Substrate Recognition by a Yeast 2′-Phosphotransferase Involved in tRNA Splicing and by Its *Escherichia coli* Homolog†

Michelle A. Steiger,‡,§ Ryszard Kierzek,⊥,# Douglas H. Turner,⊥ and Eric M. Phizicky*,‡

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642, Department of Chemistry, University of Rochester, Rochester, New York 14627, and Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

Received July 2, 2001; Revised Manuscript Received September 18, 2001

ABSTRACT: The final step of tRNA splicing in *Saccharomyces cerevisiae* requires 2′-phosphotransferase (Tpt1) to transfer the 2′-phosphate from ligated tRNA to NAD, producing mature tRNA and ADP ribose-1″-2″-cyclic phosphate. To address how Tpt1 protein recognizes substrate RNAs, we measured the steady-state kinetic parameters of Tpt1 protein with 2′-phosphorylated ligated tRNA and a variety of related substrates. Tpt1 protein has a high apparent affinity for ligated tRNA ($K_{m,RNA}$, 0.35 nM) and a low turnover rate (k_{cat}, 0.3 min^{-1}). Tpt1 protein recognizes both tRNA and the internal 2′-phosphate of RNAs. Steady-state kinetic analysis reveals that as RNAs lose structure and length, $K_{m,RNA}$ and k_{cat} both increase commensurately. For a 2′-phosphorylated octadecamer derived from the anticodon stem-loop of ligated tRNA, $K_{m,RNA}$ and k_{cat} are 5- and 8-fold higher, respectively, than for ligated tRNA, whereas for a simple substrate like pApA^{p}pA, $K_{m,RNA}$ and k_{cat} are 430- and 150-fold higher, respectively. Tpt1 is not detectably active on a trimer with a terminal 5′- or 3′-phosphate and is very inefficient at removal of a terminal 2′-phosphate unless there is an adjacent 3′-phosphate or phosphodiester. The $K_{m,NAD}$ for Tpt1 is substrate dependent: $K_{m,NAD}$ is 10 μM with ligated tRNA, 200 μM with pApA^{p}pA, and 600 μM with pApApA^{p}. Preliminary analysis of KptA, a functional Tpt1 protein homologue from *Escherichia coli*, reveals that KptA protein is strikingly similar to yeast Tpt1 in its kinetic parameters, although *E. coli* is not known to have a 2′-phosphorylated RNA substrate.

Splicing is one of many tRNA processing steps in the yeast *Saccharomyces cerevisiae* and other eukaryotes (*1*). All nuclear encoded, eukaryotic tRNAs undergo cleavage of the 5′-leader by RNase P (*2*), removal of the 3′-trailer by nucleases (*3*), addition of CCA to the cleaved 3′-end by terminal nucleotide transferase (*4*), modification of specific bases (*5*, *6*), and aminoacylation, which allows the efficient transfer of the tRNA from the nucleus to the cytoplasm (*7*, *8*). Additionally, a subset of tRNA genes in eukaryotes, including *S. cerevisiae* and humans, encode introns which must be removed by tRNA splicing (*9*, *10*). In *S. cerevisiae*, 59 of the 272 nuclear encoded tRNA genes contain a short intron (14−60 bases long) located one base 3′ of the anticodon (*9*).

tRNA splicing in yeast is a three-step process, involving six essential polypeptides and three nucleotide cofactors (*9*). First, the four-subunit tRNA endonuclease excises the intron (*11*−*13*). Then tRNA ligase (Rlg1) uses both GTP and ATP to modify the cleaved ends of the exons and join them to form ligated tRNA with a 2′-phosphate at the splice junction (*14*−*16*). Finally, 2′-phosphotransferase (Tpt1) transfers the 2′-phosphate from tRNA to NAD, via an intermediate that is ADP-ribosylated on the 2′-phosphate, to produce mature tRNA and ADP-ribose-1″-2″ cyclic phosphate (Appr>p) (*17*−*21*). This role of Tpt1 is supported by the observation that conditional *tpt1* mutants accumulate 2′-phosphorylated tRNAs as the cells stop growing under nonpermissive conditions (*20*).

Tpt1 protein may act on another substrate in yeast in addition to ligated tRNAs, since tRNA ligase is implicated in the unusual splicing of *HAC1* mRNA which occurs during the unfolded protein response (*22*). An *rlg1*−*100* ligase mutant specifically impairs *HAC1* splicing and the unfolded protein response (*22*), and analysis of *HAC1* mRNA splicing in vitro (*23*) reveals the presence of a splice junction 2′-phosphate (*24*). Thus, it seems likely that Tpt1 participates in dephosphorylation of *HAC1* mRNA. In addition, the presence of a functional homologue of Tpt1 (KptA) in *Escherichia coli* suggests there may be other substrates for this family of proteins, since there is no known 2′-phosphorylated RNA substrate in such bacteria (*25*).

To explore substrate recognition by Tpt1 protein, we have investigated the kinetics of the reaction with a battery of potential substrates, including 2′-phosphorylated RNAs that

† Supported by NIH Grants GM 52347 to E.M.P. and GM 22939 to D.R.T.

* To whom correspondence should be addressed: Eric Phizicky, Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Box 712, Rochester, NY 14642. Telephone: (716) 275−7268, Fax: (716) 271−2683, E-mail: eric_phizicky@urmc.rochester.edu.

‡ Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester.

⊥ Department of Chemistry, University of Rochester.

Polish Academy of Sciences.

§ Present address: Department of Molecular and Cellular Biology and Howard Hughes Medical Institute, University of Arizona, Tucson, AZ 85721

10.1021/bi011388t CCC: $20.00
Published on Web 10/24/2001

range in size from trinucleotides to full-length tRNA, as well as RNAs that are phosphorylated at other positions. An earlier preliminary survey indicated that Tpt1 protein could remove an internal 2′-phosphate from both ligated tRNA and an oligomer, but was less efficient with oligonucleotides bearing a terminal 2′-, 3′-, or 5′-phosphate (*26*, *27*). The analysis presented here indicates a high apparent affinity of Tpt1 protein for ligated tRNA ($K_{m,RNA} = 0.35$ nM) and a low turnover rate ($k_{cat} = 0.3$ min^{-1}). Further analysis reveals that the tRNA, the 2′-phosphate, and the immediate flanking groups contribute to the high apparent affinity of Tpt1 protein for ligated tRNA. Tpt1 protein efficiently removes an internal 2′-phosphate from simple substrates such as ApA^{p}pA, although with a ~430-fold increase in $K_{m,RNA}$ and a 150-fold increase in k_{cat}. Efficient removal of the terminal 2′-phosphate requires the presence of an adjacent 3′-phosphate or phosphodiester group. Oligomers with only a terminal 3′- or 5′-phosphate are not detectably dephosphorylated by Tpt1. Initial analysis indicates that KptA is similar to Tpt1 in its substrate specificity.

