Cell, Vol. 51, 887–898, December 24, 1987, Copyright © 1987 by Cell Press

The Telomere Terminal Transferase of Tetrahymena Is a Ribonucleoprotein Enzyme with Two Kinds of Primer Specificity

Carol W. Greider and Elizabeth H. Blackburn
Department of Molecular Biology
University of California
Berkeley, California 94720

Summary

We have analyzed the de novo telomere synthesis catalyzed by the enzyme telomere terminal transferase (telomerase) from Tetrahymena. Oligonucleotides representing the G-rich strand of telomeric sequences from five different organisms specifically primed the addition of TTGGGG repeats in vitro, suggesting that primer recognition may involve a DNA structure unique to these oligonucleotides. The sequence at the 3′ end of the oligonucleotide primer specified the first nucleotide added in the reaction. Furthermore, the telomerase was shown to be a ribonucleoprotein complex whose RNA and protein components were both essential for activity. After extensive purification of the enzyme by a series of five different chromatographic steps, a few small low abundance RNAs copurified with the activity.

Introduction

Precise recognition of nucleic acids is often carried out by enzymes that contain both RNA and protein components. Ribonucleoproteins (RNPs) have been shown to be involved in protein synthesis, ribosomal RNA processing, mRNA splicing, tRNA maturation, initiation of mitochondrial DNA replication, mitochondrial RNA processing, and phage DNA packaging (reviewed in Nomura et al., 1974; Altman et al., 1982; Padgett et al., 1986; Wong and Clayton, 1986; Chang and Clayton, 1987; Guo et al., 1987). For some of these RNPs, the RNA components provide specificity to the reaction by base pairing with the substrate (Shine and Dalgarno, 1975; Zhuang and Weiner, 1986; Parker et al., 1987). The recognition that RNA can act catalytically has led to the recent increase in the number of known RNP-catalyzed reactions (reviewed in Cech and Bass, 1986). We report here that an RNP is involved in synthesizing the telomeric sequences found at the ends of Tetrahymena macronuclear chromosomes.

Telomere replication poses special problems for the linear chromosomes of eukaryotes. The known biochemical properties of DNA polymerases, 5′ to 3′ polymerization and the requirement for a primer, predict that a linear DNA molecule would lose sequences from the ends with each round of replication (Watson, 1972). Thus, telomeres must utilize special mechanisms to ensure that all genetic information is transmitted with each cell division. Telomeric sequences necessary for the protection and replication of the ends of chromosomes have been defined. Strikingly, many features of these telomeric sequences are conserved between even distantly related organisms. Most notably, the telomeres contain a variable number of tandemly repeated, short G + C rich sequences, the G-rich strand of which is always oriented 5′ to 3′ toward the chromosome terminus (reviewed in Blackburn, 1984).

A number of theoretical models have been proposed to account for the complete replication of telomeres (Cavalier-Smith, 1974; Holmquist and Dancis, 1979; Bateman, 1975; reviewed in Blackburn and Szostak, 1984). Various mechanisms that would account for complete telomere replication have been shown to be utilized by linear eukaryotic viral genomes to overcome the end replication problem (Baroudy et al., 1982; DeLange et al., 1986; reviewed in Stillman, 1983). However, recent molecular data on telomeric structure have shown that a novel mechanism appears to be used by eukaryotic chromosome ends. Several aspects of both the structural and dynamic properties of telomeres led to the proposal that telomere replication involves nontemplated addition of telomeric repeats onto the ends of chromosomes (Shampay et al., 1984). Such nontemplated addition of sequences would allow a dynamic equilibrium to be established, in which chromosome shortening due to incomplete replication is balanced by de novo sequence addition (Shampay and Blackburn, 1987). Some of the properties that led to the proposal of this model include the heterogeneous size of telomeric restriction fragments, the gradual increase in the number of telomeric repeats when trypanosomes and Tetrahymena are kept in log phase growth (Bernards et al., 1983; Larson et al., 1987), and the terminal addition of yeast telomeric sequences when Tetrahymena and Oxytricha telomeres are transformed into yeast (Shampay et al., 1984; Pluta et al., 1984).

We have previously reported the identification of an activity in Tetrahymena cell extracts that adds telomeric repeats onto appropriate telomeric sequence primers in a nontemplated manner. The only substrates required for the reaction are a single-stranded telomeric sequence oligonucleotide, and dGTP plus dTTP. Repeats of the Tetrahymena telomeric sequence TTGGGG are added, 1 nucleotide at a time, onto the 3′ end of the input primer (Greider and Blackburn, 1985). The properties of this telomere terminal transferase, which we abbreviate here telomerase, suggest that it is the enzyme required for telomere elongation in vivo.

As for most reactions involving nucleic acid recognition, the nontemplated addition of telomeric sequences must be a highly specific reaction. We have begun characterizing and purifying the telomerase enzyme in order to investigate the mechanisms controlling the specificity of the reaction. We report here that the telomerase has two distinct specificities: a presumably structural recognition of the G-rich oligonucleotides that prime repeat addition and sequence recognition of the 3′ end of these oligonucleotides. Furthermore, the telomerase is an RNP. We propose that the RNA component(s) of telomerase may play

Table 1. Sequence of Oligonucleotides Tested for the Ability to Prime Elongation

Oligomer 5′→3′	Abbreviation	Prime Repeats? Yes	Prime Repeats? No	Lane
TTGGGG	$(TTGGGG)_1$		X	1
TTGGGGTTGGGG	$(TTGGGG)_2$	X		2
TTGGGGTTGGGGTTGGGG	$(TTGGGG)_3$	X		3
TTGGGGTTGGGGTTGGGGTTGGGG	$(TTGGGG)_4$	X		4
TGTGTGGGTGTGTGGGTGTGTGGG	Yeast	X		5
TTAGGGTTAGGGTTAGGG	$(TTAGGG)_3$	X		6
TTTTGGGGTTTTGGGGTTTTGGGGGTTTTGGGG	$(TTTTGGGG)_4$	X		7
CCCCAAAACCCCAAAACCCCAAAACCCCAAAA	$(CCCCAAAA)_4$		X	8
CCCCAACCCCAACCCCAACCCCAA	$(CCCCAA)_4$		X	9
AAGCTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG	Hind-T_4G_4	X		10
GGAGCCACTATCGACTTCGCGATCATGGCGACCA	pBR-primer		X	11
CCAACCCCAACCCCAACTCAACGGG	Tel1-junct.		X	12
----------	No oligo		X	13
TAACCCCAACTCAATGGGGAACCCGTTGAG	Tel1-end		X	14
GGGAGGAGGGGGGAGAGGGGGGAGGGGGGA	Dictyo 1	X		15
AGGGGGGGAGAGGGGGGAGGGGGG	Dictyo 2	X		16

The nucleotide sequence and the abbreviation used for each of the oligonucleotides assayed in Figure 1 are shown. The ability of the oligonucleotides to prime repeat additions is indicated.

a role in specifying the sequence of the added telomeric TTGGGG repeats, in recognizing the structure of the G-rich telomeric sequence primers, or in both.

Results

A Variety of G-Rich Telomeric Oligonucleotides Prime the Addition of TTGGGG Repeats

We reported previously that single-stranded oligonucleotides corresponding to the telomeric sequences of both Tetrahymena and the yeast Saccharomyces cerevisiae primed the addition of TTGGGG repeats by the telomerase activity of Tetrahymena in vitro (Greider and Blackburn, 1985). To define further the primer requirements for the telomeric elongation reaction, we tested oligonucleotides corresponding to the sequences of the G-rich telomeric strand from five different organisms. Table 1 lists the sequences of the oligonucleotides tested. Figure 1 shows that G strand oligonucleotides from Tetrahymena (lanes 2–4), Saccharomyces (lane 5), Trypanosoma (lane 6), and Oxytricha (lanes 7 and 10) all primed the addition of tandem repeats of the 6 base Tetrahymena telomeric sequence TTGGGG. In the separate experiment shown in Figure 1, lanes 15 and 16, two oligonucleotides with Dictyostelium telomeric sequences also primed addition of TTGGGG repeats in vitro. In Figure 1, lane 15, although the periodicity of the extension products was less marked, large amounts of long products were synthesized. In contrast, several nontelomeric oligonucleotides were inactive as primers (Figure 1, lanes 11, 12, and 14). While the C-rich strand telomeric sequence oligonucleotides did not prime the addition of long tracts of TTGGGG repeats, some [^{32}P]dGTP was incorporated into short products (Figure 1, lanes 8 and 9). At present we do not know the origin of these small products. Finally, no repeats were synthesized in the absence of added oligonucleotide primer (Figure 1, lane 13).

We also tested the ability of the telomerase to recognize Tetrahymena telomeric sequence oligonucleotides of various lengths. Figure 1, lanes 1–4, show that the hexamer d(TTGGGG) was not sufficient to prime the TTGGGG repeat addition, while $d(TTGGGG)_2$ and $d(TTGGGG)_3$ primed repeat synthesis, although with reduced efficiency compared with $d(TTGGGG)_4$. Table 1 summarizes the ability of various oligonucleotides to prime repeat addition in these experiments.

