavage product visible in this and subsequent panels result from processing of the substrates longer at the 5' end by 1 or 2 nt due to heterogeneity of the transcription start site selection. (C) Upstream cleavage products of the dH3\* and mH2a/RI pre-mRNAs (lanes 1,2) were incubated with the 3'hExo (lanes 3,4) or a *Drosophila* nuclear extract (lanes 5,6). (D) Cleavage products of the mutant dH3\* pre-mRNAs (indicated in E) generated in a *Drosophila* nuclear extract and separated in a high-resolution polyacrylamide gel. Mutated nucleotides are indicated by lowercase letters. The cleavage product of the wild-type dH3\* pre-mRNA is shown in lane 1. The unprocessed input pre-mRNAs are not shown. (E) The sequence of the pre-mRNA substrates analyzed in D. The major and minor cleavage sites are marked with large and small arrowheads, respectively.

(Scharl and Steitz 1994; Furger et al. 1998; Dominski et al. 2002b). To characterize the differences in the 3' end, we isolated the cleavage products generated in mouse or *Drosophila* nuclear extracts and treated these products with the baculovirus-expressed 3'hExo (Dominski et al. 2003a), a 3'-5' exonuclease that removes the single-stranded tail from

histone mRNAs containing a hydroxyl group at the 3' end but not a phosphate (our unpubl. results). Incubation of each processing product with 50 pmol of 3'hExo resulted in formation of identical 44-nt RNAs ending at the base of the stem, demonstrating that *Drosophila* processing product is sensitive to the exonuclease and hence must terminate with a hydroxyl group (Fig. 1C, lanes 3,4). To test whether *Drosophila* nuclear extract contains a 3' exonucleolytic activity capable of removing 1 nt from the cleavage product ending five nucleotides after the stem-loop, we incubated the purified mouse cleavage product in the *Drosophila* nuclear extract. After 90-min incubation in the presence of EDTA, this product was unchanged (Fig. 1C, lanes 5,6). Based on these results we conclude that the upstream cleavage product generated in *Drosophila* nuclear extracts is shorter by 1 nt than the cleavage product of the mouse processing and contains a 3' hydroxyl group (Fig. 1E).

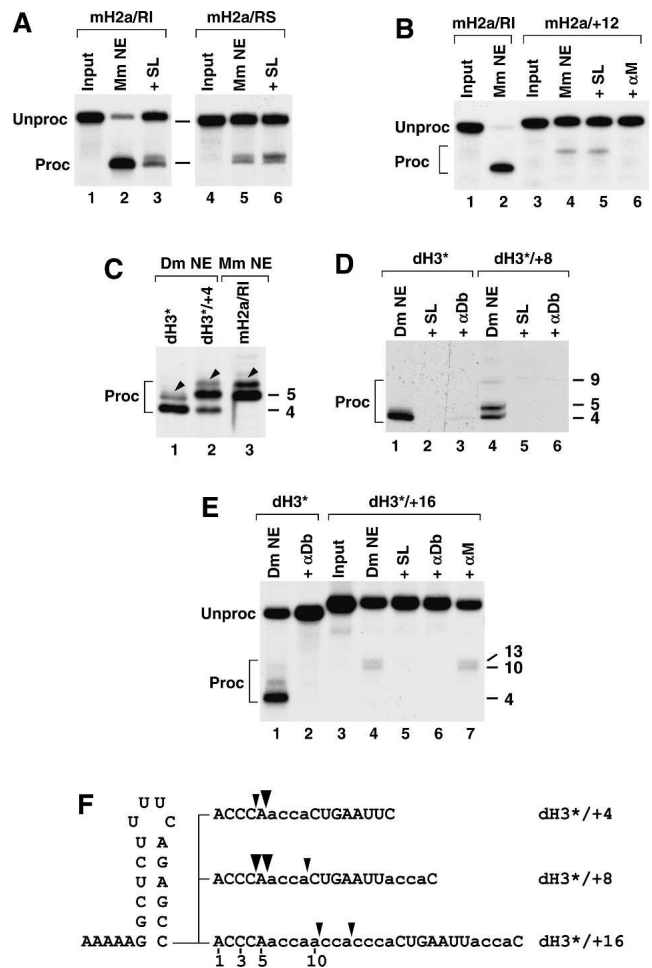
Mammalian nuclear extracts preferentially cleave histone pre-mRNAs after an adenosine and less efficiently after a cytosine (Scharl and Steitz 1994; Furger et al. 1998). Processing of the *Drosophila*-specific pre-mRNAs and the mouse H2a pre-mRNA in *Drosophila* nuclear extracts after the cytosine 4 nt 3' of the stem-loop was surprising since there is an adenosine 1 nt further downstream (Fig. 1A). To test whether *Drosophila* processing has a different nucleotide preference than mammalian processing, we created mutants of the dH3\* pre-mRNA by changing the ACCCA to ACaaA (the sequence in the genuine *Drosophila* histone H3 pre-mRNA), ACaCA, ACCaA, or ACCuA. The ACaaA pre-mRNA was predominantly cleaved after the adenosine 4 nt downstream of the stem-loop (position +4). Processing of this substrate also generated a minor product with the cleavage site after the adenosine at +3 (Fig. 1D, lane 2). In contrast, the ACaCA pre-mRNA was predominantly cleaved after the adenosine at +3, with a significant amount of the substrate being cleaved after the next cytidine (Fig. 1D, lane 3). Both the ACCaA and the ACCuA pre-mRNAs were processed at the normal site, after the fourth nucleo-

tide, and processing of the ACCaA pre-mRNA was more efficient than processing of the ACCuA or the wild-type dH3\* pre-mRNAs (Fig. 1D, lanes 4–6). Thus, *Drosophila* processing has a strong preference to cleave pre-mRNA after an adenosine located 4 nt downstream of the stem-loop. Efficient cleavage also occurs after an adenosine at position +3 but not after an adenosine located at position +5 (Fig. 1E).

**Effects of increasing the distance between the stem-loop and the HDE on *Drosophila* processing**

Removal of SLBP from a mammalian nuclear extract or sequestering SLBP by addition of an excess of the stem-loop RNA has a variable effect on 3' end processing of mammalian histone pre-mRNAs and this effect depends on the sequence of the HDE. Processing of pre-mRNAs containing an HDE that allows only weak base-pairing with the U7 snRNA is completely inhibited in the absence of SLBP, whereas processing of pre-mRNAs with strong complementarity to U7 snRNA proceeds under these conditions with only partially reduced efficiency (Streit et al. 1993; Spycher et al. 1994; Dominski et al. 1999). In vitro processing of the mouse H2a/RI pre-mRNA is reduced but not abolished in the presence of the excess stem-loop RNA (Fig. 2A, lanes 2,3). The presence of the stem-loop RNA, in addition to reducing the processing efficiency at the major site after the ACCCA, allowed cleavage at an additional site, which is located 2 nt further downstream, as determined in a high-resolution gel (not shown). The same reduction in processing efficiency and activation of the cryptic site was achieved by reversing the sequence of the stem in the pre-mRNA, abolishing binding of SLBP to the substrate (Fig. 2A, lane 5). The efficiency of processing of this substrate was not further reduced by addition of the stem-loop RNA, indicating that the reverse stem mutation and addition of the stem-loop competitor have the same effect on processing, preventing interaction between SLBP and the pre-mRNA (Fig. 2A, lane 6).

