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The DNase activity of RNase T and its application to DNA cloning

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ABSTRACT

RNase T is one of eight distinct 3′→5′ exoribonucleases present in *Escherichia coli*. The enzyme plays an important role in stable RNA metabolism, including tRNA end turnover and 3′ maturation of most stable RNAs because it is the only RNase that can efficiently remove residues near a double-stranded (ds) stem. In the course of study of its specificity and mechanism, we found that RNase T also has single-strand-specific DNase activity. Purified RNase T degrades both single-stranded (ss)RNA and ssDNA in a non-processive manner. However, in contrast to its action on RNA, RNase T binds ssDNA much more tightly and shows less sequence specificity. As with RNA, DNA secondary structure strongly affects its degradation by RNase T. Thus, RNase T action on a dsDNA with a single-stranded 3′-extension efficiently generates blunt-ended DNA. This property of RNase T suggested that it might be a useful enzyme for blunt-ended DNA cloning. We show here that RNase T provides much higher cloning efficiency than the currently used mung bean nuclease.

INTRODUCTION

RNase T is one of eight distinct 3′→5′ exoribonucleases that have been identified in *Escherichia coli* (1). It belongs to a large exonuclease superfamily characterized by a common motif consisting of four invariant acidic residues (2,3), which in DNA polymerase were shown to form the exonuclease activity site (4,5). RNase T plays an important role in stable RNA metabolism, including tRNA end turnover (6) and 3′ maturation of most stable RNAs (7–9). Although multiple RNases contribute to many of these processes, RNase T is the only enzyme that can efficiently remove residues near the double-stranded (ds) stem present in most stable RNAs.

Recently, Viswanathan *et al*. (10) reported that RNase T has 3′→5′ DNase activity against single-stranded (ss)DNA, and that in high copy it can suppress the UV repair defects of an *E.coli* mutant deficient in the ssDNA exonucleases, RecJ exonuclease, exonuclease I and exonuclease VII (11). Independently, in a study of RNase T specificity and mechanism, we have also observed this single-strand-specific DNase activity.

In this paper, we confirm and extend the observations of Viswanathan *et al*. (10). We show that purified RNase T degrades ssDNA very efficiently, but under certain conditions, it is also able to completely degrade double-stranded (ds)DNA. Moreover, the action of RNase T on dsDNA with single-strand 3′-extensions can be controlled such that predominantly blunt-ended DNA is generated. Application of this property of RNase T to blunt-end DNA cloning has proven more efficient than currently available procedures.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase and mung bean nuclease were obtained from New England Biolabs. Calf intestine alkaline phosphatase was purchased from Promega. [γ-^{32}P]ATP (6000 Ci/mmol) was from Dupont-New England Nuclear. Sequagel, for single nucleotide resolution analysis, was purchased from National Diagnostics. Supercompetent cells were products of Invitrogen. RNase T was kindly provided by Dr Zhongwei Li, and was overexpressed and purified from *E.coli* cells as previously described (12,13).

Preparation of single-strand substrates

All the oligonucleotides used for activity analyses were 17 nt in length. These included the homopolymers, dA_{17}, dT_{17} and dC_{17}, and Oligo1 [d(5′-CCCCACCACCATCACTT-3′)], Oligo2 [d(5′-AAGTGATGGTGGTGGGG-3′)], and an RNA oligonucleotide, Oligo3 [(5′-CCCCACCACCAUCACUU-3′)], with the same sequence as Oligo1. Oligo3 was deprotected by TBA/THF and was gel purified. DNA oligonucleotides were either gel purified or were used directly. They were quantitatively phosphorylated at their 5′-ends using [γ-^{32}P]ATP and polynucleotide kinase. The labeled oligonucleotides were purified by passage through a G25 spin column after first boiling for 10 min or after phenol/chloroform treatment.

