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Telomerase activity in normal and malignant hematopoietic cells

(telomere dynamics/chromosome structure/cell immortalization/leukemia)

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ABSTRACT **Bone marrow and peripheral blood leukocytes from 19 leukemia patients were found to contain telomerase activity detectable by a PCR-based assay. Telomerase was also detectable in nonmalignant bone marrow and peripheral blood leukocytes from normal donors, including fractions enriched for granulocytes, T lymphocytes, and monocytes/B cells. Semiquantitative comparison revealed considerable overlap between telomerase activities in samples from normal subjects and leukemia patients, confounding evaluation of the role of telomerase in this disease. These data indicate that human telomerase is not restricted to immortal cells and suggest that the somatic expression of this enzyme may be more widespread than was previously inferred from the decline of human telomeres.**

Human telomeres undergo progressive shortening during the development of somatic tissues. Telomeric decline was first discerned from the shorter terminal restriction fragments in peripheral blood cell DNA compared with germline DNA (1–3); additional evidence indicates that telomere attrition accompanies the formation of most human tissues (for review see ref. 4). The loss of terminal sequences can also be witnessed directly during *in vitro* culture of a variety of primary cells, including bone marrow leukocytes, T cells, and B cells (5–8).

A possible mechanism for the decline of human telomeres is the somatic repression of telomerase, an RNA-dependent DNA polymerase that is thought to maintain telomeres by adding telomeric TTAGGG repeats to the 3′ ends of human chromosomes (9, 10). Without telomerase, telomeres are predicted to shorten with cell divisions through replication-dependent sequence loss at DNA termini (11). In agreement with this idea, telomerase activity has not been detected thus far in extracts from a variety of normal human somatic tissues, whereas extract from testis yielded active telomerase (12). Telomerase expression in testis was anticipated from the long telomeres in sperm DNA, the longer telomere length in sperm of older men, and the maintenance of telomeric DNA over generations (1–3, 13).

It has been proposed that immortalization of somatic human cells involves a mechanism to halt telomere shortening and avert the deleterious consequences of uncapped chromosome ends (ref. 5; reviewed in ref. 14). In experimental tests of this idea, telomere stabilization accompanied immortalization in virally transformed primary human embryonic kidney (HEK) cells, keratinocytes, and B cells (8, 15, 16). In simian virus 40-transformed HEK cells and in Epstein–Barr virus-transformed B cells, telomerase was not detectable before the cultures entered crisis. Furthermore, the postcrisis stabilization of telomeres was accompanied by a dramatic increase in telomerase activity. Hence, it has been suggested that telomere dynamics are primarily regulated at the level of telomerase expression.

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Recent evidence suggests that changes in telomere dynamics play a role in the malignant transformation of human cells *in vivo* (for review see ref. 17). Since tumor telomeres are usually shorter than the telomeres in neighboring normal tissues, telomeric attrition appears to continue in the early stages of tumorigenesis (3, 18), and the resulting telomere loss has been suggested to contribute to the destabilization of cancer chromosomes (19). However, many human tumors contain high levels of telomerase activity at the time of diagnosis, suggesting that telomerase contributes to later stages of tumor progression (12, 20). One possibility is that cells with higher telomerase activity have a selective growth advantage due to improved stability of chromosomes with restored telomeres.

To determine whether telomerase is activated in hematologic malignancies, we measured telomerase activity in extracts of leukocytes from normal donors and leukemia patients. Our data indicate that telomerase is expressed in normal human bone marrow and peripheral blood cell populations. Quantitative estimates did not reveal a significant increase in telomerase activity in bone marrow cells from leukemia patients compared with those from normal donors. These findings are relevant to the diagnostic and therapeutic value of telomerase and raise questions about the role of telomerase in normal and transformed human cells.

