Auxin Induction of Cell Cycle Regulated Activity of Tobacco Telomerase*

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Telomerase activity was measured at each phase of the cell cycle in synchronized tobacco (Nicotiana tabacum) BY-2 cells in suspension culture with the use of the telomeric repeat amplification protocol assay. The activity was low or undetectable at most phases of the cell cycle but showed a marked increase at early S phase. The induction of telomerase activity was not affected by the S phase blockers aphidicolin (which inhibits DNA polymerase α) or hydroxyurea (which inhibits ribonucleotide reductase), but it was prevented by olomoucine, an inhibitor of Cdc2/Cdk2 kinases that blocks G1-S cell cycle transition. These results suggest that the induction of telomerase activity is not directly coupled to DNA replication by conventional DNA polymerases, but rather is triggered by the entry of cells into S phase. Various analogs of the plant hormone auxin, including indole-3-acetic acid, α-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid, potentiated the increase in telomerase activity at early S phase; the growth-inactive analog 2,3-dichlorophenoxyacetic acid, however, had no such effect. Potentiation by indole-3-acetic acid of the induction of telomerase activity was dose dependent. Together, these data indicate that telomerase activity in tobacco cells is regulated in a cell cycle-dependent manner, and that the increase in activity at S phase is specifically inducible by auxin.

Telomeres are nucleoprotein complexes present at the ends of linear eukaryotic chromosomes that preserve chromosomal integrity and are essential for genomic stability and function. The telomeric DNA in most eukaryotes, including fungi, ciliates, plants, and mammals, consists of tandem arrays of short G-rich repeats (typically, six to eight nucleotides) that are markedly conserved throughout evolution (1). Although telomeres in normal vertebrate somatic cells shorten progressively at a rate of 50 to 200 base pairs per cell division, because of the inability of conventional DNA polymerases to replicate 3’-terminal sequences, telomere length is maintained in unicellular eukaryotes, germ line cells of multicellular organisms, and many human cancer cells (2). The primary mechanism for generating and maintaining chromosome ends relies on the action of telomerase, a ribonucleoprotein complex that contains a specialized reverse transcriptase subunit and which catalyzes the addition of telomeric repetitive sequence to the chromosome ends with a segment of its integral RNA component serving as a template (3, 4).

Evidence indicates that the expression of telomerase activity is closely related to processes of development and differentiation. Telomerase activity in immortal cells has been shown to be down-regulated in response to the induction of phenotypic differentiation (5–7). Recent molecular analysis of a human telomerase reverse transcriptase showed that its expression is down-regulated in response to an inducer of differentiation (8). The expression of telomerase in normal human T lymphocytes is also both developmentally regulated and induced by cell activation (9). Moreover, human telomerase activity during embryogenesis is regulated by transcription of the human telomerase reverse transcriptase gene and alternative splicing of its transcript (10). Both telomere replication and telomerase activity are also regulated according to the cell cycle (11, 12). Telomerase activity increases in association with both cytokine-induced proliferation and cell cycle activation induced by antigen receptor stimulation in lymphocytes (13–16). Furthermore, telomerase induction has been linked to signal transduction by protein kinase C (PKC)† during T cell activation (17).

In contrast to telomerases in yeast, ciliates, and mammals, which have been extensively studied, limited information is available on plant telomerases; in particular, the components of the plant enzyme remain uncharacterized at the molecular level. Chromosome ends in most plant species comprise tandem repeats of the heptanucleotide 5’-TTTAGG-3’ (18, 19). Telomerase activity that exhibits conserved characteristics of the enzyme has been detected in various plant species and shown to be abundant in meristematic tissues, developing tissues such as embryos and anthers, and undifferentiated cells in suspension culture; activity was low or undetectable in differentiated tissues of mature plants (20–22). Recently, more detailed analyses of telomerase activity and telomere length in various plant tissues have shown them to be precisely regulated during ontogeny (23, 24). These observations suggest that the expression of telomerase might be linked to cellular proliferation capacity; however, little is known of the expression of plant telomerase during the cell cycle or its regulation.

