Characterization of 3’hExo, a 3’ Exonuclease Specifically Interacting with the 3’ End of Histone mRNA

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The 3’ end of mammalian histone mRNAs consisting of a conserved stem-loop and a terminal ACCCA interacts with a recently identified human 3’ exonuclease designated 3’hExo. The sequence-specific interaction suggests that 3’hExo may participate in the degradation of histone mRNAs. ERI-1, a Caenorhabditis elegans homologue of 3’hExo, has been implicated in degradation of small interfering RNAs. We introduced a number of mutations to 3’hExo to identify residues required for RNA binding and catalysis. To assure that the introduced mutations specifically target one of these two activities of 3’hExo rather than cause global structural defects, the mutant proteins were tested in parallel for the ability both to bind the stem-loop RNA and to degrade RNA substrates. Our analysis confirms that 3’hExo is a member of the DEDDh family of 3’ exonucleases. Specific binding to the RNA requires the SAP domain and two lysines located immediately to its C terminus. 3’hExo binds with the highest affinity to the wild-type 3’ end of histone mRNA, and any changes to this sequence reduce efficiency of binding. 3’hExo has only residual, if any, 3’ exonuclease activity on DNA substrates and localizes mostly to the cytoplasm, suggesting that in vivo it performs exclusively RNA-specific functions. Efficient degradation of RNA substrates by 3’hExo requires 2’ and 3’ hydroxyl groups at the last nucleotide. 3’hExo removes 3’ overhangs of small interfering RNAs, whereas the double-stranded region is resistant to the enzymatic activity.

Mammalian histone mRNAs end with a highly conserved and unique stem-loop structure followed by a single-stranded ACCCA sequence (1). The 3’ end of histone mRNAs is specifically recognized by two proteins, the stem-loop-binding protein (SLBP) and 3’hExo (2). Binding of SLBP requires the nucleotides at the 5’ side of the stem-loop, whereas binding of 3’hExo requires the single-stranded ACCCA at the 3’ end. In addition, binding of each protein requires specific nucleotides in the stem and the loop. SLBP and 3’hExo can bind the 3’ end of histone mRNA either individually or simultaneously, forming a ternary complex (2). SLBP stimulates binding of 3’hExo to the stem-loop and allows 3’hExo to form a complex with subopti-

mal RNA targets, raising the possibility that the two proteins make direct contact with each other in the ternary complex (2). Binding of SLBP to the stem-loop in histone mRNA precursors is required for formation of the correct 3’ end of histone mRNAs in the nucleus (3). SLBP bound to the stem-loop accompanies mature histone mRNA to the cytoplasm, where it stimulates histone mRNA translation (4).

The half-life of histone mRNAs is greatly reduced in response to completion or inhibition of DNA replication, resulting in rapid disappearance of histone mRNAs from the cytoplasm and cessation of histone production (5). The stem-loop structure is both necessary and sufficient for the selective degradation of histone mRNA and confers the same type of regulation on other mRNAs when introduced at their 3’ end (6). Sequence-specific and tight binding of 3’hExo to the 3’ end of histone mRNAs simultaneously with SLBP makes this protein a primary candidate for an exonuclease that participates in the rapid degradation of histone mRNAs. However, it has been recently suggested that 3’hExo (7) and its putative Caenorhabditis elegans homologue, ERI-1 (8), might play a role in down-regulation of RNA interference by degrading small interfering RNAs (siRNAs).²

Crystallographic studies of the 3’ exonuclease domain of 3’hExo in complex with rAMP demonstrated that 3’hExo is a member of a DEDD superfamily of 3’ exonucleases (9) that includes both RNases and DNases (10). Among the most prominent members of the family are RNase T, poly(A)-specific ribonuclease, the exosome component PM-Scl 100-kDa autoantigen, and the proofreading subunits of DNA polymerases (10). Interestingly, based on data base searches 3’hExo and ERI-1 appear to be part of a group of closely related but uncharacterized putative exonucleases present in a number of eukaryotes. To get some insight into the molecular function of 3’hExo and its homologues, we carried out experiments to further define the specificity of this enzyme and the regions required for its activity.

