Exonucleolytic degradation of double-stranded RNA by an activity in *Xenopus laevis* germinal vesicles

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We have identified, in extracts from *Xenopus laevis* germinal vesicles, a 5′ exonuclease activity that cleaves double-stranded RNA (dsRNA). Features of the 5′ ends of dsRNAs determine whether the strands are symmetrically or asymmetrically degraded. The activity hydrolyzes in the 5′ to 3′ direction, releasing 5′-mononucleotides processively, favoring strands with 5′-monophosphate termini; molecules with capped ends are resistant to digestion. Because of its ability to processively digest dsRNA to mononucleotides, we have named the exonuclease Chipper, which could cooperate or compete with Dicer (an endonuclease that produces molecules with a 5′-phosphate) in the processing of dsRNA.

RNA molecules of many shapes and sizes perform diverse informational, structural, and catalytic roles in living cells. Most cellular RNA is single-stranded, but some forms of double-stranded RNA (dsRNA) performing very specific functions have been recognized (1, 2). In higher eukaryotic cells, a variety of enzymes recognize and process these dsRNAs.

An enzyme that recognizes dsRNA, Dicer (3), is characterized by two RNase III domains that cleave dsRNA into ≈21-bp-long duplexes. Dicer sits at the intersection of the pathways of short interfering RNAs (siRNAs) (4) and small temporal RNAs (stRNAs) (5). siRNAs are thought to be generated by Dicer from any dsRNA of sufficient size; stRNAs, on the other hand, are generated from stem/loop precursors that are explicitly encoded in the genome. Whereas the siRNA precursors have complete base-pairing, the stRNA precursors have variously placed bulges and loops. These differences must be accommodated by Dicer in recognition and cleavage. Both strands of the siRNAs are detectable after processing by Dicer, but only one strand of the stRNAs is preserved, the other probably being quickly destroyed after it is produced. Other proteins that cooperate with Dicer may contribute to the differences observed between the two pathways. siRNAs provide sequence information that allows a specific mRNA to be targeted for degradation, whereas stRNAs regulate the translation of specific mRNAs (6, 7). Several laboratories have recently cloned many additional stRNA-like RNAs, subsequently termed microRNAs or miRNAs, from *Caenorhabditis elegans*, *Drosophila*, and human cells (8–12). Although the functions of these diverse RNAs remain mysterious, it seems likely that they, like the regulatory RNAs let-7 and lin-4, are transcribed as hairpin RNA precursors, which are processed to their mature forms with Dicer's participation (13–15). Dicer or other endonucleases are presumably involved in the processing of exogenous hairpin RNAs (16–21) that regulate gene expression. Dicer and some of the proteins required for the silencing of gene expression in response to exogenous dsRNA RNA interference (RNAi) in the cytoplasm, are also implicated in silencing of heterochromatin in the nucleus (22, 23).

The ADARs are adenosine deaminases that convert adenosines (A) to inosines (I) within dsRNAs (24, 25), resulting in pre-mRNA editing. Because I is read as G by the translation machinery, the deamination reaction effectively represents an A to G transition and, therefore, has the potential to alter the coding sequence of an mRNA. Because the I·U pair has lower stability than the A·U pair, ADARs (26), as a consequence of the deamination reaction, also unwind the duplex RNA (25).

Mammals have evolved robust systems for responding to dsRNAs, specifically as an antiviral defense. In somatic cells, dsRNA activates a variety of responses. Predominant among these is the PKR response (27). PKR is a protein kinase that is activated by dimerization in the presence of dsRNA. dsRNA also activates 2′-5′ oligoadenylate polymerase, the product of which is an essential cofactor for a nonspecific ribonuclease, RNase L.

Germinal vesicles (GVs), readily prepared from *Xenopus laevis*, have provided a convenient source of enzyme activities for the study of eukaryotic nucleic acid metabolism (28). GV extracts yield a concentrated source of strictly nuclear enzymes whose activities can be assayed by using suitably designed substrates. Such assays define activities that provide a basis for modeling potential regulatory pathways as well as starting points for enzyme purification. Here we describe activities in GV extracts that (*i*) processively digest one strand of dsRNAs to mononucleotides and (*ii*) selectively digest parts of suitably nicked stRNA precursors. We have named the processive exonucleolytic activity Chipper, without knowing its molecular nature.

