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Telomerase activity in human ovarian carcinoma

(telomere/ovarian cancer/cell immortalization/aging)

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ABSTRACT **Telomeres fulfill the dual function of protecting eukaryotic chromosomes from illegitimate recombination and degradation and may aid in chromosome attachment to the nuclear membrane. We have previously shown that telomerase, the enzyme which synthesizes telomeric DNA, is not detected in normal somatic cells and that telomeres shorten with replicative age. In cells immortalized *in vitro*, activation of telomerase apparently stabilizes telomere length, preventing a critical destabilization of chromosomes, and cell proliferation continues even when telomeres are short. *In vivo*, telomeres of most tumors are shorter than telomeres of control tissues, suggesting an analogous role for the enzyme. To assess the relevance of telomerase and telomere stability in the development and progression of tumors, we have measured enzyme activity and telomere length in metastatic cells of epithelial ovarian carcinoma. We report that extremely short telomeres are maintained in these cells and that tumor cells, but not isogenic nonmalignant cells, express telomerase. Our findings suggest that progression of malignancy is ultimately dependent upon activation of telomerase and that telomerase inhibitors may be effective antitumor drugs.**

Telomeres contain both DNA and protein that together appear to stabilize the ends of eukaryotic DNA (reviewed in refs. 1–3). The DNA component of telomeres is generally characterized by a G-rich strand composed of a simple tandemly repeated sequence (TTAGGG in humans) (1–3). The correct sequence of this repeat is required for telomere function, since addition of telomeric DNA harboring a mutated telomeric sequence to the ends of the endogenous *Tetrahymena* telomeres lead to telomere length instability and death (4). In addition, it now appears that the length of these repeats may play a role in the lifespan of yeast (5) and human cells (6–12). A number of studies have shown conclusively that telomere length decreases with both *in vitro* and *in vivo* division of human cells (6–12). Since cultured fibroblasts still contain telomeric repeats at senescence, it was proposed that shortening of telomeres to a critical size may act as a mitotic clock, signaling a cell cycle exit (reviewed in ref. 13).

In contrast to normal cells, transformation *in vitro* subverts normal growth control, yielding populations with extended but still finite lifespan, although immortal clones may emerge at low frequency (14–17). We have previously shown that telomere shortening continues during the extended lifespan of transformed cells until crisis, where some chromosomes may have actually lacked TTAGGG repeats (9). Interestingly, during this period the frequency of dicentric chromosomes dramatically increased. Clones overcoming crisis exhibited a stabilization of telomere length and frequency of dicentrics, apparently due to the activation of telomerase (9), the enzyme which elongates telomeres *de novo* (4, 18–20). Thus, telomerase may have restored telomere function by the addition of TTAGGG repeats and thereby permitted continued cell division in immortal clones (9). If telomerase is required for immortalization *in vitro*, it may also be necessary during tumorigenesis. We reasoned that tumor metastases or recurrent tumors, arising from repeated clonal expansion, should contain immortal cells with stable telomeres and telomerase activity and that if telomerase activation were a late event, telomeres would be short. Indeed, in some cancers it appears as though telomere length decreases with increasing tumor grade (21, 22), and tumors with very short telomeres have been detected in a variety of cancers (7, 21–26, 35). We report that late-stage ovarian carcinoma tumor cells maintain short stable telomeres both *in vitro* and *in vivo* and that telomerase is specifically activated in tumor cells, but not in normal somatic cells.

MATERIALS AND METHODS

Cell Culture. 293 CSH cells, a subline of 293 (27), were obtained from B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and cultured in Joklik medium supplemented with 5% fetal bovine serum. Ovarian carcinoma cell lines HEY and SKOV-3 (American Type Culture Collection) and normal ovarian epithelium (see below) were cultured in E3 medium [a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 supplemented with epidermal growth factor (5 ng/ml), insulin (5 μg/ml), transferrin (10 μg/ml), phosphoethanolamine (50 μM), ethanolamine (50 μM), and 3% fetal bovine serum]. The nonmalignant fraction of ascites cells were maintained in α minimal essential medium (αMEM) supplemented with 10% fetal bovine serum, whereas the tumor fraction and lines derived from them were cultured in E3 medium. Some cell lines established were tested for the ability to grow in serum-free medium (E3 medium with 0.3% bovine serum albumin replacing serum).