The cleavage site in mammalian pre-mRNA is determined by the position of the HDE; moving the HDE away from the stem-loop results in a corresponding shift of the cleavage site and in a reduction of processing efficiency (Fig. 2B, lanes 2,4; Scharl and Steitz 1994, 1996). This observation suggests that U7 snRNP functions as a molecular ruler in processing (Scharl and Steitz 1994), and binding of U7 snRNP to the HDE, in contrast to binding SLBP to the stem-loop, is the critical event for determining the cleavage site in mammalian histone pre-mRNA 3' end processing. A possible explanation for the reduced processing efficiency of the mutant pre-mRNAs was that increasing the spacing between the stem-loop and the HDE abolishes an interaction between SLBP and U7 snRNP required for stabilizing the U7 snRNP on the pre-mRNA. Indeed, the residual processing of H2a/+12, which contains a 12-nt



**FIGURE 2.** Effects of increasing the distance between the stem-loop and the HDE on *Drosophila* processing. (A) The mouse histone mH2a/RI pre-mRNA (lanes 1–3) or its derivative mH2a/RS with the stem sequence reversed (lane 4–6) was incubated in a nuclear extract from mouse myeloma cells in the absence (lanes 2,5) or presence (lanes 3,6) of an excess of stem-loop RNA. (B) The mH2a/RI pre-mRNA (lanes 1,2) or the mH2a/+12 pre-mRNA that contains a 12-nt insertion (F) between the stem-loop and the HDE (lanes 3–6) was incubated in a mouse nuclear extract under control conditions (lanes 2,4), in the presence of excess stem-loop RNA (lane 5), or in the presence of the  $\alpha$ M 2'O-methyl oligonucleotide blocking the mouse U7 snRNA (lane 6). (C) The dH3\* pre-mRNA (lane 1), the dH3\*/+4 pre-mRNA (lane 2), or the mH2a/RI pre-mRNA (lane 3) was incubated in the indicated nuclear extracts and the cleavage products analyzed in a high-resolution polyacrylamide gel. The minor band migrating over the major cleavage product (arrow heads) is a result of processing of a 1-nt-longer pre-mRNA. The unprocessed input pre-mRNAs are not shown. (D) The dH3\* pre-mRNA (lanes 1–3) and the dH3\*/+8 pre-mRNA (lanes 4–6) were incubated in a *Drosophila* nuclear extract under control conditions (lanes 1,4) or in the presence of excess stem-loop RNA (lanes 2,5) or the  $\alpha$ Db oligonucleotide (lanes 3,6). The cleavage products were analyzed in a high-resolution polyacrylamide gel. The unprocessed input pre-mRNAs are not shown. (E) The dH3\* pre-mRNA (lanes 1,2) and the dH3\*/+16 pre-mRNA (lanes 3–7) were incubated in a *Drosophila* nuclear extract under control conditions (lanes 1,4) or in the presence of excess stem-loop RNA (lane 5), the  $\alpha$ Db oligonucleotide (lane 6), or the  $\alpha$ M oligonucleotide complementary to the mouse U7 snRNA (lane 7). (F) Sequences of the dH3\* mutant pre-mRNAs analyzed in C–E and cleavage sites detected in the processing reactions.

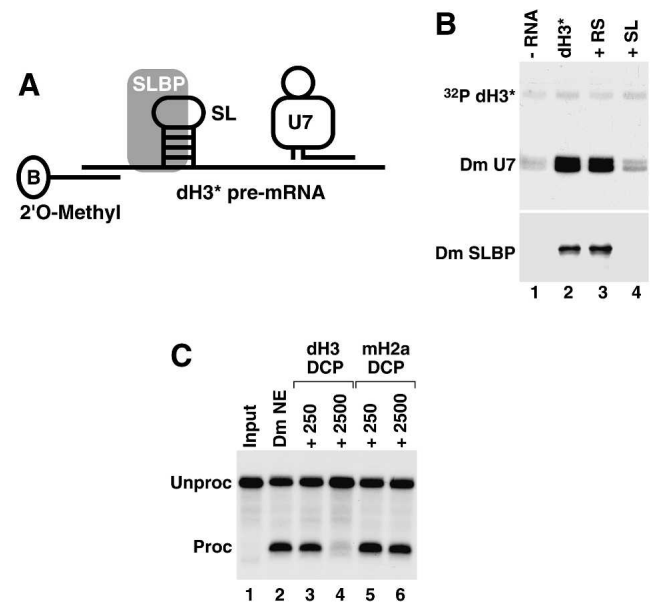
insertion within the EcoRI sequence (Dominski et al. 1999), is SLBP independent, since it is not affected by addition of a molar excess of the stem-loop RNA but is fully inhibited by the  $\alpha$ M 2'-O-methyl oligonucleotide, which is complementary to the first 17 nt of the mouse U7 snRNA (Fig. 2B, lanes 5,6).

We determined whether the same rules apply to histone pre-mRNA processing in *Drosophila* nuclear extracts. We increased the distance between the stem-loop and the HDE by inserting 4, 8, or 16 nt after the ACCCA of the dH3\* pre-mRNA and tested cleavage of the resulting pre-mRNAs in *Drosophila* nuclear extract. The inserted sequence consisted of adenosines and cytidines and provided favorable cleavage sites at a constant distance from the HDE (Fig. 2F). Overall processing of the dH3\*/+4 pre-mRNA was as efficient as processing of the parental dH3\* pre-mRNA and generated two products: the minor product that was identical to the product of processing of the dH3\* pre-mRNA, hence resulting from cleavage after the cytidine at +4, and the major product that was 1 nt longer, resulting from cleaving the dH3\*/+4 pre-mRNA after the ACCCA (Fig. 2C, lanes 1,2). Increasing the distance between the two sequence elements by 8 nt (Fig. 2F) yielded a similar result (Fig. 2D, lane 4). Processing of the resultant dH3\*/+8 occurred with equal efficiency at the normal +4 cleavage site and after the ACCCA (Fig. 2D, lane 4). There was also a small amount of a product resulting from cleavage after an adenosine at the position +9. Processing at all three sites was dependent on both SLBP and U7 snRNP, since it was abolished by excess of the stem-loop RNA or a 2'-O-methyl oligonucleotide,  $\alpha$ Db (Fig. 5A, below), complementary to 20 nt of the *Drosophila* U7 snRNA (Fig. 2D, lanes 4–6). The shift of the processing site by only 1 nt together with retaining efficient processing at the original site upon inserting 4 and 8 nt was in clear contrast to mammalian processing, which is strongly determined by the position of the HDE, and insertions as small as 4 nt in the H2a/RI pre-mRNA result in the complete shift of the cleavage site by the corresponding number of nucleotides (Dominski et al. 1999).

In contrast to the 4- and 8-nt insertions, insertion of 16 nt into the dH3\* pre-mRNA (Fig. 2F) abolished processing at the normal site and activated two weak cleavage sites further downstream (Fig. 2E, lane 4). Although we have not precisely mapped the cleavage site in dH3\*/+16, comparison with the processing product of the mouse mH2a/+12 pre-mRNA generated in the mouse nuclear extract indicates that in both pre-mRNAs the cleavage sites have moved up to 9–12 nt from the regular cleavage site (not shown). Interestingly, while processing of the mH2a/+12 pre-mRNA in a mouse nuclear extract was independent of SLBP (Fig. 2B, lanes 4,5), processing of the dH3\*/+16 pre-mRNAs remained sensitive to excess of stem-loop RNA (Fig. 2E, lane 5), indicating that binding of SLBP to the stem-loop is indispensable in *Drosophila* processing even if the cleavage site is located 13 nt distal to the stem-loop.

