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Cell cycle-dependent modulation of telomerase activity in tumor cells

(cancer/telomeres/G2M/ribonucleoprotein)

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ABSTRACT **Telomerase is a ribonucleoprotein complex that is thought to add telomeric repeats onto the ends of chromosomes during the replicative phase of the cell cycle. We tested this hypothesis by arresting human tumor cell lines at different stages of the cell cycle. Induction of quiescence by serum deprivation did not affect telomerase activity. Cells arrested at the G_1/S phase of the cell cycle showed similar levels of telomerase to asynchronous cultures; progression through the S phase was associated with increased telomerase activity. The highest level of telomerase activity was detected in S-phase cells. In contrast, cells arrested at G_2/M phase of the cell cycle were almost devoid of telomerase activity. Diverse cell cycle blockers, including transforming growth factor β1 and cytotoxic agents, also caused inhibition of telomerase activity. These results establish a direct link between telomerase activity and progression through the cell cycle.**

Telomeres form the ends of eukaryotic chromosomes consisting of an array of tandem repeats of the hexanucleotide 5′-TTAGGG-3′. They are believed to protect the ends of chromosomes against exonucleases and ligases, to prevent the activation of DNA-damage checkpoints, and to counter the loss of terminal DNA segments that occurs when linear DNA is replicated (for reviews, see refs. 1–5). Telomerase, a ribonucleoprotein enzyme, utilizes its own RNA as a template to add the hexanucleotide to the ends of replicating chromosomes (2). Telomerase activity has been detected in the vast majority of human tumors, but only in a few normal somatic cells (6–13). Differentiation of tumor cells is associated with a pronounced downregulation of telomerase activity (13).

Neoplastic transformation is a concomitant of the accumulation of multiple genetic changes that contribute to loss of fidelity in the processes involved in replication, repair, and segregation of the genome. In normal cells, fidelity is maintained by the coordinated activity of cyclin-dependent kinases, cell-cycle checkpoint controls, and the repair pathway (for a review, see ref. 14). The cell cycle involves coordination of a variety of macromolecular syntheses, assemblies, and movements. The progression of cells through the cell cycle is tightly regulated by positive and negative signals (15). Progressive loss of telomeres in normal cells is believed to activate sensing mechanism(s) responsible for cellular senescence (1).

If telomerase adds TTAGGG repeats to replicating chromosomes, it must be expressed at the S phase of the cell cycle. We developed a simple nonradioactive method to quantify the telomerase reaction products. By using this method, we examined the telomerase activity in human tumor cell lines by synchronizing cells at different stages of the cell cycle and demonstrate that the telomerase activity is regulated in a cell cycle-dependent manner. Withdrawal from the cell cycle did not inhibit the telomerase activity. In contrast, as the cell progresses through the cell cycle, maximum telomerase activity was detected in the S phase, with barely-detectable levels observed at the G_2/M phase.

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MATERIALS AND METHODS

Cell Lines. The human cell lines used in the study, SW480 colon carcinoma cells, MDA435 breast carcinoma cells, DU145 prostate carcinoma cells, MCF-7 breast carcinoma cells, HOS-MNNG osteosarcoma cells, 293 immortal kidney cells, and WI38 dermal fibroblasts, were obtained from the American Type Culture Collection and maintained as recommended by the American Type Culture Collection. Human smooth muscle cells (early passage) were obtained from Clonetics (San Diego).

Chemicals and Growth Factors. 5-Fluorouracil, methotrexate, doxorubicin, nocodazole, thymidine, and aphidicolin were from Sigma. Human transforming growth factor β1 (TGF-β1) was from R & D Systems. PicoGreen was obtained from Molecular Probes.

