Bacterial double-stranded RNA-specific RNase III recognizes the A-form of an RNA helix with little sequence specificity. In contrast, baker yeast RNase III (Rnt1p) selectively recognizes NGNN tetraloops even when they are attached to a B-form DNA helix. To comprehend the general mechanism of RNase III substrate recognition, we mapped the Rnt1p binding signal and directly compared its substrate specificity to that of both Escherichia coli RNase III and fission yeast RNase III (PacI). Rnt1p bound but did not cleave long RNA duplexes without NGNN tetraloops, whereas RNase III indiscriminately cleaved all RNA duplexes. PacI cleaved RNA duplexes with some preferences for NGNN-capped RNA stems under physiological conditions. Hydroxyl radical footprints indicate that Rnt1p specifically interacts with the NGNN tetraloop and its surrounding nucleotides. In contrast, Rnt1p interaction with GAAA-capped hairpins was weak and largely unspecific. Certain dual specificity of substrate recognition was exhibited by PacI but not by bacterial RNase III. E. coli RNase III recognized RNA duplexes longer than 11 bp with little specificity, and no specific features were required for cleavage. On the other hand, PacI cleaved long, but not short, RNA duplexes with little sequence specificity. PacI cleavage of RNA stems shorter than 27 bp was dependent on the presence of an UU-UC internal loop two nucleotides upstream of the cleavage site. These observations suggest that yeast RNase IIIIs have two recognition mechanisms, one that uses specific structural features and another that recognizes general features of the A-form RNA helix.

The RNase III family consists of a growing number of enzymes that includes at least 33 bacterial and 22 eukaryotic enzymes (1–3). The members of this family regulate gene expression by processing and degrading cellular RNAs (1, 2, 4–6). Based on protein structure, the RNase III family has been divided into 4 sub-classes (1). Class I includes all bacterial enzymes that possess both the classical nuclease domain and a dsRNA binding domain (dsRBD). The class II enzymes are distinguished by the presence of an N-terminal extension and includes fungal RNase IIIIs such as Saccharomyces cerevisiae Rnt1p (7) and Schizosaccharomyces pombe PacI (8). The class III enzymes possess two nuclease domains and include both plant and vertebrate enzymes. Class IV contains all Dicer-like enzymes involved in RNA-mediated interference and are distinguished by the presence of both N-terminal helicase and PAZ domains (6, 9). The homology between the different RNase IIIIs varies between 20 to 84% depending on their evolutionary distance, suggesting a low level of primary structure conservation (1). This relatively low degree of conservation probably reflects the species specificity of RNase III, which prevents genetic complementation between members of the RNase III family (10).

Despite the high selectivity of RNase III cleavage in vivo, many of the enzymes have been shown to cleave RNA duplexes with low sequence complexity in vitro (11–14). Moreover, except for Rnt1p, no conserved substrate structure has been identified (15, 16). Unlike other RNase IIIIs, Rnt1p recognizes RNA capped by a conserved NGNN tetraloop and cleaves at a fixed distance from the loop (15). Genetic and biochemical analyses indicate that Rnt1p binding and cleavage are regulated by reactivity epitopes grouped into three boxes. These are the initial binding and positioning box, which is located at the tetraloop, the binding stability box adjacent to the tetraloop, and the cleavage efficiency box near the cleavage site (17). Alterations of the sequence within the initial binding and positioning box and binding stability box inhibit cleavage and reduce binding, whereas alterations in the cleavage efficiency box inhibit cleavage without affecting the binding efficiency. The second nucleotide of the initial binding and positioning box is the only universally conserved nucleotide. The presence of any nucleotide other than G reduces binding and blocks cleavage (17), indicating that unlike other RNase IIIIs, the Rnt1p primary binding site is an NGNN tetraloop and not an RNA helix (16). In fact, Rnt1p can use the tetraloop to bind RNA helix or direct cleavage within the DNA strand of a DNA/RNA hybrid, indicating that the presence of an A-form RNA helix is not essential at the site of cleavage. It is not clear if Rnt1p retains the capacity to cleave duplex RNA nor whether or not other eukaryotic RNase IIIIs also recognize specific sequence or structural features beyond the form of the RNA helix.
mechanism of RNase III cleavage, we compared the cleavage specificity of S. cerevisiae Rnt1p to that of Escherichia coli RNase III and S. pombe PacI using a fixed set of RNA substrates. Rnt1p binds to RNA duplexes with variable efficiencies but cleaved only substrates capped with NGNN tetraloops under physiological conditions. E. coli RNase III recognized all RNA substrates tested in a similar fashion and was only inhibited by the presence of specific antideterminant sequences. PacI cleaved long RNA duplexes more efficiently than Rnt1p, but unlike E. coli RNase III, the cleavage of short RNA duplexes was restricted to RNAs that contained specific sequence and structural features. Our results suggest that, unlike prokaryotic RNase III, eukaryotic enzymes have evolved a mechanism by which RNA with specific structural features is posi-
tively selected, thereby bypassing the requirement for long RNA duplexes.

