# Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts

(G₁ cyclins/quiescence/cellular senescence/mitogen)

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**ABSTRACT**     The human *CCND1* cyclin D1/*PRAD1* gene was previously identified by a genetic screen for G₁ cyclin function in *Saccharomyces cerevisiae* and also was identified as the putative *BCL1* oncogene. However, its role in human cell proliferation is not known. To determine if expression of human D-type cyclin genes correlates with the state of cell growth, we examined the level of mRNAs for *CCND1* and a related gene, *CCND3*, in normal human diploid fibroblasts (HDF). The levels of both mRNAs decrease upon serum depletion or at high cell densities. Following stimulation of quiescent fibroblasts with serum, the mRNA levels increase gradually to a peak at about 12 hr, prior to the onset of S phase. Induction of cyclin gene expression by serum is reduced concomitantly with the decline in FOS induction in aging HDFs, suggesting a possible relationship to the decrease in the proliferative response to mitogens during cellular senescence. Cycloheximide partially blocks the induction of *CCND1* and *CCND3* gene expression by serum, suggesting that both *de novo* protein synthesis-dependent and -independent pathways contribute to induction. Treatment of HDFs with defined growth factors suggests a correlation between CCND mRNA induction and DNA synthesis. However, induction of these genes is not sufficient for the transition from quiescence through G₁ into S phase.

The proliferation of eukaryotic cells is controlled at specific points in the cell cycle, particularly at the G₁ to S and the G₂ to M transitions. Through a combined biochemical and genetic approach, protein complexes with protein kinase activity have been identified as key components that regulate these transitions. In the G₂ to M transition, the cdc2/CDC28 protein is the catalytic subunit of the kinase, and its binding to mitotic cyclins is a prerequisite for kinase activation (1). Similar control mechanisms seem to be involved in the G₁ to S transition. In budding yeast, the accumulation of the G₁ cyclins CLN1, CLN2, and CLN3 is rate limiting for CDC28 protein kinase activity and passage through a point in G₁ termed START, and deletion of all the yeast *CLN* genes causes a lethal G₁ arrest (2). Mammalian cells have an analogous commitment point for entry into S phase, which has been termed the "restriction point" (3). The high conservation of the cell-cycle-control machinery in eukaryotic cells suggests that, like yeast, mammalian cells possess G₁ cyclins.

In an effort to identify mammalian G₁ cyclins, human cDNA libraries were screened for cDNAs with the ability to rescue a *Saccharomyces cerevisiae* mutant lacking functional *CLN* genes (4–6). One of the isolated clones encoded a cyclin termed cyclin D1 that was distinct in structure from previously described cyclins (4). The gene encoding cyclin D1, *CCND1*, is the human homolog of a mouse *Ccn1* gene called

"CYL1" for cyclin-like (7), which was isolated as a cDNA clone from mouse macrophages stimulated by colony-stimulating factor 1 (CSF-1). The gene encoding human cyclin D1 was also identified as *PRAD1*, a gene rearranged in parathyroid tumors (8). *PRAD1* is closely linked to *BCL1* (9), a locus activated by rearrangement in some B-cell tumors where *PRAD1* is also overexpressed, suggesting that *PRAD1* may be the *BCL1* oncogene. The finding that deregulated expression of this cyclin gene may contribute to tumorigenicity suggests that cyclin D1/*PRAD1* could be involved in the control of cell proliferation.

Subsequently, two additional genes, *CCND2* and *CCND3*, encoding human D-type cyclins D2 and D3 were isolated by low-stringency hybridization and PCR techniques (10, 11). These genes share high sequence similarity with their murine counterparts (called "CYL2" and "CYL3" in refs. 7 and 12). All three human D-type cyclin genes encode small [33–34 kilodalton (kDa)] proteins that share an average of 57% identity over the entire coding region and 78% in the cyclin box. In this study, we asked how the expression of human D-type cyclin genes relates to the state of cell growth. As suggested by the expression of mitotic cyclins, which peak at the G₂ to M transition, if D-type cyclins are indeed G₁ cyclins, their expression might peak in late G₁ and be rate-limiting for the G₁ to S transition. Therefore, we examined the level of D-type cyclin transcripts in quiescent human diploid fibroblasts (HDFs) stimulated with serum. We found that transcription of the *CCND1* and *CCND3* genes is induced by serum with kinetics characteristic of delayed early genes, peaking at 12 hr after stimulation, prior to the onset of S phase. Induction of CCND1 and CCND3 mRNAs correlates with the state of the cell growth but is not sufficient for the transition from quiescence through G₁ into S phase.