Preparation of dsDNA substrates

dsDNA substrates were prepared from plasmid pUC19 DNA by a series of restriction digestions, dephosphorylations and 5′-^{32}P-labeling. Thus, to prepare dsDNA with 3′ extensions, pUC19 DNA was first digested with *Pvu*II and dephosphorylated. The *Pvu*II fragments were isolated on an agarose gel and ^{32}P-labeled at their 5′-ends. The labeled fragments were

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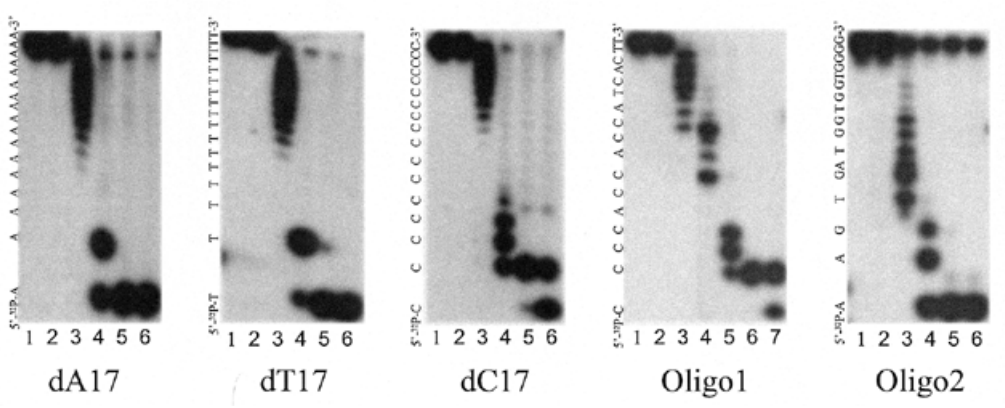

Figure 1. RNase T action on ssDNA. Reactions were carried out in 10 µl mixtures under conditions described in Materials and Methods and containing 40–80 pmol of substrate. For all substrates: lane 1, untreated substrate; lane 2, no RNase T. For all substrates except Oligo1: lane 3, 4×10^{-5} U RNase T; lane 4, 4×10^{-4} U; lane 5, 4×10^{-3} U; lane 6, 4×10^{-2} U. For Oligo1: lane 3, 10^{-4} U; lane 4, 4×10^{-4} U; lane 5, 10^{-3} U; lane 6, 10^{-2} U; lane 7, 10^{-1} U. Samples were incubated for 30 min at 37°C, and reactions terminated with 2 vol of ice-cold 96% formamide, 10 mM EDTA. After boiling for 10 min, samples were chilled on ice and analyzed on a 20% denaturing polyacrylamide gel. It should be noted that a small fraction of Oligo2 was inactive as a substrate.

purified by passage through a G25 spin column after phenol/chloroform treatment. They were then digested with various restriction enzymes (*Hae*II, *Kpn*I, *Pst*I, *Sac*I and *Sph*I) to generate dsDNA substrates with different 3′ extension sequences. To prepare dsDNA substrates containing 5′ extensions, pUC19 DNA was first digested with *Eco*RI and the fragment produced was ^{32}P-labeled at its 5′-end. The labeled fragment was then digested with a second restriction enzyme (*Pvu*II) to generate short DNA fragments containing 5′ extensions on one end and blunt termini at the other end.

RNase T assay

Unless otherwise stated, RNase T reactions contained 50 mM Tris–acetate (pH 8.0), 50 mM potassium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol and 1 mg/ml bovine serum albumin (RNase-free). The effects of ionic strength were examined by varying the potassium acetate concentration. When required, RNase T samples were diluted in reaction buffer immediately before use. After RNase T treatment, samples were analyzed by separating the ^{32}P-labeled products to single nucleotide resolution on denaturing polyacrylamide gels. Quantitative data were obtained by phosphorimaging of the gels. One unit of RNase T activity is defined as the amount of enzyme that releases 1 µmol of mononucleotide per hour.

Formation of blunt-end DNA and cloning

Blunt-end DNA cloning was examined using plasmid pUC18. pUC18 DNA was digested with the restriction enzyme *Pst*I to generate DNA with 3′-extensions. Samples were treated with either RNase T or mung bean nuclease and then recircularized with T4 DNA ligase. The efficiency with which blunt ends had been formed was determined from the amount of newly created *Bsr*FI sites in the recircularized DNA. The efficiency with which RNase T forms blunt ends could also be assessed by cloning the recircularized DNA into supercompetent cells that require α-complementation for β-galactosidase activity. In the cloning experiments, blue/white selection was applied by adding X-gal to the YT/ampicillin plates. Blue and white colonies were counted separately. White colonies were further analyzed by determining the amount of the second *Bsr*FI site in the plasmid present in the cells.

