assembled with polyglutamic acid. Thus, our current data indicate that histone H1 has an important function in transcriptional repression, but with the S-190–assembled chromatin the participation of the nucleosomal cores relative to that of H1 has increased when compared to results of previous studies in which the cores were static rather than active.
11. J. D. Gralla, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3078 (1985).
12. Previous studies of transcription from either chromatin templates or H1-DNA complexes showed that GAL4-VP16 activates transcription more effectively than GAL4(1–94) (*4, 14*), but transcriptional activation by GAL4(1–147) was not examined.
13. G. E. Croston, L. A. Kerrigan, L. M. Lira, D. R. Marshak, J. T. Kadonaga, *Science* **251**, 643 (1991).
14. G. E. Croston, P. J. Laybourn, S. M. Paranjape, J. T. Kadonaga, *Genes Dev.* **6**, 2270 (1992).
15. These observations are similar to those of previous studies in which potent activation of transcription by GAL4(1–147) was apparent in vitro (*7, 8*), although higher levels of transcription in vitro by GAL4-VP16 relative to GAL4(1–147) have also been observed with different experimental systems (*7, 9*). Other treatments that reduced basal transcription, such as the inclusion of the S-190 extract (in the absence of exogenously added histones, in which case chromatin assembly does not occur), reduction of the template concentration (by a factor of 5 or 25), or addition of nonspecific competitor DNA, had little effect on the relative potency of GAL4-VP16 versus GAL4(1–147) (*17*). It therefore appears that the effects were not the result of a nonspecific decrease in transcriptional efficiency. In addition, transcriptional inhibition by excess GAL4-VP16, commonly known as squelching [G. Gill and M. Ptashne, *Nature* **334**, 721 (1988)], is not observed with the *Drosophila* basal transcription factors (R. T. Kamakaka and J. T. Kadonaga, unpublished data).
16. A. Reik, G. Schutz, A. F. Stewart, *EMBO J.* **10**, 2569 (1991); A. Schmid, K.-D. Fascher, W. Hörz, *Cell* **71**, 853 (1992); J. E. Morgan and J. P. Whitlock, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11622 (1992); E. Verdin, P. Paras Jr., C. Van Lint, *EMBO J.* **12**, 3249 (1993).
17. M. J. Pazin and J. T. Kadonaga, unpublished data. Reproductions of unpublished data are available on request.
18. C. Wu, *Nature* **286**, 854 (1980); S. A. Nedospasov and G. P. Georgiev, *Biochem. Biophys. Res. Commun.* **92**, 532 (1980).
19. The conclusion that nucleosomal cores flank the GAL4 binding sites is based on the data shown in Fig. 3 together with the following results: First, by indirect end-labeling analysis with low-resolution agarose gels, we have observed a region of protection flanking the GAL4 binding sites, interpreted to be due to positioned nucleosomes, when probes from either side of the GAL4 binding sites were used and when the GAL4 sites were moved 1000 bp upstream of the transcription start site (*17*). Second, micrococcal nuclease digestion and primer extension analyses (at nucleotide resolution) of the chromatin in the presence or absence of GAL4 derivatives further supported the conclusion that nucleosomal cores flank the GAL4 binding sites (*17*).
20. We use the term "nucleosome reconfiguration" to denote changes in chromatin structure, but we do not intend to suggest that this alteration is necessarily a disruption, dissolution, or unfolding of the nucleosomes, or a displacement of the core histones. For example, some of the changes in chromatin structure may involve sliding or translational repositioning of the nucleosomes to another location on the DNA template. In addition, in the studies of factor-mediated nucleosome positioning, we cannot discern whether the GAL4 derivatives induce the adjacent positioning of nucleosomes by exclusion of the nucleosomes from the DNA segment that is occupied by the factors or by directed placement of the nucleosomes next to the factors.
21. T. Tsukiyama, P. B. Becker, C. Wu, *Nature* **367**, 525 (1994).
22. J. Côté, J. Quinn, J. L. Workman, C. L. Peterson, *Science* **265**, 53 (1994); H. Kwon, A. N. Imbalzano, P. A. Khavari, R. E. Kingston, M. R. Green, *Nature* **370**, 477 (1994); A. N. Imbalzano, H. Kwon, M. R. Green, R. E. Kingston, *ibid.*, p. 481.
23. P. B. Becker and C. Wu, *Mol. Cell. Biol.* **12**, 2241 (1992); G. C. Glikin, I. Ruberti, A. Worcel, *Cell* **37**, 33 (1984).
24. M. J. Fedor and R. D. Kornberg, *Mol. Cell. Biol.* **9**, 1721 (1989); R. H. Morse, *Science* **262**, 1563 (1993); J. D. Axelrod, M. S. Reagan, J. Majors, *Genes Dev.* **7**, 857 (1993); G. Cavilli and F. Thoma, *EMBO J.* **12**, 4603 (1993).
25. Chromatin assembly, in vitro transcription, and primer extension analysis were performed as described previously, except that the chromatin templates were not subjected to purification by sucrose gradient sedimentation before transcription with the soluble nuclear fraction as a histone-deficient source of basal transcription factors (*2, 3, 8*). The S-190 extract alone is not competent for basal transcription (*17*). Moreover, in control reactions containing S-190 and the soluble nuclear fraction, from which the ribonucleoside 5′-triphosphates were omitted, transcription was not observed (*17*). Hence, these extracts are not contaminated with endogenous ribonucleoside 5′-triphosphates. Quantitation of the reverse transcription products was done with a Phosphorimager (Fuji or Molecular Dynamics). All experiments were done a minimum of two times (but more commonly about four times) to ensure reproducibility of the data. In the course of these experiments, we used three S-190 extracts, two preparations of soluble nuclear fraction, three preparations of GAL4-VP16, two preparations of core histones, two preparations of histone H1, and several plasmid preparations. Thus, the results were consistently reproducible with different preparations of reagents. The plasmid pGIE-0 (M. Bulger and J. T. Kadonaga, unpublished data) contains five tandem consensus GAL4 binding sites immediately upstream of the adenovirus E4 minimal promoter (TATA box and RNA start site; from −38 to +250 relative to the RNA start site) and closely resembles pG_5E4T (*7*). The plasmid pIE-0 is identical to pGIE-0 except that it does not contain GAL4 binding sites.
26. Sephadex G-25 SF (Pharmacia) was equilibrated with buffer [12.5 mM Hepes-KOH (pH 7.5), 5% (v/v) glycerol, 0.05 mM EDTA, and 50 mM KCl]. Chromatin (300 μl, containing 3 μg of DNA) was applied to 3 ml of resin and subjected to chromatography in the same buffer. The chromatin eluted in the void volume.
27. We are grateful to H. Weintraub for inspiration and enlightenment regarding the role of chromatin structure in the regulation of gene expression. We thank B. Zimm, B. Emerson, E. Blackwood, J. Tyler, C. George, M. Bulger, S. Paranjape, and A. Wurster for critical reading of the manuscript. J.T.K. is a Presidential Faculty Fellow. Supported by grants to J.T.K. from NIH, NSF, and the Council for Tobacco Research.