TTGGGG Sequence Addition Is Correctly Initiated at Different 3′ Ends

We showed previously that the distinct 6 base banding pattern seen in our assays is due to preferential pausing or chain termination before T residues. This pattern makes it possible to visualize the TTGGGG sequence added onto the 3′ end of the primer. When the 24 base oligonucleotide with the yeast telomeric sequence shown in Table 1 was used as a primer, an apparent 1 base upward shift in the repeat pattern was seen compared with the repeat pattern primed by the 24 base Tetrahymena oligonucleotide (Figure 1, lanes 4 and 5; Figure 2, lanes 1 and 2; see also Greider and Blackburn, 1985). The 3′ end of the yeast oligonucleotide has a T followed by 3 Gs (. . . . TGGG-OH), while all of the 3′ ends of the Tetrahymena telomeric oligonucleotides shown in Figure 1 have a T followed by 4 Gs (. . . . TGGGG-OH). We predicted that the shift in the banding pattern was due to the addition of an extra G onto the 3′ end of the primer to complete the first TGGGG sequence before additional TTGGGG repeats were added (Greider and Blackburn, 1985). To test whether it is the 3′ end sequence of the yeast primer and not some other feature of this oligonucleotide that causes the observed shift in banding pattern, Tetrahymena sequence oligonucleotides having either 3 or 4 Gs at the 3′ end were used to prime elongation in vitro. Figure 2, lanes 3 and 4, show that the banding pattern of the repeats added to the 18 base Tetrahymena telomeric sequence oligonucleotide $dG(TTGGGG)_2TTGGG$ was shifted upward by 1 base rel-

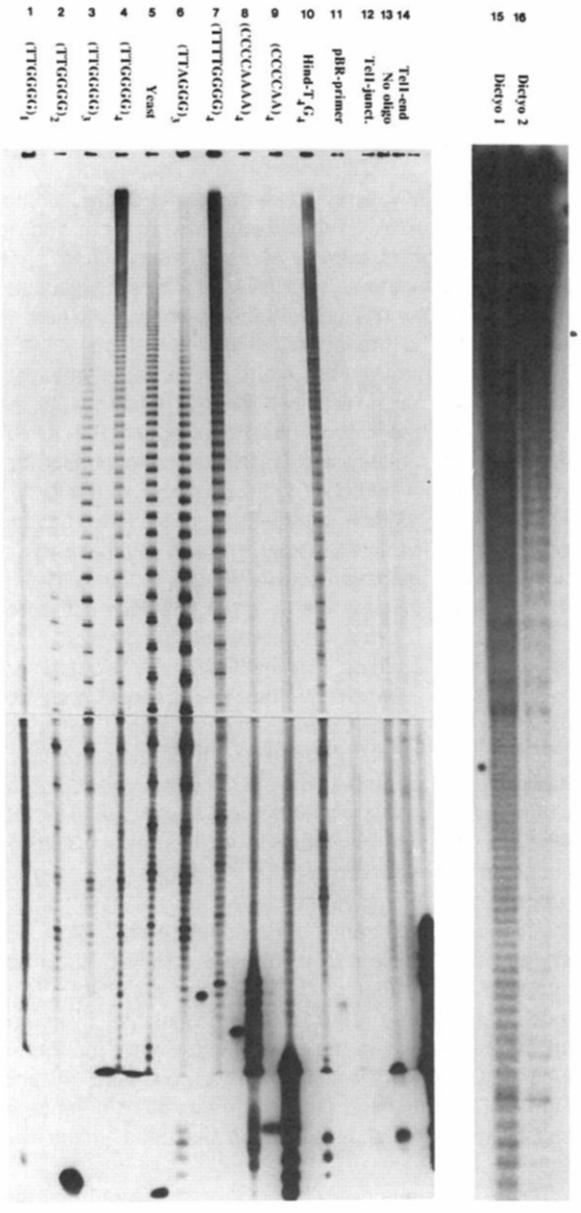

Figure 1. G-Rich Telomeric Sequence Oligonucleotides with Different Primary Sequences Prime Elongation by Telomerase

Each of the indicated oligonucleotides was assayed in the standard assay using telomerase that had been purified over a gel filtration column (see Experimental Procedures). The reactions contained 0.1 μg of oligonucleotide primer. Each of the primers was also 5′ end–labeled with ^{32}P and run in the lane to the left of each reaction to serve as a marker for the size of the input primer. The short products in lane 14 probably result from DNA polymerase activity on the oligonucleotide that has internal secondary structure. The Dictyostelium oligonucleotides shown in lanes 15 and 16 were assayed under the same conditions except that the telomerase used was purified over both gel filtration and heparin agarose columns (see Experimental Procedures). The autoradiograph of the repeat pattern synthesized using the Dictyostelium oligonucleotides was exposed more than 10 times longer than those shown in lanes 1–14.

ative to the pattern synthesized using the 18 base oligonucleotide d(TTGGGG)$_3$. In addition, the telomerase was able to correctly recognize the primer d(GGGGTT)$_3$ so that the banding pattern was shifted 4 bases relative to that primed by d(TTGGGG)$_3$ (Figure 2, lanes 3 and 5).

Finally, when the trypanosome sequence d(TTAGGG)$_3$ was used as a primer (Figure 1, lane 6; Figure 2, lane 6), a 1 base shift was again seen relative to d(TTGGGG)$_3$. This shift is most apparent by comparison of Figure 2, lanes 4 and 6, in which the banding patterns line up. However, in the lower part of the gel it is apparent that the products primed by d(TTAGGG)$_3$ are offset relative to those primed by the other oligonucleotides containing only G and T residues. This slight offset in the banding pattern is due to the fact that the 18 base trypanosome sequence oligonucleotide migrates slightly faster than the corresponding 18 base Tetrahymena sequence oligonucleotide under the conditions used in this experiment (E. Henderson, personal communication). Taking this migration difference into account, the similarity of the patterns in Figure 2, lanes 4 and 6, indicates that the A in the TTAGGG sequence is not recognized as equivalent to the G residue in the sequence TTGGGG. Thus, TTAGGG is equated with TTGGG rather than TTGGGG at the 3′ end of a primer. Taken together, the experiments in Figures 1 and 2 suggest that although a unique primary sequence does not appear to be required for primer recognition, the sequence at the 3′ end of the primer determines the next nucleotides to be added (see Discussion).

Telomerase Has an Apparent Molecular Weight of 200–500 Kd

The results described above suggest that the telomerase shows specificity not only for the sequences added but also for at least two aspects of the primer structure. To study these specificities further, we began biochemical characterization of the enzyme. Crude S100 extracts were first fractionated by gel filtration chromatography. Figure 3 shows a typical sizing column profile. The activity of the S100 starting material is shown in the lane marked Start. Comparing the starting material to the active fractions from this column (fractions 10, 12, 14, and 16), it is apparent that purification leads to an increase in activity, suggesting that inhibitors were being removed from the enzyme. Enzyme activity reproducibly eluted with an apparent molecular weight between 200 and 500 Kd. It is unlikely that the large size was attributable to nonspecific aggregation of proteins, since the same elution profile was seen even when up to 500 mM NaCl was added (data not shown). In the course of purifying the telomerase from crude extracts, we noted a marked sensitivity to salts. Exposure to NaCl, KCl, NH_4Cl, sodium acetate, or potassium phosphate at concentrations above 500 mM inactivated the telomeric elongation activity. In addition, all attempts to precipitate the enzyme with 25% or more ammonium sulfate resulted in the complete loss of activity. Removing the salt by dialysis or gel filtration did not restore activity (data not shown).

RNA and Protein Components Are Required for Telomerase Activity

The salt sensitivity and the large size of the enzyme suggested that the telomerase may be a complex containing

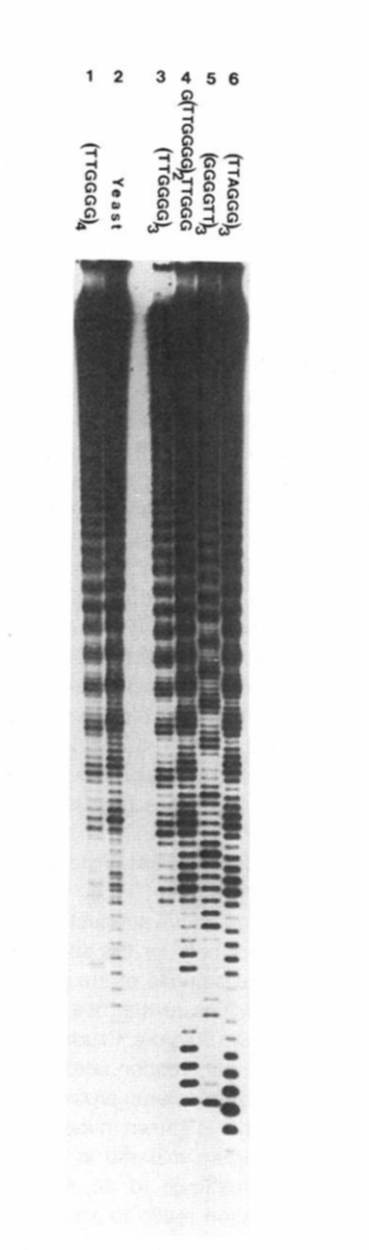

Figure 2. The 3′ End Sequence of the Primer Determines the Next Nucleotides Added

