RNA (1997), 3:1182–1193. Cambridge University Press. Printed in the USA.

Substrate structure requirements of the Pac1 ribonuclease from *Schizosaccharomyces pombe*

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ABSTRACT

The Pac1 ribonuclease of *Schizosaccharomyces pombe* is a member of the RNase III family of double-strand-specific ribonucleases. To examine RNA structural features required for efficient cleavage by the Pac1 RNase, we tested a variety of double-stranded and hairpin RNAs as substrates for the enzyme. The Pac1 RNase required substrates that have a minimal helix length of about 20 base pairs. The enzyme cut both strands of the helix at sites separated by two base pairs. However, Pac1 was also able to make a single-stranded cleavage within an internal bulge of an authentic *Escherichia coli* substrate at the same site chosen by RNase III. Pac1 efficiently degraded the structurally complex adenovirus VA RNA_I, but was inactive against the short HIV-1 TAR RNA hairpin. These results indicate that the Pac1 RNase prefers straight, perfect helices, but it can tolerate internal bulges that do not distort the helix severely. Like its homologue from *Saccharomyces cerevisiae*, the Pac1 RNase cleaved at two in vivo RNA processing sites in a hairpin structure in the 3′ external transcribed spacer of the *S. pombe* pre-rRNA, suggesting a role for the enzyme in rRNA maturation.

Keywords: double-stranded RNA; fission yeast; pre-rRNA processing; RNase III

INTRODUCTION

The Pac1 RNase of the fission yeast *Schizosaccharomyces pombe* belongs to the RNase III family of double-strand-specific ribonucleases (dsRNases) (Dunn, 1982; Xu et al., 1990; Iino et al., 1991; Court, 1993; Rotondo et al., 1995; Rotondo & Frendewey, 1996). The members of the RNase III family share several conserved primary structure elements that include regions required for catalysis and a dsRNA binding domain always found at their carboxyl termini (Court, 1993; Kharrat et al., 1995; Rotondo et al., 1995; Nicholson, 1996; Rotondo & Frendewey, 1996). The RNase III family takes its name from the founding member, which was first purified from *Escherichia coli* as an activity that degraded synthetic dsRNAs to small oligonucleotides (Robertson et al., 1968; Schweitz & Ebel, 1971; Crouch, 1974; Robertson & Dunn, 1975). RNase III was found subsequently to be an important RNA processing enzyme for rRNA and mRNA precursors (Dunn & Studier, 1973; Young & Steitz, 1978) and to participate in mRNA turnover (Court, 1993), conjugative DNA transfer (Koraimann et al., 1993), and antisense RNA-mediated regulation (Blomberg et al., 1990; Gerdes et al., 1992). *E. coli* RNase III usually makes staggered cuts in both strands of a double-helical RNA, but, in some cases, it cleaves once in a single-stranded bulge in the helix (Robertson, 1982; Chelladurai et al., 1993). The enzyme functions as a dimer that binds symmetrically on either side of its cleavage sites (Li & Nicholson, 1996). This arrangement requires approximately two turns of the A form helix, or about 20 base pairs (bp), which is consistent with the binding properties of dsRNA-binding domains (Manche et al., 1992; Bycroft et al., 1995; Bevilacqua & Cech, 1996).

Although there have been many reports of RNase III-like dsRNase activities in eukaryotic cells (Ohtsuki et al., 1977; Grummt et al., 1979), and there are at least four RNase III-like orfs that can be deduced from known eukaryotic DNA sequences (Rotondo & Frendewey, 1996), only two true eukaryotic RNase III homologues have been characterized in detail: the Pac1 RNase of *S. pombe* and the Rnt1 RNase of *Saccharomyces cerevisiae*. The budding yeast enzyme is essential for viability and, like its bacterial counterpart, it is responsible for the initial cleavages in the pre-rRNA processing pathway (Abou Elela et al., 1996). The Rnt1

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RNase cleaves in the helical stems of two extended hairpin structures found in the 5′ and 3′ external transcribed spacer (ETS) regions of the rRNA precursor (pre-rRNA). The *S. pombe* Pac1 RNase is also essential for viability (Iino et al., 1991). The *pac1*$^+$ gene was first identified by virtue of its ability to induce sterility when overexpressed (Xu et al., 1990; Iino et al., 1991) and was later shown to be an extragenic, multi-copy suppressor of a mutant that maintains reduced steady-state levels of several small nuclear RNAs (Rotondo et al., 1995). These effects of overexpression suggested roles for the Pac1 RNase in sexual development and snRNA synthesis, but its actual biological function remains to be established.

We have purified the Pac1 RNase and determined its general biochemical properties (Rotondo & Frendewey, 1996). To better understand the biological role and enzymatic characteristics of the Pac1 RNase, we tested a variety of natural and synthetic RNAs as substrates for the Pac1 RNase. These experiments established the range of dsRNA structure that the enzyme will tolerate. They also identified a native substrate for the Pac1 RNase—a hairpin structure in the *S. pombe* pre-rRNA 3′ETS that is the site of RNA processing events in vivo.

RESULTS

Minimum length dsRNA substrate for the Pac1 RNase

We previously described the characterization of a recombinant form of the *S. pombe* Pac1 RNase that is a highly active, double-strand-specific endoribonuclease (Rotondo & Frendewey, 1996). The enzyme converted long dsRNA substrates into short oligonucleotides and efficiently cleaved a hairpin RNA having a 25-bp stem. To define better the minimal helical length required for efficient cleavage by the Pac1 RNase, we tested a series of dsRNAs of varying lengths as substrates. These RNAs were prepared by annealing complementary ^{32}P-labeled single-stranded RNAs, removing the unpaired portions by ribonuclease digestion, and purifying the dsRNAs by gel electrophoresis (see Materials and Methods). Figure 1 shows the Pac1 RNase cleavage of the dsRNAs compared with a hairpin substrate, R1.1 RNA (see Fig. 2, described below). The Pac1 RNase efficiently degraded the longer dsRNAs (63–132 bp) to smaller fragments of approximately 10–30 nt. Pac1 cleavage of the 30-bp RNA resulted in two major products, one of which co-migrated with the 17-bp test substrate. Because the RNAs shown in Figure 1 were electrophoresed under conditions that did not denature the dsRNAs (Saccomanno & Bass, 1994), the cleavage pattern of the 30-bp substrate implies that the enzyme made one double-strand break near the middle of the RNA. The 17-bp test dsRNA was completely refractory to cleavage by the Pac1 RNase despite the

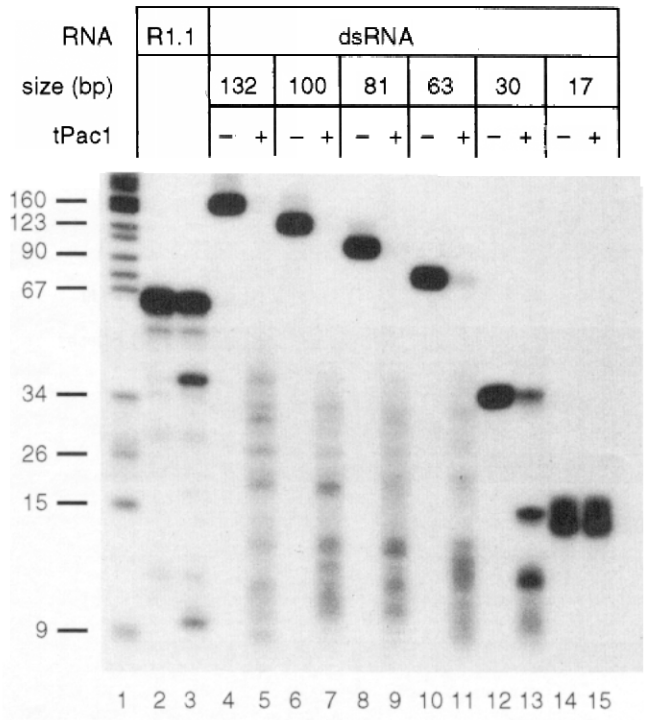

FIGURE 1. Minimal length Pac1 RNase substrate. High specific activity ^{32}P-labeled dsRNAs of varying lengths (indicated in bp above lanes 4–15) were incubated at 30 °C for 10 min in 20-μL reactions containing 30 mM Tris-HCl, pH 7.6, 25 mM KCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$ either without (–) or with (+) Pac1 RNase (tPac1) at 2 nM. For comparison, low specific activity ^{32}P-labeled R1.1 RNA (see Fig. 2) was incubated without (lane 2) or with (lane 3) Pac1 RNase in identical reactions. In each reaction, 15,000 cpm of RNA was used; therefore, concentrations varied from 45 pM for the 132-bp RNA to 250 pM for the 17-bp RNA. The concentration of R1.1 RNA was 27 nM. Reactions were stopped and one fourth of each was analyzed directly by electrophoresis on a 10% polyacrylamide/7 M urea gel run under nondenaturing conditions (see Materials and Methods) followed by autoradiography. ^{32}P-labeled dsDNA fragments from an *Msp* I digestion of pBR322 were co-electrophoresed as size markers (lane 1, sizes of selected fragments in bp indicated to the left of the lane).

approximately eightfold molar excess of enzyme over RNA used in this reaction. These results indicate that the minimum length perfect helix that is able to be cleaved by the Pac1 RNase lies between 17 and 30 bp. Efficient cleavage by Pac1 of a hairpin RNA having a 25-bp stem (Rotondo & Frendewey, 1996) further narrows the range of the minimal substrate to between 25 and 17-bp, which is very similar to that obtained for *E. coli* RNase III (Dunn, 1982; Robertson, 1982).