Materials and Methods

DNA Templates. The description of the DNA templates is detailed in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

RNA Synthesis. RNAs were prepared by using one of the following Ambion (Austin, TX) kits, as recommended by the company: SP6-MEGAscript, T7-MEGAshortscript, and SP6-mMESSAGE mMACHINE. Transcripts were internally labeled with [α-^{32}P]UTP [800 Ci (1 Ci = 37 GBq)/mmol; Amersham Pharmacia]. Cold RNAs were internally trace-labeled at a specific activity 100-fold lower than that of the labeled ones. After phenol extraction and purification by electrophoresis on polyacrylamide/urea gels, RNAs were annealed by mixing equimolar amounts in 50 mM Tris·HCl, pH 7.5/300 mM NaCl/1 mM EDTA, heated at 68°C for 1 h, and slowly cooled for >1 h to 37 or 25°C, depending on the length of RNAs. The dsRNAs were ethanol precipitated and purified on a nondenaturing polyacrylamide gel. The sense strand of PreLet7 U27 5′-UUGGAATATTACCACCGGTGAACTATGCAATTTTCTACCTTACCGGC-3′ was purchased from Dharmacon (Lafayette, CO). Synthetic RNA was gel purified after deprotection according to the manufacturer's directions.

To generate 5′-hydroxyl RNAs, the transcripts were dephosphorylated by using calf intestinal alkaline phosphatase (Roche Diagnostics). To generate 5′-phosphate RNAs, phosphorylation was performed with T4 PNK (United States Biochemical) according to the manufacturer's directions.

Activity Assay. GV extracts were prepared as described (28). Assays (12 μl) contained 10 μl of GV extract and 8.3 or 4.2 nM ^{32}P- labeled

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Abbreviations: ADARs, adenosine deaminases that convert adenosines to inosines within dsRNAs; as, antisense strand; as*, ^{32}P-labeled as; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; s, sense strand; s*, ^{32}P-labeled s; siRNA, short interfering RNA; stRNA, small temporal RNA; GV, germinal vesicle.

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dsRNA (as indicated) at specific activity of 10^5 cpm/pmol in JB70 buffer (10 mM Hepes-KOH, pH 7.5/70 mM NH_4Cl/7 mM $MgCl_2$/2.5 mM DDT/10% glycerol/0.1 mM EDTA). After incubation at 25°C for the indicated time, reactions were stopped by adding 18 μl of water, 3 μl of 5% SDS, and 3 μl of proteinase K (1 mg/ml), followed by 30 min incubation at 37°C. After the addition of 1 μg of yeast tRNA and NaCl to 0.2 M, RNA was extracted once with phenol/chloroform (1:1), precipitated with ethanol, and analyzed in either a denaturing 6% polyacrylamide gel or a nondenaturing 10% polyacrylamide gel.

Analysis of Degradation Products. Ethanol-soluble products were analyzed by TLC. The ethanol of each sample was evaporated, and the solid was resuspended in 10 mM sodium acetate, pH 5.3, and spotted on to PEI-cellulose F (Merck). The solvent used was composed of saturated $(NH_4)_2SO_4$, 0.1 M sodium acetate, pH 6.0, and isopropyl alcohol (mixed in the ratio 79:19:2). The identity of the spot was verified by the migration of unlabeled standards.

Image Analysis. Quantitative image analysis was done by using a Molecular Dynamics Model Storm (860) PhosphorImager with IMAGEQUANT software, Version 4.2.

Results

Specific Degradation of One Strand in dsRNA. We transcribed *in vitro*, using SP6 and T7 RNA polymerases, the entire mouse profilin 1 gene (29), from the initial AUG to the stop codon. ^{32}P-labeled sense and unlabeled antisense (ds1, s*-as) were annealed, as were unlabeled sense and ^{32}P-labeled antisense RNAs (ds1, s-as*). The ds1 RNAs are 425 nt long and present overhangs of 9 and 5 nt at their respective 5′ ends (Fig. 1*A*). Because only one strand is labeled in each of the ds1 types, we can observe the fate of each strand of the duplex when ds1 RNAs are incubated with an extract derived from *Xenopus* oocyte GVs(28).

To analyze the products, we used a polyacrylamide gel system under conditions in which ds1 RNA is not denatured and runs considerably faster than single-stranded RNA (ssRNA) (Fig. 1*A*). When the label is in the sense strand (ds1, s*-as), after incubation with the GV extract, 50 ± 10% of the radioactivity becomes ethanol-soluble (average of 21 different experiments) and the residual radioactivity appears as a smear on the gel (Fig. 1*A*, lanes 1 and 2). In contrast, when the label is present in the antisense strand (ds1, s-as*), after incubation with the GV extract, 10 ± 6% of the radioactivity becomes ethanol-soluble (average of 21 different experiments) and the majority of the radioactivity migrates in the gel as full-length ssRNA (Fig. 1*A*, lanes 3 and 4). A significant amount of radioactivity appears as a smear. These data suggest that the GV extract contains an activity that degrades only one strand, the sense strand of a ds1 RNA molecule, and that, as a consequence, the antisense strand is released from the duplex. The activity is specific for the duplex; Fig. 1*A* (lanes 5–8) shows that single-stranded s* and as* RNAs are not degraded. The smear of radioactivity, observed with both ds1/s*-as and ds1/s-as*, presumably represents a spectrum of intermediates generated by the ADARs. The conversion of adenine to inosine results in regional melting of dsRNA that retards migration of duplex RNA to an extent roughly proportional to the extent of modification (25). We have detected up to 20% of deamination in the RNA corresponding to the smear (data not shown). We also found that, if the products of the reaction are run under denaturing conditions, the radioactivity of the smear migrates as full-length ssRNA (data not shown).

