Critical Aspartic Acid Residues in Pseudouridine Synthases*

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The pseudouridine synthases catalyze the isomerization of uridine to pseudouridine at particular positions in certain RNA molecules. Genomic database searches and sequence alignments using the first four identified pseudouridine synthases led Koonin (Koonin, E. V. (1996) Nucleic Acids Res. 24, 2411–2415) and, independently, Santi and co-workers (Gustafsson, C., Reid, R., Greene, P. J., and Santi, D. V. (1996) Nucleic Acids Res. 24, 3756–3762) to group this class of enzyme into four families, which display no statistically significant global sequence similarity to each other. Upon further scrutiny (Huang, H. L., Pookanjanatavip, M., Gu, X. G., and Santi, D. V. (1998) Biochemistry 37, 5135–5141), the Santi group discovered that a single aspartic acid residue is the only amino acid present in all of the aligned sequences; they then demonstrated that this aspartic acid residue is catalytically essential in one pseudouridine synthase. To test the functional significance of the sequence alignment in light of the global dissimilarity between the pseudouridine synthase families, we changed the aspartic acid residue in representatives of two additional families to both alanine and cysteine: the mutant enzymes are catalytically inactive but retain the ability to bind tRNA substrate. We have also verified that the mutant enzymes do not release uracil from the substrate at a rate significant relative to turnover by the wild-type pseudouridine synthases. Our results clearly show that the aligned aspartic acid residue is critical for the catalytic activity of pseudouridine synthases from two additional families of these enzymes, supporting the predictive power of the sequence alignments and suggesting that the sequence motif containing the aligned aspartic acid residue might be a prerequisite for pseudouridine synthase function.

All organisms chemically modify their RNA after transcription, and the isomerization of uridine to its C-glycoside isomer pseudouridine (Ψ) is the most prevalent modification, Fig. 1 (1). This isomerization is catalyzed by the pseudouridine synthases, enzymes that display specificity for U residues at particular positions in certain RNA molecules, a specificity that can range from handling a single specific site to mild promiscuity (2–7). Physiological ramifications resulting from the lack of Ψ at particular locations have recently become evident, mandating a fuller understanding of Ψ generation in particular and RNA modification generally.

In Escherichia coli, severe growth inhibition results from disruption of rluD (formerly denoted sfhB or yflI), which encodes the Ψ synthase responsible for isomerization of U residues at positions 1911, 1915, and 1917 of 23 S RNA (6, 7). In eukaryotes, Steitz and co-workers (8) have elegantly demonstrated that the presence of Ψ in the U2 small nuclear RNA is required for proper assembly of the spliceosome, work that relied on the inhibition of the responsible Ψ synthase(s) by U2 transcripts containing 5-fluorouridine. Such inhibition of Ψ synthases by RNA containing 5-fluorouracil is well preceded (9–11), and this inhibition may account for a secondary mode of action of the anticancer drug 5-fluorouracil, which primarily acts by inhibiting thymidylate synthase (12). Consistent with 5-fluorouracil cytotoxicity resulting from Ψ synthase inhibition is a long string of observations concerning cell lines treated with both 5-fluorouracil and thymidine (eliminating the need for thymidylate synthase). This treatment affects many RNA-mediated events, including disruption of rRNA maturation (13, 14), disruption of pre-mRNA splicing (15–17), and, perhaps, reducing translational accuracy (18). The link is thus established between Ψ synthases and critical RNA-mediated cellular processes, the disruption of which can lead to dire consequences.

One such consequence is likely the X-linked human disease dyskeratosis congenita. Young men suffering from this disease have blotchy skin, poor dental health, sparse hair (including a lack of eyebrows), and evanescent nails; these men also tend to develop gastrointestinal tumors and suffer bone marrow failure (19). The gene responsible for dyskeratosis congenita has recently been identified and encodes a protein dubbed dyskerin, which contains a nuclear localization sequence and has two stretches of amino acids highly similar to the E. coli Ψ synthase TruB (20). Although the detected similarity to TruB is in itself rather weak evidence for concluding that dyskerin is a Ψ synthase, the sequence alignments of the known Ψ synthases support that assessment (see below), and probing the functional significance of these alignments was the purpose of the experiments described in this communication. A brief presentation of the alignments is, therefore, imperative before recounting and discussing the experiments.

