Effects of Reverse Transcriptase Inhibitors on Telomere Length and Telomerase Activity in Two Immortalized Human Cell Lines

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Received 17 May 1995/Returned for modification 6 July 1995/Accepted 10 October 1995

The ribonucleoprotein telomerase, a specialized cellular reverse transcriptase, synthesizes one strand of the telomeric DNA of eukaryotes. We analyzed telomere maintenance in two immortalized human cell lines: the B-cell line JY616 and the T-cell line Jurkat E6-1, and determined whether known inhibitors of retroviral reverse transcriptases could perturb telomere lengths and growth rates of these cells in culture. Dideoxyguanosine (ddG) caused reproducible, progressive telomere shortening over several weeks of passaging, after which the telomeres stabilized and remained short. However, the prolonged passaging in ddG caused no observable effects on cell population doubling rates or morphology. Azidothymidine (AZT) caused progressive telomere shortening in some but not all T- and B-cell cultures. Telomerase activity was present in both cell lines and was inhibited in vitro by ddGTP and AZT triphosphate. Prolonged passaging in arabinofuranyl-guanosine, dideoxyinosine (ddI), dideoxyadenosine (ddA), didehydrothymidine (d4T), or phosphonoformic acid (foscarnet) did not cause reproducible telomere shortening or decreased cell growth rates or viabilities. Combining AZT, foscarnet, and/or arabinofuranyl-guanosine with ddG did not significantly augment the effects of ddG alone. Strikingly, with or without inhibitors, telomere lengths were often highly unstable in both cell lines and varied between parallel cell cultures. We propose that telomere lengths in these T- and B-cell lines are determined by both telomerase and telomerase-independent mechanisms.

Telomeres, the ends of eukaryotic chromosomes, are characterized by an array of tandemly repeated short DNA repeat units (3), with the sequence TTAGGG in humans and other mammals (33). These essential telomeric sequences are added to chromosomal DNA ends by telomerase (14; reviewed in reference 4), a cellular ribonucleoprotein reverse transcriptase which uses a sequence within its RNA moiety to template the repeats added to chromosome ends (15, 31, 40, 41). The specialized DNA-protein complexes formed by these repeats (6, 13) are thought to be important for still poorly understood interactions within the nucleus that affect nuclear division and chromosome maintenance (5) and protect the ends of chromosomes from fusion (30) and potential degradation by exonucleases (13).

During successive rounds of DNA replication, an inevitable progressive loss of genetic information is predicted to occur, because DNA polymerase is unable to complete synthesis of the ends of linear DNA (reviewed in references 3 and 10). By polymerizing DNA onto the chromosome termini, telomerase counterbalances this terminal DNA attrition. Functional telomerase has been demonstrated to be essential for normal telomere maintenance in the ciliated protozoan *Tetrahymena thermophila* and the yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. In *T. thermophila*, a particular mutant RNA, which prevents correct telomerase polymerization in vitro (12), causes telomere shortening and senescence (11a, 12, 46). Similarly, deleting or disrupting the RNA moiety of telomerase in the budding yeasts *K. lactis* and *S. cerevisiae* or the *EST1* gene in *S. cerevisiae* causes progressive telomere shortening and cellular senescence (28, 31, 41). These results established that in these organisms, which can grow indefinitely, telomere

maintenance is essential for long-term viability and telomerase is required for normal telomere maintenance. However, preventing normal telomerase-mediated maintenance has also revealed telomerase-independent mechanisms for telomere maintenance in yeasts. In *est1⁻* cells that survive deletion of the *EST1* gene, *RAD52*-dependent recombination takes place between internal and terminal telomeric repeat tracts separated by the Y' class of subtelomeric elements, regenerating sufficient terminal telomeric repeat tracts for chromosomal maintenance (27). The *K. lactis* genome lacks internal telomeric repeat tracts, precluding the type of pathway seen in *est1⁻* *S. cerevisiae* cells. Instead, deletion of the telomerase RNA gene, *TER1*, of *K. lactis* has uncovered a second pathway of non-telomerase-mediated telomeric DNA replenishment, involving recombination and/or gene conversions between terminal telomeric repeats in the *ter1⁻* survivors (31a). Heterologous telomeres introduced into *S. cerevisiae* also exhibit recombination between the introduced telomeric sequences (44). These results raised the possibility that non-telomerase-mediated pathways play important roles in other systems.

Telomerase activity has been detected in various immortalized human and mouse cell lines, as well as tumor and germ line cells and some normal somatic cell types (6a, 36; reviewed in reference 10). In early studies, it was found that certain primary human somatic cells in culture lacked detectable telomerase activity (8) and that telomeres from these cells decreased in length during cell divisions (8, 17; reviewed in reference 10). These cells eventually reached a state in which they ceased to divide ("crisis"), but in the tiny fraction of cells that for unknown reasons survive crisis and become "immortal," telomerase activity became detectable. In the subsequent cell divisions, telomere lengths stabilized and sometimes increased (8, 23). Therefore, it was proposed that telomerase activity may be required for cell immortalization in vitro and that interfering with telomere length regulation by inhibiting telomerase may be a basis for cancer therapy (9, 17).

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In several studies, attempts have been made to relate telomere lengths to in vitro telomerase activity and cell growth. However, various contradictory results have prevented the emergence of any straightforward relationship between these properties. Although telomerase activity has been detected by in vitro assays of extracts from many immortalized cell lines (8, 22), an immortal human fibroblast cell line with no detectable telomerase activity has been reported (34). An artificially constructed marked telomere and a natural telomere analyzed in this cell line showed highly variable and unstable lengths as the cells were propagated, yet the chromosome bearing the marked telomere was stable and there was no correlation of loss rates of this chromosome with shortening of its marked telomere (34). The lack of detectable telomerase and the patterns of telomere length variability strongly suggested that a non-telomerase-mediated mechanism was acting to maintain the telomeres in this cell line.

Telomeres in cancer cells are often significantly shorter than in normal somatic tissue (9, 11, 18, 21, 39). Hence, it was suggested that because of their increased numbers of divisions, cancer cells that may initially lack telomerase lose more telomeric repeats than do surrounding somatic tissue cells and that when telomerase is reactivated in these cancer cells, telomere lengths stabilize, albeit at a shorter length (8, 9). However, in some tumor samples, the reported telomere lengths were much greater than those of normal surrounding tissues, while other tumor samples showed no changes compared with normal donor cells (19, 35, 39). Reports of telomere lengths in immortalized cell lines have given variable results. Rogalla et al. (37) reported decreased mean telomere length in immortalized cells compared with cells from the originating tumor. On the other hand, telomeres in some immortalized HeLa cell lines can be very long (>20 kb) (11). While many malignant tumors have detectable telomerase activity, some do not (19, 22). Nilsen et al. (35) reported that malignant hematopoietic (acute leukemia) cells could be either positive or negative for telomerase activity but that the telomere lengths were no different in both classes. All these results suggest that the relationship between telomerase activity and telomere length is not a simple one.

Our initial goal was to determine whether telomere length maintenance could be perturbed in immortalized human cells expressing telomerase activity and, if so, whether this would lead to cellular senescence. We have shown previously that telomerase activity from *T. thermophila* can be inhibited in vitro by chain-terminating nucleoside triphosphate analogs known to inhibit retroviral reverse transcriptases. Some of these analogs, including azidothymidine (AZT), caused telomere shortening in vivo in *T. thermophila* (42). AZT also inhibited the developmentally programmed de novo telomere addition of this organism.