Cleavage of an *E. coli* RNase III substrate

The shared structural features and biochemical properties of *E. coli* RNase III and the *S. pombe* Pac1 RNase indicate clearly that they are homologous proteins (Xu et al., 1990; Iino et al., 1991; Rotondo et al., 1995; Rotondo & Frendewey, 1996). To examine whether the bacterial and yeast enzymes recognize similar substrates, we compared the cleavage of an authentic

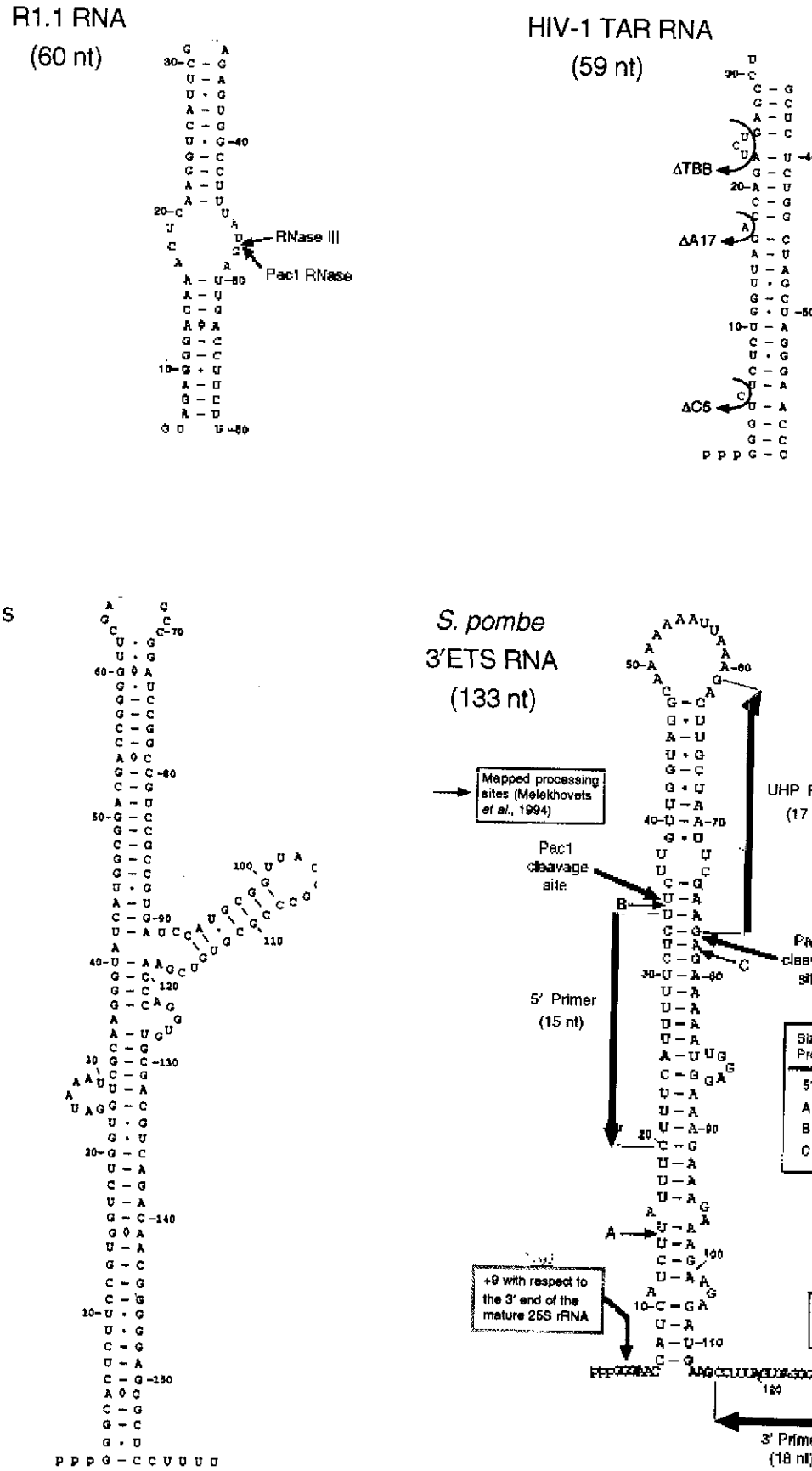

FIGURE 2. *(Legend on facing page.)*

E. coli substrate by RNase III and Pac1. The substrate, R1.1 RNA (Fig. 2), was derived from a T7 phage polycistronic transcript (Dunn & Studier, 1973) and consists of two 10-bp helices separated by an asymmetric bulge that maintains some helical character (Schweisguth et al., 1994). Earlier work demonstrated that RNase III cleaves R1.1 RNA at the site indicated by the arrow in Figure 2 (Dunn, 1976; Chelladurai et al., 1993). The single cut produces a larger 5′ product and a shorter 3′ product (Fig. 3, lane 2). The mobilities of these products relative to the xylene cyanol and bromophenol blue dye markers and the heterogeneity of the 3′ product were as reported previously (Chelladurai et al., 1993). The Pac1 RNase produces the identical cleavage pattern with R1.1 RNA (Fig. 3, compare lane 2 and 3). Thus, Pac1 recognizes the same cleavage site as RNase III on an authentic *E. coli* substrate.

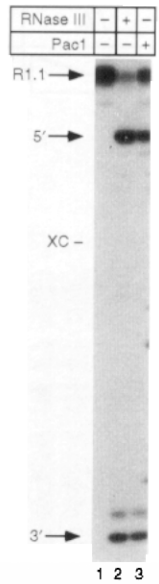

FIGURE 3. Cleavage of the R1.1 RNA by *E. coli* RNase III and the *S. pombe* Pac1 RNase. [α-^{32}P]UTP-labeled R1.1 RNA at 80 nM was incubated for 10 min at 30 °C without enzyme (lane 1) or with either *E. coli* RNase III (lane 2) or Pac1 RNase (lanes 3) at a concentration of 8 nM, with respect to enzyme monomer, in 10-μL reactions. Buffer and salt conditions for RNase III (lane 2) were those described by Li et al. (1993). Pac1 (lane 3) and control (lane 1) reactions contained 30 mM CHES, pH 8.5, 25 mM KCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$. The reactions were stopped, heat-denatured, and one half of each was analyzed directly by electrophoresis on a 15% polyacrylamide/7 M urea gel followed by autoradiography. The positions of the R1.1 substrate, the 5′ and 3′ cleavage products, and the xylene cyanol (XC) dye are given to the left.

Test of VA and TAR RNAs as substrates for the Pac1 RNase

To further examine the structural requirements for Pac1 substrate recognition, we tested the adenovirus VA RNA_I and HIV-1 TAR RNA as substrates. These RNAs are essentially hairpins, but they carry extensions and asymmetric bulges that are likely to perturb their helical backbones (Fig. 2). VA RNA_I is a 160-nt RNA polymerase III transcript that inhibits the double-stranded-RNA-activated protein kinase and is necessary for viral replication (Mathews & Shenk, 1991). The most recently refined model for VA RNA_I suggests that the short stem in the central domain folds back on the unpaired bulges, causing a bend in the RNA (Ma & Mathews, 1996). TAR is a short (59 nt) hairpin found at the 5′ end of all HIV-1 transcripts. It is the target of the transcriptional transactivator Tat (Kessler et al., 1991; Cullen, 1993), a small basic protein that binds to the trinucleotide bulge (labeled TBB for Tat-binding bulge in Fig. 2) near the apical end of the helix. TAR RNA also has two single nucleotide bulges at C5 and A17.

