Natural Science Report, Ochanomizu University, Vol. 47, No. 2 (1996)
お茶の水女子大学 自然科学報告 第47巻 第2号

TELOMERE AND TELOMERASE IN HUMAN LEUKOCYTES AND IMMORTAL CELL LINES

Yukiko Shimada, Mariko Nakano and
Kimiko Murakami-Murofushi

Department of Biology, Faculty of Science, Ochanomizu University
(Received November 8,1996)

Summary

Telomere length and telomerase activity were determined in peripheral blood leukocytes isolated from normal donors and human, immortal leukemic cell lines derived from different cell sources. Telomeres in normal leukocytes had shorten with age of donors but no telomerase activity was detected in these cells. An apparent shortening of telomeres was observed in most of immortal cells, and they showed telomerase activity with a high processivity. The expression of telomerase may be required as a critical step for tumorgenesis, but telomere length may not be a determinants of malignancy of cancer cells. The enzyme activitywas sensitive to pretreatment with RNase A and proteinase K, suggesting the nature of this enzyme as a ribonucleoprotein.

Introduction

Telomerase is a highly specialized telomere terminal transferase which was first identified by Greider and Blackburn in the cell free extracts of ciliates *Tetrahymena* (1). This enzyme was shown to be a ribonucleoprotein (2), and the essential RNA component of the enzyme seemed to be the template for the synthesis of telomeric repeats, TTGGGG(3). The telomerase activity was also identified by Morrin in HeLa cell extracts(5),and this catalyzed the addition of a 6-nucleotide repeats to oligonucleotide primer consisting of a human telomeric repeat sequence, TTAGGG, and it was also shown to be a ribonucleoprotein. Recently, Counter *et al.* demonstrated telomere shortening, expression of telomerase activity and chromosome rearrangements in human embryonic kidney cells after transformation with SV40 or Ad5 (6), and they suggested the activation of telomerase would necessarily occur during the cell immortalization. Counter *et al.* showed that a late-stage ovarian carcinoma tumor cells maintained short stable telomeres both *in vivo* and *in vitro*, and that telomerase was specifically induced in ovarian carcinoma cells, but not in normal ovarian epithelium(7). They also measured telomere length and telomerase activity throughout the life span of human B lymphocytes transformed by Epstein-Barr virus, and reported that only clones in which telomeres were stabilized by activation of telomerase could continue to proliferate indefinitely, *i.e.*, became immortal (8). These findings suggest that the progression of malignancy of cancer cells is dependent on the activation of telomerase. Recently, Kim *et al.* reported the specific association of telomerase activity with 98 of 100 immortal cells and 90 of 101 biopsies from tumor tissues (9). In this manuscript, we report the expression of telomerase activity in human leukemic cell lines derived from different cell sources, and we suggest that an activation of telomerase may be required as a critical step for the tumorgenesis of leukocytes.

Materials and Methods

Cells. Human leukemia cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). HL60 was derived from acute myelogenous leukemia (APL), U937 was from histocytic lymphoma (HL), K562 from chronic myelogenous leukemia (CML-bc), Reh was established from B cell leukemia,Namalwa from a patient with Barkitt lymphoma (B cell), and CMK was from megakaryotic leukemia (10, 11).