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Specific Association of Human Telomerase Activity with Immortal Cells and Cancer

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Synthesis of DNA at chromosome ends by telomerase may be necessary for indefinite proliferation of human cells. A highly sensitive assay for measuring telomerase activity was developed. In cultured cells representing 18 different human tissues, 98 of 100 immortal and none of 22 mortal populations were positive for telomerase. Similarly, 90 of 101 biopsies representing 12 human tumor types and none of 50 normal somatic tissues were positive. Normal ovaries and testes were positive, but benign tumors such as fibroids were negative. Thus, telomerase appears to be stringently repressed in normal human somatic tissues but reactivated in cancer, where immortal cells are likely required to maintain tumor growth.

Telomeres are specialized structures at the ends of eukaryotic chromosomes that appear to function in chromosome protection, positioning, and replication (*1, 2*). In vertebrates, telomeres consist of hundreds to thousands of tandem repeats of the sequence TTAGGG and associated proteins (*2, 3*). Analysis of chromosome terminal restriction fragments (TRFs) provides the composite lengths of all telomeres in a cell population (*4–6*). In all normal somatic cells examined to date, TRF analysis has shown that the chromosomes lose about 50 to 200 nucleotides of telomeric sequence per cell division (*4–6*), consistent with the inability of DNA polymerase to replicate the ends of linear DNA (*7*). This shortening of telomeres has been proposed to be the mitotic clock by which cells count their divisions (*8*), and a sufficiently short telomere may be the signal for replicative senescence in normal cells (*5, 6, 9*). In contrast, all immortal cells examined to date show no net loss of telomere length or sequence with cell division, suggesting that maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely (*10–12*).

Telomerase is a ribonucleoprotein that synthesizes telomeric DNA onto chromo-

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somal ends using a segment of its RNA component as a template (*13, 14*). Telomerase activity can be measured in vitro by a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (*13, 15*). In extracts of human cells and tissues, telomerase activity was identified in nine immortal cell lines and in ovarian carcinoma, but was not detected in four normal somatic cell cultures or in normal tissues adjacent to the carcinoma (*10–12, 15, 16*). Together with TRF analysis, these results suggest that telomerase activity is directly involved in telomere maintenance, linking this enzyme to cell immortality.

To extend these findings to a broad range of human cells and tissues, we developed improved methods for extraction and detection of telomerase activity. With previous methods, reliable telomerase extraction by hypotonic swelling and physical disruption of cells required at least 10^7 to 10^8 cells, and the extraction efficiency varied between cell types (*10, 15*). Thus, a detergent lysis method (*12*) was improved to allow more uniform extraction of telomerase activity even at low cell numbers (*17*). The conventional activity assay was used with all telomerase specificity controls (*13, 15*) to demonstrate authentic telomerase activity in the detergent extracts.

To increase the sensitivity, speed, and efficiency of detecting telomerase activity, we developed a polymerase chain reaction (PCR)–based assay designated TRAP (for telomeric repeat amplification protocol) (Fig. 1). In the TRAP assay, telomerase synthesizes extension products, which then serve as the templates for PCR amplification (Fig. 1A). Reaction conditions suitable for both telomerase and Taq polymerase activities (*18*) were devised to allow the TRAP assay to be performed in a single reaction tube (Fig. 1B). Multiple control experiments demonstrated that a positive signal in the TRAP assay required a ribonucleoprotein in an immortal cell extract capable of extending the TS oligonucleotide with three or more TTAGGG repeats, validating the assay for specific detection of telomerase activity (Fig. 1C).