Figure 4 shows that the Pac1 RNase efficiently cleaved VA RNA_I at multiple sites (lanes 1–5)—nearly all of the RNA was converted to products in 10 min at a substrate to enzyme ratio of 5:1 (lane 2). The extent of cleavage did not increase when the Pac1 concentration was raised eightfold, indicating that the reactions were complete and the products were resistant to further degradation. In contrast, TAR RNA was completely resistant to cleavage by the Pac1 RNase, even at enzyme concentrations that were in excess of substrate (Fig. 4, lanes 6 to 10). Longer incubations at higher Pac1 concentrations under a variety of ionic conditions failed to produce significant cleavage of TAR RNA (data not shown). To examine the effects of the un-

FIGURE 2. Test substrate RNAs. Secondary structure models are depicted for four RNAs tested as substrates for the *S. pombe* Pac1 RNase. R1.1 RNA (upper left) is part of an authentic *E. coli* RNase III processing signal in a T7 phage transcript. Arrows mark the sites of in vitro cleavages by *E. coli* RNase III (black) and the *S. pombe* Pac1 RNase (purple). HIV-1 RNA (upper right) is the hairpin formed by the first 59 nt of the transactivation response region at the 5′ end of all HIV-1 transcripts. Curved red arrows circumscribe nucleotides deleted in variant forms of TAR RNA (designated as Δ followed by the deleted nucleotide) tested as substrates for the Pac1 RNase; TBB stands for Tat-binding bulge. Adenovirus VA RNA_I (lower left) is modeled according to Ma and Mathews (1996). *S. pombe* 3′ETS RNA is derived from the potential hairpin structure in the 3′ETS region of the *S. pombe* pre-rRNA. Nucleotide numbering is from the 5′ end of the RNA. Nucleotides shown in blue are complementary to parts of the transcription template not derived from *S. pombe* sequence. The second G in the RNA represents a position that lies 9 nt downstream of the 3′ end of the mature 25S rRNA. Green arrows (A, B, and C) show the positions of in vivo processing sites mapped by Melekhovets et al. (1994); the sizes of expected products for cleavages at these sites are given in the box on the right. Purple arrows identify the sites of in vitro cleavage by the Pac1 RNase. Red arrows lie adjacent to sequences complementary to oligodeoxynucleotide primers used for product identification and primer extension mapping of the cleavage sites.

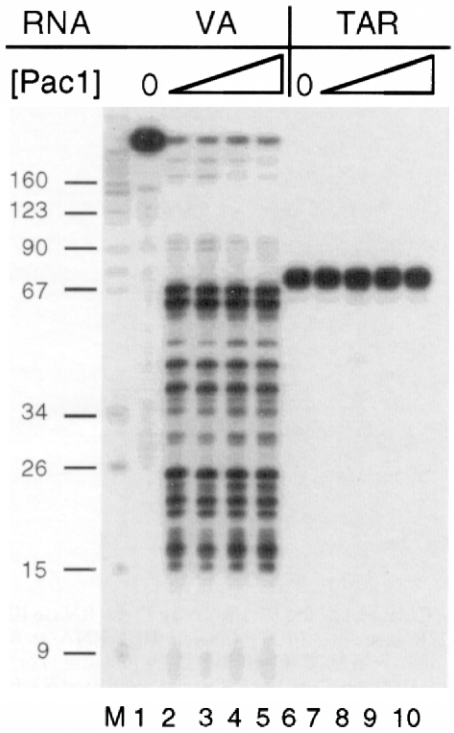

FIGURE 4. Test of adenovirus VA RNA_I and HIV-1 TAR RNA as substrates for the Pac1 RNase. [α-^{32}P]GTP-labeled VA (lanes 1–5) and TAR (lanes 6–10) RNAs at 35 nM were incubated for 10 min under standard conditions either without (lanes 1 and 6) or with Pac1 RNase (lanes 2–5 and 7–10). Triangles represent twofold increases in Pac1 concentration between 7 and 56 nM. Reactions were stopped, and one half of each was heat-denatured and analyzed directly by electrophoresis on a 12% polyacrylamide/7 M urea gel. DNA markers (lane M, sizes on left) are as in Figure 1.

paired nucleotides in TAR RNA, we synthesized variants in which C5, A17, and the TBB were deleted (indicated by the curved red arrows in the Fig. 2) either singly or in combination. None of these alterations converted the TAR RNA into an efficient substrate for the Pac1 RNase. Removal of the TBB did allow Pac1 to make a double-strand break in the modified TAR RNAs, but the rate of cleavage was extremely slow compared with that for the similarly sized R1.1 RNA and other dsRNAs (data no shown). Thus, for reasons that are not obvious, HIV-1 TAR RNA is inherently resistant to cleavage by both the Pac1 RNase and *E. coli* RNase III (Gunnery et al., 1990).

Pac1 RNase cleaves a hairpin structure in the *S. pombe* pre-rRNA 3′ETS

E. coli RNase III makes double-strand cuts in helices that sequester the large and small subunit RNAs in the pre-rRNA (Young & Steitz, 1978). Likewise, the *S. cerevisiae* Rnt1 RNase cleaves hairpin structures in the 5′ and 3′ ETS sequences of the pre-rRNA at or near mapped processing sites (Abou Elela et al., 1996). To test the hypothesis that the Pac1 RNase has a homologous function in fission yeast, we synthesized a 130-nt RNA derived from a part of the *S. pombe* 3′ETS that can be modeled as a large, imperfect hairpin (Fig. 2). The method of synthesis resulted in the incorporation of 1 nt at the 5′ end and 19 nt at the 3′ end that are not found in the *S. pombe* 3′ETS (see Materials and Methods). The 3′ end of the mature 25S RNA lies 9 nt upstream of the second G of the 3′ETS substrate. Melekhovets et al. (1994) mapped three putative in vivo processing sites within the hairpin (labeled A, B, and C in Fig. 2). If Pac1 cleaved our 3′ETS substrate at these sites, we would expect four products of 14, 20, 44, and 55 nt (see box at right of RNA in Fig. 2).

Upon incubation with the Pac1 RNase, the internally labeled 3′ETS RNA was converted to three predominant products, indicating two cleavages (Fig. 5A, lanes 2–4). The approximate sizes of the products relative to DNA markers—55 nt for the upper band, 45 nt for the middle band, and 35 nt for the lower band—were consistent with cuts near processing sites B and C. The largest band appeared to correspond to the expected 3′ product, the middle band to the upper hairpin (UHP) fragment, and the smallest band to the 5′ portion of the 3′ETS RNA (see Fig. 2). We confirmed these assignments by demonstrating that the cleavage products could serve as primer extension templates with the 3′, UHP, and 5′ primers shown in Figure 2. The sizes of the cDNAs obtained matched the sizes of the RNA products (data not shown). The cleavage pattern implied that the Pac1 RNase did not cut at processing site A (Fig. 2), which would have produced a fourth product of 14 nt. We confirmed this conclusion by Pac1 cleavage of a 5′-labeled substrate, which produced a ~35 nt product that co-migrated with the smallest fragment from cleavage of the internally labeled RNA (Fig. 5B).

Thus, the Pac1 RNase makes two specific cleavages in the *S. pombe* pre-rRNA 3′ETS substrate at or near two of the presumptive in vivo processing sites. In marked contrast, the Pac1 RNase did not recognize and accurately cleave the homologous *S. cerevisiae* substrate (Abou Elela et al., 1996), but instead degraded it into a heterogeneous collection of fragments (Fig. 5A, lanes 5–8). A reciprocal experiment in which the *S. pombe* 3′ETS was tested as a substrate for the Rnt1 RNase produced an analogous result (S. Abou Elela & M. Ares, pers. comm.). In addition, the *S. pombe pac1*$^+$ gene and the *S. cerevisiae RNT1* gene do not function in the heterologous yeast cells (S. Abou Elela, D. Frendewey, & M. Ares, unpubl. results). Therefore, despite their clearly homologous structures and the similarities of their 3′ETS substrates, the two yeast enzymes have different substrate recognition determinants.