EXPERIMENTAL PROCEDURES

Expression and Mutagenesis of 3’hExo—The ³⁵S-labeled 3’hExo was synthesized in rabbit reticulocytes using the transcription and translation-coupled system (TNT), as suggested by the manufacturer (Promega). SLBP and the wild-type and mutant forms of 3’hExo were overexpressed using the baculovirus expression system (Invitrogen). Briefly, the cDNAs for each protein were cloned into a pFastBac plasmid and used to

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² The abbreviations used are: siRNA, small interfering RNA; SL, stem-loop.
generate the recombinant viruses. For preparative purification, a 200-ml culture of Sf9 insect cells was infected with each virus and the His-tagged proteins were purified by chromatography on nickel-agarose. Point mutations and internal deletions within the 3’hExo coding region were generated using the QuickChange site-directed mutagenesis kit (Stratagene).

**RNA**s—The 31-nucleotide stem-loop (SL) RNA and its derivative containing biotin at the 5’ end (SLbi) were synthesized by Dharmacon. The sequence of these RNAs is shown in Fig. 1A. The sequences of the 48-nucleotide H2a and 86-nucleotide pre-H2a RNAs, both generated by T7 transcription, were published previously (2). H2a mutant RNAs containing various deletions or mutations at the 3’ end were generated by T7 transcription using appropriately modified DNA templates. The following single-stranded RNA oligonucleotides were ordered from Dharmacon and used as substrates for 3’hExo (written in 5’-3’ orientation): CGUCACUUACGGAUCCGAAGCUUUGCCAdC (2’H), CGUCACUUACGGAUCCGAAGCGUGCCAddC (2’3’H), AUUGUUAUGUGAGACUGUUG (siRNA-S), CAGUCCACCAUAUAAACAUUU (siRNA-A).

**RNA Labeling**—RNA substrates were labeled at the 5’ end with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP. RNAs generated by T7 transcription prior to labeling were treated with calf intestinal phosphatase to remove the 5’ phosphate. The pre-H2a RNA (50 ng) was labeled at the 3’ end with [5’-32P]pCp (Amersham Biosciences) using T4 RNA ligase (Ambion) as suggested by the manufacturer.

**Partial KOH Hydrolysis of the Pre-H2a RNA**—1 ng of the 5’ labeled pre-H2a RNA (50,000 counts/min) was dissolved in 200 µl of 40 mM KOH and incubated at room temperature for 5 min. The solution was neutralized with 5 µl of 3 M potassium acetate, pH 5.5, and 20 µl of 1 M Tris, pH 8.0. The RNA was precipitated with ethanol and dissolved in 10 µl of water.

**3’hExo Binding Assays**—Mobility shift assay was carried out using 25 pmol of baculovirus-expressed 3’hExo and/or SLBP and 50 fmol of 5’ labeled SL RNA in a total volume of 10 µl. In the pulldown binding assay, 35S-labeled 3’hExo was bound to either the SLbi RNA or to various H2a RNA mutants pre-annealed to a biotinylated oligonucleotide complementary to the first 17 nucleotides of the RNA. Both assays were carried out as previously described (2).

**3’hExo Enzymatic Assay**—The exonuclease activity of 3’hExo was tested using 1, 5, or 25 pmol of baculovirus-expressed protein as indicated for each experiment and 50 fmol of 32P-labeled RNA substrates in a total volume of 10 µl containing of 135 mM KCl, 50 mM Tris, pH 8, 2.5 mM MgCl2, 2.5% glycerol, and 1 µg/ml bovine serum albumin. In some reactions, 10 mM EDTA was used to inhibit the enzymatic activity of 3’hExo. The reactions were incubated at 37 °C for 30 min, and the RNA was separated in 8% denaturing polyacrylamide gels and detected by autoradiography.