Here we report the effects of several inhibitors of retroviral reverse transcriptases on the telomere length and cell growth properties of two immortalized human lymphoid cell lines, the B-cell line JY616, derived from a B-cell lymphoma, and the T-cell line Jurkat E6-1, derived from a human T-cell leukemia. While telomeres in both cell lines reproducibly were progressively shortened by prolonged passaging in the nucleoside analog dideoxyguanosine (ddG), there was no detectable senescence or change in cell growth rates. In addition, passaging in the presence of 100 μ M AZT caused marked progressive telomere shortening over several weeks in some but not all T- and B-cell cultures, again without changing cell growth rates. We show that telomerase activity is present in both cell lines and is inhibited strongly by ddGTP and less strongly by AZT-5'-triphosphate (AZT-TP). Hence, we propose that the in vivo

shortening of telomeres in the presence of ddG or AZT may be attributable to inhibition of telomerase activity by these analogs within the cell.

These studies also revealed unexpectedly high degrees of telomere length variation between parallel cell cultures and dynamic changes in telomere lengths during logarithmic-phase growth in both the T- and B-cell lines with or without inhibitors. We propose that telomeres in the two immortalized lymphoid cell lines examined here are acted on by a combination of telomerase and telomerase-independent mechanisms.

MATERIALS AND METHODS

Manufacturers of reverse transcriptase inhibitors. Arabinofuranyl-guanosine (Ara-G; discontinued), ddI, and ddG were obtained from Calbiochem. ddA, ddGTP, ddTTP, foscarnet, dideoxythymidine (d4T) and dimethyl sulfoxide (DMSO) were obtained from Sigma. AZT was obtained from Boehringer-Mannheim. AZT-TP supplied as a 1-mg/ml solution in sterile water, was obtained from Raymond F. Schinazi through the AIDS Research and Reference Reagent program, Division of AIDS, National Institute of Allergy and Infectious Diseases.

Cell culture. JY616 cells, an immortalized human B-cell lymphoma cell line generously supplied by Joel Goodman, Department of Microbiology and Immunology, University of California, San Francisco, were maintained in RPMI 1640–0.025 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)–10% fetal calf serum supplemented with 100 U of penicillin per ml plus 100 μ g of streptomycin per ml or with 50 μ g of gentamicin per ml at 37°C under 5% CO₂. For each experiment, culture aliquots were maintained in parallel as separate passaging lineages (for exceptions, see below) after the initial aliquoting of the stock culture. Cultures were grown in six-well plates, 5 ml per well in duplicate, with analogs added to the medium to final working concentrations immediately before passaging. Cells were counted with a hemacytometer and transferred every 7 to 10 days (five to seven mean population doublings [MPDs]), with 3×10^4 cells per well seeded into fresh medium containing analog or control medium. In some cases, cell viability was checked before harvesting by using trypan blue stain during counting. For all cultures counted in this way, the average viability was greater than 90%. Remaining cells were pelleted and stored at –80°C until processed for analysis of DNA. All cultures in a given experiment were initially split from the same cell stock. In some cases, cultures were lost over time because of contamination or problems with the growth media. In these cases, the cultures were reseeded at the appropriate density with cells from the corresponding duplicate culture. Only contiguous passages of each culture have been used in this work. Jurkat E6-1 cells (ATCC TIB152), an immortal human T-cell leukemia cell line generously supplied by Art Weiss, Department of Medicine, University of California, San Francisco, were maintained essentially identically to the B cells, in RPMI 1640 (no HEPES)–10% fetal calf serum, with penicillin-streptomycin or gentamicin, seeded at 6×10^4 cells per well. All cultures were split from the same cell stock at the start of the experiment, with two exceptions: RPMI-only control B4 was lost because of contamination at passage 3, and RPMI-only control A4 was lost at passage 4. Both were replaced with the corresponding passage of RPMI-only control A1 and subsequently carried as separate cultures, as noted in the legend to Fig. 9.

To determine the highest concentration of each inhibitor that was nontoxic and that did not affect cell viability or growth, each cell line was cultured in duplicate as described above in various concentrations (in twofold dilutions) of the inhibitor for 1 week (five to seven MPDs). The highest concentration of each analog which did not cause a decrease in cell growth rate measured after 1 week or cause changes in cell morphology was chosen for use throughout the duration of the experiment. For the JY616 line, these concentrations were 10 μ M ddG, 100 μ M AZT, 12 μ M Ara-G, 10 μ M ddA, 300 μ M foscarnet, 50 μ M d4T, 2.5 μ M ddI, and DMSO at a final concentration of 0.01% as the solvent control for ddG and Ara-G. Two RPMI medium-only cultures were also maintained as controls. For the Jurkat cell line, the subcytotoxic inhibitor concentrations determined in this way were 30 μ M ddG, 100 μ M AZT, 0.05 μ M Ara-G, 37.5 μ M ddA, 600 μ M foscarnet, 75 μ M d4T, and DMSO at a final concentration of 0.008% as the solvent control for ddG and Ara-G. Eight RPMI medium-only cultures were also maintained as controls.

Genomic DNA was prepared by incubating cell pellets in 3 ml of lysis buffer (0.1 M NaCl, 10 mM Tris [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K [Boehringer Mannheim] per ml) overnight at 55°C, followed by phenol extraction and ethanol precipitation. DNA pellets were resuspended in TE (10 mM Tris [pH 7.6], 1 mM EDTA [pH 8.0]), and restriction digests were done with either *RsaI* plus *HinfI* as described previously (9) for JY616 cells except those shown in Fig. 1B and C or *MseI* plus *MnlI* for the JY616 cells shown in Fig. 1B and C and all Jurkat E6-1 cells. *MseI* plus *MnlI* was determined empirically to give shorter and therefore clearer terminal restriction fragments from these cell lines than *RsaI* plus *HinfI* in Southern blots with hybridization to a telomeric probe. Approximately 1 μ g of digested DNA per lane was loaded onto a 0.8% agarose gel. Southern blotting was performed by standard methods (38), and blots were hybridized with the α -³²P-labeled oligo-

nucleotide (TTAGGG)₃ at 42°C for 3 to 18 h. Prior to restriction digestion, a sample of each DNA was electrophoresed to verify its integrity. Telomere lengths were measured with an LKB 2202 Ultrascan densitometer, with the center of the peak taken as the mean telomeric restriction fragment length. For several data sets, telomere lengths were also calculated as described previously (17) following scanning of the Southern blot autoradiograms with a digital scanner (Alpha Innotech Corp.).

Preparation of S-100 cell extracts. Extracts were prepared essentially as described previously (8) with minor modifications. Briefly, approximately 6×10^8 cells growing in suspension were collected by centrifugation for 10 min at 3,000 rpm ($1,500 \times g$) in a Sorvall GSA fixed-angle rotor, rinsed twice in cold phosphate-buffered saline (PBS) (without Ca^{2+} or Mg^{2+}), and centrifuged for 3 min at 3,000 rpm ($1,500 \times g$). The final pellet was rinsed in hypotonic lysis buffer (Hypo buffer, consisting of 10 mM HEPES [pH 8.0], 3 mM KCl, 1 mM MgCl_2 , 1 mM dithiothreitol, 0.1 mM Pefabloc SC [Boehringer Mannheim], 10 U of RNasin (Promega) per ml, 1 mM leupeptin [Boehringer Mannheim], and 10 mM pepstatin A [Boehringer Mannheim]) and centrifuged at 1,800 rpm ($600 \times g$) in a Sorvall HB-4 swinging-bucket rotor, and the final pellet was resuspended in 0.75 volume of Hypo buffer. After incubation on ice for 10 min, the cells were transferred to a 7-ml ice-cold Dounce homogenizer and homogenized on ice with a tight-fitting (B type) pestle. After a further 30 min on ice, the suspension was centrifuged for 10 min at 10,000 rpm ($16,000 \times g$) in a Sorvall HB-4 swinging-bucket rotor (this step was omitted for the JY616 cells; instead, the extract remained on ice for an additional 20 min). The supernatant was harvested and centrifuged for 1 h at 48,000 rpm ($100,000 \times g$) in a Beckman TL100 mini-ultracentrifuge TLA100.2 fixed-angle rotor at 4°C. The supernatant was harvested, and glycerol was added to a 20% final concentration for storage at -80°C until DEAE columns were prepared.