## The role of SLBP and the HDE in *Drosophila* 3' end processing

SLBP functions in mammalian histone pre-mRNA processing by facilitating recruitment of U7 snRNP to the pre-mRNA (Streit et al. 1993; Spycher et al. 1994; Dominski et al. 1999). We previously used an anti-SLBP antibody to precipitate processing complexes containing the U7 snRNP formed in a mouse nuclear extract on the mouse H2a-614 pre-mRNA (Dominski et al. 1999). However, we were unable to directly demonstrate that SLBP stimulates binding of the U7 snRNP to the pre-mRNA, since depleting or sequestering SLBP from the nuclear extract precludes subsequent precipitation of processing complexes by anti-SLBP. Recently we developed a new approach for isolating proteins (Dominski et al. 2003a) or processing complexes associated with histone pre-mRNAs (Dominski et al. 2003b) that allows direct testing of the role of SLBP in recruitment of the U7 snRNP. In this method, a pre-mRNA is annealed to an adapter 2'-O-methyl oligonucleotide containing biotin on the 3' end (Fig. 3A).

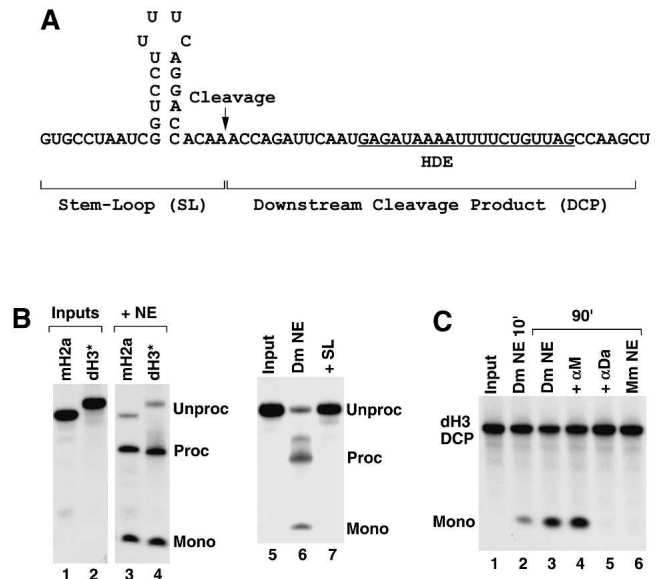


**FIGURE 3.** The role of SLBP and the HDE in *Drosophila* processing. (A) The scheme for purification of processing complexes containing SLBP and U7 snRNP assembled on the dH3\* pre-mRNA. The adapter 2'-O-methyl oligonucleotide complementary to the first 17 nt of the pre-mRNA contains biotin (B) on the 3' end. (B) Detection of the *Drosophila* U7 snRNA (Dm U7) by Northern blotting (top) and SLBP (Dm SLBP) by Western blotting (bottom) in *Drosophila* processing complexes formed on the dH3\* pre-mRNA in the absence of any competitor RNA (lane 2), in the presence of the reverse stem RNA (lane 3), or the stem-loop RNA (lane 4). The background amount of Dm SLBP and Dm U7 snRNA bound to streptavidin beads in the absence of the dH3\* pre-mRNA is shown in lane 1. A small amount of a radioactive dH3\* pre-mRNA ( $^{32}$ P dH3\*) was added to each processing reaction to monitor the efficiency of isolation of substrate RNA and to control for ethanol precipitation. (C) Processing of the dH3\* pre-mRNA in a *Drosophila* nuclear extract under control conditions (lane 2) or in the presence of a 250 or 2500 M excess of the downstream cleavage product (DCP) from the dH3 pre-mRNA (lanes 3,4) or the mouse H2a-614 pre-mRNA (lanes 5,6).

The oligonucleotide is complementary to the first 17 nt of the pre-mRNA and formation of the duplex does not interfere with processing reaction but allows subsequent recovery of the processing complexes on streptavidin beads. We previously successfully used this method to isolate and identify the *Drosophila* U7 snRNA (Dominski et al. 2003b) and to determine binding affinities of 3'Exo to various mutants of the mature H2a-614 mRNAs (Dominski et al. 2003a).

To form processing complexes we incubated 50 ng of the dH3\* pre-mRNA annealed to the 2'-O-methyl oligonucleotide with either 250  $\mu$ L of a normal *Drosophila* nuclear extract or the same amount of extract preincubated with a large excess of the stem-loop RNA to sequester all SLBP. As a negative control we used the same preparation of the nuclear extract preincubated with the reverse stem RNA (RS) unable to bind SLBP. Each sample contained a small amount of radioactively labeled dH3\* pre-mRNA to monitor recovery of the substrate. The amount of SLBP and U7 snRNA in processing complexes was determined by using Western and Northern blotting, respectively. Upon brief incubation at room temperature with the *Drosophila* nuclear extract, the dH3\* pre-mRNA formed processing complexes containing SLBP and the U7 snRNA, whereas only trace amounts of both factors were detected on streptavidin beads in the absence of the bulk of unlabeled dH3\* pre-mRNA (Fig. 3B, lanes 1,2). Addition of a large molar excess of the RS RNA did not affect the amount of both factors detected in the processing complexes assembled on the dH3\* pre-mRNA (Fig. 3B, lane 3). However, in the presence of the SL RNA, only background amounts of both SLBP and the U7 snRNA were collected on streptavidin beads, whereas the amount of the labeled dH3\* pre-mRNA was constant, indicating that the pre-mRNA was efficiently recovered from the nuclear extract (Fig. 3B, lane 4). Based on these results we conclude that *Drosophila* SLBP is essential for binding of U7 snRNP to histone pre-mRNA.

The HDE from the mouse H2a-614 pre-mRNA at 50-fold molar excess fully inhibited processing of the H2a-614 pre-mRNA, whereas the HDE from the mouse H1t pre-mRNA at the same concentration had no effect on processing of this pre-mRNA (Dominski et al. 1999). This result demonstrated that the HDE from the H2a-614 pre-mRNA, but not from the H1t pre-mRNA, can efficiently interact with the U7 snRNP in the absence of the stem-loop. We tested an ability of the dH3-specific HDE to compete processing of the dH3\* pre-mRNA. As a competitor in this experiment we used a 48-nt RNA corresponding to the downstream cleavage product (DCP) generated during processing of the *Drosophila* H3 pre-mRNA (Fig. 4A). The dH3 DCP RNA begins with the nucleotide that follows the cleavage site in the genuine *Drosophila* H3 pre-mRNA and contains the entire U7 binding site including the purine core GAGA. As a control we used the DCP generated during processing of the mouse H2a-614 pre-mRNA (Materials and Methods). This substrate is processed in *Drosophila* nuclear



**FIGURE 4.** Degradation of the *Drosophila* DCP by a 5'-3' exonuclease. (A) The sequence of the genuine *Drosophila* H3 pre-mRNA encompassing the stem-loop and the HDE. The arrow indicates the cleavage site. The upstream and the downstream cleavage products are indicated. (B) Processing of the uniformly labeled mH2a/RI (lane 3) or dH3\* pre-mRNA (lanes 4,6,7) in a mouse or a *Drosophila* nuclear extract, respectively, showing generation of the upstream cleavage product and the release of mononucleotides from degradation of the DCP. (C) Degradation of the dH3-specific DCP incubated during the indicated time in a *Drosophila* nuclear extract (lanes 2-5) under control conditions (lanes 2,3) or in the presence of the  $\alpha$ M or  $\alpha$ Da oligonucleotides (lanes 4,5). The dH3 DCP incubated for 90 min in a mouse nuclear extract is shown in lane 6.

extracts with very low efficiency (Dominski et al. 2002b) and forms a weaker duplex with the *Drosophila* U7 snRNP than does the *Drosophila* H3 pre-mRNA (Dominski et al. 2003b). The dH3 DCP only slightly reduced processing of the labeled dH3\* pre-mRNA at 250-fold molar excess but nearly completely inhibited processing of this pre-mRNA at 2500 molar excess (Fig. 3C, lanes 3,4). Even at this higher concentration, the mouse H2a-614 DCP had only a slight effect on processing of the dH3\* substrate (Fig. 3C, lanes 5,6). Altogether, these results demonstrate that in *Drosophila* processing, the HDE separated from the stem-loop cannot efficiently interact with the U7 snRNA, and SLBP plays the key role in recruiting the U7 snRNP to the pre-mRNA.