**RESULTS**

**3’hExo Prefers the ACCCA at the 3’ End**—We have previously shown that in the presence of EDTA 3’hExo can bind to the 3’ end of histone mRNAs both alone and simultaneously with SLBP (2). Fig. 1A presents a simplified model of how SLBP and 3’hExo bind to the target sequence consisting of a highly conserved SL followed by ACCCA. SLBP recognizes primarily the 5’ side of the SL with an important role played by two adenosines located 2 and 3 nucleotides upstream of the stem, whereas 3’hExo binds to the 3’ side of the structure with an important role played by the ACCCA terminus. We analyzed the role of the ACCCA terminus in the binding of 3’hExo in more detail using a pulldown assay as previously described (2). In this assay, 35S-labeled 3’hExo was incubated in the presence of EDTA with 25 pmol of 48-nucleotide wild-type H2a RNA (Fig. 1B) or its various derivatives pre-annealed to a 2’O-methyl adaptor oligonucleotide containing biotin at the 3’ end. The RNA-protein
FIGURE 2. Mapping regions of 3′ hExo required for RNA binding. A, the schematic domain organization and amino acid sequence of 3′ hExo. The SAP and 3′ exonuclease domains are underlined, and other regions conserved between 3′ hExo and ERI-1 are overlined. The DEDDh core residues and other conserved amino acids of the 3′ exonuclease domain are indicated with arrowheads. B, binding of the 35S-labeled wild-type (WT) 3′ hExo or the ∆SAP mutant to either 25 or 100 pmol of the SLbi RNA. Lanes 1 and 5 contain 30% of the input. The amount of the radioactive 3′ hExo collected on streptavidin beads in the absence of RNA is shown in lane 2. C, degradation of 50 fmol of the 5′ labeled pre-H2a RNA by 5 pmol of the baculovirus-expressed WT or ∆SAP variants of 3′ hExo in the presence of 2.5 mM Mg2+. The reactions were prepared in a total volume of 10 μl and incubated for 30 min at 37 °C. The RNA was separated in an 8% denaturing polyacrylamide gel and visualized by autoradiography. A 43-nucleotide product is accumulated due to the presence of the stem-loop structure blocking the enzyme. The 48-nucleotide H2a RNA generated by 3′ hExo lacks any known RNA binding domain (Fig. 2A). The SAP domain (Fig. 2D) has been defined as a 35-residue motif containing an invariant glycine and a conserved distribution of hydrophobic, polar, and bulky amino acids (11). The SAP domain is present in a number of eukaryotic proteins in conjunction with other domains that link these proteins with RNA or DNA metabolism (11). The functional role of the SAP domain is unknown, although it has been suggested that it facilitates binding of proteins to double-stranded DNA (11). In 3′ hExo, the SAP domain exists together with the 3′ exonuclease domain (Fig. 2A). This organization is typical of orthologues of 3′ hExo in other vertebrates and is also found in C. elegans ERI-1 (T02441), the only known invertebrate protein that contains both the SAP domain and the 3′ exonuclease domain (8).

We synthesized a 35S-labeled ∆SAP mutant lacking the SAP domain (amino acids 76–110) and analyzed its ability to bind 25 or 100 pmol of the SLbi RNA containing biotin at the 5′ end (Fig. 1B). No detectable amount of mutant protein was collected on streptavidin beads, demonstrating that deletion of the SAP domain abolished binding of 3′ hExo to the SLbi RNA (Fig. 1C, lane 2), whereas in the absence of RNA no protein was absorbed on the beads (lane 1). Progressive shortening of the ACCCA region resulted in a strong reduction of binding of 3′ hExo to the RNA (lanes 3–5). A significant reduction in the efficiency of binding was also caused by extending the 3′ end with CUAG (+4) or 38 nucleotides (+38), as in the pre-H2a RNA (lanes 7–8). In addition to changing the length of the 3′ single-stranded tail, we replaced the entire ACCCA sequence with AUUUU. Binding of 3′ hExo to this RNA was reduced compared with the wild-type RNA by a factor of 20 (lane 10). Therefore, in agreement with our previous results (2), the ACCCA sequence at the end of histone mRNA is optimal for 3′ hExo binding.

Role of the SAP Domain and the Interdomain Spacer in RNA Binding—The interaction between 3′ hExo and the 3′ end of histone mRNA was surprising because 3′ hExo lacks any known RNA binding domain (Fig. 2A). The SAP domain (Fig. 2D) has been defined as a 35-residue motif containing an invariant glycine and a conserved distribution of hydrophobic, polar, and bulky amino acids (11). The SAP domain is present in a number of eukaryotic proteins in conjunction with other domains that link these proteins with RNA or DNA metabolism (11). The functional role of the SAP domain is unknown, although it has been suggested that it facilitates binding of proteins to double-stranded DNA (11). In 3′ hExo, the SAP domain exists together with the 3′ exonuclease domain (Fig. 2A). This organization is typical of orthologues of 3′ hExo in other vertebrates and is also found in C. elegans ERI-1 (T02441), the only known invertebrate protein that contains both the SAP domain and the 3′ exonuclease domain (8).