MATERIALS AND METHODS

Human Leukocyte Fractions. Granulocytes and peripheral blood mononuclear cells (PBMCs) were obtained respectively from the pellet and interface of Ficoll-Paque (Pharmacia)-separated, heparinized peripheral blood from normal volunteer donors. Erythrocytes were depleted from the granulocyte fractions by hypotonic lysis. PBMCs were further fractionated by standard procedures involving rosetting with neuraminidase-treated sheep erythrocytes: T cells were separated from non-T cells among the PBMC population on the basis of their binding to sheep erythrocytes via CD2. The remaining mononuclear cells, consisting of monocytes, B cells, and a trace population of dendritic cells, are referred to as the monocyte/B-cell fraction. Resting T lymphocytes were further purified by elution over nylon wool columns. Bone marrow samples were obtained as "found specimens" from normal donors already undergoing bone marrow harvesting for allogeneic transplantation and were used as bulk bone marrow mononuclear cells from the Ficoll-Paque interface. Bone marrow or peripheral blood was also obtained as found specimens from patients with myeloid and lymphoid malignancies. Bulk mononuclear cell fractions from Ficoll-Paque interfaces were used in either case without further fractionation. Patients were at various stages of disease, pre- or post-allogeneic bone marrow transplant, and receiving various or no therapies at the time of sample

Abbreviations: PBMC, peripheral blood mononuclear cell; TRAP, telomeric-repeat amplification protocol.

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collection. Samples were obtained in accordance with institutional guidelines.

Cell Extracts. S100 fractions (21) or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) detergent extracts (12) were prepared from isolated bone marrow and peripheral blood cells. These methods yielded detectable telomerase activity in all but 2 of the 27 leukemia extracts. The two negative extracts may represent experimental inactivation of the enzyme, since preparations obtained at different time points from the same patients yielded active telomerase. Protein concentrations were determined by the Bradford assay (Bio-Rad) with bovine serum albumin as a standard. Independent confirmation of protein concentration was provided by Western blot analysis using an antibody which recognizes the members of the heterogeneous nuclear ribonucleoprotein (hnRNP) D group (22). Western blot analysis also indicated the absence of protein degradation in the extracts.

Telomerase Assays. Initial detection of telomerase in leukemia extracts employed the PCR-based telomeric-repeat amplification protocol (TRAP) described by Kim *et al.* (12), using the oligonucleotides TS and CX as forward and reverse primers, respectively, and incorporation of [α-^{32}P]dGTP into the PCR products as a means of monitoring the reaction. In later assays, the PCR products were detected through the use of end-labeled TS primer, which gave essentially the same results. The latter method was found to be preferable for the detection of low levels of telomerase, because the sensitivity and signal-to-noise ratio were considerably improved. In brief, TRAP assays were performed as follows: the TS primer was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, isolated over Sephadex G-25, and diluted with nonradioactive TS to a specific activity of $\approx 5 \times 10^6$ cpm/μg. Telomerase was allowed to extend an excess of the TS primer (0.1 μg) for 15 min at 22°C in 50 μl of the buffer described by Kim *et al.* (12), which includes *Taq* DNA polymerase. RNase digestions were performed by adding 0.5 μg of RNase A (Pharmacia; heat-inactivated at 65°C for 20 min) to the extension-step mixture. After the telomerase extension reaction, the tubes were incubated at 65°C for 3 min and 0.1 μg of the reverse primer [CX (12) or, in later reactions, 5′-GGCGCGACCCTAACCCTA-ACCCTA-3′] was added to each reaction mixture. PCR was performed for 27 cycles with a 30-sec denaturation step at 94°C, 30-sec annealing step at 50°C, and 1-min extension step at 72°C. Products were resolved by electrophoresis in nondenaturing 15% polyacrylamide gels (19:1 acrylamide/*N,N′*-methylenebisacrylamide weight ratio) in 45 mM Tris base/45 mM boric acid/1 mM EDTA for 1800 V·hr. Gels were fixed and exposed without drying to PhosphorImager (Molecular Dynamics) screens for 5–48 hr. TRAP assays were scored as negative when products could not be visualized after a 48-hr exposure. It should be noted that the length of the products does not necessarily reflect the processivity of telomerase; some additional elongation of the telomerase products with 6-nt increments can occur as a consequence of staggered annealing of the reverse PCR primer.