#### Degradation of the *Drosophila* DCP by a 5'-3' exonuclease

Following cleavage of mammalian histone pre-mRNA, the 3' downstream cleavage product (DCP) is subjected to exonucleolytic degradation by an activity that is resistant to EDTA and dependent on U7 snRNP (Walther et al. 1998). Synthetic RNAs encompassing the DCP from the mouse H4-12 pre-mRNA (Walther et al. 1998) or the

mouse H2a-614 pre-mRNA (our unpubl. results) are also rapidly degraded in mammalian nuclear extracts by the same activity, demonstrating that the exonucleolytic degradation of the DCP can be uncoupled from the endonucleolytic cleavage.

We analyzed the fate of the DCP generated during processing of the uniformly labeled dH3\* pre-mRNA in *Drosophila* nuclear extracts. After 90-min incubation, in many preparations of the *Drosophila* nuclear extract the dH3\* substrate was cleaved to form the upstream product containing the stem-loop, whereas the downstream cleavage product was not detectable and the remaining radioactivity was present as mononucleotides (Fig. 4B, lane 4). The radioactive mononucleotides were also generated during processing of the uniformly labeled mouse H2a/RI pre-mRNA in a mouse nuclear extract (Fig. 4B, lane 3). Accumulation of the mononucleotides during processing of the dH3\* pre-mRNA was inhibited by the presence of the SL RNA (Fig. 4B, lane 7) or an oligonucleotide blocking *Drosophila* U7 snRNA (not shown) and thus was strictly related to the processing activity and not a result of nonspecific degradation of the dH3\* pre-mRNA. We tested whether 5'–3' exonucleolytic activity can degrade a synthetic DCP from the *Drosophila* H3 pre-mRNA (Fig. 4A). The 39-nt dH3 DCP RNA was labeled at the 5' end and incubated in a *Drosophila* nuclear extract under the same conditions as used for histone pre-mRNA processing and the release of the radioactive mononucleotide and disappearance of the input RNA monitored in denaturing gels. A small amount of the mononucleotide was generated after 10-min incubation at room temperature, and after 90 min ~50% of the input was degraded (Fig. 4C, lanes 2,3). The degradation was inhibited by the  $\alpha$ Da oligonucleotide complementary to the first 17 nt of the *Drosophila* U7 snRNA but was unaffected by the same concentration of the  $\alpha$ M complementary to the first 17 nt of the mouse U7 snRNA (Fig. 4C, lanes 4,5). Thus, release of the mononucleotide was dependent on the ability of the *Drosophila* U7 snRNP to bind the dH3 HDE. The *Drosophila* H3 DCP was stable during a 90-min incubation in a mouse nuclear extract (Fig. 3D, lane 6), and the mouse H2a-614 specific DCP was not degraded in the *Drosophila* nuclear extract (not shown), further demonstrating that degradation of the RNA substrate is U7 dependent and is not catalyzed by nonspecific nucleases resistant to EDTA. These results demonstrate that degradation of the DCP by the U7-dependent activity is a universal feature of 3' end processing of histone pre-mRNAs that has been conserved between vertebrates and invertebrates.

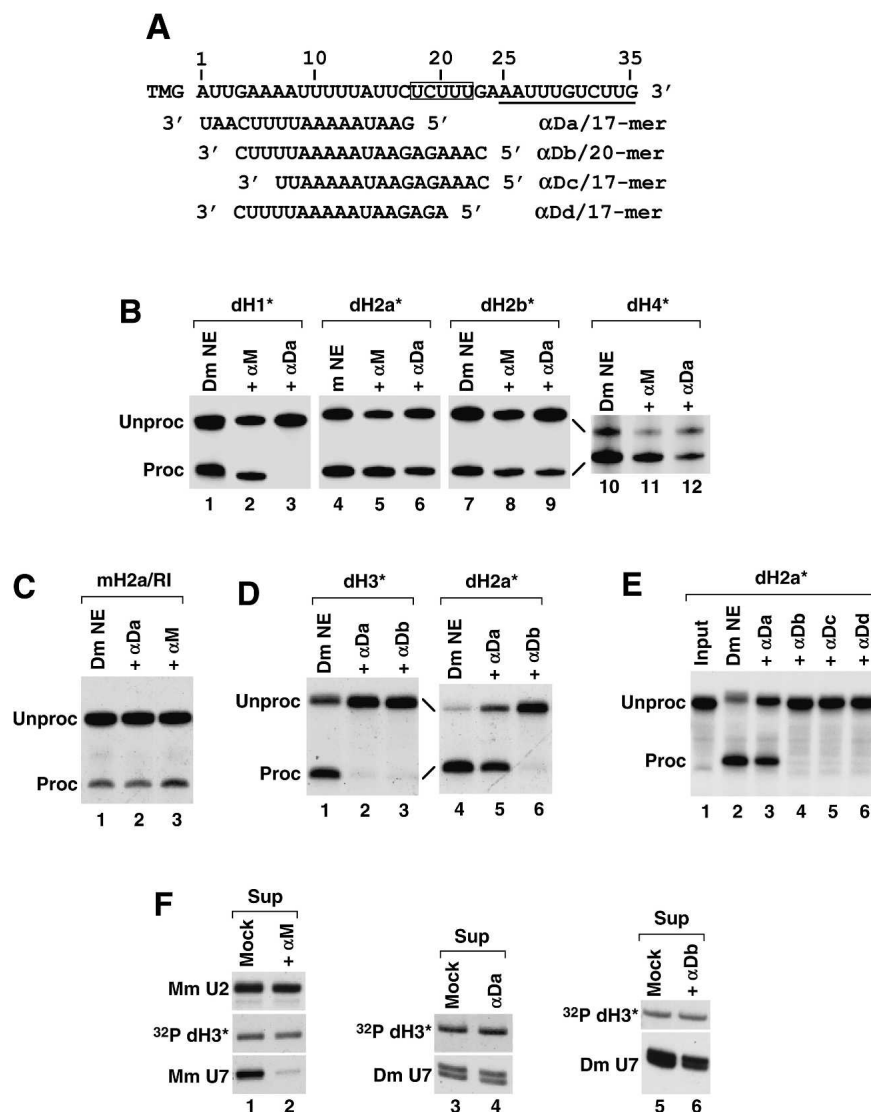
#### Effects of 2'O-methyl oligonucleotides complementary to the *Drosophila* U7 snRNA on 3' end processing