MATERIALS AND METHODS

Enzymatic Assays—Recombinant Rnt1p and the catalytically impaired mutant Rnt1p D247R, PacI, and Rnt1p for 20 min at 30 °C. The RNA products were then fractionated on 20% denaturing PAGE. The substrate secondary structures are illustrated on the left. The black, gray, and open arrowheads represent the Rnt1p, PacI, and bacterial RNA III cleavage sites, respectively. The alkaline hydrolysis ladder of a model substrate is shown on the left of the gel. The average cleavage of the different enzymes observed in three independent experiments relative to that of Rnt1p cleavage of R31D is indicated on the bottom. B, cleavage of long intermolecular RNA duplexes with different RNase III. An excess of 5'-end-labeled 106-bp-long RNA duplex of random sequence was incubated with 20 nM recombinant Rnt1p, PacI, and E. coli RNase III in different KCl concentrations. The resulting bands were fractionated on 4% nondenaturing PAGE. The positions of the gel origin (Ori), dsRNA, single-stranded RNA (ssRNA), and the product (P) are indicated on the right. C, mapping the cleavage products of long RNA duplexes. Cleavage reactions in the presence of an excess of protein of the 106 RNA duplex were performed as described in B but were fractionated using 12% denaturing PAGE. Increasing concentrations of Rnt1p varying between 10 and 160 mM were incubated with the RNA duplex in 10 mM KCl. The RNA molecular weight marker is shown on the left. The positions of the substrate (S) and the products (P1, P2, and P3) are indicated on the right of the gel. D, sequence of the 106 RNA duplex used to test RNase III cleavage. The cleavage positions corresponding to each product (P1, P2, and P3) are shown in small brackets. Positions that resisted cleavage by Rnt1p (black boxes), PacI (gray boxes), and RNase III (open boxes) were determined using a high resolution sequencing gel.

FIG. 2. Members of the RNase III family cleave long RNA duplexes with varying efficiencies. A, in vitro cleavage of long RNA hairpins with different RNase III. Long 33-bp RNA stems capped with AGUC (R31D) and GAAA (R31D-G3A) tetraloops were 5'-end-labeled and then cleaved with excess bacterial RNase III, PacI, and Rnt1p for 20 min at 30 °C. The RNA products were then fractionated on 20% denaturing PAGE. The substrate secondary structures are illustrated on the left. The black, gray, and open arrowheads represent the Rnt1p, PacI, and bacterial RNA III cleavage sites, respectively. The alkaline hydrolysis ladder of a model substrate is shown on the left of the gel.

The average cleavage of the different enzymes observed in three independent experiments relative to that of Rnt1p cleavage of R31D is indicated on the bottom. B, cleavage of long intermolecular RNA duplexes with different RNase III. An excess of 5'-end-labeled 106-bp-long RNA duplex of random sequence was incubated with 20 nM recombinant Rnt1p, PacI, and E. coli RNase III in different KCl concentrations. The resulting bands were fractionated on 4% nondenaturing PAGE. The positions of the gel origin (Ori), dsRNA, single-stranded RNA (ssRNA), and the product (P) are indicated on the right. C, mapping the cleavage products of long RNA duplexes. Cleavage reactions in the presence of an excess of protein of the 106 RNA duplex were performed as described in B but were fractionated using 12% denaturing PAGE. Increasing concentrations of Rnt1p varying between 10 and 160 mM were incubated with the RNA duplex in 10 mM KCl. The RNA molecular weight marker is shown on the left. The positions of the substrate (S) and the products (P1, P2, and P3) are indicated on the right of the gel. D, sequence of the 106 RNA duplex used to test RNase III cleavage. The cleavage positions corresponding to each product (P1, P2, and P3) are shown in small brackets. Positions that resisted cleavage by Rnt1p (black boxes), PacI (gray boxes), and RNase III (open boxes) were determined using a high resolution sequencing gel.
FIG. 3. Hydroxyl radical protein footprinting of Rnt1p-RNA complexes. Trace amounts of R31D (A) and R31D-G3A (B) RNAs were 3' end-labeled and incubated with 0, 0.4, 1.2, or 4.8 μM recombinant Rnt1p D247R in the absence (N) or presence of hydroxyl radical-generating reagents on ice. The RNA was extracted, loaded on a 20% denaturing PAGE, and visualized by autoradiography, and the bands were quantified.
indicates RNA incubated with 4.8 (pH 7.5), 100 mM KCl, 0.1 mM DTT, and 0.1 mM EDTA (pH 7.5)) and incubated on ice for 5 min. The hydroxyl radical generating mix was at least three times.

Molecular Analyst Software (Bio-Rad). All experiments were repeated the dried gels were exposed to film. The bands were quantified using the nol blue in formamide). The samples were loaded on 20% PAGE, and pending in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue) and then incubated in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue). The RNA was ethanol-precipitated and resuspended in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue). The RNA was ethanol-precipitated and resuspended in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue).

Hydroxyl Radical Footprinting Assays—Hydroxyl radical footprinting was carried out as described (25) with several modifications. Re-combinant Rnt1p (D247R) was purified as described above, but the final dialysis was performed against MOPS dialysis buffer (30 mM MOPS (pH 7.5), 500 mM KCl, 0.1 mM EDTA (pH 7.5), and 0.1 mM DTT) to ensure the absence of glycerol from the reaction and stored at −80 °C. In the reaction tube 28 fmol of 3′-end labeled RNA were mixed with 0.4–4.8 μM Rnt1p (D247R) in 17 μl of 1× MOPS buffer (20 mM MOPS (pH 7.5), 100 mM KCl, 0.1 mM DTT, and 0.1 mM EDTA (pH 7.5)) and incubated on ice for 5 min. The hydroxyl radical generating mix was prepared by the addition of 1 μl of 40 mM ferrous ammonium sulfate and 80 mM Na2EDTA (pH 8.0) and 1 μl of 40 mM sodium ascorbate and 1 μl of 2.4% hydrogen peroxide (H2O2) to the reaction tube. The reactions were carried out on ice for 4 min and were quenched by adding 4 μl of 100 mM thioareua. The RNA was ethanol-precipitated and resuspended in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue) and then incubated in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue). The RNA was ethanol-precipitated and resuspended in RNA loading dye (0.05% xylene cyanol and 0.05% bromphenol blue).

