

# The effects of nucleoside analogs on telomerase and telomeres in *Tetrahymena*

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## ABSTRACT

The ribonucleoprotein enzyme telomerase is a specialized type of cellular reverse transcriptase which synthesizes one strand of telomeric DNA, using as the template a sequence in the RNA moiety of telomerase. We analyzed the effects of various nucleoside analogs, known to be chain-terminating inhibitors of retroviral reverse transcriptases, on *Tetrahymena thermophila* telomerase activity *in vitro*. We also analyzed the effects of such analogs on telomere length and maintenance *in vivo*, and on vegetative growth and mating of *Tetrahymena* cells. Arabinofuranyl-guanosine triphosphate (Ara-GTP) and ddGTP both efficiently inhibited telomerase activity *in vitro*, while azidothymidine triphosphate (AZT-TP), dideoxyinosine triphosphate (ddITP) or ddTTP were less efficient inhibitors. All of these nucleoside triphosphate analogs, however, produced analog-specific alterations of the normal banding patterns seen upon gel electrophoresis of the synthesis products of telomerase, suggesting that their chain terminating and/or competitive actions differ at different positions along the RNA template. The analogs AZT, 3'-deoxy-2',3'-didehydrothymidine (d4T) and Ara-G in nucleoside form caused consistent and rapid telomere shortening in vegetatively growing *Tetrahymena*. In contrast, ddG or ddl had no effect on telomere length or cell growth rates. AZT caused growth rates and viability to decrease in a fraction of cells, while Ara-G had no such effects even after several weeks in culture. Neither AZT, Ara-G, acycloguanosine (Acyclo-G), ddG nor ddl had any detectable effect on cell mating, as assayed by quantitation of the efficiency of formation of progeny from mated cells. However, AZT decreased the efficiency of programmed *de novo* telomere addition during macronuclear development in mating cells.

## INTRODUCTION

Telomeres, the ends of eukaryotic chromosomes, are essential for chromosome stabilization and complete replication [reviewed in 1–3]. Telomeric DNA typically consists of simple tandemly

repeated sequences characterized by clusters of G residues on the DNA strand at each 3' end of the duplex chromosomal DNA [4]. In ciliates such as *Tetrahymena thermophila*, and human cells, telomeric DNA is replicated and maintained, and new telomeres are added to chromosomes, by telomerase [reviewed in 5]. This unusual ribonucleoprotein enzyme functions as a specialized reverse transcriptase activity, synthesizing telomeric DNA repeats using a template sequence in the telomerase RNA [6–9]. Telomerase activities have been identified in other ciliates besides *Tetrahymena*, and in cultured mammalian cells as well as some mammalian tissues [reviewed in 5]. In each case repeats of the species-specific telomeric sequence, which in *Tetrahymena* is  $(T_2G_4)_n$ , are added by the telomerase activity.

We tested the effects of reverse transcriptase inhibitors on telomerase activity from *Tetrahymena thermophila* both *in vitro* and *in vivo*. Retroviral reverse transcriptases, such as that of HIV, are inhibited both *in vivo* and *in vitro* by chain-terminating dideoxynucleoside triphosphate substrates (ddNTPs), and by other nucleoside triphosphate analogs including azidothymidine triphosphate (AZT-TP) [reviewed in 10–12]. Previous *in vitro* results indicated that dideoxy-GTP (ddGTP) and ddTTP also efficiently chain-terminate elongation by *Tetrahymena* telomerase [13]. We therefore extended these studies to include other nucleoside analogs and to perform a more detailed characterization of the effects of these chain-terminating analogs on the telomerase reaction. Each round of synthesis of the hexameric repeat  $T_2G_4$  by the *T. thermophila* telomerase involves six successive polymerizations along the intrinsic RNA template of telomerase (see below). Here we show that these six polymerization steps are differentially affected by the nucleoside analog inhibitors, suggesting that at each position on the template the polymerization complex has different properties.

The nucleoside analogs tested *in vitro*, and other known reverse transcriptase inhibitors, were tested for their effects on telomere maintenance in *Tetrahymena thermophila* *in vivo*. An essential function of telomerase is to counterbalance the progressive shortening of the chromosome from its ends which would result from rounds of semi-conservative DNA replication [reviewed in 1]. Previous work has shown that a particular mutation in the telomerase RNA in *Tetrahymena* cells leads to telomere shortening and a severe cellular senescence phenotype [9]. In

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the yeast *Saccharomyces cerevisiae*, mutation or deletion of the *EST 1* gene causes gradual shortening of telomeres and eventually senescence [14]. In this system, as with the alteration of the telomerase RNA in *Tetrahymena* [9], loss of telomeric DNA sequences below an apparent lower threshold also coincides with the senescence phenotype. In certain mammalian cells shortening of telomeric DNA has been correlated with cellular senescence [15]. Hence we predicted that an inhibitor acting specifically on telomerase in dividing cells would similarly cause telomere loss, and thus eventual cell death after several cell divisions. Here we show that although some analogs which inhibited telomerase activity *in vitro* caused telomere shortening in dividing *Tetrahymena* cells, only AZT at high concentrations caused both telomere shortening and eventual reduced viability of a subset of the dividing cells. In mating cells, none of the analogs tested irreversibly disrupted progeny cell formation, but AZT decreased the efficiency of new telomere formation.

## MATERIALS AND METHODS

### *Tetrahymena* strains and cell culture conditions

*Tetrahymena thermophila* strains SB210(VI) and PB9R(II), where numbers in parentheses indicate mating type, were maintained as stocks at room temperature in 1% PPYS [1% proteose peptone (Difco), 0.1% yeast extract (Difco) and 0.0015% Sequestrene (Ciba-Geigy)]. The nucleoside analogs Acyclo-G, AZT and d4T were purchased from Sigma, and Ara-G, ddG and ddI were purchased from Calbiochem. Stocks were passaged every three to four weeks. All experiments were begun using small cultures (25 ml or 50 ml) in 2% PPYS or thymine-deficient 'Isobroth' (Isosensitest broth, Oxoid, USA) in 250 ml flasks inoculated from stationary stock cells and allowed to grow overnight at 30°C on a gyratory shaker (100 rpm). After harvesting cells at various time points in each experiment, cells were pelleted and stored at -80°C until processed for DNA analysis.