## MATERIALS AND METHODS

**Cell Cultures and Reagents.** HeLa, A-431, HepG2, WI-38, and Hs68 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum. H9 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. Growth factors were obtained from Upstate Biotechnology (Lake Placid, NY). Phorbol 12-tetradecanoate (TPA) and cycloheximide (CHX) were obtained from Sigma. Forskolin and isobutylmethylxanthine (IBMX) were from Calbiochem.

**Plasmid Constructions.** pSPD1 was constructed by inserting a *Pst* I–*Pvu* II fragment (−258 to +134) of the *CCND1* genomic clone G6 (10) into pSP65. pBSD3 carries an *Eco*RI–*Pst* I fragment containing the first 182 base pairs (bp) of the CCND3 cDNA clone H34 (10) inserted into pBS. The human

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Abbreviations: HDF, human diploid fibroblast; CSF-1, colony-stimulating factor 1; CHX, cycloheximide; TPA, phorbol 12-tetradecanoate 13-acetate; IBMX, isobutylmethylxanthine; MPD, mean population doublings; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor.

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c-*fos* (*FOS*) antisense plasmid (kindly provided by M. Greenberg) contains the *Bss*HII–*Xmn* I fragment (−100 to +488) of the *FOS* gene (13) inserted into pSP64. To produce template for run-off transcripts, pSPD1 was linearized at the *Hin*dIII site, and pBSD3 and *FOS* plasmids were linearized at the *Eco*RI site of the vector polylinker sequence.

**RNA Isolation and Analysis.** Cytoplasmic RNA was prepared from cells by the Nonidet P-40 method and treated with DNase I (14). Uniformly labeled riboprobes for cyclin D1 and FOS RNAs were prepared with SP6 RNA polymerase, and the riboprobe for cyclin D3 was prepared with T3 RNA polymerase (15). Equal amounts of RNA were dissolved in 30 $\mu$l of 40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA containing $5 \times 10^5$ cpm (Cerenkov) of each probe as indicated, denatured at 85°C for 5 min, and hybridized at 60°C overnight. A digestion mix (0.3 ml) of 10 mM Tris, pH 7.5/0.3 M NaCl/5 mM EDTA/40 $\mu$g of RNase A per ml/2 $\mu$g of RNase T1 per ml was added and incubated at 16°C for 45 min. Digestion was terminated by addition of 20 $\mu$l of 10% SDS and 5 $\mu$l of proteinase K at 10 mg/ml, followed by incubation at 37°C for 30 min. Samples were phenol/chloroform-extracted, ethanol-precipitated, and electrophoresed on 8% polyacrylamide/8 M urea gels. Gels were visualized by autoradiography and quantitated by exposing the dried gels to phosphor screens and analyzing with a Molecular Dynamics Phosphor-Imager equipped with ImageQuant software. Relative changes in the levels of specific mRNAs were highly consistent within the experimental series.

**Metabolic Labeling and Cell Extract Analysis.** Cells were labeled for 3 hr with 45 $\mu$Ci (1 $\mu$Ci = 37 kBq) of [$^{35}$S]methionine (1129 Ci/mmol) per ml of methionine-free media. Extracts from whole cells were prepared by several cycles of freezing and thawing as described (16), and 20 $\mu$g of proteins were separated on 0.1% SDS/10% polyacrylamide gels. The gel was either stained with Coomassie blue or processed for fluorography.

**Flow Cytometric Analysis.** Cells ($10^6$) were resuspended in 0.5 ml of phosphate-buffered saline containing 1% fetal calf serum and 1 mM EDTA, fixed by gradual addition of ice-cold 80% ethanol while mixing, and stored for 1 hr at 4°C. The fixed cells were resuspended in the same buffer and stained for 30 min at 37°C with propidium iodide (Sigma) at 50 $\mu$g/ml in the presence of RNase A at 0.1 mg/ml. The fluorescence intensities of the samples were measured by quantitative flow cytometry with an Epics C system (Coulter).