To map precisely the Pac1 cleavage sites in the 3′ETS, we performed primer extension reactions with the 3′ and UHP primers (Fig. 2) on the purified 3′ and UHP products. The cDNAs produced by these reactions were

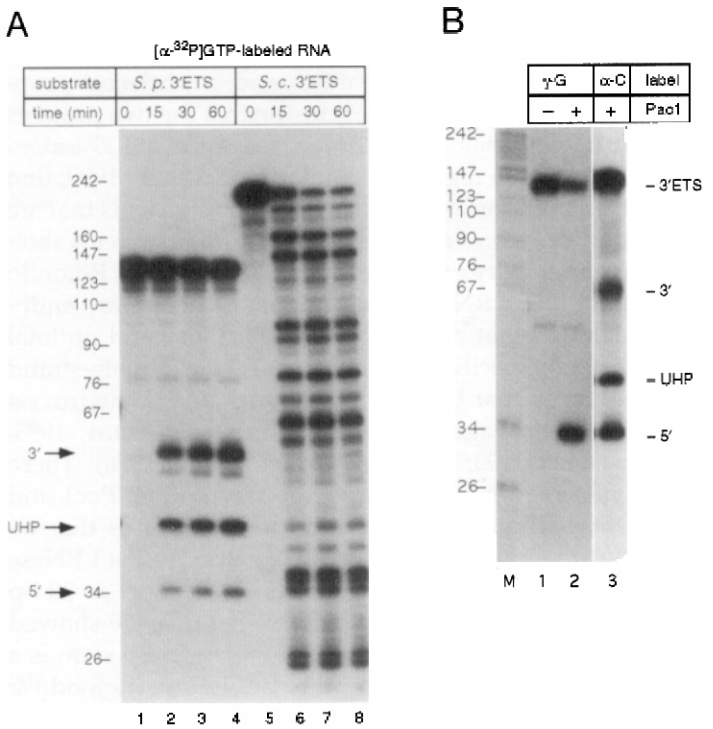

FIGURE 5. Cleavage of 3'ETS RNA by the Pac1 RNase. **A:** Comparison of the *S. pombe* and *S. cerevisiae* 3'ETS substrates. The 133-nt *S. pombe* 3'ETS RNA (lanes 1–4) and a 217-nt transcript containing the Rnt1 cleavage sites in the *S. cerevisiae* 3'ETS (lanes 5–8) were incubated with the Pac1 RNase under standard conditions plus polycytidylic acid for the times indicated above each lane. The [α-^{32}P]GTP-labeled RNAs were present at the following concentrations: *S.p.* 3'ETS, 100 nM; *S.c.* 3'ETS, 70 nM. One fourth of each reaction was heat-denatured and analyzed directly by electrophoresis on a 10% polyacrylamide/7 M urea gel. The positions of *Msp* I fragments of pBR322 co-electrophoresed as size markers are given on the left with their corresponding sizes in nucleotides. Arrows in the left panel indicate the positions of the 3', upper hairpin (UHP), and 5' cleavage products. **B:** Identification of the 5' cleavage product. 3'ETS RNA labeled at its 5' end with [γ-^{32}P]GTP was incubated at 16 nM without (lane 1) or with Pac1 RNase (lane 2) under standard conditions plus polycytidylic acid for 60 min and then analyzed by co-electrophoresis with the cleavage products of a Pac1 reaction with an [α-^{32}P]CTP-labeled *S. pombe* 3'ETS (at 100 nM, lane 3). Lanes 1 and 3 were not immediately adjacent in the original gel. Substrate and cleavage products are indicated in the right panel. Size markers as in Figure 1 with sizes given in nucleotides were run in lane M.

co-electrophoresed with DNA sequencing ladders generated with the same 5'-end-labeled primers on the PCR product used as the template for transcription of the 3'ETS RNA. Both primer extension reactions produced two products that differed by one nucleotide in length (Fig. 6, lanes 5 and 12). The lengths of the cDNAs extended from the UHP primer indicated two 5' ends at U35 and U34 for the UHP cleavage product. Similarly, extension of the 3' primer mapped two 5' ends for the 3' cleavage product at A78 and G77. The double 5' ends for the 3' and UHP products were inconsistent with the apparent homogeneity of the sizes of the cleavage products when analyzed directly (Fig. 5). Because we observed that primer extensions on the 3'ETS substrate RNA with either the UHP primer (Fig. 6, lane 6) or a primer complementary to the last 39 nt of the 3'ETS (data not shown) mapped the 5' end one nucleotide beyond the transcription initiation site, the upper bands of the doublets may have been the result of addition of an extra, nontemplated nucleotide.

To resolve whether the double 5' ends mapped by the primer extensions reflected true heterogeneity in the cleavage sites or were the result of a peculiarity in the reverse transcription reactions, we determined the 5' ends of the 3' and UHP products by direct nucleotide analysis of the RNAs. Digestion with RNases A, T1, and T2 of the Pac1 3' cleavage product of an [α-^{32}P]GTP-labeled 3'ETS RNA released a labeled nucleotide that co-chromatographed with an adenosine-3',5'-bisphosphate (pAp) standard by 2D-TLC (Fig. 7A). We did not detect pGp, which runs below and slightly to the right of Gp in the 2D-TLC system (Nishimura, 1979). PEI cellulose TLC confirmed that pAp was the only labeled bisphosphate nucleotide released from the 3' product (data not shown). Secondary digestion of the purified pAp product with nuclease P1 (Willis et al., 1986; Rotondo & Frendewey, 1996) released radioactive orthophosphate (P_i, Fig. 7C), indicating that the pAp was labeled at its 3' position by nearest neighbor transfer from G. These results indicate that Pac1 makes a single cut between G77 and A78, one nucleotide 5' of site C mapped by Melekhovets et al. (1994) (Fig. 2). Because the primer extension results predict two products with pG77p and pA78p 5' termini (Fig. 6, lane 12), the larger primer extension product appears to be an artifact.

An identical analysis was performed to map the other cleavage site. Complete digestion of the UHP product from Pac1 cleavage of an [α-^{32}P]CTP-labeled substrate produced a labeled pUp nucleotide (Fig. 7B). This established the 5' end of the UHP product at U35, in agreement with the shorter primer extension product (Fig. 6). To test for a second UHP product with a 5' end at U34, as indicated by the primer extension mapping (Fig. 6, lane 5), we performed a 5'-end-group analysis on an [α-^{32}P]UTP-labeled UHP product. If Pac1 cleavage of the 3'ETS RNA produced a UHP product with a 5' end at U34, then total digestion should release a pUp in which both phosphates are labeled. 2D-TLC analysis of the digestion products revealed a labeled pUp spot, which, upon purification and secondary digestion with nuclease P1, was partially converted to uridine-5'-monophosphate; labeled P_i was not released (Fig. 7C). These results establish a single 5' cleavage at

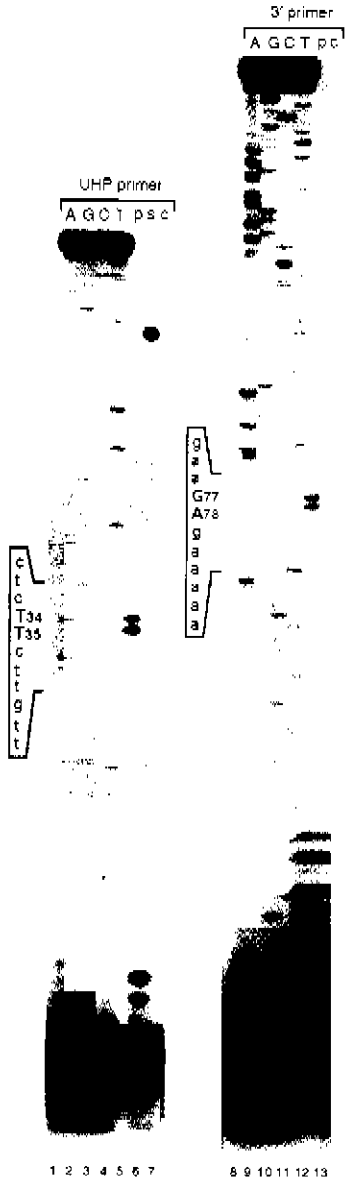

FIGURE 6. Primer extension mapping of the Pac1 RNase cleavage sites on the 3'ETS RNA. Reverse transcription reactions were performed with the purified UHP (lane 5) and 3' products (lane 12) shown in Figure 5A as templates. The UHP and 3' primers (see Fig. 2) were used with their complementary products. Letters above lanes 5–7, 12, and 13 refer to the templates: either a cleavage product (p, lanes 5 and 12), the 3'ETS substrate (s, lane 6), or *E. coli* tRNA as a control (c, lanes 7 and 13). (The 3' primer was not able to extend from the 3'ETS substrate RNA.) Primer extension reactions were electrophoresed alongside DNA sequencing ladders generated with the same end-labeled primers used in the primer extension reactions: UHP primer, lanes 1–4; 3' primer, lanes 8–11. Complementary sequences surrounding the ends of the extension products are given on the left of each panel, with the mapped 5' ends of the UHP and 3' cleavage products identified by numbered upper-case nucleotides (refer to Fig. 2).

the in vivo processing site B (Melekhovets et al., 1994) between U34 and U35. In summary, the Pac1 RNase cleaves the 3'ETS RNA between U34 and U35 and between G77 and G78, as indicated by the purple arrows in Figure 2.