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2B, lanes 6 and 7). To determine whether deletion of the SAP domain resulted in global misfolding of 3’hExo rather than specifically affecting RNA binding, we expressed the ΔSAP protein in the baculovirus system and tested the ability of the mutant protein to degrade the 5’ labeled pre-H2a RNA. This 86-nucleotide RNA contains the internally located stem-loop, which prevents complete degradation of the RNA by 3’hExo, resulting instead in the formation of a 43-nucleotide product terminating with the stem. This product is 5 nucleotides shorter than the H2a RNA generated in a mouse nuclear extract from the pre-including the stem. This result demonstrates that removal of the SAP domain does not cause gross structural changes in 3’hExo and is also consistent with the fact that most nucleases of the DEDD family do not contain the SAP domain and yet display enzymatic activity.

To identify amino acids of the SAP domain critical for RNA binding, we substituted selected amino acids with alanines. The SAP domain of 3’hExo contains two positively charged amino acids, Lys99 and Lys104, and two tyrosines, Tyr109 and Tyr110, that are conserved in ERI-1 but not present in other SAP domain proteins (Fig. 2D). Suggesting that these residues may participate in sequence-specific RNA binding. Mutant proteins containing alanine at position 92 (K92) or 104 (K104) retained the wild-type efficiency to bind the SLBi RNA in the pulldown assay (Fig. 2D, lanes 5 and 9). No effect on binding was observed when the two mutations were combined in one K92 + K104 protein (Fig. 2D, lane 11). Alanine substitution of Lys99, which is present in nearly all known SAP domains, reduced the ability of 3’hExo to bind the SLBi RNA only slightly (Fig. 2D, lane 7). Simultaneous substitution of two tyrosines at positions 109 and 110 with alanines (2Y mutant) had a similar small effect on binding (Fig. 2D, lane 13). Overall, these initial experiments failed to identify residues of the SAP domain that are involved in RNA binding, suggesting that the critical specificity determinants are provided by other amino acids of the domain and/or that simultaneous change of many amino acids may be required to adversely affect RNA binding activity of 3’hExo.

Recently, the crystal structure of 3’hExo in complex with the SL RNA was solved, and it demonstrated that the SAP domain is indispensable for binding to the RNA and that a crucial role is played by arginine at position 105.3 Guided by these studies, we substituted this residue with alanine. This single change was sufficient to abolish binding of 3’hExo to the SL RNA (Fig. 2E, lanes 6 and 7), whereas substitution of another arginine in position 96, which according to the crystal structure is not involved in RNA binding, had no effect (not shown).

We next replaced the Lys111 and Lys112 with alanines, thus generating the 2K mutant protein. These two lysines are located in the interdomain spacer that separates the SAP domain from the 3’ exonuclease domain. The 35S-labeled 2K protein when tested in the pulldown assay did not detectably interact with 25 pmol of the SLBi RNA (Fig. 2D, lane 15). The baculovirus-expressed 2K protein in a wide range of concentrations retained normal enzymatic activity, indicating that the 2K mutation did not affect global folding of the exonuclease (Fig. 2F, lane 4, and not shown).

We also mutated the YYDY1 and ELRINEK sequences flanking the exonuclease domain (Fig. 2A). Mutations within these sequences, including alanine substitutions and partial or complete deletions, significantly reduced or abolished binding to the SLBi RNA (not shown). However, these mutations had also a negative effect on the enzymatic activity of 3’hExo (not shown), suggesting that they may partially disrupt the overall structure of the protein rather than specifically affect RNA binding.

Residues of 3’hExo Required for the Enzymatic Activity—The 3’ exonuclease domain of 3’hExo is located between amino acids 133 and 311. Based on the amino acid sequence of this domain, we tentatively classified 3’hExo as a member of the DEDD family of 3’ exonucleases (2). All members of this family are characterized by the presence of the four invariant acidic residues required for catalysis (10). The DEDD family has been divided in two subfamilies depending on the presence of either a conserved histidine (DEDDh subfamily) or tyrosine (DEDDy subfamily). Structural studies of the exonuclease domain of 3’hExo complexed with rAMP in the presence of Mg2+ classified 3’hExo as a member of the DEDDh subfamily and identified the following invariant residues of the signature sequence: Asp134, Glu136, Asp234, Asp298, and His293 (9) (Fig. 2A). The four acidic residues coordinate two magnesium cations and together with histidine at position 293 provide a platform for hydrolytic cleavage of RNA substrates in the 3’–5’ direction (9).