By using TLC, the products of the degradation were found to be 5′-monophosphates. The ethanol-soluble material, derived from the digestion of a dsRNA labeled with [α-^{32}P]UTP, was analyzed by TLC using 5′-NMP and 3′-NMP markers. Fig. 1*B* shows that the sole product of digestion of ds3 RNA (Fig. 2*A*) is uridine 5′-monophosphate. We did not observe any partially degraded single strands. This result suggests that the activity is an exonuclease and that it does not dissociate from the substrate before digestion has been completed. Because of its ability to processively convert one strand of dsRNA into mononucleotides, we named the exonuclease Chipper.

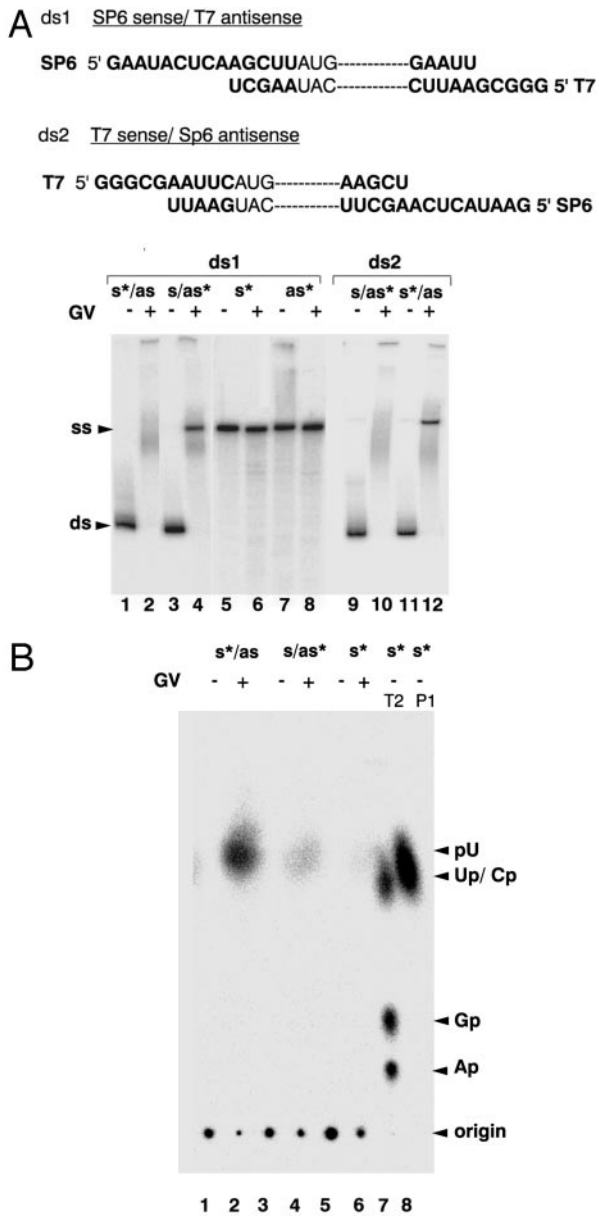

Fig. 1. Asymmetric strand degradation of dsRNA. (*A*) The fate of each strand of two duplex RNAs (ds1 and ds2) after incubation with GV extract was analyzed by 8 M urea/6% PAGE. One hundred femtomoles of the indicated substrates containing uniformly [α-^{32}P]UTP-labeled sense strand (lanes 1, 2, 11, and 12) or antisense strand (lanes 3, 4, 9, and 10) were incubated for 90 min with GV extract (lanes 2, 4, 10, and 12) or buffer (lanes 1, 3, 9, and 11). Lanes 5–8 show the incubation in the extract (lanes 6 and 8) or in buffer (lanes 5 and 7) of 100 fmol of labeled sense or antisense ssRNA. Arrowheads indicate the position of full-length ss- and dsRNA in the gel. (*B*) To determine whether the products of degradation were 5′- or 3′-monophosphates, the ethanol-soluble material was analyzed by TLC. One hundred femtomoles of ds3 (Fig. 2*A*, a), containing sense- or antisense-labeled strand and 100 fmol of labeled sense ssRNA were incubated with GV extract (lanes 2, 4, and 6) or buffer (lanes 1, 3, and 5). The nucleoside 3′-phosphate and nucleoside 5′-phosphate markers were obtained by treatment of uniformly labeled sense strand with RNase T2 (lane 7), or with nuclease P1 (lane 8), respectively. Arrows indicate the marker positions on the plate.