Telomerase Assay. Cells were treated with various agents for the indicated length of time, and total cellular extracts were made according to the protocol of Kim *et al.* (6) by using the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Freeze-thaw lysates in buffer B (see ref. 6) minus CHAPS were made by dry-ice-ethanol and 37°C incubation for 3 cycles of 5 min each, followed by centrifugation at 12,000 × *g* for 15 min, and the supernatant was used to measure telomerase activity. The PCR-based telomeric repeat amplification protocol (TRAP) was used to determine telomerase activity as described (13). A serial dilution (10-fold) of the protein extracts from each cell line was made, and an appropriate range of protein concentrations was selected that produced a linear response.

Telomerase reaction products were quantitated by using a double-stranded DNA-specific fluorescent dye, PicoGreen, as recommended by the manufacturer. Following the TRAP assay without ^{32}P-dNTP in triplicate, 45-μl aliquots of TRAP reaction products were transferred to 96-well plates and an equal volume of diluted PicoGreen (1:200) was added. The fluorescence was measured by using the Cytofluor Fluorescence Measurement System 2300 (Millipore). The excitation wavelength was 485/20 nm and the emission intensity was measured at 530/25 nm. The background fluorescence (from extract-free TRAP assay) was subtracted from the sample values. Routine controls for both qualitative and quantitative

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FACS, fluorescence-activated cell sorter; TRAP, telomeric repeat amplification protocol; TGF-β1, transforming growth factor β1.

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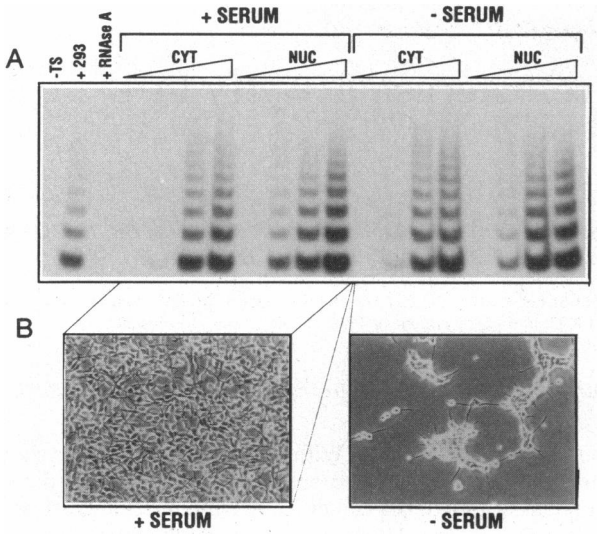

FIG. 1. Exiting from cell cycle does not inhibit telomerase activity. (*A*) 293 cells were plated in growth medium in duplicate dishes, and 24 h later one set of dishes was washed free of medium and incubated in basal medium minus serum for 72 h. Nuclear (Nuc) and cytoplasmic (Cyt) extracts were made from these cells, and increasing amounts of extracts (0.01, 0.1, 1.0, and 3.0 μg of protein) were analyzed for telomerase activity by TRAP assay. −TS, control for the TRAP assay without the nontelomeric primer; +293, 1 μg of CHAPS extract from 293 cells as positive control for the TRAP assay; +RNase A, 293 cells CHAPS extract pretreated with RNase A (200 μg/ml) to inactivate the RNA component of telomerase. (*B*) Photomicrograph of 293 cells cultured in growth medium (+serum) and in basal medium minus serum (−serum). (×24.6)

TRAP included (*i*) omitting nontelomeric primer, TS (6) and (*ii*) pretreatment of the protein extract with RNase A (200 μg/ml). Each experiment was repeated two to four independent times with cells at various passages.

Cell Cycle Analysis. Cell synchronization was achieved by different agents. Quiescence was induced by serum deprivation for 72 h as confirmed by thymidine incorporation. Cells were synchronized at the G_1/S phase by sequential treatment with 5 mM thymidine for 20 h and 5 μg/ml aphidicolin for 16 h (16). S-phase synchronization was achieved by treatment with hydroxyurea (0.3 mM) for 32 h. G_2/M-phase synchronization was obtained by treatment with 0.4 μg/ml nocodazole for 16–20 h, and nonadherent cells were analyzed (mitotic shakeup). The percentage of the cells in different cell cycle phases was determined by fluorescence-activated cell sorter (FACS) analysis of the DNA content as described (16, 17).