For analysis of cell viability and macronuclear DNA from cultures containing AZT grown in Isobroth, Ara-G grown in 2% PPYS, or controls lacking analog, at various time points during vegetative divisions SB210 and PB9R cells were counted and plated separately at 50–500 cells/ml in 24-well plates (Falcon) and grown at 30°C without shaking. DMSO (Fisher) was the solvent for Ara-G and was added to 1% in 2% PPYS as a control; Isobroth alone was used as the control for AZT. Every 2–6 days cells were transferred using a multi-pronged replicator into 1 ml of fresh medium with or without analog, and the remaining cells were harvested.

For analysis of macronuclear DNA from vegetative cultures containing d4T in Isobroth, ddG in 2% PPYS, or ddI in 2% PPYS, or controls lacking the analog (DMSO to 1% in 2% PPYS as the control for ddG, 2% PPYS alone for ddI, and Isobroth alone as control for d4T), duplicate cultures of SB210 cells were inoculated at 100 cells/ml into broth containing varied amounts of analog, and grown at 30°C with shaking. 500–2000 cells were transferred to fresh medium every 2–4 days, and the remainder harvested.

For analysis of vegetative growth rates and macronuclear DNA from single-cell transfer cultures containing the nucleoside analogs AZT or Ara-G, strain SB210 cells were counted and added to the appropriate medium plus analog (Ara-G to 10 µM or 1 mM, or DMSO to 1% as a control in 1% PPYS; AZT to 10 µM or 1 mM in Isobroth, or no addition as a control) and

plated in 96-well plates, 100 µl per well at an average density of 1 cell per well. Five plates were prepared for each analog or control. Wells were scored for cell growth and plates were replica plated every 1–2 days (Ara-G and DMSO control plates) or every 2–4 days (AZT and Isobroth control plates) to maintain approximate inoculation densities of 1–10 cells per well for each passage. Occasionally individual wells were passaged by hand (1 µl inoculated per well using a pipettor) into several blank wells, to expand the number of live wells per plate as slow-growing single-cell cultures were lost over time due to low probability of being transferred at each passage. After passaging, cells were pooled for harvesting.

For analysis of rDNA species from cells mated in the presence of nucleoside analogs, 50 ml overnight cultures (2% PPYS) of cells of the two different mating types were starved by pelleting cells and resuspending in an equal volume of Dryl's solution before returning to a 30°C shaking incubator for 18 hours. (1 × Dryl's solution = 0.5 g Na citrate, 0.16 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.14 g Na<sub>2</sub>HPO<sub>4</sub> per liter, plus 0.30 g CaCl<sub>2</sub>·2H<sub>2</sub>O per liter). Equal numbers of cells of each mating type were mixed, plated at an average density of 4.5 × 10<sup>6</sup>/well into 6-well plates and allowed to conjugate for 6 hours, 30°C without shaking. Mock-conjugated SB210 cells were treated identically but not mixed with PB9R cells. At 6 hours the cultures were checked for pairing (>90%, except SB210 controls) and either 1 ml Dryl's solution or 2% PPYS containing the nucleoside analog, or DMSO to 1% or no addition as controls, were added slowly with gentle swirling. Cultures were returned to 30°C for an additional 18 hours before harvesting.

For analysis of progeny formation, cycloheximide (CHX) sensitivity of cells was determined after conjugation in the presence of analog. Twenty-four hours after mixing, cells were diluted in Dryl's solution, counted and plated at 1 cell per well in 96-well plates in 1% PPYS without analog. Cells were grown for 4 days in a humid chamber at 30°C, without shaking. Cells were then replica plated into 1% PPYS plus 15 µg/ml cycloheximide, allowed to grow for four days before scoring, and the percent CHX-resistant wells was calculated.

### DNA isolation and analysis

Total cellular DNA was prepared essentially as described previously [16] except that the Hoechst 33258–CsCl gradient purification step was omitted.

Restriction digests, agarose gel electrophoresis, transfer of DNA to Nytran filters (Schleicher and Schuell), and hybridization with <sup>32</sup>P-nick-translated or random-primed probes were carried out using standard procedures [17]. Telomere length was analyzed as described previously for *Tetrahymena* [18].

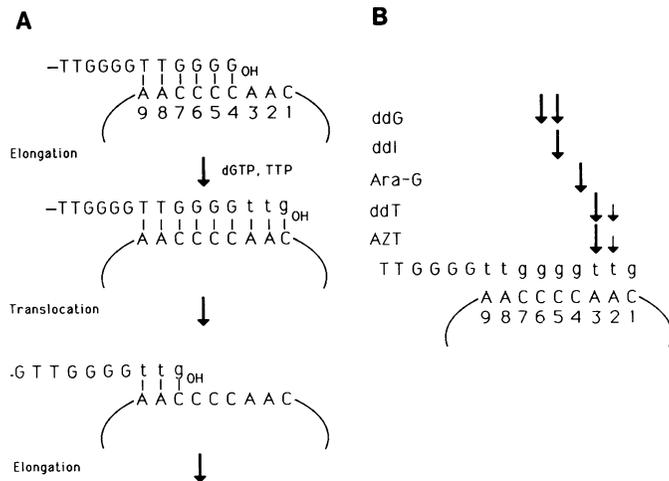
For PCR analysis for the presence of one telomeric end of the 11 kb rDNA, DNA was isolated at 24 hours from cells conjugated in the presence or absence of inhibitor. 1.25 µM each of the telomeric primer (C<sub>4</sub>A<sub>2</sub>)<sub>4</sub> and a 25-mer specific for the rDNA, located 1371 nucleotides from the 5' end of the rDNA ('5' primer': 5' GTGGCTTACACAAAATCTAAGCGC 3'), were used as described previously [19]. PCR was performed on total cell DNA preparations from five of the mating experiments using two or three different DNA concentrations (over a four- to ten-fold dilution range) for all the inhibitors tested, as well as with DNA from the control matings lacking added inhibitor. As controls for the presence of DNA, identical reactions were done using '3' micronuclear' primers: 9610 nucleotides from the 5' end (5' CAAAAGAATTCAAGATTTGATTTAAAA 3'), and

10300 nucleotides from the 5' end (5' CAATAATGTATT-AAAAATATGCTACTTATGCATTATC 3') of the single micronuclear copy of the rDNA.