**RESULTS**

**Rnt1p Binds RNA Duplexes Lacking an NGNN Tetraloop—** Binding assays show that the Rnt1p dependence for the present NGNN tetraloop, the interaction with RNA hairpins is proportional to the length of the RNA stem (16, 17). Mutations that alter the NGNN sequence block the binding of Rnt1p to substrates with 9-bp stems (17) but only reduce that to substrates with 24-bp stems by 2–4-fold (15, 19). This suggests that Rnt1p may still retain the capacity to directly bind RNA duplexes in a manner analogous to other dsRNA-binding proteins. To assess the importance of the NGNN tetraloop for substrate recognition by Rnt1p, we constructed RNA hairpins with different stem lengths or tetraloop sequences and tested their binding and cleavage by Rnt1p (Fig. 1). Two hairpins with identical 33-bp-long stems were constructed with either an AGUC (R31D) or a GAAA tetraloop (R31D-G3A). A similar third hairpin possessing a 27-bp stem capped with an AGUC tetraloop (R31–27) was also constructed to test the effect of the stem length on Rnt1p binding and cleavage. As shown in Fig. 1A, R31–27 and R31D, both of which possess the conserved NGNN tetraloop, bound efficiently to Rnt1p with apparent Kd values of 3.3 ± 0.3 and 2.5 ± 0.3 μM, respectively. In contrast, Rnt1p bound to R31D-G3A 2–3-fold less efficiently, with an apparent Kd of 7.0 ± 0.5 μM. This indicates that Rnt1p can bind RNA stems in the absence of the NGNN tetraloop, albeit inefficiently. Interestingly, the number of ribonucleoprotein complexes formed by Rnt1p binding varied depending on the stem length. Substrates shorter than 27 bp and capped with an NGNN tetraloop formed one complex with Rnt1p, whereas NGNN capped substrates longer than 27 bp formed two complexes with Rnt1p (Fig. 1A). At protein concentrations ranging from 1 to 3 μM the 33-bp substrate R31D formed a single ribonucleoprotein complex with Rnt1p (C1). At concentrations higher than 3 μM, all of the free RNA is shifted, and a second slower migrating RNA complex (C2) is observed. This suggests that in the presence of NGNN the C1 complex is more stable and/or forms earlier than C2. In contrast the C2 complex was much more abundant than the C1 complex when the GAAA-capped substrate (R31D-G3A) was incubated in the presence of the same concentration of Rnt1p. This indicates that the C1 complex is favored or stabilized by the presence of the NGNN tetraloop, whereas the C2 complex formation is tetraloop-independent and requires the presence of a long RNA duplex.

Cleavage by Rnt1p was tested by incubating recombinant Rnt1p with 32P-labeled versions of the different substrates in the presence of Mg2+. Rnt1p efficiently cleaved R31–27 and R31D (data not shown and Fig. 1B) but not R31D-G3A, which lacks the NGNN tetraloop (Fig. 1B). Even at low monovalent salt concentrations, which normally permit the cleavage of weak substrates (17, 20), R31D-G3A was not cleaved by Rnt1p. Because the binding reactions are conducted at 4 °C, whereas the cleavage reactions are performed at 30 °C, it is possible that the ribonucleoprotein complex formed between Rnt1p and R31D-G3A (Fig. 1A) does not form in the cleavage reaction. To eliminate this possibility we performed a cleavage reaction using preformed ribonucleoprotein complexes. R31D-G3A and R31D were bound to Rnt1p, and the gel fragments containing either the C1 or C2 complexes were incubated with Mg2+ to initiate cleavage. As shown in Fig. 1C, complexes formed between Rnt1p and R31D were immediately cleaved, whereas those formed with R31D-G3A remained uncleaved even after 20 min of incubation. These results show that Rnt1p binding to RNA does not directly lead to cleavage and that the NGNN tetraloop is necessary not only for binding efficiency but also to correctly position the RNA for cleavage.

**Members of the RNase III Family Cleave dsRNA with Varying Efficiencies and Sequence Specificity—** Although RNase IIIIs other than Rnt1p have been shown to cleave generic dsRNA, their affinities for an NGNN-capped RNA stem have never been tested. To determine whether or not Rnt1p NGNN cleavage dependence is shared with other RNase IIIIs, we compared E. coli RNase III, S. pombe PacI, and Rnt1p cleavage of R31D and R31D-G3A (Fig. 2A). As expected, Rnt1p only cleaved R31D, whereas bacterial RNase III cleaved both substrates to the same extent regardless of the sequence of the tetraloop. PacI cleaved R31D 12 bp from the tetraloop, and R31D-G3A cleaved both 12 bp and at an additional cleavage site located 17 bp from the tetraloop. At high salt concentrations the PacI cleavage of R31D was also more efficient than that of R31D-G3A, and cleavage 12 bp from the tetraloop was preferred only in the presence of NGNN (data not shown). Thus, at least in the context of certain RNA stems, the presence of NGNN tetraloop may influence the selection of PacI cleavage site. These data