Quantitation of TRAP Assays. For each extract, initial TRAP reactions were performed with various amounts of protein to identify conditions under which the TRAP reactions were not inhibited. In addition, each extract was assayed in the presence of 0.1 μg of HeLa cell protein in order to monitor for inhibitory activities. Once optimal conditions were established, TRAP reactions were quantitated with a PhosphorImager. The signal intensity of each band of the TRAP ladders were measured individually and corrected for the background (signal in an identically sized rectangle above each band). The adjusted signals of the products in each lane were then summed to yield arbitrary units of activity. These values were compared with the TRAP products obtained with known amounts of HeLa extract assayed in parallel. The relative specific telomerase activity in each extract is expressed as a percentage of the specific activity obtained with HeLa extract. By assaying the HeLa standard and the experimental samples at similar protein concentrations (0.5–1.0 μg per assay), extensive extrapolation was usually not required to calculate relative specific activities. When it was necessary to compare different amounts of experimental and HeLa protein, the data in Fig. 3*B* were used to adjust the HeLa specific activity. Statistical analysis on the relative telomerase activities in normal leukocytes and chronic myeloid leukemia samples was performed with the χ^2 test for independence.

RESULTS

Detection of Telomerase Activity in Normal and Malignant Hematopoietic Cells. We used a modified version of the TRAP assay (12) which permits detection of *in vitro* telomerase products (see *Materials and Methods*). A 5′-end-labeled substrate primer to which telomeric repeats can be added by telomerase was incubated with cellular extracts. A reverse primer then was added and PCR was carried out, resulting in amplification of telomerase extension products. Using this method, we assayed telomerase activity in bone marrow or peripheral blood leukocytes from 19 patients with leukemia. An example of the results is displayed in Fig. 1, where the typical 6-nt TRAP ladders of variable intensity are detected in assays of 5 chronic and 5 acute leukemias. As expected from the RNA template dependence of telomerase, RNase A treatment interfered with the generation of TRAP products (Fig. 1). With this assay, 25 of 27 extracts from patients with active leukemia showed telomerase activity (Table 1 and see *Materials and Methods*). Weak telomerase activity could also be detected in bone marrow extracts from 3 leukemia patients in hematologic and cytogenetic remission (Table 1).

Telomerase activity was also detected in 6 of 9 bone marrow and 2 of 2 PBMC preparations from normal donors (Fig. 2 and

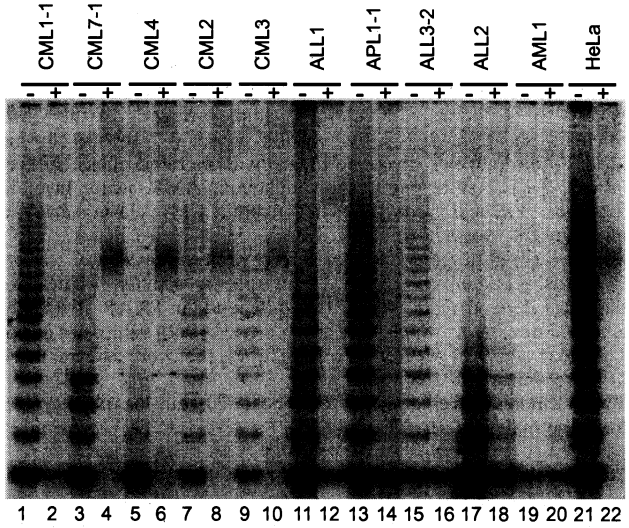

FIG. 1. Detection of telomerase activity in leukemia extracts. Cell extracts without (−) or with (+) RNase treatment were assayed for telomerase activity by a modified version of the TRAP protocol and fractionated in a nondenaturing 15% polyacrylamide gel. The smallest band detected represents the PCR product of the TS primer elongated with three telomeric repeats. Lanes 1–10, chronic myeloid leukemias; lanes 11–20, acute leukemias (for details on the leukemia samples, see Table 1); lanes 21 and 22, HeLa extract. Treatment of the extracts with RNase A (+ lanes) diminished the formation of TRAP products. The protein amounts used per assay varied from 0.5 to 1.0 μg. The telomerase activity in AML1 (lane 19) was detectable in a longer exposure of this gel. The origin of the faint heterogeneous band migrating in the middle of the gel in some of the lanes is unclear. This band is occasionally observed with RNase A-treated extracts and has no obvious relationship to the TRAP products.