**Analysis of DNA Synthesis.** Cells were labeled with 3 $\mu$Ci of [$^3$H]thymidine (34 Ci/mmol) per ml of media for 24 hr, rinsed in phosphate-buffered saline, and then pelleted. Cell pellets were resuspended in 0.25 ml of 0.3 M NaOH and incubated on ice for 15 min. An equal volume of 20% trichloroacetic acid was added, and incubation was continued on ice for 15 min. The extracts were then passed through Whatman GF/A glass fiber filters and washed with ice-cold 10% trichloroacetic acid followed by ice-cold 90% ethanol. Filters were dried and quantitated by liquid-scintillation counting.

## RESULTS

**Genes Encoding Cyclins D1 and D3 Are Differentially Expressed in Transformed and Primary Human Cells.** As a first step to study the expression of D-type cyclins, various cell types were surveyed to determine whether the cyclin genes are expressed ubiquitously or in a restricted pattern. Cytoplasmic RNA isolated from growing cells was examined by RNase protection analysis with probes specific for each of the cyclin mRNAs. Although there were differences in the levels of expression, cyclin D1 RNA was expressed in most cells tested, with the exception of T-cell lines (Fig. 1, lane 4). In contrast, cyclin D3 was expressed in all cells examined (Fig. 1 and data not shown). Although this exposure does not
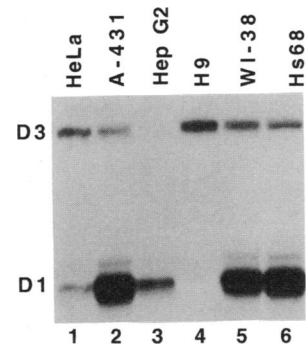


FIG. 1. Relative abundance of cyclin D1 and D3 mRNAs from transformed (epitheloid carcinoma HeLa, epidermoid carcinoma A-431, hepatocellular carcinoma HepG2, T-cell lymphoma H9) and primary (fibroblast WI-38 and Hs68) human cells. cDNA probes specific for D1 and D3 mRNA were hybridized to 10 $\mu$g of cytoplasmic RNA and subjected to RNase protection analysis. The probe fragments protected by D1 and D3 mRNA are indicated on the left.

show D3 RNA in HepG2 cells (Fig. 1, lane 3), it was detectable on a longer exposure of the gel. In contrast to the widely distributed pattern of expression for cyclins D1 and D3, cyclin D2 expression was highly restricted. It was detectable in two embryonal carcinomas (Tera-1 and Tera-2) and in the T-cell line H9, barely detectable in HDFs, and undetectable in a variety of other cell lines (unpublished work). In addition, the levels of D1, D2, and D3 mRNAs varied relative to each other among the different cell types. Therefore, D-type cyclin genes are differentially expressed.

**Kinetics of Induction of D1 and D3 Gene Expression by Serum in HDFs.** To determine if the abundance of cyclin mRNAs changes upon reentry of quiescent cells into the cell cycle, primary fibroblast Hs68 cells were made quiescent by a 48-hr serum starvation and then were restimulated with 10% serum. RNA was prepared from the cells at various times after serum stimulation and analyzed by RNase protection analysis with probes for D1, D3, and FOS mRNAs (Fig. 2 *Upper*). The FOS mRNA peaked at 30 min after stimulation and was undetectable by 4 hr. In contrast, the levels of D1 and D3 cyclin mRNAs increased gradually, peaking at about 12 hr after stimulation, and then declined slowly over the next 26 hr. Similar studies conducted in WI-38 cells gave identical results (data not shown). Nuclear run-on assays showed that the transcription rate of D1 and D3 cyclin genes increased in response to serum, but not enough to account for the increase in the steady-state mRNA levels (data not shown). Therefore, posttranscriptional regulation may also contribute to the increase in the levels of D1 and D3 mRNAs.

To determine whether induction of cyclin gene expression coincided with cell-cycle progression, flow cytometric analysis was performed on the serum-stimulated cells (Fig. 2 *Lower*). Ninety-two percent of the serum-starved cells were in $G_1$ phase as estimated by DNA content. When the cells were stimulated with 10% serum, the population of cells in S phase peaked 20 hr after stimulation. Labeling with [$^3$H]thymidine for 1-hr intervals confirmed the peak time point of S phase to be 20 hr after stimulation (data not shown). Thus, the levels of D1 and D3 cyclin mRNAs peak prior to the onset of DNA synthesis.