Suspension cultures of tobacco BY-2 cells have proved valuable for various studies on the plant cell division cycle because they can be highly synchronized (25, 26). BY-2 cells have also been shown to possess a high level of telomerase activity (21). We have now shown that expression of telomerase activity in synchronized BY-2 cells is largely restricted to early S phase, indicating cell cycle-dependent regulation, and that expression...
is triggered on entry into S phase from G1. Tobacco telomerase activity was also shown to be inducible by the phytohormone auxin, which promotes cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Plant Cells and Culture Conditions—**Suspensions cultures of tobacco BY-2 (Nicotiana tabacum L. cv. bright yellow-2) cells were maintained on a rotary shaker (120 rpm) at 27 °C in 100 ml of Murashige-Skoog salt medium (27) supplemented with 0.5 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-DCA) (Wako, Osaka, Japan). For auxin treatment of BY-2 cells, 2,4-DCA, indole-3-acetic acid (IAA) (Sigma), α-naphthaleneacetic acid (Wako), and the DNA was cross-linked to the membrane by exposure to ultraviolet light. The incorporation of BrdUrd (Sigma) and alkaline phosphatase-conjugated antibodies to linked immunosorbent assay based on a mouse monoclonal antibody to BrdUrd into newly synthesized DNA was quantified with an enzyme-

**Synchronization of BY-2 Cells—**Cells cultured for 7 days, most of which had not proliferated, were subjected to synchronization. A portion (10 ml) of the suspension culture was transferred to 90 ml of fresh Murashige-Skoog medium supplemented with 0.5 μg/ml 2,4-DCA and 0.4 μg/ml aphidicolin (Sigma). The cells were incubated for 24 h, washed three times with 200 ml of ice-cold 3% (w/v) sucrose, incubated for 4 h in 100 ml of fresh Murashige-Skoog medium, and, after the addition of 70 μl of 6 μm propyzamide (Sigma) (final concentration), returned for another 4 h. The cells were washed with sucrose solution and then resuspended in 100 ml of fresh Murashige-Skoog medium for analyses. A portion (1 ml) of the culture was sampled every 2 h and the total cell number counted. For determination of the cell division index (the number of mitotic cells as a fraction of the total number of cells), cell nuclei were stained with 4',6-diamidino-2'-phenylindole dihydrochloride (Sigma) and the number of dividing cells was counted under a fluorescence microscope.

**Cell Cycle Arrest and Monitoring of DNA Synthesis—**The cell cycle blockers aphidicolin, propyzamide, and olomoucine (Sigma) were dissolved in dimethyl sulfoxide (Sigma) and the DNA was cross-linked to the membrane by exposure to ultraviolet light. The incorporation of BrdUrd (Sigma) and alkaline phosphatase-conjugated antibodies to linked immunosorbent assay based on a mouse monoclonal antibody to BrdUrd (Sigma) was added to a final concentration of 30 μM to synchronized cell cultures immediately after their release from M phase arrest by propyzamide. During cell cycle progression, 1 ml of culture was sampled every 2 h. The cells in each sample were fixed with 70% (v/v) ethanol and then immediately after their release from M phase arrest by propyzamide.

**Quantitative Assay of Telomerase Activity in BY-2 Cells—**To quantitate the telomerase activity in BY-2 cells and to standardize comparisons of activity among samples, we performed TRAP assays with various dilutions of cell extracts. Telomerase activity was highest when the assay was performed with 0.5 μg of extract protein (Fig. 1). It decreased approximately in accordance with the dilution factor as the amount of extract protein was reduced below 0.5 μg; however, activity also decreased as the amount of extract protein was increased above 0.5 μg, possibly due to the presence in the cell extract of an inhibitor of telomerase or of the Taq DNA polymerase used in the assay. We therefore used 0.2 μg of extract protein for all subsequent TRAP assays.

**Cell Cycle Dependence of Telomerase Activity—**BY-2 cells were synchronized by consecutive exposure to aphidicolin and propyzamide, after which total cell number, cell division index (Fig. 2A), DNA synthesis (Fig. 2B), and telomerase activity (Fig. 2, C and D) were monitored. The coordinated time courses of total cell number, cell division index, and DNA synthesis after release from propyzamide-induced M phase arrest indicated that the cells were highly synchronized for at least 14 h. The maximal cell division index during this period was approximately 0.7 at 4 h after release from propyzamide block. The telomerase activity of cell extracts was maximal 8 h after release from M phase arrest, a time corresponding to early S phase; activity was low or undetectable at other phases of the cell cycle, with the exception of the intermediate level apparent immediately after removal of propyzamide. These data thus indicated that tobacco telomerase activity is regulated according to the phase of the cell cycle.