To confirm that the aspartic acids at positions 234 and 298 are required for the exonuclease activity of 3’hExo, we substituted each of the two residues with an alanine. This approach was previously used to identify catalytic residues in two other 3’ exonucleases of the DEDD family (12, 13). The two mutant proteins, D234 and D298, were expressed in the baculovirus system and tested for the ability to degrade the 5’ labeled 86-nucleotide pre-H2a RNA. The wild-type 3’hExo (25 pmol) converted all the input RNA into the 43-nucleotide intermediate (Fig. 3A, lane 3), whereas the two mutant proteins at this high protein concentration were inactive, thus confirming the importance of aspartic acids in positions 234 and 298 for catalysis (Fig. 3A, lanes 5 and 7). Human 3’hExo and its vertebrate orthologues as well as C. elegans ERI-1 contain another conserved aspartic acid, located in 3’hExo at position 198. Mutation of this residue to alanine did not abolish the enzymatic activity of 3’hExo, and at 25 pmol the D198 mutant protein displayed a comparable activity to that of the wild-type protein (Fig. 3A, lanes 3 and 4). At lower protein concentrations, the D198 mutant was less active than the wild-type 3’hExo (Fig. 4E, lanes 2 and 3). Substitution of a methionine 235 with alanine eliminated the exonuclease activity of 3’hExo (Fig. 3A, lane 6). Based on the presence of this methionine, the vertebrate orthologues of 3’hExo and C. elegans ERI-1 have been tentatively classified as members of a new DEMD subfamily rather than the DEDD subfamily (8).

We next asked whether the baculovirus-expressed mutant proteins enzymatically inactive because of mutations within the conserved residues retain normal ability to bind to the 31-nucleotide SL RNA in the mobility shift assay. The SL RNA was

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3 Y. Cheng and D. Patel, personal communication.
identical to the SLbi but was lacking biotin, thus allowing labeling at the 5' end (Fig. 1, A and B). In the presence of 10 mM EDTA both 3'hExo and SLBP form a binary complex with the SL RNA, although the interaction of the RNA with 3'hExo is not as strong as with SLBP (Fig. 3B, lanes 2 and 3). A ternary complex with the SL RNA is formed in the presence of SLBP and 3'hExo (Fig. 3B, lane 4). The same ability to form a binary and a ternary complex in the presence of EDTA was observed for the D234 protein (Fig. 3B, lanes 5 and 6) and D298 protein (not shown, see also Fig. 3C), demonstrating that these two 3'hExo mutants retain normal ability to interact with the SL RNA and SLBP. Surprisingly, the M235 protein was unable to interact with the SL RNA or to efficiently form a ternary complex containing SLBP (Fig. 3B, lanes 7 and 8), suggesting that mutation of Met^{235} leads to global changes in the 3'hExo structure that abolish both binding and catalytic functions of the protein. A significant amount of the SL probe incubated with the M235 was trapped in the well during electrophoresis (not shown), suggesting protein aggregation and further supporting the notion that mutation of methionine 235 results in protein misfolding. We also tested the ability of the ΔSAP mutant expressed in the baculovirus system to form the binary and ternary complexes. In agreement with the results of the pull-down assay (Fig. 2, B and E), this mutant was unable to interact with the SL RNA and to efficiently form the ternary complex (Fig. 3B, lanes 9 and 10).

SLBP also forms a stable complex with the SL RNA in the presence of 2.5 mM Mg^{2+} (Fig. 3C, lane 2). As previously shown (2), under these conditions 3'hExo degrades the SL RNA, precluding detection of a binary complex (Fig. 3C, lane 3). SLBP prevents degradation of the SL RNA by 3'hExo in the presence of Mg^{2+}, thus allowing detection of a stable ternary complex containing the two proteins (Fig. 3C, lane 4) (2). As expected, the enzymatically inactive D298 protein formed a stable binary complex with the SL RNA also in the presence of magnesium ions and efficiently cooperated with SLBP in forming a ternary complex (Fig. 3C, lanes 5 and 6).