Telomerase-positive extracts from an immortal cell line (human 293 kidney cells) were produced routinely from 10^5 cells as assessed by TRAP assay (Fig. 1D, lane 3), with a current lower limit of 10^4 cells for CHAPS extraction (lane 4) (*19*). A quantity of extract representing 10^3 cells (1% of an extract from 10^5 cells) reproducibly gave a clear, positive signal in the TRAP assay (lane 3) with a current lower limit of 10^2 cells for detection of telomerase activity at 27 PCR cycles (lane 4). A similar level of detection resulted from serial titration of a single extract (Fig. 2B). Together the improvements in extraction efficiency and assay sensitivity increase detectability of telomerase activity by a factor of 10^4. Mixing of immortal cells and normal somatic cells prior to extraction, even at a ratio of $1:10^4$, does not interfere with the lower limit of detection. Detection of telomerase activity in 10^2 immortal cells (Fig. 1D, lane 4), but not in 10^5 BJ normal somatic cells (lane 1), indicates that the difference in activity be-

Table 1. Telomerase activity in normal and immortal cells (*29*).

Tissue of origin	Cell type	Telomerase activity (no. positive/ no. tested)
Skin	Tumor	8/8
Skin	Normal	0/5
Connective	Tumor	1/1
Joint	Normal	0/1
Adipose	Tumor	1/1
Breast	Tumor	22/22
Breast	Normal	0/8
Lung	Tumor	18/18
Lung	Transformed	2/3
Lung	Normal	0/3
Stomach	Tumor	1/1
Pancreas	Tumor	3/3
Ovary	Tumor	5/5
Cervix	Tumor	3/3
Cervix	Normal	0/1
Uterus	Normal	0/1
Kidney	Tumor	8/8
Kidney	Transformed	1/1
Bladder	Tumor	3/3
Bladder	Normal	0/1
Colon	Tumor	7/7
Prostate	Tumor	2/2
Prostate	Transformed	0/1
Prostate	Normal	0/2
CNS	Tumor	3/3
Retina	Transformed	1/1
Blood	Tumor	9/9

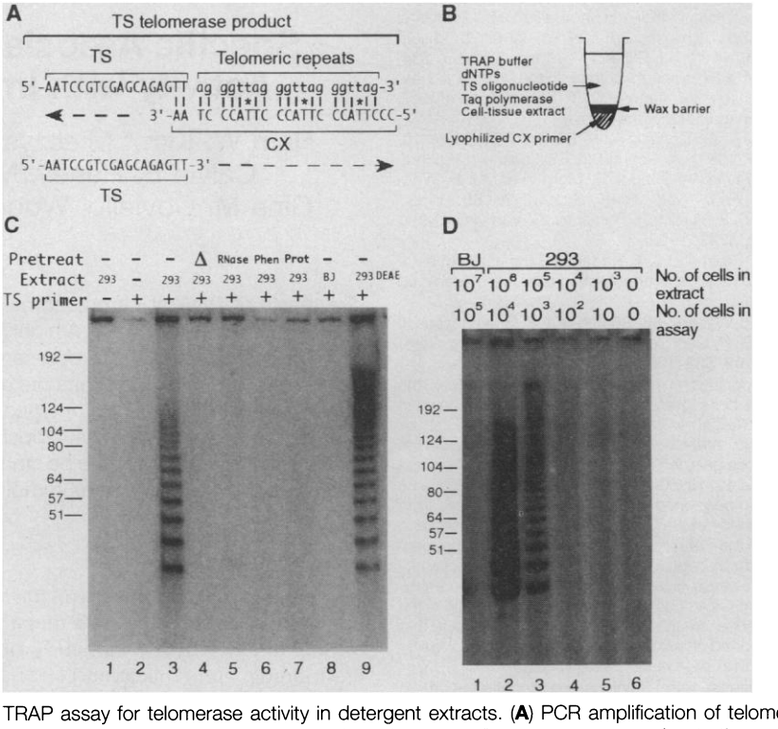

Fig. 1. TRAP assay for telomerase activity in detergent extracts. (**A**) PCR amplification of telomerase extension products. Telomerase synthesizes telomeric repeats (lowercase sequence) onto the nontelomeric oligonucleotide TS (5′-AATCCGTCGAGCAGAGTT-3′). Such telomerase products are specifically amplified by PCR with the downstream primer CX [5′-$(CCCTTA)_3$CCCTAA-3′] and the upstream primer TS (*26*). DNA synthesis by PCR (broken arrows) and optimal annealing of the CX primer (vertical lines) are shown. Asterisks indicate designed mismatches in CX that reduce primer interaction (*27*). (**B**) Single-tube arrangement of the TRAP assay. The CX primer is initially separated from the rest of the reaction mix by a wax barrier (*18*). (**C**) Specificity of TRAP assay for telomerase activity. Lane 1, control omitting TS oligonucleotide; lane 2, control omitting cell extract; lane 3, TRAP assay of immortal 293 cell extract; lane 4, heat-treated 293 extract (*28*); lane 5, RNase-pretreated 293 extract; lane 6, phenol-extracted 293 extract; lane 7, protease-pretreated 293 extract; lane 8, normal fibroblast BJ cell extract; and lane 9, extract enriched for telomerase by DEAE chromatography (*25*). Molecular sizes (in base pairs) of DNA markers (Marker V, Boehringer Mannheim) are shown on the left. (**D**) Limits of detergent extraction and TRAP detection. In each lane, 1% of the total extract (1 μl of 100 μl) was assayed. Lane 1, extract from 10^7 normal fibroblast BJ cells; lanes 2 to 5, extracts from 10^6, 10^5, 10^4, and 10^3 immortal 293 cells, respectively; and lane 6, control assay with lysis buffer only (*19*).

tween immortal and mortal cells is at least 1000-fold.