Isolation of Cells. Ascitic fluid was withdrawn from ovarian carcinoma patients at the time of diagnostic laparotomy or by subsequent paracentesis (28) and centrifuged to obtain a cell pellet as described (29). Leukocytes were obtained from the pooled buffy coats of three normal males, and normal ovarian epithelium was obtained from the surface of the ovary (30).

Fractionation of Ascites Cells. Fibroblasts and mesothelial cells (the nonmalignant fraction) were separated from the tumor cells based on their ability to adhere to plastic more readily than tumor cells or most leukocytes (31, 32). Specifically, cells were resuspended in αMEM supplemented with 10% fetal bovine serum and left undisturbed for 12–18 hr in culture plates. Nonadhering tumor cells were removed and expanded in culture in E3 medium, whereas adherent nonmalignant cells were expanded in αMEM with 10% fetal

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Abbreviation: TRF, terminal restriction fragment.

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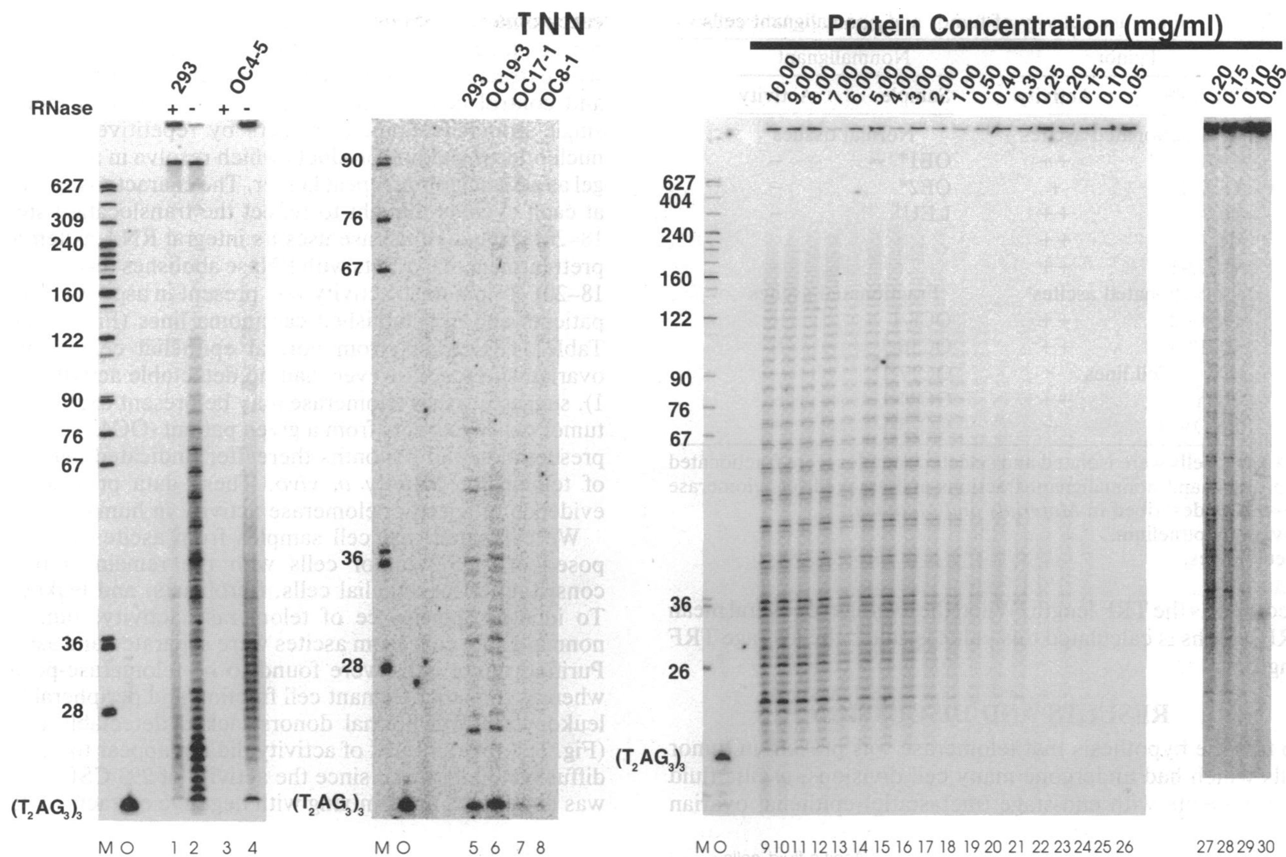

