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EXPRESSION OF TELOMERASE ACTIVITY ASSOCIATED WITH TRANSFORMATION OF HUMAN FIBROBLASTS

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Summary

Telomerase activity and telomere length in normal and immortalized human fibroblasts were determined. In normal cells, no telomerase activity was detected, but after transformation of these cells with SV40 or ^{60}Co, cells changed into immortal and an apparent telomerase activity expressed. Telomere length of these cells were determined and a distinct shortening of telomeres was observed in one case of transformants,and in other cases, telomere elongation was observed. DNA polymerase α activity was also determined, and high activity was detected in the cells having long telomeres, but low activity was detected in the cells bearing short telomeres. The expression of telomerase may be required as a critical step for tumorgenesis, but telomere length may not be a determinants of malignancy of tumor cells.

Introduction

Recently, telomerase has been paid a great attention because of its correlation with tumorgenesis. 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 contained RNA component (2) as the template for the synthesis of telomeric repeats,TTGGGG (3). Telomerase activity was also identified in HeLa cells by Morrin (5), and this enzyme activity catalyzed the addition of nucleotide repeats to oligonucleotide primer consisting of a human telomeric repeat sequence, TTAGGG, and it was also shown to be a ribonucleoprotein. Counter *et al.* demonstrated telomere shortening, expression of telomerase activity and chromosome rearrangements in human embryonic kidney cells after transformation with SV40 (6), and 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 in definitely, *i.e.* became immortal(8). These findings suggest that the activation of telomerase may necessarily occur during the tumorgenesis and the progression of malignancy of cancer cells is dependent on the activation of telomerase. Recently, Kim *et al.* reported the association of telomerase activity with 98 of 100 immortal cells and 90 of 101 biopsies from tumor tissues (9) and strongly suggested the specific correlation between telomerase activa-

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tion and tumorgenesis. In this manuscript, we report the expression of telomerase activity in transformed human fibroblasts followed by immortalization, and existing no correlation between telomere length and telomerase activity. Then we suggest that an activation of telomerase may be required as a critical step for the tumorgenesis of common normal cells, but telomere length may not be a determinants of malignancy of tumor cells..

Materials and Methods

Cells. Human fibroblasts, TIG-3, WI-38, IMR-90, KMS-6 were cultured in MEM supplemented with 10% fetal calf serum (FCS), and they were transformed with SV40 (TIG-3sv, WI-38sv, IMR-90sv) or ^{60}Co (KMST-6) (10).

DNA isolation and Southern blot analysis. Cells were lyzed and digested by proteinase K followed by DNA extraction with phenol/chloroform (11). 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 (12), after which they were hybridized with a [^{32}P]-labeled probe. The membranes were prehybridized for 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 oligonucleotide probe,5'-[^{32}P](TTAGGG)$_7$ telomeric probe, Alu(BLUR-8) and alphoid sequences (pSP3), were hybridized for 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 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 timeat -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.

Preparation of S100 extracts. Cell extracts, designated as S100 extracts, were prepared from >10^8 cells basically 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 conc. 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) with a slight modification. Twenty five microlitters of S100 extracts were assayed in a final volume of 40 μl. Standard assay conditions were follows: 50 mM Tris-acetate, pH 8.2, 20mM 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) [α-^{32}P]dGTP 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 preflashed film at -80°C with enhancing screen for 4 days.

Assay of DNA polymerase α. DNA polymerase α activity was assayed using 170 μg protein of S-100 extracts in the reaction mixture (100 μl) containing 10 μg of activated DNA, 25 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 20 μM dCTP, dGTP, dATP, 2 μM (1.6 μCi) [^{3}H]TTP, 1.0 mM ATP, 2mM 2-ME, 40 mM NaCl, 400 μg/ml BSA, and 10% glycerol. The reaction was carried out at 37°C for 30 min, and stopped by addition of equal vol. of 200 mM Tris-HCl, pH 8.0, 1% SDS, 1 M NaCl, 50 mM EDTA and 4 mM ATP. One hundred μl aliquots of reaction mixture were spotted onto filter discs, then fixed and washed 4 times with 10% and 5% TCA, successively. Then the radioactivities were counted in liquid scintilation fluid.

Results and Discussion

Immortalization of human fibroblasts by transformation with SV40 or ^{60}Co. Transformed cells, TIG-3sv, WI-38sv, IMR-90sv and KMST-6, could survive after more than 100 times cell division. This indicates that they have become immortal (data not shown).