**Effects of Cell Cycle Blockers on Telomerase Activity—**To investigate whether the increase in tobacco telomerase activity at early S phase is directly coupled with DNA replication, we examined the effects of S phase blockers on telomerase activity in synchronous BY-2 cells. Despite the complete inhibition of DNA synthesis, cells treated with aphidicolin, a specific inhibitor of DNA polymerase α, showed no increase in telomerase activity similar to that apparent with untreated cells (Fig. 3, A and B). Likewise, treatment of cells with hydroxyurea, an inhibitor of ribonucleotide reductase, did not affect the pattern of telomerase activity (Fig. 3C). These results indicate that the above two inhibitors of S phase progression do not block the increase in telomerase activity. In contrast, exposure of cells to

**RESULTS**

**Preparation of Cell Extract—**Samples (50 μl) of cell cultures (10× to 10× cells/ml) were mixed with 450 μl of an ice-cold solution containing 10 mM Hapes-KOH (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, and 1 mM dithiothreitol, and then centrifuged at 15,000 rpm for 3 min at 4 °C. The cells were resuspended in 50 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA, 0.1 mM 4-(aminoethyl)-benzenesulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS detergent, and 10% glycerol, and were homogenized with a plastic cell scraper in an Eppendorf tube. The resulting cell lysate was incubated on ice for 30 min and then centrifuged at 15,000 rpm for 20 min at 4 °C; the resulting supernatant (cell extract) was either used immediately for assay of telomerase activity or stored at −80 °C. The protein concentration of the cell extract was determined with the Bio-Rad protein assay reagent, with bovine serum albumin as the standard.

**Assay of Telomerase Activity—**Telomerase activity in cell extracts was assayed with the polymerase chain reaction-based telomerase repeat amplification protocol (TRAP) as described previously (28), but with minor modifications. The TS21 primer (5'-GACAATCCTGGTCCAGTAAGTT-3') and the 5'-digoxigenin-labeled antisense telomeric repeat primer DTPR (5'-AACCGCTTAA-3') were used as forward and reverse oligonucleotide primers, respectively; both were obtained from Nippon Gene Research Lab (Miyagi, Japan). For TRAP assays, 2 μl of cell extract were incubated for 20 min at 24 °C with 38 μl of elongation buffer (20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM of each deoxynucleoside triphosphate, T4G32 protein (0.125 mg/ml), bovine serum albumin (0.1 mg/ml)) containing 0.1 μg of TS21 primer. As a control, RNase A (0.5 μg) (Amersham Pharmacia Biotech), which had been rendered free of DNAase activity by heating at 90 °C for 10 min, was added to the elongation reaction mixture in order to confirm the specific telomerase activity that is abolished by degrading RNA component of the enzyme. Elongation was terminated by heating the reaction mixture at 95 °C for 10 min. Ten microliters of polymerase chain reaction mixture (10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1% Triton X-100, 1.5 mM MgCl₂, 50 μM of each deoxynucleoside triphosphate, 0.2 units of Taq DNA polymerase (TOYOBO, Tokyo, Japan)) containing 0.01 μg of DPTPR primer were then added to 10 μl of the elongation mixture at 95 °C, so that the polymerase chain reaction was initiated under “hot-start” conditions. After initial incubation at 95 °C for 3 min, polymerase chain reaction was performed for 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s. The amplification products were separated by nondenaturing polyacrylamide gel electrophoresis on a 10% gel, transferred to a Hybond N+ membrane with the use of a semidry electroblootlapparatus (Biocraft, Tokyo, Japan), and, detected with the use of a chemiluminescent digoxigenin detection system (Roche Molecular Biochemicals, Mannheim, Germany). Ladders of DNA bands on x-ray film were scanned with a Scanning Imageer (Molecular Dynamics, Sunnyvale, CA) and band intensity was quantitated with the use of Imagequant NT software (Molecular Dynamics).