The positions of the tetraloops are shown on the right of the gels, and the position of the alkaline hydrolysis ladder is on the left. Diagrams showing the ribose residues protected from hydroxyl radical are on the left. The black, gray, and open circles indicate, respectively, sites of strong, moderate, and weak protection. The gray boxes, which indicate the protected regions, are shown below the gels. The histograms were obtained using Gel Doc (Bio-Rad). The protection pattern obtained corresponds to the direct counts (cpm) obtained by Instant Imager (data not shown). The black line represents the pattern obtained with RNA incubated alone, and the gray line indicates RNA incubated with 4.8 μM Rnt1p. The experiments were repeated four times, and the error margin ranged from 10 to 15%.
indicate that *E. coli* RNase III is not affected by the tetraloop composition, whereas in context both yeast RNase IIIs are influenced by the tetraloop sequence (albeit to different degrees).

The inability of Rnt1p to cleave a 33-bp stem capped with GAAA tetraloop (Fig. 1) was surprising given earlier results showing that Rnt1p could cleave RNA duplexes like other RNase IIIs (7). To determine the source of this apparent discrepancy we tested the capacity of Rnt1p to cleave 106-bp RNA duplexes of random sequences and compared it to those of *E. coli* RNase III and PacI. The cleavage was performed in the presence of an excess of either RNA (10:1) or protein (100:1) in either 150 mM KCl (physiological salt conditions for *S. cerevisiae*) or 10 mM KCl dilution (Figs. 2, B and C). In the presence of an excess RNA Rnt1p did not cleave the RNA duplex even at low salt concentrations. In contrast, both *E. coli* RNase III and PacI cleaved the same substrate to completion in all salt conditions (Fig. 2C). Under single turnover conditions Rnt1p cleaved the RNA only in the presence of high protein concentrations (Fig. 2C). This result clearly indicates that, unlike RNase III and PacI, Rnt1p has only a rudimentary ability to cleave dsRNA and suggests that Rnt1p has lost the ability to directly cleave an RNA duplex in vivo.

To examine whether RNase IIIs cleave dsRNA randomly or if they prefer specific sequences, we mapped the cleavage sites for all three enzymes using a high resolution denaturing gel completed with different RNA markers (17, 21) (Fig. 2C, D). If the cleavage is random and the minimum product size is 11 nt, as suggested by the crystal structure of bacterial RNase III, we expect all three enzymes to cleave long dsRNA every 11 bp in all sequence combinations, leading to a final product of 11 nt. As shown in Fig. 2C, none of the three enzymes produced 11-nt final products. In fact most of the products of *E. coli* RNase III and Rnt1p digestion were 30 nt long, whereas most PacI products were 60 nt long (very little 30-nt products were detected with PacI). Following the cleavage reaction at different times (data not shown) allowed us to identify potential cleavage sites that are in fact not cut by each enzyme (Fig. 2D). In general all enzymes preferred areas with low G-C content. The most sensitive in this regard was PacI, which did not cleave sites with two consecutive G-C bp. The least sensitive was *E. coli* RNase III, which was only affected by a stretch of 7 G-C bp. This indicates that all three enzymes are inhibited to varying extents by the presence of G-C bp at the cleavage site. These results show that *E. coli* RNase III has low sequence specificity but does not cleave dsRNA randomly and that yeast RNase IIIs

---

**Fig. 4. Yeast and bacterial RNase IIIs have different specificities for short RNA hairpins.** A, diagrams representing the secondary structures of the different substrates used to test the specificities of the different RNase IIIs. The open boxes indicate the positions of *E. coli* RNase III distal and proximal boxes shown previously to influence RNA cleavage (13). The gray boxes indicate bp that inhibit *E. coli* RNase III and/or Rnt1p cleavage. The black, gray, and open arrowheads indicate cleavage sites for Rnt1p, PacI, and RNase III, respectively. B, internally labeled substrates (R31D, R31–27, R31L) or 5' end-labeled substrates (R31L-3 and R31-U5L) were incubated in the absence (Neg) or the presence of excess Rnt1p (10 nM), RNase III (10 nM), or PacI (80 nM) in reaction buffer containing Mg²⁺ for 20 min at 30 °C. After incubation, the reaction products were directly loaded on 12% PAGE, and the bands were visualized using Instant Imager. The RNA marker (M) is indicated on the left. The products were mapped using an alkaline ladder and nuclease P1 digestions (data not shown). The cleavage rates were determined under single turnover conditions (ST) and are presented as fractional velocities relative to R31D when cleaved by Rnt1p. The velocities are an average of three experiments. Errors in values of velocities are within ±0.05.
Identification of Rnt1p-protected Ribose Residues by Hydroxyl Radicals—To determine how Rnt1p binds its substrates, we mapped the binding pattern of RNA either with or without excess protein with 10 nM Rnt1p, PacI, or RNase III in the presence of Mg\(^{2+}\) and 10 mM KCl. Cleavage products were loaded on 20% denaturing PAGE and visualized using Instant Imager. The cleavage rates are presented as fractional velocities relative to that of the original substrate (P3E) when cleaved by PacI under single turnover conditions (ST) and are the average of three experiments. The positions of the alkaline ladder are indicated on the left. The black, gray, and open arrowheads indicate the Rnt1p, PacI, and RNase III cleavage sites, respectively. S indicates products produced by a single cleavage on one side of the helix. Errors in the velocities values are within ±0.05.
Fig. 6. Specific structural motif dictates cleavage by PacI. A and C represent the structure of the different mutations engineered in the natural PacI substrate. The gray boxes indicate the positions of the different mutations introduced in P3E. B and D show cleavage in the presence of excess protein of the different 5’ end-labeled substrates by Rnt1p, Pac1, and RNase III in 10 mM KCl. Neg, absence of excess protein. The cleavage
the conserved NGNN tetraloop using chemical footprinting. Hydroxyl radicals are small, highly reactive, and are insensitive to both sequences and secondary structures but clearly detect tertiary structure and protein protection. As shown in Fig. 3A, specific and strong footprints were seen within the tetraloop sequence and at the cleavage site. Footprints were consistently strong near the tetraloop, and the strongest footprints were detected at position 38 and 39 (Supplemental Fig. 1). Nucleotides at positions 38 and 39 were also shown by mutagenesis and chemical interference to be crucial for cleavage by Rnt1p (17). In general, the protection pattern of R31D was consistent with the previously determined minimum substrate length of 18 bp. Protection of nucleotides at the 3' end of the NGNN tetraloop were more pronounced than those at the 5' end of the tetraloop, consistent with asymmetrical binding of Rnt1p. The level of protections increased as Rnt1p concentration increased and reached a maximum in the presence of 1.2 μM Rnt1p. In contrast protections were not detected with R31D-G3A, which lacks the NGNN tetraloop, in any Rnt1p concentrations (Fig. 3B and Supplemental Fig. 1). The absence of protection is not due to the inability of Rnt1p to bind R31D-G3A, as indicated by mobility shift assay performed under the same conditions used for chemical footprinting (data not shown). This result suggests that Rnt1p binding in the absence of NGNN tetraloop is either too heterogeneous or too weak to produce specific footprint. We conclude the Rnt1p specifically interacts with sequences adjacent to the tetraloop and near the cleavage site in an NGNN dependent manner.