**In vitro assays of telomerase activity**

Synthetic oligomers were prepared as described previously [13]. Extracts were prepared as described by Blackburn *et al.* [20]. A standard telomerase assay contained 50% by volume of heparin-agarose-purified telomerase, 25 μM TTP, 1.25 μM

<sup>32</sup>P-labeled dGTP (400 Ci/ mMol, Amersham), 1 μM oligo (either (T<sub>2</sub>G<sub>4</sub>)<sub>4</sub> or (T<sub>2</sub>G<sub>4</sub>)<sub>2</sub> mixed with water and heated at 90°C for two minutes and cooled at 30°C for 10 minutes), and 0.1 μl RNasin (40 U/ml, Promega) in a no-salt buffer [21]. AZT-triphosphate was generously provided by Dr Wayne Miller (Burroughs Wellcome, NC). Ara-G-triphosphate was purchased from Calbiochem and ddNTPs from Sigma. Reaction mixes were kept on ice until ready for use, and then mixed into tubes containing analog for incubation at 30°C for thirty minutes. The reaction products were analyzed essentially as described by Greider and Blackburn [6]. For quantitative assays, aliquots of the reaction mixture were spotted in triplicate onto DE81 paper and washed as described [13]. Incorporation of <sup>32</sup>P label from either <sup>32</sup>P-TTP or <sup>32</sup>P-dGTP was measured to monitor reaction rates. Backgrounds were not subtracted for each time point, and instead the incorporation of radioactivity from the [α-<sup>32</sup>P]-dNTP precursor is plotted directly as the mean of each triplicate reaction in Figure 2. For visualization of the elongation reaction products, samples were heated to 95°C for 2 minutes and cooled on ice before loading onto a 12% polyacrylamide/8 M urea gel.

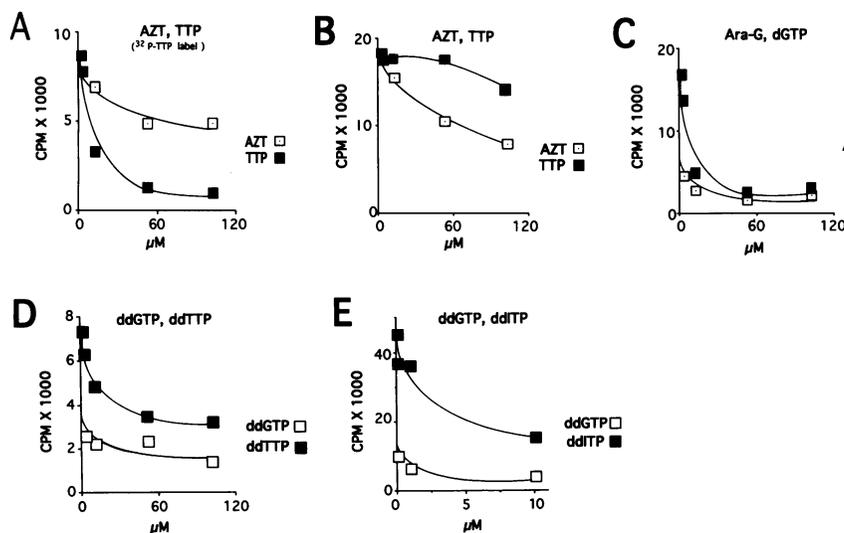


**Figure 1.** Mechanism and inhibition of the telomerase reaction. **A.** The templating portion of the telomerase RNA is shown with residues numbered 1 (5') through 9 (3'), corresponding to positions 43–51 in the whole RNA [6]. The oligonucleotide primer with the sequence T<sub>2</sub>G<sub>4</sub>T<sub>2</sub>G<sub>4</sub> binds to the template by the base-pairing shown. Elongation followed by template translocation are thought to occur as indicated. **B.** Positions of major chain termination on the telomerase RNA template by different nucleoside triphosphate analogs. The telomerase RNA template sequence is shown as in A. Arrows indicate the position of maximal chain termination for each nucleoside triphosphate analog shown.

**RESULTS**

**The effects of chain-terminating nucleoside triphosphate analogs on *Tetrahymena* telomerase activity in vitro**

The model for the reaction carried out by the telomerase from *Tetrahymena* is shown in Figure 1A. This ribonucleoprotein enzyme synthesizes TTGGGG repeats onto the 3' end of a suitable DNA primer by copying a template sequence in the RNA moiety of the enzyme. The residues in the templating region are numbered 1 to 9 (5' to 3' along the RNA, corresponding to positions 43–51 in the telomerase RNA sequence) [6]. The standard telomerase assay used in this work consists of incorporation of dGTP and TTP substrates, one triphosphate [α-<sup>32</sup>P]-labeled, into synthesized DNA in the reaction shown in Figure 1A. The DNA primer is either 1 μM (T<sub>2</sub>G<sub>4</sub>)<sub>4</sub> or (T<sub>2</sub>G<sub>4</sub>)<sub>2</sub> (saturating primer concentrations [21]), under conditions in which



**Figure 2.** Nucleoside analog triphosphates inhibit incorporation of <sup>32</sup>P label in the standard telomerase assay. The effect of adding increasing concentrations of the analog, unlabeled dGTP or unlabeled TTP on the incorporation of labeled nucleotides was measured using the standard quantitative telomerase reaction assay (see Materials and Methods). Radioactivity incorporated (cpm) was plotted against the concentration of competitors indicated in each panel. **A.** Labeled with 1.25 μM [α-<sup>32</sup>P]TTP, plus 10 μM dGTP. **B–E.** Labeled with [α-<sup>32</sup>P]dGTP, plus 25 μM unlabeled TTP.

the overall rate of incorporation of label was determined previously to be linear over time (data not shown).