*Drosophila* U7 snRNA is unusual in having a long 5' region that can potentially base pair with the HDE over a 24-nt region. At the 3' end of this region (nt 18–22) there is a

UCUUU sequence, which is complementary to the purine core of the HDE and is conserved in vertebrate and sea urchin U7 snRNAs. A 2'O-methyl oligonucleotide complementary to the first 17 nt of the U7 snRNA ( $\alpha$ Da), which does not overlap with the UCUUU sequence (Fig. 5A), inhibits processing of the dH3\* pre-mRNA (Fig. 5E), providing evidence that the base-pairing between the U7 snRNA and the HDE is essential in *Drosophila* processing (Dominski et al. 2003b). We tested the effect of the same oligonucleotide on processing of the substrates containing the HDE from the other four *Drosophila* histone pre-mRNAs: H1, H2a, H2b, and H4 (Fig. 1A). As a negative control we used a 2'O-methyl oligonucleotide complementary to the first 17 nt of the mouse H2a-614 pre-mRNA ( $\alpha$ M). Surprisingly, processing of only one of these pre-mRNAs, dH1\*, was inhibited by the  $\alpha$ Da oligonucleotide, whereas processing of the three other substrates, dH2a\*, dH2b\*, and dH4\* pre-mRNAs, was only partially reduced (Fig. 5B). Processing of the mouse H2a/RI pre-mRNA in the *Drosophila* nuclear extract was not affected by this oligonucleotide (Fig. 5C).

The sensitivity of dH3\* and dH1\* pre-mRNAs and the resistance of the four other pre-mRNAs were independent of the cell line (Kc or S2) or batch of *Drosophila* nuclear extract, suggesting that it reflects a fundamental difference in how U7 snRNP recognizes these two groups of substrates. We tested three additional 2'O-methyl oligonucleotides:  $\alpha$ Db complementary to a 20-nt region of the *Drosophila* U7 snRNA that extends into the UCUUU conserved sequence;  $\alpha$ Dc, a 17-mer with the same 5' end as  $\alpha$ Db; and  $\alpha$ Dd, a 17-mer with the same 3' end as  $\alpha$ Db (Fig. 5A). Each of these oligonucleotides inhibited processing of the dH2a\* pre-mRNA (Fig. 5D, lane 6; Fig. 5E, lanes 4–6) and the mouse H2a/RI pre-mRNA (not shown).

One possibility explaining the failure of a large molar excess of the  $\alpha$ Da oligonucleotide to inhibit processing of dH2a\*, dH2b\*, dH4\*, and the mouse H2a/RI pre-mRNAs was that the region located closer to the 5' end of the *Drosophila* U7 snRNA is bound by proteins or structured and thus inaccessible to the oligonucleotide until the processing reaction has been initiated. We determined whether a large molar excess of the  $\alpha$ Da and  $\alpha$ Db oligonucleotides could efficiently deplete the U7 snRNA from a *Drosophila* nuclear extract. As a negative control we carried out a parallel experiment in the absence of any oligonucleotide. We also tested the ability of the  $\alpha$ M oligonucleotide to deplete the mouse U7 snRNA from a mouse nuclear extract. Mouse or *Drosophila* nuclear extracts were incubated with the appropriate oligonucleotide, each containing biotin on the 5' end, followed by absorption of the oligonucleotide and the bound U7 snRNP on streptavidin beads. The efficiency of depletion was determined by analyzing the amount of the U7 snRNAs remaining in each supernatant using Northern blotting with either mouse- or *Drosophila*-specific probes. Incubation of the mouse nuclear extract



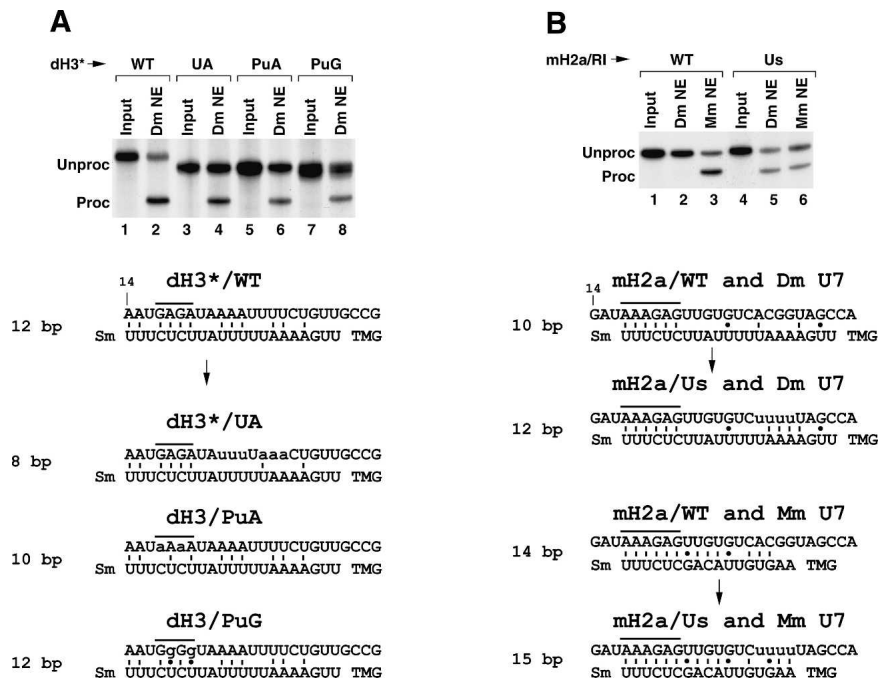
**FIGURE 5.** Variable effects of 2'-O-methyl oligonucleotides complementary to the *Drosophila* U7 snRNA on *Drosophila* histone pre-mRNA processing. (A) The sequence of the first 35 nt of the *Drosophila* U7 snRNA (written in 5'-3' orientation) extending from the 5' terminal tri methyl guanosine cap (TMG) to the end of the Sm binding site (underlined) and the sequences of the four 2'-O-methyl oligonucleotides complementary to the 5' end of the U7 snRNA (written in 3'-5' orientation). The highly conserved UCUUU sequence in the U7 snRNA is boxed. (B) Processing of the dH1\*, dH2a\*, dH2b\*, and dH4\* histone pre-mRNAs in a *Drosophila* nuclear extract under control processing conditions (lanes 1,4,7,10) or after preincubation of the nuclear extract with either the  $\alpha$ M (lanes 2,5,8,11) or the  $\alpha$ Da oligonucleotide (lanes 3,6,9,12). (C) Processing of the mouse histone H2a/RI pre-mRNA in a *Drosophila* nuclear extract under control processing conditions (lane 1) or after preincubation of the nuclear extract with either the  $\alpha$ Da (lane 2) or the  $\alpha$ M oligonucleotide (lane 3). (D) Processing of the dH3\* and dH2a\* pre-mRNAs in a *Drosophila* nuclear extract under control processing conditions (lanes 1,4) or after preincubation of the nuclear extract with either the  $\alpha$ Da (lanes 2,5) or the  $\alpha$ Db oligonucleotide (lanes 3,6). (E) Processing of the dH2a\* pre-mRNA in a *Drosophila* nuclear extract under control processing conditions (lane 2) or after preincubation of the nuclear extract with the indicated oligonucleotides complementary to the *Drosophila* U7 snRNA (lanes 3-6). Lane 1 contains the input pre-mRNA. (F) The indicated biotinylated 2'-O-methyl oligonucleotides were incubated with either a mouse (lane 1) or a *Drosophila* (lanes 4,6) nuclear extract and collected on streptavidin beads. The level of U7 snRNA left in the supernatant was determined by Northern blotting. In the mock experiments (lanes 1,3,5), the nuclear extracts were preincubated with streptavidin beads in the absence of any oligonucleotide. A small amount of the labeled pre-mRNA was added to the extract to control for the efficiency of precipitation ( $^{32}$ P dH3\*). The mouse U2 snRNA (Mm U2) was detected by Northern blotting to determine the specificity of depletion of the mouse U7 snRNA (Mm U7) in the mouse nuclear extract (lanes 1,2).