Table 1. Telomerase activity in hematologic malignancies

Patient	Telomerase detection* 0.1 μg	1.0 μg	10 μg	Specific telomerase activity†
	Chronic myeloid leukemias			
CML1	+	+	+	4.5 ± 3.1
CML2	ND	+	+	1.0 ± 0.9
CML3	+	−	−	0.7, 1.8
CML4	−	−	+	6.5 ± 5.1
CML5	+	+	−	5.2, 9.5
CML6	−	+	+	0.6 ± 0.3
CML7-1	+	+	+	18.0 ± 4.9
CML7-2	+	+	+	ND
CML7-3	−	+	−	ND
CML8-1	−	+	+	17.0 ± 15.0
CML8-2	−	+	+	2.1 ± 2.1
CML9-1	+	+	+	0.8 ± 0.4
CML9-2	−	+	−	ND
CML10-1	−	+	−	20.0 ± 19.0
CML10-2	−	+	−	ND
CML11-1	−	+	+	<0.1
CML11-2	−	−	+	ND
	Acute leukemias			
ALL1	+	+	+	107.0 ± 95.5
ALL2	−	−	+	76.0 ± 28.4
ALL3-1	−	+	+	ND
ALL3-2	+	+	+	39.1 ± 7.2
ALL3-3	+	+	+	ND
APL1-1	+	+	+	28.0 ± 18.2
APL1-2	+	+	+	ND
AML1	+	+	−	0.3, 1.2
	Leukemias in remission			
CML12-1‡	+	+	−	<0.1
ALL4	−	+	+	0.3, 1.8
APL2‡	−	+	+	ND

All extracts were derived from bone marrow except extracts CML8-1, CML9-2, and CML10-1 and -2, which were prepared from peripheral blood. The numbers used with the disease abbreviations do not indicate disease stage, but rather are a numeric designation for an individual patient (first digit) and the sample order (second digit, after hyphen) for a patient with multiple samples. Staging of the CML patients was as follows: 1–3, first chronic phase; 4–7, second chronic phase; 8, third chronic phase; 9–11, early accelerated phase. Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; ND, not determined.

*Shown for 0.1, 1.0, and 10 μg of extract protein per assay.

†Given as a percentage of the HeLa specific activity. Values are estimates derived from two to five independent TRAP assays per extract. For extracts which were assayed twice, both values are given. The mean and SD are given only for extracts that were assayed three or more times.

‡Showed molecular evidence of chromosomal translocations typical for these malignancies.

Table 2), prompting further analysis of normal primary leukocytes. Three subfractions of peripheral blood leukocytes were examined in more detail (Fig. 2 and Table 2). For six normal donors, we found telomerase activity in both the monocyte/B-cell and the T-cell fractions from peripheral blood. In addition, granulocytes from five of six donors were telomerase positive. For each of these extracts, the TRAP products displayed the characteristic 6-nt ladder and the telomerase activity was diminished by mild digestion with RNase A (Fig. 2). We note that the distribution of telomerase products was often different in extracts with lower relative specific activities (e.g., a disproportionately strong fourth band). Whether this altered pattern is due to a difference in telomerase activity remains to be determined.

These data indicated that qualitative detection of telomerase activity *per se* was not obviously correlated with hematologic disease, necessitating a quantitative evaluation of these samples.