MATERIALS AND METHODS

Preparation of RNA Molecules. Unspliced yeast pre-tRNA^{Phe} was transcribed in vitro from a plasmid containing the end-matured portion of the gene, using either 400 or 0.4 μCi [α-^{32}P] ATP (548 or 0.67 Ci/mol ATP, respectively) and T7 RNA polymerase, as described (*28*). *E. coli* tRNA^{Phe} was similarly transcribed with 4 μCi [α-^{32}P] ATP (6.7 Ci/mmol ATP) from a DNA construct encoding mature *E. coli* tRNA^{PHE} (*29*). Ligated tRNA, bearing a splice junction 2′-phosphate, was made by treating the precursor tRNA with partially purified tRNA endonuclease and tRNA ligase for 30 min at 30 °C as previously described (*26*).

All small RNA substrates were chemically synthesized and structurally analyzed as described (*30*). These RNAs were 5′-end labeled with 5 μCi [γ-^{32}P] ATP (from 3000 to 0.83 Ci/mmol, as needed) and 2 units polynucleotide kinase (3′-phosphatase free) in buffer (Boehringer Mannheim) at 37 °C for 30 min, and labeled substrates were separated from unlabeled substrates by chromatography on silica thin layer plates (J. T. Baker) resolved in solvent containing *n*-propanol/NH_4OH/H_2O (55:35:10, v/v/v), followed by autoradiography and elution in water.

Purification of Tpt1-His$_6$ and KptA-His$_6$ Protein. A 250 mL culture of *E. coli* BL21(DE3) cells transformed with a pET24b vector containing *TPT1* (strain EMP1201-1) was grown at 37 °C to $A^{600} = 0.4$, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h, harvested, and resuspended in 3 mL of sonication buffer (20 mM Tris 7.5, 1 M NaCl, 10% glycerol, 4 mM $MgCl_2$, 0.5 mM β-mercaptoethanol) containing 1 mg/mL lysoyzme and protease inhibitors (2 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.5 mM diisopropylfluorophosphonate). A crude extract was made by sonication of the mixture (6 times for 10 s, with ice incubations of 1 min between sonication rounds) followed by centrifugation to remove debris. To purify Tpt1, extracts were diluted with an equal volume of sonication buffer lacking NaCl and mixed for 30 min with 1 mL of Talon resin (Clontech) for immobilized metal ion chromatography, IMAC.[1] Then the IMAC resin was washed twice in batch by low speed centrifugation with 10 mL of buffer, packed into a column, washed with an additional 5 to 10 column volumes of buffer, and protein was eluted with 6 mL of buffer containing 100 mM imidizole (pH 7.73). The peak protein-containing fractions were dialyzed into buffer containing 20 mM Tris (pH 7.5), 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl, and 50% glycerol, assayed for protein concentration using the Bradford assay (*31*), and stored at −20 °C. KptA-His$_6$ is prepared in the same manner as Tpt1-His$_6$ protein, except KptA is expressed from a pET24a vector (strain EMP 1213).

2′-Phosphotransferase Reactions. 2′-phosphotransferase was assayed for 30 min unless otherwise indicated (*19*), at 30 °C with 1 mM NAD for yeast Tpt1 protein, and at 37 °C with either 1 or 20 mM NAD for *E. coli* KptA protein. For each assay, dilutions of protein were used that resulted in less than 10% conversion of substrate to products. Products from ligated tRNA and most oligonucleotides were separated on polyethyleneimine cellulose thin-layer chromatography plates developed in solvent containing 2 M sodium formate (pH 3.5). Products from reactions with octadecamer, octamer, and pApApA^p were separated on silica plates developed in solvent containing *n*-propanol/NH_4OH/H_2O (55:35:10, v/v/v). Kinetic measurements for octadecamer and pApApA^p were performed with mixtures of labeled and unlabeled RNA substrates to obtain the desired final concentrations of RNA in the enzymatic reaction. Effective inhibitory concentrations of competing substrates (IC_{50}) were measured with labeled 2′-phosphorylated ligated tRNA at 150 pM and a range of concentrations of unlabeled substrates.

Results were quantitated by analysis on a phosphorimager (Molecular Dynamics) and plotted with SigmaPlot, version 4.0. For yeast Tpt1, concentrations and k_{cat} values are adjusted assuming that the protein is 50% pure. For KptA, the maximum velocity (V_{max}) is expressed as nmoles min^{-1} (ng of total protein)$^{-1}$ in the reaction, based on the concentration of the purified fraction (0.36 μg of total protein/μL, determined by Bradford assay), and the dilution used in the experiments. Unless otherwise indicated, K_m refers to the measured K_m for an RNA substrate.

Equilibrium Gel Filtration Column. A 12-cm Sephadex G-75 column was equilibrated with buffer containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, 2.5 mM spermidine, 0.1 mM DTT, 0.2 mM EDTA, 100 μg/mL BSA, and 200 nM [5′-^{32}P]pApA^{p}pA. Reaction mixtures containing 3.25 μM Tpt1 and varying concentrations of pApA^{p}pA were incubated at 30 °C for 20 min in the same buffer, applied to the column, and chromatographed at 4 °C in the same buffer. Fractions were collected and counted to evaluate binding of Tpt1 to pApA^{p}pA. For the data in Figure 5, $K_D = [P_f][R_f]/[PR] = [P_t - PR][R_f]/[PR] = (3250 - 2000\text{ nM})(200\text{ nM})/(2200 - 200\text{ nM}) = 125$ nM, where $[P_f]$ and $[R_f]$ are the concentrations of free Tpt1 protein and RNA, respectively, and [PR] is the concentration of the protein·RNA complex.