RESULTS

As part of an ongoing study of the exoribonuclease RNase T, we have been investigating its substrate specificity and mechanism of action. For this purpose, we synthesized a variety of RNA oligonucleotides of differing lengths and base composition to test as substrates. As expected, based on its *in vivo* specificity, RNase T could act on RNA duplexes with 3′ extensions, removing residues in the extension up to the double-stranded stem. Interestingly, RNase T could also digest ssRNAs 10–20 nt in length. Molecules shorter or longer than this length were much poorer substrates (unpublished observations). Inasmuch as RNase T displayed sequence similarity to that of DNA exonucleases, we also examined whether it had any activity against DNA oligonucleotides. To our surprise, DNA was an active substrate of the enzyme, and it is this activity and its practical uses which are the subjects of this paper. While this work was in progress, Viswanathan *et al.* (10) reported that RNase T could also act on high molecular weight ssDNAs as a 3′→5′ exonuclease.

RNase T action on ssDNA

Under the same conditions used for RNase assays, RNase T was found to be quite active on ssDNA oligonucleotides. As shown in Figure 1, the stepwise shortening of the 5′-^{32}P-labeled substrates indicates that its mode of action on DNA is the same as that on RNA, acting as a 3′→5′ exonuclease. Moreover, the distribution of products of varying lengths seen with all the substrates (lanes 3) supports a non-processive or random mode of attack, as previously suggested for RNA substrates (12). It is also evident that while RNase T showed relatively little sequence specificity, dC_{17} and short dC oligonucleotides were digested somewhat more slowly than their dA and dT counterparts at every enzyme concentration

tested. Likewise, Oligo1, containing multiple C residues, was digested more slowly than its complement, Oligo2. Nevertheless, given sufficient enzyme, each of the substrates could be digested completely, resulting in a 5′-^{32}P-labeled mononucleotide.

Interestingly, DNA is a significantly better substrate for RNase T than is RNA (Table 1). K_m values for the various DNA substrates were in the 0.2 μM range, while those for tRNA and Oligo3, the RNA equivalent of Oligo1, were ~10 μM, or 50-fold higher. Furthermore, the V_{max} values for DNA were 3- to 5-fold higher than those for RNA. These data explain why at any given substrate and enzyme concentration, DNA is degraded by RNase T much more effectively than is RNA.

Table 1. Kinetic constants with various substrates

Substrate	K_m (μM)	V_{max} (μmol/h/mg protein)
dT_{17}	~0.2	~800
dC_{17}	~0.5	~800
Oligo1	~0.2	~1100
Oligo2	~0.2	~600
Oligo3	>10	>200
tRNA[a]	4	300

[5′-^{32}P]DNA or RNA oligonucleotides were incubated with RNase T under conditions leading to 10–20% reduction of the original substrate. Substrate concentrations were determined by UV absorption at 260 nm using the following absorption coefficients: dT_{17}, 13.9×10^4 M^{-1} cm^{-1}; dC_{17}, 12.2×10^4; Oligo1, 14.7×10^4; Oligo2, 17.5×10^4; Oligo3, 14.9×10^4. Oligo3 data were estimated based on only a partial saturation curve because of the high concentrations required to reach saturation.

[a]Taken from Li *et al*. (13).