DEAE column chromatography of S-100 extracts. A 0.5-ml volume of DEAE-agarose (Bio-Rad Bio-Gel agarose, 100-200 mesh) was equilibrated in ice-cold Hypo buffer, and 0.5 ml of extract was loaded at 4°C. The column was washed with 2 volumes of Hypo buffer followed by 2 volumes of Hypo buffer plus 0.2 M NaCl and eluted with 1 ml of 0.3 M NaCl in Hypo buffer. The eluate was concentrated at 4°C in a Microcon 30 microconcentrator (Amicon) as specified by the manufacturer and brought up to a convenient working volume in 0.3 M NaCl in Hypo buffer, approximately a twofold concentration. Glycerol was added to 20% for storage at -80°C if assays were to be performed later.

Conventional telomerase assays. For the standard telomerase assay, DEAE-purified extract was diluted in Hypo buffer to a convenient working volume so that the final salt concentration was approximately 0.04 M NaCl. A 20- μl aliquot of this dilution was mixed with 20 μl of a $2\times$ reaction mixture and incubated for 2 h at 30°C . The final concentrations in the $2\times$ reaction mixture were 2 mM dATP, 2 mM TTP, 5 μM total dGTP including 50 μCi of [$\alpha\text{-}^{32}\text{P}$]dGTP (800 Ci/mmol; Amersham), 2 μM (TTAGGG)₃, 3 mM MgCl_2 , 1 mM spermidine, 5 mM 2-mercaptoethanol, 50 mM potassium acetate, and 50 mM Tris acetate (pH 8.5). In reaction mixtures containing ddGTP or AZT-TP, telomerase extract was added to the $2\times$ cocktail on ice and this mixture was added to chilled reaction tubes containing analog. The reaction was then started by placing tubes at 30°C . For reaction mixtures in which the concentration of TTP was varied, TTP was omitted from the $2\times$ cocktail and added separately to each reaction tube before addition of enzyme. RNase controls were done by adding 0.7 μl of RNase A (10 mg/ml) and 0.6 μl of 2-mercaptoethanol to the tube before addition of extract. The 40- μl reactions were stopped by adding 50 μl of 0.1-mg/ml RNase A in 20 mM Tris HCl [pH 7.5]–10 mM EDTA to each tube and incubating the tubes for 10 min at 37°C . Reaction mixtures were deproteinized by adding proteinase K (Boehringer Mannheim) at 0.3 mg/ml in 10 mM Tris HCl (pH 7.5)–0.5% SDS, 50 μl per tube, incubating the tubes at 37°C for 15 min, and performing phenol-chloroform extraction and ethanol precipitation with glycogen (Calbiochem) as the carrier. DNA products were separated on a 10% polyacrylamide–7 M urea sequencing gel at 1,700 V for 2 to 3 h with $0.6\times$ Tris-borate-EDTA. Dried gels were exposed on a Molecular Dynamics phosphorimaging screen and analyzed on a Molecular Dynamics phosphorimager. Typical exposures were 1 week. Dried gels were also exposed on Kodak X-AR5 film for up to 30 days.

TRAP assays. TRAP assays were performed essentially as described by Kim et al. (22) with the following minor changes: cells were pelleted, resuspended in 300 μl of PBS, and frozen at -80°C . The cells were thawed and incubated on ice for 30 min in 60 μl of $5\times$ TRAP lysis buffer {[2.5% 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate [CHAPS], 50 mM Tris HCl [pH 7.5], 5 mM MgCl_2 , 5 mM EGTA, 25 mM β -mercaptoethanol, 50% glycerol] plus 2 μl of 100 mM Pefabloc (Boehringer Mannheim)}. Cell debris was pelleted for 20 min at $16,000 \times g$ at 4°C . Supernatants were aliquoted and flash frozen on dry ice before storage at -80°C .

Each telomerase reaction volume was 25 μl above the wax barrier (22), at an extract concentration corresponding to 100 cells per assay. Control assays were performed on samples of each cell type to determine that bands visualized on a 10% polyacrylamide–7 M urea sequencing gel were due to telomerase activity. These included preincubation for 20 min at 37°C with 10 μg of RNase A as a control for non-telomerase-mediated incorporation of the ^{32}P label or with RNase plus RNasin (40 U), phenol extraction of the cell extract before the assay, omission of the cell extract from the assay, and assaying with only the CX or the TS primer (22) (data not shown). Reaction products were labeled with [$\alpha\text{-}^{32}\text{P}$]dGTP and [$\alpha\text{-}^{32}\text{P}$]TTP. Each set of reactions included duplicate reaction

mixtures containing 10 μg of RNase A in the 25 μl above the wax barrier to control for non-telomerase-produced background. One-tenth of each total reaction mixture (5 μl plus 5 μl of formamide loading dye) was run on the gel.

Duplicate reactions were performed to ensure reproducibility of the reaction and of gel loading. Gels were phosphorimaged for 1 to 2 days. They were then exposed to Kodak X-AR5 film for up to 1 week. For the quantitations of TRAP assays shown in Fig. 6, reaction products were separated on a denaturing 10% polyacrylamide gel and quantitated by phosphorimaging, measuring the total phosphorimage units (pixels) for each lane in a central region of the gel. The mean pixels of duplicate RNase-treated reactions were subtracted as background from the mean of each of the other duplicate reactions. The 0 μM inhibitor reactions were considered to be 100% total telomerase activity, and the results were plotted as a percentage of total incorporation against the concentration of inhibitor (micromolar) present in the telomerase reactions.

RESULTS

ddG causes progressive telomere shortening in two immortalized cell lines. Duplicate cultures of the immortalized JY616 B-cell line were tested with several viral reverse transcriptase inhibitors (24): the nucleoside analogs AZT, Ara-G, ddG, ddI, ddA, d4T and a nonnucleoside viral reverse transcriptase inhibitor, foscarnet (a triphosphate analog). Concentrations of each inhibitor used were determined in initial tests (see Materials and Methods). For each inhibitor, the concentration chosen was the highest that did not cause a significant change in cell growth rate or morphology over five to seven population doublings. For each culture, every 5 to 7 days, cells were counted to determine cell growth rates (as MPDs), cell morphologies were monitored, and cells were passaged quantitatively to maintain them in logarithmic growth conditions. Telomere length distributions were analyzed at a series of time points during the serial passaging. Lengths of terminal restriction fragments (TRFs), determined by Southern blotting analyses with a telomeric sequence probe, were used as the measure of telomere lengths, as described previously (18). Consistent with previous measurements of telomere lengths in human cells (17, 18, 26), the data presented here indicate that the great majority of length fluctuations were attributable to different numbers of telomeric TTAGGG repeats rather than to different complex sequences added to ends, since we used frequently cutting restriction enzymes to measure TRFs.

Figure 1A shows the results of one experiment with JY616 cells. After 10 weeks of passaging in the presence of ddG (plus the 0.01% DMSO added as the solvent for ddG or Ara-G), a marked telomere shortening (a $\sim 3.2\text{-kb}$ drop in mean TRF length) was seen compared with the control 0.01% DMSO culture (a $\sim 1.2\text{-kb}$ drop) (Fig. 1A, compare lane 9 with lane 12). However, over the 10-week passaging period, the mean telomere length also declined in all the cultures in this experiment (e.g., a $\sim 2.2\text{-kb}$ drop in RPMI medium alone), although the change after 10 weeks was greatest with ddG present (Fig. 1A, compare lane 9 with lanes 3, 6, 12, and 15; also see Fig. 3, JY4 panel).