Telomerase activation in transformed human fibroblasts. In all cases tested, apparent telomerase activity was detected in immortal fibroblast cells (Fig.1). 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. In contrast, normal fibroblasts, TIG-3, WI-38, IMR-90 and KMS-6, did not show detectable telomerase activity even when four times as much protein were incubated for longer period (180 min). Thus, the telomerase activation was observed in all transformants tested.

Telomere length in normal and immortalized fibroblasts. Telomere length of human normal and immortalized fibroblasts were measured, and detected a shortening of telomere length in one case (TIG-3sv), and elongation in other several cases after transformation (WI-38sv, IMR-90sv, and KMST-6) (Fig.2). Thus, the telomere length in transformants did not always correlate with cellular tumorgenesis.

DNA polymerase α acitivity in normal and transformed human fibroblasts. Activity of DNA polymerase α in fibroblast cells were determined, and high activity was detected in the transformants having long telomeres, but low activity was detected in the transformed cells with short telomeres (Fig.3). The meaning of these correlation between telomere length and DNA polymerase activity has not yet been clarified.

These observations support that the expression of telomerase may be required as a critical step for tumorgenesis, but telomere length may not be a determinants of malignancy of tumor cells. Telomerase activation may depend on multiple mutational events which are only achieved after many cell divisions,and tumor cells lose telomeric region of chromosomal

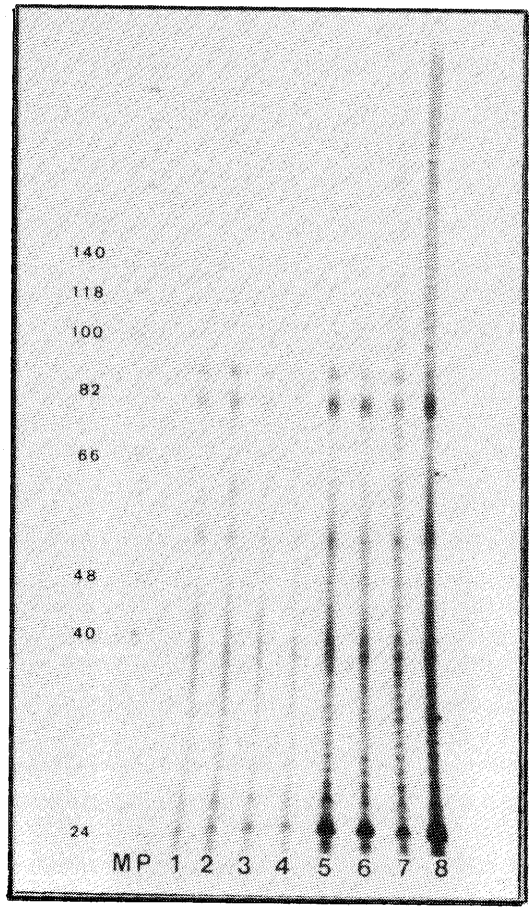

Fig. 1. Telomerase activation in transformed human fibroblasts. S100 extracts prepared from normal and transformed human fibroblasts were assayed for telomerase activity. Assay was carried out according to Materials and Methods. Protein content in each extract was 170 μg per assay. M, size marker; P, [^{32}P]-labeled primer, $(TTAGGG)_4$; lane 1, TIG-3; lane 2, WI-38; lane 3, IMR-90; lane 4, KMS-6; lane 5, TIG-3sv; lane 6, WI-38sv; lane 7, IMR-90sv; lane 8, KMST-6.

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.