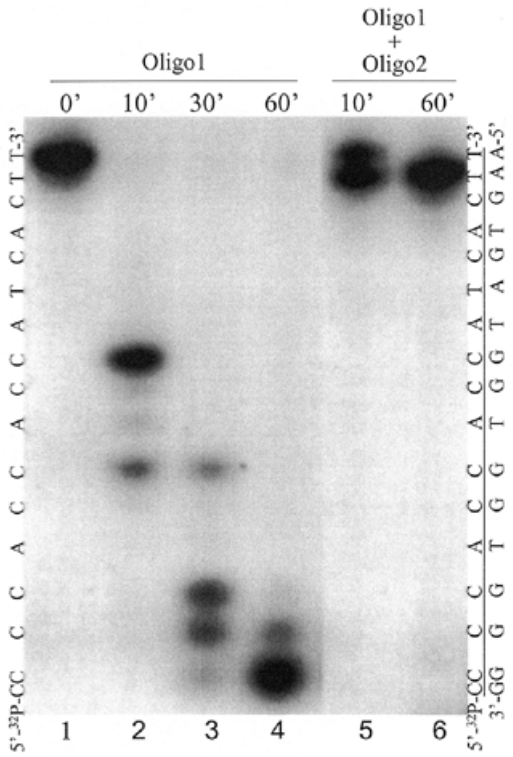

Figure 2. RNase T action on dsDNA. Reactions were carried out in 20 μl mixtures as described in Materials and Methods except that 0.3 M potassium acetate was present. Aliquots of 100 pmol of [5′-^{32}P]Oligo1 and, when present, 120 pmol of Oligo2 were added. The mixture of Oligo1 and Oligo2 in 0.3 M potassium acetate was boiled for 5 min and then slowly cooled to room temperature to anneal the two strands prior to adding to the reaction mixture. Samples were incubated for the indicated times at 37°C with 2×10^{-3} U of RNase T. Following incubation, samples were treated and analyzed as in Figure 1.

RNase T action on dsDNA

In contrast to its efficient digestion of the ssDNA oligomers (Fig. 1), RNase T action on dsDNA was extremely limited. Thus, as shown in Figure 2, a mixture of the complementary oligonucleotides, Oligo1 and Oligo2, was almost totally resistant to digestion by RNase T. Over a period of 60 min, only the 3′-terminal T residue from Oligo1 (from an AT base pair) was removed from the dsDNA (lane 6). Under the same conditions, Oligo1 by itself was digested completely (lane 4). This experiment was carried out in the presence of 0.3 M potassium acetate to maintain the stability of the short dsDNA. The inability of RNase T to digest dsDNA mimics its action on RNA during RNA processing in which only single-stranded 3′-extensions are removed from RNA precursors.

To determine how RNase T would act on longer DNA substrates, restriction enzymes were used to generate a variety of dsDNAs from pUC19 carrying 3′ or 5′ overhangs, as described in Materials and Methods. Treatment of these substrates containing 4-nt 3′ extensions with RNase T led to complete removal of the four single-stranded nucleotides in each case (Fig. 3). To ensure that, in fact, 4 nt were removed from each substrate, each was also digested with sufficiently low levels of RNase T such that each of the intermediate length products could be visualized on high resolution gels. This allowed direct measurement of the number of nucleotides that had been removed, and confirmed that for each 3′-extended substrate examined in Figure 3, 4 nt were removed (data not shown). In contrast to these substrates, the *Eco*RI product, carrying a 5′ extension, was not a substrate for RNase T based on quantitative comparisons of lanes 11 and 12 by phosphorimager analysis (Fig. 3). These data show that RNase T can effectively remove 3′ tails from dsDNA and can do so in buffers optimal for various restriction enzymes. Moreover, removal of the 3′ extension from the *Pst*I substrate was effective in a variety of commercial restriction enzyme buffers (data not shown).

The ability of RNase T to cleanly remove 3′ tails from dsDNA was strongly dependent on salt concentration and the amount of enzyme present (Fig. 4). Thus, in the absence of added potassium acetate (lanes 2 and 7), RNase T digested past the 4-nt extension and into the duplex region of the DNA, in fact, completely digesting the DNA at the higher enzyme level (lane 2). At the lower level of RNase T (lanes 7–11), digestion of the double-stranded region could be prevented by addition of 50 mM salt (lane 8). However, with 5-fold the amount of RNase T, 200 mM potassium acetate was needed to prevent