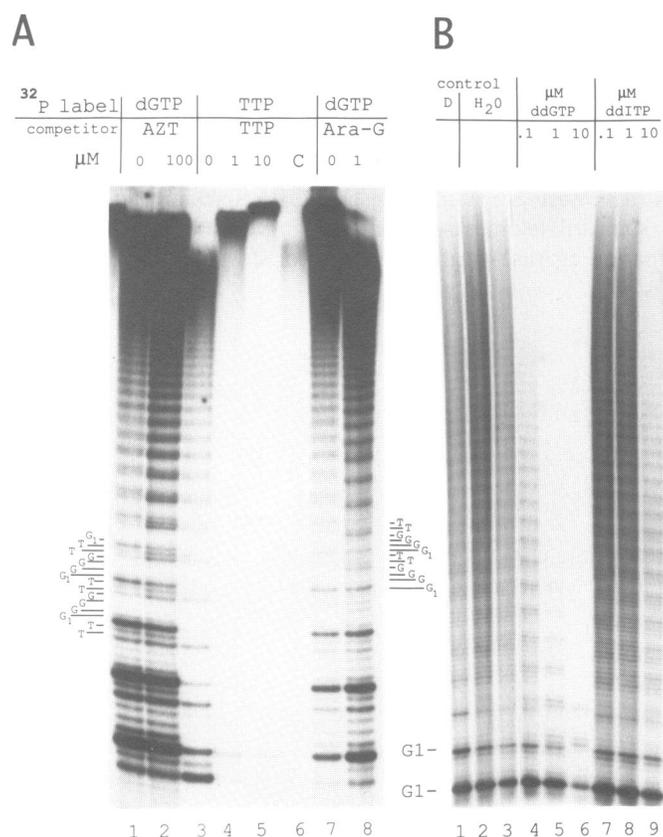
The effect of adding increasing amounts of AZT-triphosphate (AZT-TP) to the standard assay for telomerase activity is shown in Figure 2A. A series of control reactions using unlabeled TTP added at the same concentrations as the AZT-TP was run in parallel (Figure 2A). The unlabeled TTP inhibited incorporation of the  $^{32}\text{P}$ -labeled TTP by simple competition, allowing the  $K_m^{\text{app}}$  for TTP to be determined as  $\sim 5 \mu\text{M}$ . Compared with addition of unlabeled TTP competitor, AZT-TP had only a modest quantitative effect on the incorporation of  $^{32}\text{P}$ -labeled TTP (Figure 2A). Since AZT incorporation leads to chain termination (see below), this result indicated that AZT-triphosphate competes less efficiently for telomerase than TTP. Similar results were obtained when incorporation of  $^{32}\text{P}$ -dGTP instead of  $^{32}\text{P}$ -TTP was monitored (Figure 2B), with 50% inhibition occurring at  $\sim 80 \mu\text{M}$  AZT-TP.

The analog arabinofuranyl-guanosine triphosphate (Ara-GTP) significantly reduced overall incorporation even at very low concentrations (50% inhibition at  $0.7 \mu\text{M}$  Ara-GTP; Figure 2C). From parallel experiments in which unlabeled dGTP was added as competitor (Figure 2C), the  $K_m^{\text{app}}$  for dGTP under these reaction conditions was found to be  $1\text{--}2 \mu\text{M}$ ; thus Ara-GTP potentially competes as well as unlabeled dGTP. However, as incorporation of Ara-G causes chain termination, each Ara-G residue incorporated is expected to have a greater impact on total incorporation than each dGTP residue incorporated.

We also tested the effects of dideoxynucleoside triphosphates (ddNTPs) on the telomerase reaction. As shown previously for telomerase [13] and many other reverse transcriptases, ddNTPs are recognized by the enzyme and incorporated, causing chain termination. Consistent with previous qualitative analyses of *Tetrahymena* and human telomerases [13, 22], ddGTP and ddTTP each inhibited the incorporation of labeled  $^{32}\text{P}$ -GTP into elongation products (Figure 2D and E). ddGTP was a much more efficient inhibitor than ddTTP: under these reaction conditions 50% inhibition occurred at  $<0.1 \mu\text{M}$  and  $5 \mu\text{M}$  ddGTP and ddTTP, respectively. As observed previously for *Tetrahymena* telomerase [13], no significant effects were seen with either ddC-TP or ddATP (data not shown). In addition, dideoxyinosine triphosphate (ddITP) inhibited telomerase (Figure 2E), although less efficiently than ddGTP, with 50% inhibition occurring at  $3 \mu\text{M}$  ddITP.

The size distributions and profiles of labeled products were analyzed by denaturing polyacrylamide gel electrophoresis. Consistent with the expectation for a chain-terminator, the proportion of longer telomerase products was decreased in the presence of AZT compared with cold TTP competitor controls (Figure 3A; compare lanes 1 and 2 with lanes 3–5), and in the presence of Ara-G (Figure 3A; lanes 7 and 8), ddGTP and ddITP (Figure 3B).

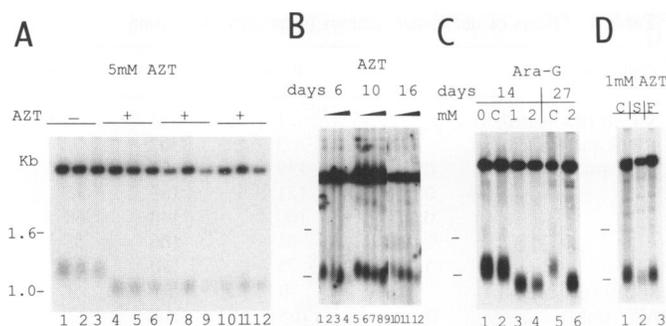
Each nucleoside triphosphate analog produced distinctive and characteristic patterns of chain termination, as shown by analysis of the shifts in the banding patterns of the elongation products. Previous results allow the bands seen to be assigned to copying of known positions on the template [21, M.Lee, D.Gilley and E.H.B., unpublished results]. AZT-triphosphate increased the relative intensities of the bands corresponding to the incorporation of the two T residues (copying the A residues at positions 2 and 3 on the template RNA; see Figure 1A). This change in banding pattern is consistent with simple chain termination at both positions. Similar effects were seen with ddTTP [13] (data not