Cleavage Requirements of Short RNA Hairpins by Yeast and Bacterial RNase III—Because the most natural substrates of bacterial and yeast RNase IIIIs are short hairpins, we decided to compare the Rnt1p, PacI, and E. coli RNase III cleavage of RNA hairpins of various sizes and sequence. All hairpins used in Fig. 4 have the same upper 5-bp stem-loop sequence, whereas the length and sequence of the lower stems vary. Cleavage was tested in the presence of an excess of protein and a low monovalent salt concentration to detect both efficient and inefficient cleavages. Rnt1p cleaved substrates with stems varying from 33 to 16 bp at identical sites 14 and 16 bp from the tetraloop with similar efficiencies. The sole exception was R31L-27, which was previously shown to impair cleavage because of the presence of a 4 G-C-bp stretch at the cleavage site (17). Changing this G-C-bp stretch to A-U bp (R31L-3U5L) restored cleavage. In contrast, E. coli RNase III-cleaved substrates with 33-bp (R31D) and 27-bp (R31–27) long stems but not substrates with 19-bp (R31L) and 16-bp (R31L-3) stems. Unlike Rnt1p, E. coli RNase III cleavage was not linked to a fixed distance from the tetraloop and instead correlated with the presence of C-G bp within the distal and proximal boxes 2 and 9 bp from the cleavage site, as shown previously (13). Changing this sequence to A-U (R31L-3U5L) partially restored cleavage. E. coli RNase III cleavage of R31L-3U5L was less efficient than that with Rnt1p. Surprisingly, PacI only cleaved R31D and R31L-3U5. This lack of cleavage cannot be explained on the basis of stem length because the 16-bp-long R31L-3U5 was cleaved, whereas the 27-bp-long R31–27 was not. The presence of inhibitory sequences like G-C bp alone cannot explain PacI inability to cleave R31–27 since it has the same sequence as R31D except for 6 bp distal to the cleavage site. In addition, alteration of the number or position of the G-C bp within the R31–27 stem failed to restore cleavage (data not shown). This suggests that PacI cleavage of hairpins shorter than 27 bp is much more sequence-specific than previously believed (14). Together, the results indicate that E. coli RNase III and Rnt1p substrate selectivity is not affected by changes in the substrate length, whereas the PacI sequence specificity dramatically increases as the substrate length decreases.