with the  $\alpha$ M oligonucleotide reduced the amount of the U7 snRNA to  $\sim$ 20% of the amount detected in the control supernatant (Fig. 5F, lanes 1,2). The amount of the U2 snRNA (Mm U2) in each supernatant was similar, indicating that the  $\alpha$ M oligonucleotide specifically removed the mouse U7 snRNA. In contrast, the  $\alpha$ Da oligonucleotide did not reduce the amount of the *Drosophila* U7 snRNP in the *Drosophila* nuclear extract (Fig. 5F, lanes 3,4), and the  $\alpha$ Db removed only  $\sim$ 50% of the U7 snRNP (Fig. 5F, lanes 5,6). Thus, the region near the 5' end of the U7 snRNA in *Drosophila* U7 snRNP is not readily accessible.

### Effects of mutations within the HDEs of the dH3\* and H2a/RI pre-mRNAs on 3' end processing

A feature of the extended 5' end of the *Drosophila* U7 snRNA is a stretch of four adenosines followed by five uridines (Fig. 5A). All the *Drosophila* HDEs contain a stretch of uridines, and all, except H2b pre-mRNA, contain an adjacent stretch of adenosines, which could potentially base pair to this region of the U7 snRNA. We previously showed that two 3-nt mutations within the HDE of the dH3\* replacing the AAA or the UUU located 23-25 and 27-29 nt downstream of the stem-loop with the complementary sequences had no effect on processing (Dominski et al. 2002b). When these two mutations were combined in the dH3\*/UA pre-mRNA, processing was less efficient but not abolished, suggesting that base-pairing in this region is not essential for processing (Fig. 6, lane 4). In contrast, a 3-nt mutation within the AGA purine core (nt 18-20 downstream of the stem-loop) nearly completely inhibited processing (Dominski et al. 2002b). To investigate what features of the purine core are important for processing, we changed the GAGA in the dH3\* pre-mRNA (overlined in Figs. 6, 7) to either four adenosines or four guanosines and tested the resulting mutant pre-mRNAs, dH3/PuA and dH3/PuG, for processing in a *Drosophila* nuclear extract. Both





**FIGURE 6.** Effects of mutations within the HDEs of the dH3\* and H2a/RI pre-mRNAs on 3' end processing. (A) Processing of the uniformly labeled wild-type dH3\* histone pre-mRNA (lanes 1,2) and its mutants, UA, PuA, and PuG (lanes 3–8), in a *Drosophila* nuclear extract. The sequence of the pre-mRNAs and their likely base-pairing with the *Drosophila* U7 snRNA are shown at the *bottom*. GU base pairs are indicated with dots. In each duplex, the pre-mRNA sequence beginning with the 14th nucleotide following the stem-loop is shown at the *top* and the U7 snRNA sequence is shown at the *bottom*. The purine core is overlined and the mutated nucleotides are written in lowercase letters. (B) Processing of the wild-type mouse H2a/RI pre-mRNA (lanes 1–3) and the mutant mH2a/Us pre-mRNA containing four Us in the HDE (lanes 4–6) in a *Drosophila* (lanes 2,5) or a mouse (lanes 3,6) nuclear extract. Likely base-pairing schemes between the pre-mRNAs and either the *Drosophila* U7 snRNA or mouse U7 snRNA are shown at the *bottom*.

mutations in the purine core of the HDE had a moderate effect on processing, reducing efficiency from 60% for the wild type to ~25% for each mutant pre-mRNA (Fig. 6A). Thus, the presence of both adenosines and guanosines in the purine core of the HDE is important for maximum efficiency of processing.

The mouse H2a/RI pre-mRNA is processed in *Drosophila* nuclear extracts with very low efficiency. The HDE of this pre-mRNA also contains the purine-rich core, which can efficiently base pair with the *Drosophila* U7 snRNA. However, the mouse H2a/RI pre-mRNA can only form a total of 10 bp with the *Drosophila* U7 snRNA, compared to the 12 bp formed by the dH3\*. In particular the mouse H2a HDE cannot base pair with the stretch of adenosines and uridines in the *Drosophila* U7 snRNA. We replaced the ACGG in the mouse H2a HDE with four uridines to allow additional base-pairing to the 5' end of the *Drosophila* U7 snRNA (Fig. 6B). This nucleotide substitution should increase the number of base pairs formed between the pre-mRNA and the *Drosophila* U7 snRNA from 10 to 12 without disrupting the base-pairing with the mouse U7 snRNA. We compared processing of the original mouse H2a/RI pre-mRNA

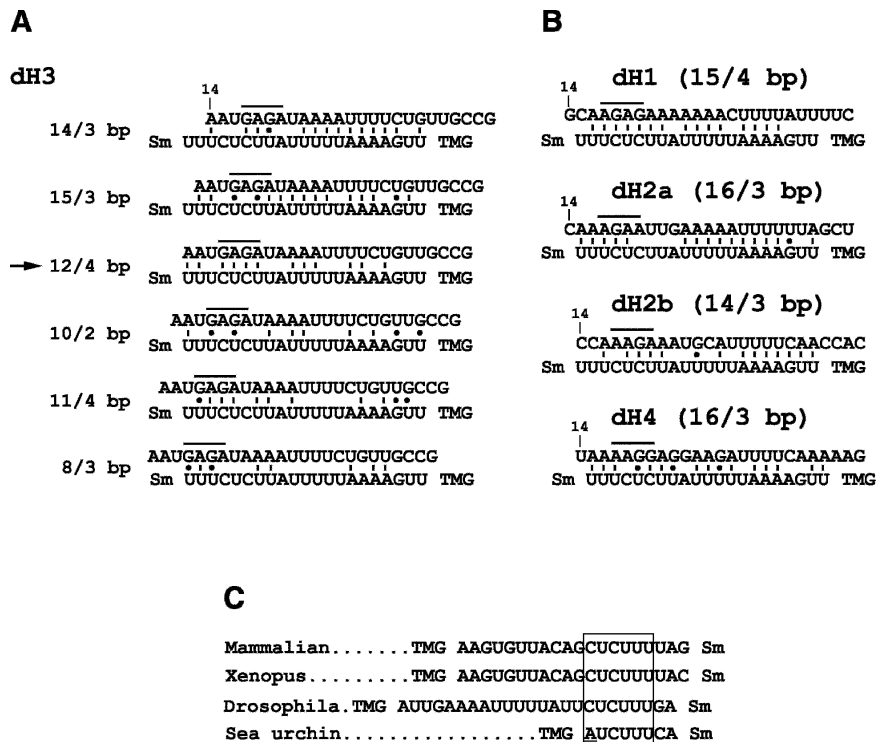
and the mutant mH2a/Us pre-mRNA in both *Drosophila* and mouse nuclear extracts. The wild-type H2a/RI pre-mRNA was not detectably processed in this preparation of a *Drosophila* nuclear extract, although the same preparation of the nuclear extract processed ~45% of the mutant pre-mRNA with the improved base-pairing to the 5' end of the *Drosophila* U7 snRNA (Fig. 6B, lanes 2,5). In a mouse nuclear extract the wild-type H2a/RI pre-mRNA was processed with ~60% efficiency, and substitution of the ACGG with the UUUU reduced the efficiency to 35% (Fig. 6B, lanes 3,6), possibly because the mutation incorporated an additional mismatch to the duplex with the mouse U7 snRNA or the UUUU sequence in the HDE is less favorable in mammalian processing. Thus, by increasing the base-pairing potential of the mouse H2a pre-mRNA to the *Drosophila* U7 snRNA without significantly changing the base-pairing potential of the mutant pre-mRNA to the mouse U7 snRNA, we created a substrate that, unlike any of the wild-type *Drosophila* histone pre-mRNAs, undergoes efficient processing in both *Drosophila* and mouse nuclear extracts.