The oligonucleotides indicated above each lane were assayed under standard conditions using 0.1 μg of primer and telomerase that had been purified over both gel filtration and heparin agarose columns. The oligonucleotides used were 5′-TTGGGGTTGGGGTTGGGGTTGGGG-3′ (lane 1), 5′-TGTGTGGGTGTGTGGGTGTGTGGG-3′ (lane 2), 5′-TTGGGGTTGGGGTTGGGG-3′ (lane 3), 5′-GTTGGGGTTGGGGTTGGG-3′ (lane 4), 5′-GGGGTTGGGGTTGGGGTT-3′ (lane 5), and 5′-TTAGGGTTAGGGTTAGGG-3′ (lane 6).

a nucleic acid component. To test whether the telomerase contained an essential nucleic acid, we pretreated active fractions from a gel filtration column with either micrococcal nuclease (MNase) or RNAase A. The nuclease activity of each of these enzymes abolished the telomeric elongation activity. Figure 4A, lane 1, shows the activity of an untreated telomerase fraction. When increasing amounts of MNase were preincubated with telomerase fraction containing EGTA, normal elongation activity was seen (Figure 4A, lanes 3–6). Since MNase requires Ca^{2+}, the nuclease was inactivated under these conditions. In contrast, when active fractions were preincubated in the presence of MNase and Ca^{2+}, no TTGGGG repeats were synthesized (Figure 4A, lanes 7–10). The sensitivity of telomerase to MNase could be due to the degradation of either DNA or RNA. To determine which of these nucleic acids was required, extracts were pretreated with RNAase A alone and, in controls, with RNAase A plus the placental RNAase inhibitor (RNasin). Pretreatment with RNAase A alone destroyed the activity (Figure 4A, lanes 16–18). Pretreatment with RNAase A and RNasin simultaneously did not affect enzyme activity (Figure 4A, lanes 19–22), except at the highest concentration of added RNAase A (lane 22). Presumably, at this RNAase concentration the RNasin was not sufficient to inactivate all of the added RNAase A. In addition, pretreatment of an active fraction with oxidized, inactive RNAase A protein (Sigma) also did not affect the elongation activity (Figure 4A, lanes 11–14). These results indicate that it is the nuclease activity of MNase and RNAase A, and not simply the physical presence of these proteins, that inactivates the telomerase.

The RNAase sensitivity that we observed was not limited to these relatively crude fractions from gel filtration columns. The results of the treatment of telomerase that had been further purified over either two or four different columns (see below) with RNAase A are shown in Figure 4B. The profile of inactivation with increasing RNAase concentration was very similar for these two enzyme preparations (Figure 4B, lanes 1–4 and 9–12). Again, at the highest concentration of RNAase A added, the RNasin was not sufficient to completely inactivate the ribonuclease activity (Figure 4B, lanes 5 and 13). Although the amount of RNAase required to completely inactivate the telomerase activity in the experiments shown in Figures 4A and 4B was not the same (see Figure 4A, lane 16, and Figure 4B, lanes 3 and 11), we attribute this difference in sensitivity to the differing conditions used in the two experiments.

The experiments described above suggested that the telomerase contains an essential RNA component. Alternative interpretations were ruled out by the following control experiments. First, the sensitivity of the telomerase to nucleases could be due to inhibition of the elongation reaction by the products of nuclease digestion. To test this possibility, extensively purified telomerase preparations (see Experimental Procedures) were treated with RNAase A, digestion was stopped with RNasin, and equal amounts of this extract were mixed with untreated samples of the same enzyme preparation. The mixtures were then assayed for telomerase activity. No inhibitory effects of the nuclease-treated extracts on the activity of telomerase were seen (data not shown).

Second, to test whether the absence of the TTGGGG repeat ladder in RNAase-treated extracts was due to the degradation of the telomeric repeats by our RNAase preparation, we treated samples with RNAase A either before or after elongation reactions had been carried out. The telomeric repeats synthesized in vitro were completely

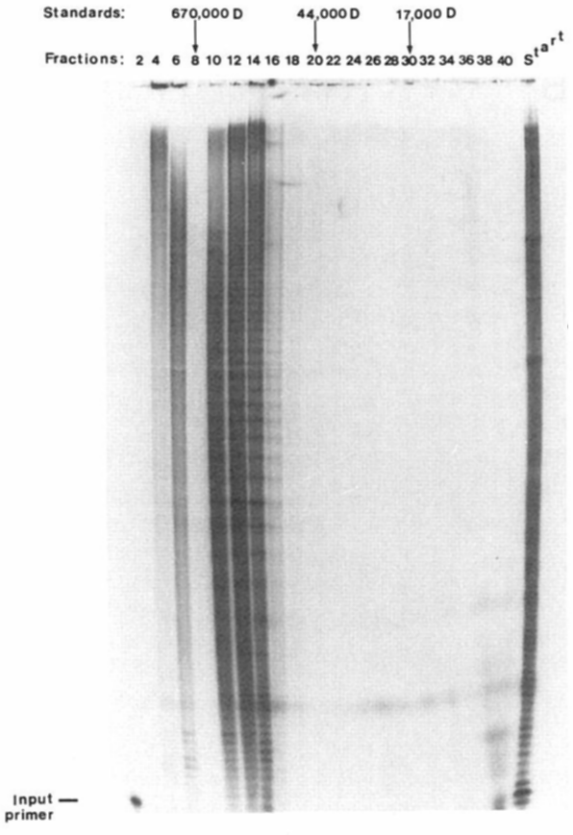

Figure 3. Gel Filtration Chromatography of Telomerase

One milliliter of active S100 fraction was run on a 38 ml Biorad A 5.0 m gel filtration column. Fractions containing 550 μl were collected, and every other fraction was assayed in the standard assay. The elution volume of the molecular weight standards (Biorad) are shown. The proteins used as markers were thyroglobulin (670,000 daltons), ovalbumin (44,000 daltons), and myoglobin (17,000 daltons).

stable to treatment with RNAase A at concentrations similar to those used in Figure 4 (data not shown). We have also confirmed our earlier results (Greider and Blackburn, 1985), that the pretreatment of telomerase with proteinase K inactivates the enzyme (data not shown). We conclude that telomerase contains RNA and protein components that are both essential for activity.

Simple Addition of RNA Does Not Restore Enzyme Activity

The nuclease sensitivity we observed could be due to removal of a specific RNA component of the enzyme or to a nonspecific requirement for the presence of RNA. To distinguish between these two possibilities, extracts were treated with MNase, EGTA was added to inhibit further nuclease activity, and various RNA preparations were added back to the reactions. Figure 5 shows that addition of neither RNA from an active extract purified over two columns (lane 2), total RNA from an S100 fraction of the Tetrahymena extract (lane 3), nor total E. coli tRNA (lane 4) restored the elongation activity of a MNase-treated extract. Figure 5, lane 5, is a control reaction to which no RNA was added. When the same RNA preparations were added to untreated, active extracts, no inhibitory effect of these RNA preparations was seen on the telomerase activity (Figure 5, lanes 6–9). Additional control experiments showed that the RNAs added back to the MNase-treated extracts remained intact throughout the reaction period (data not shown). Thus, simple addition of RNA back to nuclease-treated extracts was not sufficient to restore activity, suggesting that a specific RNA is essential for telomerase activity.

Specific Small RNAs Copurify with the Telomere Terminal Transferase Activity

The large size of the enzyme along with its protease sensitivity and requirement for a specific RNA all suggest that the telomerase is an RNP. To identify the RNA species that reproducibly copurified with the activity, the enzyme was extensively purified. Active fractions pooled from a gel filtration column and stored frozen in liquid nitrogen were further purified by a series of four chromatographic steps. Because the telomerase was somewhat unstable, after thawing the gel filtration column fractions, all columns were routinely run and assayed on the same day. At each step, the salt cuts containing telomerase activity (determined by pilot experiments) were pooled and loaded directly onto the next column. Figure 6A shows the activity profile for the column series: gel filtration, heparin agarose, hydroxylapatite, DEAE agarose, and spermine agarose (see Experimental Procedures). Equal volumes of the eluates from the first four columns and fractions collected from the last column were all assayed in the standard assay. Protein gels of the most purified active fractions showed 10–12 bands by silver staining (data not shown). To identify the RNA species that copurified with the activity, the same column fractions were phenol-extracted and the RNA was post-labeled with [^{32}P]pCp using T4 RNA ligase. The 3′ end–labeled RNAs were then run on a 7 M urea, 6% polyacrylamide gel alongside DNA molecular weight markers (Figure 6B).