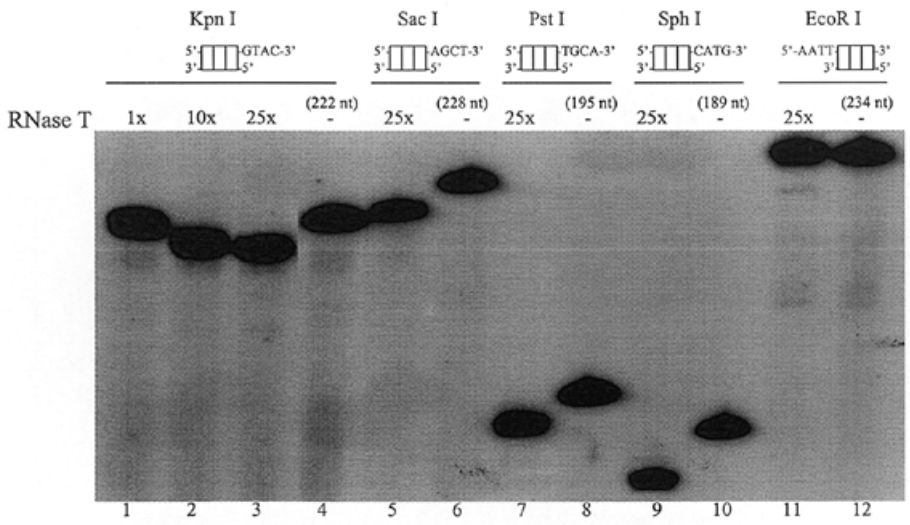

Figure 3. RNase T action on dsDNA with 3′ or 5′ extensions. The various duplex DNA substrates, shown in the figure, were prepared by treatment of a small *Pvu*II fragment of pUC19 (322 bp) with the indicated restriction enzyme. The 5′-^{32}P label was present on the longer strand whose length is indicated. Approximately 50 nM of each substrate was incubated for 30 min at 37°C with or without RNase T. In lanes 1 and 2, 1.1×10^{-2} and 1.1×10^{-1} U of RNase T were present; all other lanes with RNase T contained 2.7×10^{-1} U. Following incubation, samples were treated as in Figure 1 and analyzed on a 4% denaturing polyacrylamide gel.

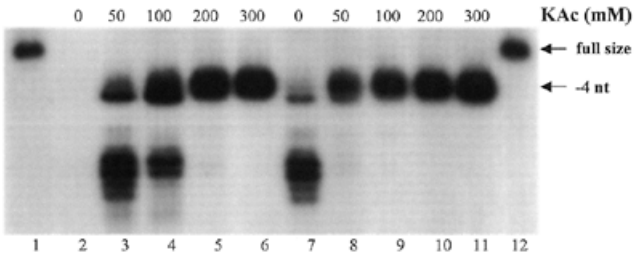

Figure 4. Effect of salt on RNase T action on dsDNA with a 3′ extension. A *Pst*I-digested substrate (133 nt length) was treated with RNase T at various concentrations of potassium acetate. Substrate was present at ~50 nM. Lanes 1 and 12, no added RNase T; lanes 2–6, 5.4×10^{-2} U of RNase T; lanes 7–11, 1.1×10^{-2} U of RNase T. The potassium acetate concentrations are shown at the top of each lane. Samples were incubated for 30 min at 37°C, treated as in Figure 1 and analyzed on a 6% denaturing polyacrylamide gel.

digestion in the duplex region (lane 5). Apparently, at low salt, there is sufficient 'breathing' of the duplex DNA structure to allow RNase T to act, and even a slow rate of digestion can become significant when sufficient enzyme is present. Nevertheless, these data indicate that conditions can be found under which RNase T can essentially quantitatively generate blunt-end DNA from restriction fragments, raising the possibility that this enzyme might prove useful for DNA cloning.

Feasibility of RNase T for DNA cloning

To evaluate whether RNase T could be used to prepare substrates for blunt-end DNA cloning, a feasibility experiment was developed that involved recircularizing plasmid DNA that had been cleaved with a restriction enzyme and trimmed with RNase T. The experimental design is presented in Figure 5. *Pst*I digestion of the sequence 5′-ACCTGCAGGC-3′ in the polycloning site of pUC18 leads to ends with 4-nt 3′ extensions (TGCA). Exact removal of these 4-nt overhangs and rejoining of the blunt ends generated will result in the new sequence 5′-ACCGGC-3′ at the joint site. This sequence, which is a *Bsr*FI restriction site, provides a simple method to measure the accuracy of RNase T in generating blunt-end DNA. Moreover, since the removal of 4 nt results in inactivation of the *lacZ* gene in pUC18, blue/white selection affords a simple means to assess the overall efficacy of the procedure.