These methods were used to measure telomerase activity in dividing cultures of various immortal cell lines and normal somatic cells derived from different tissues and individuals. This survey included a total of 100 immortal cell lines and 22 normal somatic cell cultures from 18 different tissues (Fig. 2A and Table 1). As opposed to other enzymes involved in DNA synthesis, telomerase is not growth-regulated: None of the actively proliferating normal somatic cell cultures displayed detectable telomerase activity in the TRAP assay. Of the 100 immortal cell lines, 94 were tumor-derived lines and 6 were cell lines transformed with viral oncoproteins. All of the 94 tumor lines contained telomerase activity. However, two lines transformed with SV40 T antigen tested negative for telomerase activity. This result was unexpected as TRF analysis had shown that these cells have substantially longer telomeres than the normal cells from which they were derived.

It has been suggested that telomere length might be an indirect measure of telomerase activity in a cell population (*20*). To test this hypothesis, we determined TRF length (*5*) for several of the immortal cell lines assayed for telomerase activity. Although most of the cell lines had short TRFs (<4 kb), some had intermediate (4 to 10 kb) or long TRFs (>10 kb). TRF length did not correlate with the levels of telomerase activity measured in these cells and thus is not a reliable marker for telomerase activity.

The difficulty in establishing cell lines from tumor samples has led many investi-

Table 2. Telomerase activity in human tumors and tissues. Germline tissue (ovary and testis) and almost all advanced tumors were positive for telomerase activity, whereas tissue adjacent to tumors was generally negative. However, rapidly proliferating benign growths such as leiomyomas (fibroids) were telomerase negative. Tumor tissue of all eight colon carcinomas exhibited telomerase activity, whereas extracts of adjacent colonic muscosa without overt tumor tissue, benign tubular adenoma, and a colonic polyp were negative. Although telomerase activity was not detected in some tumor tissue samples (for example, three of four node-negative breast cancers), some tissue samples that were apparently tumor free had telomerase activity. We believe that tumor cells can be present in macroscopically normal adjacent tissues of cancer patients with advanced disease, even if they are not detected by routine pathological examination. All eight normal prostatic tissues examined were negative for telomerase activity, whereas two adenomatous carcinomas of the prostate were positive. Because prostate cancer can be found localized to a small, peripheral region of the prostate gland, we confirmed by pathologic examination that the tissue adjacent to the sample contained tumor cells. Of 10 samples of benign prostatic hyperplasia (BPH), 1 was positive for telomerase activity. This was unexpected, because there does not appear to be a direct correlation between the presence of BPH and the development of prostate cancer. This patient may have had prostate cancer that was not detected microscopically. Telomerase activity was detected in 3/5 samples of prostatic intraepithelial neoplasia type 3 (PIN3). Although it is not known whether prostatic intraepithelial neoplasia (PIN) is an obligate precursor of adenocarcinoma, there is circumstantial evidence for the premalignant nature of PIN (*30*).

Tissue type	Telomerase activity (no. positive/ no. tested)	Tissue type	Telomerase activity (no. positive/ no. tested)
Fetal testis	2/2	Normal breast tissue (from noncancer patients)	0/8
Adult testis	1/1	Prostate cancer	2/2
Fetal ovary	2/2	Prostatic intraepithelial neoplasia type 3	3/5
Ovarian follicle	1/1	Benign prostatic hyperplasia	1/10
Gastrointestinal malignancies		Normal prostatic tissue	0/8
Hepatocellular carcinoma*	1/1	Neuroblastoma	5/5
Colon cancer	8/8	Brain tumors	6/8
Adjacent colonic tissue	0/7	Lung small-cell carcinoma	4/4
Colonic tubular adenoma	0/1	Rhabdomyosarcoma	1/1
Colonic polyp	0/1	Leiomyosarcoma	3/3
Squamous cell carcinoma (head and neck)	14/16	Leiornyoma (fibroids)	0/11
Adjacent tissue	6/16	Normal myometrium	0/10
Wilms tumor	6/6	Hematological malignancies	
Adjacent kidney tissue	2/6	Acute lymphocytic leukemia	14/16
Breast cancer (ductal and lobular, node positive)	18/20	Chronic lymphocytic leukemia	2/2
Breast cancer (axillary node negative)	1/4	Lymphoma (adult)	5/5
Adjacent tissue	2/20		

*Needle biopsy, frozen 3 months.