RESULTS

Withdrawal from Cell Cycle Does Not Affect Telomerase Activity. 293 cells cultured in the presence or absence of serum had similar telomerase activity in both the cytoplasm and the nucleus (Fig. 1*A*), despite significant growth inhibition when serum was withdrawn (Fig. 1*B*). The level of telomerase activity in the serum-deprived culture did not change upon restimulation with serum or cytokines such as interleukin 1α, tumor necrosis factor α, and epidermal growth factor (not shown). These agents, however, did induce transcription factors including nuclear factor-κB and Sp1, demonstrating that the serum-deprived cultures were capable of reentering the cell cycle. Identical results were obtained with DU145 prostate carcinoma, MDA435 breast carcinoma, and HOS-MNNG

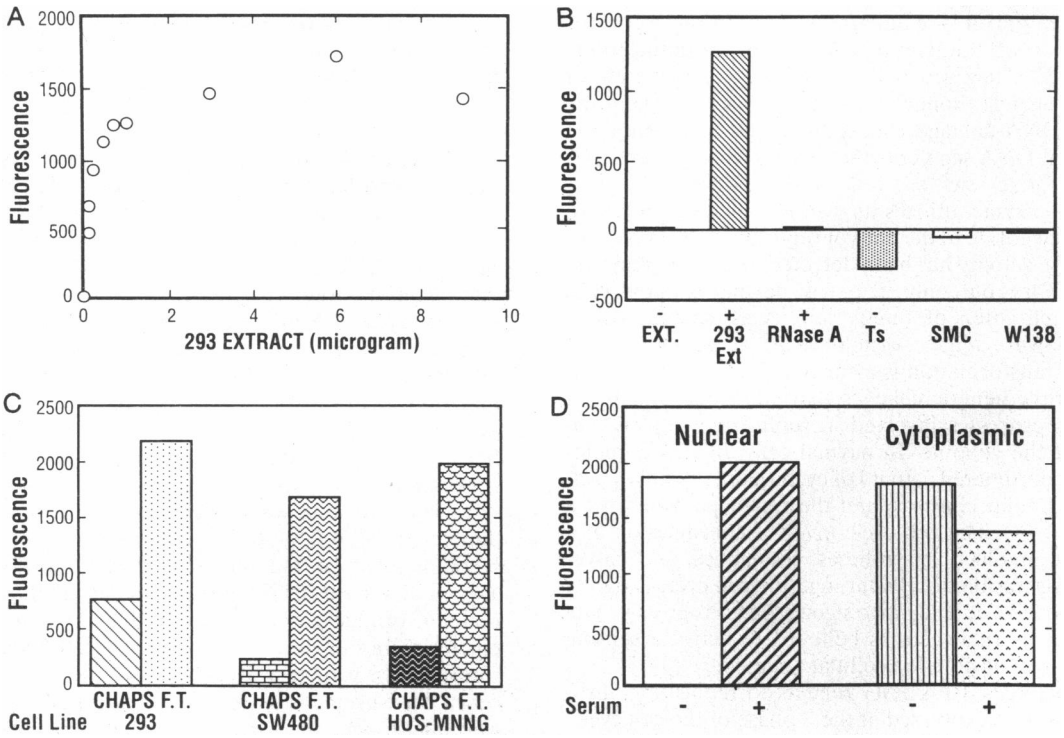

FIG. 2. Quantitation of telomerase reaction products. (*A*) Linearity of TRAP quantitation by PicoGreen assay. CHAPS extract from 293 cells (0–9 μg of protein) were used in the TRAP assay, and the amplification products were quantified by PicoGreen. (*B*) Specificity of PicoGreen quantitation assay. CHAPS extracts (1 μg) from 293 cells (telomerase positive), smooth muscle cells, and WI38 fibroblasts (both telomerase negative) were analyzed by TRAP assay for telomerase activity, and the products were analyzed by PicoGreen assay. −Ext, minus extract control; +293 Ext, positive control; +RNase A, extract pretreated with RNase A; −TS, minus nontelomeric primer TS; SMC, WI38, extracts from smooth muscle cells (early passage) and WI38 fibroblasts. (*C*) A simple method to isolate telomerase activity. Extracts from 293 cells, SW480 cells, and HOS-MNNG cells were made following the CHAPS protocol (6) or by freeze-thaw cycle (F.T., as indicated in *Materials and Methods*) and equal amounts of protein (0.5 μg) were analyzed simultaneously by TRAP-PicoGreen assay for telomerase activity. (*D*) Quantitation of telomerase activity in nuclear and cytoplasmic extracts of 293 cells. Extracts from Fig. 1 were analyzed by TRAP-PicoGreen assay.

osteosarcoma tumor cells (not shown). These results indicate that quiescent tumor cells do retain telomerase activity.