DISCUSSION

The *S. pombe* Pac1 RNase and *E. coli* RNase III share a number of structural features and biochemical properties that support the conclusion that the two proteins are homologous. Their primary sequences are 25% identical over the regions of similarity (Xu et al., 1990; Iino et al., 1991), and mutations in two amino acids that are absolutely conserved in all RNase III-like proteins abolish the activity of both enzymes (Court, 1993; Rotondo et al., 1995). RNase III and the Pac1 RNase are endonucleases that require Mg^{2+} for activity and optimal cleavage specificity, and both execute double-strand cleavages that leave 5' phosphates and 3' hydroxyls on the cleavage products (Dunn, 1982; Court, 1993; Li et al., 1993; Rotondo & Frendewey, 1996). These similarities extend to substrate recognition. Pac1 and RNase III are double-strand-specific RNases that require a similar minimal length of helix. The Pac1 RNase efficiently cleaved simple dsRNAs, as short as 30 bp but failed to cut a 17-bp dsRNA. Because we showed previously that a hairpin RNA with a 25-bp stem is a very efficient substrate for the Pac1 RNase (Rotondo & Frendewey, 1996), Pac1's minimal substrate length is in the range of 17–25 bp, which is essentially the same as *E. coli* RNase III (Dunn, 1982; Robertson, 1982). This size restriction could reflect the binding properties of the dsRNA-binding domain found at the C-terminus of all members of the RNase III family. Double-stranded RNA-binding domains bind one turn of A-form RNA helix, approximately 11 bp (Manche et al., 1992; Bycroft et al., 1995; Bevilacqua & Cech, 1996). *E. coli* RNase III binds its substrate as a symmetrical dimer on either side of the cleavage sites (Li & Nicholson, 1996), an arrangement that rationalizes the requirement for a helix whose size is approximately two helical turns.

E. coli RNase III usually makes two staggered cuts in both strands of its dsRNA substrates, but, in rare instances, the enzyme will cleave once in a single-stranded bulge within a helix (Robertson, 1982). An example of such a cleavage occurs in the R1.1 processing site between mRNAs of the polycistronic T7 phage early transcript (Dunn & Studier, 1973). RNase III cleaves a single phosphodiester bond in the 3' half of the asymmetric bulge of the R1.1 processing signal (Nicholson et al., 1988). Remarkably, we found that the Pac1 RNase cuts the R1.1 RNA at the same site. Thus, the *S. pombe* enzyme has conserved the ability to recognize the structural signature in R1.1 that directs a single-strand cleavage at a defined site. If the bulge in the R1.1 RNA is closed with Watson-Crick base pairs, Pac1 is no longer able to recognize the R1.1 site, but instead makes a double-strand cut closer to the base of the stem (Rotondo & Frendewey, 1996, and unpubl. results). *E. coli* RNase III, on the other hand, retains the ability to cleave at the in vivo processing site in the closed-helix form of the R1.1 RNA (Chelladurai et al.,

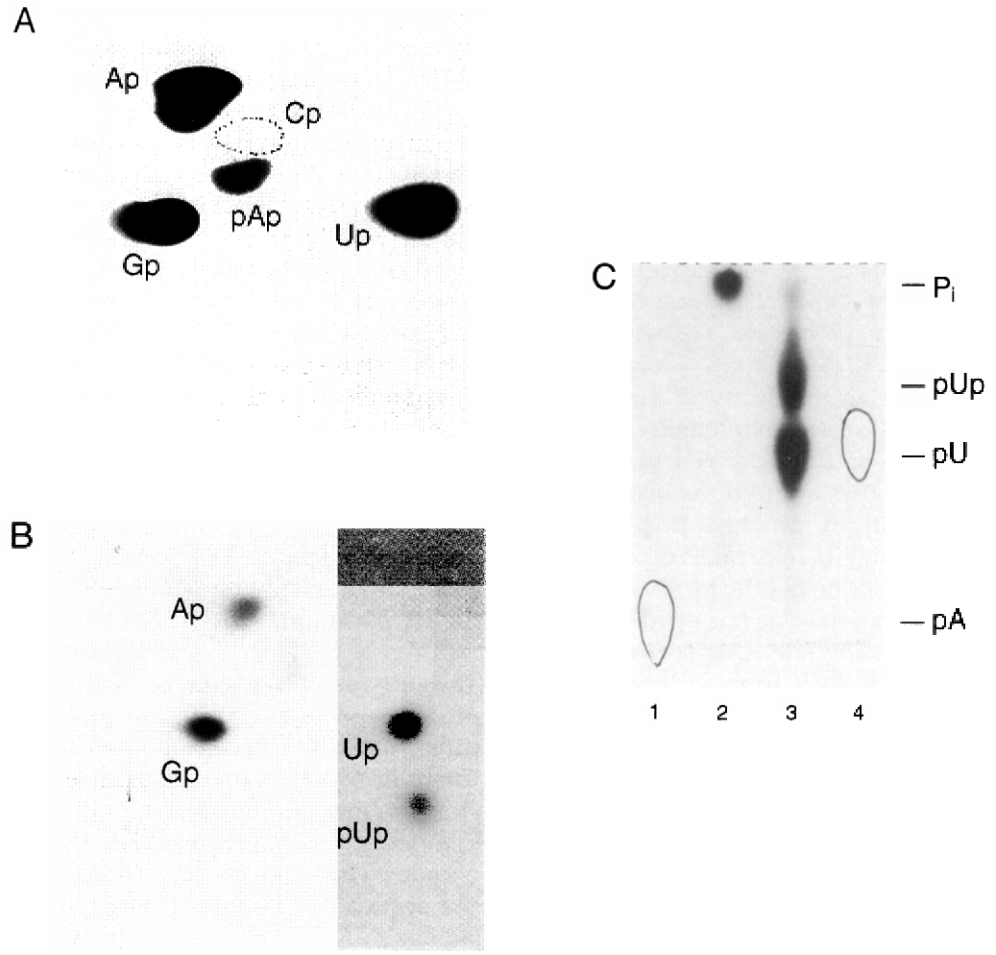

FIGURE 7. 5′ End group analysis of Pac1 RNase cleavage products. Gel-purified products were digested to completion with RNases A, T1, and T2; nucleotides were separated by cellulose 2D-TLC (**A,B**) and visualized by autoradiography. Radioactive spots are identified as nucleoside-3′-monophosphates (e.g., Cp) or nucleoside-3′,5′-bisphosphates (e.g., pAp). **A:** 3′ Cleavage product of an [α-^{32}P]GTP-labeled 3′ETS RNA. The position of an unlabeled Cp standard is shown to indicate its separation from the radioactive pAp spot. **B:** UHP cleavage product from an [α-^{32}P]CTP-labeled 3′ETS RNA. **C:** Secondary analysis. The pAp spot (panel A) from the [α-^{32}P]GTP-labeled 3′ETS 3′ cleavage product (lane 2) and the pUp spot (not shown) from an [α-^{32}P]UTP labeled 3′ETS UHP product (lane 3) were purified, digested with nuclease P1, and the digestion products separated by cellulose TLC in the second dimension solvent. Unlabeled AMP and UMP standards were run in lanes 1 and 4, respectively. Radioactive products and unlabeled standards are identified on the right: pU and pA, nucleoside-5′-monophosphates; P_i, orthophosphate. Dashed line at top indicates the solvent front.

1993). These results illustrate the somewhat different mechanisms for substrate recognition or cleavage positioning employed by the yeast and bacterial enzymes.