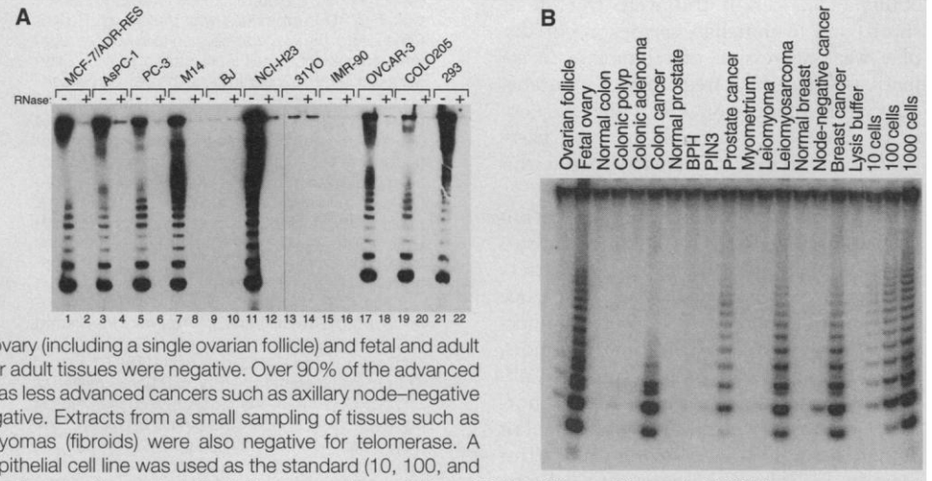

Fig. 2. TRAP assays of human cells and tissues. (**A**) TRAP assays of 10^5 cell equivalents per reaction. Even-numbered lanes, extracts pretreated with RNase. Lanes 1 and 2, breast carcinoma line MCF-7/ADR-RES; lanes 3 and 4, pancreatic carcinoma line AsPC-1; lanes 5 and 6, prostate carcinoma line PC-3; lanes 7 and 8, melanoma line M14; lanes 9 and 10, normal foreskin fibroblast BJ; lanes 11 and 12, lung carcinoma line NCI-H23; lanes 13 and 14, normal stromal fibroblast 31YO; lanes 15 and 16, normal lung fibroblast IMR-90; lanes 17 and 18, ovarian carcinoma line OVCAR-3; lanes 19 and 20, colon carcinoma line COLO205; and lanes 21 and 22, transformed kidney line 293. (**B**) Normal germline tissues such as ovary (including a single ovarian follicle) and fetal and adult testis (Table 2) were telomerase-positive, but other adult tissues were negative. Over 90% of the advanced tumors surveyed were telomerase-positive, whereas less advanced cancers such as axillary node–negative breast tumors were generally (but not always) negative. Extracts from a small sampling of tissues such as a colonic polyp, a tubular adenoma, and leiomyomas (fibroids) were also negative for telomerase. A telomerase-positive immortalized human breast epithelial cell line was used as the standard (10, 100, and 1000 cell equivalents per assay), and lysis buffer as the negative control. Each tissue sample consisting of 50 to 100 mg of frozen (−80°C) tissue was washed in ice-cold wash buffer (*17*) then homogenized with 200 μl of lysis buffer (4°C) in Kontes tubes with matching pestles rotated at 450 rpm. After 25 min at 4°C, the lysate was centrifuged at 16,000*g* for 20 min at 4°C and the supernatant frozen in liquid N_2 and stored at −80°C. A sample of the extract (6 μg of protein) was used for each telomerase assay. After 30 min at room temperature, the reaction mixture (*18*) was heated at 90°C for 90 s and subjected to 31 PCR cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s.

gators to question whether cancer cells in vivo are immortal (*21*). However, the detection of telomerase activity in tumor cells obtained from ascitic fluid of patients with metastatic ovarian carcinoma (*11*) suggests that at least some cancer cells may indeed be immortal. This hypothesis can be tested by examining the correlation between telomerase activity and tumor progression in a number of different human tumor types. However, the lack of primary human tumor samples of sufficiently large size severely limits the ability to measure telomerase activity by the conventional assay. Also, in contrast to tumor cell lines, primary tumors are rarely homogeneous and often consist of mixtures of tumor cells with surrounding stromal tissue. With the conventional telomerase assay, a threefold excess of normal cells did not inhibit telomerase activity of cultured tumor cells. With the TRAP assay, telomerase could be detected if 1 in 10^4 cells was immortal. These results indicated that analysis of primary tumor material for telomerase activity would be feasible. Normal somatic and germline tissues were obtained at autopsy from individuals who died of natural causes. As predicted, telomerase activity was easily detected in germline tissue but not in 50 other adult tissues examined (*22*). TRAP assays performed on a wide range of normal and tumor tissues indicated that 90 of 101 malignant tumor samples specifically expressed telomerase (Fig. 2B and Table 2). These data confirm the stringent repression of telomerase in normal somatic tissue and suggest that malignant progression may depend on the activation of telomerase (*23*, *24*).

It is possible that in long-lived species such as humans, repression of telomerase in somatic tissues evolved to reduce the probability of cancer. If this were true, some short-lived mammalian species might display weak repression of telomerase in somatic tissues, a high frequency of spontaneous cell transformation, and a high frequency of cancer on a per cell, per year basis. Additional studies are needed to test this hypothesis.