Isolation of peripheral blood cell fractions. Normal peripheral blood mononuclear cells (PBMC), monocytes, T cells and B cells were obtained from blood from five consenting healthy volunteers aged in the 20s to 60s. Heparinized blood was layered onto Mono-Poly Resolving Medium (ICN Biomedicals Ltd., Irvine, Scotland) and centrifuged at 1,000 x g for 30 min. This step resulted in the separation of mononuclear cells into the top band and polymorphonuclear cells into the second band (12). PBMC were collected, washed and incubated at 2 x 10^6 cells/ml for 2 h at 37°C in Petri dishes containing RPMI-1640 medium with 10% FCS (RPMI-10%). The recovered PBMC were washed six times with RPMI-10% to remove non-adherent cells. The adherent cells were gently scraped off and incubated at a density of 5 x 10^6 cells/ml for 45 min at 37°C in a test tube containing RPMI-10% with 0.4 mg/ml carbonyl iron (Sigma Chemical Co., St.Louis, MO). Cells which ingested or adhered to the iron particles were collected with a magnet (MPC-6; Dynal, Skyen, Norway). The procedure provided a monocyte-enriched population which was judged to be 95% pure by esterase staining (13) and 90% viable by trypan blue staining. The nonadherent cells were allowed to form rosettes with neuraminidase-treated sheep erythrocytes at 4°C for 1 h, and the rosetting T cells and natural killer cells were separated from the other PBMC by pelletting through a Ficoll-Conray gradient. The sheep erythrocytes were lyzed with 0.17 M NH_4Cl at 4°C for 15 min, followed by an addition of RPMI-10%, and enriched T cells and natural killer cells were pelletted. The nonrosetting cells were collected as B cell fraction, and the T and B cell fractions were subsequently suspended at a cell density of 5 x 10^7/ml in RPMI-10%, then incubated at 4°C for 30 min with 50 μg/ml each of OKT3 (anti-CD3; Ortho Diagnostic Systems, Raritan, NJ) and B4 (anti-CD19; Coulter Immunology, Hialesh, FL) monoclonal antibodies. Then the cells were washed three times with cold RPMI-10% and incubated at 4°C for 30 min with goat anti-mouse IgG-coated magnetic particles (Dynabeads M-450; Dynal) with gently rotation. The rosetted cells and beads were suspended in 5 ml of RPMI-10% and they were attached to a samarium cobalt magnet (MPC-6), then the rosetted cells were washed seven times with RPMI-10%. The beads were detached by incubating of the cells at a density of 5 x 10^5/ml in RPMI-10% at 37°C for 20 h. The beads were removed by magnetic attraction,and the cells were washed with RPMI-10% and stained directly for flow cytometric analysis (14). By flow cytometry, the phenotypes of the purified T and B cells were detected as $CD3^+$ (>95%) and $CD20^+$ (>90%), respectively.

DNA isolation and Southern blot analysis. Cells were lyzed and digested by proteinase K followed by DNA extraction with phenol/chloroform (15). Five μg of genomic DNA were digested with *Hinf* I (Takara Co., Kyoto, Japan) according to the supplier's recommendation. The fragments were loaded to a 0.8% agarose gel and transferred to nylon membranes (Hybond N., Amersham Japan, Tokyo, Japan) according to the method described by Southern (16), after which they were hybridized with a [^{32}P]-labeled probe.The membranes were prehybridized for 16-24 h at 65°C in a solution containing 5 x SSC, 4 x Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The oligo-

nucleotide probe, 5'-[^{32}P](TTAGGG)$_7$ telomeric probe, Alu(BLUR-8) and alphoid sequences (pSP3),were hybridized for 16-24 h at 50°C in 5 x SSC and then washed at 50-60°C with 4 x SSC. The hybridization solution for latter two oligonucleotide sequences contained 10% dextran sulfate in addition to the prehybridization mixture. Hybridization was allowed to proceed for 24 h at 65°C, and then the filters were washed for 30 min at the same temperature in 0.5 x SSC and 0.5% SDS. Prehybridization and hybridization for the triplet repeat probe $(CAC)_5$ were performed in 5 x SSPE (0.9 M NaCl,50 mM $NaPO_4$,5 mM EDTA, pH 7.7), 5 x Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA for 16-24 h at 45°C, then the filters were washed for 30 min in 0.5 x SSC and 0.5% SDS at room temperature. The membranes were exposed to X-ray film (Eastman Kodak Co., Rochester,NY) for an appropriate length of time at -80°C with an intensifying screen. Each lane was scanned with a computer-controlled densitometer (Shimadzu CS-9000; Shimadzu, Kyoto) to determine the amount of telomeric DNA and the mean TRF length.

Fluorescence in situ hybridization (FISH). FISH was performed based on the protocol of Pinkel *et al.* (17) with a slight modification. The synthetic oligonucleotides $(TTAGGG)_6$ were labeled with biotin-16-dUTP (Boehringer) using terminal deoxynucleotidyl transferase(BRL) as recommended by the manufacturer. Slides were pretreated with RNase (100 μg/ml) in 2 x SSC for 1 h at 37°C, and then treated with proteinase K (40 ng/ml in 20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$) for 7 min at 37°C, followed by the fixation in 4% paraformaldehyde for 10 min. The slides were denatured for 2 min at 70°C in 70% formamide in 2 x SSC and dehydrated through an ethanol series. Hybridization was carried out for 17 h at 37°C in 50% formamide, 2 x SSC, 10% dextran sulfate, BSA (1 μg/10 μl), sonicated *E. coli* DNA (50 μg/ml), with 10 ng probe per slide. The slides were washed in 50% formamide in 2 x SSC for 10 min at 37°C followed by washes in 4 x SSC three times at 37°C. FITC-avidin and three rounds of amplification with biotinylated goat anti-avidin antibody(Vector laboratories) were used to detect hybridization signals. Chromosomes were counterstained with propidium iodide (0.5 μg/ml) in antifadeglycerol solution.