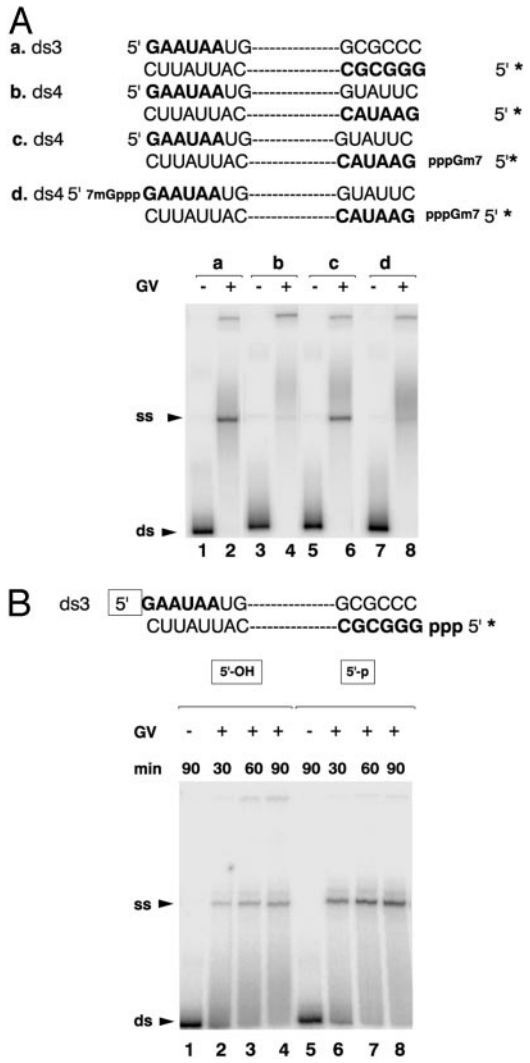

Fig. 2. Properties of Chipper. (*A*) One hundred femtomoles of the indicated substrates with a labeled antisense strand and containing a m7GpppG cap at the 5′ end of the antisense strand alone (lanes 5 and 6) or at the 5′ ends of both strands (lanes 7 and 8) were incubated with GV extract (lanes 2, 4, 6, and 8) or buffer (lanes 1, 3, 5, and 7). The reaction products were analyzed in 8 M urea/polyacrylamide gels as in Fig. 1. (*B*) The indicated substrates (100 fmol) with the antisense strand labeled and containing at the 5′ end of the sense strand a hydroxyl group (lanes 1–4) or a phosphate group (lanes 5–8) were incubated with GV extract (lanes 2–4, 6, 7, and 8) or buffer (lanes 1 and 5). The reactions were stopped at the indicated times and the RNA products were extracted and analyzed in an 8 M urea/polyacrylamide gel.

Information for the Degradation Resides in the Terminal Sequences of the Duplex. An alternative substrate was generated by transcribing the sense strand of the profilin 1 gene with T7 polymerase and the antisense strand with SP6 polymerase. This substrate, ds2, differs from ds1 with respect only to the 5′ overhang sequences derived from the promoters. We produced two types of ds2 RNA molecules, s*-as and s-as*, each of which has only one labeled strand. Fig. 1*A* (lanes 9–12) shows that also in the case of this substrate, only one strand was degraded. As in the case of ds1 RNA, the strand synthesized by the SP6 polymerase is degraded, whereas the one synthesized by the T7 polymerase is not. We conclude, therefore, that the information for the asymmetric degradation is not contained in the profilin gene coding sequence but, rather, in the sequences that flank it (in bold in Fig. 1*A*).

This conclusion is supported by results obtained by using two other dsRNAs, 330 nt in length, produced by annealing of T7 and SP6 transcripts of part of the first exon of a human gene coding for the GPR37 receptor (30). These two dsRNAs are also subject to asymmetric degradation, in which the degraded strand is always the one transcribed by the SP6 polymerase (data not shown).

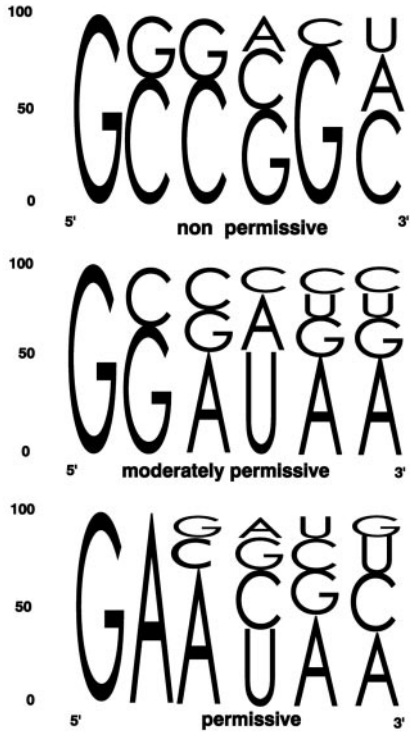

Fig. 3. Sequence composition of the hexamers. Source data for the pictogram are available in Table 1.The height of each letter is proportional to the frequency of the indicated nucleotide in the three classes of sequences.