**Figure 3.** The effect of nucleoside triphosphate analogs on banding patterns of telomerase reaction products *in vitro*. **A.** Telomerase reactions were performed under standard conditions (see Materials and Methods) in the presence or absence of the indicated nucleoside triphosphate analogs. Unlabeled TTP competitor was also analyzed as a control, with (lanes 3–5) and without ('C', lane 6) primer in the reaction mix. Products were analyzed on a denaturing polyacrylamide gel. G<sub>1</sub> indicates bands corresponding to copying template position C<sub>1</sub> (see Fig. 1). Reading up the gel from each G<sub>1</sub> band, the nucleotides added in the next elongation cycle are G (copying position C<sub>6</sub>, following translocation) G (position C<sub>5</sub>), G (C<sub>4</sub>), T (A<sub>3</sub>), and T (A<sub>2</sub>). **B.** Standard telomerase reactions (see Materials and Methods) were performed in the presence of ddGTP (lanes 4–6), ddITP (lanes 7–9), or DMSO (lane 1). DMSO was the solvent for ddGTP and at the highest concentration tested (1%) showed no effect on the reactions compared with control reactions run without analog or DMSO (control lanes 2–3). Products were analyzed on a denaturing polyacrylamide gel.

shown). We interpret this to mean that AZT-triphosphate was recognized by the enzyme and incorporated into the correct positions in the growing telomeric sequence, causing chain termination. However, it has been shown previously that slowing the incorporation of TTP at position 2 increases the dissociation of the product of copying position 3 [21]. Therefore increased dissociation, caused by competition with TTP and a slower reaction rate with AZT-triphosphate at position 2, may have contributed to the even greater band intensity at template position 3 relative to position 2. Although G residues can be incorporated at four positions on the template (see Figure 1A), the strongest chain termination by Ara-GTP was at position 4 (Figure 3A, lanes 7 and 8), by ddGTP, at positions 6 and 5 (Figure 3B, compare lane 1 with lanes 4 to 6), and by ddITP, at position 5 (Figure 3B, lanes 7–9).

Figure 1B summarizes schematically the effects of the various triphosphate analogs on polymerization at each of the six positions along the template. There was no obvious relationship between



**Figure 4.** The effects of nucleoside analogs on telomere length *in vivo*. Genomic DNA was digested with *Pst*I and *Bam*HI and the rDNA telomeres analyzed as described previously [18]. A nick-translated [ $\alpha$ - $^{32}$ P]-labeled plasmid containing a 3' rDNA fragment was used as probe. The telomeric *Pst*I fragment from the rDNA is between the 1.6 and 1.0 kb markers, indicated as lines on the left side of each panel. The constant 2.8 kb band is the adjacent internal *Pst*I rDNA fragment [18]. **A.** A clone of *Tetrahymena thermophila* was grown in 2% PPYS in the absence (-) and three clones in the presence (+) of 5 mM AZT. Each set of three lanes shows the results for a single cell clone grown vegetatively and transferred after 3 days (lanes 1, 4, 7, 10), 10 days (lanes 2, 5, 8, 11) and 16 days (lanes 3, 6, 9, 12). **B.** AZT concentration-dependent shortening of telomeres. Log phase cells were grown in thymine-deficient broth (Isobroth) plus AZT. DNA was made from cells sampled at 6, 10, and 16 days. Shortened telomere lengths remained constant between 6 and 16 days in culture. Lanes 1, 5 and 9, no AZT controls; lanes 2, 6 and 10, 0.01 mM AZT; lanes 3, 7 and 11, 0.1 mM AZT; lanes 4, 8 and 12, 1 mM AZT. **C.** Cells grown vegetatively in 2% PPYS with no addition (lane 1), with 1% DMSO, the solvent for Ara-G ('C', lanes 2 and 5), and with Ara-G (lane 3, 1 mM; lanes 4 and 6, 2 mM) at 14 and 27 days in culture. **D.** Analysis of telomere length in single-cell cultures grown in Isobroth plus 1 mM AZT (lanes 2 and 3) segregated into two classes based on growth rate: 'slow' ('S', 0-1 doubling per day, lane 2) or 'fast' ('F', 2-4 doublings per day, lane 3). DNA from a control culture grown in Isobroth without AZT (2-4 doublings per day) is indicated ('C', lane 1). Several cultures were pooled in order to obtain sufficient DNA for analysis.

the efficiency of a nucleoside analog as an inhibitor and the position of its maximal chain termination on the template. For example, the potent inhibitors ddG- and Ara-G-triphosphates caused maximal chain termination at different positions on the telomerase RNA template.

#### Effects of nucleoside analogs on cell viability and telomere length of vegetatively growing *Tetrahymena*: mass transfer experiments

At least one alteration of the *T. thermophila* telomerase RNA template region causes telomere shortening in dividing cells, leading to cellular senescence [9, M. Lee, D. Gilley and E. H. B., unpublished results], possibly by directly inhibiting telomerase activity [M. Lee, D. Gilley and E. H. B., unpublished results]. Because the triphosphate forms of the analogs AZT, Ara-G, ddT, ddG and ddI each inhibited telomerase *in vitro*, some potentially, we tested whether the nucleoside form of such analogs caused *in vivo* changes in telomere length, or senescence, when supplied in the growth medium of dividing cells.