Preparation of S100 extracts. S100 extracts were prepared from >10^8 cells as described by Morrin (5) and Counter *et al.*(6) with a slight modification. Cells were collected and washed with phosphate-buffered saline (PBS) and rinsed in extraction buffer (10 mM HEPES, pH 8.0, 3 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 10 U/ml of RNasin). After the incubation at 0°C for 10 min, the sample was homogenized and incubated further at 0°C for 30 min, then centrifuged at 40,000 x g for 2 min at 2°C. One-fiftieth volume of 5 M NaCl was added and centrifuged at 150,000 x g for 1 h at 2°C. Glycerol was added to a final concentration of 10% and stored at -80°C. Protein contents of S100 extracts were determined by Bradford assay (Bio-Rad).

Telomerase assay. Assay of telomerase was performed basically according to Morrin (5) and Counter *et al.* (6). Twenty five microlitters of S100 extract were assayed in a final volume of 40 μl. Standard assay conditions were follows: 50 mM Tris-acetate, pH 8.2, 20 mM KCl, 5 mM 2-mercaptoethanol (2ME), 3.5 mM $MgCl_2$, 1 mM EDTA, 2 mM spermidine, 4 mM dATP, 4 mM dTTP, 2 μM $(TTAGGG)_3$ primer, 2.5 μM (50 μCi) [a-^{32}P]dGTP(800 Ci/mmol), and 20% glycerol. The mixture was incubated at 30°C for 90 min, and reaction was stopped by addition of 50 μl of 20 mM EDTA and 10 mM Tris-HCl(pH 7.5) containing 0.1 mg/ml RNase A, followed by incubation at 37°C for 15 min. Fifty μl of proteinase K solution (0.3 mg/ml in 10 mM Tris-HCl, pH 7.5, and 0.5% SDS) were added and incubated at 40°C for 15 min followed by successive extraction with phenol/chloroform (1:1), phenol/

chloroform/isoamyl alcohol (24:24:1), and chloroform. Then, carrier tRNA was added and the DNA was precipitated with 500 μl of ethanol at -20°C. The pellets were resuspended in 2 μl of formamide loading dye, boiled for 1 min,chilled on ice and loaded onto an 8% polyacrylamide-7 M urea sequencing gel and run at 1,800 V for 2 h using 0.6 x TBE buffer. Gels were dried and exposed to Kodak XAR-5 pre-flashed film at -80°C with enhancing screen for 4 days.

Results and Discussion

Telomere reduction in normal peripheral leukocytes and immortal leukemic cell lines. Telomere length of human normal peripheral blood mononuclear cells (PBMC) and immortalized leukemic cell lines are measured. A shortening of telomere length of normal blood cells was observed with age of the donors (Fig.1), and the telomeric DNA of 5 out of 6 leukemic cell lines were apparently smaller than that of normal cells (Figs.2 & 3). When the filters those had been hybridized to $(TTAGGG)_7$ were stripped and rehybridized to pSP3 or BLUR-8,no marked differences were observed in the size of the repetitive DNA sequences found in the leukemic cells (Fig.3b), indicating that the DNA changes were specific to the telomeric regions. No loss of telomeric DNA did occur as a result of general degradation during DNA preparation.

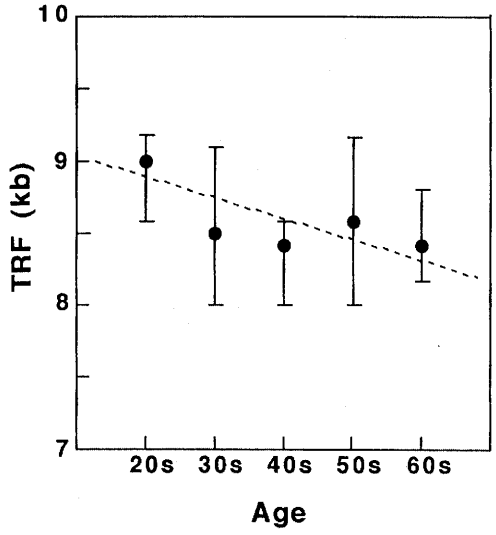

Fig.1. Change of length of telomeric repeats (TRF,terminal restriction fragment) with ageing in human normal peripheral blood mononuclear cells (PBMC). The autoradiographs were analyzed with an automatic autoradiographic scanner to determine the peak fractions of the telomeric repeats in PBMC.