The delay in induction of cyclin genes relative to *FOS* raised the possibility that the activation of D1 and D3 gene expression might require *de novo* protein synthesis. To test this possibility, RNA levels were measured in cells stimulated with serum in the presence of CHX (Fig. 3). The induction of D1 and D3 cyclin mRNAs by serum was inhibited by ≈60% by CHX. Another protein synthesis inhibitor, puromycin, gave a similar result (data not shown). Considering that the induction of other secondary-response genes is
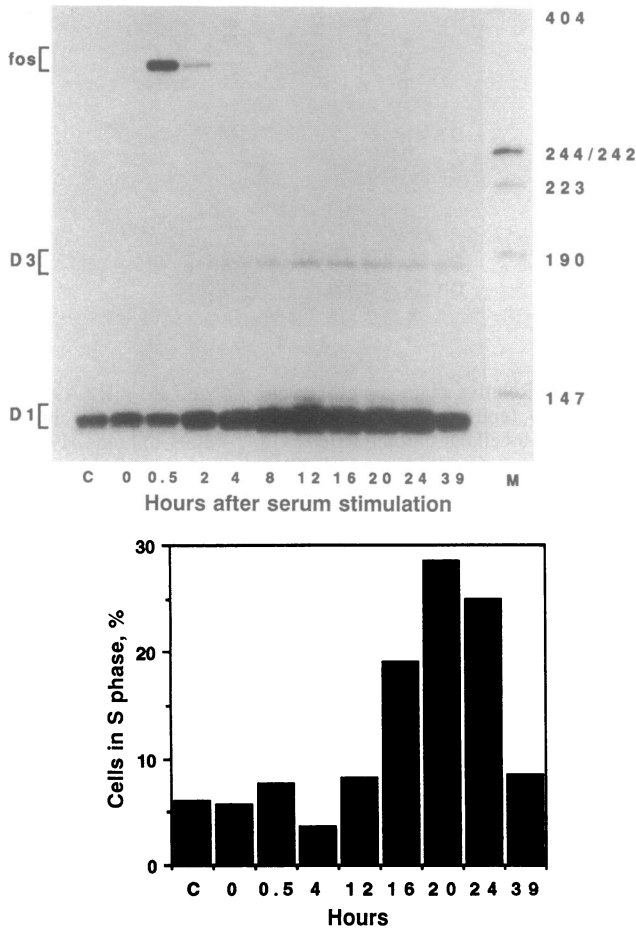
FIG. 2. Time course for the induction of D1 and D3 cyclin gene expression. Hs68 cells were treated with 10% serum after serum starvation for 48 hr. (*Upper*) At the times indicated, 5 μg of cytoplasmic RNA was prepared and analyzed by RNase protection assay using cDNA probes for FOS, cyclin D1, and cyclin D3 mRNAs. Lanes: C, proliferating cells without serum starvation; M, *Msp* I-digested pSP64 as size markers (in nucleotides). (*Lower*) At each time point after serum stimulation, cells were harvested for flow cytometric analysis of DNA content. The percentage of cells in the S phase of the cycle was calculated (17) and plotted.

completely blocked by CHX (18, 19), the partial block of serum induction of the cyclin genes by CHX is rather unusual. Therefore, we determined the extent of protein synthesis inhibition by labeling a duplicate set of cells with [$^{35}$S]methionine and preparing whole-cell extracts.
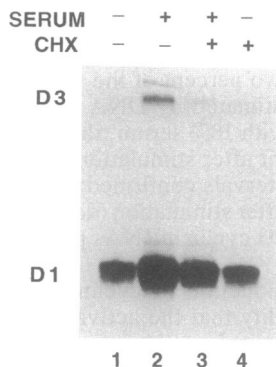


FIG. 3. Effect of CHX on the induction of cyclin D1 and D3 gene expression by serum. After serum starvation for 48 hr, Hs68 cells were treated for 12 hr with 10% serum alone or in the presence of CHX (35 μg/ml) or with CHX alone. Cytoplasmic RNA (5 μg) was analyzed for D1 and D3 transcripts by RNase protection.

Coomassie blue staining of the gel confirmed that the same amount of protein was loaded in each lane, and fluorography of the gel showed that protein synthesis was undetectable in CHX-treated cells (data not shown). Thus, cyclin D gene induction might occur through two pathways, one that requires protein synthesis and another that does not. However, the formal possibility exists that a very low level of protein synthesis occurs that could not be detected by $^{35}$S-labeling but that was sufficient to stimulate cyclin gene expression slightly. Nevertheless, the full induction of D1 and D3 cyclin genes by serum requires synthesis of new proteins, suggesting that immediate early gene products might be required for the expression of cyclin genes.