In the experiment described above it was evident that only a few of the small RNAs initially present in the active gel filtration fraction (Figure 6B, lane 1) copurified with the telomerase over these five columns (Figure 6B, lane 16). In similar purification experiments the same profile of RNAs was seen to reproducibly copurify with the telomerase activity. Figure 7 shows an experiment in which a different five column series was used (gel filtration, heparin agarose, DEAE agarose, Cibacron-Blue agarose, and spermine agarose). The activity and RNA profiles from the last columns of this series are shown in Figures 7A and 7B, respectively. The activity eluted off the spermine agarose column in fractions 10 and 12 (Figure 7A, lanes 6 and 7), while most of the RNAs initially loaded onto the column eluted in later fractions (Figure 7B, lanes 12, 13, and 14). Because the enzyme is inactivated and we believe it may dissociate at salt concentrations above 500 mM, we have focused our attention on only those RNAs that show a positive correlation with telomerase activity at the lowest salt concentrations. By comparing the two experiments in Figures 6 and 7 and several similar column series (data not shown), it is apparent that the

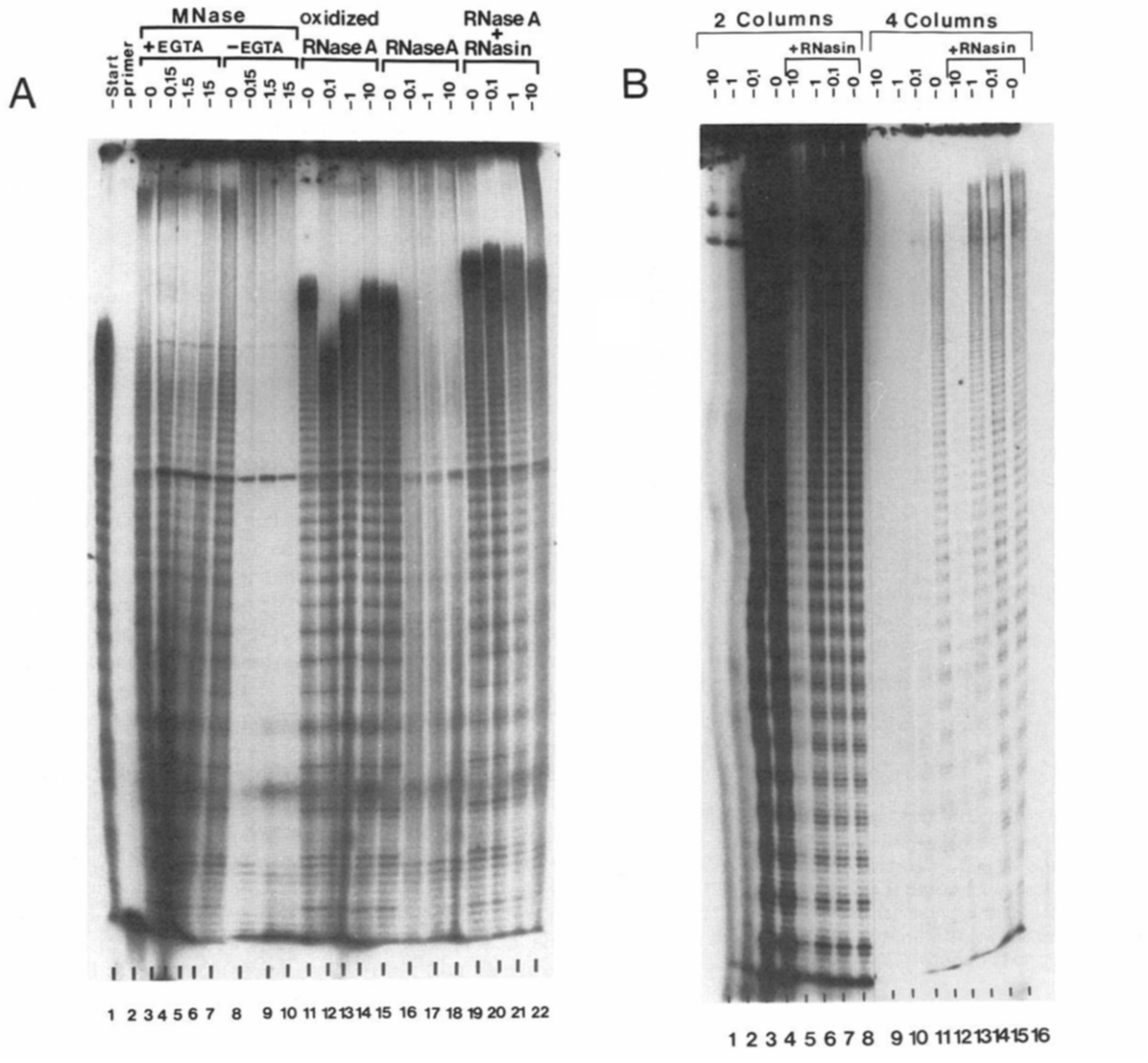

Figure 4. Nuclease Sensitivity of Telomerase

(A) Lane 1 shows activity of the untreated gel filtration fraction used in this experiment. In lane 2, 5′ end–labeled $(TTGGGG)_4$ primer was run as a size marker. Lanes 3–10 show the pretreatment of 20 μl of active telomerase with 0, 0.15, 1.5, or 15 U of MNase in the presence (lanes 3–7) or absence (lanes 8–10) of 10 mM EGTA. Lanes 4–11 show pretreatment with an oxidized, inactive form of RNAase A (Sigma) at 0, 0.1, 1, or 10 μg per ml of telomerase fraction. Lanes 15–22 show the activity of the telomerase after pretreatment with 0, 0.1, 1, or 10 μg per ml (final concentration) of RNAase A in the presence (lanes 19–22) or absence (lanes 15–18) of 100 U of RNasin (Promega Biotec).

(B) Telomerase purified over two columns (gel filtration and heparin agarose) or four columns (gel filtration, heparin agarose, hydroxylapatite, and DEAE agarose) was pretreated with RNAase A at 0, 0.1, 1, or 10 μg/ml, final concentration (lanes 1–4 and 9–12). In lanes 5–8 and 13–16, 100 U of RNasin was added before treatment with the RNAase A. The difference in the concentration of RNAase required to inactivate telomerase activity in this experiment and that shown in (A) may be due to differences in purity of the telomerase and the fact that different RNAase A and RNasin stocks were used.

RNA species running near the DNA markers of 154 and 75 bases are always found in the active fractions (arrowheads on Figure 6B and 7B). While these RNA species were also among those that eluted with higher salt (Figure 7, lanes 12–15), the lack of enzyme activity in these fractions may be attributed to the inhibitory effects of the high salt concentrations in these fractions. From these experiments the RNAs of approximately 154 and 80 nucleotides appear to be the most likely candidates for the RNA component(s) of the telomerase. Preliminary T4 kinase labeling of heparin agarose fraction RNA did not reveal additional RNA species (data not shown). However, additional experiments are necessary to rule out the possibility that other RNAs may be undetected by the RNA labeling conditions.

Discussion

We have investigated the specificities of the telomere terminal transferase (telomerase) of Tetrahymena. Initially

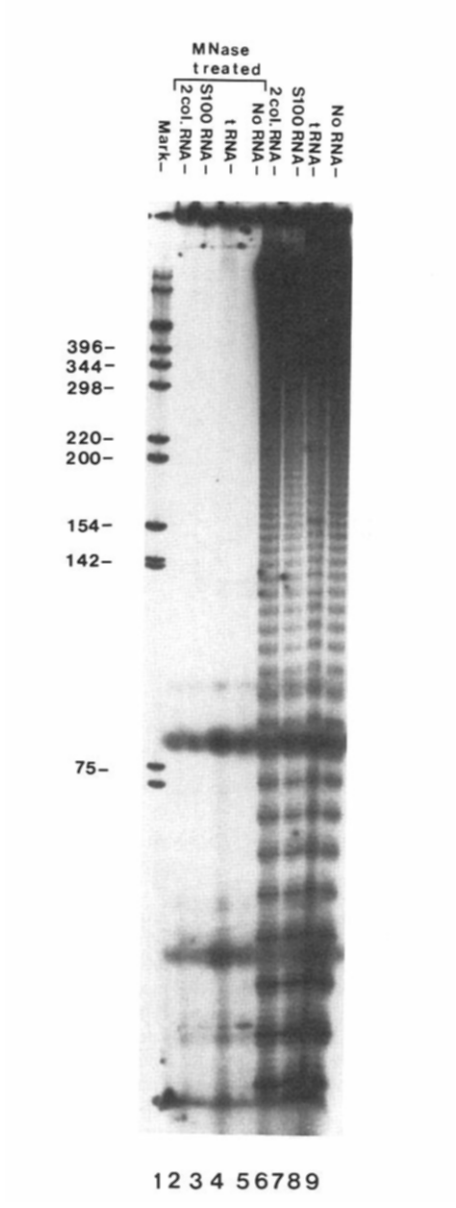

Figure 5. Addition of RNA to MNase-Treated Extracts Does Not Restore Elongation Activity

Active telomerase purified over both gel filtration and heparin agarose columns was either treated with 0.15 U of MNase per μl of extract (lanes 2–5) or incubated in the absence of MNase (lanes 6–9). After inactivation of the MNase with EGTA and addition of 5 mM $MgCl_2$, three different RNA preparations were added to both nuclease-treated and untreated telomerase fractions. The RNAs added were as follows: RNA extracted telomerase purified over both gel filtration and heparin agarose columns (two column RNA) (lanes 2 and 6), RNA prepared from an S100 telomerase fraction (lanes 3 and 7), and total E. coli tRNA (BRL) (lanes 4 and 8). To lanes 5 and 9, DEP-treated H_2O containing no RNA was added. Lane 1 shows molecular weight markers. The sizes of the DNA markers (initially double-stranded) loaded onto the denaturing gel are shown for clarity. The diffuse labeled bands in all the lanes above and below the 75 base marker are contaminants of the [^{32}P]dGTP preparation. They were often seen in the absence of added extract (data not shown).

we defined which sequences would function as oligonucleotide primers for elongation. The fact that the reaction is highly specific for oligonucleotides with G-rich telomeric sequences suggests that some feature, other than primary sequence, common to these different oligonucleotides is recognized by the enzyme. All of the oligonucleotides that were able to prime elongation by the telomerase have been found to self-associate into specific intramolecular structures that migrate rapidly and anomalously in nondenaturing gels (E. Henderson et al., 1987). The oligonucleotides that do not prime repeat addition (see Table 1) do not assume these unique DNA conformations. The physical interactions governing the novel DNA structure formed by these telomeric sequences have been analyzed in detail for the oligonucleotide $(TTGGGG)_4$ (E. Henderson et al., 1987).