## DISCUSSION

We carried out *in vitro* studies using nuclear extracts from *Drosophila* Kc cells and mouse myeloma cells to compare 3' end processing of histone pre-mRNAs in *Drosophila* and mammalian systems. Our studies demonstrate that although *Drosophila* and mammalian histone pre-mRNA processing occur with similar chemistry and both require SLBP and the U7 snRNP, the two mechanisms differ significantly in the relative importance of these *trans*-acting factors and in the specification of the cleavage site.

### The stem-loop and SLBP play a dominant role in *Drosophila* processing

*Drosophila* nuclear extracts cleave histone pre-mRNAs after the fourth nucleotide following the stem-loop and prefer an adenosine preceding the cleavage site. Consistent with this, all natural *Drosophila* histone pre-mRNAs contain an adenosine in this position. If the fourth nucleotide is changed to a pyrimidine, cleavage is also efficient after an adenosine at the third position but not after an adenosine located 5 nt



**FIGURE 7.** Potential base-pairing schemes between *Drosophila* pre-mRNAs and the *Drosophila* U7 snRNA. (A) Several possible base-pairing alignments between the dH3-specific HDE (top sequence) and the first 21 nt of the *Drosophila* U7 snRNA (bottom sequence). The sequence of the HDE starts 14 nt downstream of the stem-loop (10 nt after the cleavage site). The purine core located between nt 17 and 20 is overlined. The Sm indicates the Sm binding site and the TMG indicates the tri-methyl guanosine cap at the 5' end of the U7 snRNA. There are two additional nucleotides between the last indicated nucleotide of the U7 snRNA and the Sm binding site. Due to the proximity to the Sm binding site these nucleotides are unlikely to base pair with the pre-mRNA and are not included. The arrow indicates the most favorable alignment that forms a relatively stable duplex and contains the highest number of base pairs between the purine core of the HDE and the UCUUU sequence of the U7 snRNA. (B) The most favorable alignments between the HDE from the four remaining *Drosophila* histone pre-mRNAs, H1, H2a, H2b, and H4, and the *Drosophila* U7 snRNA. The numbers in parentheses indicate the total number of base pairs within the duplex and the number of the base pairs formed between the purine core of the HDE and the CUCUUU sequence of the U7 snRNA, respectively. (C) The sequences located between the trimethyl guanosine (TMG) cap and the Sm binding site of the U7 snRNAs from evolutionarily distant organisms. The highly conserved CUCUUU sequence is boxed. The adenosine in the sea urchin U7 snRNA departing from the consensus is underlined.

downstream of the stem-loop, i.e., at the site exclusively utilized during mammalian processing. Sea urchin histone mRNAs, the only other invertebrate histone mRNAs with the characterized 3' ends, terminate with an ACCA consensus sequence (Birnstiel and Schaufele 1988). Thus, cleavage after the fourth nucleotide following the stem-loop may be a general feature of 3' end processing of invertebrate histone pre-mRNAs. Both *Drosophila* and mammalian processing machineries are similar in their extreme resistance to EDTA, generation of a 3' hydroxyl group at the end of the upstream cleavage product, and degradation of the downstream cleavage product by a U7 snRNP dependent activity. These results suggest that both processing machineries utilize the same or a highly related cleavage factor in 3' end processing of histone pre-mRNAs.

In mammalian processing, the site of cleavage is determined by the position of the HDE, and moving the HDE, and, hence, the U7 snRNP, away from the stem-loop by as few as 4 nt results in a corresponding shift of the cleavage site (Scharl and Steitz 1994, 1996; Dominski et al. 1999). This observation led to the hypothesis that U7 snRNP recruits the cleavage factor to the pre-mRNA and acts as a molecular ruler to specify the cleavage site (Scharl and Steitz 1994, 1996). SLBP bound to the stem-loop facilitates binding of the U7 snRNP to the HDE but does not play a direct role in recruitment of the cleavage factor. Consistent with this model, removal of SLBP, or using a substrate that cannot bind SLBP, reduces processing activity but does not abolish it.

In contrast to mammalian processing, processing of *Drosophila* histone pre-mRNA is absolutely dependent on SLBP. In addition, increasing the distance between the stem-loop and the HDE by 4 or 8 nt in *Drosophila* histone pre-mRNA moved the cleavage site only 1 nt upstream from its normal position and did not abolish processing at the normal site. Larger insertions between the stem-loop and the HDE resulted in low efficiency cleavage further away from the stem-loop, but cleavage at these sites was still dependent on SLBP. This is in direct contrast to mammalian histone processing, where cleavage at the distant sites is independent of SLBP. Thus, in *Drosophila* processing the U7 snRNP does not function as a molecular ruler, but instead SLBP plays the critical role in

specifying the cleavage site.

To explain the observed differences between processing in *Drosophila* and mammalian nuclear extracts, we propose that within the *Drosophila* processing complex SLBP tightly interacts with the U7 snRNP, and this interaction is essential for bringing the U7 snRNP to the pre-mRNA. The two factors remain associated even if their respective binding sites are separated by a larger distance, likely by looping out the inserted nucleotides. The mutant pre-mRNAs are preferentially cleaved close to the stem-loop, reflecting the critical role of SLBP in forming the processing complex, although the precise position of the cleavage site and efficiency of processing depends on the size of the insert. In mammalian processing, the region between the stem-loop and the HDE is either rigidified, thus precluding looping

out the inserted nucleotides, as previously suggested (Scharl and Steitz 1994, 1996), or the interaction between SLBP and the U7 snRNP is relatively weak and disrupted by larger insertions, so binding of the U7 snRNP to the pre-mRNA depends solely on the base-pairing interaction. It is likely that in *Drosophila* processing the cleavage factor is recruited to histone pre-mRNA by interaction with both the U7 snRNP and SLBP, and neither factor is competent to carry out this function individually.

### The 5' end of *Drosophila* U7 snRNA is not accessible

In mammalian nuclear extracts processing of histone pre-mRNAs is efficiently inhibited by relatively short 2'-O-methyl oligonucleotides complementary to the 5' end of the mammalian U7 snRNA (Cotten et al. 1991). These oligonucleotides, including a 10-mer, are also very efficient in depleting the U7 snRNP from nuclear extracts and were successfully used to affinity purify U7 snRNP from mammalian cells, demonstrating that the 5' end of the mammalian U7 snRNA is readily accessible (Smith et al. 1991; Pillai et al. 2001, 2003). In contrast, two relatively long oligonucleotides,  $\alpha$ Da, complementary to the first 17 nt of the *Drosophila* U7 snRNA, and  $\alpha$ Db, complementary to nt 4–23, were not effective in depleting the U7 snRNP from *Drosophila* nuclear extracts. These results suggest that the 5' end of U7 snRNA is not accessible in the *Drosophila* U7 snRNP.