Permissive Sequences for Degradation. The results described suggest that information for strand selection by Chipper resides in the precise 5′-terminal sequence. We produced several variants of ds1 RNA to determine which components of the terminal sequences are recognized by Chipper. To begin, note that $GAAUA_{C\text{-}A}$ signals degradation and $GGGCG_{A\text{-}C}$ does not. The former is at the 5′ end of the SP6 transcript and the latter at the 5′ end of the T7 transcript. These sequences, provided they are at the 5′ end of a strand of a long duplex such as ds3, also operate when they are completely paired [Fig. 2*A*, a (lanes 1 and 2)]. GGGCGC and GGGCGA, if present at the 5′ ends of the duplex, fail to signal degradation. When incubated with the GV extract, this nonpermissive duplex is unwound, presumably by the ADARs, but neither strand is degraded (data not shown). In contrast, a duplex presenting GAAUAA or GAAUAC at the 5′ end of both strands (ds4) is symmetrically degraded. Fig. 2*A* (b, lanes 3 and 4) shows the degradation of the antisense strand in ds4 (s-as*). The same result is obtained with ds4 (s*-as; data not shown). Fig. 2*A* (c, lanes 5 and 6), on the other hand, shows that if the antisense strand is capped, then it is not degraded. The sense strand is degraded, and the antisense one is released and migrates accordingly in the gel. When both strands are capped, no degradation occurs [Fig. 2 *A* (d, lanes 7 and 8)] and presumably more of the duplex is available for modification by ADARs (Fig. 2*A*, compare lanes 4 and 8). We conclude that molecules with a capped end are resistant to digestion by Chipper and that both degradation and resistance to degradation are determined by features of the 5′ ends of both dsRNA strands. These experiments also show that the GV extracts are relatively free of nonspecific

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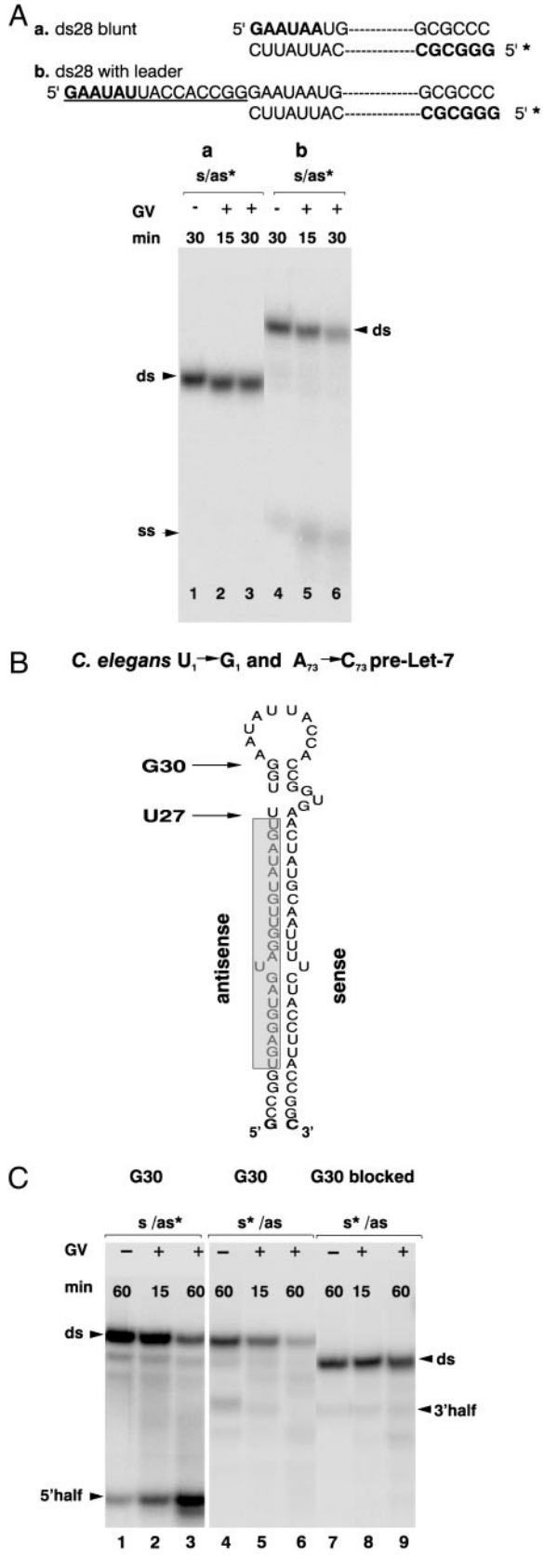