The double-stranded oligonucleotide $(CCCCAA)_4 \cdot (TTGG)_4$ was inactive as a primer for elongation, and over a range of concentrations of $(TTGGGG)_4$ tested, only a very small percentage of the primer present in the reaction was elongated (data not shown). These data are consistent with the hypothesis that a specific structure formed by telomeric G strand sequences is recognized for elongation. There is evidence that on purified native telomeres this strand is present as a single strand overhang in several organisms (Klobutcher et al., 1981; E. Henderson, personal communication). Therefore this unusual G strand structure may function in vivo as the recognition signal for telomeric elongation. Preliminary experiments from our laboratory also indicate that purified natural telomeres from Tetrahymena can prime elongation by the telomerase (E. Henderson, personal communication).

Another striking feature of the telomeric elongation reaction is the precision with which the TTGGGG repeats are synthesized in vitro. Preliminary experiments indicate that the repeat addition has a distributive nature (data not shown). We have also shown previously that the enzyme can stop at any nucleotide in the TTGGGG repeats to produce the 1 base ladder seen in the reaction (Greider and Blackburn, 1985). Thus if the enzyme dissociates and reassociates on the growing polymer, in order to synthesize repeats of TTGGGG and not irregular permutations of that sequence, the telomerase must accurately complete the most 3′ TTGGGG sequence, given 3′ ends terminating at any point within the repeat. We have shown that this accurate recognition occurs using oligonucleotide primers that have different 3′ ends within the TTGGGG repeat sequence.

From the observations discussed above, we believe that the telomerase has two distinct specificity components. First, the enzyme must recognize the structure of the telomeric substrate. Second, the sequence at the 3′ end of the primer must be recognized to allow the correct next nucleotide to be added. Both the structural and the 3′ end recognition are required since a non-G-rich oligonucleotide with 3 Gs at its 3′ end is not elongated (Figure 1, lane 12).

The two different specificities described here for the telomerase reaction are analogous to those of tRNA nucleotidyltransferase. This RNA processing enzyme recognizes the structure of many different tRNAs and adds the 3′ CCA sequence to those that do not have it. The tRNA binding subsite of tRNA nucleotidyltransferase is

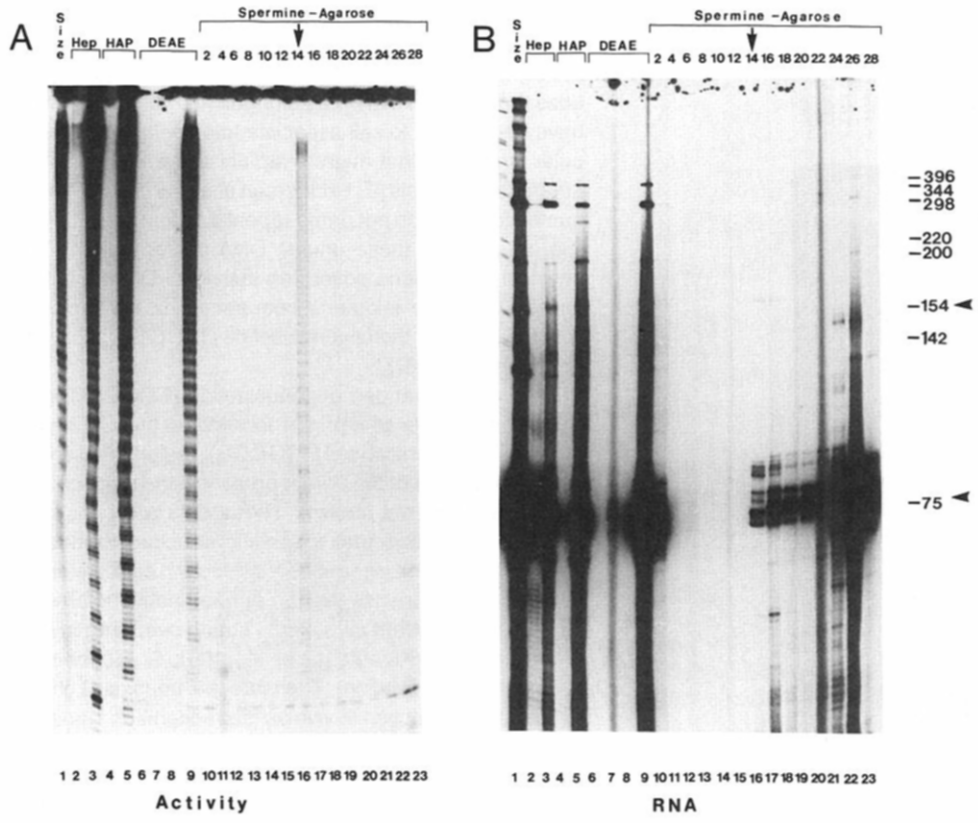

Figure 6. Small RNAs Copurify with the Telomerase over Five Columns

(A) Telomerase was purified over the following column series: gel filtration, heparin agarose, hydroxylapatite, DEAE agarose, and spermine agarose, as described in Experimental Procedures. The standard activity assay was carried out on the following fractions: pooled active gel filtration fraction (lane 1, Size), flowthrough and 100 mM NaCl cut from heparin agarose (lanes 2 and 3, respectively), flowthrough and 100 mM potassium phosphate salt cut from hydroxylapatite (lanes 4 and 5, respectively), flowthrough from DEAE agarose (lane 6), TMG and TMG plus 100 mM NaCl washes of the DEAE column (lanes 7 and 8, respectively), and TMG plus 350 mM NaCl eluate from DEAE agarose (lane 9). In lanes 10–23, 1 ml fractions from the spermine agarose were collected and every second fraction was assayed for activity. The fractions were eluted from the column by the addition of TMG buffer containing the NaCl concentrations listed below. Fractions 1–6 represent the flowthrough as the column was loaded. At fraction 7 the 350 mM NaCl wash was applied to the column, at fraction 13 500 mM NaCl was applied to the column, and at fraction 20 700 mM NaCl was applied.

(B) RNA was extracted from 300 μl of each of the fractions shown in (A) and 3′ end–labeled with [^{32}P]pCp (see Experimental Procedures). The radiolabeled RNA was separated by electrophoresis on a 40 cm, 6% polyacrylamide, 7 M urea gel. The RNA samples run in lanes 5, 6, and 7 were dialyzed to remove the phosphate before the labeling reaction was carried out. The arrowheads mark the RNA species that eluted in the active fractions.

distinct from the nucleotide binding subsite. The addition of CCA occurs onto the 3′ end of the tRNA held in the acceptor binding site when CTP or ATP is correctly bound at the appropriate nucleotide binding site. tRNAs having the structure tRNA, tRNA-C, or tRNA-C-C are correctly completed to tRNA-C-C-A, indicating that the terminal residue plays an important role in enzyme recognition (reviewed in Deutscher, 1983). Steps analogous to those of the tRNA nucleotidyltransferase can be envisioned to occur in the telomerase reaction, except that the addition of multiple repeats must be accounted for. After primer recognition, the 3′ end is positioned in an addition site such that the frame of the last repeat is established; dGTP and dTTP residues are then added until a complete TTGGGG repeat is synthesized, then the growing polymer is translocated to allow the synthesis of additional repeats. The translocation step could also occur after the addition of each individual nucleotide.

In Tetrahymena all cloned examples of telomeres contain perfect T_2G_4 repeats, with the exception of only 1 T_2G_5 in 300 T_2G_4 repeats from 11 telomeres sequenced in our laboratory (Budarf and Blackburn, 1987). However, the telomeric sequences of Saccharomyces and Dictyostelium are irregular, containing either 1, 2, or 3 Gs followed by a T in Saccharomyces or 1 to 8 G's followed by an A in Dictyostelium (Shampay et al., 1984; Emery and Weiner, 1981). This irregularity suggests there may be less stringent controls of the translocation step or the 3′ end recognition step by the telomeric elongation machinery of these organisms. In Paramecium the telomeric sequences contain a mixture of T_2G_4 and T_3G_3 repeats where the 6 base periodicity is maintained, yet the sequence varies slightly (Forney and Blackburn, 1987; Baroin et al., 1987). Similarly, in Plasmodium the telomeric heptamer repeats are mixtures of T_3AG_3 and T_2CAG_3 (Ponzi et al., 1985). The maintenance of the telomeric re-