A 3'OH Is Required for Efficient Degradation of RNA Substrates by 3'hExo—To determine whether 3'hExo is sensitive to the nature of the 3' end of the substrate, we tested a number of RNAs containing various groups at the 3' terminal nucleotide. We generated a ladder of degradation products by treating the 5' labeled pre-H2a RNA with either KOH, which leaves a cyclic 2'-3' phosphate, or nuclease S1, which leaves a 3' hydroxyl (14). The products of alkaline hydrolysis were resistant to 3'hExo, whereas the full-length RNA present in the same reaction mixture was shortened to the 43-nucleotide intermediate by the enzyme, indicating that the presence of the cyclic phosphate inhibits the 3'hExo activity (Fig. 4A, lanes 5 and 6). As expected, the RNA partially degraded by S1 nuclease was efficiently shortened to the 43-nucleotide intermediate (Fig. 4A, lanes 3 and 4). To further demonstrate that a phosphate group at the 3' end prevents enzymatic degradation, we ligated [5'-32P]pGp to the 3' end of the pre-H2a RNA, thus generating an 87-nucleotide RNA substrate labeled near the 3' end and terminating with a 3' phosphate. This RNA was resistant to 25 pmol of 3'hExo alone and became degraded by this amount of
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FIGURE 4. RNAs with modified 3’ ends as substrates for 3’hExo. A, the ability of 25 pmol of the baculovirus-expressed 3’hExo to degrade RNA intermediates (total 50 fmol) generated by partial S1 digestion (lane 4) or partial KOH hydrolysis (lane 6) of the 5’ labeled 86-nucleotide pre-H2a RNA. Degradation of the untreated pre-H2a RNA (Untreat) by 3’hExo is shown in lane 2. The input RNA substrates are shown in lanes 1, 3, and 5, B, the ability of 25 pmol of the baculovirus-expressed 3’hExo to degrade 50 fmol of the pre-H2a RNA labeled at the 3’ end by [5’-32P]pCp. The RNA was incubated in the presence of 2.5 mM Mg2+ with 10 units of calf intestinal phosphatase alone (lane 2) or simultaneously with both enzymes (lane 4). The samples were prepared in a total volume of 10 μl and processed as described for Fig. 2C. The input RNA substrate is shown in lane 1. The ability of 25 pmol (left panel) or 1 pmol (right panel) of the baculovirus-expressed variants of 3’hExo to degrade 50 fmol of the 5’ labeled 28-nucleotide RNA/2’ H terminating with a 2’ deoxycytidine. The reactions were carried out in a total volume of 10 μl containing 2.5 mM Mg2+. D, the ability of 25 pmol of the baculovirus-expressed WT 3’hExo to degrade 50 fmol of the 5’ labeled 28-nucleotide RNA/2’ H terminating with a deoxyxycytidine. The reactions were carried out in a total volume of 10 μl with either 2.5 mM Mg2+ (lane 2) or 10 mM EDTA (lane 3). E, the ability of 1 pmol of the baculovirus-expressed variants of 3’hExo to degrade 50 fmol of the 5’ labeled pre-H2a RNA in the presence of 2.5 mM Mg2+.

the enzyme only after simultaneous addition of calf intestinal phosphatase that removes the terminal phosphate (Fig. 4B).

We next tested whether 3’hExo can degrade a 5’ labeled RNA terminating with a 2’ deoxynucleotide. We used an unstructured 28-mer consisting of 27 ribonucleotides extended at the 3’ end by a 2’ deoxycytidine (RNA/2’ H). The wild-type enzyme at 25 pmol efficiently degraded this substrate, whereas the D298 mutant protein was inactive (Fig. 4C, lanes 2 and 4). In the presence of 1 pmol of the wild-type 3’hExo, the degradation of the RNA/2’ H was very inefficient (Fig. 4C, lane 6), and only a portion of the input was converted to a 23-nucleotide intermediate as determined by using high resolution gel electrophoresis (not shown). The same amount of 3’hExo degraded the majority of the pre-H2a RNA to the 43-nucleotide product (Fig. 4E, lane 2), indicating that the presence of 2’ hydro-
gen at the last nucleotide slows down RNA degradation. The reason for the accumulation of the 23-nucleotide RNA is unclear. The presence of two uridines at the 3’ end of this RNA may indicate that 3’hExo at lower concentrations is inefficient in degrading short RNAs when it encounters a tract of uridines. We have previously observed generation of an intermediate ending with uridines during degradation of the stem-loop RNA (2).