In conclusion, the development of improved assay methods has permitted a survey of telomerase activity in a wide variety of human cells and tissues. The presence of telomerase in 98 of 100 cultured immortal cell lines, 90 of 101 primary tumors, and adult germline tissues, and the absence of detectable telomerase in 22 normal somatic cell cultures and 50 normal or benign tissues, provide strong support for hypotheses linking telomerase to cell immortality. Expression of telomerase in almost all advanced malignancies tested suggests that immortal cells are likely required to maintain tumor growth. These methods should facilitate a further understanding of telomerase biology, potentially leading to diagnostic and therapeutic applications.

REFERENCES AND NOTES

1. E. H. Blackburn and J. W. Szostak, *Annu. Rev. Biochem.* **53**, 163 (1984); V. A. Zakian, *Annu. Rev. Genet.* **23**, 579 (1989).
2. E. H. Blackburn, *Nature* **350**, 569 (1991).
3. R. K. Moyzis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6622 (1988); J. Meyne, R. Ratliff, R. Moyzis, *ibid.* **86**, 7049 (1989).
4. C. B. Harley, A. B. Futcher, C. W. Greider, *Nature* **345**, 458 (1990).
5. R. C. Allsopp *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10114 (1992).
6. H. Vaziri *et al.*, *Am. J. Hum. Genet.* **52**, 661 (1993).
7. J. D. Watson, *Nature New Biol.* **239**, 197 (1972); A. Olovnikov, *J. Theor. Biol.* **41**, 181 (1973).
8. C. B. Harley, *Mutat. Res.* **256**, 271 (1991).
9. N. D. Hastie *et al.*, *Nature* **346**, 866 (1990); J. Lindsey, N. McGill, L. Lindsey, D. Green, H. Cooke, *Mutat. Res.* **256**, 45 (1991); W. E. Wright and J. W. Shay, *Trends Genet.* **8**, 193 (1992).
10. C. M. Counter *et al.*, *EMBO J.* **11**, 1921 (1992).
11. C. M. Counter, H. W. Hirte, S. Bacchetti, C. B. Harley, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2900 (1994).
12. C. M. Counter, F. M. Botelho, P. Wang, C. B. Harley, S. Bacchetti, *J. Virol.* **68**, 3410 (1994).
13. C. W. Greider and E. H. Blackburn, *Cell* **43**, 405 (1985); *Nature* **337**, 331 (1989).
14. G.-L. Yu, J. D. Bradley, L. D. Attardi, E. H. Blackburn, *Nature* **344**, 126 (1990).
15. G. B. Morin, *Cell* **59**, 521 (1989).
16. K. R. Prowse, A. A. Avilion, C. W. Greider, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1493 (1993).
17. Cells were washed once in phosphate-buffered saline, pelleted at 10,000*g* for 1 min at 4°C, resuspended in ice-cold wash buffer [10 mM Hepes-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol], pelleted again, and resuspended at 10^4 to 10^6 cells per 20 μl of ice-cold lysis buffer [10 mM tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS (Pierce), 10% glycerol]. The suspension was incubated 30 min on ice and then centrifuged for 30 min in a microultracentrifuge (100,000*g*, 4°C). The supernatant was removed, quick-frozen on dry ice, and stored at −70°C. Protein concentrations were typically 5 to 10 mg/ml, and the telomerase activity was stable to multiple freeze-thaws.
18. Assay tubes were prepared by lyophilizing 0.1 μg of CX primer onto the bottom of the tube and sealing it with 7 to 10 μl of molten wax (Ampliwax, Perkin-Elmer). After the wax was allowed to solidify at room temperature, the tubes were stored at 4°C. Fifty-microliter TRAP reactions above the wax barrier contained 20 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 μM deoxynucleoside triphosphates, 0.1 μg of TS oligonucleotide, 1 μg of T4g32protein (Boehringer Mannheim), bovine serum albumin (0.1 mg/ml), 2 U of Taq DNA polymerase (Boehringer Mannheim), and 1 to 2 μl of a CHAPS cell extract. For radiolabeling of products, 0.2 to 0.4 μl of [α-^{32}P]dGTP (deoxyguanosine triphosphate) or [α-^{32}P]dCTP (deoxycytidine triphosphate) (10 μCi/μl, 3000 Ci/mmol) were added to the reaction. After 10 min at 23°C for extension of oligonucleotide TS by telomerase, tubes were transferred to a thermal cycler for 27 rounds at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The CX primer (0.1 μg) was liberated when the wax barrier melted at ~70°C. One-half of the reaction was analyzed by electrophoresis in 0.5× tris-borate EDTA on 15% polyacrylamide nondenaturing gels.
19. For extraction of different quantities of cells, the volume of lysis buffer was kept constant at 100 μl. The limit of detection is a function of the TRAP assay conditions used and should be considered as a practical limit under the given set of conditions rather than as an absolute limit.
20. E. Hiyama, K. Hiyama, T. Yokoyama, T. Ichikawa, Y. Matsuura, *Jpn. J. Cancer Res.* **83**, 159 (1992); P. Nurnberg, G. Thiel, F. Weber, J. T. Epplen, *Hum. Genet.* **91**, 190 (1993); H. S. Schwartz, G. A. Dahir, M. G. Butler, *Cancer Genet. Cytogenet.* **71**, 132 (1993); C. Mehle, B. Ljungberg, G. Roos, *Cancer Res.* **54**, 236 (1994).
21. A. C. Stamps, B. A. Gusterson, M. J. O'Hare, *Eur. J. Cancer* **28A**, 1495 (1992).
22. The presence of telomerase in testes and ovaries is consistent with the concept that germline cells are functionally immortal. Telomeres from sperm DNA do not decrease with age of the donor (as do somatic cells), suggesting a mechanism for maintaining telomere length (*5*).
23. C. B. Harley *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **59**, in press.
24. Candidate hematopoietic stem cells show evidence of functional decline and loss of telomeric DNA with age in vitro and in vivo [see H. Vaziri *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9857, (1994)], but the question of cell mortality or immortality in human stem cells warrants additional studies.
25. G. B. Morin, *Nature* **353**, 454 (1991).
26. Oligonucleotides representing discrete telomerase extension products, for example, TS+4 (TS plus four telomeric repeats), were used to develop specific amplification conditions. Even under high stringency, staggered annealing of the downstream primer occurred (for example, annealing by three of the four repeats). Hence PCR amplification of a discrete telomerase extension product yielded a 6-nucleotide (nt) ladder of PCR products increasing in size up to the limit of gel resolution. Thus, TRAP assay products do not directly reflect the distribution of telomerase products generated in the assay, and the interaction between the upstream and downstream primers must be prevented.
27. Because telomerase is known to extend oligonucleotides of nontelomeric sequence (*25*), such an oligonucleotide substrate (TS) was used to avoid artifacts resulting from PCR primer complementarity. As further precautions to avoid primer interaction, mismatches in the downstream primer CX, single-stranded binding protein T4g32, hot start PCR, and an annealing temperature of 50°C were used (*18*). The product formation in the TRAP assay is absolutely dependent on the presence of TS and CX oligonucleotides. Absence of either oligonucleotide does not lead to formation of a TRAP product. Under these conditions specific amplification occurs only if the oligonucleotide substrate has been extended with three or more TTAGGG repeats resulting in a 6-nt ladder of TRAP assay products extending from 40 nt (first amplifiable telomerase product) up to the limit of gel resolution.
28. Pretreatments were heat (65°C for 10 min); ribonuclease (RNase) [10-μl extract plus 0.5 μg of RNase (deoxyribonuclease-free; Boehringer Mannheim) for 10 min at 23°C]; phenol (1:1 extraction); and protease [50-μl extract plus 5 μg of Bromelain (Boehringer Mannheim) for 10 min at 37°C, followed by protease removal according to the manufacturer's protocol]. All non-RNase–treated extracts were also incubated for 10 min at 23°C prior to the assay. Control assays omitting the CX oligonucleotide were negative.
29. The specific immortal cell lines and normal cell cultures are listed by tissue of origin. Skin: melanoma (LOXIMVI, M14, Malme-3M, UACC-62, SK-MEL-28, SK-MEL-2, SK-MEL-5, UACC-257), normal fibroblasts (GFS, S37b, Malme-3, BJ), and normal keratinocytes (primary foreskin); connective: fibrosarcoma (HT-1080); joint: normal synovial fibroblast (HSF); adipose: liposarcoma (SW872); breast: adenocarcinoma (MCF7, MCF-7/ADR-RES, MDA-MB-231), ductal carcinoma (T 47 D, MDA-MB-435), carcinoma (MDA-MB-157, MDA-MB-175-VI, MDA-MB-436, MDA-MB-468, ZR-75-1, ZR-75-30, UACC-812, UACC-893, BT-20, BT-474, BT-483, BT-549, HS578T, SK-BR-3, SCC70, SCC38, SCC202), and normal epithelial and stromal cells (HME: 15, 17, 31, 32, 35); lung: carcinoma (NCI-H522, NCI-H23, A549, EKVX, 1299, H146, H69, NCI-H460, H358, H182, H322, H226, HOP62, H510A, H128, NCI-H209, NCI-H446, NCI-H82), SV40 T antigen–transformed fibroblasts (IDH4, SW26-IG, SW-26-C4), and normal fetal fibroblasts (GFL, IMR-90, Wi38);