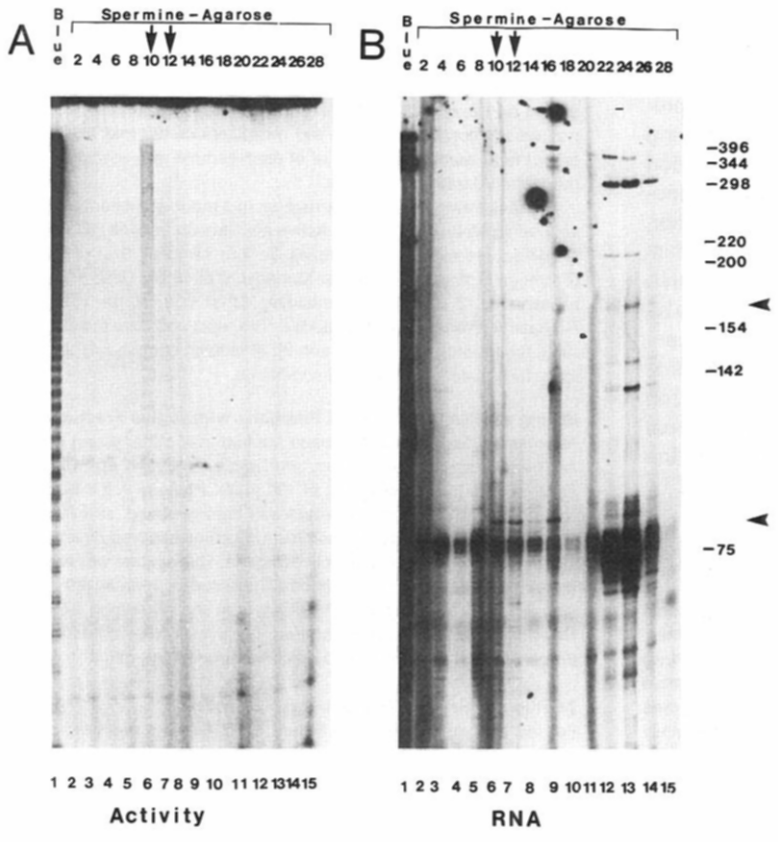

Figure 7. Small RNAs Copurify with Telomerase over a Different Five Column Series

(A) Telomerase was purified over the following column series: gel filtration, heparin agarose, DEAE agarose, Cibacron-Blue agarose, and spermine agarose. The activity profile from the last two columns is shown. In lane 1 pooled activity eluted with 400 mM NaCl plus 1% Triton X-100 eluate from Cibacron-Blue agarose (lane labeled Blue) was assayed. The salt concentration of this fraction was slightly less than 400 mM since the end of the previous 100 mM wash was included in the pooled material. In lanes 2–15, 900 μl fractions from the spermine agarose column were collected and every other fraction was assayed in the standard assay. The column was eluted with TMG buffer plus 1% Triton X-100 containing the NaCl concentrations indicated below. Fractions 1–7 represent the flowthrough from the loading of the column. At fraction 8 a 400 mM NaCl wash was applied to the column, at fraction 13 500 mM NaCl was applied, and at fraction 20 700 mM NaCl was applied.

(B) RNA was prepared from 300 μl of each fraction described above, labeled with [^{32}P]pCp, and run on a 40 cm, 6% polyacrylamide, 7 M urea sequencing gel. The molecular weight markers (not shown) are indicated to the right of the figure.

peat length, with some variation in the sequence, suggests that these organisms may provide insights as to how the telomerase specifies these parameters.

In addressing the question of the specificity of the telomerase, we discovered that this enzyme contained an essential RNA component. The RNAs copurifying with the telomerase are very minor species of the small RNAs found in Tetrahymena. From preliminary estimates, comparing known amounts of pCp-labeled 5S RNA with the telomerase fraction RNAs, there may be as few as 1000 molecules per cell. Even given the inaccuracy inherent in this kind of quantification, the finding is somewhat surprising since a single Tetrahymena macronucleus contains 10^4–10^5 telomeres. During vegetative growth of Tetrahymena, on average 1 TTGGGG repeat is added per telomere per generation (Larson et al., 1987). Thus it is possible that only a small number of telomerase molecules may be required in Tetrahymena in vivo.

The RNA of telomerase may simply provide a scaffold for the assembly of proteins in the active enzyme complex; however, the RNA may play many possible roles in the telomeric elongation reaction. RNA functions in ribosomes to provide both structure and specificity (reviewed in Nomura et al., 1974; Shine and Dalgarno, 1975). The RNA components of the signal recognition particle and RNAase P play structural and catalytic roles, respectively, in the reactions of these RNPs (Walter and Blobel, 1982; Guerrier-Takada et al., 1983). Of the snRNAs involved with pre-mRNA splicing (reviewed in Sharp, 1987; Maniatis and Reed, 1987; Padgett et al., 1986), U1 and U2 RNAs provide specificity to the splicing reaction by base pairing with the pre-mRNA substrate (Zhuang and Weiner, 1986; Parker et al., 1987). In addition, the RNA component of the mitochondrial endoribonuclease involved in primer formation for mitochondrial DNA replication (Chang and Clayton, 1987) may provide specificity to the cleavage reaction since it has the potential to base pair with the RNA that is cleaved (D. Chang and D. Clayton, personal communication).

It is tempting to speculate that the RNA component of the telomerase might be involved in determining the sequence of the telomeric repeats that are synthesized and/or the specific primer recognition. If the RNA of telomerase contains the sequence CCCCAA, this sequence could act as an internal guide sequence like that used to position the growing polymer in the polymerization reaction catalyzed by the self-splicing Tetrahymena rDNA intron (Zaug and Cech, 1986). If the RNA of telomerase provides an internal template, the mechanism of repeat synthesis must involve either a translocation step or the dissociation and reassociation of the enzyme to produce the polymers that are typically longer than 400 nucleotides under the in vitro assay conditions. As noted above, the irregular repeats of Saccharomyces, Dictyostelium, Plasmodium, and Paramecium may be the result of relaxed requirements for the translocation step or the dissociation and reassociation of the enzyme with the growing polymer. The irregular repeats could also result

from a mixed population of enzymes with internal template RNAs of slightly different sequences.

Alternatively, the RNA may be directly involved in the primer recognition reaction. RNAase P cleaves many pre-tRNA substrates with different primary sequences, relying on the tertiary structure unique to tRNAs for recognition (reviewed in Altman et al., 1982). Since the RNA component of RNAase P can carry out this cleavage reaction alone in vitro (Guerrier-Takada et al., 1983), the essential aspects of substrate recognition must lie within the RNA itself. Similarly, the RNA component of telomerase may be directly involved in recognizing the unique three-dimensional structure of the G-rich telomeric oligonucleotide primers. Finally, by analogy with the self-splicing intron enzyme (Zaug and Cech, 1986), the RNA component of telomerase could have a direct role in catalyzing the TTGGGG repeat polymerization.

Experimental Procedures

Extract Preparation

Tetrahymena strains SB210 and SB1969 (kindly provided by Ed Orias) were grown and mated as previously described (Greider and Blackburn, 1985). All extracts were made from mated cells 9 hr after mixing cells to initiate mating. Buffers were made with diethyl pyrocarbonate (DEP)-treated H_2O and then autoclaved; 2-mercaptoethanol was added after autoclaving, and PMSF was added to 100 μM immediately before use from a 100 mM stock in 100% ethanol. Cells were spun down, washed once with Dryls solution, (1.7 mM sodium citrate, 1.2 mM NaH_2PO_4, 1 mM Na_2HPO_4, and 2 μM $CaCl_2$), resuspended in 3 volumes of TMG (10 mM Tris [pH 8.0], 1 mM $MgCl_2$, 10% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM PMSF) per volume of packed cells, RNasin (Promega Biotec) was added to a concentration of 10 U/ml, and the cells were lysed with continuous stirring at 4°C for 20 min by the addition of 0.1 volume of 2% NP40 in TMG. The cell lysate was then centrifuged in an SW41 rotor at 35,000 rpm for 60 min at 4°C. The supernatant of this spin was designated S100 and was used in subsequent purification steps. The pellet fractions were found to contain no telomerase activity and were discarded.

In Vitro Reaction Conditions

The in vitro reactions were modified from Greider and Blackburn (1985). A standard assay contained 20 μl of a 2× reaction mix and 20 μl of an enzyme fraction. The 2× reaction mix consisted of 4 μl of 10× reaction buffer, 20 mM dTTP, 2 μM dGTP, 0.8 μg $(TTGGGG)_4$ oligonucleotide (unless some other oligonucleotide was specified), 0.5 U of RNasin, and 1 μl (10 μCi) of [α-^{32}P]dGTP (specific activity, 3000 Ci/mmol). The 10× reaction buffer contained 500 mM Tris (pH 8.5), 1 M sodium acetate, 20 mM $MgCl_2$, 1 mM spermine, 10 mM spermidine, and 50 mM 2-mercaptoethanol. After mixing the enzyme preparation to be assayed with the reaction mix, samples were incubated at 30°C for 60 min. The reaction was then stopped by the addition of 100 μl of stop mix (20 mM EDTA and 10 mM Tris–HCl [pH 7.5]), then extracted once with 100 μl of 50:50 phenol, chloroform. The supernatant was removed to a new tube, 140 μl of 5 M ammonium acetate and 2 μg of carrier tRNA were added, and the nucleic acid was precipitated by the addition of 900 μl of 95% ethanol. The precipitation was carried out at room temperature for 10 min, and the samples were spun for 15 min in an Eppendorf microcentrifuge. The supernatant was removed with a drawn out Pasteur pipette, and the pellets were dried in a vacuum desiccator for 20 min. The pellets were then resuspended in 2.5 μl of sequencing loading mix (90% deionized formamide, 1× TBE, 0.2% xylene cyanol, and 0.2% bromphenol blue) and loaded onto a 6% polyacrylamide, 7 M urea sequencing gel. The 0.3 mm thick, 40 cm long gel was then run at 1500 V for 2 hr, until the bromphenol blue dye was at the bottom.