In addition to the wild-type 3’hExo and the D298 mutant protein we tested the D198 protein. At 1 pmol, this mutant protein compared with the wild-type 3’hExo displayed moderately reduced activity on both the RNA/2’ H and the pre-H2a substrates (Fig. 4C, lane 7; Fig. 4E, lane 3). We next tested an RNA oligonucleotide identical to the RNA/2’ H but terminating with a deoxycytidine (RNA/2’3’H). This substrate compared with the RNA/2’ H was more resistant to the wild-type 3’hExo, and in the presence of the highest concentration of the enzyme (25 pmol) and 2.5 mM Mg2+ only ~30% of the input was completely degraded (Fig. 4D, lane 3). No degradation was observed in the presence of 10 mM EDTA inhibiting the enzyme (Fig. 4E, lane 2). Collectively, these results demonstrate that the nature of the chemical modification at the 2’ and 3’ ribose of the last nucleotide greatly affects the ability of 3’hExo to degrade RNA substrates and a phosphate exerts a particularly inhibitory effect.

Our earlier studies failed to demonstrate that 3’hExo is active on substrates consisting entirely of deoxynucleotides and thus suggested that 3’hExo is an RNA-specific exonuclease (2). We have tested a number of additional single-stranded DNA substrates and detected a very limited 3’ exonuclease activity with some longer oligonucleotides only in the presence of high enzyme concentrations (25 pmol and more). Further studies are required to determine whether 3’hExo under certain circumstances can indeed function as DNase.

3’hExo Can Remove the 2-Nucleotide Overhangs from siRNAs—Recent genome-wide screening for proteins potentially involved in RNA interference suggested that ERI-1, a C. elegans homologue of 3’hExo, may be involved in degrading siRNAs (8). Surprisingly, both 3’hExo and ERI-1, when expressed in rabbit reticulocytes, were reported to efficiently
degrade double-stranded RNAs and to be inactive on 21-nucleotide single-stranded RNA substrates (8). To test the ability of the baculovirus-expressed 3′hExo to degrade siRNAs, we carried out a number of experiments using two 21-nucleotide single-stranded RNAs, siRNA-S and siRNA-A, which upon annealing formed a 19-nucleotide double-stranded region extended on each side by 2-nucleotide single-stranded RNA. Generation of an 18-nucleotide product is likely because of increased efficiency in formation of the double-stranded RNA. Increasing the amount of the complementary RNA resulted in more labeled substrate being protected, likely because of increased efficiency in formation of the double-stranded RNA. Generation of an 18-nucleotide product indicates that 3′hExo, in addition to removing the 2-nucleotide overhangs, can also remove the first nucleotide of the double-stranded regions. In the presence of 1 pmol of 3′hExo the pre-annealed RNA was stable, indicating that the enzyme at this low concentration is unable to remove 3′ overhangs (Fig. 5C, lanes 5 and 6), although it can partially degrade the single-stranded RNA under these conditions (Fig. 5C, lanes 2–4).

**DISCUSSION**

The highly conserved 26-nucleotide sequence at the 3′ end of mammalian replication-dependent histone mRNAs containing a 6-base pair stem and a 4-nucleotide loop followed by ACCCA is specifically recognized by two proteins, the stem-loop binding protein (SLBP) and a protein with 3′ exonuclease activity designated 3′hExo (2). Whereas SLBP is known to play multiple roles in biogenesis and metabolism of histone mRNAs (1), the role of 3′hExo remains unknown. Here we carried out a number of experiments to further characterize this exonuclease.