After Penhoet and co-workers (9) and Ofengand and co-workers (2–4) cloned the first four Ψ synthase genes, both Koonin (21) and Santi and co-workers (22) undertook alignments and data base searches with these Ψ synthases. Both studies found statistically insignificant identity between the four proteins over their entire length, but both studies also
Critical Aspartic Acid Residues in Ψ Synthases

FIG. 1. a, isomerization of U to Ψ in RNA. b, U residues in E. coli tRNA<sup>Ψ</sup><sub>Ψ</sub> isomerized to Ψ by the Ψ synthases TruA, TruB, and RluA.

Fig. 2. One region of similarity shared by the first four cloned Ψ synthases (RsuA, TruA, RluA, and TruB) and dyskerin (20), adapted from Koonin (21) and Gustafsson et al. (22). The D denotes the critical aspartic acid residue noted by Huang et al. (7). Note that the glycine five amino acids downstream of the D is not present in all identified homologs of these proteins. The numbering excludes His<sub>6</sub> tags added to the N terminus to simplify purification.

TruA (Asp-60) was mutated to alanine, asparagine, glutamate, serine, and lysine, and all of the mutant TruA proteins were catalytically inactive although still able to bind tRNA with near wild-type affinity (27). The critical catalytic participation of the aligned aspartic acid residue strongly supports the functional significance of the aligned sequence motifs. Fig. 2 shows the region, which Koonin (21) named motif II, containing the aligned aspartic acid residue, including the first four cloned Ψ synthases and dyskerin, which shares strong similarity with TruB in this region (20). Interestingly, this motif was also identified in both deoxyxycytidine triphosphate deaminase (catalyzes dCTP → dUTP) and deoxyuridine triphosphatase (catalyzes dUTP → dUMP). Based on this observation, Koonin hypothesized that this stretch of amino acids was involved in uridine binding (21), which appears to conflict with the ability of the TruA mutants to bind tRNA (27). This seeming contradiction between prediction and experimental results and the weak similarity between the four families of Ψ synthases dictated further testing of the alignment generally and the conserved aspartic acid residue specifically. To this end, we undertook the mutation of the aligned aspartic acid residues in two other Ψ synthases of different families, RluA and TruB.

EXPERIMENTAL PROCEDURES

General—Hen egg white lysozyme was purchased from Sigma. Activated charcoal (Norit SA-3) was purchased from Aldrich. Nucleoside triphosphates and competent JM109(DE3) E. coli cells were purchased from Promega Corp. (Madison, WI). The restriction enzyme BstNI was purchased from New England Biolabs (Beverly, MA). Isopropyl-β-D-thiogalactopyranoside, HEPES, and Tris were purchased from Roche Molecular Biochemicals. Oligonucleotides were purchased from the Great American Gene Company (Ramona, CA). QuikChange™ site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA). [5-3H]UTP was purchased from Amersham Pharmacia Biotech. Prime RNase inhibitor was purchased from Promega Corp. (Madison, WI). The restriction enzyme BstNI was purchased from New England Biolabs (Beverly, MA). Isopropyl-β-D-thiogalactopyranoside, HEPES, and Tris were purchased from Roche Molecular Biochemicals. Oligonucleotides were purchased from the Great American Gene Company (Ramona, CA). QuikChange™ site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA).

Plasmids—The plasmid p67CF23, which contains the gene for E. coli tRNA<sup>Ψ</sup><sub>Ψ</sub> behind a T7 promoter, was a generous gift from O. Uhlenbeck (28). Plasmids containing the genes truB (2) and rluA (3) in PET15b vectors were generously provided by J. Ofengand. Because the reports of these plasmids do not assign them names, they will be referred to as p<sup>Ψ</sup>55 (containing truB) and p<sup>Ψ</sup>746 (containing rluA). The plasmid p<sup>Ψ</sup>77-911Q for expression of bacteriophage T7 RNA polymerase gene was a gift from T. Shraer, and the overexpressed T7 RNA polymerase was isolated as described (29). All of these overexpressed proteins have a His<sub>6</sub> tag fused to their N terminus to simplify purification.