Nuclease Digestions

MNase digestions were carried out as follows: $CaCl_2$ was added to 160 μl of active fraction to a final concentration of 1 mM. The fraction was then split into eight 20 μl aliquots, and 1 μl of 100 mM EGTA was added to four of the aliquots. Next, either 0, 0.15, 1.5, or 15 U of MNase (Worthington) in TMG was added to all eight test fractions, and the samples were incubated at 37°C for 15 min. The samples were then placed on ice, and 1 μl of 200 mM EGTA was added to those samples not already containing EGTA. Two microliters of 100 mM $MgCl_2$ was added to all samples, and 20 μl of each sample was assayed under the standard assay conditions.

RNAase treatments were carried out in a manner similar to that described for MNase digestions. Active gel filtration fraction (160 μl) was aliquoted into eight 20 μl samples. To four samples, 3 μl of RNasin (Promega Biotec or Boehringer Mannheim) at 40 U/μl was added. To all samples, 2 μl of TMG containing either 0, 1, 10, or 100 μg/ml RNAase A (Worthington) was added. The reactions were incubated at room temperature for 15 min, and 20 μl of each sample was assayed for activity using the standard conditions.

Mixing of RNAase A–Treated Fractions with Active Fractions

Telomerase (300 μl) that had been purified over either sizing column and heparin agarose or sizing column, heparin agarose, and spermine agarose was treated with 3 μl of 100 μg/ml RNAase A for 15 min at room temperature. Sixty micoliters was then removed, and 360 U of RNasin was added. Twenty microliters was then assayed directly, and 10 μl was mixed with 10 μl of active fraction. This mixture was assayed under the standard assay conditions. Untreated telomerase (20 μl) was also assayed as a control. The RNA from the remaining 240 μl of RNAase-treated telomerase and from 240 μl of untreated fraction was prepared, 3′ end–labeled, and run on a sequencing gel.

Addition of RNA to MNase-Treated Extracts

For the experiment shown in Figure 5, RNAs were prepared as described below. RNA from either 400 μl of S100 extract or telomerase purified over both gel filtration and heparin agarose columns was extracted (see below) and precipitated. The pellets were resuspended in 20 μl of DEP-treated H_2O. Telomerase extract (500 μl) purified over gel filtration and heparin agarose was thawed, and 5 μl of 100 mM $CaCl_2$ was added. One hundred microliters was removed, 60 U of MNase was added to the remaining 400 μl, and both samples were incubated at 37°C for 10 min. The samples were then set on ice, EGTA was added to 2.5 mM, and $MgCl_2$ was added to 5 mM. The MNase-treated extract was split into four 100 μl samples, and the untreated extract was split into four 20 μl samples. Four microliters of each telomerase RNA preparation (see below) was added to an aliquot of the MNase-treated enzyme, and 1 μl of each was added to aliquots of the untreated controls. One microliter of tRNA at 8 mg/ml was also added to a MNase-treated sample, and 0.4 μl of tRNA was added to the untreated sample. As a control, 4 μl or 1 μl of DEP-treated H_2O was added instead of RNA to aliquots of MNase-treated and untreated enzyme, respectively. Twenty microliters of each of the eight samples was assayed in the standard assay. The remaining 80 μl of MNase-treated fractions plus RNA was incubated with reaction buffer in the absence of [^{32}P]dGTP. After 60 min at 30°C, the RNAs were extracted from the mock-reacted samples, labeled, and run on a sequencing gel. All the RNAs added back to the nuclease-treated extract remained intact throughout the reaction period.

Column Chromatography

All columns were run at 4°C using cold, sterile buffers to which 2-mercaptoethanol and fresh PMSF had been added. The purification protocol described here involves chromatography over a preparative gel filtration column and freezing of the fractions after assaying to determine which contain activity. In pilot purification procedures we found that freezing and thawing telomerase preparations in subsequent stages in the preparation lead to large losses in activity. Therefore all columns, after the gel filtration step, started with freshly thawed gel filtration fractions and were run consecutively without assaying for activity until the last column was fractionated. Each of the columns run in the purification schemes shown in Figures 3, 6, and 7 are described below.

Gel Filtration

Analytical gel filtration columns were run using Biorad A 5.0 m agarose beads according to the manufacturers specifications. The beads were equilibrated with TMG, degassed, and poured into a 48 cm × 0.78

cm^2 column. The columns were calibrated and checked for even packing by prerunning known molecular weight standards and following the OD_{280} eluted from the column. The molecular weight of the standards eluted from the column was confirmed by running protein gels on the peak fractions. The Bio-rad sizing column calibration kit used for the calibration contained γ-globulin as one of the size markers. As the γ-globulin did not run with its predicted molecular weight of 68,000 dalton, this protein was not used to calibrate the molecular weight of the telomerase. For the molecular weight determination, 1 ml of S100 Tetrahymena extract was loaded onto the column and eluted with a flow rate of 100 μl/min. Fractions (500 μl) were collected, and every other fraction was assayed for telomerase activity. Attempts to determine the molecular weight of enzyme that had previously been purified over several columns were all unsuccessful. When purified telomerase was run over a gel filtration column no activity was ever recovered. We are currently trying to stabilize the purified enzyme before gel filtration.

For preparative gel filtration, Pharmacia Sephacryl S-500 polyacrylamide beads were preequilibrated according to the manufacturers specifications. After equilibration and degassing, a 1 m × 19.6 cm^2 column was poured. The column was calibrated with the same markers described above to identify the region where the telomerase was expected to elute. Tetrahymena S100 fraction (60–100 ml) was loaded onto the column and eluted at a flow rate of 2 ml/min. One liter of flowthrough was collected, and then 300 2 ml fractions were collected. Every tenth fraction was assayed for telomerase activity, and all 300 fractions were frozen in liquid nitrogen as soon as all fractions had been collected.

Heparin Agarose

For an analytical heparin agarose column preparation, such as those shown in Figures 6 and 7, 6 ml of active gel filtration fractions was loaded onto a 3 ml heparin agarose (Biorad) column, preequilibrated in TMG. Neighboring active gel filtration fractions were thawed and pooled, PMSF was added to 0.1 mM, and RNasin was added to 400 U per ml of extract. The pooled fractions were then loaded onto the heparin agarose column at a flow rate of about 0.1 ml/min. The flowthrough was collected, and the column was washed with 6 ml of TMG. The telomerase was eluted with 6 ml of TMG plus 100 mM NaCl, again at a flow rate of about 0.1 ml/min. This heparin agarose fraction was stored frozen in liquid nitrogen, or an aliquot was removed and the remainder was loaded onto either a DEAE agarose or a hydroxylapatite column. Preparative heparin agarose columns were scaled up directly from this procedure.

Hydroxylapatite

The 100 mM NaCl in TMG eluate from heparin agarose was loaded onto a 1.5 ml hydroxylapatite (Calbiochem Fast Flow) column equilibrated with TMG plus 100 mM NaCl. The flowthrough was collected, the column was washed with 6 ml of TMG plus 100 mM NaCl, and the telomerase was eluted with 4 ml of 100 mM potassium phosphate buffer (pH 8.0). A sample of this eluate was removed for later assay, and the remainder was loaded onto a DEAE agarose column.

DEAE Agarose

The eluted fractions from either a heparin agarose column or a hydroxylapatite column were loaded onto a 2 ml DEAE agarose (Biorad) column. If the fraction from heparin agarose was to be loaded, the column was equilibrated with TMG plus 100 mM NaCl, the flowthrough was collected, the column was washed with 6 ml of TMG plus 100 mM NaCl, and the activity was eluted with 4 ml of TMG plus 350 mM NaCl. If the fraction from a hydroxylapatite column was to be loaded, the column was equilibrated with 100 mM potassium phosphate buffer (pH 8.0). After the hydroxylapatite fraction was loaded onto the column and the flowthrough collected, the column was washed extensively to remove the phosphate buffer (this buffer interfered with the subsequent RNA analysis). The first wash was with 6 ml of TMG, and the second wash was with 8 ml of TMG plus 100 mM NaCl. The telomerase was then eluted with 4 ml of TMG plus 350 mM NaCl.

Cibacron-Blue Agarose

A 2 ml Affigel Blue (Biorad) column was equilibrated with TMG plus 350 mM NaCl. The pooled 350 mM NaCl eluate from DEAE agarose was loaded, the flowthrough was collected, and the column was washed with 6 ml of TMG plus 350 mM NaCl. The telomerase activity was then eluted with 4 ml of a buffer containing TMG, 400 mM NaCl, and 1% Triton X-100 (Sigma). The salt concentration at which the telomerase eluted from the Affigel Blue column varied slightly in five different experiments, depending on the purity of the loaded material.