RESULTS

Overproduction and Purification of Recombinant Tpt1 Protein. Active yeast Tpt1 protein is efficiently purified from *E. coli* when overexpressed as a C-terminal Tpt1-His$_6$ fusion

[1] Abbreviations: IMAC, immobilized metal ion chromatography; Appr>p, adenosine diphosphate ribose 1″-2″-cyclic phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

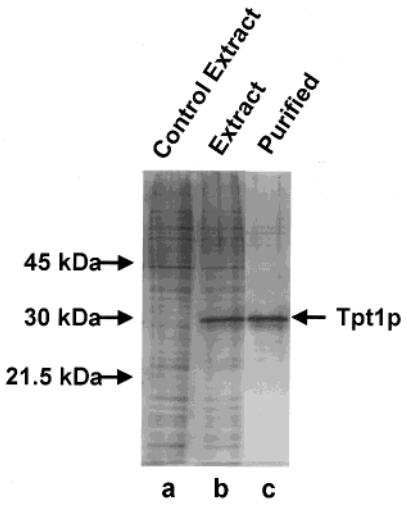

FIGURE 1: Analysis of Tpt1-His_6 protein purification. Samples containing 0.4 μg of protein were resolved on a 12% SDS polyacylamide gel and visualized by silver staining. Lane a, control extract; lane b, extract from cells overproducing Tpt1 protein; lane c, purified Tpt1 protein. Migration of standards is shown at the left.

protein from a pET24 vector. Induction of Tpt1 expression results in the appearance of a predominant 30-kDa polypeptide in extracts, as determined by SDS−PAGE, and as expected based on the size of Tpt1 protein produced in yeast (Figure 1, lanes a and b; data not shown). Tpt1 protein purified from these *E. coli* extracts by immobilized metal ion chromatography is about 50% pure, based on visual inspection of polypeptides resolved on SDS−PAGE and stained with silver (Figure 1, lane c). This level of polypeptide purity is similar to that obtained after purification of Tpt1 from yeast cells (*19*); data not shown). Additionally, the two sources of protein have similar specific activities using our standard assay conditions with ligated tRNA (*19*): 2.0×10^8 units/mg for recombinant Tpt1 protein produced in *E. coli*, and 1.4×10^8 units/mg for Tpt1 purified from yeast.

Steady-State Kinetic Parameters of Tpt1 Protein with 2′-Phosphorylated Ligated tRNA. Tpt1 catalyzes a two-substrate reaction involving both RNA and NAD. Thus, for investigation of the kinetic parameters with respect to RNA, reactions were performed in the presence of 1 mM NAD, which is a saturating concentration for Tpt1 (see below).

We measured the steady-state kinetic parameters of Tpt1 protein with its natural substrate, 2′-phosphorylated ligated $tRNA^{Phe}$, which was prepared by in vitro transcription from template DNA, followed by endonucleolytic cleavage and ligation (see Materials and Methods). The linearity of the Tpt1 reaction over time with ligated tRNA substrate is illustrated in Figure 2A. The Michaelis Menten plot shown in Figure 2B yielded a K_m of 0.35 nM for ligated $tRNA^{Phe}$, and a relatively low k_{cat} of 0.13 min^{-1}. The kinetic parameters for ligated tRNA were measured multiple times, resulting in similar K_m values ($K_m = 0.35$ nM $\pm$ 0.1) but variable k_{cat} values ($k_{cat} = 0.1$ to 0.3 min^{-1}, Table 1). The variability observed in k_{cat} is likely due to either different amounts of contaminants in the gel-purified tRNA or to progressive degradation of ligated tRNA due to radioactive decay. The overall catalytic efficiency, k_{cat}/K_m, is 1.4×10^7 M^{-1} s^{-1}, which is relatively fast considering the low k_{cat}, only 10−100 fold below the diffusion-limited rate of 10^8-10^9 M^{-1} s^{-1}.

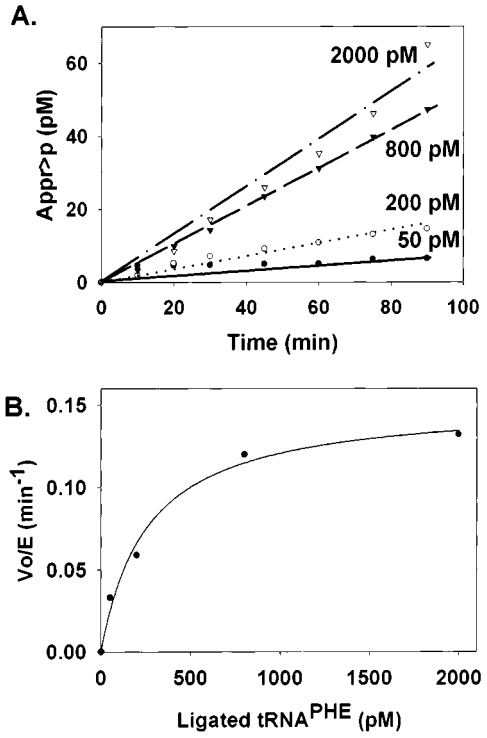

FIGURE 2: Kinetics of reaction with ligated tRNA and Tpt1 protein. (A) Time courses of the phosphotransferase reaction with ligated tRNA. Reaction mixtures of 60 μL, prepared as described in Materials and Methods, contained 4.25 pM Tpt1 protein, 1 mM NAD and ligated tRNA as indicated, and were incubated at 30 °C. Aliquots were taken at the time points, and products were separated by thin-layer chromatography and quantitated as described in Materials and Methods. (B) Rate of phosphotransferase as a function of ligated tRNA concentration. Rates determined from the data in panel A are plotted against the concentration of ligated tRNA.

Tpt1 Protein Recognizes tRNA. The high apparent affinity of Tpt1 protein for ligated tRNA could be directed by the 2′-phosphate on the RNA or by the tRNA. The ability of Tpt1 protein to recognize tRNA was examined by measuring inhibition of its activity by mature *E. coli* $tRNA^{Phe}$, which has canonical tRNA structure, and by intron-containing yeast pre-$tRNA^{Phe}$, which has the same structure as mature yeast $tRNA^{Phe}$, except in the region of the intron (*32*, *33*). Both mature *E. coli* $tRNA^{Phe}$ and yeast pre-$tRNA^{Phe}$ inhibit Tpt1 activity measured at 150 pM ligated tRNA, with IC_{50} values (the inhibitor concentration where 50% of the reaction is inhibited) of 25 and 10 nM, respectively (Figure 3, data not shown). The inhibition is apparently competitive since in the presence of 75 nM pre-$tRNA^{Phe}$, the apparent K_m increases 2.5-fold, but k_{cat} is unaffected (data not shown). Thus, Tpt1 protein can recognize and bind tRNA. The oligoribonucleotide, ApGpApUpUpUpApC, which lacks any obvious secondary structure and has no terminal phosphate, also serves as an inhibitor of the phosphotransferase reaction with ligated tRNA substrate. However, the measured IC_{50} is 50 μM, indicating that Tpt1 protein only weakly recognizes relatively short RNAs without structure (Figure 3).