Duplicate cultures of *T. thermophila* cells were maintained in log-phase growth conditions by serial passaging in the presence of varying concentrations of nucleoside analogs. Growth rates and cell morphology were monitored, and telomere length was analyzed at a series of time points during the serial passaging. AZT at 5 or 10 mM added to a thymine-poor medium (Isobroth medium; see Materials and Methods) strongly inhibited cell growth and killed cells within a day, and thus at these concentrations acted in a manner suggestive of immediate toxicity

to cells, rather than of senescence. However, up to 1 mM AZT in Isobroth did not decrease cell growth rate or cause gross senescence of cultures maintained by subculture of  $\sim 10^3$  cells per transfer, over a 50-day period of continuous growth and subculturing of these cell cultures (150-250 cell generations, from growth rate measurements). In similar mass transfer experiments, no effects on overall cell doubling rate, morphology or long term viability were observed with cells grown in rich medium (2% PPYS) containing up to 5 mM AZT or 2 mM Ara-G (the highest concentrations tested that did not cause immediate toxicity).

Telomere lengths were monitored by Southern blotting analysis. The ribosomal RNA genes (rDNA) are present as high copy linear minichromosomes in *Tetrahymena* macronuclei, and the rDNA telomeres comprise 30-50% of the total macronuclear telomeres in the cell. The mean lengths of all the macronuclear telomeres are regulated in concert [18]; therefore, measuring rDNA telomere length can be used to assess telomere length regulation in this system [18]. The telomeres of cells grown vegetatively in 5 mM AZT in 2% PPYS medium were reproducibly shortened by an average of 170 base pairs within 3 days of growth in AZT, compared with the control cultures grown in 2% PPYS in the absence of the drug (Figure 4A). Telomere shortening caused by AZT was concentration-dependent (Figure 4B), with at least 50% of the maximal shortening effect occurring at 10  $\mu$ M AZT, the lowest concentration tested. For each AZT concentration tested, the full telomere length decrease was seen within 3 days of culturing in the presence of the drug (15-30 cell divisions), but after this initial length adjustment, at each drug concentration telomeres thereafter showed no statistically significant further shortening over time, and mean telomere length consistently remained static for at least 28 days of passaging by mass transfers (Figure 4B and data not shown). Similar degrees of shortening (an average of 170 bp), and timing of telomere shortening, were produced by 1 or 2 mM Ara-G in 2% PPYS (Figure 4C). After 5 days ( $\sim 16$  generations) in culture, the thymine analog d4T (10  $\mu$ M to 1 mM in Isobroth medium) produced significantly shortened telomeres only at 1 mM, the highest concentration tested. In contrast, after 5 days of subculturing ( $\sim 50-60$  cell generations), up to 1 mM ddG or ddI in 2% PPYS produced no changes in telomere length compared with control cultures (data not shown).

#### Effects of nucleoside analogs on cell viability and telomere length of vegetatively growing *Tetrahymena*: single cell transfer experiments

Recent findings with *est 1*<sup>-</sup> yeast cells have shown that a subset of *est 1*<sup>-</sup> cells in a population can escape the senescence phenotype caused by lack of function of the *EST 1* gene product [23]. Because at least some of the analogs tested strongly inhibited telomerase *in vitro*, and caused rapid telomere shortening *in vivo* in the mass transfer experiments, we explored the possibility that telomere maintenance was in fact being disrupted *in vivo* by these analogs. We reasoned that our failure to find any evidence of progressive telomere shortening or senescence might be attributable to a subset of the cell population that escapes an inhibitory effect of the analog on telomerase or other activities normally required for telomere maintenance. Under our mass transfer subculturing regime, in which about  $10^3$  cells were transferred per passage, if, for example, a fraction as small as  $\sim 1\%$  of the cells escaped senescence, and if their growth advantage was sufficiently high compared with cells losing

telomeres, they could become the predominant population in any cell passage and we would not have detected any obvious growth phenotype.

To test whether a senescing subpopulation of cells had been missed, vegetatively dividing *Tetrahymena* cells were again grown in the presence and absence of analog: 10  $\mu$ M and 1 mM AZT in Isobroth, and 10  $\mu$ M and 1 mM Ara-G in 1% PPYS. However, for subculturing, cells were now plated at an average of 1–10 cells per well, instead of the  $\sim 10^3$  cells/transfer in the mass transfer experiments described above. For each analog and its control, cells were plated out in this manner for 30 consecutive days (90–150 cell generations) for the 10  $\mu$ M analog concentration, and 16 consecutive days (50–80 cell generations) for the 1 mM analog concentration.

For cells grown in 10  $\mu$ M AZT, 10  $\mu$ M Ara-G and 1 mM Ara-G, over the course of the experiment no changes in the plating efficiencies were observed compared with control cultures grown without nucleoside analog. However, with the 1 mM AZT cultures, monitoring growth rates of cells maintained by single cell transfers in this way allowed us to identify two general growth classes, which we designated as slow (0–1 cell doublings per day) and fast (2–4 cell doublings per day). Furthermore, monitoring the cells remaining in wells after transfers had been made showed that the slow cells lost viability over time. Throughout the course of the transfers, slow cells appeared from formerly fast cell wells, but not *vice versa*.

Telomere length was analyzed at intervals. To obtain sufficient DNA for Southern analysis, slow growing cells were pooled from several microtiter wells, and their telomere length distribution compared with that of pooled fast cells. In Isobroth lacking AZT, telomeres were 165 bp shorter (mean length, as measured by densitometric analysis) than in cells grown in 1% PPYS medium lacking analog. Mean telomere length did not change over the course of the experiment (data not shown). In the pooled slow or fast cells grown in 1 mM AZT in Isobroth, the mean telomere length was a further 60–70 bp shorter, and the length distribution was tighter (Figure 4D). This mean length and length distribution were indistinguishable from those of cells grown in 1% PPYS plus 1 mM Ara-G. The significance of these results is discussed below.

#### Effects of nucleoside analogs on progeny formation by conjugated *Tetrahymena* cells

Production of progeny cells from mated *Tetrahymena* requires development of new macronuclei. Macronuclear development involves developmentally programmed, site-specific fragmentation of germline chromosomes into linear subchromosomes, whose ends are healed by *de novo* addition of telomeres. Telomerase has been shown not only to elongate pre-existing telomeres *in vivo* during vegetative cell divisions [21], but also to add telomeric DNA directly onto non-telomeric sequences during this developmentally-controlled chromosome healing [8]. Because of this immediate requirement for telomere addition to fragmented DNA, we reasoned that this *de novo* telomere addition might be more sensitive to telomerase inhibition than telomere maintenance during vegetative growth.