**Results**

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plant hormone auxin induces cell proliferation and is thought to be required both for the G1-S phase transition in the cell cycle. Similar results have been obtained with various organisms and cell lines, including primary human epithelial cells (33), synchronized Chinese hamster ovary cells (34), and certain tumor cell lines (17, 19), as well as for the induction of telomerase activity in synchronized BY-2 cells (35). We also demonstrated that the effect of IAA on telomerase activity at early S phase requires typical auxin activity, we investigated the effects of various synthetic auxins. Like IAA, both 2,4-DCPA and α-naphthaleneacetic acid at a concentration of 1 μM also increased by 2–3-fold the telomerase activity of cells at S phase (Fig. 5A). In contrast, 2,3-DCPA, a growth-inactive analog of 2,4-DCPA, did not increase telomerase activity. We also demonstrated that the effect of IAA on telomerase activity was dose dependent (Fig. 5B); the effect was apparent at IAA concentrations of 1 and 10 μM but not at concentrations of 0.01, 0.1, or 100 μM. This observation is consistent with previous results showing that IAA is biologically active at concentrations between 1 and 10 μM (31, 32). Thus, the induction of telomerase activity at early S phase appears to be a specific biological effect of auxin.

**DISCUSSION**

We have shown that telomerase activity in synchronized BY-2 cells is specifically induced during early S phase of the cell cycle. Similar results have been obtained with various organisms and cell lines, including primary human epithelial cells (33), synchronized Chinese hamster ovary cells (34), and certain tumor cell lines (17, 19), as well as for the induction of telomerase activity. Meanwhile, a cytokinin class phytohormone, 6-benzylaminopurine, which is also known to induce cell division was also tested; however, it showed no significant effect on the telomerase activity (data not shown).

**Specificity of Auxin Induction of Tobacco Telomerase Activity—** Synthetic auxins structurally distinct from IAA, including both biologically active and inactive forms, have been synthesized (30). To examine whether the induction of telomerase activity by IAA at S phase requires typical auxin activity, we investigated the effects of various synthetic auxins. Like IAA, both 2,4-DCPA and α-naphthaleneacetic acid at a concentration of 1 μM also increased by 2–3-fold the telomerase activity of cells at S phase (Fig. 5A). In contrast, 2,3-DCPA, a growth-inactive analog of 2,4-DCPA, did not increase telomerase activity. We also demonstrated that the effect of IAA on telomerase activity was dose dependent (Fig. 5B); the effect was apparent at IAA concentrations of 1 and 10 μM but not at concentrations of 0.01, 0.1, or 100 μM. This observation is consistent with previous results showing that IAA is biologically active at concentrations between 1 and 10 μM (31, 32). Thus, the induction of telomerase activity at early S phase appears to be a specific biological effect of auxin.

**Regulation of Tobacco Telomerase by Auxin and the Cell Cycle**

**Cell cycle-dependent control of telomerase activity in synchronous BY-2 cells.** A, total cell number (circles) and cell division index (squares) were determined at various times after release from propyzamide-induced M phase arrest. Data are mean ± S.D. of triplicate determinations from a single experiment. B, DNA synthesis was monitored in the synchronized cells by measuring the incorporation of BrdUrd. Data are expressed relative to the value for cells incubated for 10 h. C and D, detection (C) and quantitation (D) of telomerase activity in synchronized cells. Data in D are expressed relative to the value for cells incubated for 8 h; the right-most lane represents an RNase control for the cell extract derived from the cells incubated for 8 h.
telomerase RNA transcription in budding yeast (35). In contrast, a naturally synchronized culture of Physarum plasmodia exhibited maximal telomerase activity during late S phase, immediately before the completion of DNA replication (36). Our data also revealed that BY-2 cells exhibited an intermediate level of telomerase activity immediately after release from M phase arrest. This latter observation may result from an increase in telomerase activity induced by washing or subculturing of the cells or it might reflect induction of activity at M phase; telomerase is active in extracts of Xenopus laevis germ-line cells in both S and M phases of the cell cycle (37). Together, these various studies indicate that regulation of telomerase activity during the cell cycle represents a conserved mechanism for maintaining telomere length.

Exposure of synchronous BY-2 cells to aphidicolin, an inhibitor of DNA polymerase γ, did not block the induction of telomerase activity at S phase. Telomerase induction during T cell activation also occurs in the presence of aphidicolin (17), indicating that DNA synthesis is not required for this effect. In the ciliate Euplotes, aphidicolin treatment resulted in a general

![Figure 3](image1.png)

**Fig. 3. Effects of cell cycle blockers on BY-2 telomerase activity.** Synchronous BY-2 cells were cultured in the absence (A) or presence of 15 μM aphidicolin (B), 0.2 mM hydroxyurea (C), or 30 μM olomoucine (D) for the indicated times after release from propyzamide-induced M phase arrest. Incorporation of BrdUrd into newly synthesized DNA (top panels) and telomerase activity (bottom panels) were measured. BrdUrd incorporation is expressed relative to that for cells incubated for 8 h.