Tpt1 Protein Efficiently Dephosphorylates Oligoribonucleotides with an Internal 2′-Phosphate. We further examined the dependence of Tpt1 protein activity on RNA structure and length by measuring kinetic parameters for synthetic 18,

Table 1: Kinetic Values at 30 °C for Substrates with Internal 2′-phosphates

substrate	K_m (nM)	IC_{50} (nM) vs. ligated $tRNA^{PHE}$	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
ligated $tRNA^{PHE}$ (2′−P)	0.35 ± 0.10		0.10 to 0.30	1.4×10^7
octadecamer, p*ACAAGACUGUAA^{p}AUCUUG	1.7 ± 0.3	5.3	2.4 ± 0.4	2.4×10^7
octamer, p*GUAA^{p}AUCU	20.0 ± 6.0	25	19 ± 10.0	1.6×10^7
trimer, p*ApA^{p}pA	150 ± 75	200	44 ± 20	5×10^6
p*ApU^{p}pU	480	600	27 ± 4.0	9.4×10^5
p*UpU^{p}pU	325 ± 75	500	8.8 ± 4.0	4.5×10^5

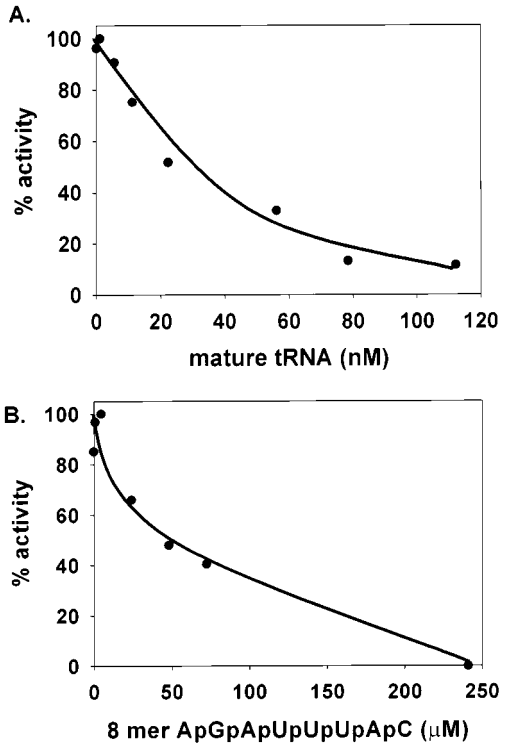

FIGURE 3: Effect of competitor RNAs on rate of Tpt1 reaction. The rate of the Tpt1 reaction with 150 pM ligated tRNA was measured in the presence of varying amounts of mature *E. coli* $tRNA^{Phe}$ (A) or of the 8-mer AGAUUUAC (B). Reaction mixtures were prepared and analyzed as described in the legend of Figure 2A, and contained 21.5 pM Tpt1 protein, 1 mM NAD, and competitor RNA as indicated.

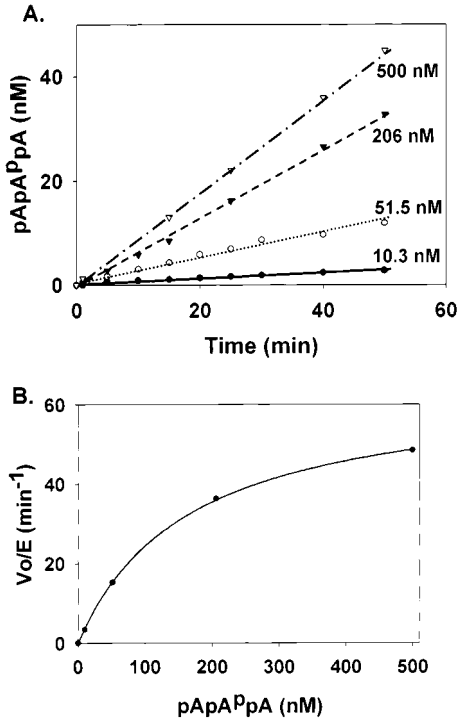

FIGURE 4: Kinetics of reaction with pApA^{p}pA and Tpt1 protein. (A) Time course of the phosphotransferase reaction with pApA^{p}pA. Reaction mixtures of 50 μL contained 21.4 pM Tpt1, 1 mM NAD, and the indicated concentration of pApA^{p}pA, and were incubated at 30 °C for the indicated time before products were separated by thin-layer chromatography, and quantitated as described in Materials and Methods. (B) Rate of phosphotransferase as a function of pApA^{p}pA. Rates determined from the data in panel A are plotted against the concentration of pApA^{p}pA.

8, and 3-mer 2′-phosphorylated RNAs that are derived from the anticodon stem−loop of spliced $tRNA^{Tyr}$. The 18-mer (pApCpApApGpApCpUpGpUpApA^{p}pApUpCpUpUpG) has the expected structure of the anticodon stem−loop of $tRNA^{Tyr}$, as determined by Tm experiments (*30*), whereas the octamer (pGpUpApA^{p}pApUpCpU) and the trimer (pA-pA^{p}pA) are not predicted to have structure.

A comparison of the kinetic parameters for these small RNAs with those for full length ligated tRNA is shown in Table 1. Figure 4A shows the linearity over time of the phosphotransferase reaction with the substrate pApA^{p}pA, and Figure 4B is the Michaelis−Menten plot generated from these data. For all other substrates, we have used a 30-min time point to determine rates. The trimer pApA^{p}pA is the simplest of these RNAs and is still an effective substrate for Tpt1, as measured by k_{cat}/K_m. Although the K_m for pApA^{p}pA is increased 430-fold relative to that for ligated tRNA, there is also an accompanying 150-fold increase in k_{cat}, which results in a k_{cat}/K_m value that is only decreased 3-fold from that of ligated tRNA (See Table 1 and Figure 4B). The kinetic parameters for the 2′-phosphorylated octamer and octadecamer are intermediate between those of trimer and ligated tRNA, while retaining similar k_{cat}/K_m values. Thus, the 2′-phosphorylated octamer has a K_m of 20 nM and a k_{cat} of 19 min^{-1}, values which are each ∼60-fold higher than those for ligated tRNA but still 2−8-fold lower than those for pApA^{p}pA. The kinetic parameters measured for the octadecamer are much more similar to those observed with ligated tRNA. Its K_m of 1.7 nM and k_{cat} of 2.4 min^{-1} are only 5- and 8-fold higher, respectively, than the corresponding values for ligated tRNA. Thus, the stem−loop structure of the octadecamer appears to contain most of the elements required for Tpt1 recognition and activity with ligated tRNA.

We also investigated the effect of neighboring sequence on Tpt1 protein activity. Since the 5′-splice junction nucleotide of yeast tRNAs is always a purine, it was possible that Tpt1 protein might require a purine at that position. Table 1 shows a comparison of the activity of Tpt1 between pApA^{p}pA and two trimers with uridine residues surrounding the 2′-phosphate: pApU^{p}pU and pUpU^{p}pU. As shown in

Table 2: Kinetic Values at 30 °C for Substrates that Vary in 2′-Phosphate Chemical Environment

substrate	K_m (nM)	IC_{50} (nM) vs. spliced $tRNA^{PHE}$	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
p*ApA^{p}pA	150 ± 75	200	44 ± 20	5×10^6
p*ApApAp	ND[a]	>400 000	ND	<0.9[b]
p*ApApA^p	40 000	50 000	0.34 ± 0.04	1.4×10^2
p*ApApA^{p}p	650	800	9.0	2.3×10^5
p*ApApA^{p}pOCH$_3$	3,000	2,500	70.4	3.9×10^5

[a] ND, not detected. [b] Estimated as described in text.