Quantitation of Telomerase Activity. The currently used TRAP assay to measure telomerase activity is essentially a qualitative assay, and only extreme differences in the telomerase levels are interpretable. Hence, we next developed a simple method to quantify the telomerase reaction products by using a double-stranded DNA-specific fluorescent dye, PicoGreen (Fig. 2). The intensity of fluorescence was linear with reference to the protein concentration (0–1 μg), reaching saturation at concentrations greater than 1 mg (Fig. 2*A*). The specificity of this assay is shown in Fig. 2*B*; the fluorescent intensity was negligible in samples (*i*) without the protein extract, (*ii*) with RNase A pretreatment, (*iii*) without nontelomeric primer TS, and (*iv*) telomerase-negative cells (including early passage human smooth muscle cells and WI38 human fibroblasts). The fluorescent intensity of the reaction products was linear with reference to the amount of the products and was abolished by DNase treatment of the TRAP reaction products (not shown). Comparison of CHAPS extract with a simple freeze-thaw extract (see *Materials and Methods*) by quantitation showed 2- to 8-fold increase in the telomerase activity in diverse tumor cell lines (Fig. 2*C*).

Application of this assay for quantitative measurement of the telomerase activity in extracts of serum-deprived 293 cells (Fig. 2*D*) showed similar levels in the nucleus of the serum-deprived cultures; in proliferating cells (plus serum), slightly elevated nuclear telomerase activity was detected with a corresponding decrease in the cytoplasmic telomerase activity. These results confirmed that when analyzed in tandem, the qualitative and quantitative TRAP assays are complementary. Hence, we used this method to quantify the telomerase activity in subsequent experiments.

Telomerase Activity Is Cell-Cycle Regulated. Although withdrawal from the cell cycle by serum deprivation did not appreciably alter the relative telomerase levels in tumor cells, telomerase activity may fluctuate at distinct stages of the cell cycle. SW480 colon carcinoma cells were arrested at the G_1/S, S, or G_2/M phase by thymidine/aphidicolin, hydroxyurea, or nocodazole treatment, respectively, as indicated in *Materials and Methods*. After cells were released from the block by washing them free of the agents (0 time) and replating for 12 and 24 h, telomerase activity was measured (Fig. 3). Cells arrested at the G_1/S phase had similar telomerase activity to the asynchronous cells. Upon reentry into the cell cycle, these cells showed a gradual increase in telomerase activity (Fig. 3 *A* and *B*). The FACS profile of the SW480 cells arrested at different stages of the cell cycle followed by 24-h release is shown in Fig. 3*C*. Cells arrested at the S phase had the highest level of telomerase activity; upon progression through the cell cycle, they showed a gradual decrease in telomerase activity.