Parallel experiments were conducted comparing RNase T and the commonly used endonuclease, mung bean nuclease. The mung bean nuclease had been pretested to determine its optimal concentration for maximum efficiency in generating transformants. In a typical experiment (Table 2), RNase T treatment yielded over 30 times as many white colonies as could be obtained with optimal levels of mung bean nuclease, indicating that RNase T is much more effective in generating DNA ends that can be religated. Thus, RNase T overcomes the problems associated with the use of mung bean nuclease, i.e. either too much degradation due to breathing of ends or too little digestion such that blunt ends are not generated. Restriction digestion of plasmids prepared from randomly selected white colonies revealed that 70% of those obtained with RNase T had acquired a second *Bsr*FI site indicating accurate removal of the 4-nt 3′ extension and generation of blunt ends. A similar percentage (60%) of the white colonies obtained with mung bean nuclease treatment also had plasmids with two *Bsr*FI sites, but considering the low yield of white colonies recovered after this treatment, overall, mung bean nuclease is as much as

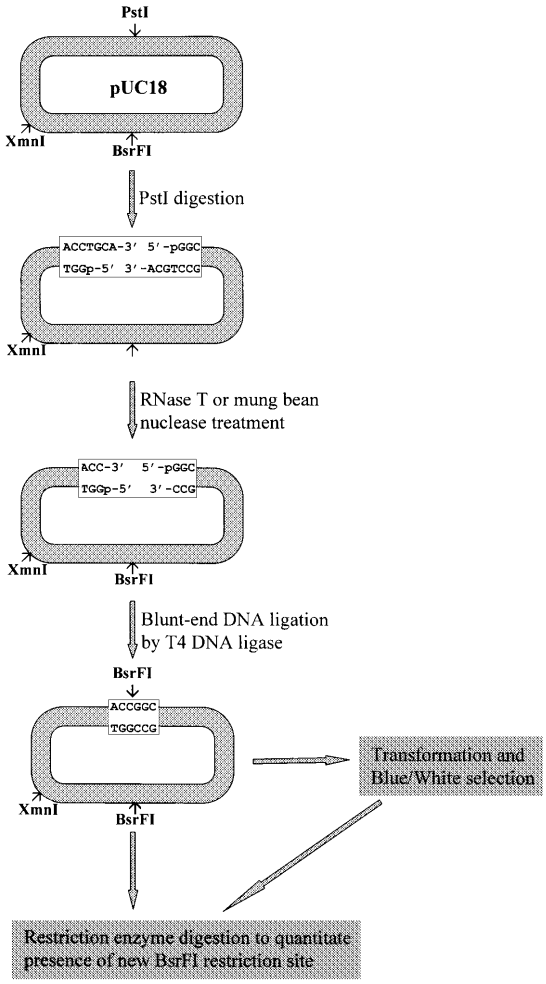

Figure 5. Experimental design for comparing blunt-end cloning with RNase T and mung bean nuclease. The relative positions of the *Pst*I, *Xmn*I and the original and newly created *Bsr*FI sites in pUC18 are indicated.

40-fold less effective than RNase T. Thus, these data indicate that RNase T is the preferred enzyme for use in blunt-end DNA cloning.

DISCUSSION

Considerable evidence has accumulated indicating that RNase T is an important enzyme for 3′ maturation of various stable RNAs, and that it is essential for maturation of 5S and 23S rRNA (7–9) and for end-turnover of tRNA (6). All of these RNA species have a common structural feature—an RNA duplex formed between their 5′- and 3′-ends and a single-strand 3′ extension (8). Although multiple enzymes can contribute to removal of extra 3′ residues in RNA precursors, RNase T is the only enzyme that can efficiently remove residues near a double-stranded stem.

To examine RNase T substrate specificity, and to determine whether the duplex structural feature is a requirement for RNase T action, we used chemically synthesized RNA and DNA oligonucleotides to mimic various structural features of the RNA precursors. Surprisingly, we found that in addition to its RNase activity, RNase T can also degrade ssDNA very efficiently. Similar to its action on RNA, RNase T action on DNA substrates is non-processive. However, assuming that the K_m values for RNA and DNA in these one-substrate reactions reflect dissociation constants, RNase T binding to ssDNA is much tighter than that to RNA. RNase T can also act on dsDNA, but this activity is highly dependent on buffer conditions. Thus, RNase T can degrade dsDNA to completion when the salt concentration is low, but not under high salt conditions. This makes the enzyme ideal for removing single-stranded 3′ tails to generate blunt-ended DNA.