**Induction of D1 and D3 Cyclin Gene Expression Correlates with the State of Cell Growth.** Since D-type cyclins may have a role in growth control, we hypothesized that the level of expression of D1 and D3 cyclin genes might reflect the extent of cell proliferation. We examined this prediction using two different approaches. First, cells were plated at subconfluent levels and allowed to grow until arrested by contact inhibition (Fig. 4A). The levels of D1 and D3 mRNAs were high in exponentially growing cells and declined sharply as cells approached confluence. In a second experiment, serum was withdrawn from exponentially growing cells, and RNA was prepared at various times (Fig. 4B). The levels of D1 and D3 mRNAs declined by 70% after 7 hr of serum deprivation. Thus, HDFs respond to growth inhibitory signals such as cell contact or the absence of growth factors by decreasing the expression of the cyclin D genes. That proliferating cells showed higher levels of D1 and D3 mRNAs is consistent with a role for D-type cyclins in growth control.

**Differential Induction of D1 and D3 Cyclin Gene Expression by Serum in Young and Old HDFs.** With time in culture, HDFs undergo profound molecular and physiologic changes and finally lose the ability to respond to growth-stimulatory signals (20). Because senescent HDFs have competent growth factor receptors (21), the block to proliferation must lie between the stimulation of membrane receptors and the initiation of S phase. Therefore, to determine if a decline of D-type cyclin gene expression correlates with this block to proliferation, we examined D1 and D3 mRNA levels in old cells stimulated with serum. When young [mean population doublings (MPD) = 30] and old (MPD = 76) serum-deprived fibroblasts were refed with serum, FOS mRNA rose to much higher levels in young compared with old fibroblasts (Fig. 5, lanes 2 and 5), which is in agreement with previous studies
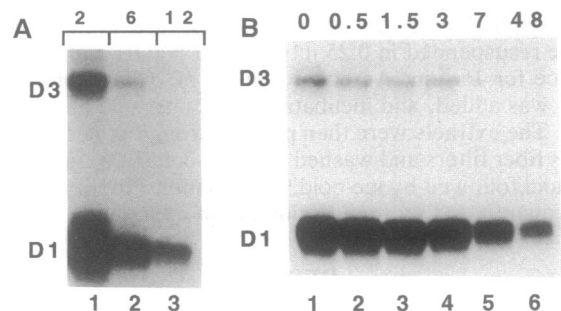


FIG. 4. The expression of D1 and D3 cyclin genes in growth-arrested human diploid fibroblasts by contact inhibition and serum starvation. (A) Hs68 cells were plated in DMEM containing 10% serum in 10-cm culture dishes at 2 × 10$^5$ cells per dish. Cytoplasmic RNA was prepared 2, 6, and 12 days after seeding as shown at the top of lanes. Two days before each RNA preparation, cells were given fresh medium. Equal amounts (5 μg) of RNA were analyzed by RNase protection assay. (B) Hs68 cells in asynchronous growth (lane 1) were fed serum-free medium, and cytoplasmic RNA was prepared at the indicated hours after serum withdrawal shown at the top of lanes. Equal amounts (5 μg) of RNA were analyzed by RNase protection assay.

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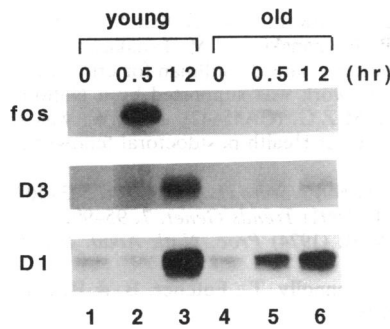*Proc. Natl. Acad. Sci. USA 89 (1992)* 9913



FIG. 5. Differential induction of D1 and D3 cyclin gene expression in young and old fibroblasts. Young (MPD 30) and old (MPD 76) fibroblasts were treated with 10% serum for 30 min or 12 hr after serum starvation for 48 hr. Cytoplasmic RNA (5 μg) was analyzed by RNase protection assay with cDNA probes for FOS, cyclin D1, and cyclin D3 mRNAs. The same gel was exposed for 3 hr (D1) and 12 hr (D3 and FOS).

(22, 23). Similarly, young cells expressed higher levels of D1 and D3 cyclin mRNAs than did old fibroblasts in response to serum (Fig. 5, lanes 3 and 6). Thus, the decline in the ability of aging HDFs to proliferate in response to normally mitogenic extracellular stimuli correlates with a decline in the induction of cyclin gene expression.