FIG. 1. Telomerase activity in cells from ascitic fluid. Telomerase activity in extracts prepared and assayed at a protein concentration of ≈11 mg/ml is shown for 293 CSH cells (lane 2) and unfractionated ascites cells from patient OC4 (lane 4; patients were assigned individual codes followed by the paracentesis number). For lanes 1 and 3, RNase was added to the extracts prior to the addition of [α-^{32}P]dGTP. Activity was detected in extracts from 293 CSH cells (lane 5), and the tumor (T, lane 6) but not the nonmalignant (N, lanes 7 and 8) cell fractions from ascites. Protein concentration in the tumor cell extract was 3.3 mg/ml, and in the normal cell extracts 2.1 and 1.9 mg/ml, respectively. Consequently, the 293 cell extract was diluted and assayed at 1.5 mg/ml for proper comparison with the negative extracts. Lanes 9–26 show a serial dilution of 293 CSH cell extract. Lanes 27–30 show the concentration range 0.2–0.05 mg/ml at greater sensitivity. Exposure to PhosphorImager screens was 1 week in all cases. Lane M, size markers (lengths in nucleotides at left); lane O; ^{32}P-labeled (TTAGGG)$_3$ oligonucleotide.

bovine serum. Cells fractionated in this manner were used to prepare S100 extracts for assaying telomerase activity (see below). Highly pure tumor cells for DNA analysis were obtained by fractionating ascites cells through two consecutive cycles of differential attachment. Additionally, attempts were made to establish some of these pure tumor cells in culture. Sometimes it was possible to obtain enough cells for analysis by filtering ascites cells through a 30-μm nylon mesh (Spectrum), which retains tumor clumps but not the smaller leukocytes, fibroblasts, or mesothelial cells. Filters were backwashed to yield essentially only tumor cell clumps (29, 31). Results with this technique were similar to those obtained by fractionating cells by differential attachment.

Preparation of S100 Extracts. Extracts were prepared from $>10^8$ cells from fresh material (leukocytes and unfractionated ascites cells) and cultured cells (nonmalignant and tumor cell fractions, normal ovarian epithelium, and cell lines HEY, SKOV-3, and 293 CSH) as described (9). Protein concentrations were determined by Bradford assay (Bio-Rad) and enzymatic activity was determined by the level of DNA polymerase activity (9).