Because the decline in telomere length in cells grown without inhibitors was unexpected, the experiments were repeated with the JY616 B-cell line and a different cell line, the Jurkat E6-1 T-cell line. Three separate additional complete experiments, each with duplicate culture sets, were performed with the JY616 cells. In each experiment, an initial stock of each cell line was split into multiple culture aliquots, which were maintained in parallel in log-phase growth conditions by serial passaging in the presence of each reverse transcriptase inhibitor or in control medium lacking an inhibitor. Analogs were tested individually or in certain combinations. Figures 1B and C and Fig. 2 show representative data from one set of Southern blotting analyses, in which JY616 cells were passaged in either medium alone (RPMI panel), 0.01 or 0.03% DMSO, or various inhibitors as indicated on the panels. The Southern blots

FIG. 1. Telomere lengths of JY616 cells grown in the presence of ddG and other analogs. DNAs from serial passages of JY616 cells grown in the absence or presence of analogs or DMSO were restriction digested with *RsaI* and *HinfI* (A) or *MseI* and *MnlI* (B and C), run on a 0.8% agarose gel, and Southern blotted with the ^{32}P -labeled human telomeric oligonucleotide (TTAGGG)₃ as a probe. Numbers above each lane represent the week of passage for the sample. Size markers (in kilobases) are shown to the left of each panel. RPMI lanes are medium-only controls for AZT cultures. DMSO was the solvent for ddG and Ara-G, and DNA preparations from cells grown in medium containing 0.01% DMSO (lanes 10 to 12 in panel A and lanes 7 to 12 in panel B) were used as controls for ddG- or Ara-G-containing cultures; 0.03% DMSO (lanes 1 to 5) in panel C was the control for these two analogs in combination. (A) Lanes: 1 to 3, RPMI medium-only control; 4 to 6, 100 μM AZT; 7 to 9, 10 μM ddG plus 0.01% DMSO; 10 to 12, 0.01% DMSO (control for ddG plus DMSO); 13 to 15, 12 μM Ara-G. (B and C) A separate experiment in which analogs were used in combination, starting from a different stock culture of JY616 cells from that used in panel A. (B) Lanes: 1 to 6, RPMI medium-only control; 7 to 12, 0.01% DMSO as control for ddG plus foscarnet or Ara-G plus foscarnet; 13 to 18: 10 μM ddG plus 300 μM foscarnet. (C) Lanes: 1 to 5, 0.03% DMSO as control for ddG plus Ara-G; 6 to 10, 10 μM ddG plus 12 μM Ara-G; 11 to 14: 12 μM Ara-G plus 300 μM foscarnet; 15 to 18, 10 μM ddG plus 12 μM Ara-G plus 300 μM foscarnet (G/A/F).

shown in Fig. 1B and C were probed with the telomeric ^{32}P -labeled oligonucleotide (TTAGGG)₃, and telomeric mean lengths and distributions were measured by scanning densitometry of the autoradiograms. Figure 2 graphically shows the mean telomere length data over time from this representative set of passaging series, along with the corresponding number of MPDs for each culture.

In every experiment, 10 μM ddG reproducibly caused progressive telomere shortening beyond that seen in control cultures lacking ddG. Figure 3 shows the results for 26 separate passaging series in which telomere lengths were compared at the beginning of the passaging and after the number of MPDs indicated for each culture. The only case in which more telomere shortening occurred than in the parallel culture maintained in ddG was in one culture containing AZT (Fig. 3, JY5 panel). However, in these and all other JY616 cultures, the MPD rates remained similar to each other and did not change significantly over any of the passaging series (see Fig. 2 and 7 for examples; also see below).

In similar experiments, the same inhibitors were tested individually on Jurkat T cells. Again, the highest concentration of each analog which did not cause cytotoxicity after five to seven MPDs was chosen for use as described in Materials and Methods. The cytotoxicity levels for several of the analogs were markedly different for the JY616 B cells and the Jurkat T cells. However, although the concentrations of inhibitors used on the B- and T-cell lines in the time course experiments were different, they were functionally equivalent in terms of being twofold below their detectable cytotoxic levels.

Most of the analogs tested caused no reproducible telomere shortening in Jurkat cells, even after prolonged culture (~ 100 MPDs) in the presence of the analog (data not shown). As described below, Jurkat cell telomere length distributions were highly variable over time and between cultures. However, 30 μM ddG reproducibly caused telomere shortening in duplicate Jurkat T-cell cultures. In a typical result, at the beginning of the experiment the TRF lengths formed a broad distribution ranging from ~ 13 to ~ 2 kb, whereas after 142 MPDs in ddG, they ranged from ~ 8 to ~ 2 kb (data not shown). Again, in all cultures, cell growth rates remained unchanged throughout the entire passaging series.

Inhibition of telomerase activity in vitro by ddGTP and AZT-TP. Because ddG caused TRFs to shorten reproducibly in both the JY616 B-cell and Jurkat T-cell lines and, as described below, AZT had variable effects on telomere lengths, we tested whether telomerase activity from these cells was inhibited in vitro by ddGTP and AZT-TP. A new partial purification protocol for human telomerase and conventional in vitro telomerase reactions (see Materials and Methods) were used to assay for telomerase activity in both cell lines. The TTAGGG repeat elongation products of telomerase were fractionated by polyacrylamide gel electrophoresis. An RNase-sensitive, primer-dependent 6-nucleotide repeat banding pattern was seen for both cell lines (Fig. 4A, compare lanes 17 and R; Fig. 4B, compare lanes 19 and R). Thus, telomerase activity was present in both cell lines. In the reactions with the Jurkat cell fractions, in addition to the RNase-sensitive telomerase products, a few heavily labelled bands appeared at and above the

FIG. 2. Telomere lengths and growth rates of JY616 cells grown in the presence of combinations of analogs, DMSO, or medium-only controls. Densitometry analyses of Southern blots shown in Fig. 1B and C were performed. The center of the broad peak of telomeric DNA hybridization in each lane was used to calculate the average TRF length in kilobases for each cell population. MPDs (right y axis) are shown as open circles plotted against days in culture (x axis). Mean TRF lengths in kilobases (left y axis) are shown as solid squares plotted against days in culture (x axis).

position of the input telomeric repeat oligonucleotide primer (Fig. 4B). These bands were not RNase sensitive (Fig. 4B, compare lanes 19 and R) and therefore may result from addition of [32 P]dG residues to the DNA primer by terminal deoxynucleotidyl transferase, which is overexpressed in some acute lymphocytic T-cell leukemias (29).

ddGTP was an efficient *in vitro* inhibitor of both these human cell telomerase activities, causing 50% inhibition of overall product formation at 1 μ M ddGTP in the presence of 5 μ M dGTP, 2 mM TTP, and 2 mM dATP, as measured by phosphorimager analysis (Fig. 4A, lanes 1 to 6; Fig. 4B, lanes 1 to 8; Fig. 5A, left panel). Interestingly, in contrast to the effect of ddGTP on *T. thermophila* telomerase *in vitro* (14, 42), the inhibition of the telomerase activity from these human cells was not accompanied by alterations in the banding patterns of the products seen in gel electrophoresis (Fig. 4) or in a decreased ratio of longer to shorter products (Fig. 5B). Changes in the relative intensities of the bands corresponding to the positions of dG residues in the TTAGGG repeats, with a concomitant shift to shorter products, would have been expected for chain termination events. Hence, these results are consistent with those reported previously for human HeLa cell telomerase by Morin (32), who proposed that while ddGTP is efficiently recognized, it is not itself incorporated into the newly forming DNA. This differs from the incorporation of dideoxynucleotides and other chain-terminating analogs by *T. thermophila* telomerase (42).