RNA Elements Required for S. pombe RNase III Cleavage—Faced with the unexpected substrate selectivity of PacI shown in Fig. 4, we wanted to determine whether or not a conserved sequence is required for the cleavage of natural PacI substrates. To date only two natural PacI substrates have been reported, the stem-loop structure found at the 3' end of U2 small nuclear RNA and the stem-loop structure found at the end of the rRNA 3' external transcribed spacer. The overall structures of these substrates are very different, but both exhibit an internal loop of similar sequence (UU-UC for rRNA 3' external transcribed spacer and UC-UU for U2), and the stem of both substrates has a G-C or G-U bp 9 bases above of the internal loop. Although two substrates are hardly enough to deduce conserved features, we decided to see if these or other features of the natural substrates affect PacI cleavage. We constructed a variety of substrates based on the sequence of the rRNA 3' external transcribed spacer (P3E) and compared their cleavages by PacI, Rnt1p, and E. coli RNase III (Fig. 5). To directly test PacI sequence specificity we reversed either the complete stem sequence (P3E-F) or the first G-C bp of the stem conserved in both U2 and the rRNA 3' end (P3E-1CG). As expected, PacI cleaved P3E efficiently two nucleotides below the conserved internal loop, as shown previously (22). In contrast, P3E-F and P3E-1CG were not cleaved at the predicted site. Instead, very low amounts of cleavage were detected within the single-stranded terminal and internal loops, indicating that the first G-C bp below the terminal loop is required for both cleavage efficiency and specificity. Rnt1p did not cleave any of PacI substrates due to the absence of the conserved NGNN tetraloop. E. coli RNase III cleaved P3E but not P3E-F or P3E-1CG. To evaluate the single-stranded RNA terminal loop for cleavage by PacI, we base-paired the nucleotides of the terminal loop to create a longer stem capped with an AAUU tetraloop (P3E-1PL). PacI cleaved P3E-1PL more efficiently than the wild type substrate at the predicted cleavage site, indicating the terminal loop is not required for either cleavage efficiency or specificity. As expected E. coli RNase III but not Rnt1p cleaved P3E-1PL efficiently. We examined the effect of the Rnt1p-specific NGNN tetraloop on the cleavage of PacI natural stem sequence by changing the P3E terminal loop to either AGUC (P3E-A) or GAAA (P3E-G) tetraloops and testing for cleavage. PacI cleaved both substrates with similar efficiencies and at the predicted cleavage sites, indicating that cleavage specificity is enforced by the stem sequence and not the tetraloop. Rnt1p cleaved P3E-A, but not P3E-G, whereas E. coli RNase III cleaved both substrates with similar efficiencies. We evaluated the requirement for the internal loop by base-pairing the loop sequence of P3E-A and P3E-G, producing substrates P3E-AP and P3E-GP, respectively (Fig. 5). Surprisingly, removing the internal bulge blocked all cleavage by PacI while enhancing cleavage by E. coli RNase III. Rnt1p inefficiently cleaved P3E-AP and did not cleave P3E-GP. Changes of the sequence and reducing the G-C contents of the stem did not restore cleavage by PacI (Supplemental Fig. 2). This result

rates are an average of three experiments and are presented as fractional velocities relative to that of the parent substrate (P3E) under single turnover conditions (ST). The black, gray, and open arrowheads indicate the Rnt1p, PacI, and RNase III cleavage sites, respectively. S indicates products produced by a single cleavage on one side of the helix. The RNA molecular weight marker is shown on the left. The products were mapped using an alkaline hydrolysis ladder and nuclease P1 digestion.
shows that PacI, but not Rnt1p or E. coli RNase III, requires an internal loop for cleavage of short RNA substrates.

To determine whether PacI recognizes the sequence or the structure of the internal loop, we either reversed the sequence of the internal loop (P3E-ABF) or changed it to AA-AG (P3E-ABR) (Fig. 6A). PacI failed to cleave both substrates, indicating that the sequence of the internal loop is essential for cleavage (Fig. 6B). Rnt1p did not efficiently cleave either substrate, whereas E. coli RNase III cleaved both substrates but preferred P3E-ABR. To test if the internal loop determines the cleavage site, as is the case with the Rnt1p tetraloop, we moved it 2 bp below its original position and increased the stem length by 2 bp so as to maintain the distance from the loop to the stem ends (P3E-ABD). As shown in Fig. 6, C and D, P3E-ABD cleavage was shifted 2 bp below the original cleavage site, indicating that the cleavage occurs at a fixed distance from the internal loop. However, the cleavage efficiency of P3E-ABD was only 18% of that observed with P3E, suggesting that other elements are required for efficient cleavage. We noticed that the distance between the internal loop and the G-C bp, shown to be important for cleavage by PacI (Fig. 5), is changed in P3E-ABD (Fig. 6). To test if the distance between the G-C bp and the internal loop is required for cleavage, we produced a substrate that restores the distance between these two elements (P3E-ABD-3GC). As shown in Fig. 6D, the efficiency of the cleavage was greatly enhanced, and the cleavage site remained two bp downstream of the wild type site. Cleavage was not enhanced by introducing additional elements, like the U-A bp found three nucleotides from the internal loop (P3E-ABD-3GC/9UA), found in the wild type substrate (Fig. 6). Moving the internal loop to a lower position along the RNA stem inhibited cleavage by E. coli RNase III and did not significantly affect Rnt1p cleavage, indicating that the effect of the tetraloop is specific to PacI. These data indicate that unlike bacterial RNase III, PacI (like Rnt1p) recognizes specific sequence and structural motifs and uses this to determine the cleavage site. We conclude that bacterial RNase III, PacI, and Rnt1p possess distinct substrate specificity. Indeed, substrates can be designed such that they can be cleaved by only one enzyme (Supplemental Fig. 3).