Table 1, the RNAs with U residues surrounding the 2′-phosphate have a 2- to 3-fold increase in K_m (and IC_{50}) relative to that of pApA^{p}pA, and a k_{cat} value that is 20−60% of the value with pApA^{p}pA, leading to a k_{cat}/K_m value that is 9−20% of the value with pApA^{p}pA. We conclude that Tpt1 has a modest preference for 2′-phosphorylated RNA substrates that are flanked by purine residues, relative to flanking pyrimidine residues.

Tpt1 Protein Is Specific for RNA Substrates with 2′-Phosphates. A previous preliminary survey of Tpt1 protein showed that it could act more efficiently on RNAs bearing an internal 2′-phosphate than on RNAs with a terminal 5′- or 3′-phosphate (*26*, *27*). We rigorously examined this result with [5′-^{32}P]pApApAp, which has terminal 5′- and 3′-phosphates, but no 2′-phosphate. Use of this substrate allows us to monitor removal of either phosphate by Tpt1 protein: removal of the 5′-phosphate results in production of labeled Appr>p, and removal of the 3′-phosphate causes a change in the chromatographic mobility of the labeled RNA. Tpt1 protein did not detectably use pApApAp as a substrate during a 30-min incubation (see Table 2), even at high enzyme concentrations (up to 6 μM Tpt1, compared to 22 pM Tpt1 normally used with the substrate pApA^{p}pA). Similarly, with 150 pM ligated tRNA substrate, Tpt1 activity was not inhibited by pApApAp at concentrations up to 400 μM. A maximum k_{cat}/K_m of 0.9 M^{-1} min^{-1} was calculated for pApApAp by assuming that the K_m for pApApAp was above 5 μM, and that a 1% conversion of substrate to product would be detectable. Thus, as measured by k_{cat}/K_m, Tpt1 protein is less that 2×10^{-5}% as active with the substrate pApApAp as with pApA^{p}pA.

Efficient RNA Substrates with a Terminal 2′-Phosphate Have a 3′-Phosphoryl Group Adjacent to the 2′-phosphate. We previously reported that Tpt1 protein did not efficiently remove a terminal 2′-phosphate from an RNA substrate (*27*). Reexamination of this result with pApApA^p reveals that this RNA is a very weak substrate for Tpt1, with a 270-fold higher K_m and a 125-fold lower k_{cat} than that observed with pApA^{p}pA. This corresponds to a k_{cat}/K_m for pApApA^p that is 0.003% of the value for pApA^{p}pA, a factor of ~34 000 (see Table 2).

We extended this analysis to determine the chemical environment necessary for the terminal 2′-phosphate to serve as an efficient Tpt1 protein substrate. To this end, we compared the kinetic parameters of synthetic RNAs with a terminal 2′-phosphate flanked by a hydroxyl (pApApA^p) to those of a substrate with its terminal 2′-phosphate adjacent to a 3′-phosphate (pApApA^{p}p) or to a methoxy phosphate (pApApA^{p}p$_{OCH_3}$). Surprisingly, both RNAs with a phosphoryl group at the 3′-position adjacent to the 2′-phosphate are much more efficient substrates than the RNA with just a terminal 2′-phosphate (Table 2). For both of these substrates, the overall catalytic efficiency, as measured by k_{cat}/K_m, is 2000−3000-fold greater than for the RNA with only a terminal 2′-phosphate, and as much as 5% the value for pApA^{p}pA. Thus, substrates with a terminal 2′-phosphate are much more effective when they have a phosphate, a phosphodiester, or a nucleotide at the neighboring 3′-position. We note that for pApApA^{p}p we confirmed that the terminal 2′-phosphate was removed, rather than the 3′-phosphate. To this end, we did a large-scale phosphotransferase reaction with pApApA^{p}p and analyzed the product RNA after RNase T2 or P1 nuclease treatment by thin-layer chromatography (*34*). As expected, Ap, rather than A^p, was produced by RNase T2, and pA, rather than pA^p, was produced by P1 nuclease.

Most of the Tpt1 Protein Is Active and Binds pApA^{p}pA with a Low K_D. One concern about the kinetic characterization of Tpt1 is that the observed k_{cat} values might be artificially lower because of an unexpectedly low fraction of active protein. This could occur if, for example, the protein was largely inactivated during purification. To address this question, we measured binding of Tpt1 protein to pApA^{p}pA by equilibrium gel filtration (*35*). In different experiments, increasing concentrations of pApA^{p}pA were incubated with the same concentration of Tpt1 (3.25 μM), and then free and Tpt1-bound pApA^{p}pA were resolved on a Sephadex G-75 column equilibrated and run in buffer containing 200 nM pApA^{p}pA. As shown in Figure 5A,B, this column effectively separates Tpt1-bound pApA^{p}pA (the peak in fractions 40 to 60) from unbound pApA^{p}pA (the peak or trough in fractions 95 to 110). Quantitation of the first peak reveals that at least 60% of the Tpt1 binds pApA^{p}pA, assuming that each Tpt1 molecule binds one RNA and that the contaminants in the preparation do not appreciably bind RNA. Thus, a substantial fraction of the Tpt1 protein is active for binding and, presumably, also catalysis. We note also that Tpt1 binds pApA^{p}pA quite effectively. As shown in Figure 5C, a plot of the peak or trough area of the free pApA^{p}pA as a function of pApA^{p}pA concentration in the incubated sample indicates that at 2.2 μM pApA^{p}pA there would be no resulting peak or trough of free pApA^{p}pA from the column. Thus, under these conditions, the concentration of free pApA^{p}pA in the incubation mixture is 200 nM, which corresponds to a K_D of 125 nM if all the Tpt1 protein is active (see Materials and Methods) and a correspondingly lower K_D if less is active. This maximum K_D is similar to the measured K_m of 150 nM for this substrate.