Telomerase Assay. The assay was performed as described (9) with minor modifications. In brief, reaction mixtures containing 0.5 volume of S100 extract, 3.5 mM $MgCl_2$, 1 mM spermidine, 5 mM 2-mercaptoethanol, 50 mM potassium acetate, 2.5 mM EGTA, 50 mM Tris acetate (pH 8.5), 1 μM telomere primer (TTAGGG)$_3$, 3.1 μM [α-^{32}P]dGTP (800 Ci/mmol; 1 Ci = 37 GBq), 2 mM dTTP and 2 mM dATP were incubated at 30°C for 1 hr. As a control, RNase A was added to parallel reactions before the addition of [α-^{32}P]dGTP. Reactions were terminated by treatment with RNase A followed by proteinase K (9). Unincorporated [α-^{32}P]dGTP was removed with Nick Spin columns (Pharmacia). Products were resolved by electrophoresis in a sequencing gel and exposed to a PhosphorImager screen (Molecular Dynamics). Extracts were considered positive (++) if the characteristic 6-nucleotide pattern was detected in a 7-day exposure, and weakly positive (+) or negative (−) if detected or not detected, respectively, after 2 weeks. Protein concentration in all extracts was >1 mg/ml—i.e., 10 times the lowest concentration at which activity was detected in control 293 CSH extracts. Dilution of 293 CSH extract was performed with 1× hypobuffer (9) with the addition of NaCl to a final concentration of 0.1 M.

Analysis of DNA. DNA was isolated, digested with restriction enzymes *Hin*fI and *Rsa* I, and quantitated by fluorometry from $>10^7$ cells of unfractionated ascites or nonmalignant or tumor ascites fractions or cultured cells as described (6, 9–11). Restriction enzyme digestion liberates terminal restriction fragments (TRFs), which are composed of telomeric DNA (TTAGGG) at the most distal end, followed by subtelomeric DNA consisting of degenerate TTAGGG and other unrelated repetitive DNA (26, 33). TRFs were resolved in agarose gels, hybridized with the telomere-specific ^{32}P-labeled (CCCTAA)$_3$ probe, and visualized on film as described (9–11). The mean TRF length was determined from the values obtained from densitometric scanning of at least two autoradiographs over the size range 2–21 kbp (9–11). For simplicity, this value is

Table 1. Telomerase activity of tumor and nonmalignant cells

Tumor		Nonmalignant	
Sample	Activity	Sample	Activity
Unfractionated ascites		Normal tissues	
OC1-1	++	OE1*	–
OC2-1	+	OE2*	–
OC4-1	++	LEU†	–
OC4-5	++		
OC23-1	++		
Fractionated ascites		Fractionated ascites	
OC18-2	++	OC8-1	–
OC19-3	++	OC16-7	–
Cell lines		OC17-1	–
HEY	++	OC24-1	–
SKOV-3	+	OC25-1	–

Ascites cells were isolated and selected samples were fractionated into tumor and nonmalignant fractions and assayed for telomerase activity as described in *Materials and Methods*.
*Ovarian epithelium.
†Leukocytes.

recorded as the TRF length. When the average of several mean TRF lengths is calculated this value is called the average TRF length.

RESULTS AND DISCUSSION

To test the hypothesis that telomerase was present in tumor cells which had undergone many cell divisions, ascitic fluid from patients with end-stage (metastatic) epithelial ovarian carcinoma was obtained by diagnostic laparotomy or therapeutic paracentesis. Assays for telomerase were performed with extracts from ascites cells, ovarian carcinoma cell lines, and control cells. A telomerase-positive extract elongates single-stranded telomere primers by repetitive addition of nucleotides, yielding products which resolve in a sequencing gel as a 6-nucleotide repeat ladder. The characteristic pausing at each cycle is thought to reflect the translocation step (4, 18–20). Since telomerase uses its integral RNA as template, pretreatment of extracts with RNase abolishes this pattern (4, 18–20). Telomerase activity was present in ascites cells from patients and in established carcinoma lines (Fig. 1, lane 4; Table 1). Extracts from normal epithelial cells from the ovarian surface, however, had no detectable activity (Table 1), suggesting that telomerase may be present exclusively in tumor cells. Extracts from a given patient (OC4, Table 1), at presentation and 8 months thereafter, indicated persistence of telomerase activity *in vivo*. These data provide direct evidence of specific telomerase activity in human tissue.