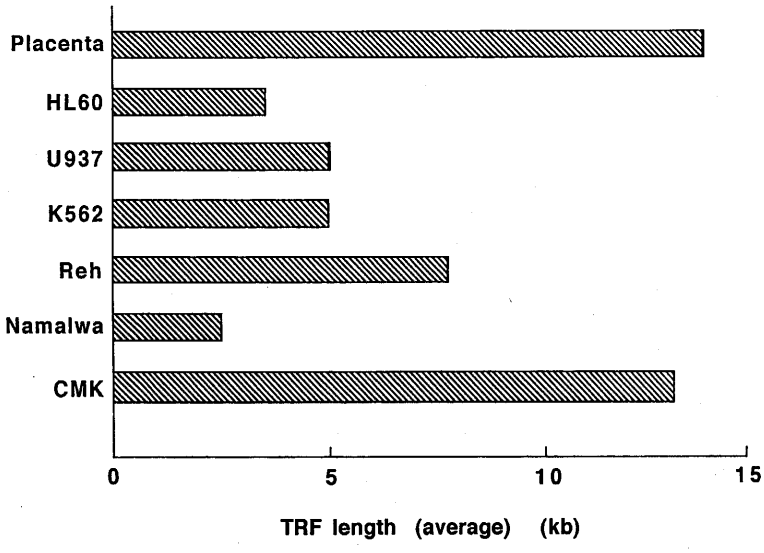

Fig.2. Average length of telomeric repeats (TRF) in immortalized leukemic cells determined with an automatic autoradiographic scanner.

To determine whether the telomere reduction was simply due to cellular immaturity and whether differences in PBMC composition could influence the telomeric DNA size, the telomeric DNA sizes in different cell populations from normal blood cells were compared (Fig.4).

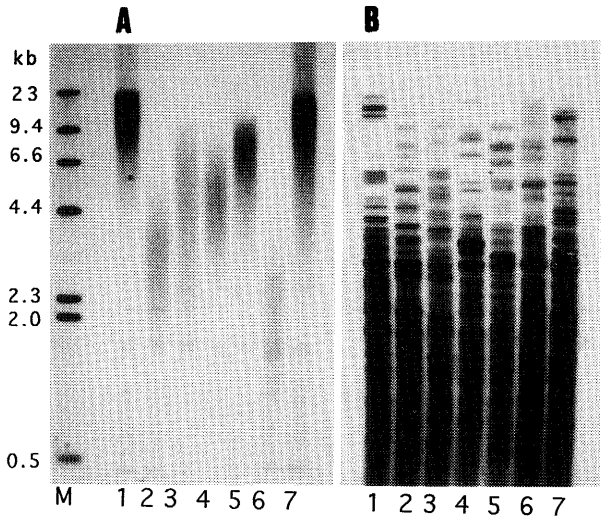

Fig.3. Southern blot analysis of leukemic cell lines. DNA from leukemic cells and from a human placenta was digested with Hinf I and hybridized with the DNA probe to the (A) telomere sequence $(TTAGGG)_7$, (B) after which the filter was rehybridized with the minisatellite probe $(CAC)_5$. Lane 1, placenta; lane 2, HL60; lane 3, U937; lane 4, K562; lane 5, Reh; lane 6, Namalwa; lane 7, CMK; M, size marker.