AZT-TP inhibited telomerase activity from both the immortalized JY616 B-cell and Jurkat T-cell lines *in vitro*. As with ddGTP, overall product synthesis was decreased without a significant alteration of the banding pattern of elongation products (Fig. 4A, lanes 7 to 17; Fig. 4B, lanes 9 to 19). However, consistent with previous results from *T. thermophila* telomerase (42), AZT-TP was a much less potent inhibitor of telomerase from these human cell lines than was ddGTP, with

FIG. 3. Change in mean TRF lengths (Δ TRFL) of DNA from JY616 cells grown in the presence or absence of reverse transcriptase inhibitors. Telomere lengths (kilobases) for each of 26 cultures grown with or without analogs were measured by scanning densitometry at an early passage (1 to 3 weeks in culture) and at a later time point when each culture was approximately the same age. Changes in lengths are shown as histogram bars. The number of MPDs elapsed is shown under each bar. Histogram bars: stippled, RPMI medium alone; black cross-hatched, AZT; horizontally hatched, d4T; gray, foscarnet; diagonally hatched, Ara-G plus DMSO; vertically hatched, Ara-G plus foscarnet plus DMSO; black, DMSO; white, ddG plus DMSO. The three panels represent three separate experiments. JY4 is the experiment shown in Fig. 1A. Duplicate cultures are shown for several analogs and controls in experiments JY5 and JY6.

FIG. 4. Lymphoid cell telomerase activity. Telomerase reaction product profiles are shown from conventional telomerase assays (see Materials and Methods) with DEAE-purified S100 extracts from either JY616 cells (A) or Jurkat E6-1 cells (B), performed in the presence of the indicated concentrations of ddGTP (0, 0.5, 1, or 10 μM) plus 2,000 μM TTP or 100 μM AZT-TP with different concentrations of TTP (20, 100, or 2,000 μM). The reaction products were separated on denaturing 10% polyacrylamide gels. Concentrations of ddGTP and TTP are indicated above the lanes. Lane R in each panel indicates RNase-pretreated reactions. The input primer (TTAGGG)₃, labeled by addition of a dG residue with terminal deoxynucleotidyl transferase, is included as a 19-nt size marker (lane M and arrow).

100 μM AZT-TP causing significant inhibition (80%) of product synthesis only when the TTP concentration was lowered to 20 μM (Fig. 4A, lanes 7 and 8; Fig. 4B, lanes 9 and 10; Fig. 5A, right panel).

We tested whether telomerase activity extracted from cells that had undergone up to 38 passages in ddG or 16 passages in AZT had become resistant to either inhibitor. The *in vitro* TRAP assay (22) was used as described in Materials and Methods. Figure 6A shows an example of an autoradiogram of such TRAP assays, performed in the presence of different ddGTP concentrations, for telomerase from JY616 cells. The results of this and similar assays of JY616 cell telomerase, assayed after different numbers of passages in ddG or in control medium containing 0.01% DMSO, and Jurkat T-cell telomerase, after prolonged passaging in AZT or the control medium (see below), were plotted graphically (Fig. 6B and C). There was no

loss of sensitivity to ddGTP or AZT-TP after passaging in the respective analog (Fig. 6B and C). Similar results were obtained with Jurkat cells after passaging for 91 days in ddG (data not shown). Hence, there was no evidence for any selection for a subset of cells whose telomerase had mutated to become resistant to either ddGTP or AZT-TP.

Highly variable telomere lengths in the B- and T-cell lines. A previous report (8) indicated that telomere lengths were relatively stable over many generations in an immortalized human embryonic kidney cell line (the HA1-IM 293 line). In contrast, highly variable and stochastic patterns of telomere length changes were apparent in both of the immortalized human cell lines analyzed here. In all the experiments performed, the cell growth conditions were closely controlled to maintain logarithmic growth rates. Despite these precautions, telomere length variability was very marked not only within a culture during its propagation for prolonged periods but also between cultures passaged in parallel. We observed large changes in mean telomere lengths as well as in length distributions, with different types of changes being characteristic for each cell line.

In JY616 cells, as shown in Fig. 1, the entire population of telomeric restriction fragments usually consisted of a single broad peak. Hence, at any one time point, telomeres fell primarily into a unimodal length distribution about a single common mean length. However, the mean telomere length both increased and decreased over time in many cultures, including those lacking any inhibitor. Furthermore, for the most part, these changes were different between duplicate cultures propagated in parallel after seeding with aliquots from the same initial stock culture. The only exceptions to this nonreproducibility were the B- and T-cell cultures grown in the presence of ddG, which invariably showed progressive telomere shortening, as described above. Figure 7 shows a sampling of the variety of telomere length dynamics observed in JY616 cells cultured for up to 281 days.

Many of the JY616 cultures exhibited both gradual (~ 10 to 40 bp/MPD) and rapid (~ 200 to 400 bp/MPD) stochastic decreases in telomere length. An example of progressive gradual decrease was seen in one AZT passaging series (Fig. 7, AZT panel). Between passages 5 and 19 (days 36 to 148), the mean TRF length gradually decreased by ~ 20 bp/MPD, leading to an overall telomere shortening of ~ 1.6 kb. In an experiment in which a culture was grown in 10 μM ddG for 281 days, as with all other ddG cultures, the telomeres shortened (Fig. 7, ddG panel). The rate of loss was ~ 73 bp/MPD between passages 5 and 9 (days 20 to 36) in this passaging series. At 120 days, the culture was split in two and the remainder of the passaging series was continued in either ddG alone or ddG plus 100 μM AZT. The telomeres remained short in both passaging series. The growth rates were slightly but not significantly different for the cultures in ddG alone versus those in ddG plus AZT (Fig. 7, 10 μM ddG panel). With Ara-G or foscarnet alone, as with AZT alone, telomere shortening was seen sporadically (although not reproducibly [see below]). However, adding Ara-G and/or foscarnet together with ddG caused no additive or synergistic effects on telomere shortening or changes in long-term cell growth rates (Fig. 2 and 3). One d4T culture showed small decreases and increases in mean telomere length during the entire experiment but no consistent long-term trend toward either shortening or lengthening (Fig. 7, d4T panel).

With foscarnet, in one culture between passages 5 to 15 (from days 36 to 117, corresponding to 66 MPDs), the mean TRF also decreased by ~ 15 bp/MPD (Fig. 7, left foscarnet panel), whereas in the duplicate foscarnet culture, there was a very gradual overall decrease of ~ 4 bp/MPD between passages

FIG. 5. Inhibition of lymphoid cell telomerase activity by ddGTP and AZT-TP. (A) Conventional telomerase assays were performed with DEAE-purified S-100 extracts from either JY616 or Jurkat E6-1 cells (see Materials and Methods). The intensities of the first four strong bands with 6-base periodicity above the input primer size were measured by phosphorimaging. After subtraction of backgrounds, the total phosphorimager units (pixels) of the sum of all four bands in each lane are plotted (y axis), setting the values for the control reactions at 100%. Data shown for Jurkat cell and JY616 cell telomerase reactions with ddGTP are the average for three experimental duplicate sets. Data for JY616 cell telomerase with AZT-TP represent one experimental duplicate set. (B) Quantitation of each of the first four strong bands with 6-base periodicity above the input primer size in the telomerase reaction product profiles. Conventional telomerase assays were performed as in panel A with different concentrations of ddGTP. Bands 1 and 4 are the shortest and longest product measured, respectively.

5 and 31 (Fig. 7, right foscarnet panel). Decreases in telomere lengths of ~ 50 bp/MPD have been reported in primary and immortalized human fibroblast cell lines lacking detectable telomerase (8, 17, 34). Thus, these gradual decreases in length in the JY616 cells were less than would have been predicted from the absence of telomerase-mediated maintenance.