We estimated that cell samples from ascites were composed of ≈95% tumor cells with the remaining fraction consisting of mesothelial cells, fibroblasts, and leukocytes. To identify the source of telomerase activity, tumor and nonmalignant cells from ascites were separated and assayed. Purified tumor cells were found to be telomerase-positive, whereas the nonmalignant cell fraction and peripheral blood leukocytes from normal donors lacked detectable activity (Fig. 1; Table 1). Lack of activity did not appear to be due to diffusible inhibitor(s), since the activity of 293 CSH extracts was not altered upon mixing with negative extracts (data not

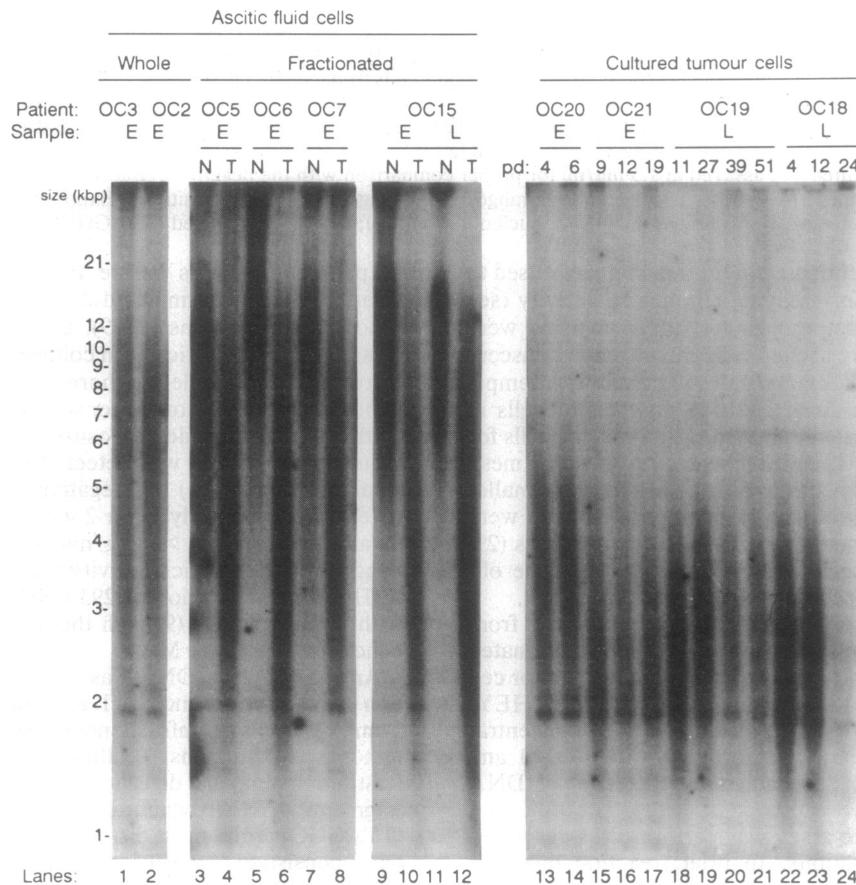

FIG. 2. TRF lengths in cells from ascitic fluid. Southern hybridization with the telomere-specific probe $(CCCTAA)_3$, which detects TRFs, is shown for genomic DNA isolated from unfractionated ascites cells (lanes 1 and 2) and nonmalignant cell (N, lanes 3, 5, and 7) and highly pure tumor cell (T, lanes 4, 6, and 8) fractions. DNA from nonmalignant and tumor cells from the same patient was analyzed at presentation [early (E), lanes 9 and 10] and just prior to death [late (L), lanes 11 and 12]. Tumor cells from both early and late paracentesis were cultured and analyzed at the indicated population doublings (pd) (lanes 13–24).