Inasmuch as the salt concentration of the bacterial cytoplasm is high (up to ~0.5 M), it is unlikely that RNase T can degrade dsDNA *in vivo*. This does not exclude the possibility that *in vivo* RNase T may participate in DNA metabolism, especially with the help of other proteins. In fact, the *rnt* gene encoding RNase T is part of a two gene operon, which also contains downstream *lhr*, a putative ATP-dependent helicase (14,15). The *lhr* gene product could help to generate ssDNA for RNase T function. It has already been shown that RNase T can act as a high copy suppressor of UV sensitivity in strains deficient in ssDNA exonuclease activity (11).

Among the DNA exonuclease activities found in *E.coli*, the RNase T DNase activity is quite similar to the activity of the proof-reading subunit of DNA polymerase III (ε subunit or DnaQ protein). Both activities act 3′→5′ on ssDNA in a non-processive manner with 5′ mononucleotides as the product. RNase T action on dsDNA under low ionic strength conditions

Table 2. Comparison of blunt-end DNA cloning with mung bean nuclease and RNase T

Enzyme	Total colonies	White colonies (%)	Plasmids in white colonies with two *Bsr*FI sites
Mung bean nuclease	~1100	147 (13%)	6/10
RNase T	~6600	~5000 (75%)	14/20

pUC18 DNA (1 μg) in 10 μl of NE buffer 2 was digested with *Pst*I for 1 h at 37°C and then treated with either 2 U of mung bean nuclease for 20 min at room temperature or 5×10^{-2} U of RNase T for 30 min at 37°C. After purification, DNA was recircularized with phage T4 DNA ligase overnight at room temperature. An aliquot of 1 μl of each ligation mixture was used for transformation. Plasmid DNA, prepared from white colonies, was examined for the presence of a second *Bsr*FI restriction site using *Bsr*FI and *Xmn*I. In the absence of ligation, 6 white and 137 blue colonies were obtained with mung bean nuclease, and 24 white and 8 blue colonies were obtained with RNase T. Although the absolute number of colonies differed among experiments, in three experiments with mung bean nuclease the percentage of white colonies remained nearly constant, varying from 12 to 18%, and in five experiments with RNase T the percentage varied from 75 to 96%.

may also resemble DNA polymerase III action on mismatched 3′-primer termini. The differences are that DnaQ protein is not known to act on RNA substrates or DNA dinucleotides, whereas RNase T acts on both DNA and RNA substrates and can degrade DNA dinucleotides to mononucleotides, albeit slowly. DnaQ requires the DNA polymerase III α subunit to stimulate its DNase activity by providing better substrate binding. Similarly, an RNase T mutant (C168S) deficient in dimerization (13), which has very low RNase activity, is also deficient in DNase activity (data not shown). This may indicate that an RNase T monomer needs another monomer to stimulate its activity, possibly by providing better substrate binding in an analogous fashion to the α subunit of DNA polymerase III.

In fact, RNase T is a close homolog of the DNA polymerase III proof-reading subunit (2). Both of them belong to the exonuclease superfamily (3). However, while DnaQ has orthologs widely distributed among the bacteria, RNase T orthologs have only been found in the γ division of proteobacteria that includes such organisms as *Escherichia*, *Hemophilus*, and *Vibrio* (unpublished work). This raises the interesting question of why RNase T evolved to participate in so many important functions in RNA metabolism, and possibly also in DNA metabolism, in such a limited group of bacteria.

Although the biological function of the DNase activity of RNase T is not yet clear, a useful practical application of this activity is described here. Under high salt conditions, RNase T can trim single-strand 3′ extensions to generate predominantly blunt-end dsDNA. This property of RNase T was tested in a DNA cloning experiment which showed that RNase T is much more effective than mung bean nuclease for this purpose. Normally, mung bean nuclease is used in conjunction with Klenow fragment DNA polymerase, and greater yields may be obtained when both enzymes are present. However, use of only a single enzyme, RNase T, would be much more convenient. More importantly, if precise removal of 3′ extra residues is required, or if only one end of the DNA needs to be trimmed so that the ligation direction could be controlled for better cloning efficiency, RNase T would be a much better choice than mung bean endonuclease.

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