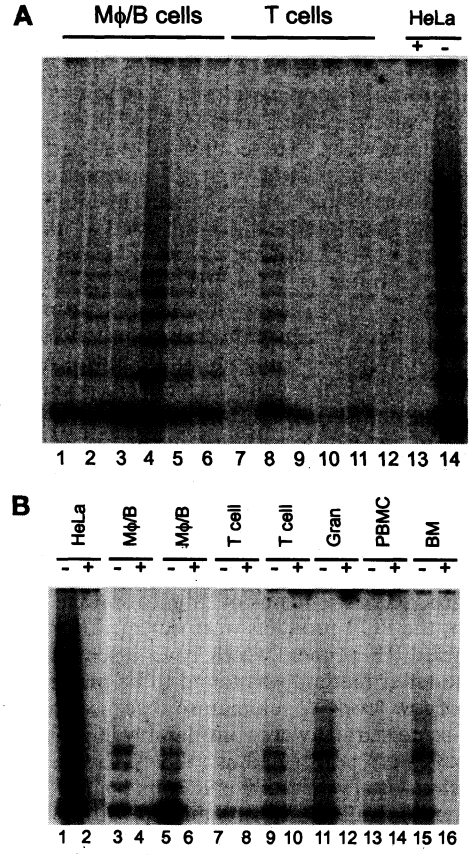

FIG. 2. Telomerase activity in normal primary hematopoietic cells. (*A*) Telomerase activity in fractions of leukocytes enriched for B cells and monocytes (Mø/B) isolated from six normal individuals (lanes 1–6) and fractions enriched for T cells from five normal individuals (lanes 7–11); the T-cell fraction from a sixth donor is shown in lanes 7 and 8 of *B*. Each lane contained the TRAP products obtained with extract from 5×10^4 cells. Lane 12, no extract, lanes 13 and 14, TRAP products obtained with extract from 5×10^3 HeLa cells with (lane 13) or without (lane 14) RNase treatment. (*B*) RNA-dependent TRAP reactions with extracts from fractionated leukocytes. Lanes 1 and 2, extract from 7×10^3 HeLa cells; lanes 3–6, two Mø/B cell fractions; lanes 7–10, two T-cell fractions; lanes 11 and 12, granulocyte fraction (Gran); lanes 13 and 14, bulk PBMCs; lanes 15 and 16, 2×10^3 bulk bone marrow mononuclear cells (BM). All extracts were derived from normal donors. Even-numbered lanes show products of reactions with RNase-treated extracts (+).

Comparison of *in Vitro* Telomerase Activities. To determine whether the results of the TRAP assay could be evaluated in a quantitative manner, we varied the amount of HeLa extract per assay from 0.05 to 25 μg of protein (representing 1×10^3 to 5×10^5 cells) and measured the total incorporation of labeled primer into PCR products (Fig. 3*A*). The incorporation of labeled primer into TRAP products was roughly proportional to the amount of protein added when 0.1–1 μg of protein was assayed (Fig. 3*B* and data not shown). However, at higher concentrations of HeLa cell extract (2.5–25 μg per reaction), the TRAP reactions appeared to be inhibited (Fig. 3*B*). This inhibitory effect occurred in part at the level of the PCR; it was also observed with several of the leukemia extracts (see Table 1).

To derive an estimate of the telomerase activity in extracts from normal and malignant cell populations, we adopted the strategy detailed in *Materials and Methods*. In brief, each extract was initially assayed at various protein concentrations to identify conditions at which the TRAP products were

Table 2. Telomerase activity in normal leukocytes

Cell fraction	Telomerase detection frequency*	Specific telomerase activity†
PBMC	2/2	
PBMC-3		3.6 ± 2.8
Bone marrow	6/9	—
Granulocyte	5/6	
Gran-1		3.0 ± 1.6
Gran-2		8.5, 9.8
Gran-4		13.9 ± 8.3
Gran-5		20.7, 35.0
Gran-6		3.2, 7.9
Monocyte/B cell	6/6	
Mϕ/B-1		4.8, 17
Mϕ/B-2		0.2, 0.9
Mϕ/B-5		3.9 ± 2.9
Mϕ/B-6		22.2 ± 15.4
T cell	6/6	
T-2		14.0, 30.9
T-3		33.0, 53.0
T-4		3.1, 8.5
T-5		2.1 ± 1.2
T-6		4.9 ± 3.9

Individual extracts are indicated by an abbreviation and a number which refers to the donor (e.g., extracts Gran-6 and T-6 represent granulocytes and T cells from the same donor).