Reexamination of the sequence data by Santi and co-workers (27) led to the insight that among all of the Ψ synthases and their identified homologs, a single residue, an aspartic acid, is found aligned in all sequences. This aspartic acid residue in
nal cycling program. Because the Robocycler moves sample tubes between heating blocks maintained at different temperatures, additional time is required for the sample to achieve thermal equilibrium than with a single block thermal cycler (in which the sample temperature equilibrates as the block is adjusted to the new temperature). The final temperature profile used was: incubating overnight against 20 mM triethanolamine buffer, pH 8.1, containing NTPs (4 mM each), MgCl₂ (24 mM), spermidine (1 mM), NaCl (300 mM) and imidazole (3 mM). All subsequent steps were performed in 50 mM sodium phosphate buffer (10 ml), pH 8.0, containing 0.5% Triton X-100 (0.01%), Prime RNase inhibitor (125 units), GMP (16 mM), and T7 RNA polymerase (0.1 mg/ml). After 8 h at 37 °C, the lysate was centrifuged for 3 min), the pelleted resin was resuspended in the same volume of buffer initially used for cell resuspension and packed into a column. The column was washed (three times, 7 ml each) with 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM NaCl (final solution, 300 mM; final [RNA] = 10 mM). After 10 min at room temperature, an aliquot of [5-3H]UTP (0.1 mM) was added to 1 ml of a concentrated solution of a [5-3H]uridine (0.54 mCi/mmol) for UTP (27). This [5-3H]RNA was typically diluted with unlabeled tRNA transcript to afford [5-3H]RNA of suitable specific activity for the Ψ synthase assays.

Ψ Synthase Activity Assay—The assay for Ψ synthase activity was a slight modification of the tritium release assay reported by Nurse et al. (2), which measures the liberation of tritium from C5 when labeled U in tRNA is isomerized to C5H4U, which measures the liberation of tritium from C5 when labeled U in tRNA is transaminated to form [5-3H]uridine. After 5 min at 37 °C, reaction was initiated by addition of a small volume (<5 μl) of a concentrated solution of Ψ synthase (final concentration, 20 nm to 2 μl). Aliquots (95 μl) were removed periodically (30 s to 30 min) and quenched by dilution into 0.1 x HCl (1 ml) containing Norit-SA3 (12% w/v). Mixtures were centrifuged for 5 min at maximum speed in a microcentrifuge, and the supernatants were filtered through a plug of glass wool. The pellet was washed twice by resuspension in 0.1 x HCl (1 ml), followed by centrifugation. The supernatants from these washes were separately passed through the glass wool plug and combined with the supernatants from the first wash. An aliquot (1 ml) of the combined filtrate was mixed with Scintisafe Econo 2 scintillation fluid (10 ml) and subjected to scintillation counting.

Filter Binding Assay to Determine Dissociation Constants of tRNA from Ψ Synthases—A solution of trnAΨ synthase containing [5-3H]uridine (0.54 μl, 5.55 μCi/mmol tRNA, 16 C/mmol) was added to a solution of Ψ synthase (5–50 μl) in 50 mM HEPES buffer, pH 7.5, containing 100 mM KH₂PO₄ (final solution, 300 mM; final [RNA] = 10 mM). After 10 min at room temperature, an aliquot (95 μl) of each mixture was very slowly filtered through a 25 mm cellulose nitrate membrane filter (0.45 μm; Whatman, Maidstone, United Kingdom), which were prewetted with the HEPES buffer. The filter was rinsed rapidly with 25 mM potassium phosphate buffer, pH 7.4 (two times, 1 ml each). After air-drying, the filter was placed into a scintillation vial with scintillation fluid (5 ml), shaken vigorously, and then counted in a liquid scintillation counter. Each protein concentration provided three filter binding data points, and the computer program GraphPad InPlot was used to plot the data and fit them to the binding curve $e_{\text{ss}} = \frac{[\text{tRNA}]_{\text{max}}}{(e_{\text{ss}} - e_{\text{eq}}) + [\text{tRNA}]_{\text{ss}}}$, where $e_{\text{ss}}$ is the total enzyme concentration, $e_{\text{eq}}$ is the concentration of Ψ synthase bound to enzyme, $e_{\text{ss}}$ is the dissociation constant for the enzyme-tRNA complex. The use of this simplified binding curve is allowed because the concentration of tRNA (10 mM) is 1% of the lowest enzyme concentration (1 μM), so that the free enzyme concentration essentially equals the total enzyme concentration.