stomach: gastric carcinoma (KATO-III); pancreas: ductal carcinoma (SU.86.86) and adenocarcinoma (AsPC-1, Capan-1); ovary: carcinoma (OVCAR-3, OVCAR-5, IGROV-1, SK-OV-3) and adenocarcinoma (OVCAR-8); cervix: carcinoma (HeLa S3, C-33 A, HT-3) and normal primary epithelial cells; uterus: normal primary endometrial cells; kidney: carcinoma (A498, CAKI-1, 786-O, ACHN, RxF393, TK-10, SN-12-C, UO-31) and adenovirus 5–transformed embryonic kidney cells (293); bladder: carcinoma (5637), transitional cell carcinoma (T24), squamous carcinoma (SCaBER), and normal fetal (FHs 738B1); colon: adenocarcinoma (COLO 205, SW-620, HCT-116, HT-29, HCT-15, HCC-2998, KM12R); prostate: adenocarcinoma (PC-3, DU 145), SV40-transformed BPH fibroblasts (BPH-1), normal stromal fibroblasts (31YO), and BPH fibroblasts (S52); central nervous system (CNS): carcinoma (U251, SNB-75) and glioblastoma (SF268); retina: SV40-transformed pigmented epithelium (RPE28SV4); blood: leukemia (Molt4, HEL, SR-WJU, CCRF-CEM, RPMI-8226), T cell leukemia (Jurkats), acute promyelocytic leukemia (HL-60), chronic myelogenous leukemia (K-562), and histiocytic lymphoma (U-937).