*Telomerase was assayed by TRAP assay on 0.1–10 μg of extract protein. Detection frequency is expressed as the number of extracts that yielded telomerase activity at one or more protein concentrations/total number of extracts tested.

†Given as a percentage of HeLa specific activity. For extracts that were assayed twice, both values are given. For extracts that were assayed three or more times, the mean and SD are given.

proportional to the amount of protein added. Quantitation was subsequently performed under conditions where the TRAP assay did not appear to be inhibited or saturated, and the resulting activity was compared with a HeLa standard assayed in parallel. The resulting rough estimates of the relative specific activities suggested that normal leukocytes yielded extracts with less telomerase activity than HeLa cell extracts (Table 2 and Fig. 2). The specific telomerase activities ranged from <1% to ≈40% of the HeLa standard for bulk or fractionated peripheral blood leukocytes. The source of the variation in telomerase activity in extracts from different individuals is unclear. Similar variation was observed when the telomerase activities were compared on a per-cell basis (Fig. 2*A* and data not shown). There was no direct correlation between the activity levels in the different leukocyte fractions obtained from any one donor. Specific telomerase activities in bone marrow samples were not determined. However, on a per-cell basis, bulk bone marrow leukocytes contained on the order of 1–10% of the telomerase activity of HeLa cells (Fig. 2*B* and data not shown).

The relative telomerase activities in leukemia samples also varied considerably (see Table 1). For the chronic myeloid leukemias, no obvious correlation was found between telomerase activity and the stage of the disease (Table 1) or other clinical parameters (data not shown). In addition, there was no significant difference between telomerase activities in the chronic myeloid leukemia extracts and the normal leukocyte samples tested in this study ($P = 0.5$; see *Materials and Methods*). It is not excluded that the acute leukemias have slightly elevated levels of telomerase. However, additional data will be required to assess changes in telomerase activity in this disease.

DISCUSSION

Our data demonstrate that telomerase is expressed in normal primary human cells. Telomerase activity was detected by the PCR-based TRAP assay in extracts of bone marrow and peripheral blood leukocytes and in fractionated circulating cell populations enriched for T cells, monocytes and B cells, and granulocytes. The levels of telomerase activity in these cell populations varied substantially, from <1% to ≈40% of the specific activity of HeLa cell protein, which is a rich source of telomerase. TRAP assays had previously failed to reveal telomerase activity in a variety of normal somatic cells (12). Perhaps telomerase is more extensively repressed in the cell types used in the preceding studies than in the hematopoietic lineages analyzed here. In addition, the improved sensitivity of the assay and the availability of fresh primary cells may have facilitated our detection of somatic telomerase expression.