Strikingly, cells arrested at the G_2/M phase showed almost no telomerase activity. The inhibition was maintained for 12 h following release from the G_2/M block. At 24 h, telomerase levels were similar to those of cells arrested at the S phase. Nocodazole (0.4 mg/ml) did not directly inhibit the telomerase activity *in vitro*, suggesting that the decline of telomerase activity in the G_2/M blocked cells must be cell mediated. An almost complete lack of telomerase activity in G_2/M-synchronized cells was seen in two other tumor cell lines, MCF-7 and MDA435 (breast carcinoma cells; not shown). Mixing of extracts from G_2/M-arrested SW480 cells with extracts from asynchronous cells did not affect telomerase activity. This suggests that no inhibitor was present in the extracts of cells synchronized at G_2/M.

Effect of TGF-β1 and Cytotoxics on Telomerase Activity. TGF-β1, a growth inhibitory polypeptide, arrests the cell cycle in susceptible cells in middle to late G_1 phase (18). We tested whether the growth arrest mediated by TGF-β1 is reflected in telomerase inhibition. MDA435 breast carcinoma cells were treated with TGF-β1 for 1 week and telomerase activity was measured (Fig. 4 *A* and *B*). Growth inhibition by TGF-β1 in these cells was accompanied by a marked reduction in telomerase activity. We next tested the effects of cytotoxic agents including methotrexate and 5-fluorouracil, which block cells at the S phase (19), and doxorubicin, which blocks cells predominantly at the G_2/M phase (20). SW480 colon carcinoma cells were treated for 72 h with these agents, and telomerase activity was measured (see Fig. 4 *C* and *D*). Treatment with doxorubicin abolished the telomerase activity, similar to the nocodazole treatment in the nonadherent cells. Treatment with methotrexate and 5-fluorouracil caused a marked reduction of telomerase activity despite lack of toxicity at 72 h. None of these agents directly inhibited the telomerase activity *in vitro*, suggesting that the inhibition must be cell mediated.