Rapid stochastic decreases in mean TRF lengths were observed in some cultures of JY616 cells. In 12 μM Ara-G, in just one passage (between passages 9 and 10; days 64 to 76) the mean TRF length of the entire telomere population dropped by approximately 380 bp/MPD (Fig. 7, Ara-G panel). A less extreme drop was seen in another Ara-G culture (~ 75 bp/MPD over passages 6 to 8; days 43 to 58 [data not shown]).

Rapid stochastic increases in TRF length of some or all of the entire telomere population were also observed; in one example of JY616 cells passaged in DMSO, a mean TRF increase of ~ 240 bp/MPD for the entire telomere population was observed between the third and fifth passages (data not shown). In an interesting example (Fig. 7, left foscarnet panel),

initially the majority of the population of telomeres steadily lost approximately 1 kb of telomeric DNA over 66 MPDs (~ 15 bp/MPD). Then in a single passage of 6.5 MPDs (between passages 14 and 15), a large telomere subpopulation suddenly gained almost 4 kb in length, an increase of ~ 600 bp/MPD. At this point, this cell culture abruptly exhibited a bimodal distribution of telomere lengths, after which the population of longer telomeres gradually shortened again. Southern blotting analysis of this passaging series is shown in Fig. 8. In contrast, the duplicate culture grown in foscarnet did not exhibit any notable changes in mean telomere length or length distribution (Fig. 7, right foscarnet panel).

With Jurkat T cells, in most cultures the mean TRF lengths increased dramatically as the cells were propagated in log-phase growth conditions (Fig. 9, lanes 1 to 5, 6 to 10, 21 to 25, and 26 to 30). As described above, the only reproducible exception to the general telomere lengthening was with ddG, in which telomeres invariably steadily shortened. In several cultures, the overall hybridization patterns also became more

FIG. 6. Inhibition by ddGTP and AZT-TP of telomerase activity from cells passaged in the presence or absence of ddG or AZT. (A) Denaturing 10% polyacrylamide gel showing reaction products of TRAP assays (22) of telomerase performed with different ddGTP concentrations. TRAP assays (see Materials and Methods) were performed in duplicate on extracts from JY616 cells passaged in the presence of 0.01% DMSO for 30 weeks. The indicated concentrations of ddGTP were added to the telomerase reaction mixtures. These concentrations were previously determined to cause no inhibition of the PCRs when added to the PCR mixture only following the telomerase reaction (data not shown). RNase A was added to duplicate telomerase reaction mixtures as a control for non-telomerase-mediated incorporation of the 32 P label (+R lanes). The amount of extract used per reaction represented approximately 100 cells, with 1/10 of the total reaction mixture loaded onto a denaturing 10% polyacrylamide gel. (B and C) TRAP assays were performed in duplicate as in panel A, with approximately 100 cells per reaction, and contained the indicated concentrations of ddGTP or AZT-TP added to the telomerase reaction mixture. These concentrations were previously determined to cause no inhibition of the PCR. (B) JY616 cells grown in 10 μ M ddG for one passage ($\square$) and in 10 μ M ddG for 12, 30, and 38 passages ($\blacksquare$) or in 0.01% DMSO for one passage ($\triangle$) or 30 passages ($\blacktriangle$). (C) Jurkat E6-1 cells grown for 16 passages in 100 μ M AZT ($\blacksquare$) or RPMI control medium ($\blacktriangle$). The two Jurkat cultures grown in AZT are each passage 16 of the same passaging series shown in Fig. 9, lanes 17 to 20 and 26 to 30, and the control RPMI culture is passage 16 of a passaging series similar to that shown in Fig. 9, lanes 7 to 10.

complex, suggesting that multiple subpopulations of telomeres with widely separated mean lengths were generated, each subpopulation having a relatively tight distribution about its mean length, so that the telomeres now fell into discrete length subsets (Fig. 9, lanes 9 and 10 and lanes 28 to 30). In one culture grown in 100 μ M AZT, one modal telomere size class, which shortened in concert with the rest of the telomeres, decreased in mean TRF length from $\sim$ 12 to $\sim$ 8 kb over 15 passages, a shortening rate of $\sim$ 50 bp/MPD (Fig. 9, lanes 16 to 20). The entire telomere population in this culture showed the same steady shortening over the entire 15 passages (92 MPDs) monitored. This shortening was clearly distinguishable from the length variations in control cultures: without AZT or ddG no similar steady shortening was seen in any of the total of 24 different Jurkat E6-1 cell-passaging series analyzed. In contrast, telomeres in the duplicate AZT culture (Fig. 9, lanes 26 to 30) showed the same general increase in telomere lengths as did several of the control cultures (compare with Fig. 9, lanes

21 to 25). However, the sensitivity to AZT-TP of telomerase activity from both cultures remained the same as that for the control culture (Fig. 6).

Extreme telomere shortening without cell senescence. The shortest mean TRF lengths occurred in JY616 cells grown in the presence of ddG or Ara-G. Examples were found in which the mean TRF lengths were $\sim$ 1.1 kb (ddG, foscarnet, plus Ara-G), $\sim$ 1.6 kb (ddG plus foscarnet), $\sim$ 1.3 kb (another culture grown in ddG plus foscarnet), and $\sim$ 1.4 kb (a culture grown in ddG plus foscarnet plus Ara-G) (Fig. 2 and 3 and data not shown). Furthermore, the broad band comprising the total telomeric fragment population was clearly visible down to 1.0 kb at these and several other points in these experiments (for example, Fig. 1B, lane 17 and Fig. 1C, lanes 10 and 18). Because the hybridization signal obtained with the short oligonucleotide probe (TTAGGG) $_3$ underestimates the numbers of telomere molecules in proportion to the shortness of the telomere tract (see references 8 and 26 for discussion), the mean telomere length was also calculated as described previously (17) to correct for this underestimate. As expected, even lower estimates were obtained for the mean telomere lengths (data not shown), and the very short ($\sim$ 1.0-kb) telomeric restriction fragments often made up a significant proportion of the total telomeres present in the cell population.

The TRFs include subtelomeric DNA, which contains tracts of degenerate, TTAGGG-cross-hybridizing sequences (1, 7). Because it is not known whether these act as functional TTAGGG tracts, the presence of these degenerate tracts normally prevents exact length determination of the functional tracts on natural human telomeres. An estimate for the minimum telomeric TTAGGG-hybridizing tracts in a human embryonic kidney cell line at crisis was made with the same *RsaI-HinfI* digestion of human genomic DNA used here, by quantitating the TTAGGG repeat hybridization signals as a function of TRF length. At crisis, the mean TRF length fell to its observed minimum of $\sim$ 3.4 kb, and the average length of TTAGGG-hybridizing repeat tracts was $\sim$ 1.5 kb in these cells (8). Hence, the shortest TRFs in the JY616 cells were considerably shorter than those reported previously for natural telomeres. This may reflect the different cell types used in the different studies or, possibly, differences in growth conditions between this and other studies.

In an experiment in which JY616 cell cultures were maintained in parallel in continuous log-phase growth by frequent passaging for up to 281 days in either RPMI medium alone, 0.01% DMSO, or various inhibitors, the shortest mean telomere lengths observed were in 12 μ M Ara-G ($\sim$ 1.6 kb; Fig. 7), and 10 μ M ddG ($\sim$ 1.7 kb; Fig. 7). After these shortest telomeres were observed, the mean telomeric fragment lengths increased slightly and then stabilized at $\sim$ 2.1 and $\sim$ 2.0 kb, respectively. These stabilized lengths represented relative net losses of $\sim$ 0.9 and $\sim$ 1.3 kb, respectively, from the measured starting lengths. The loss, gain, and stabilization of these telomeres were reminiscent of the dynamics seen in the small fraction of primary cells which overcome cell cycle controls to become immortalized (8, 23). However, in the situation reported here, these telomere length changes were not accompanied by any significant decreases in cell population doubling rates or increases in the percentage of trypan-blue-positive cells, which might have indicated increased cell death.