Fig. 4. Chipper cleaves short dsRNAs only if they have a permissive 5′ leader. (*A*) One hundred femtomoles of the indicated substrates (*a* and *b*) with uniformly labeled antisense strand were incubated with buffer (lanes 1 and 4), or in the extract for 15 (lanes 2 and 5) or 30 (lanes 3 and 6) min. The products of incubation were analyzed by gel electrophoresis in a nondenaturing 10% polyacrylamide gel. (*B*) Stem–loop structure of *C. elegans* preLet-7-RNA. The G1 to U1 and A73 to C73 substitutions required for *in vitro* transcription of the precursor are in bold. Arrows indicate the different 5′ ends of the sense let-7 strand of the two dsRNAs used in the experiments described below. (*C*) Asymmetric strand degradation of dsRNA G30. Release after incubation with GV extract (lanes 2 and 3) or with buffer (lane 1) for the indicated times of the 5′ half molecule from 50 fmol of double-stranded precursor containing the uniformly ^{32}P-labeled 5′ half (nondenaturing 10% gel). Lanes 4–9 show that degradation of the sense strand depends on the sequence at its 5′ end. Fifty femtomoles of dsRNA substrates G30 (lanes 4–6) and G30 blocked (lanes 7–9), containing the uniformly ^{32}P-labeled 3′ half, were incubated with GV extract (lanes 5, 6, 8, and 9) or buffer (lanes 4 and 7) for the indicated times. Arrows indicate the position in the gel of dsRNAs, and 5′ and 3′ half molecules.

RNases targeting either long ds- or long ssRNA under these conditions.

To identify new permissive sequences, we utilized a tester duplex characterized by the sequence GGGCGC at the 5′ end of one strand, which is resistant to degradation. The sequence to be probed is placed at the 5′ end of the other strand and the release of the blocked strand from the duplex is measured. There are intrinsic limitations to this type of analysis. Having synthesized RNA *in vitro*, with either SP6 or T7 RNA polymerases, the first position in the transcript must always be occupied by G, whereas the second cannot be occupied by U. We have nonetheless been able to conclude that there are many sequences that permit degradation, although they operate with different efficiencies.

In Table 1, which is published as supporting information on the PNAS web site, we have assigned 27 hexamers to three categories, which are as follows: (*i*) sequences that do not support degradation; (*ii*) moderately permissive sequences; and (*iii*) permissive sequences. Two conclusions can be drawn from analysis of Table 1. First, AU-rich sequences are, in general, more permissive than GC-rich sequences. Second, the closer a position is to the 5′ end of a strand, the more important it is in the determination of permissiveness. For example, an A at position 2 is a stronger determinant than an A at position 6.

Fig. 3 shows the sequence composition preferences relative to each of the three categories listed in Table 1. For example, the sequences that do not support degradation never contain an A at position 2 or either an A or a U at position 3. Permissive sequences, on the contrary, are characterized by a strong preference for A at positions 2 and 3. The intrinsic limitations imposed by the requirements for *in vitro* RNA synthesis allowed us to test only a restricted set of hexamers but, presumably, many other permissive sequences do exist. In what follows we show, for example, that the hexamer UUGGAA is strikingly permissive.

Molecules with a 5′-Phosphate Are the Preferred Substrates for Chipper. The finding that capping one strand of the duplex protects that strand from degradation agrees with the conclusion that Chipper is a 5′ exoribonuclease. Molecules with a 5′-phosphate are the preferred substrates for an entire family of eukaryal 5′-exoribonucleases (31); those with a 5′-OH, a 5′-triphosphate, or a capped end are much more resistant to digestion by any of them. To determine whether Chipper also prefers a 5′-phosphate group, we used the substrate ds3/s-as* RNA. We produced two types of duplexes. The former is characterized by a hydroxyl group at the 5′ end of the degradable strand (Fig. 2*B*, lanes 1–4) and the latter presents a phosphate group in that position (Fig. 2*B*, lanes 5–8).

Fig. 2*B* shows a time course of the release of the as* strand from the duplexes. The best substrate for Chipper contained a 5′-phosphate group; it was significantly better than the one characterized by three phosphates at the 5′ end (data not shown). The worst substrate of all contained a 5′-hydroxyl. This interpretation is the most straightforward because the reactions are being done in extracts that contain many proteins, so it seems there could be an

alternate explanation. For example, it seems possible that a molecule with a 5′-hydroxyl is the best substrate for another RNA-binding protein, so there is more competition with Chipper under these conditions.

A 5′ Leader Is Required for Efficient Cleavage of Short dsRNAs. In the experiments reported above, the length of the dsRNA molecules was >100 bp. These long dsRNAs, provided that permissive sequences are present at the 5′ end of one or both strands, are all excellent substrates for Chipper, even when the two strands are completely paired.