The Apparent Affinity for NAD Changes with Different RNA Substrates. The steady-state kinetic parameters determined for NAD indicate that the apparent affinity for NAD is dependent on the RNA substrate. In the presence of nearly saturating concentrations of RNA substrate, the $K_{m,NAD}$ with ligated tRNA is 10 μM, whereas that with pApA^{p}pA is 200 μM (see Figure 6). This 20-fold difference in $K_{m,NAD}$ is observed when comparing two RNA substrates that differ 430-fold in $K_{m,RNA}$. The relationship between $K_{m,NAD}$ and $K_{m,RNA}$ was further tested by comparing the $K_{m,NAD}$ of pApApA^p with that of pApA^{p}pA, since the $K_{m,RNA}$ of pApApA^p is 270-fold higher than that of pApA^{p}pA. In the presence of subsaturating concentrations of the two RNAs (11 μM pApApA^p and 105 nM pApA^{p}pA) the observed $K_{m,NAD}$ is 660 μM with pApApA^p, and 100 μM with pApA^p-

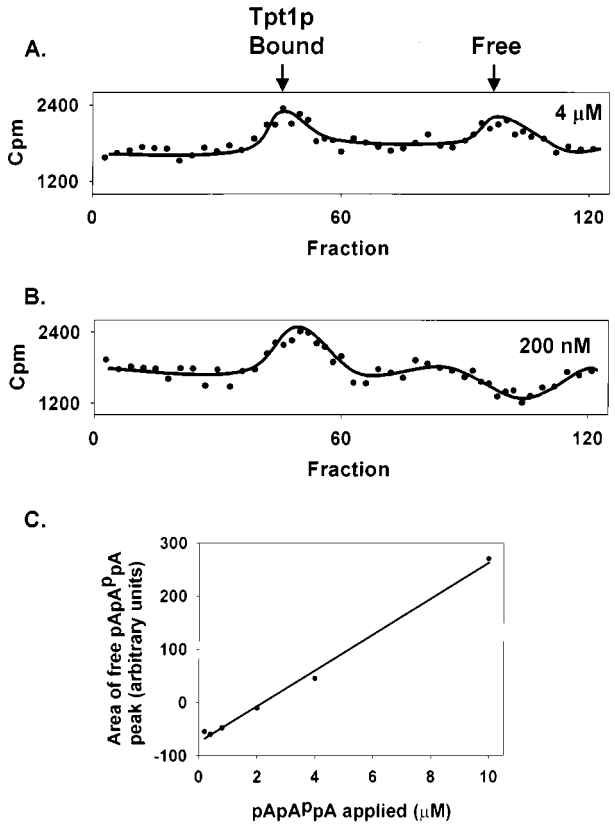

FIGURE 5: Equilibrium gel filtration of Tpt1 protein with pApA^{p}pA. (A) A 100 μL reaction mixture containing 3.25 μM Tpt1 protein and 4 μM pApA^{p}pA was applied to a Sephadex G-75 column equilibrated in buffer containing 200 nM pApA^{p}pA, and resolved in the same buffer, as described in Materials and Methods. Fractions of 250 μL were collected, 150 μL of which was counted. (B) Same as in panel A, but with 200 nM pApA^{p}pA in the incubation mixture. (C) A plot of the area of the free pApA^{p}pA peak (or valley) versus the concentration of pApA^{p}pA in the reaction mixture loaded on the column.

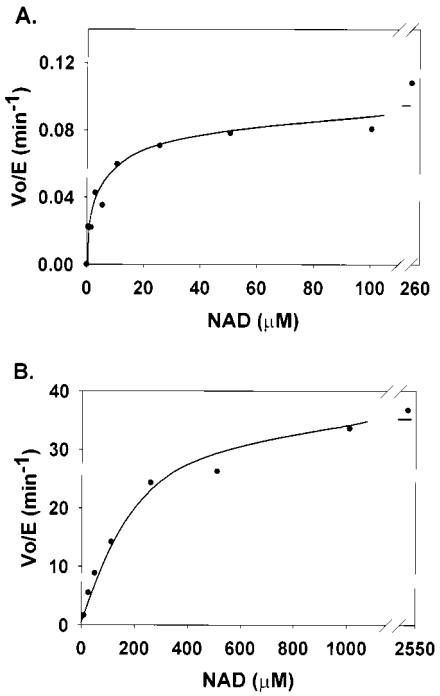

FIGURE 6: Effect of substrate RNA on kinetics of reaction with Tpt1 and NAD. (A) Kinetics of Tpt1 reaction with ligated tRNA and varying concentrations of NAD. Reaction mixtures of 10 μL contained 1.1 nM ligated tRNA substrate, 42.5 pM Tpt1 protein and NAD as indicated, and were incubated for 30 min at 30 °C and analyzed. (B) Kinetics of Tpt1 reaction with pApA^{p}pA and varying concentrations of NAD. Reaction mixtures of 10 μL contained 3.5 μM pApA^{p}pA substrate, 42.5 pM Tpt1 protein, and NAD as indicated, and were incubated for 30 min at 30 °C and analyzed.

pA (data not shown). Thus, although the $K_{m,NAD}$ appears generally higher with RNA substrates with higher $K_{m,RNA}$ values, the nature of the cooperativity observed for the $K_{m,NAD}$ is not immediately obvious.

KptA Protein Is Similar to Tpt1 in Its Kinetic and Substrate Specificity Properties. We have also done a preliminary analysis of the *E. coli* homologue of Tpt1, called KptA. KptA, like Tpt1, transfers the splice junction 2′-phosphate of ligated tRNA to NAD in vitro and can substitute for the essential function of Tpt1 in yeast, but there is no known 2′-phosphorylated RNA in *E. coli* that might be a substrate (*25*). To test further for functional homology between KptA and Tpt1, we initiated an analysis of KptA specificity and kinetics.

We report the kinetic parameters for KptA in terms of V_{max}/protein rather than k_{cat}, because visual inspection of silver stained fractions indicated that KptA was only approximately 10% pure, and there is no independent gauge of the fraction of the total protein that is active. KptA kinetic parameters were determined at 1 mM NAD, which is the $K_{m,NAD}$ with ligated tRNA and not at a saturating concentration of NAD. This was done to limit the amount of intermediate formed during the course of reactions. Unlike Tpt1 protein, KptA protein accumulates significant amounts of this intermediate (RNA that is ADP-ribosylated on the 2′-phosphate) with high concentrations of NAD (*21*; S. Spinelli and E. Phizicky unpublished observations). The limited amount of intermediate that does accumulate at 1 mM NAD is quantitated as product in our treatment of the data. We note that independent measurements of kinetic parameters of KptA for 4 RNAs at 20 mM NAD altered the results by less than a factor of 4, and did not alter the trend (see Table 3).