**Expression of D1 and D3 Genes Is Not Sufficient for the Transition from Quiescence Through $G_1$ into S Phase.** The observation that the D1 and D3 cyclin genes are induced by serum (Fig. 2) raises questions concerning the identity of the inducer within the serum and the intracellular mediators of the signal. To identify specific growth factors able to induce cyclin gene expression, quiescent cells were treated with various agents, and cyclin mRNA levels and DNA synthesis
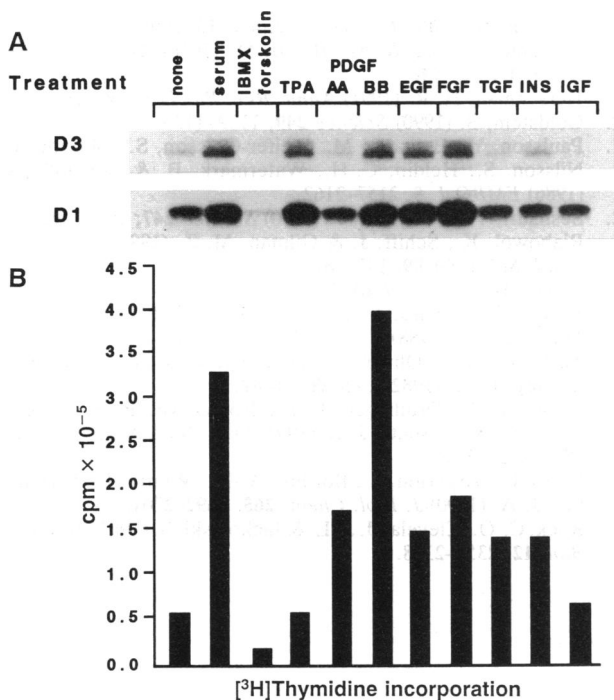


FIG. 6. The induction of D1 and D3 cyclin gene expression by specific growth factors. Hs68 cells were starved for 48 hr and treated for 12 hr with 10% serum, IBMX and forskolin, TPA, the AA or BB form of platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor β (TGF), insulin (INS), or insulin-like growth factor 1 (IGF). (*A*) Cytoplasmic RNA (5 μg) was analyzed by RNase protection assay. (*B*) [³H]Thymidine incorporation at 24 hr was measured as described in text.

were measured (Fig. 6). Treatment with 10% serum resulted in an increase in D1 and D3 mRNA levels and [³H]thymidine incorporation, as seen previously. In contrast, upon treatment with IBMX, an inhibitor of cyclic nucleotide phosphodiesterases, and forskolin, an activator of adenylate cyclase, both the cyclin mRNA levels and [³H]thymidine incorporation declined, suggesting that activation of the cAMP pathway leads to down-regulation of cyclin gene expression and to growth arrest. Treatment with the BB form of PDGF, EGF, or FGF resulted in an increase in D1 and D3 mRNA levels as large as that produced by treatment with serum (Fig. 6*A*). Similarly, treatment with the phorbol ester TPA increased cyclin mRNA levels, suggesting that activation of the protein kinase C pathway can cause the induction of cyclin genes. However, whereas treatment with the BB form of PDGF induced DNA synthesis to the same extent as serum, treatment with EGF or FGF produced only a moderate increase, and treatment with TPA produced no change in thymidine incorporation as compared with the control (Fig. 6*B*). Treatment with insulin-like growth factor 1 showed no change in both cyclin mRNA levels and thymidine incorporation. Treatment with the AA form of PDGF, transforming growth factor β, or insulin failed to induce cyclin mRNAs but resulted in small increases in thymidine incorporation. The observation that treatment with TPA induces cyclin gene expression with no effect on DNA synthesis suggests that induction of D cyclin genes is not sufficient for entry into S phase.