This difference is also seen in the action of the two enzymes on adenovirus VA RNA_I and HIV-1 TAR RNA. Both of these modified hairpin RNAs are recognized by proteins that possess dsRNA-binding domains (Mathews & Shenk, 1991; Gatignol et al., 1993), but neither is a substrate for *E. coli* RNase III (Gunnery et al., 1990; Mellits et al., 1990). In contrast, VA RNA_I, despite its complex tertiary structure (Ma & Mathews, 1996), was cut efficiently at multiple sites by the Pac1 RNase. The terminal and apical stems of VA RNA_I each contain at least 20 bp of uninterrupted helix that are likely to offer sites for Pac1 cleavage. HIV-1 TAR RNA, on the other hand, was completely refractory to cleavage by the Pac1 RNase. The inability of Pac1 to cut TAR RNA persisted over a range of monovalent and divalent cation concentrations and pH (data not shown). Unlike *E. coli* RNase III (Dunn, 1976), we have found that the cleavage site preference of the Pac1 RNase for any substrate we have tested is not affected by changes in ionic conditions (with the exception of replacing Mg^{2+} with Mn^{2+}). Rather, the enzyme is most active at low ionic strength and inhibited gradually at higher salt concentrations (Rotondo & Frendewey, 1996). The inability of Pac1 to cleave TAR RNA is not explained entirely by the three asymmetrical bulges that interrupt its hairpin structure and permit at the most only 11 bp of uninterrupted helix. Removal of the bulged nucleotides, which was expected to result in a perfectly helical hairpin RNA with a 24-bp stem, allowed only a very inefficient cleavage by the

Pac1 RNase (data not shown). This result is perplexing given that a very similar hairpin RNA, the closed-helix form of R1.1 RNA, is an excellent Pac1 substrate (Rotondo & Frendewey, 1996). The bulge-less TAR RNA appears to assume a shape that prevents efficient recognition by the Pac1 RNase. The peculiar structure of the HIV-1 TAR RNA may have evolved as a protection against destruction by RNase III-like dsRNases.

E. coli RNase III and its *S. cerevisiae* homologue, the Rnt1 RNase, catalyze the initial cleavages in the pre-rRNA processing pathway (Robertson, 1982; Abou Elela et al., 1996). The Rnt1 RNase makes double-strand breaks in two hairpin elements in the 5′ and 3′ ETS regions of the pre-rRNA. In a similar manner, the Pac1 RNase cleaved a synthetic RNA derived from the *S. pombe* 3′ETS at or near two of the three in vivo processing sites in the hairpin structure (Melekhovets et al., 1994). One of the in vitro cleavages occurs at the exact position assigned for one of the processing sites, whereas the other Pac1 cut lies one nucleotide to the 5′ side of a second processing site. Our sequencing of 3′ETS DNA amplified by PCR from a cloned rDNA repeat revealed an additional A, in the long stretch of A's in the apical loop, relative to the sequence reported by Melekhovets et al. (1994). Since these authors mapped the in vivo processing sites by S1 nuclease protection against a sequencing ladder, the extra A could account for the one-nucleotide disparity with our results. Because the Pac1 RNase, like RNase III, makes double-stranded cuts that are staggered by 2 bp (D. Frendewey, unpubl. results), we believe the in vitro cleavages reflect the true in vivo processing sites, rather than the 3-bp stagger of the B and C sites of Melekhovets et al. (1994). The close correspondence between the in vitro cleavages and the in vivo processing sites strongly implies that the Pac1 RNase is the enzyme responsible for these cleavages in *S. pombe* cells. Given its homology with the Rnt1 RNase, we also expect that Pac1 will cleave at sites within the 5′ETS, although these processing sites have not yet been mapped in fission yeast.

The Pac1 RNase did not cut at in vivo processing site A (Fig. 2), which lies 21-nt downstream of the mature end of the 25S RNA. This region of the 3′ETS hairpin contains three asymmetric bulges that might prevent Pac1 cleavage. This cleavage specificity may reflect Pac1's true in vivo behavior, or it could be a consequence of improper folding of our artificial substrate, which might also explain the poor cleavage efficiency of the 3′ETS substrate compared with the R1.1 RNA. Alternatively, because U8 RNA has been shown to be required for 3′ETS cleavage in *Xenopus* oocytes (Peculis & Steitz, 1993), the Pac1 RNase might require a small nucleolar ribonucleoprotein cofactor for optimal activity, substrate recognition, or cleavage specificity, perhaps by promoting proper folding of the 3′ETS.

The *S. cerevisiae* and *S. pombe* RNase III homologues have conserved the bacterial enzyme's function in pre-rRNA processing, but the eukaryotic RNase III enzymes are likely to have other functions. Inactivation of Rnt1p in *S. cerevisiae* causes growth arrest long before mature rRNA is depleted (Abou Elela et al., 1996), which suggests at least one other essential function for the enzyme. Our isolation of the *pac1*$^+$ gene as an extragenic, multi-copy suppressor of the *snm1* mutant, which maintains reduced levels of several snRNAs, suggested a role for the Pac1 RNase in snRNA synthesis (Potashkin & Frendewey, 1990; Rotondo et al., 1995). This prediction has been supported recently by the observation that the Rnt1 RNase is required for U5 snRNA processing in *S. cerevisiae* (Chanfreau et al., 1997). The ability of *pac1*$^+$ to cause sterility when overexpressed in *S. pombe* suggests a role in sexual development (Xu et al., 1990; Iino et al., 1991; Rotondo et al., 1995). In an intriguing parallel, RNase III cleavage of the R1 plasmid gene 19 mRNA controls the conjugal transfer of DNA in *E. coli* (Koraimann et al., 1993). Thus, RNase III-like enzymes may play a role in sexual processes in both prokaryotic and eukaryotic organisms. We found a second RNase III-like open reading frame in *S. pombe* that maps near the *ste1* locus on chromosome I (Hoheisel et al., 1993; Rotondo & Frendewey, 1996). This unusual orf has the potential to encode a 1,374-amino acid protein having an RNase III-like sequence at its carboxyl terminus and a sequence similar to RNA helicases in its large amino-terminal domain. A homologous orf exists in the *Caenorhabditis elegans* genome (Rotondo et al., 1995; Rotondo & Frendewey, 1996). The characterization of these enzymes and the identification of their substrates should reveal novel and important functions for RNase III family members in cellular growth and differentiation.

MATERIALS AND METHODS

Materials

The Pac1 RNase used for all experiments carried polyhistidine and epitope tags at its N-terminus and was purified as described previously (Rotondo & Frendewey, 1996). Purified *E. coli* RNase III (Li et al., 1993) and an oligodeoxynucleotide template for the in vitro transcription of R1.1 RNA (Chelladurai et al., 1993) were the generous gifts of A. Nicholson (Wayne State, Detroit). The pT7VA and pEM-7 plasmids for the synthesis of adenovirus VA RNA$_I$ and HIV-1 TAR RNA, respectively, were kindly provided by M. Mathews (UMDNJ—New Jersey Medical School, Newark). A clone of the 10.4-kb *Hind* III fragment containing an entire *S. pombe* rDNA repeat (Schaack et al., 1982) was the generous gift of M. Yanagida (Kyoto, Japan). Oligodeoxynucleotides were synthesized by the NYU Pathology oligo service and Oligos Etc. (Wilsonville, Oregon). The following materials are listed with their suppliers: pBluescript-SK(−), Stratagene; ^{32}P-labeled nucleotides,

New England Nuclear; mono- and diphosphate nucleotide markers, Sigma; nucleoside triphosphates, Pharmacia; RNases A and T2 and nuclease P1, Calbiochem; RNase T1 and bacteriophage T3 and T7 RNA polymerases, Ambion; DNase I, Promega; shrimp alkaline phosphatase, United States Biochemical; AmpliTaq DNA polymerase, Perkin-Elmer Cetus; DNA restriction endonucleases, T4 polynucleotide kinase, CircumVent thermal cycle sequencing kit, and DNA size markers (pBR322 digested with *Msp* I), New England Biolabs; SuperScript II RNase H$^-$ reverse transcriptase, Gibco BRL; cellulose TLC plates, Kodak; PEI-cellulose TLC plates, Selecto Scientific (Norcross, Georgia); autoradiographic film, Kodak and Fuji.