Surprisingly, the  $\alpha$ Da 2'-O-methyl oligonucleotide abolished processing of the dH3\* and dH1\* pre-mRNAs but did not significantly affect processing of the other three *Drosophila* histone pre-mRNAs. Three additional oligonucleotides complementary to the regions of the U7 snRNP located closer to the Sm binding site effectively blocked processing of all five histone pre-mRNAs. We do not understand why processing of only two *Drosophila* pre-mRNAs was affected by the  $\alpha$ Da oligonucleotide and which features of the HDEs make processing of the *Drosophila* pre-mRNAs either sensitive or resistant to this oligonucleotide. Selective inhibition of processing by the  $\alpha$ Da oligonucleotide depending on the type of pre-mRNA used in the reaction suggests that blocking of the U7 snRNA must occur during processing. One possibility is that the U7 snRNP is initially recruited to the pre-mRNA solely by SLBP bound to the pre-mRNA, and later this interaction is followed by formation of a duplex between the HDE and the U7 snRNA, as a result of unmasking of the 5' end of U7 snRNA. The  $\alpha$ Da oligonucleotide might block binding of the U7 snRNA to the HDE in the dH1\* and dH3\* pre-mRNAs, but not in the other pre-mRNAs, during this later step, while the other oligonucleotides block binding to all the HDEs.

Overall, our studies indicate that the structure of the 5' end of the *Drosophila* U7 snRNA and the mechanism of its initial interactions with the HDE differ significantly from the recognition of the HDE in processing of mammalian histone pre-mRNAs.

### Base-pairing between U7 snRNA and HDE in *Drosophila* processing

In vitro processing of all five *Drosophila* histone pre-mRNAs is absolutely dependent on SLBP (Dominski et al. 2002b). Here we demonstrated that SLBP is essential for recruitment of the U7 snRNP to the pre-mRNA. The necessity of SLBP for recruitment of the U7 snRNP to the *Drosophila* pre-mRNAs suggests that either *Drosophila* HDEs are unable to form a strong duplex with the U7 snRNA or that the interaction of the U7 snRNP with the SLBP/pre-mRNA complex is necessary to promote base-pairing by making the 5' end of U7 snRNA accessible.

Both the 5' end of the *Drosophila* U7 snRNA and *Drosophila* HDEs are AU rich, allowing a number of possible base-pair schemes for making a duplex between the two RNAs. We hypothesize that the most likely alignment used during processing is the one that allows formation of the largest number of base pairs between the purine core of the HDE and the CUCUUU sequence in the U7 snRNA and not necessarily the alignment, which allows formation of the overall most stable duplex (Fig. 7). The CUCUUU sequence is highly conserved among all known U7 snRNAs and is involved in recognition of the purine core in sea urchin and mammalian histone pre-mRNAs. A 3-nt mutation within the purine core of the dH3\* pre-mRNA abolished processing (Dominski et al. 2002b), whereas a 6-nt mutation within the AU-rich region immediately downstream of the purine core only partially inhibited processing. These results support our interpretation that base-pairing between the U7 snRNA and the purine core is critical, whereas formation of additional base in other regions increases the efficiency of *Drosophila* processing. It is also possible that the base-pairing interaction is limited to the purine core and the CUCUUU sequence in the U7 snRNA, whereas the AU-rich sequences in the U7 snRNA and the HDE are brought together by protein-protein interactions.

We demonstrated that the HDE of the dH3\* pre-mRNA can abolish processing of the full-length substrate, presumably by sequestering the U7 snRNP, only when present at very high concentrations. Interestingly, this weak interaction of *Drosophila* HDEs with the U7 snRNP is sufficient to recruit a 5'-3' exonuclease that specifically degrades the downstream cleavage product in a U7 dependent manner. Thus, the endonucleolytic cleavage must require much stronger binding of the U7 snRNP to the pre-mRNA, while degradation of the DCP by an exonuclease may require only loose association of the HDE with the U7 snRNP.

### Conclusions

The most notable difference between histone pre-mRNA processing in *Drosophila* and mammalian nuclear extracts is the absolute dependence of *Drosophila* processing on SLBP

and the role of SLBP in specifying the cleavage site close to the stem-loop. The *Drosophila* U7 snRNP does not function as a molecular ruler in processing and this feature most likely reflects a critical role of SLBP in recruiting the cleavage factor as well as the U7 snRNP, to histone pre-mRNA. Our data suggest that SLBP and the U7 snRNP may form a tight complex on the histone pre-mRNA, and this complex remains stable even in the presence of large insertions between the stem-loop and the HDE.

The similarities in the chemistry of the cleavage reaction, including preference for an adenosine preceding the cleavage site and generation of the 3'OH group in the presence of EDTA, as well as degradation of the downstream cleavage product by a U7-dependent 5'–3' exonuclease suggest that the cleavage factor has been conserved between *Drosophila* and mammalian processing. It will be of interest to determine whether there are factors unique to only one of these two types of organisms emphasizing long evolutionary distance and the divergence between vertebrates and invertebrates.

## MATERIALS AND METHODS

### RNA

RNA oligonucleotides were synthesized by Dharmacon. The sequences of the 2'-O-methyl oligonucleotides complementary to the *Drosophila* U7 snRNA are shown in Figure 5. Other 2'-O-methyl oligonucleotides had the following sequences (written in 5'–3' orientation):

AAAGAGCUGAACACUU ( $\alpha$ M),  
CGAGCUCGAAUUCGCC (adaptor oligonucleotide with biotin on the 3' end),  
ACCAGAUUCAUGAGAUAAAAUUUCUGUUAGCCA  
AGCU (*Drosophila* H3 DCP), and  
CUGAAUCAGUAAAAGAGUUGUGUCACGGUAGCCAA  
GCU (mouse H2a-614 DCP).

*Drosophila* and mouse-specific pre-mRNA substrates were generated by T7 transcription. In most cases the pre-mRNA substrates were first synthesized in the presence of unlabeled nucleotides, gel purified, dephosphorylated by calf intestinal phosphatase, and labeled at the 5' end using T4 polynucleotide kinase (NEB) and [<sup>32</sup>P]- $\gamma$ ATP. Internally labeled RNA substrates were synthesized by incorporation of [<sup>32</sup>P]- $\alpha$ UTP, as described (Dominski et al. 1999).

### Nuclear extract preparation and histone pre-mRNA processing

Nuclear extracts were prepared from *Drosophila* Kc cells and mouse myeloma cells, and processing of histone pre-mRNAs was carried out as previously described (Dominski et al. 1995, 2002b). Each processing reaction contained in a final volume of 10  $\mu$ L the following: 7.5  $\mu$ L nuclear extract (10 mg/mL protein), 20 mM EDTA (pH 8), and 0.1 pmol of a radioactively labeled substrate

(Dominski et al. 1999, 2002b). *Drosophila* and mouse processing reactions were incubated for 90 min at 25°C (room temperature) or 32°C, respectively. The reactions were then treated with 5  $\mu$ g of proteinase K, diluted with 4 volumes of 7 M urea dye, and the processing products analyzed in 8%/7 M polyacrylamide gels.

### Formation of *Drosophila* processing complexes

*Drosophila* processing complexes were assembled and isolated as described (Dominski et al. 2003b). The mouse and *Drosophila* U7 snRNAs were analyzed as described (Dominski et al. 1999, 2003b).

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