## DISCUSSION

Control of the commitment to DNA synthesis in mammalian cells is likely to be more complex than in yeast (24, 25). In human cells, in addition to D-type cyclins (4, 8, 10, 12), at least two other cyclins, C- and E-types (5, 6), have been suggested to function as $G_1$ cyclins. Although the function of each $G_1$ cyclin and their interrelationships are not yet clear, it is likely that the function of the CLN-type cyclins in yeast is fulfilled by multiple $G_1$ cyclins in higher eukaryotes. Moreover, the presence of multiple members within the same type of cyclin might reflect the more varied modes of proliferation control in different cell types of higher eukaryotes. Specifically, particular cyclins could be regulated by specific extracellular signals in a cell-type-specific manner. Consistent with this model, we found that D-type cyclin genes are differentially expressed among various cell types. Furthermore, the cyclin D2 gene is differentially expressed in two different T-cell lines: it is highly expressed in H9 cells but undetectable in Jurkat cells (unpublished work). Since H9 and Jurkat cells represent distinct stages of T-cell differentiation (26), the expression of D-type cyclin genes might be differentially regulated even within the same type of cells depending on the differentiation state. It is noteworthy that in HDFs where both D1 and D3 cyclin genes are expressed, growth factors induce these cyclins with the same kinetics (Fig. 6 and unpublished work), suggesting that common signal transduction pathways and/or common regulatory elements are used to effect coordinate expression of these genes.

Characteristic of a delayed early gene, the kinetics of human cyclin D1 gene induction by serum in HDFs is comparable to those of mouse D1 homolog by CSF-1 in murine macrophages (7). However, there is a significant amount of D1 mRNA present in serum-starved HDFs, whereas D1 mRNA is undetectable in CSF-1-deprived murine macrophages. Furthermore, although D1 mRNA induction was only partially blocked by CHX in HDFs, D1 mRNA induction was completely blocked in murine macrophages. These differences could be explained simply by differences in the sensitivities of the assays for cyclin expression or by

differences in the cell types and growth factor treatments used for the study of human and mouse D-type cyclins. Murine macrophages are dependent only on CSF-1 for their proliferation and survival (27). These cells die within 36 hr when deprived of CSF-1. In contrast, Hs68 cells do not lose their viability when grown in serum-free medium for several days and retain the ability to reenter the cell cycle upon serum stimulation (unpublished work). Therefore, D1 mRNA induction and degradation in murine macrophages may be more tightly associated with the dependency of the cells on mitogens than cyclin D1 mRNA in HDFs.

Our studies using HDFs show that expression of D-type cyclin genes correlates with the state of cell growth. This is also supported by elutriation experiments with cycling HeLa cells, which showed that D1 and D3 cyclin mRNA levels cycle weakly with a peak during $G_1$ (unpublished work). Similar results were obtained with HeLa cells and fibroblasts that were blocked by drug treatment and then released (ref. 8 and unpublished work). Consonant with the decrease in the expression of D1 and D3 cyclins in HDFs under unfavorable growth conditions (Fig. 4), the decrease in the induction of cyclin gene expression by serum in aging HDFs (Fig. 5) may be one of a series of events involved in the growth arrest associated with senescence. In senescent cells there is repression of two types of temporally separated growth-related genes, *FOS* and cyclin genes (22, 23, 28) (Fig. 5). In contrast, an increase in the level of cAMP induces *FOS* expression in HDFs (23) but inhibits D1 and D3 cyclin gene expression (Fig. 6), indicating that *FOS* and cyclin D gene expression are not always linked. Like HDFs, CSF-1-dependent murine macrophages are also arrested by cAMP analogs in the $G_1$ phase of the cell cycle (29). Since cAMP does not block either CSF-1-activated signaling pathways or several immediate responses to CSF-1, including the induction of Fos and Myc mRNA (29), cAMP-mediated growth arrest might occur later in $G_1$ (30). The effects of cAMP on the expression of cyclin D genes in macrophages are unknown. Since these genes are delayed early genes and may be involved in the $G_1$ to S transition, they might be specifically targeted for repression by cAMP. Therefore, we suggest that in CSF-1-dependent murine macrophages and HDFs, cAMP might act directly on the cyclin D genes to repress their expression and that down-regulation of $G_1$ cyclins by cAMP might be an important event contributing to the $G_1$ block.

Our data suggest that induction of D-type cyclin gene expression is not sufficient to induce DNA synthesis. It is possible that D-type and other types of cyclins function either together or separately during $G_1$ and at the $G_1$ to S transition and that cooperativity among different types of cyclins is required for the onset of DNA synthesis. It is also possible that the function of D-type cyclins is at the point of entry into the proliferative cycle, the $G_0/G_1$ boundary, and that other cyclins are required to traverse the $G_1$ phase and induce DNA synthesis. These may be induced by signal transduction pathways that are distinct from those that induce D-type cyclin gene expression.

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