Test for Deaminating Activity—An assay mixture was prepared as described above except that the volume was doubled. A mutant Ψ synthase was added (final concentration, 100 nM), and after 30 min, half of the reaction mixture was quenched and processed as described above. To the other half of the reaction mixture, wild-type Ψ synthase was added (final concentration, 100 nM); after an additional 30 min of incubation, the reaction mixture was worked up as described above.

RESULTS

Characterization of the Ψ Synthase Mutants—The site-directed mutagenesis proceeded smoothly, affording the D48A and D48C mutant TruB and D64A and D64C mutant RluA. By virtue of an N-terminal His6 tag, all four mutant enzymes were purified to very near homogeneity by chromatography over a column of Ni-NTA resin. No major differences were noted in the yield, isolation, or storage of the mutant enzymes versus the wild-type enzymes.

Nitrocellulose binding assays (30) were used to probe the ability of the wild-type and mutant Ψ synthases to bind substrate. A small concentration of [3H]tRNA was incubated with an increasing concentration of enzyme, and protein was adsorbed to nitrocellulose filters, which were subjected to liquid scintillation counting to quantitate bound [3H]tRNA. A representative binding curve is shown in Fig. 3. Binding of [3H]tRNA by enzyme plateaus at 70% of the tritium present, which cor-
Critical Aspartic Acid Residues in ψ Synthases

The tritium release assay utilizes charcoal to adsorb tRNA, and any free uracil produced during incubation of a mutant enzyme with tRNA transcript would also be adsorbed. We therefore tested whether or not the mutant enzymes release free uracil at a rate comparable to the rate of glycosidic bond cleavage by the wild-type ψ synthases. Half of an incubation of [5-3H]tRNA with mutant ψ synthase was withdrawn and processed while wild-type ψ synthase was added to the other half, which was processed later. In all cases, the incubation with wild-type enzyme resulted in release of the theoretical maximum amount of tritium from [5-3H]tRNA, as did a control incubation with wild-type enzyme and substrate (Fig. 5).

The mutant ψ synthases described here were generated and characterized to probe the value of the sequence alignments (21, 22) of the first identified ψ synthases. The ready availabil-

**Table I**

| Enzyme       | $K_d$ for [3H]tRNA (μM) |
|--------------|-------------------------|
| TruB wild-type | 8.8 ± 2.0               |
| TruB D48A    | 1.6 ± 0.4               |
| TruB D48C    | 1.3 ± 0.2               |
| RluA wild-type | 4.0 ± 1.1               |
| RluA D64A    | 1.8 ± 0.9               |
| RluA D64C    | 1.2 ± 0.5               |

**Table II**

Activity assays for wild-type and mutant TruB and RluA

Release of tritium from [3H]tRNA (500 nM, 76 Ci/mol U) was used to monitor the reaction. After a 5-min (TruB) or 10-min (RluA) incubation at 37 °C, the reaction was quenched by addition of charcoal in dilute acid. Similar data (not shown) have been obtained at other enzyme and tRNA concentrations and at different tRNA specific activities.

| Identity | Concentration | 3H released | Percentage of wild-type |
|----------|---------------|-------------|-------------------------|
| TruB     | Background    | 0           | 40 ± 4                  |
|          | Wild-type     | 20          | 1098 ± 20               |
|          | D48A          | 20,000      | 38 ± 4                  |
|          | D48C          | 20,000      | 52 ± 4                  |
| RluA     | Background    | 0           | 40 ± 4                  |
|          | Wild-type     | 20          | 592 ± 6                 |
|          | D64A          | 200         | 38 ± 4                  |
|          | D64C          | 200         | 36 ± 4                  |

**Fig. 3.** Representative nitrocellulose filter binding assay to measure the affinity of ψ synthases for [3H]tRNA. The data are for TruB D48C. Each point is the average of three determinations, and the size of the dots approximates the standard deviation. The line is the fit of the data to a binding isotherm. The plateau at 70% tritium bound is consistent with the amount of competent substrate present in this preparation of [3H]tRNA.