Synthesis of RNA substrates

Double-stranded RNAs of various lengths were prepared according to Manche et al. (1992). Briefly, the *Pvu* II fragment of pBS-SK(−) containing the opposing T3 and T7 promoters separated by the polylinker region was digested in separate reactions with *Apa* I, *Xho* I, *Eco*R I, *Bam*H I, and *Not* I, and the template DNAs were purified by the Gene Clean procedure (Bio 101). High specific radioactivity (approximately 125 Ci/mmol) [α-^{32}P]UTP-labeled RNAs were transcribed (Rotondo & Frendewey, 1996) from both the T3 and T7 promoters of the *Pvu* II template and from the T7 promoters of the smaller templates. The T3 transcript from the *Pvu* II template was annealed to each of the T7 transcripts and the unpaired ends removed by digestion with RNases A and T1. The hybrids were gel-purified (Saccomanno & Bass, 1994) and stored in diethyl pyrocarbonate-treated distilled water at −20 °C.

R1.1 RNA was synthesized from partially double-stranded oligodeoxynucleotide templates (Milligan & Uhlenbeck, 1989) in low specific radioactivity (approximately 1–2 Ci/mmol) reactions. VA RNA$_I$ was transcribed from pT7VA digested with *Dra* I (Mellits et al., 1990). TAR RNA was transcribed from pEM-7 digested with *Afl* II (Gunnery et al., 1990) or from oligodeoxynucleotide templates consisting of a T7 promoter fused to sequences complementary to TAR RNA or the deletion derivatives described in Results. The complementary T7 promoter strand for these templates was 5′-TAATACGACTCACTATAG-3′. The template for the transcription of the *S. pombe* 3′ETS RNA was produced by PCR amplification of a portion of the 3′ETS genomic region (Melekhovets et al., 1994) with the following primers: T7-3′ETS, 5′-taatacgactcactata·gGGAACCATCATCTTATTTCTT-3′; and T3-3′ETS, 5′-aattaaccctcactaaa·ggCTTCATCTCTTCTTTCT TTC-3′. Lower-case letters indicate the T7 or T3 promoters with the transcription initiation sites preceded by dots; uppercase letters are rDNA sequences. The PCR product was gel-purified (Frendewey et al., 1990). The template for the synthesis of the *S. cerevisiae* 3′ETS RNA (generous gift of S. Abou Elela and M. Ares) was a plasmid linearized by cleavage with *Eco*R I. This template produced a run-off transcript of 217 nt that contains the 3′ end of the 25S rRNA and part of the 3′ETS (Abou Elela et al., 1996). Internally labeled radioactive *S. pombe* or *S. cerevisiae* 3′ETS RNAs were transcribed from the T7 promoter in the presence of [α-^{32}P]-nucleotide triphosphates. 5′-End-labeled *S. pombe* 3′ETS RNA was produced in a transcription reaction that incorporated [γ-^{32}P]-GTP as the only labeled nucleotide. All radioactive RNA substrates were purified and quantified as described previously (Rotondo & Frendewey, 1996).

dsRNase Assays

Pac1 RNase reactions were performed as described previously (Rotondo & Frendewey, 1996). Unless otherwise indicated, standard conditions were 30 mM CHES, pH 8.5, 5 mM $MgCl_2$, 1 mM DTT, and 8 nM Pac1 at 30 °C in a volume of 10 μL. Polycytidylic acid, which stimulates the activity of the Pac1 RNase (Rotondo & Frendewey, 1996), was included at 1 μM in some reactions as indicated in the figure legends. Reactions were stopped by dilution with an equal volume of 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 12 mM EDTA, and analyzed directly by electrophoresis on polyacrylamide (19:1, acrylamide: bisacrylamide)/7 M urea gels run in 1× Tris-borate-EDTA buffer (Sambrook et al., 1989) at the gel percentages indicated in the figure legends. The products were detected by autoradiography at −70 °C with an intensifying screen. Double-stranded substrates (Fig. 1) were applied directly to the gels (no heat-denaturation) and electrophoresed at low voltage to maintain the temperature near ambient. This procedure did not disrupt the dsRNA hybrids despite the urea in the gel (Saccomanno & Bass, 1994; Rotondo & Frendewey, 1996). DNA markers were run in the same manner so that dsRNA sizes were compared to dsDNA standards. Single-stranded RNAs and their accompanying DNA markers were heat-denatured at 75 or 95 °C immediately prior to electrophoresis. ^{32}P-labeled DNA size markers (*Msp* I-digested pBR322) were prepared by dephosphorylation with shrimp alkaline phosphatase, according to the manufacturer's instructions, followed by re-phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989).

Cleavage site mapping

The 5′ ends of Pac1 cleavage products were analyzed as previously described (Rotondo & Frendewey, 1996). Radioactive nucleotides were separated by two-dimensional cellulose TLC (Nishimura, 1972) or by one-dimensional PEI-cellulose TLC in 1.75 M ammonium formate, pH 3.5 (Randerath et al., 1980), or 1 M LiCl (Greer, 1994). 3′- or 5′-Monophosphate and 3′,5′-bisphosphate nucleotides were identified by co-chromatography with unlabeled markers (visualized by ultraviolet absorbance) or by comparison with published TLC maps (Nishimura, 1979). To map the Pac1 cleavage sites on the 3′ETS RNA by primer extension, we performed reverse transcription reactions on gel-purified cleavage products at 45 °C with SuperScript II reverse transcriptase according to the manufacturers instructions for first-strand cDNA synthesis. Five micrograms of *E. coli* tRNA were included in all reactions. Primers were labeled at their 5′ ends with [γ-^{32}P]-ATP and T4 polynucleotide kinase (Sambrook et al., 1989). The 3′ cleavage site was mapped by extension of a primer (5′-ATTAACCCTCACTAAAGG-3′) complementary to the 3′ end of the 3′ cleavage product, whereas the 5′ cleavage site was mapped by extension of a primer (5′-CTTCGAATTAGCAAGTC-3′) complementary to the 3′ end of the upper hairpin cleavage product (see Fig. 2). The 3′ ends of the primer extension products were determined by co-electrophoresis alongside DNA sequencing lad-

ders of the 3'ETS PCR product generated by thermal cycle sequencing with Vent (exo$^-$) DNA polymerase according to the manufacturer's instructions and with the same labeled primers used in the primer extension reactions. To avoid mapping errors, an effort was made to match the ionic conditions of the primer extension samples to those of the DNA sequencing reactions prior to electrophoresis. A primer (5'-AGAGAAAAATGAAAG-3') complementary to the 3' end of the 5' cleavage product (see Fig. 2) was used to confirm the identity and size of the product by primer extension.

ACKNOWLEDGMENTS

We thank A. Nicholson for the generous gift of purified *E. coli* RNase III and for oligodeoxynucleotide templates for the synthesis of the R1.1 RNA. We are grateful to S. Abou Elela and M. Mathews for providing templates for the synthesis of *S. cerevisiae* 3'ETS RNA, adenovirus VA RNA$_I$, and HIV-1 TAR RNA. We appreciate the advice and suggestions of M. Ares concerning the 3'ETS experiments and the information provided prior to publication by G. Chanfreau. This work was supported by the New York University Medical Center.

Received July 3, 1997; returned for revision July 30, 1997; revised manuscript received August 7, 1997

REFERENCES

Abou Elela S, Igel H, Ares M Jr. 1996. RNase III cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site. *Cell 85*:115–124.

Bevilacqua PC, Cech TR. 1996. Minor-groove recognition of double-stranded RNA by the double-stranded RNA-binding domain from the RNA-activated protein kinase PKR. *Biochemistry 35*:9983–9994.

Blomberg P, Wagner EGH, Nordström K. 1990. Control of replication of plasmid R1: The duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically in vivo and in vitro by RNase III. *EMBO J 9*:2331–2340.

Bycroft M, Grünert S, Murzin AG, Proctor M, St. Johnston D. 1995. NMR solution structure of a dsRNA binding domain from *Drosophila* staufen protein reveals homology to the N-terminal domain of ribosomal protein S5. *EMBO J 14*:3563–3571.

Chanfreau G, Abou Elela S, Ares M Jr., Guthrie C. 1997. A role for RNase III in yeast snRNA 3'-end processing. *Genes Dev* (in press).

Chelladurai B, Li H, Zhang K, Nicholson AW. 1993. Mutational analysis of a ribonuclease III processing signal. *Biochemistry 32*:7549–7558.

Court D. 1993. RNA processing and degradation by RNase III. In: Brawerman G, Belasco J, eds. *Control of mRNA stability*. New York: Academic Press. pp 70–116.

Crouch RJ. 1974. Ribonuclease III does not degrade deoxyribonucleic acid-ribonucleic acid hybrids. *J Biol Chem 249*:1314–1316.