Spermine Agarose

For loading the 350 mM NaCl eluate from DEAE, a 2 ml spermine agarose (Sigma) column was equilibrated with TMG plus 350 mM NaCl. Fractions (1 ml) were collected representing the flowthrough, 7 ml of TMG plus 350 mM NaCl wash, 7 ml of TMG plus 500 mM NaCl elution, and 7 ml of TMG plus 700 mM NaCl elution. If the telomerase fraction from an Affigel Blue column was to be loaded, the spermine agarose column was equilibrated with TMG, 400 mM NaCl, and 1% Triton X-100. Fractions (900 μl) were then collected representing the flowthrough, 5 ml of TMG, 400 mM NaCl, and 1% Triton X-100 wash, 8 ml of TMG, 500 mM NaCl, and 1% Triton X-100 elution, and 8 ml of TMG, 700 mM NaCl, and 1% Triton X-100 elution. The fractions were assayed along with samples from all of the previous columns. All of the fractions were then stored frozen in liquid nitrogen.

RNA Preparation and [^{32}P]pCp End Labeling

To analyze the RNA composition of column fractions, RNA was usually extracted from 300 μl of each fraction. The fractions, stored frozen in liquid nitrogen, were thawed, and to 300 μl, 30 μl of a 10% SDS, 100 mM EDTA solution and 50 μg of proteinase K were added. The samples were incubated at 37°C for 10 min, then extracted twice with phenol, chloroform. To the final supernatant, 30 μl of 3 M sodium acetate and 0.5 μg of carrier λ DNA (BRL) were added, and the nucleic acids were precipitated by addition of 900 μl of absolute ethanol and incubation at −70°C for 30 min. After spinning down the precipitate, the pellets were dried and resuspended directly in the RNA labeling mix. In the case of fractions containing potassium phosphate from the hydroxylapatite column, the phosphate pellet was resuspended in TE and dialyzed against TE for several hours. The RNA was then ethanol-precipitated and dried down for labeling (this dialysis procedure often led to loss of some RNA). Each RNA labeling reaction contained 50 mM HEPES (pH 8.3), 10 mM $MgCl_2$, 3 mM DTT, 10% glycerol, 15% DMSO, 6 μM ATP, 1 U of RNasin, 1 U of T4 RNA ligase (Pharmacia or New England Biolabs), and 10 μCi of [^{32}P]pCP (Amersham) at 3000 Ci/mmol. The RNA pellets were resuspended in this mix, and the reactions were incubated for 10–14 hr at 4°C. After incubation, the reactions were ethanol-precipitated by the addition of 200 μl of 250 mM ammonium acetate and 600 μl of absolute ethanol at room temperature. The labeled RNA was spun down in a microcentrifuge for 15 min and dried in a vacuum desiccator. The pellets were resuspended in 2.5 μl of sequencing gel loading dye (90% formamide, 1× TBE, 0.2% xylene cyanol, and 0.2% bromphenol blue) and loaded onto a 6% polyacrylamide, 7 M urea sequencing gel. The gels (0.3 mm thick, 40 cm long) were run at 1500 V for 2 hr until the bromphenol blue dye ran off the bottom.

Acknowledgments

We thank Bruce Stillman and Adrian Krainer for suggesting the blue agarose and spermine agarose columns, Bruce Malcolm for rapid synthesis of the oligonucleotides, and Jeff Reynolds for his assistance with making figures. We also thank Marietta Dunaway and members of the Blackburn lab for critical reading of the manuscript. This work was supported by grant GM 26259 from the National Institutes of Health to E. H. B.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 24, 1987; revised October 13, 1987.

References

Altman, S., Guerrier-Takada, C., Frankfort, H. M., and Robertson, H. D. (1982). RNA processing nucleases. In Nucleases, S. M. Linn and R. J. Roberts, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 243–274.

Baroin, A., Prat, A., and Caron, F. (1987). Telomeric site position heterogeneity in macronuclear DNA of Paramecium primaurelia. Nucl. Acids Res. *15*, 1717–1728.

Baroudy, B. M., Venkatesan, S., and Moss, B. (1982). Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell *28*, 315–324.

Bateman, A. J. (1975). Simplification of the palindromic telomere theory. Nature *253*, 379.

Bernards, A., Michels, P. A. M., Lincke, C. R., and Borst, P. (1983). Growth of chromosomal ends in multiplying trypanosomes. Nature *303*, 592–597.

Blackburn, E. H. (1984). Telomeres: do the ends justify the means?. Cell *37*, 7–8.

Blackburn, E. H., and Szostak, J. W. (1984). The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. *53*, 163–194.

Budarf, M., and Blackburn, E. H. (1987). S1 nuclease sensitivity of double stranded telomeric DNA sequences. Nucl. Acids Res. *15*, 6273–6292.

Cavalier-Smith, T. (1974). Palindromic base sequences and replication of eukaryotic chromosome ends. Nature *250*, 467–470.

Cech, T. R., and Bass, B. L. (1986). Biological catalysis by RNA. Annu. Rev. Biochem. *55*, 599–629.

Chang, D. D., and Clayton, D. A. (1987). A mammalian mitochondrial RNA processing activity contains nucleus-encode RNA. Science *235*, 1178–1184.

DeLange, A. M., Reddy, M., Scraba, D., Upton, C., and McFadden, G. (1986). Replication and resolution of cloned poxvirus telomeres in vivo generates linear minichromosomes with in tact viral hairpin termini. J. Virol. *59*, 249–259.

Deutscher, M. P. (1983). tRNA nucleotidyltransferase and the -C-C-A terminus of transfer RNA. In Enzymes of Nucleic Acid Synthesis and Modification. Vol. II, RNA Enzymes, S. T. Jacobs, ed. (Boca Raton: Florida: CRC Press), pp. 159–177.

Emery, H. S., and Weiner, A. M. (1981). An irregular satellite sequence is found at the termini of the linear extrachromosomal rDNA in Dictyostelium discoideum. Cell *26*, 411–419.

Forney, J. D., and Blackburn, E. H. (1987). Developmentally controlled telomere addition in wild type and mutant Paramecia. Mol. Cell. Biol., in press.

Guo, P., Erickson, S., and Anderson, D. (1987). A small viral RNA is required for in vitro packaging of bacteriophage U 29 DNA. Science *236*, 690–694.

Greider, C. W., and Blackburn, E. H. (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell *43*, 405–413.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell *35*, 849–857.

Henderson, E., Hardin, C. C., Wolk, S. K., Tinoco, I., Jr., and Blackburn, E. H. (1987). Telomeric DNA oligonucleotides form novel intramolecular structures containing guanine·guanine base pairs. Cell, this issue.

Holmquist, G. P., and Dancis, B. (1979). Telomere replication, kinetochore organizers and satellite DNA evolution. Proc. Natl. Acad. Sci. USA *76*, 4566–4570.

Klobutcher, L. T., Swanton, M. T., Donini, P., and Prescott, D. M. (1981). All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3′ terminus. Proc. Natl. Acad. Sci. USA *78*, 3015–3019.

Larson, D. D., Spangler, E. A., and Blackburn, E. H. (1987). Dynamics of telomere length variation in Tetrahymena thermophila. Cell *50*, 477–483.

Maniatis, T., and Reed, R. (1987). The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature *325*, 673–678.

Nomura, M., Tissieres, A., and Lengyel, P. (1974). Ribosomes (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S., and Sharp, P. A. (1986). Splicing of messanger RNA precursors. Annu. Rev. Biochem. *55*, 1119–1150.

Parker, R., Silicano, P. G., and Guthrie, C. (1987). Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell *49*, 229–239.

Pluta, A. F., Dani, G. M., Spear, B. B., and Zakian, V. A. (1984). Elaboration of telomeres in yeast: recognition and modification of termini from Oxytricha macronuclear DNA. Proc. Natl. Acad. Sci. USA *81*, 1475–1479.

Ponzi, M., Pace, T., Dore, E., and Frontali, C. (1985). Identification of a telomeric DNA sequence in Plasmodium berghei. EMBO J. *4*, 2991–2995.

Shampay, J., and Blackburn, E. H. (1987). Generation of telomere length heterogeneity in S. cerevisiae. Proc. Natl. Acad. Sci. USA, in press.

Shampay, J., Szostak, J. W., and Blackburn, E. H. (1984). DNA sequences of telomeres maintained in yeast. Nature *310*, 154–157.

Sharp, P. A. (1987). Splicing of messenger RNA precursors. Science *235*, 766–771.

Shine, J., and Dalgarno, L. (1975). Determination of cistron specificity in bacterial ribosomes. Nature *254*, 34.

Stillman, B. W. (1983). The replication of adenovirus DNA with purified proteins. Cell *35*, 7–9.

Szostak, J. W., and Blackburn, E. H. (1982). Cloning yeast telomeres on linear plasmid vectors. Cell *29*, 245–255.

Walter, P., and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature *299*, 691–698.

Watson, J. D. (1972). Origin of concatameric T4 DNA. Nature New Biol. *239*, 197–201.

Wong, T. W., and Clayton, D. A. (1986). DNA primase of human mitochondria is associated with structural RNA that is essential for enzymatic activity. Cell *45*, 817–825.

Zaug, A. J., and Cech, T. R. (1986). The intervening sequence RNA of Tetrahymena is an enzyme. Science *231*, 470–475.

Zhuang, Y., and Weiner, A. M. (1986). A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. Cell *46*, 827–835.