Table 3: Kinetic Values at 37 °C for KptA Protein and Various RNA Substrates

substrate	K_m (nM)	V_{max}/protein (fmol/min/ng) @ 1 mM NAD (@ 20 mM NAD)	V_{max}/K_m/protein ($l{-}g^{-1}\ s^{-1}$) at 1 mM NAD
ligated tRNA^{PHE}	4 ± 2	0.26 (0.60)	1.1
octadecamer	150 ± 30	99 (220)	11
octamer	250 ± 100	170 (670)	11
trimer, p*ApA^{p}pA	700 ± 300	280 (510)	6.7
p*UpU^{p}pU	1800	1030	9.5
p*ApApA^{p}	ND[a]	ND	ND
p*AAA^{p}p	1300 ± 200	1.4	0.018
p*AAA^{p}pOCH$_3$	3800 ± 1000	45	0.2

[a] ND, not detected.

The kinetic parameters of partially purified KptA are listed in Table 3. They are strikingly similar to those with Tpt1 protein, in three ways: First, the K_m values for RNA substrates observed with KptA are similar in value and vary in the same way as those seen with Tpt1; thus, for KptA the lowest K_m is obtained with ligated tRNA substrate (4 vs 0.36 nM with Tpt1), and progressively higher K_m values are

observed with octadecamer, 8-mer, pApA^{p}pA trimer and then pUpU^{p}pU (Table 3). Second, the increase in K_m as the size and structural complexity of the RNA decreases is accompanied by a corresponding increase in V_{max}/protein. Third, KptA protein does not detectably transfer a terminal 5′-, 3′-, or 2′-phosphate, but an RNA with a terminal 2′-phosphate adjacent to a phosphate or methoxyphosphate is a much improved substrate (Table 3). No dephosphorylation of pApApA^{p} (or of pApApAp) is detected during 30-min reactions, even with large amounts of KptA protein (0.36 μg, as compared to 0.58 ng of protein used for pApA^{p}pA) and NAD at 20 mM in the reactions (as compared to 1 mM).

We note that the estimated k_{cat} value of KptA for ligated tRNA is about 0.065 min^{-1} with NAD at 1 mM, and 0.15 min^{-1} with NAD at 20 mM, based on our estimate of 10% purity of the protein. Thus, our reactions with saturating amounts of ligated tRNA yielded 2 (or 4.5) turnovers at low (or high) NAD concentrations, assuming all of the KptA protein in the preparation is active.

DISCUSSION

The data presented here begins to define elements of the RNA and of the chemical environment around the 2′-phosphate that are important for Tpt1 protein recognition and activity. Tpt1 protein has a low $K_{m,RNA}$ (0.35 nM) and a low k_{cat} (0.1−0.3/min) with its known physiological substrate, 2′-phosphorylated ligated tRNA (Table 1). Examination of a battery of RNAs shows two additional features of Tpt1 recognition. First, Tpt1 efficiently uses smaller and less structured RNAs with an internal 2′-phosphate; however, as the substrate becomes shorter and less structured, both the $K_{m,RNA}$ and k_{cat} increase in nearly corresponding amounts (Table 1). Thus, the 2′-phosphorylated octadecamer, which resembles the anticodon stem−loop of tRNA^{TYR} with a splice junction 2′-phosphate, is kinetically very similar to ligated tRNA ($K_{m,RNA}$ 5-fold higher and k_{cat} 8-fold higher), and the trimer pApA^{p}pA has a 430-fold higher $K_{m,RNA}$ and a 150-fold higher k_{cat} value but still has only a marginal change in $k_{cat}/K_{m,RNA}$. Second, Tpt1 protein is highly specific for RNAs with an internal 2′-phosphate (Table 2). The terminal 2′-phosphate of pApApA^{p} is removed with only 0.003% of the catalytic efficiency of the internal 2′-phosphate of ApA^{p}pA; however, the addition of a flanking phosphate or methoxyphosphate drastically increases $k_{cat}/K_{m,RNA}$ to within 20-fold of the value for pApA^{p}pA. Additionally, Tpt1 protein does not detectably remove terminal 5′- or 3′-phosphates.

This extreme substrate specificity for RNAs with internal 2′-phosphates is consistent with the known role of Tpt1 protein, removal of splice junction 2′-phosphates from ligated tRNAs generated by tRNA ligase during splicing (*14*, *15*), as demonstrated previously by investigation of conditional *tpt1* mutants (*20*). The $K_{m,RNA}$ of 0.35 nM with ligated tRNA indicates that Tpt1 protein finds its cellular substrate through its high apparent affinity for ligated tRNA, although the significant inhibition with pre-tRNA and tRNA (IC_{50} = 25 nM) demonstrates that these molecules could modulate function in vivo. The $K_{m,RNA}$ for ligated tRNA substrate is in the same range as the K_D of 0.1 nM of tRNA ligase for tRNA half-molecules (*36*) and somewhat lower than the K_m of 30 nM of tRNA endonuclease for pre-tRNA substrates (*28*) and $K_{m,RNA}$ of 350 nM of yeast phenylalanine tRNA synthetase for its cognate tRNA (*37*). Since Tpt1 protein has a comparable low $K_{m,RNA}$ for other RNAs with an internal 2′-phosphate (150 nM for a trimer, and 20 nM for an octamer), it seems possible that Tpt1 protein could also function to remove the 2′-phosphate from *HAC1* mRNA after splicing by Ire1 endonuclease and tRNA ligase (*22*−*24*).

The direct correspondence between k_{cat} and K_m for RNA substrates (see Table 1) may imply that product release is rate limiting, as observed for the catalytic component of RNase P (*38*, *39*) and the Tetrahymena ribozyme (*40*, *41*). In this case, the k_{cat} would reflect the off-rate of the enzyme•product complex. Alternatively, there may be another rate-limiting step such as chemistry when structured RNAs such as tRNA are employed. We noted above that the measured turnover rate is not the result of a large fraction of inactive enzyme, since our equilibrium gel filtration binding experiments indicate that 60% of the Tpt1 can bind RNA. This result is consistent with the independent observation with a Tpt1 variant enzyme that at least 75% of the enzyme is kinetically active (M. Steiger and E. Phizicky, unpublished observation).

The data suggest a significant contribution of both the 2′-phosphate and the tRNA to recognition and binding by Tpt1 protein. tRNA is presumed to be important because pre-tRNA and *E. coli* tRNA^{Phe} have IC_{50} values of 10−25 nM, about 3000-fold less than the IC_{50} of the nonphosphorylated octamer (50 μM). Similarly the 2′-phosphate is important for recognition based on the 2000-fold difference between the IC_{50} of the nonphosphorylated octamer (50 μM) and of the 2′-phosphorylated octamer (25 nM, which is similar to its $K_{m,RNA}$ of 20 nM). An additional level of complexity within the Tpt1 reaction mechanism is suggested by the cooperativity indicated by the differences in $K_{m,NAD}$ with ligated tRNA, pApA^{p}pA, or pApApA^{p} (Figure 6). At this time, the relationship between Tpt1 protein−RNA binding and Tpt1 protein−NAD binding is unclear.