In all the experiments, in spite of the dramatic telomere losses in JY616 cells, no senescence phenotype was observed for the cell population, even after continuous logarithmic phase growth in the presence of 10 μ M ddG for up to 238 mean population doublings (Fig. 1, 2, and 7 and data not shown). Likewise, Jurkat cells maintained in logarithmic-phase

FIG. 7. Telomere lengths and growth rates of JY616 cells grown in the presence of analogs for up to 281 days. Growth rates and TRF lengths were calculated and plotted as in Fig. 2. MPDs (right y axis) are shown as open circles plotted against days in culture (x axis). Mean lengths of TRFs (in kilobases) (left y axis) are shown as solid squares plotted against days in culture (x axis). In the 10 μ M ddG panel, the culture was split in two, and beginning at day 120, passaging was continued in 10 μ M ddG alone ($\blacksquare$, telomere lengths; $\circ$, MPDs) or in 10 μ M ddG plus 100 μ M AZT ($\blacksquare$, telomere lengths; $\odot$, MPDs). In the left foscarnet panel, in which two distinguishable telomere subpopulations were seen, the mean length shown is that of the major subpopulation.

growth for ~ 100 MPDs in the presence or absence of inhibitors showed no decrease in growth rate or changes in cell morphology (data not shown). At the times during propagation when telomeres reached minimal lengths, as discussed above, there were no detectable changes in cell viabilities or population doubling rates. Hence, even extreme loss of telomeric DNA in these immortalized cell lines did not correlate with morphological changes, senescence, or cell death.

DISCUSSION

Human telomere shortening and telomerase inhibition by the nucleoside analog ddG. We have shown here that the nucleoside analog ddG reproducibly causes progressive telomere shortening in two immortalized human lymphoid cell lines, the B-cell line JY616 and the T-cell line Jurkat E6-1. This analog was the only reverse transcriptase inhibitor tested that consistently caused this effect in every culture duplicate of both cell lines. The progressive loss of telomeric DNA observed

with ddG and in some cultures with AZT is predicted to occur in the absence of telomerase (reviewed in references 3 and 10) and resembles that initially seen after disruption of the telomerase RNA gene in *T. thermophila* and the budding yeasts *K. lactis* and *S. cerevisiae* (12, 31, 41, 46). Similar gradual shortening was reported in some subclones of an immortalized human fibroblast line which lacks detectable telomerase (34) and in human primary fibroblasts in culture, which also lacked detectable telomerase activity, as they approached senescence (8). However, telomerase activity was present in cell-free fractions of both the cell lines studied here and was efficiently inhibited in vitro by ddGTP. No effects on cell doubling rates, viability, or morphology occurred at the ddG concentrations used here, suggesting that other cellular DNA polymerases or DNA metabolic enzymes were not significantly perturbed. These results suggest that the progressive telomere shortening in all cultures grown in ddG is caused by inhibition of telomerase itself. The in vitro results further suggest that in human cells, ddG may cause its telomere-shortening effects by binding

FIG. 8. Telomere lengths of JY616 cells grown in the presence of 300 μ M foscarnet. DNAs from serial passages of JY616 cells grown in the presence of 300 μ M foscarnet were restriction digested with *Rsa*I and *Hinf*I, separated on a 0.8% agarose gel, and Southern blotted with the 32 P-labeled human telomeric oligonucleotide (TTAGGG)₃ as the probe. Numbers above each lane represent the week of passage for each sample. S indicates DNA from the originating stock cell culture, not treated with analog. Kilobase size markers are shown to the left of the panel. The corresponding graph is shown in Fig. 7, left foscarnet panel.

to and competing for the nucleoside triphosphate-binding site of telomerase rather than by being incorporated and causing chain termination, since no evidence was found for incorporation of chain-terminating ddG residues *in vitro*. Hence, these findings provide evidence that telomere maintenance in human cells in culture can be perturbed by inhibition of telomerase activity by an exogenously added nucleoside analog.

In spite of significant telomere losses, the two established immortalized cell lines studied here continued to thrive. No senescence or changes in overall cell growth rates were observed even after continuous passaging under log-phase growth conditions for almost 1 year in the presence of ddG. Specifically, a significant finding was that no changes in doubling rates were detected at the points at which telomeres first reached minimum lengths. Telomerase remained sensitive to ddGTP *in vitro*. These results suggest that the degree of disruption of telomerase in these cell lines was insufficient to promote cellular senescence.

The ability of immortalized JY616 B cells to maintain telomeres at short lengths for prolonged periods in the presence of ddG, with no apparent decrease of population doubling rates, is consistent with observations in other systems suggesting that telomere length is regulated. Telomeres in many organisms do not normally fall below specific minimum lengths. During cell divisions in several of the same reverse transcriptase inhibitors tested here, *T. thermophila* telomeres decreased to a minimum threshold length below which no telomeres were detectable (42). Other studies of human telomeres are also indicative of a critical lower length limit below which telomere associations and fusions may increase (reviewed in reference 10). However, the exact minimum length for the TTAGGG repeat tract in

human telomeres is unclear. In all the JY616 cultures, there appeared to be a minimum threshold length for the TRFs of ~ 1.0 kb. Hence, if the subtelomeric sequences in the TRFs of this cell line are similar to those in the embryonic kidney cell line analyzed by Counter et al. (8), this may represent as little as a few hundred base pairs of telomeric TTAGGG repeats. Such a short length is consistent with the very short telomeric TTAGGG repeat tracts reported at the end of an artificially constructed telomere in an immortalized human fibroblast cell line lacking detectable telomerase (34). Lacking associated tracts of degenerate TTAGGG-cross-hybridizing repeats, this telomere allowed accurate measurement of its minimal telomeric TTAGGG repeat tract length. At times, this was as little as a few hundred base pairs or less, with no apparent accompanying changes in cell growth rates or chromosome loss (34).

Analysis of telomere length regulation in cells of the yeast *K. lactis* synthesizing variant telomeric sequences has provided strong evidence that interactions with the telomeric DNA-protein complex regulate telomerase action at the chromosome end (31). The predicted consequence of this feedback mechanism is the selective addition of telomeric repeats to telomeres bearing shorter telomeric tracts, bringing their net lengths up to the level of the average population. While no direct information is available about the mechanism of such regulation in human cells, our results suggest that similar feedback could operate in the B- and T-cell lines maintained in ddG. In addition, as discussed below, sporadic mass shortening of the entire telomere population in several cultures also occurred, with no accompanying changes in cell population doubling rates or cell morphology and no evidence for senescence. The lack of senescence or cell death when the telomeres reached very short lengths and the stabilization of telomeres after that point suggest that to effectively overcome the regulation of telomere repeat addition, it will be necessary to perturb telomere length more severely than was accomplished by the inhibitors in this study.