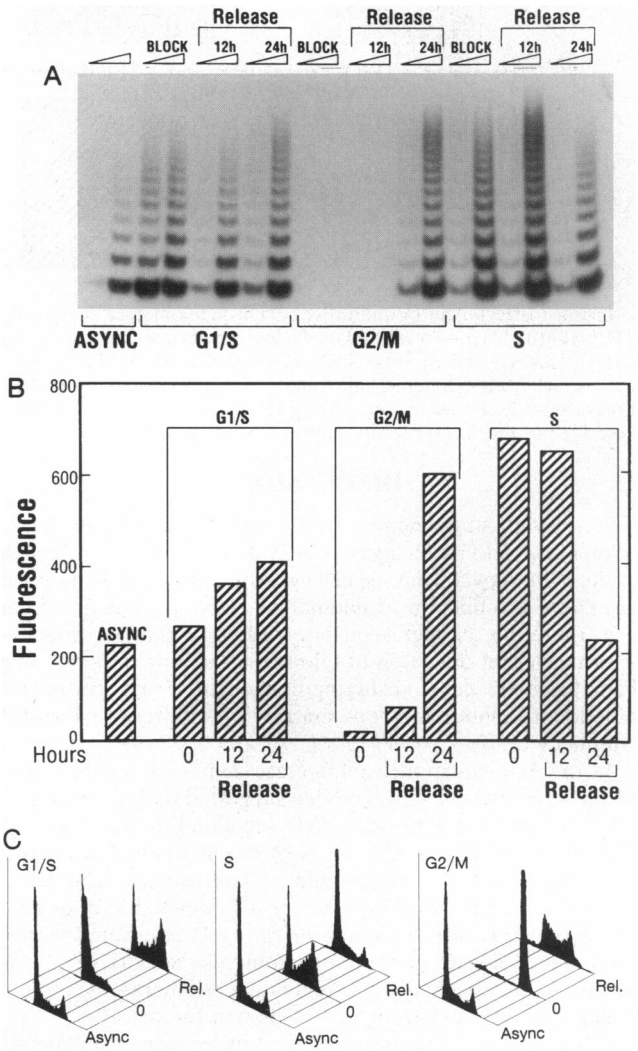

FIG. 3. Cell-cycle regulation of telomerase activity. Freeze-thaw extracts of SW-480 colon carcinoma cells asynchronized (Async) or blocked at G_1/S (thymidine/aphidicolin), G_2/M (nocodazole), or S phase (hydroxyurea) followed by release for 12 and 24 h as described, were analyzed for telomerase activity by TRAP assay (*A*) and TRAP-PicoGreen quantitation (*B*). In *A*, 0.1 and 1.0 μg of protein was used. In *B*, 0.2 μg of protein was used. (*C*) FACS profile of asynchronized (Async), synchronized (0 time), and 24-h released (Rel) cultures are shown. Cell-cycle distribution at 0 time: G_1/S block, 34% G_0/G_1, 64% S, and 2% G_2/M; S block, 27% G_0/G_1, 47% S, and 23% G_2/M; and G_2/M block, 1% G_0/G_1, 4% S, and 90% G_2/M. In comparison, the distribution of asynchronized cultures was 63% G_0/G_1, 28% S, and 10% G_2/M.