Cullen BR. 1993. Does HIV-1 Tat induce a change in viral initiation rights? *Cell 73*:417–420.

Dunn JJ. 1976. RNase III cleavage of single-stranded RNA. Effect of ionic strength on the fidelity of cleavage. *J Biol Chem 251*:3807–3814.

Dunn JJ. 1982. Ribonuclease III. In: Boyer PD, ed. *The enzymes, vol 15, part B*. New York: Academic Press. pp 485–499.

Dunn JJ, Studier FW. 1973. T7 early RNAs and *Escherichia coli* ribosomal RNAs are cut from large precursor RNAs in vivo by ribonuclease III. *Proc Natl Acad Sci USA 70*:3296–3300.

Frendewey D, Barta I, Gillespie M, Potashkin J. 1990. *Schizosaccharomyces* U6 genes have a sequence within their introns that matches the B box consensus of tRNA internal promoters. *Nucleic Acids Res 18*:2025–2032.

Gatignol A, Buckler C, Jeang KT. 1993. Relatedness of an RNA-binding motif in human immunodeficiency virus type 1 TAR RNA-binding protein TRBP to human P1/dsI kinase and *Drosophila* Staufen. *Mol Cell Biol 13*:2193–2202.

Gerdes K, Nielsen A, Thorsted P, Wagner EG. 1992. Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable hok, srnB and pndA effector messenger RNAs. *J Mol Biol 226*:637–649.

Greer CL. 1994. Processing of transfer RNA precursors. In: Higgins SJ, Hames BD, eds. *RNA processing, a practical approach, vol II*. Oxford: IRL Press. pp 173–209.

Grummt I, Hall SH, Crouch RJ. 1979. Localisation of an endonuclease specific for double-stranded RNA within the nucleolus and its implication in processing ribosomal transcripts. *Eur J Biochem 94*:437–443.

Gunnery S, Rice AP, Robertson HD, Mathews MB. 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. *Proc Natl Acad Sci USA 87*:8687–8691.

Hoheisel JD, Maier E, Mott R, McCarthy L, Grigoriev AV, Schalkwyk LC, Nizetic D, Francis F, Lehrach H. 1993. High resolution cosmid and P1 maps spanning the 14 Mb genome of the fission yeast *S. pombe*. *Cell 73*:109–120.

Iino Y, Sugimoto A, Yamamoto M. 1991. *S. pombe pac1*$^+$, whose overexpression inhibits sexual development, encodes a ribonuclease III-like RNase. *EMBO J 10*:221–226.

Kessler M, Gunnery S, Laspia MF, Rice AP, Mathews MB. 1991. The role of the TAR region and Tat protein in HIV-directed gene expression. In: Haselstine WA, Wong-Staal F, eds. *Genetic structure and regulation of HIV*. New York: Raven Press. pp 75–96.

Kharrat A, Macias MJ, Gibson TJ, Nilges M, Pastore A. 1995. Structure of the dsRNA binding domain of *E. coli* RNase III. *EMBO J 14*:3572–3584.

Koraimann G, Schroller C, Graus H, Angerer D, Teferle K, Hogenauer G. 1993. Expression of gene 19 of the conjugative plasmid R1 is controlled by RNase III. *Mol Microbiol 9*:717–727.

Li HL, Chelladurai BS, Zhang K, Nicholson AW. 1993. Ribonuclease III cleavage of a bacteriophage T7 processing signal. Divalent cation specificity, and specific anion effects. *Nucleic Acids Res 21*:1919–1925.

Li H, Nicholson AW. 1996. Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. *EMBO J 15*:1421–1433.

Ma Y, Mathews MB. 1996. Secondary and tertiary structure in the central domain of adenovirus type 2 VA RNA$_I$. *RNA 2*:937–955.

Manche L, Green SR, Schmedt C, Mathews MB. 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol 12*:5238–5248.

Mathews MB, Shenk T. 1991. Adenovirus virus-associated RNA and translation control. *J Virol 65*:5657–5662.

Melekhovets YF, Good L, Abou Elela S, Nazar RN. 1994. Intragenic processing in yeast rRNA is dependent on the 3' external transcribed spacer. *J Mol Biol 239*:170–180.

Mellits KH, Pe'ery T, Manche L, Robertson HD, Mathews MB. 1990. Removal of double-stranded contaminants from RNA transcripts: Synthesis of adenovirus VA RNA$_I$ from a T7 vector. *Nucleic Acids Res 18*:5401–5406.

Milligan JF, Uhlenbeck OC. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol 180*:51–62.

Nicholson AW. 1996. Structure, reactivity, and biology of double-stranded RNA. *Prog Nucleic Acids Res Mol Biol 52*:1–65.

Nicholson AW, Niebling KR, McOsker PL, Robertson HD. 1988. Accurate in vitro cleavage by RNase III of phosphorothioate-substituted RNA processing signals in bacteriophage T7 early mRNA. *Nucleic Acids Res 16*:1577–1591.

Nishimura S. 1972. Minor components in transfer RNA: Their characterization, location, and function. *Prog Nucleic Acids Res Mol Biol 12*:49–85.

Nishimura S. 1979. Chromatographic mobilities of modified nucleotides. In: Schimmel P, Söll D, Abelson J, eds. *Transfer RNA: Structure, properties, and recognition*. New York: Academic Press. pp 551–552.

Ohtsuki K, Groner Y, Hurwitz J. 1977. Isolation and purification of double-stranded ribonuclease from calf thymus. *J Biol Chem 252*:483–491.

Peculis BA, Steitz JA. 1993. Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell 73*:1233–1245.

Potashkin J, Frendewey D. 1990. A mutation in a single gene of *Schizosaccharomyces pombe* affects the expression of several snRNAs and causes defects in RNA processing. *EMBO J 9*:525–534.

Randerath K, Gupta RC, Randerath E. 1980. ^{3}H and ^{32}P derivative methods for base composition and sequence analysis of RNA. *Methods Enzymol 65*:638–680.

Robertson HD. 1982. *Escherichia coli* ribonuclease III cleavage sites. *Cell 30*:669–672.

Robertson HD, Dunn JJ. 1975. Ribonucleic acid processing activity of *Escherichia coli* ribonuclease III. *J Biol Chem 250*:3050–3056.

Robertson HD, Webster RE, Zinder ND. 1968. Purification and properties of ribonuclease III from *Escherichia coli*. *J Biol Chem 243*:82–91.

Rotondo G, Frendewey D. 1996. Purification and characterization of the Pac1 ribonuclease of *Schizosaccharomyces pombe*. *Nucleic Acids Res 24*:2377–2386.

Rotondo G, Gillespie M, Frendewey D. 1995. Rescue of the fission yeast snRNA synthesis mutant *snm1* by overexpression of the double-strand-specific Pac1 ribonuclease. *Mol Gen Genet 247*:698–708.

Saccomanno L, Bass BL. 1994. The cytoplasm of *Xenopus* oocytes contains a factor that protects double-stranded RNA from adenosine-to-inosine modification. *Mol Cell Biol 14*:5425–5432.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual, 2nd ed.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schaak J, Mao J, Söll D. 1982. The 5.8S RNA gene sequence and the ribosomal repeat of *Schizosaccharomyces pombe*. *Nucleic Acids Res 10*:2851–2864.

Schweisguth DC, Chelladurai BS, Nicholson AW, Moore PB. 1994. Structural characterization of a ribonuclease III processing signal. *Nucleic Acids Res 22*:604–612.

Schweitz H, Ebel JP. 1971. A study of the mechanism of action of *E. coli* ribonuclease III. *Biochimie 53*:585–593.

Willis I, Frendewey D, Nichols M, Hottinger-Werlen A, Schaack J, Söll D. 1986. A single base change in the intron of a serine tRNA affects the rate of RNase P cleavage in vitro and suppressor activity in vivo in *S. cerevisiae*. *J Biol Chem 261*:5878–5885.

Xu HP, Riggs M, Rodgers L, Wigler M. 1990. A gene from *S. pombe* with homology to *E. coli* RNase III blocks conjugation and sporulation when overexpressed in wild type cells. *Nucleic Acids Res 18*:5304.

Young RA, Steitz JA. 1978. Complementary sequences 1700 nucleotides apart form a ribonuclease III cleavage site in *Escherichia coli* ribosomal precursor RNA. *Proc Natl Acad Sci USA 75*:3593–3597.