First, the nucleoside analogs were tested for effects on progeny formation, to test whether they irreversibly inhibited macronuclear development. We mated two strains of *T. thermophila* which are sensitive to cycloheximide, but are genetically constructed so that their progeny after mating are resistant to cycloheximide (CHX). Because generation of progeny expressing

**Table 1.** Effects of nucleoside analogs on progeny formation

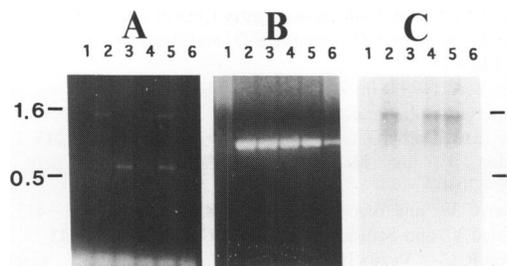
Cell treatment	# Chx-R	# Total	%Chx-R
SB210 (not mated)	1	215	0.5
PB9R (not mated)	3	307	1
AZT (mM)			
0	139	21	66
0.01	121	169	72
0.1	100	148	68
1.0	91	166	55
5.0	75	120	63
SB210 (not mated)	0	57	0
AZT (mM)			
0	165	214	77
0.01	67	92	73
0.1	128	190	67
1.0	60	125	48
5.0	89	168	53
1% DMSO	84	109	77
ARA-G (mM)			
0.01	114	141	81
0.1	134	167	80
1.0	89	161	55
1% DMSO	51	75	68
ARA-G (mM)			
1.0	51	86	59
2.0	40	92	43
SB210 (not mated)	0	9	0
PB9R (not mated)	0	37	0
ddI (mM)			
0	63	75	84
0.001	59	71	83
0.01	85	96	89
0.1	83	106	78
1.0	100	110	91
1% DMSO	21	44	48
ddG (mM)			
0.001	86	102	84
0.1	73	86	85
1.0	51	66	77
ACYCLO-G (mM)			
0	36	45	80
0.017	80	107	75
0	78	116	67
0.017	101	146	69

the cycloheximide resistance marker requires successful production of a new macronucleus, cells whose macronuclear development is disrupted are killed in cycloheximide. Synchronized mated cells were treated with AZT, Ara-G, Acyclo-G, ddI or ddG at various concentrations. The analogs were introduced into mating cultures prior to macronuclear development (6 hours after mating was initiated), and remained present until its normal completion time (the 24 hour time point). At 24 hours, the treated cells and their controls were diluted and plated out in microtiter plate wells in fresh medium lacking the analog, at an average density of one cell per well, and allowed to grow for the minimum period before selection for cycloheximide-resistant progeny (see Materials and Methods). The results are shown in Table 1.

The unmated cells showed 99–100% cell death in cycloheximide, while the majority of cells mated with or without analog produced progeny that survived in cycloheximide. None of the nucleoside analogs had any statistically significant effect on progeny formation. The design of this experiment would prevent takeover of the culture by a minority population that evaded the effects of the drug, as described above. Therefore, these nucleoside analogs caused little or no irreversible disruption of macronuclear development.

#### Effect of reverse transcriptase inhibitors on *de novo* telomere formation in developing macronuclei

During development of the new macronucleus, an 11 kb rDNA molecule with telomeres at both ends is generated. This molecule,



**Figure 5.** PCR analysis of DNA from *Tetrahymena* cells conjugated in the presence of analog and starved for the duration of mating. **A.** A telomeric primer and the rDNA '5' primer' (see Materials and Methods) were used in PCR reactions with DNA from cells conjugated in the presence (lanes 3–5) or absence (lane 2) of analog to detect the addition of telomeres to the 11 kb rDNA formed during macronuclear development. A reaction was run without DNA (lane 1) as a control. Lane 3, 5 mM AZT; lane 4, 1 mM Ara-G; lane 5, 1 mM Acyclo-G. SB210 cells were mock-conjugated as a control (lane 6). The expected product is approximately 1.4 kb. **B.** The '3' micronuclear' rDNA primers (see Materials and Methods) were used on the same DNA (see Figure 5A) to demonstrate the presence and competence of the DNA samples for PCR. The expected product is 810 bp. **C.** Southern blot analysis of the 5' rDNA telomeric PCR reactions shown in panel A using a random-primed <sup>32</sup>P-labeled 5' rDNA probe confirmed that the 1.4 kb PCR product originated from the 11 kb rDNA with *de novo* added telomeres. No hybridization was seen in the no DNA control (lane 1) or the SB210 mock-conjugated control (lane 6). Lane 2, no added analog; lane 3, 5 mM AZT; lane 4, 1 mM Ara-G; lane 5, 1 mM Acyclo-G; lane 6, mock-conjugated SB210.

which is either an intermediate or a by-product of formation of the mature amplified form of the rDNA molecules [reviewed in 24–26], is present only transiently during new macronuclear development, and as such acts as a marker specific for *de novo* telomere addition *in vivo*. To determine whether reverse transcriptase inhibitors affected *de novo* formation of new macronuclear telomeres in the progeny cells, macronuclear development in mated cells was allowed to proceed as described above after addition at 6 hours of 1 mM AZT, Acyclo-G, ddG, ddI, ddT, d4T, or 0.5 mM Ara-G. As controls, the same volume of the solvent for the analog (water or DMSO) was added to the mating culture. For each inhibitor and its control, cells were either refed at 6 hrs with 2% PPYS or Isobroth, to force macronuclear development to proceed in the presence of inhibitor, or starved until 24 hrs (the duration of the analog treatment) to arrest macronuclear development at an intermediate stage [27], although rDNA amplification still proceeds in these starved cells [28]. Whole cell DNA prepared at 24 hr was analyzed by PCR for telomere addition to 11 kb rDNA (see Materials and Methods). Representative results of the PCR analysis are shown in Figure 5.