The initial analysis of KptA protein deepens the mystery of its role in bacteria (*25*). KptA is remarkably similar to Tpt1 protein in its low K_m for tRNA, the similarity of $V_{max}/K_{m,RNA}$/protein values as the $K_{m,RNA}$ rises 200−450-fold with 2′-phosphorylated trimers, and the strong preference for an RNA substrate with an internal 2′-phosphate rather than a terminal 5′-, 3′-, or 2′ -phosphate. Since KptA does not detectably act on RNAs phosphorylated at positions other than an internal 2′ position, and since there is no obvious ligase or other enzyme to generate a 2′-phosphorylated RNA substrate in *E. coli*, its role in bacteria remains enigmatic. In addition to RNAs, possible substrates of KptA protein include other phosphorylated small molecules or proteins, and the reaction catalyzed by KptA in the cell could be synthesis of Appr>p as observed for tRNA splicing (*18*), or ADP-ribosylation, as observed in the first step of the phosphotransferase reaction (*21*).

ACKNOWLEDGMENT

We thank Jane Jackman, Elizabeth Grayhack, Sherry Spinelli, and Mark Dumont for valuable discussions during the course of this work and critical comments on the manuscript.

REFERENCES

1. Wolin, S. L., and Matera, A. G. (1999) *Genes Dev. 13*, 1−10.

2. Bertrand, E., Houser-Scott, F., Kendall, A., Singer, R. H., and Engelke, D. R. (1998) *Genes Dev. 12*, 2463−8.
3. Yoo, C. J., and Wolin, S. L. (1997) *Cell 89*, 393−402.
4. Aebi, M., Kirchner, G., Chen, J. Y., Vijayraghavan, U., Jacobson, A., Martin, N. C., and Abelson, J. (1990) *J. Biol. Chem. 265*, 16216−20.
5. Bjork, G. R. (1995) in *tRNA: Structure, Biosynthesis and Function* (Soll, D., and RajBhandary, U. L., Eds.) pp 165−205, ASM Press, Washington, D. C.
6. Grosjean, H., Sprinzl, M., and Steinberg, S. (1995) *Biochimie 77*, 139−41.
7. Lund, E., and Dahlberg, J. E. (1998) *Science 282*, 2082−5.
8. Azad, A. K., Stanford, D. R., Sarkar, S., and Hopper, A. K. (2001) *Mol. Biol. Cell 12*, 1381−92.
9. Abelson, J., Trotta, C. R., and Li, H. (1998) *J. Biol. Chem. 273*, 12685−8.
10. van Tol, H., and Beier, H. (1988) *Nucleic Acids Res. 16*, 1951−66.
11. Peebles, C. L., Gegenheimer, P., and Abelson, J. (1983) *Cell 32*, 525−36.
12. Ho, C. K., Rauhut, R., Vijayraghavan, U., and Abelson, J. (1990) *EMBO J. 9*, 1245−52.
13. Trotta, C. R., Miao, F., Arn, E. A., Stevens, S. W., Ho, C. K., Rauhut, R., and Abelson, J. N. (1997) *Cell 89*, 849−58.
14. Greer, C. L., Peebles, C. L., Gegenheimer, P., and Abelson, J. (1983) *Cell 32*, 537−46.
15. Phizicky, E. M., Consaul, S. A., Nehrke, K. W., and Abelson, J. (1992) *J. Biol. Chem. 267*, 4577−82.
16. Belford, H. G., Westaway, S. K., Abelson, J., and Greer, C. L. (1993) *J. Biol. Chem. 268*, 2444−50.
17. McCraith, S. M., and Phizicky, E. M. (1991) *J. Biol. Chem. 266*, 11986−92.
18. Culver, G. M., McCraith, S. M., Zillmann, M., Kierzek, R., Michaud, N., LaReau, R. D., Turner, D. H., and Phizicky, E. M. (1993) *Science 261*, 206−8.
19. Culver, G. M., McCraith, S. M., Consaul, S. A., Stanford, D. R., and Phizicky, E. M. (1997) *J. Biol. Chem. 272*, 13203−10.
20. Spinelli, S. L., Consaul, S. A., and Phizicky, E. M. (1997) *RNA 3*, 1388−400.
21. Spinelli, S. L., Kierzek, R., Turner, D. H., and Phizicky, E. M. (1999) *J. Biol. Chem. 274*, 2637−44.
22. Sidrauski, C., Cox, J. S., and Walter, P. (1996) *Cell 87*, 405−13.
23. Sidrauski, C., and Walter, P. (1997) *Cell 90*, 1031−9.
24. Gonzalez, T. N., Sidrauski, C., Dorfler, S., and Walter, P. (1999) *EMBO J. 18*, 3119−32.
25. Spinelli, S. L., Malik, H. S., Consaul, S. A., and Phizicky, E. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14136−41.
26. McCraith, S. M., and Phizicky, E. M. (1990) *Mol. Cell. Biol. 10*, 1049−55.
27. Zillman, M., Gorovsky, M. A., and Phizicky, E. M. (1992) *J. Biol. Chem. 267*, 10289−94.
28. Reyes, V. M., and Abelson, J. (1987) *Anal. Biochem. 166*, 90−106.
29. Harrington, K. M., Nazarenko, I. A., Dix, D. B., Thompson, R. C., and Uhlenbeck, O. C. (1993) *Biochemistry 32*, 7617−22.
30. Kierzek, R., Steiger, M. A., Spinelli, S. L., Turner, D. H., and Phizicky, E. M. (2000) *Nucleosides, Nucleotides Nucleic Acids 19*, 917−33.
31. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−54.
32. Swerdlow, H., and Guthrie, C. (1984) *J. Biol. Chem. 259*, 5197−207.
33. Lee, M. C., and Knapp, G. (1985) *J. Biol. Chem. 260*, 3108−15.
34. Tyc, K., Kellenberger, C., and Filipowicz, W. (1987) *J. Biol. Chem. 262*, 12994−3000.
35. Gegner, J. A., and Dahlquist, F. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 750−4.
36. Apostol, B. L., and Greer, C. L. (1991) *Nucleic Acids Res. 19*, 1853−60.
37. Sampson, J. R., DiRenzo, A. B., Behlen, L. S., and Uhlenbeck, O. C. (1989) *Science 243*, 1363−6.
38. Reich, C., Olsen, G. J., Pace, B., and Pace, N. R. (1988) *Science 239*, 178−81.
39. Beebe, J. A., and Fierke, C. A. (1994) *Biochemistry 33*, 10294−304.
40. Herschlag, D., and Cech, T. R. (1990) *Biochemistry 29*, 10159−71.
41. Young, B., Herschlag, D., and Cech, T. R. (1991) *Cell 67*, 1007−19.

BI011388T