Table 2. TRF lengths of tumor and nonmalignant cells

Unfractionated ascites*		Fractionated ascites			Cultured tumor cells†	
			TRF length, kbp			
Sample	TRF length, kbp	Sample	Nonmalignant	Tumor	Sample	TRF length, kbp
OC1-1	3.8	OC5-1	7.0	5.0	OC18-2	3.4
OC2-1	5.5	OC6-1	9.2	5.4	OC19-3	3.4
OC3-1	5.4	OC7-1	8.0	5.4	OC20-1	4.2
OC3-2	4.4	OC8-1	7.7	4.3	OC21-1	3.3
OC4-1	4.5	OC9-1		5.2	OC5-1	4.3
		OC10-1		3.9	OC22-13	6.9
		OC11-2		3.7		
		OC12-1		3.8		
		OC13-1		5.1		
		Serial				
		OC14-1 (E)	7.3	4.1		
		OC14-5 (L)		4.7		
		OC15-1 (E)	9.4	5.0		
		OC15-5 (L)	9.3	5.2		
		OC16-1 (E)		3.9		
		OC16-2 (E)		3.4		
		OC16-7 (L)		3.9		
		OC17-1 (E)	7.7	4.3		
		OC17-15 (L)		4.7		
Average‡	4.7 ± 0.7	Average‡	8.2 ± 0.9	4.5 ± 0.6	Average‡	4.2 ± 1.4 (3.7 ± 0.5)§

TRF lengths were determined from densimetric scans of multiple autoradiographs (similar to that in Fig. 2). Average standard deviation was 0.5 kbp, with the largest deviation being 2 kbp. Samples defined as E (early) were obtained near the time of presentation, and samples L (late) near death. Paracenteses were performed 4–15 times over the course of 4–22 months.

*Since TRF length in unfractionated ascites cells is almost identical to that of the tumor fraction (4.7 versus 4.5 kbp) and since we assume that both the nonmalignant (TRF of 8.2 kbp) and tumor fractions contribute to the composite TRF distribution in proportion to their relative abundance, the fraction of tumor cells (x) can be determined in the equation $8.2(1 - x) + 4.5x = 4.7$. This yields $x = 0.95$, indicating that 95% of the ascites cells are of tumor origin, a value similar to those reported by others (32).

†TRF length was determined for each sample over at least 30 population doublings. Values were averaged since TRFs were stable in all populations.

‡Average ± SD of the mean TRF lengths of all samples.

§Average value excluding OC22-13.

shown). In 293 CSH cells, telomerase activity was approximately proportional to total protein concentration (Fig. 1, lanes 9–26) and was detectable at 0.1 mg/ml (lane 29). Thus, lack of activity in extracts from nonmalignant cells, which were assayed at 1–3 mg/ml, was most likely unrelated to low protein concentration. In support of this, extracts from the ovarian tumor cell line SKOV-3, with half the protein concentration of nonmalignant cell extracts, were positive (Table 1). The extent of primer elongation and frequency of initiations, as judged by the intensity of bands, was less in reactions with extracts from unfractionated ascites or purified tumor cells than that with 293 CSH cells (Fig. 1, compare lanes 2 and 4 for extracts assayed at the same protein concentration). This was also the case when the cell extracts were assayed for DNA polymerase (data not shown). Whether these results reflect an inherent property of tumor cells, heterogeneity of the tumor cell population, or some feature of the isolation procedures is not known.

We have previously shown that telomerase activity is associated with maintenance of telomere length in cultured human cell lines (9). To determine whether this was the case for fresh tumor cells, lengths of TRFs were measured by Southern analysis of DNA from unfractionated ascites cells, using a $(CCCTAA)_3$ telomeric probe. Consistent with results from studies of other tumors (7, 21–26), the average TRF in these cells was short (4.7 ± 0.7 kbp; Table 2). The TRF size distribution, however, was quite heterogeneous, ranging from 2 to >12 kbp (Fig. 2, lanes 1 and 2). To assess whether differences in telomere length existed among cell types, ascites cells were separated into nonmalignant (fibroblast and mesothelial cells) and tumor cell fractions. Southern analysis of DNA demonstrated that the TRF length of nonmalignant cells was much greater than that of tumor cells from the same ascites (Fig. 2; compare samples N and T; Table 2; 8.2 ± 0.9 kbp versus 4.5 ± 0.6 kbp, $P = 0.001$). Thus, TRF heterogeneity in unfractionated ascites resulted from the presence of at least two cell populations with quite different TRF lengths.