**Fig. 4.** Activity assays for wild-type and mutant TruB and RluA. Release of tritium from [3H]tRNA (500 nM, 76 Ci/mol U) was used to monitor the reaction. After a 5 min (TruB) or 10 min (RluA) incubation at 37 °C, the reaction was quenched by addition of charcoal in dilute acid. The concentration of TruB mutants was 200 nM versus 20 nM for wild-type, and the concentration of RluA mutants was 200 nM versus 20 nM for wild-type.
Critical Aspartic Acid Residues in Ψ Synthases

To assess the role of the aligned aspartic acid residue in the highly dissimilar families of Ψ synthases (Fig. 2), Asp-48 in TruB and Asp-64 in RluA were each mutated to alanine and cysteine. Alanine was chosen as a substitution to eliminate all side chain functionality while avoiding potential problems due to the conformational flexibility of glycine. The choice of cysteine was guided by a possible nucleophilic role for the aspartic acid residue, a role that may allow functional substitution of the carboxylate of aspartate by the nucleophilic thiol(ate) of the cysteine side chain. No other mutations were made because a critical role for the aligned aspartic acid residues in TruB and RluA would allow confident extrapolation from the four other synthases (Fig. 5). It can be concluded, therefore, that the aligned aspartic acid residue is, indeed, critical for Ψ synthase function.

The precise role of the critical aspartic acid residues also remains the domain of future experiments. The nitrocellulose binding assay data (Table I, Fig. 3) indicate that the mutant enzymes still bind tRNA efficiently, which argues against a large structural change. However, the CD spectra (data not shown) of the wild-type and mutant enzymes vary in the intensity of the features diagnostic for secondary structure, suggesting that either the folding or, perhaps, the degree of folding is somewhat perturbed by mutation. These differences may be a function of concentration or buffer composition or even arise from differences in interaction between the His6 tag and the rest of the protein, possibilities currently under investigation. Regardless of the cause (structural, catalytic, or both), the results presented here demonstrate that the aligned aspartic acid residues are conserved, critical for Ψ synthase function.

The proposal of Koonin (21) that the motif containing the "conserved" aspartic acid residue constituted a uridine binding sequence suggested an alternate explanation of our observations. If the aspartic acid residue were essential for binding the uracil ring, the mutant enzymes might catalyze glycosidic bond cleavage at a rate unimpaired by mutation but then lose their grip on a putative uracil(ate) intermediate (27) and release it into solution. The free uracil would be adsorbed onto the charcoal during the assay protocol, and no free tritium would be detected (as was observed). Mutation of the aligned aspartic acid, then, would convert a Ψ synthase into a "deuracilase."

To test this hypothesis, [3H]tRNA was incubated with each of the described Ψ synthase mutants. The amount of tritium released by the wild-type enzyme was the theoretical maximum for the amount of substrate present and was identical whether or not the substrate was preincubated with mutant enzymes (Fig. 5). It can be concluded, therefore, that the mu-
tant enzymes do not release uracil(ate) into solution at a rate comparable to glycosidic bond breakage in the wild-type enzymes. The possibility remains that the aligned aspartic acid residue is required for the “specific binding” of substrate tRNA necessary for precise positioning of the isomerized uridine in the active site. In this case, only “nonspecific” interaction with tRNA is being detected by the nitrocellulose binding assays. The results do, however, clearly eliminate the possibility that mutation of the aligned aspartic acid residue converts the $\Psi$ synthases into efficient deuracilases.

The results of this study also strengthen the assignment of $\Psi$ synthase function to dyskerin, the protein the absence of which leads to the human disease dyskeratosis congenita, for the similarity to TruB in another stretch of amino acids that display no statistically significant global sequence similarity to each other. In fact, the presence of the amino acid motif containing the aligned aspartic acid residue has functional significance in two $\Psi$ synthase families other than that represented by TruA, families that display no statistically significant global sequence similarity to each other. In fact, the presence of the amino acid motif may prove useful as a prerequisite for assigning $\Psi$ synthase activity to genes of unknown function.