DISCUSSION

In this study we present the first direct comparison between eukaryotic and prokaryotic RNase IIIIs with the aim of understanding the general mechanism of substrate specificity. We show that yeast RNase IIIIs may either recognize the helical A-form of an RNA helix or positively identify substrates with specific sequences or structural motifs. Yeast and bacterial RNase IIIIs recognize generic dsRNA in a similar fashion and are affected by the presence of G-C bp at or near the cleavage site (Fig. 2). Motif recognition was restricted to yeast RNase IIIIs and was not demonstrated by bacterial RNase III. The dominance of the two cleavage mechanisms is very different with S. cerevisiae Rnt1p and S. pombe PacI. Rnt1p cleavage of long and short RNA substrates was always motif-dependent at physiological conditions, whereas PacI was motif-sensitive only for the cleavage of short RNA substrates (Figs. 2, 4, and 5). Rnt1p homodimer bound and cleaved RNA stems capped with an NGNN tetraloop efficiently, whereas two homodimers were required to bind RNA lacking the NGNN tetraloop and did not induce cleavage (Fig. 1). PacI cleaved short hairpins at a fixed distance from a UU-UC internal loop found in natural substrates, and mutations in the loop sequence inhibited RNA cleavage (Fig. 6). Furthermore, mutating this internal loop sequence at the 3′ end of the 25 S rRNA blocked RNA processing in vivo (23), indicating that this loop is important for PacI cleavage in vivo as well as in vitro. The conservation of this internal loop cannot be assessed since only two substrates were identified for PacI, at the 3′ end of the 25 S rRNA (10) and at the 3′ end of U2 small nuclear RNA (22). Both substrates contain an internal loop, but the sequence is flipped in the case of U2 (CU-UU instead of UU-UC). Interestingly, the cleavage site of U2 small nuclear RNA (22) seems to occur in a position similar to that observed when the sequence of the RNA 3′ end is flipped (Fig. 5). Together these results show that yeast RNase IIIIs have evolved distinct substrate specificities and suggest that the general mechanism of dsRNA recognition of bacterial RNase III is not predominant among eukaryotic orthologues.

Rnt1p cleaves RNA stems capped with an NGNN tetraloop and recognizes the tetraloop even when it is attached to a B-form DNA helix (16). Because bacterial RNase III, like most double-stranded RNA binding proteins, recognizes A-form RNA helices with little sequence specificity (13, 24), we asked whether or not Rnt1p retained the capacity to cleave generic dsRNA. By comparing Rnt1p to PacI and E. coli RNase III we found that Rnt1p retained only a residual capacity to bind and cleave dsRNA under physiological conditions (Fig. 2). The incapacity of Rnt1p to cleave dsRNA is likely due to incorrect positioning of the RNA within the enzyme catalytic core and not to a low binding affinity. Cleavage was not detected in preformed dsRNA-Rnt1p complexes (Fig. 1), and NGNN capped substrates with similar K₅ values were cleaved by Rnt1p (17). However, Rnt1p footprints in both the presence and the absence of the NGNN tetraloop were very different, suggesting that the enzyme binds the two substrates differently. The NGNN-specific footprints suggest an interaction pattern consistent with that deduced from Rnt1p dependent chemical shift assay, chemical interference, and substrate mutagenesis (15, 17). Rnt1p footprints in the absence of the NGNN tetraloop are
similar to those produced by \textit{E. coli} RNase III (25). The protection patterns of both enzymes are equally distributed on both sides of the cleavage site. Moreover, the protection pattern is asymmetrical, reflecting the binding of antiparallel homodimer. The protection pattern that spans 13–14 nt for each subunit is consistent with the binding pattern suggested by the structure of \textit{Aquifex aeolicus} RNase III (26). In contrast, the binding of Rnt1p to substrates capped with an NGNN tetraloop protected nucleotides surrounding the tetraloop, and the whole complex appeared to be shifted upstream of the cleavage site (Fig. 3). This mode of binding suggests that Rnt1p recognizes its substrate in an asymmetric manner (Fig. 7) and explains why cleavage always occurs at a fixed distance from the tetraloop.

Recognition of a specific RNA motif is not restricted to Rnt1p but is also shared by \textit{S. pombe} PacI. PacI recognized RNA shorter than 27 bp only when a UU-UC internal loop is present, and the location of the cleavage site was dictated by the position of this loop (Figs. 5 and 6). This mode of cleavage is similar to Rnt1p NGNN-dependent cleavage and very different from the motif-independent bacterial RNase III cleavage (24). Unlike Rnt1p, PacI internal loop selectivity was salt and protein concentration-dependent, and cleavage of long dsRNA was very efficient under all conditions tested (Fig. 2). Therefore, PacI appears to be less strict than Rnt1p, but more sequence-specific than \textit{E. coli} RNase III. Comparison of the protein sequences of all three enzymes does not provide a clear indication as to how the substrate specificity was developed (1). The only obvious difference is the N-terminal domain, which is found in eukaryotic but not prokaryotic RNase IIIIs. However, deletion of this sequence did not affect Rnt1p substrate selectivity (20), whereas the Rnt1p dsRBD by itself preferentially bound to RNA capped with an NGNN tetraloop (19). This suggests that the motif specificity of eukaryotic enzymes is embedded in the dsRBD. Comparison of the dsRBD sequences shows variations in the distribution of the positively charged amino acids, which have been implicated in the RNA binding of Staufen and \textit{E. coli} RNase III dsRBDs (27, 28). Mutations in the corresponding residues of Rnt1p dsRBD may provide clues as to the origin of the substrate selectivity.