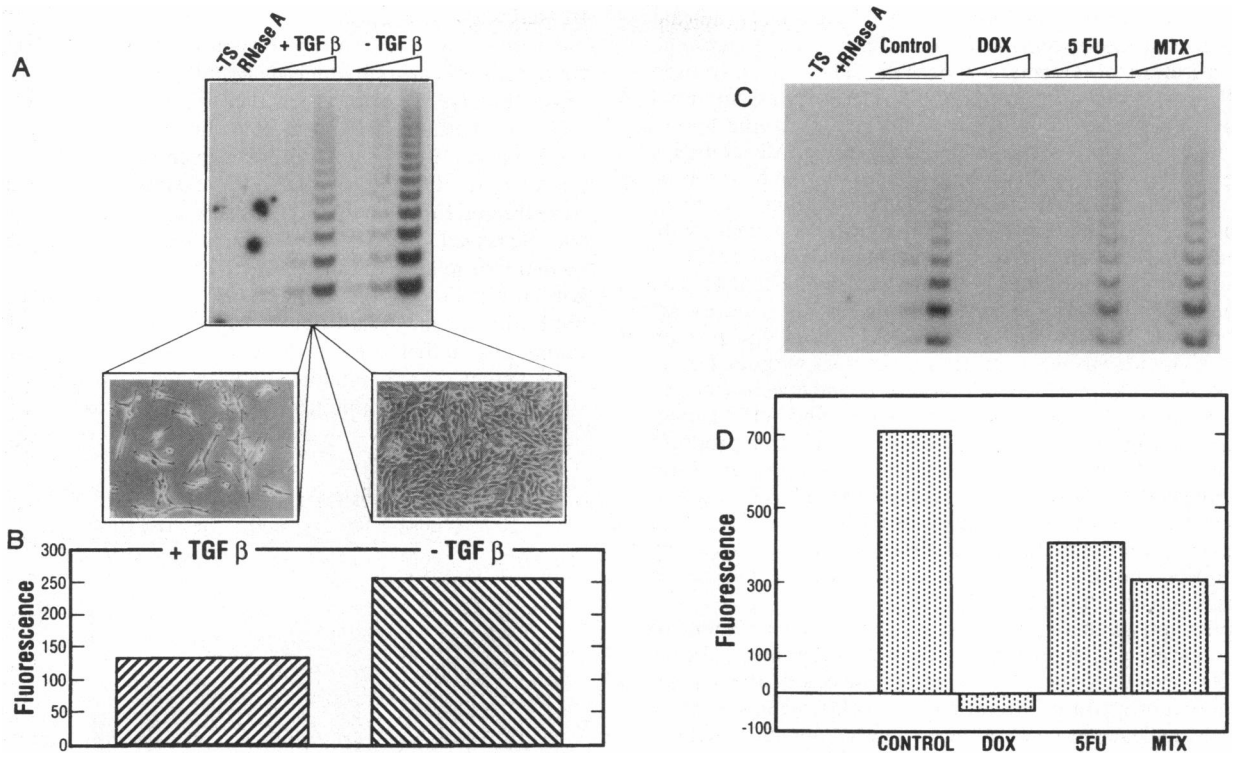

FIG. 4. Effect of antiproliferative agents on telomerase activity in tumor cells. (*A*) MDA-435 breast carcinoma cells were treated with or without TGF-β1 (10 pM) for 1 week and the freeze-thaw lysates were analyzed for telomerase activity. Photomicrograph of the cells prior to analysis. (×30.). (*B*) Telomerase activity from *A* was quantified by TRAP-PicoGreen assay using 0.5 μg of protein. (*C*) SW480 colon carcinoma cells were treated with vehicle (0.1% dimethyl sulfoxide, control), 10 μM doxorubicin (DOX), 10 μM 5-fluorouracil (FU), or 10 μM methotrexate (MTX) for 72 h. Freeze-thaw lysates were analyzed for telomerase activity by TRAP assay using 0.05, 0.2, and 1.0 μg of protein. −TS and +RNase A lanes are as described in Fig. 1. (*D*) Samples from *C* were quantified by TRAP-PicoGreen assay for telomerase activity using 0.5 μg of protein.

DISCUSSION

The present study demonstrates that in tumor cell lines, telomerase activity changes as cells progress through the cell cycle. Withdrawal from the cell cycle by serum deprivation did not cause a reduction of telomerase activity. Thus quiescent cells do retain telomerase activity, a finding that is consistent with the recent detection of telomerase activity in noncycling hematopoietic cells, including leukocytes from peripheral blood, cord blood, and bone marrow (9) and resting T and B lymphocytes (7).

In the *Xenopus* model, telomerase activity was detected in M-phase extracts (21); it has been suggested that in this model either telomerase is not cell-cycle regulated or, alternatively, that the early embryonic stages of development have active telomerase that is undetectable at later stages. The latter possibility is supported by our recent findings that in diverse immortal cells, differentiation *in vitro* was associated with a pronounced downregulation of telomerase activity (13). It is likely that the mechanism(s) regulating telomerase activity in *Xenopus* is different from that of human tumor cells.

If telomerase is required for the maintenance of telomeres, it must be active during the DNA replication stage (S phase) but not necessarily in the nonreplicative phase of the cell cycle—for example, the G_2/M phase. The present results are consistent with this prediction (see Fig. 5). As cells progress through the G_1/S phase of the cell cycle, telomerase activity gradually increases. The highest level of telomerase activity is present at the replicative S phase, with a virtual loss of activity at the G_2/M phase. However, withdrawal from the cell cycle did not cause a reduction of telomerase activity, and reentry of quiescent cells into the cell cycle mediated by cytokines and growth factors did not cause any further increase in telomerase activity. These latter results suggest that immediate early genes that are induced by these stimuli are unlikely to play a regulatory role in modulating telomerase activity.

The lack of telomerase activity in tumor cells synchronized at the G_2/M phase is striking. Two different G_2/M blockers (nocodazole and doxorubicin) caused a complete abolition of telomerase activity. Although mixing experiments with G_2/M extracts did not reveal the presence of an inhibitory molecule(s), it is tempting to speculate that one or several proteins that are expressed and/or activated during the G_2/M phase may inhibit telomerase activity. Telomerase activity was inhibited by TGF-β1, a G_1/S blocker (18), in comparison to the thymidine/aphidicolin-mediated G_1/S block, which showed no effect. Similarly, methotrexate- and 5-fluorouracil-mediated S-phase block (22) caused inhibition of telomerase activity,