![Figure 4](image2.png)

**Fig. 4. Stimulatory effect of IAA on tobacco telomerase activity at S phase.** Seven hours after release from propyzamide block, synchronous BY-2 cells were incubated for the indicated times in the absence (A) or presence (B) of 1 μM IAA or presence of IAA with olomoucine (C) and then assayed for telomerase activity.
lengthening of the G strands by telomerase and a marked increase in C strand heterogeneity (38), suggesting that the synthesis of G strands per se was not affected by aphidicolin. The S phase-specific expression of tobacco telomerase activity also was not blocked by hydroxyurea, an inhibitor of ribonucleotide reductase. Similarly, hydroxyurea did not inhibit telomerase activation or Cdk2 kinase activity in normal human T cells (14). Furthermore, methotrexate and 5-fluorouracil, both of which arrest cells at S phase, did not inhibit telomerase activity in human cancer cells (39).

Conversely, gradual telomere shortening in the absence of telomerase does not immediately affect cell cycle progression (40), suggesting that telomerase activity is not directly coupled to DNA replication. Our data indicate that telomerase activation at S phase is not linked directly to DNA replication by conventional DNA polymerases.

Olomoucine, an inhibitor of Cdc2/Cdk2 kinases that blocks plant cells at the G1-S cell cycle transition, prevented the induction of telomerase activity in synchronous BY-2 cells, suggesting that Cdc2/Cdk2 kinase activity is necessary for both expression of telomerase activity and entry into S phase. Two inhibitors of PKC, bisindolylmaleimide and H-7, were shown to inhibit telomerase activity in human nasopharyngeal cancer cells (39). Moreover, inhibition of PKC with bisindolylmaleimide prevented the increase in telomerase activity associated with activation of human T cells (17), indicating that the induction of telomerase activity is linked to signal transduction by PKC in these cells. PKC contributes to the regulation of Cdc2 activity (41) and is thought to play an important signaling role, similar to that of the mitogen-activated protein kinase cascade, in cell proliferation (42). It is therefore possible that the PKC-Cdk signaling pathway contributes to the cell cycle-dependent regulation of telomerase activity.

The phytohormone auxin augmented the induction of telomerase activity at S phase in synchronous BY-2 cells. Auxin is a key regulator of growth and development throughout the plant life cycle. Exogenous auxin can affect rates of cell division and cell elongation, and can trigger specific differentiation. Recent molecular and genetic studies of the mechanism of auxin action have shown that the hormone exerts a rapid effect on several cellular processes through a specific signal transduction pathway (43). Moreover, studies on the activation of cell division in various tissues, organs, and cell culture systems have demonstrated that Cdk expression and activity are linked to auxin signaling (44). Thus, auxin promotes cell division, which is mediated by activation of Cdc2, a key regulatory component in cell cycle control (45). Our observations that auxin promotes and olomoucine inhibits induction of telomerase activity in
BY-2 cells suggest that telomerase is a downstream target of the auxin signaling pathway that controls cell cycle progression (Fig. 6). The pattern of auxin distribution in plants mirrors that of developing tissues and of undifferentiated cells with a high proliferation capacity. Many responses to endogenous auxin depend on the redistribution or polar transport of the hormone to tissues such as meristem, shoot apex, plumule, and immature seeds. The auxin distribution pattern also closely matches the spatial expression pattern of Cdc2, which is important for meristem activity and cell proliferation during plant development (46, 47). In addition, expression of an Arabidopsis cyclin gene, CYClAt, that is induced by auxin has been shown to be restricted almost exclusively to dividing cells in root and shoot apical meristems (48). Consistent with the localized expression of the Cdc2 gene, telomerase activity is higher in immature embryos and meristematic tissues than in other tissues (49). Moreover, the length of telomeres in barley tissues decreases during differentiation, but it is increased in undifferentiated callus tissue (49).

In summary, we have shown that telomerase activity is induced at early S phase in tobacco cells. This effect was promoted by auxin and appeared to require Cdc2/Cdk2 activity. Thus, regulation of telomerase activity by auxin is likely related to the control of cell proliferation by this hormone.

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