Crystallographic studies of the 3′exonuclease domain of 3′hExo indicate that 3′hExo belongs to the DEDDh subfamily of exonucleases and that the invariant residues involved in catalysis include Asp$^{134}$, Glu$^{136}$, Asp$^{234}$, Asp$^{298}$, and His$^{293}$ (9). This classification has been confirmed by our mutational analysis demonstrating that alanine substitution of either Asp$^{234}$ or Asp$^{298}$ eliminates enzymatic activity of 3′hExo. The residues of
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The DEDDh core identified in 3′hExo by the crystallographic studies are highly conserved in all vertebrate orthologues of 3′hExo and in other putative exonucleases that share significant similarity with 3′hExo, including ERI-1 and three other homologues of this protein in C. elegans (not shown). Interestingly, all these proteins contain a highly conserved aspartate (position 198 in human 3′hExo) that is not part of the DEDDh core. Mutation of this residue to alanine did not abolish the enzymatic activity of 3′hExo but moderately reduced its ability to degrade RNA substrates.

A structure-based comparison with other known 3′ exonucleases of the DEDD family revealed that 3′hExo is more similar to DNA-specific exonucleases, including a proofreading domain of Escherichia coli DNA polymerase III, than to RNases (9). At least one enzyme of the DEDD family, E. coli RNase T, is both DNase and RNase (10, 15–17). In fact, RNase T is more active on DNA than on RNA substrates (15) and can complement defects in DNA repair in cells lacking other DNases normally involved in excising damaged nucleotides from DNA (18). This result suggests that participation in some aspects of DNA metabolism may be a natural function of RNase T in addition to its role in RNA metabolism. We demonstrated that 3′hExo is primarily an exonuclease. Single-stranded DNAs are very poor substrates, if at all, for 3′hExo and therefore unlikely to be degraded by this enzyme under physiological conditions. Efficient degradation of RNA substrates by 3′hExo requires 2′ and 3′ hydroxyl groups at the last nucleotide. The activity of two other DEDD exonucleases, poly(A)-specific ribonuclease (19) and RNase T (16), has been previously shown to depend on the presence of the 3′ hydroxyl at the last nucleotide of their substrates.

The most characteristic feature of 3′hExo is its ability to specifically and tightly bind the stem-loop at the 3′ end of histone mRNAs. Binding of 3′hExo to the stem-loop is facilitated by simultaneous binding of SLBP, which recognizes the opposite platform of the same RNA target. A detailed picture of how 3′hExo interacts with the SL RNA and how SLBP facilitates binding of 3′hExo to its RNA target will become available through structural studies of the binary and ternary complexes. Deletion of amino acids immediately flanking the 3′ exonuclease domain had a strong negative effect on RNA binding but at the same time reduced catalytic activity of 3′hExo, suggesting that these mutations at least partially affect global folding of the protein rather than target amino acids specifically involved in RNA binding. The strong inhibitory effect on RNA binding ability of 3′hExo was caused by deletion of the SAP domain or an alanine substitution of Lys111 and Lys112 located immediately to its C terminus. These two mutations did not affect enzymatic activity of 3′hExo, suggesting that both the SAP domain and the two lysines are specifically required for interaction with the SL RNA. Indeed, recent x-ray crystallographic studies of the 3′hExo-SL RNA complex demonstrated that the SAP domain is involved in binding to the RNA with the crucial role being played by arginine at position 105. Substitution of this residue with alanine was sufficient to abolish binding of 3′hExo to the SL RNA.

The sequence-specific interaction of 3′hExo with the 3′ end of histone mRNAs either alone or together with SLBP strongly suggests that 3′hExo may play a role in the rapid decay of histone mRNA at the end of S-phase. This function is consistent with the high concentration of 3′hExo in the cytoplasm, the site of histone mRNA degradation. ERI-1 has been identified by genome-wide scanning for mutants of C. elegans with enhanced RNA interference, leading to the hypothesis that one function of this exonuclease may be to degrade siRNAs (8). However, as demonstrated recently, ERI-1 exists in a complex with Dicer and, in addition to down-regulating the response to exogenous double-stranded RNAs, is required for accumulation of several endogenous siRNAs (20). Based on these studies a new model for the molecular function of ERI-1 has been proposed. In this model, inspired by the unique features of 3′hExo, ERI-1 binds to short stem-loops in a group of endogenous RNAs and removes unpaired 3′ nucleotides, thus generating a structure suitable for synthesis of double-stranded RNA species subsequently cleaved by Dicer and involved in RNA interference (20). Future studies should provide more information on molecular functions of ERI-1 and 3′hExo and help to determine whether these two proteins are related functionally in addition to sharing significant sequence similarity.

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