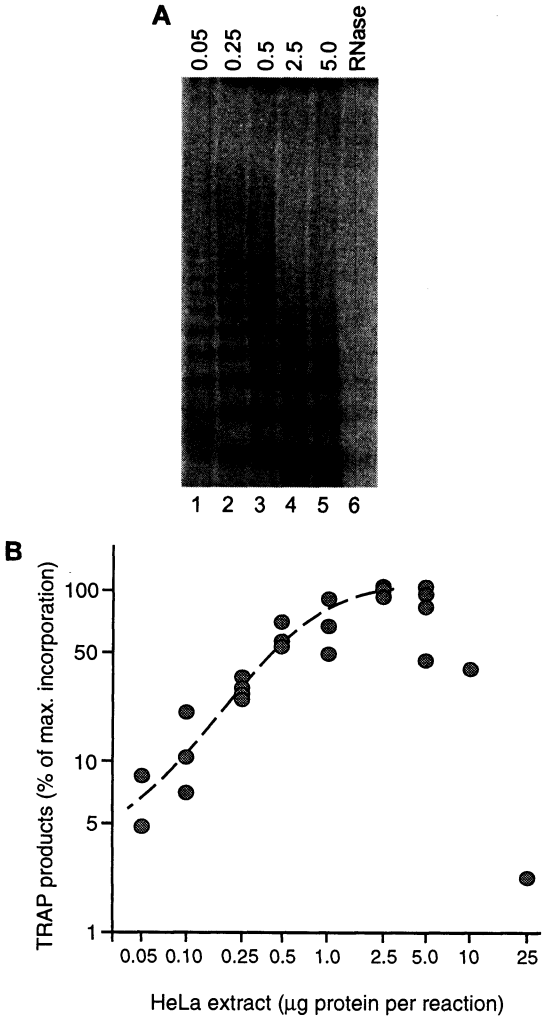

FIG. 3. Quantitation of TRAP products obtained with HeLa cell extract. (*A*) TRAP products obtained with various amounts of HeLa cell extract. Lanes 1–5, reactions with 0.05, 0.25, 0.5, 2.5, and 5 μg of HeLa extract protein; lane 6, 1 μg of protein treated with RNase A. (*B*) Relationship between TRAP product signals and amount of HeLa extract protein present in the assay. Telomerase activity in HeLa extract was determined by TRAP assay at protein levels ranging from 0.05 μg (1×10^3 cells) to 25 μg (5×10^5 cells) per assay. Total incorporation of labeled TS primer into TRAP products was measured on gels similar to the one shown in *A* (see *Materials and Methods*) and expressed as a percentage of the maximal incorporation (observed with 2.5 μg of protein). The *x* and *y* axes represent logarithmic scales with actual values (μg of protein and % activity) indicated along each axis. The TRAP products are approximately proportional to the amount of protein in the range between 0.1 μg and 1.0 μg; at higher protein concentrations the TRAP reactions are inhibited. The broken line is intended to aid in visualization of the data and does not represent a theoretical curve.

The expression of telomerase in human hematopoietic cells was unexpected because both circulating and bone marrow leukocytes show telomeric decline. Peripheral blood cell telomeres are shorter than sperm telomeres, and the telomeres of isolated bone marrow leukocytes, T cells, and B cells gradually diminish during *in vitro* culture (1–3, 6–8). Two explanations for this apparent discrepancy should be considered. One possibility is that telomerase is expressed in a small subset of total leukocytes whose stable telomeres have escaped detection. However, if telomerase is confined to such a subpopulation, our data indicate that these cells are represented in each of the leukocyte fractions we examined. The alternative explanation is that there are primary human leukocytes in which the telomeres shorten despite the presence of active telomerase. If correct, this observation may suggest that the expression of telomerase in primary human cells could be a more general phenomenon than was previously anticipated on the basis of telomeric decline in the soma.

It is not clear why telomerase should be expressed in cells with declining telomeres. One possibility is that telomerase may be needed to create the correct structure at telomere temini, either through addition of telomeric repeats or through incorporation of telomerase components into the telomeric nucleoprotein complex. Additional possibilities are that low levels of the activity are required for moderation of telomeric decline rates or for repair of telomere deletion events. Elucidation of the role of telomerase in normal hematopoietic cells is important in view of the suggested therapeutic use of telomerase inhibitors (5, 15).

In yeast, regulation of telomere length is a complex process governed by a large number of genes (23). Our data suggest that telomere shortening in human soma may not simply be due to complete repression of telomerase. Additional levels of regulation, potentially at the level of telomerase activity and/or the accessibility of the telomere terminus, are plausible. This would provide one explanation for the variation in the *in vitro* telomere loss rates in different cell types (5–8, 15, 16, 24).

With the TRAP assay, human telomerase activity had previously been detected in 98% of immortal cell lines and in 90% of primary tumors, including in 14 out of 16 acute lymphocytic leukemias (12). Our data suggest that the detection of telomerase *per se* may not be directly correlated with disease in all malignancies. Specifically, we have been unable to demonstrate a significant increase in telomerase activity in chronic myeloid leukemia patients compared with normal donors. Our data are compatible with enhanced telomerase activity in acute leukemias, but additional data on this possible increase is required. Thus, for hematologic malignancies it appears important to compare the relative levels of telomerase activity in normal and tumor cells. Recently, a similar conclusion was reached by others on the basis of detection of telomerase in normal and malignant hematopoietic cells (25). Since the TRAP assay is not easily quantitated, a more definitive assessment of the role of telomerase in leukemias and lymphomas will await the availability of molecular reagents for (activated) telomerase.

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