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

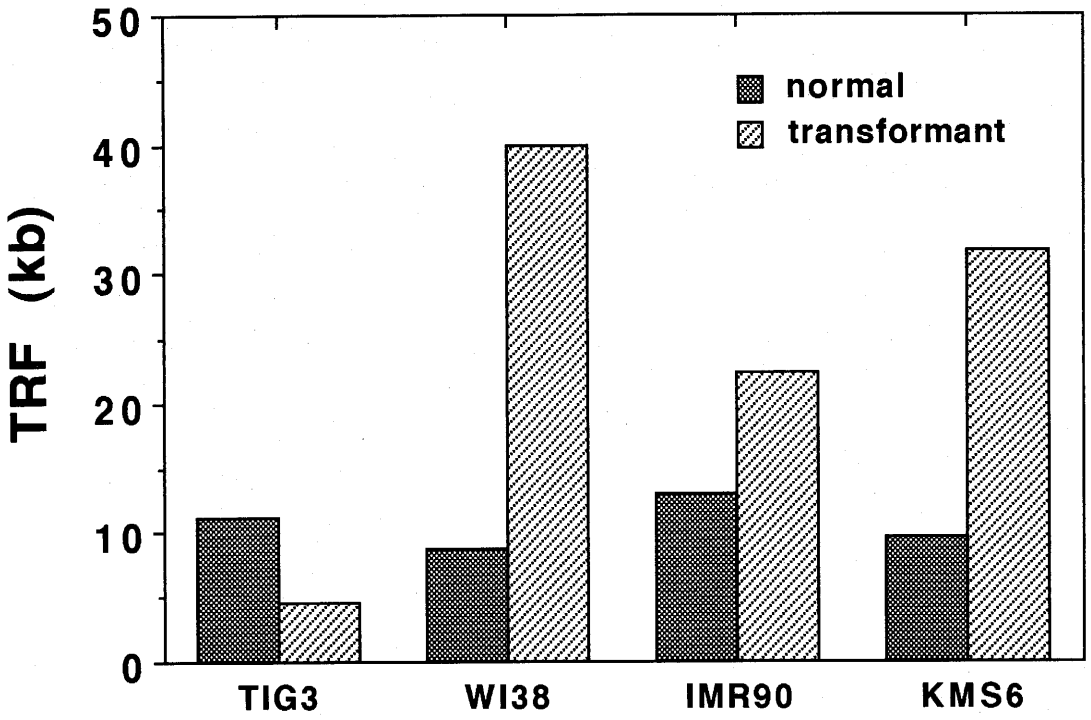

Fig.2. Average length of telomeric repeats (TRF) in human fibroblasts before and after transformation.

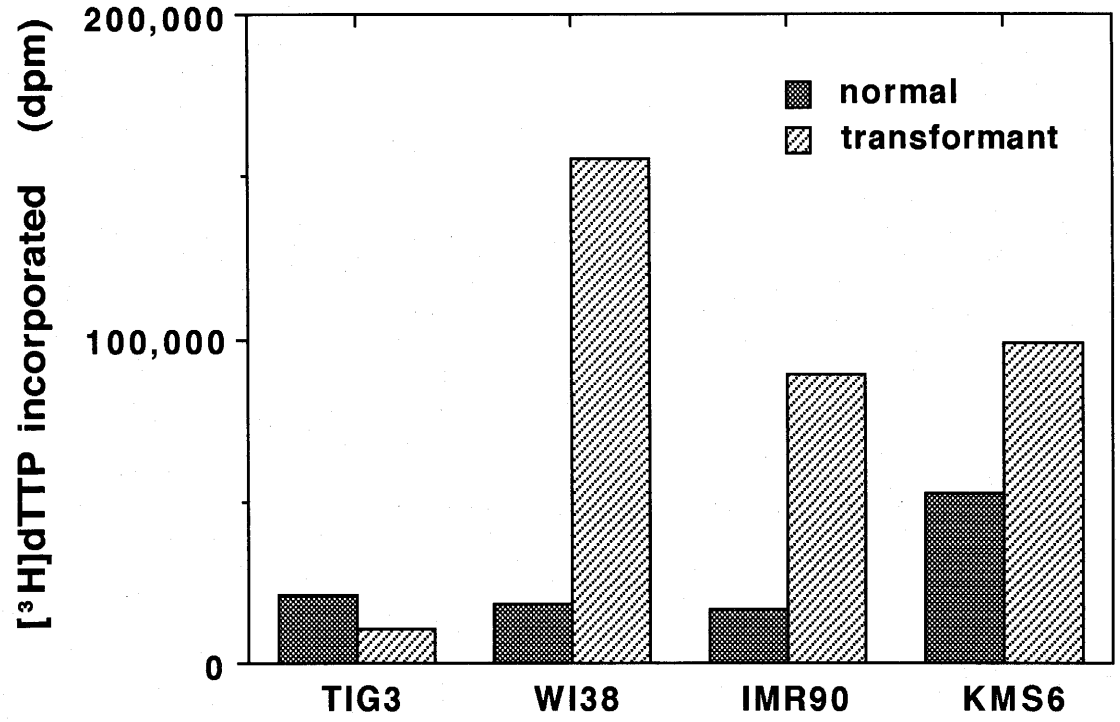

Fig.3. DNA polymerase α activity in human fibroblasts before and after transformation.

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. Kanda,N., Kaji,K., and Aikawa,E. (1994) in Saiboubaiyou (in Japanese) **20**, 204-207.
11. Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) Molecular cloning. A laboratory manual, 1st ed., Cold Spring Harbor, NY.
12. Southern,E.M. (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. **98**, 503-517.