Short dsRNAs are, in general, more difficult to study because of their instability. Fig. 4*A* (lanes 2 and 3) shows the results of an experiment with a blunt-end 28-nt-long dsRNA. Although it contains the permissive sequence GAAUAA at the 5′ end of the sense strand, it was only slightly cleaved by Chipper. Chipper's behavior corresponds to that of other enzymes, such as Dicer (4) or ADARs (32), that specifically recognize dsRNA. Their efficiency is usually critically dependent on the length of the double-stranded region of the substrate. Fig. 4*A* (lanes 5 and 6) shows that, on the contrary, when one of the two strands of a 28-nt helix has a 5′ leader with a permissive sequence at the start, the other strand is released. The leader does not need to be long; five nucleotides are sufficient (data not shown).

Thus, Chipper efficiently processes long blunt-end dsRNAs, provided their 5′-end sequences satisfy the canonical requirements, whereas it is ineffective on short blunt-end dsRNAs unless the latter present a specific 5′ leader, in which case they are cleaved with a disproportionately high efficiency compared with other duplexes of similar size. To determine whether Chipper, besides attacking perfect dsRNA, can deal with more complex secondary structures, we utilized molecules derived from small hairpin RNAs, like the let-7 precursor (5), that contain mismatches in their stems.

Prelet-7 G30 is a let-7 precursor nicked between G29 and G30, leaving a Chipper-permissive sequence on the sense strand (see Table 1 and Fig. 4*B*). When the antisense strand of G30 was labeled (Fig. 4*C*, lanes 2 and 3), the release of the 5′ half by incubation with Chipper is detected. The bands migrating between the dsRNA and the 5′ half are not intermediates of Chipper's catalyzed reaction; most of them are present in the control incubated without extract and are presumably the result of spontaneous breakage of, for example, pyrimidine-A (Y-A) bonds, which are much less stable than other phosphodiester bonds (33). Other trace bands might result from cleavage carried out by other enzymatic activities present in the extract. At any rate, only 10 ± 4% of the radioactivity (average obtained from five different experiments), both of the control and of the experimental sample, were rendered ethanol soluble after 60 min of incubation. These results indicate that the 5′ half of G30 is not degraded.

On the other hand, when the sense strand is labeled with [α^{32}P]UTP, the release of the intact 3′ half is not detected (Fig. 4*C*, lanes 5 and 6). After 15 min of incubation of the control and of the experimental sample, respectively, 8 ± 6% and 50 ± 6% of the radioactivity (average of four different experiments) became ethanol soluble. Analysis by TLC showed that the soluble radioactivity is exclusively uridine 5′-monophosphate (data not shown). When the sequence GAAUAU at the 5′ end of the 3′ fragment was changed to GCCGGU, a nonpermissive sequence (Table 1), the 3′ half was not degraded (Fig. 4*C*, lanes 8 and 9). After 15 min of incubation of the control and of the experimental sample, respectively, 7 ± 3% and 13 ± 5% of the radioactivity (average of four different experiments) become ethanol soluble. These results allow us to conclude that Chipper's activity degrades the 3′ half of prelet-7 G30 *in vitro*.

As expected, based on the properties of Chipper described previously, a capped 3′ half is not degraded and molecules with a 5′-phosphate are preferred substrates. Those with a 5′-OH or a 5′-triphosphate are much more resistant to digestion (data not shown).

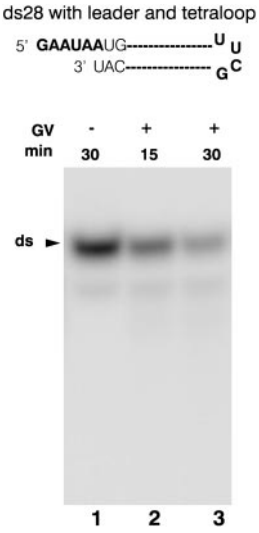

Fig. 5. Chipper is processive. One hundred femtomoles of the UUCG hairpin (ds28 with leader and tetraloop) were incubated in the extract for 15 min (lane 2) or 30 min (lane 3), and with buffer for 30 min (lane 1), and loaded on a nondenaturing 10% polyacrylamide gel.

Prelet-7 U27 (Fig. 4*B*), a molecule nicked between U26 and U27, is also asymmetrically degraded by Chipper. Fig. 7, which is published as supporting information on the PNAS web site, shows that, as a consequence of the degradation of the sense strand, the antisense strand is released. Only 13 ± 5% of the radioactivity (average of four different experiments) is rendered ethanol soluble. This result allows us to add UUGGAA to the list of permissive sequences of Table 1.