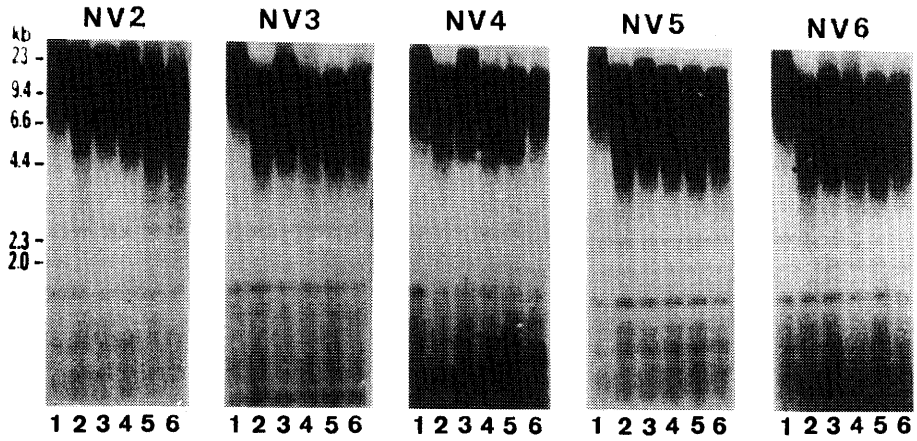

Fig.4. Southern blot analysis of four different blood cell fractions and of bone marrow $CD34^+$ hematopoietic progenitor cells from the same five healthy donors. NV2-NV6 designate normal volunteers aged from the 20s to the 60s, respectively. Lane 1, placenta; lane 2, T cells; lane 3, B cells; lane 4, polymorphonuclear leukocytes (PMN); lane 5, monocytes; lane 6, $CD34^+$ cells. DNA was digested with Hinf I and hybridized with telomere sequence.

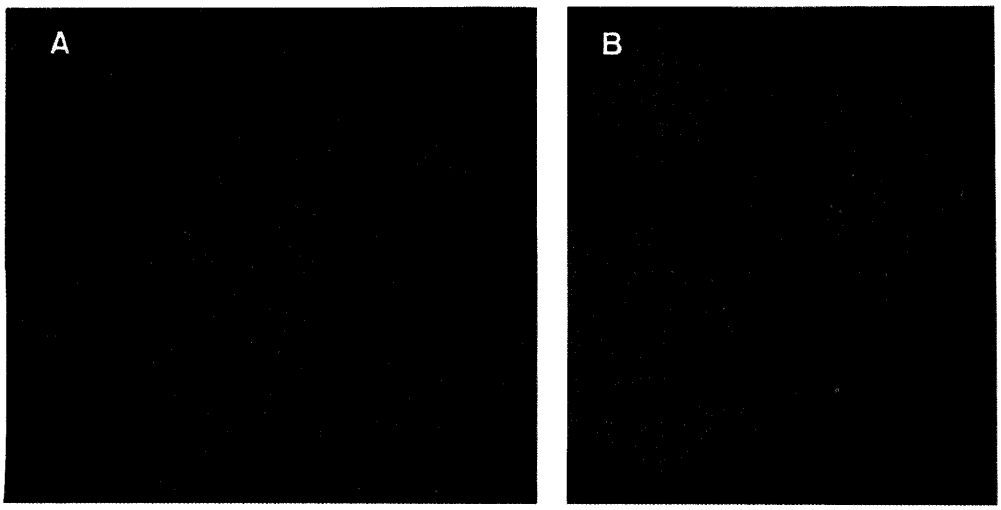

Fig.5. FISH analysis of CMK and Namalwa cells. Metaphase spreads of CMK (A) and Namalwa (B) cells which have been hybridized to the telomeric repeats $(TTAGGG)_6$. Telomeric signal at the chromosome termini was detected in almost every chromosome of CMK cells but rarely observed in Namalwa cells, consistent with the results obtained by Southrn blot hybridization.

The telomeric DNA of each of the T cells, B cells, polymorphonuclear leukocytes, monocytes, and $CD34^+$ cells from five normal donors in their age of 20s, 30s, 40s, 50s, and 60s was not markedly different.

These findings support the hypothesis that the shortening of telomeres is associated with aging of the leukocytes, and telomere shortening may have a crucial role for a tumorgenesis of blood cells. Then, the length of telomeres may be stabilized (not always short) in the tumor cells.

To examine the chromosomal localization of elongated or shortened telomeric repeats, we performed fluorescence *in situ* hybridization (FISH) using $(TTAGGG)_6$ probe to chromosome preparation of CMK and Namalwa cells which showed the typical elongation or shortening of the telomeric repeats by Southern blot hybridization (Fig.5). As expected, hybridization signals were observed at the termini of almost every chromosome in CMK cells (Fig.5A), though the faint terminal signal was detected in Namalwa cells, reflecting the decrease of telomere repeats (Fig.5B).