The amounts of 1.4 kb PCR-generated fragment diagnostic of telomere addition to the 11 kb rDNA were higher in all starved mating cells compared with refed mating cells (data not shown). However, in DNA from starved mating cells treated with 1 mM AZT, this telomere-containing PCR product was absent or greatly reduced compared with its control (6 out of 6 independent mating experiments). In 3 out of 6 mating experiments in Ara-G, telomere addition to the 11 kb rDNA was also reduced relative to control matings, but not in the other three independent mating experiments with Ara-G. Acyclo-G (four independent mating experiments), ddG, ddI, ddT and d4T (two independent mating experiments each; data not shown) had no effect on the amount of 1.4 kb PCR product. Southern blotting with an rDNA probe confirmed that the 1.4 kb telomere-containing PCR product was from the expected 11 kb rDNA sequence (Figure 5C). As positive

controls to show that the DNA used in the PCR reactions was present and competent for PCR, identical reactions were performed using primers from the 3' end of the micronuclear chromosomally-integrated single copy of the rDNA [29]. In all samples the expected 0.8 kb fragment was produced in similar quantities (Figure 5B). As expected, mock-conjugated SB210 cells showed no 1.4 kb telomeric PCR product (Figure 5A and C).

In summary, these results indicated that AZT reduced the efficiency of telomere addition to the 11 kb rDNA during macronuclear development, but without an irreversible effect on progeny cell formation.

## DISCUSSION

The results reported here show that the action of the chain terminating nucleoside triphosphate inhibitors AZT-TP, ddGTP, ddTTP, ddITP and Ara-GTP on telomerase activity *in vitro* is generally similar to their action on conventional reverse transcriptases. The low ratios of ddG or ddT needed for efficient chain termination by the telomerases from *Tetrahymena* have been noted previously [13]. These results suggest that telomerase may be more similar to reverse transcriptases than to other DNA polymerases.

Previous results have shown that *in vitro* products of the telomerases from *Tetrahymena* and other organisms have a species-specific, characteristic periodic banding pattern under similar assay conditions [7, 13, 22, 30]. By kinetic studies of the *Tetrahymena* telomerase reaction, it has been shown that this banding pattern primarily reflects different probabilities of dissociation of the elongating primer at each position, rather than pausing [21]. Here we have shown that for each triphosphate analog tested the efficiency of chain termination varies with position on the telomerase RNA template. This *in vitro* behavior provides direct experimental evidence that, as polymerization proceeds along the template, the properties of the polymerization complex (consisting of the enzyme active site, dNTP substrate and RNA template) change. Since the telomerase RNA is an intrinsic part of the telomerase ribonucleoprotein RNP, as the enzyme active site aligns with each of the individual positions on the RNA template, the geometry of the RNA within telomerase must be altered. Hence we propose that each template position constitutes a distinguishable conformation of the internal RNA template with respect to the telomerase active site.

Variants of the HIV retrovirus that are resistant to AZT have been shown to have mutant reverse transcriptases with altered amino acid sequences [11]. However, we found that AZT-TP was a relatively ineffective inhibitor of *Tetrahymena* telomerase *in vitro*. As the HIV-1 RT readily mutates to a resistant form [11], it was not unexpected to find that *Tetrahymena* telomerase is naturally somewhat resistant, although AZT did have effects on *de novo* telomere synthesis and, as discussed below, possibly on telomere maintenance. Ara-GTP and ddGTP were potent *in vitro* inhibitors of telomerase but they did not prevent telomere maintenance *in vivo*. There could be several reasons for this: for example, inefficient uptake of the analog, or poor utilization by cellular enzymes to form the triphosphate. However, at least 50% of the maximal telomere shortening was seen with a relatively low AZT concentration (10  $\mu$ M), suggesting that for this nucleoside analog, uptake is not severely limiting.

Progressive loss of telomeric DNA during cell divisions has been implicated in causing eventual cell death in dividing

*Tetrahymena* and other systems [4, 9, 14, 31]. The continual generation of a subpopulation of *Tetrahymena* cells with reduced viability during cell divisions in AZT suggests that this reduction in viability was not caused by general toxicity of AZT, because many cells continued to grow well. Although the telomere length of the cells grown in 1 mM AZT in Isobroth was indistinguishable from that of cells grown in rich PPYS medium plus 1 mM Ara-G, we suggest the following model to explain our results. Because the telomeric G<sub>4</sub>T<sub>2</sub> repeat tracts in control cells grown in Isobroth medium without added analog are already markedly shorter than those of cells grown in PPYS medium, we propose that telomere length maintenance is more easily compromised in the thymine-poor Isobroth than in the richer PPYS medium. Hence, the additional amount of telomere shortening caused by adding 1 mM AZT to Isobroth may be sufficient to reduce, continually and stochastically, a fraction of the telomeres below a critical lower threshold required for function, thus continually generating a subpopulation of cells with decreased viability. We cannot, however, rule out the possibility that toxicity of AZT progressively becomes higher in this subset of cells for unrelated reasons.

Like AZT, Ara-G and d4T caused rapid telomere shortening in vegetatively dividing cells, but there was no observable further long-term continued progressive loss of telomeric DNA over time, or senescence. Whether these analogs exerted their effects on mean telomere length directly or indirectly is unknown. In yeast, many genes, involved directly or indirectly in DNA synthesis, have been implicated in telomere length regulation [reviewed in 3]: for example, yeast mutants with temperature-sensitive DNA polymerase  $\alpha$ , helicase or thymidylate synthetase show altered telomere length regulation. Competition of the analog for enzymes in triphosphate synthesis pathways could affect triphosphate pools and hence affect rates of a variety of DNA metabolic processes. This may indirectly affect the dynamics of the lengthening and shortening processes whose balance ultimately determines mean telomere length [32].

Our results show that several nucleoside analogs inhibit one component of the telomere synthetic machinery, telomerase, and also can perturb telomere metabolism *in vivo*. However, prediction of the *in vivo* effects of these inhibitors from their effects on telomerase *in vitro* is likely to prove complex.

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