Thus, Chipper, despite the presence of mismatches, interacts with both of the nicked let-7 RNA precursors, destroying the fragment 3′ to the nick and preserving the fragment 5′ to the nick. These results are consistent with all of the canonical requirements for cleavage of short dsRNAs manifested by Chipper.

Chipper Is Processive and Able to Travel Through a Tetraloop. Exonucleases may be either distributive or processive (31). Processive exonucleases complete degradation of RNA molecules before being released. Distributive exonucleases, instead, produce a general shortening of the substrate as a function of the extent of the reaction, a fact that indicates that the enzyme dissociates from the substrate before digestion has been completed.

We never observed partially degraded single strands, suggesting that Chipper is processive. To further substantiate this conclusion, we produced a substrate consisting of a hairpin with the sequence GAAUA as 5′ leader, a double-stranded region 23-nt long, and the tetraloop UUCG (Fig. 5). Chipper degraded the hairpin entirely, without producing any intermediates, and the radioactivity that disappeared from the band corresponding to the hairpin became ethanol soluble. When the hairpin instead contained the sequence GCCGG at the 5′ end of the leader, no degradation occurred (data not shown). These results indicate that when Chipper attacks a hairpin, complete degradation occurs before the enzyme is released. These results indicate not only that Chipper is processive but also that it can travel through a tetraloop. It appears that Chipper needs dsRNA near the 5′ end to be loaded, but once loaded, it is processive even after passing through the double-stranded region into ssRNA.

Discussion

dsRNA is a potent biological effector molecule, and processing of dsRNAs and other secondary structures is now understood to play

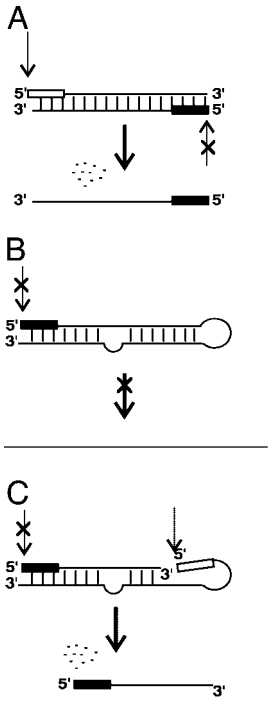

Fig. 6. Substrates and products of Chipper. (*A*) Long dsRNA with a permissive (open bar) and a nonpermissive (solid bar) sequence at the 5′ end of each strand is asymmetrically cleaved by Chipper. (*B*) Short hairpin RNA with a nonpermissive sequence is not cleaved by Chipper. (*C*) Short nicked hairpin RNA is asymmetrically cleaved by Chipper when the nick produces a 5′ leader with a permissive sequence.

a key role in the regulation of gene expression and gene silencing. Thus, any activity that is present in cells that can modify or alter the activity of dsRNA molecules is likely to be worthy of study. This paper provides an introduction to an intriguing and potentially important nuclease activity that appears to recognize a subset of duplexed RNA strands and to catalyze a processive exonucleolytic degradation. We demonstrate that an extract from *X. laevis* GVs exhibits nuclease activities, which can attack certain dsRNAs and other RNA molecules with more complex secondary structures. Under our *in vitro* conditions, Chipper is strictly processive and is able to run through a tetraloop. Whether the substrate is long or short dsRNA, perfect or with mismatches, or with a leader, we always observe the following: (*i*) GCCGGU is a nonpermissive sequence, (*ii*) a 5′-phosphate is preferred, and (*iii*) the products are 5′-monophosphates.

These results suggest that the catalytic subunit of Chipper is always the same; it is, however, possible that the subunit composition of a Chipper complex varies with the type of substrate. The catalytic subunit of Chipper could belong to the eukaryotic 5′ exoribonuclease family, which includes Xrn 1p and Rat 1p (31); however, these enzymes exhibit a strong preference for single-stranded molecules as substrates. The exquisite requirement for dsRNA manifested by Chipper could derive from its association with cooperating proteins.

Chipper reveals another level of complexity in the processing of dsRNA, summarized in Fig. 6. Fig. 6*A* shows a scenario in which Chipper destroys only one strand. If Dicer and Chipper are active in the same cellular compartment, then competition for the same substrate might be expected. For example, dsRNAs derived from centromeric heterochromatin repeats would be processed into siRNAs by Dicer (22, 23) or degraded by Chipper. Fig. 6*B* shows that short hairpin-structured RNAs are not attacked by Chipper. Fig. 6*C* shows that nicked hairpin RNAs, provided the 5′ leader presents a permissive sequence, are asymmetrically degraded by Chipper. Clearly, in the original hairpin, the loop must be located 5′ to the sequence to be destroyed.

We have also detected Chipper activity in HeLa cell nuclei and in extracts derived from the ovaries of *Parascaris equorum*. We propose that Chipper is a component of a conserved machinery for dsRNA processing in higher eukaryotes.

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