We also measured the change of telomere length in human fibroblasts before and after transformation with SV40 or ^{60}Co, and detected a shortening of telomere length in one case and elongation in other several cases after transformation (Fig.6). Telomeres in transformed cells are not always shorter than those in normal cells. So, the length of telomeres is not always correlate with cellular tumorgenesis.

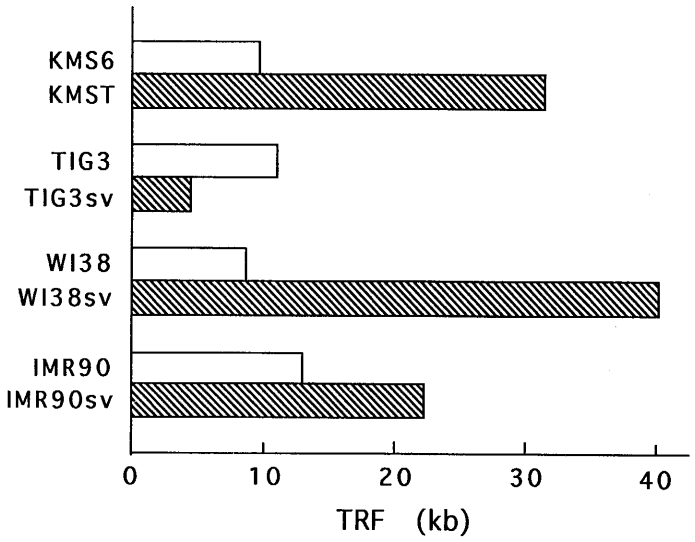

Fig.6. Average length of telomeric repeats (TRF) in human fibroblast before and after transformation: KMST, transformed with ^{60}Co; others, transformed with SV40.

Telomerase activation in leukemic cell lines. In all cases tested, high telomerase activity was detected in immortal leukemic cell lines regardless of the source of them as shown in Fig.7 (lane 1-6). The enzyme activity was sensitive to pretreatment with RNase A, proteinase K, or at high temperature, suggesting the nature of the enzyme as a ribonucleoprotein (Table I). In contrast, normal peripheral blood mononuclear cells at the age of 20s, 30s, 40s, 50s, and 60s did not show detectable telomerase activity even when twice as much protein was incubated for longer period (180 min, Table I). Thus, the telomerase activation was observed in all leukemic cell lines tested, and most of leukemic cells have shorter telomeres than those of normal cells.

We also detected the telomerase activation in very frequency in blast cells obtained from

Table 1. Telomerase activity in leukemic cell lines and normal peripheral blood cells.

Cell type	Treatment	Telomerase activity
Leukemic cells		
HL60	–	+
	RNase A	–
	Proteinase K	–
	High temp.	–
U937	–	+
	RNasa A	–
	Proteinase K	–
	High temp.	–
K562	–	+
	RNasa A	–
	Proteinase K	–
	High temp.	–
Reh	–	+
	RNasa A	–
	Proteinase K	–
	High temp.	–
Namalwa	–	+
	RNasa A	–
	Proteinase K	–
	High temp.	–
CMK	–	+
	RNasa A	–
	Proteinase K	–
	High temp.	–
Human normal peripheral blood mononuclear cells[1]		
20S	–	–
30S	–	–
40S	–	–
50S	–	–
60S	–	–

[1] Telomerase assay was performed with twice as much protein (200μg) and incubated for 180 min.

patients with acute and chronic leukemia, and also an apparent activation of telomerase was detected in human fibroblasts after transformation with SV40 or ^{60}Co (data not shown, and will be published elsewhere).

These observations support the hypothesis that the shortening of telomeres and concomitant decrease in chromosome stability may trigger an activation of telomerase. And, as Counter *et al.* suggested (7), telomerase activation may depend on multiple mutational events which are only achieved after many cell divisions, and tumor cells lose telomeric region of chromosomal DNA to a critically short length, then only cells which can maintain chromosome stability through telomerase activation may be capable to proliferate continuously. In some cases, chromosomes are stabilized with very short length of telomeres, and in other cases, telomeres are elongated with high telomerase activity.