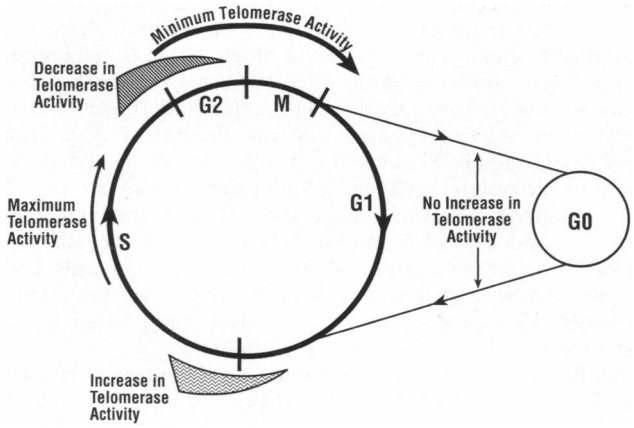

FIG. 5. Proposed model for cell-cycle regulation of telomerase activity.

whereas a hydroxyurea-mediated S-phase block did not. These results suggest that inhibition of telomerase activity by these agents is complex and may be indirect.

We have recently demonstrated that induction of differentiation in HL-60 cells by diverse agents results in a pronounced downregulation of telomerase activity (13). These inducers arrested the HL-60 cells at the G_1 phase of the cell cycle. Interestingly, when asynchronous HL-60 cells were enriched for G_1, S, and G_2/M phase cells by centrifugal elutriation (23) and analyzed for telomerase activity, no differences were seen (data not shown). This suggests that measurement of telomerase activity at distinct stages of the cell cycle may be affected by the mode of enrichment—i.e., native cells versus the use of chemical blockers. Alternatively, telomerase activity in leukemic cells is not cell cycle-dependent. This latter possibility is supported by the recent detection of telomerase activity in diverse normal hematopoietic cells (7, 9). Clarification of this awaits the molecular cloning of telomerase subunits.

Several reports have documented a functional relationship between telomerase and the cell cycle. In the ciliate *Tetrahymena*, alteration of a single nucleotide in the T4G2 telomeric repeat sequence disrupts cell division and causes cellular senescence (24). In *Saccharomyces cerevisiae*, telomeric DNA is replicated in the late S phase (25), and loss of a single telomere results in cell cycle arrest at G_2 (26). It has been postulated that telomere replication may serve as a checkpoint for completion of the S phase (3). In the macronucleus of the ciliate *Oxytricha nova*, the telomeres are bound to a heterodimeric telomere protein that is phosphorylated by cyclin-dependent kinases (27). In *Xenopus* extracts, one of the subunits is phosphorylated during the M phase and dephosphorylated at interphase (27). Telomeres are likely to play a role in nuclear architecture by localizing chromosomes to the nuclear envelope (28, 29) or to the nuclear matrix (30). Progression through the M phase involves phosphorylation of a set of proteins including histone H1, nuclear lamins, vimentin, and caldesmon. The Cdc2/cyclin A and Cdc2/cyclin B kinases are required for most of these events (for a review, see ref. 15). Thus, our results of cell cycle-dependent regulation of telomerase activity are consistent with the hypothesis that telomeric DNA synthesis, telomere structure, and telomerase function are subject to cell-cycle regulation.

Telomerase inhibitors may merit a place in cancer therapeutics (for reviews, see refs. 4, 5, and 31). Understanding the regulation of telomerase activity in tumor cells is pivotal to validating its therapeutic relevance in cancer. To date, no regulators of telomerase activity have been described, with the exception of downregulation of telomerase activity observed in differentiating tumor cells *in vitro* (13). Our present findings of cell cycle-dependent regulation of telomerase activity, and particularly the virtual abolition of telomerase activity in G_2/M-arrested tumor cells, provide a valuable clue to the regulation of telomerase in tumor cells.

Note Added in Proof. At press time, a concurrent report (32) showed telomerase activity to be constant at all stages of the cell cycle in elutriated cells, consistent with our HL-60 results. Holt *et al.*'s studies were done using a different inducer of quiescence and different means of cell-cycle arrest (elutriation), which suggests that analysis of telomerase activity depends upon the nature of cell-cycle perturbation.

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