REFERENCES

1. Grosjean, H., and Benne, R. (1998) *Modification and Editing of RNA*, ASM Press, Washington, D. C.
2. Nurse, K., Wrzesinski, J., Bakin, A., Lane, B. G., and Ofengand, J. (1995) *RNA* 1, 102–112
3. Wrzesinski, J., Nurse, K., Bakin, A., Lane, B. G., and Ofengand, J. (1995) *RNA* 1, 437–448
4. Wrzesinski, J., Bakin, A., Nurse, K., Lane, B. G., and Ofengand, J. (1995) *Biochemistry* 34, 8904–8913
5. Simos, G., Tekotte, H., Grosjean, H., Segref, A., Sharma, K., Tollervey, D., and Hurt, E. C. (1996) *EMBO* J. 15, 2270–2284
6. Raychaudhuri, S., Conrad, J., Hall, B. G., and Ofengand, J. (1998) *RNA* 4, 1407–1417
7. Huang, I. X., Ku, J., Pookanjanatavip, M., Gu, X. R., Wang, D., Greene, P. J., and Santi, D. V. (1998) *Biochemistry* 37, 15851–15857
8. Yu, Y. T., Shu, M. D., and Steitz, J. A. (1998) *EMBO* J. 17, 5783–5795
9. Kammen, H. O., Marvel, C. C., Hardy, L., and Penhoet, E. E. (1988) *J. Biol. Chem.* 263, 2253–2263
10. Samuelsson, T. (1991) *Nucleic Acids Res.* 19, 6139–6144
11. Patton, J. R. (1993) *Biochem. J.* 290, 595–600
12. Santi, D. V., McHenry, C. S., and Sommer, H. (1974) *Biochemistry* 13, 471–481
13. Wilkinson, D. S., and Pitot, H. C. (1973) *J. Biol. Chem.* 248, 63–68
14. Ghoshal, K., and Jacob, S. T. (1994) *Cancer Res.* 54, 652–656
15. Doong, S. L., and Dolnick, B. J. (1988) *J. Biol. Chem.* 263, 4467–4473
16. Sierackowska, H., Shukla, R. R., Dominski, Z., and Kole, R. (1989) *J. Biol. Chem.* 264, 19185–19191
17. Lens, H. J., Manno, D. J., Danenberg, K. D., and Danenberg, P. V. (1994) *J. Biol. Chem.* 269, 31962–31968
18. Boldin, B. J., and Pink, J. J. (1985) *J. Biol. Chem.* 260, 3006–3014
19. Luzzatto, L., and Karadimitris, A. (1998) *Nat. Genet.* 19, 6–7
20. Heiss, N. S., Knight, S. W., Vulliamy, T. J., Klauck, S. M., Wiemann, S., Mason, P. J., Poustka, A., and Dokal, I. (1998) *Nat. Genet.* 19, 32–38
21. Koonin, E. V. (1996) *Nucleic Acids Res.* 24, 2411–2415
22. Gustafsson, C., Reid, R., Greene, P. J., and Santi, D. V. (1996) *Nucleic Acids Res.* 24, 3756–3762
23. Chen, J., and Patton, J. R. (1999) *RNA* 5, 409–419
24. Conrad, J., Sun, D. H., England, N., and Ofengand, J. (1998) *J. Biol. Chem.* 273, 18562–18566
25. Lecointe, F., Simos, G., Sauer, A., Hurt, E. C., Motorin, Y., and Grosjean, H. (1998) *J. Biol. Chem.* 273, 1316–1323
26. Becker, H. F., Motorin, Y., Planta, R. J., and Grosjean, H. (1997) *Nucleic Acids Res.* 25, 4493–4499
27. Huang, I. X., Pookanjanatavip, M., Gu, X. R., and Santi, D. V. (1998) *Biochemistry* 37, 344–351
28. Peterson, E. T., and Uhlenbeck, O. C. (1992) *Biochemistry* 31, 10380–10389
29. Ichetovkin, I. E., Abramochkin, G., and Shrader, T. E. (1997) *J. Biol. Chem.* 272, 33009–33014
30. Arlison, V., HOUNTDJI, C., Robert, B., and Grosjean, H. (1998) *Biochemistry* 37, 7268–7276
31. Foster, P. G., Huang, L. X., Santi, D. V., and Stroud, R. M. (1997) *FASEB J.* 11, A862
32. Corollio, D., Blair-Johnson, M., Conrad, J., Fiedler, T., Sun, D. H., Wang, L., Ofengand, J., and Fenna, R. (1999) *Acta Crystalllogr., Section D* 55, 302–304
33. Watkins, N. J., Gottschalk, A., Neubauer, G., Kastner, B., Fabrizio, P., Mann, M., and Luhrmann, R. (1998) *RNA* 4, 1549–1568