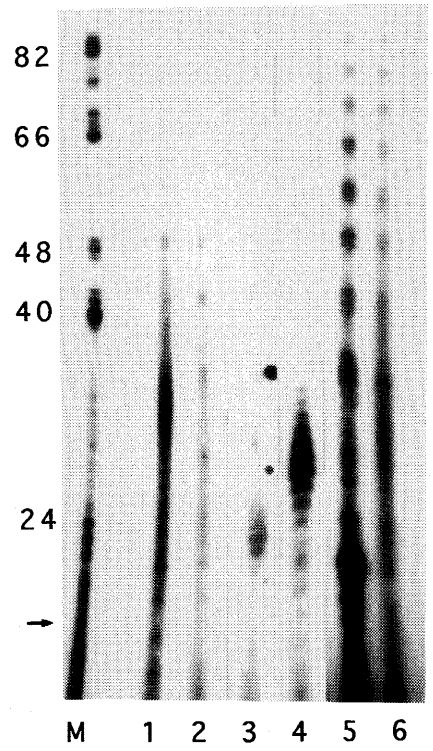

Fig.7. Telomerase activity in immortalized leukemic cells. S100 fractions prepared from leukemic cells were assayed for telomerase activity. Assay was carried out according to Materials and Methods. Protein content in each fraction was 100μg per assay. Lane 1, HL60; lane 2, U937; lane 3, K562; lane 4, Reh; lane 5, Namalwa; lane 6, CMK; M, size marker. Exposure periods were 4days (lane 1, 2, 3, 5 and 6) or 2 hours (lane 4). An arrow indicates the position of a primer, $(TTAGGG)_3$.

Acknowledgement

This work was supported in part by grant from Esso Foundation for Female Scientists.

References

1. Greider,C.W., and Blackburn,E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 43, 405-413.
2. Greider,C.W., and Blackburn,E.H. (1987) The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell 51, 887-898.
3. Greider,C.W., and Blackburn,E.H. (1989) A telomeric sequence in the RNA of *Tetrahymana* telomerase required for telomere repeat synthesis. Nature 337, 331-337.

4. Shippen-Lentz,D., and Blackburn,E.H. (1990) Functional evidence for an RNA template in telomerase. Science **247**, 546-552.
5. Morrin,G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell **59**, 521-529.
6. Counter,C.M., Avilion,A.A., LeFeuvre,C.E., Stewart,N.G., Greider,C.W., Harley,C.B., and Bacchetti,S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J. **11**, 1921-1929.
7. Counter,C.M., Hirte,H.W., Bacchetti,S., and Harley,C. (1994) Telomerase activity in human ovarian carcinoma. Proc.Natl.Acad.Sci.USA **91**, 2900-2904.
8. Counter,C.M., Botelho,F.M., Wang,P., Harley,C.B., and Bacchetti,S. (1994) Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. J. Virol. **68**, 3410-3414.
9. Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L., Coviello,G.M., Wright,W.E., Weinrich,S.L., and Shay,J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science **266**, 2011-2015.
10. Drexler,H.G., Gignac,S.M., and Minowada,J. (1994) Hematopoietic cell lines. in "Atlas of human tumor cell line 2, ed.R.J.Hay, J.G.Park, and A.Gazdar, pp.213-250, Academic Press, NY.
11. Drexler,H.G., Macleod,R.A.F., Quentmeier,H., and Steube,K. eds. (1994) DSM catalogue of human and animal cell lines, 4th ed. Braunscweig, Germany.
12. Ferrante,A., and Thong,Y.H. (1980) Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. J. Immunol. Methods **36**, 109-117.
13. Li,C.Y., Lam,K.W., and Yam,L.T. (1973) Esterase in human leukocytes. J. Histochem. Cytochem. **21**, 1-12.
14. Oshimi,K., Oshimi,Y., Yamada,O., Wada,M., Hara,T., and Mizoguchi,H. (1990) Cytotoxic T lymphocyte triggering *via* CD16 is regulated by CD3 and CD3 antigens. J. Immunol. **144**, 3312-3317.
15. Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) Molecular cloning. A laboratory manual, 1st ed., Cold Spring Harbor, NY.
16. Southern,E.M. (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. **98**, 503-517.
17. Pinkel,D., Landegent,C., Collins,C., Fuscoe,J., Seagraves,R., Lucas,J., and Gray,J. (1988) Fluorescence *in situ* hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocation of chromosome 4. Proc.Natl.Acad.Sci.USA **85**, 9138-9143.