30. M. B. Garnick, *Sci. Am.* **270**, 72 (April 1994); E. C. Jones and R. H. Young, *Am. J. Clin. Pathol.* **101**, 48 (1994).

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Assembly of Transcriptionally Active RNA Polymerase I Initiation Factor SL1 from Recombinant Subunits

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Initiation of ribosomal RNA synthesis by RNA polymerase I requires the promoter selectivity factor SL1, which consists of the TATA-binding protein, TBP, and three associated factors, TAF_Is 110, 63, and 48. Here the in vivo and in vitro assembly of functional SL1 complexes from recombinant TAF_Is and TBP are reported. Complexes containing TBP and all three TAF_Is were as active in supporting transcription from the human ribosomal RNA gene promoter as endogenous SL1, whereas partial complexes without TBP did not efficiently direct transcription in vitro. These results suggest that TAF_Is 110, 63, and 48, together with TBP, are necessary and sufficient to reconstitute a transcriptionally active SL1 complex.

Transcription of the genes for 28S and 18S ribosomal RNA by RNA polymerase I (RNA pol I) in mammalian cells is a highly regulated process that has been intensively studied for about two decades (*1*). Three components, RNA pol I, the upstream binding factor UBF, and the promoter selectivity factor SL1 are minimally required for accurate initiation of transcription from the human ribosomal promoter (*2*). UBF has been purified to homogeneity and cloned, and recombinant versions of UBF are available (*3*). Until recently the subunit composition and biochemical activities of SL1 were unknown. Recent advances in antibody affinity chromatography allowed us to purify small quantities of SL1 from HeLa (human) nuclear extracts to apparent homogeneity. These studies revealed that SL1 is composed of the TATA binding protein, TBP, and at least three associated subunits of 110, 63, and 48 kD, called TAF_Is (TBP associated factors for RNA pol I) (*4*).

Although reconstitution of SL1 with recombinant TBP and partially purified TAF_I complexes that contain the three subunits obtained by TBP antibody affinity chromatography suggested that these associated factors are important for the assembly of transcriptionally active SL1 complexes (*4*), it was not possible to determine whether these subunits were sufficient to direct promoter and RNA polymerase selectivity. Further purification and disruption of the complex and the reassembly of active SL1 proved to be impractical given the low amount of SL1 in the cell and the limited amounts of purified material obtainable. Thus, it became evident that future advances in determining the structure and function of this essential human transcription complex would require molecular cloning and high level expression of each subunit. In the accompanying paper, we describe the isolation of complementary DNAs (cDNAs) encoding each of the three human TAF_Is present in SL1 (*5*). Here, we describe the assembly of partial and complete SL1 complexes. We have assembled transcriptionally active SL1 in HeLa cells infected with recombinant vaccinia viruses expressing TAF_Is and TBP. As an alternative procedure for reconstituting SL1, we have also reconstructed stable complexes containing the three TAF_Is and TBP in vitro using purified recombinant proteins. After isolating these in vivo and in vitro assembled complexes by antibody affinity chromatography, their ability to support accurate initiation of transcription from the human ribosomal promoter in a reconstituted reaction containing UBF and RNA pol I was tested. Our results suggest that a complex containing TAF_I 110, 63, 48, and TBP is both necessary and sufficient to provide SL1 function.

Using a combination of epitope-tagged versions of the recombinant TAF_Is, we have shown that each of the subunits of SL1 can individually bind to TBP. In addition, the TAF_Is appear to contact each other in order to form a relatively stable complex that presumably involves multiple interactions. These TBP-TAF_I and TAF_I-TAF_I interactions do not require DNA (*5*). However, it was of critical importance to determine whether these protein-protein contacts resulted in a functional SL1 complex that could support transcription of the ribosomal promoter by RNA pol I. Our initial attempts to assemble functional complexes from TAF_Is expressed in *Escherichia coli* or in insect cells infected with recombinant baculoviruses, failed to produce transcriptionally active SL1. One reason for our inability to reconstitute active SL1 complexes was the difficulty in obtaining sufficiently high concentrations of the four subunits of SL1 in order to perform the assembly reactions. The bacterially expressed proteins were largely insoluble, and the amounts of soluble baculovirus expressed TAF_Is were limited. Consequently, we tried alternative means of assembling SL1 from the recombinant subunits. We approached the problem with two complementary strategies: in vivo assembly by co-expression of the subunits and in vitro assembly of denatured and renatured purified subunits.

In order to attempt in vivo assembly of the four components of SL1, we generated recombinant vaccinia viruses that would direct the production of each subunit in HeLa cells upon co-infection with the various combinations of viruses. We constructed the appropriate vaccinia virus vectors such that the recombinant TAF_Is could be distinguished from endogenous TAF_Is on the basis of differences in molecular weight and antibody specificity by fusing a unique epitope tag to each subunit. TAF_I48 was constructed with a FLAG-tag, TAF_I63 was fused to the Polyoma-Myc (PM) epitope, and TAF_I110 was linked to the hemagglutinin-peptide

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