Evidence for nonreciprocal recombination or gene conversion events. An unexpected outcome of the present studies was the high degree of variability in telomere lengths revealed in both of the human immortalized lymphoid cell lines analyzed. In the JY616 B-cell line, the stochastic changes in telomere lengths were usually concerted increases and decreases in mean length of the entire telomere population. These occurred either gradually or rapidly. Gradual concerted length changes are expected if the predominant telomere maintenance mechanism involves regulated additions by telomerase, countered by gradual telomere-shortening processes. In other human and nonhuman systems, telomeres are also globally regulated in concert (2, 8, 25, 43), with mean telomere length and length distribution being determined by the interactions of many genetic loci in *S. cerevisiae* (43; reviewed in reference 3). Over time, even slight perturbations of the relative rates of telomerase action versus DNA losses from the chromosome end will lead to gradual lengthening or shortening (reviewed in reference 3). While in the JY616 cells the trend was often toward gradual shortening (Fig. 1A, 7, and 8), this varied in rate even between duplicate cultures. In some but not all ddA and DMSO cultures, the mean TRF length increased gradually throughout the entire passaging (data not shown). These results suggest that the actions of telomerase and shortening mechanisms were modulated in a variable fashion. Since different cell culture aliquots showed marked differences in the lengths of the entire telomere population from culture to culture, small changes in cell conditions and possibly feedback between cells may have occurred in each cell population.

The sudden large drops in average telomere length, most

FIG. 9. Telomere length dynamics in Jurkat E6-1 cells grown in the absence or presence of 100 μ M AZT. DNAs from serial passages of Jurkat E6-1 cell cultures grown in the absence (lanes 1 to 15 and 21 to 25) or presence (lanes 16 to 20 and 26 to 30) of 100 μ M AZT were restriction digested with *Mse*I and *Mn*I, separated on a 0.8% agarose gel, and Southern blotted with the 32 P-labeled human telomeric oligonucleotide (TTAGGG)₃ as the probe. Numbers above each lane represent the week of passage for each sample. Kilobase size markers are shown to the left of each panel. All cultures analyzed were split from the same cell stock at the start of the experiment (except that shown in lanes 6 to 10, which was subcultured at 3 weeks from the culture shown in lanes 11 to 15) and grown under identical conditions. Each set of lanes represents a separate culture; i.e., lanes 1 to 5, lanes 6 to 10, lanes 11 to 15, and lanes 21 to 25 represent four different medium-only control cultures, respectively. Lanes 17 to 20 and lanes 26 to 30 show two separate cultures grown in 100 μ M AZT.

notably the ~ 380 -bp/MPD drop in one JY616 cell culture grown in Ara-G, were much faster than predicted from lack of telomerase activity, which has been associated with loss rates of only ~ 50 bp per MPD (8, 17, 34). In addition, the ~ 600 -bp/MPD length increase in one foscarnet culture (Fig. 7 and 8) was much greater than expected for telomerase action. Again, it was striking that in JY616 cells, usually the entire telomere population was shifted abruptly up or down in length.

The Jurkat E6-1 T-cell line showed even more complex and dynamic telomere length behavior. On the one hand, except in ddG, most telomeres in most T-cell cultures gradually lengthened during prolonged log-phase growth, with few if any telomeres detectable in the lower regions of the gels. On the other hand, the telomere population also stochastically showed sudden large changes in patterns of length distributions, with several discrete size classes often generated simultaneously. The observation of rapid, wide fluctuations in telomere lengths in an immortalized fibroblast cell line lacking detectable telomerase activity led Murnane et al. to propose that elongation of shortened telomeres can occur by nonreciprocal recombination (34). The large, rapid, stochastic increases and decreases in average telomere lengths in the B and T cells studied here are strikingly similar to the results with this fibroblast line. Telomere-telomere gene conversion and/or recombination events, often leading to massively lengthened telomeres, have

been seen in *K. lactis* cells with telomerase RNA gene deletions (31a). The nontelomerase telomere maintenance pathways operative in the descendants of the survivors of the telomerase RNA deletion event and in the human fibroblast cell line lacking detectable telomerase (34) are adequate to support indefinite and relatively rapid cell population doubling rates.

To account for the surprising degree of telomere length variability in the immortalized human B and T cells analyzed here, we propose that in these cells, telomeres are maintained through a combination of telomerase and nontelomerase mechanisms acting on telomeric DNA. Our results indicate that the relative predominance of these mechanisms is characteristic of individual cell lines and may vary within a cell line at different times. Specifically, we propose that the large length changes characteristic of the Jurkat cell cultures and seen more rarely in the JY616 cultures result from extensive nonreciprocal recombination or gene conversions between telomeres. Such mechanisms could lead to periodic homogeneous lengthening or shortening of subsets of the telomeric population. These observations suggest for the first time for human cells that nontelomerase mechanisms can be a major determinant of telomere length, even in cells in which telomerase is active, and can obscure its action. Such pathways are potentially an impediment to therapeutic approaches involving targeting telomerase activity.

An important aspect of the present study is that the strong stochastic component of telomere length variation would not have been revealed if we had not passaged and analyzed duplicate and often multiple cultures in parallel from the same initial stock. The variability in telomere populations was particularly striking because cell growth conditions were carefully controlled: MPD rates were monitored closely by cell counting, and passaging was performed quantitatively and frequently to maintain logarithmic growth rates. However, even though measured MPD rates may be constant for long periods, a characteristic of immortalized mammalian cells maintained in culture is the wide range of doubling rates of individual cells in the population. One study has shown that slowly and rapidly dividing cells within an immortalized population growing at a steady overall rate constantly give rise to cells with highly heterogeneous division rates, whose doubling rates repeatedly tend toward that of the population as a whole (16). Hence, even though we observed constant MPD rates, the superposition of inherent clonal heterogeneity onto stochastic alterations in telomere lengths may have contributed significantly to the large jumps in length seen in the populations and subpopulations of telomeres.

Effects of AZT on telomere maintenance. Stochastic variations in cell populations with variable clonal cell division rates may underlie the differences we observed between duplicate cultures grown in AZT, the only other inhibitor besides ddG that caused significant progressive telomere shortening. Although the effects were not identical from one culture aliquot to another, when telomere shortening did occur in the presence of AZT, the trend was very consistent for many passagings. Although AZT-TP was only a relatively weak inhibitor of human T- and B-cell telomerase in the *in vitro* assays used here, such assays may not directly reflect the degree of the effects in the cell.

Our results suggest that AZT-TP may exert a direct effect on human telomerase and that AZT can perturb telomere maintenance in immortalized T cells. As AZT is used clinically in the treatment of AIDS (24), its effect on a T-cell telomerase is of particular interest. It has recently been shown that normal T cells express telomerase activity (6a). Since human immunodeficiency virus type 1 infection is accompanied by large amounts of new CD4⁺ T-cell production (20, 45), sustaining this additional prolonged proliferation in response to human immunodeficiency virus type 1 infection may require telomerase activity. Therefore, although the predominance of different pathways for telomere maintenance may differ between cell types and immortalized cells in culture may differ from those *in vivo*, a possible clinical implication of these findings is that AZT may interfere with telomere maintenance and hence could compromise T-cell proliferation during human immunodeficiency virus type 1 infection in patients.

In summary, we have shown that ddG causes marked progressive telomere loss in immortalized cells in culture, probably from inhibition of telomerase activity, and our data suggest that AZT may have similar effects. However, overall cell growth rates were unchanged, and the results suggest that when telomeres become very short, they may be subject to regulation which counteracts further losses of telomeric DNA. These detailed studies of telomere length dynamics also uncovered a strong variable and stochastic component to telomere length regulation in the two immortalized cell lines analyzed. These insights into telomere length regulation may be relevant to therapeutic approaches against both cancer and human immunodeficiency virus type 1 infection.

ACKNOWLEDGMENTS

We thank Karen Kirk, Michael McEachern, and Renata Gallagher for critical comments on the manuscript and other laboratory members for helpful discussions; Joel Goodman and Art Weiss, University of California, San Francisco, for cell strains; and Raymond F. Schinazi, AIDS Research and Reference Reagent program, division of AIDS, NIAID, NIH, for AZT-5'-triphosphate.

This work was supported by grant GM26259 from the NIH to E.H.B. Laboratory facilities were provided in part by the Lucille P. Markey Charitable Trust.

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