Telomeres were shorter in tumor cells than in normal cells from the tissue of origin. In ovarian epithelial cells obtained from three nondiseased donors, TRFs were ≈12 kbp long (data not shown), somewhat longer than those in nonmalignant ascites cells or in other aged-matched somatic cells (6–8, 10, 11). If rate of telomere loss in tumor cells *in vivo* is similar to that in transformed cells *in vitro* (65 bp per population doubling) (9), the loss of ≈7.5 kbp in tumor cells relative to normal cells represents in excess of 100 doublings. This is most likely an underestimate, since tumor cell populations maintain neither 100% viability nor exponential growth (34).

Telomere length in tumor cells was stable *in vivo*. Over the course of the disease, a dozen or more ascites samples, each containing 10^9–10^{10} tumor cells, may be withdrawn from a given patient. Since TRF lengths in tumor cells from a patient at presentation and just prior to death remained essentially constant, telomere length was maintained *in vivo* over a large number of cell doublings (Fig. 2, lanes 10 and 12; Table 2; 4.3 ± 0.5 kbp for early samples versus 4.6 ± 0.5 kbp for late samples; $P = 0.5$). Although TRF size did not change appreciably during the disease in nonmalignant cells from the

ascitic fluid (Fig. 2, lanes 9 and 11; Table 2), these cells are predicted to undergo relatively few cell divisions, as judged from the rate of telomere loss of normal somatic cells *in vivo* (7, 10, 11). Similarly, when put in culture, the limited lifespan of nonmalignant cells (≈10 doublings) (31) precluded detection of telomere shortening normally associated with somatic cell proliferation (6, 8–13).

When end-stage tumor cells from 151 patients were cultured, ≈25% (41) of the samples readily established and continued proliferating for months with little or no discernible crisis, suggesting that the cells were already immortal *in vivo*. Moreover, the cells had a transformed morphology and they formed foci, and many of the clones tested grew in an anchorage-independent manner in serum-free medium. DNA was collected from six cultures every two to four population doublings, and TRF lengths were determined. Samples OC19-3, OC18-2, OC20-1, OC21-1, and OC5-1 (Fig. 2, lanes 13–24; Table 2) had a short but stable average TRF length of 3.7 ± 0.5 kbp *in vitro*, confirming our *in vivo* observations. Notably, one sample, OC22-13, contained stable telomeres of much greater length (6.9 kbp; Table 2), as occasionally reported for other tumors (7, 21–23, 26).

It is interesting that in almost all of the tumor samples analyzed, telomerase was detected after telomeres had become very short. In our studies with cells transformed *in vitro* we proposed that activation of the enzyme was most likely due to mutational event(s) which could conceivably occur at any time and telomere length (9). Although one tumor (OC22-13) had long, stable telomeres, compatible with early activation of the enzyme, elongation may have occurred *in vivo* after initial shortening, as observed in some immortalized cell lines (9, 26, 36). Whether selective pressure operates on telomerase-positive cells only when telomeres are short or whether telomerase activation occurs late in the transformation process is unknown. It is possible that short telomeres and the concomitant decrease in chromosome stability (9) trigger activation of the enzyme in rare cells. Alternatively, activation may depend upon multiple mutational events which are only achieved after many cell divisions.

We propose that tumor cells, like cells transformed *in vitro* (9, 12), lose telomeric DNA until a critically short telomere length is reached. At this stage, only cells which can maintain functional chromosome ends—for example, through the expression of telomerase—are capable of continued proliferation. Thus, drugs which specifically inhibit telomerase activity could critically limit the lifespan of late-stage tumor cells. Side effects from this therapeutic approach should be limited, since telomerase is believed to be absent or minimally expressed in somatic tissues (9, 13).

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