Rnt1p, PacI, and \textit{E. coli} RNase III recognize different features of an RNA hairpin; consequently, a distinct model substrate for each enzyme can be imagined. As shown in Fig. 7, \textit{E. coli} RNase III would preferably recognize substrates with two turns of an RNA helix (~21 bp) but could also recognize RNAs with less than two turns of an RNA helix (Fig. 4). The interaction does not involve specific motifs but instead is regulated by the presence of antideterminants (13). The binding of \textit{E. coli} RNase III is enhanced by Mg$^{2+}$ (25) and could be achieved in the absence of the dsRBD, albeit inefficiently (29), suggesting that the nuclease domain is the main source of substrate specificity. Thus, most substrates that bind are cleaved. Rnt1p could bind to RNA in a similar fashion using the dsRNA helix, but this mode of binding is not stable and does not support efficient cleavage (Figs. 1 and 2). Instead Rnt1p uses an NGNN tetraloop in combination with helical elements surrounding both the tetraloop and the cleavage site to bind and cleave its RNA substrates. PacI recognizes long dsRNAs with similar efficiency to \textit{E. coli} RNase III while cleaving short RNA substrates using specific motifs like Rnt1p. Therefore, certain eukaryotic RNase IIIIs like PacI may recognize their substrates either through the helical shape of a long RNA duplex or by recognizing a specific structural motif within short RNA hairpins.

Acknowledgments—We thank Kevin Wilson for help with the hydroxylation experiments. We are grateful to Jean-Pierre Ferreault, Dominique Fourmy, and Kevin Wilson for critical reading of this manuscript.

REFERENCES

1. Lamontagne, B., Larose, S., Boulanger, J., and Abou Elela, S. (2001) \textit{Curr. Issues Mol. Biol.} 3, 71–78
2. Nicholson, A. W. (1999) \textit{FEMS Microbiol. Res.} 23, 371–390
3. Conrad, C., and Rauthi, R. (2002) \textit{Int. J. Biochem. Cell Biol.} 34, 116–129
4. Nicholson, A. W. (1996) \textit{Prog. Nucleic Acid Res. Mol. Biol.} 52, 1–65
5. Grishok, A., and Mello, C. C. (2002) \textit{Adv. Genet.} 46, 339–360
6. Saunders, L. R., and Barber, G. N. (2003) \textit{FASEB J.} 17, 961–983
7. Abou Elela, S., Igel, H., and Ares, M., Jr. (1996) \textit{Cell} 85, 115–124
8. Iino, Y., Sugimoto, A., and Yamamoto, M. (1991) \textit{EMBO J.} 10, 221–226
9. Banerjee, D., and Slack, F. (2002) \textit{Bioessays} 24, 119–129
10. Rotondo, G., Huang, J. Y., and Frendewey, D. (1997) \textit{RNA} (N Y) 3, 1182–1193
11. Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B., and Radmark, O. (2002) \textit{EMBO J.} 21, 5864–5874
12. Zhang, H., Kolb, F. A., Brondani, V., Billy, E., and Filipowicz, W. (2002) \textit{EMBO J.} 21, 5875–5885
13. Zhang, K., and Nicholson, A. W. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} 94, 13437–13441
14. Rotondo, G., and Frendewey, D. (1996) \textit{Nucleic Acids Res.} 24, 2377–2386
15. Chanfreau, G., Buckle, M., and Jacquier, A. (2000) \textit{Proc. Natl. Acad. Sci. U. S. A.} 97, 3142–3147
16. Lebars, I., Lamontagne, B., Yushizawa, S., Abou Elela, S., and Fourmy, D. (2001) \textit{EMBO J.} 20, 7250–7258
17. Lamontagne, B., Ghazal, G., Lebars, I., Yushizawa, S., Fourmy, D., and Abou Elela, S. (2000) \textit{J. Mol. Biol.} 307, 985–1000
18. Chanfreau, G., Abou Elela, S., Ares, M., Jr., and Guthrie, C. (1997) \textit{Genes Dev.} 11, 2741–2751
19. Nagel, R., and Ares, M., Jr. (2000) \textit{RNA} (N Y) 6, 1142–1156
20. Lamontagne, B., Tremblay, A., and Abou Elela, S. (2000) \textit{Mol. Cell. Biol.} 20, 1104–1115
21. Solnner-Webb, B., Cruz-Reyes, J., and Rusche, L. N. (2001) \textit{Methods Enzymol.} 324, 378–383
22. Zhou, D., Frendewey, D., and Lobo Ruppert, S. M. (1999) \textit{RNA} (N Y) 5, 1083–1098
23. Spasov, K., Perdomo, L. I., Evakine, E., and Nazar, R. N. (2002) \textit{Mol. Cell} 9, 433–437
24. Chellaiafrui, B. S., Li, H., and Nicholson, A. W. (1991) \textit{Nucleic Acids Res.} 19, 1759–1766
25. Li, H., and Nicholson, A. W. (1996) \textit{EMBO J.} 15, 1421–1433
26. Blaszczyk, J., Tropea, J. E., Bubunenko, M., Reutzahn, K. M., Waugh, D. S., Court, D. L., and Ji, X. (2001) \textit{Structure (Camb.)} 9, 1225–1236
27. Ramos, A., Gruner, S., Adams, J., Mckiein, D. R., Proctor, M. R., Freund, S., Byrrof, M., St Johnston, D., and Varani, G. (2000) \textit{EMBO J.} 19, 997–1009
28. Kelly, S. M., and Price, N. C. (2000) \textit{Curr. Protein Pept. Sci.} 1, 349–384
29. Sun, W., Jun, E., and Nicholson, A. W. (2001) \textit{Biochemistry} 40, 14976–14984
30. Lamontagne, B., and Abou Elela, S. (2001) \textit{Methods Enzymol.} 342, 159–167
31. Tremblay, A., Lamontagne, B., Catala, M., Yam, Y., Larose, S., Good, L., and Abou Elela, S. (2002) \textit{Mol. Cell. Biol.} 22, 4792–4802