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Enhanced Ribosomal Association of p27^{Kip1} mRNA Is a Mechanism Contributing to Accumulation during Growth Arrest*

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p27^{Kip1} regulates the decision to enter into S-phase or withdraw from the cell cycle by establishing an inhibitory threshold above which G_1 cyclin-dependent kinases accumulate before activation. We have used the HL-60 cell line to study regulation of p27 as cells withdraw from the cell cycle following treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We found that the amount of p27 is maximal in G_0 cells, lower in G_1 cells, and undetectable in S-phase cells. In contrast to the protein, the amount of p27 mRNA was the same in these populations, suggesting that accumulation of p27 during the cell cycle and as cells withdraw from the cell cycle is controlled by post-transcriptional mechanisms. In S-phase cells, the degradation of p27 appears to predominate as a regulatory mechanism. In G_0 cells, there was an increase in the synthesis rate of p27. Our data demonstrate that, in G_0 cells, accumulation of p27 is due to an increase in the amount of p27 mRNA in polyribosomes.

The activation status of the cyclin-dependent kinases (CDKs)[1] regulates progression through G_1 (1). The phosphorylation of the Rb protein correlates with transition through the restriction point when cells commit irreversibly to S-phase and subsequent cell division (2). Rb is phosphorylated by cyclin D·CDK complexes early in G_1, and cyclin E·CDK2 complexes further phosphorylate and maintain the hyperphosphorylated state through the remainder of G_1 (3). A class of proteins that interact with both G_1 CDKs, called Kips (CD*K i*nhibitory *p*roteins), might coordinate these two classes of kinases and the start of S-phase. p27^{Kip1} might directly bridge the activation of cyclin E·CDK2 complexes to expression of cyclin D·CDK complexes (4, 5). p21^{Kip1} might regulate cyclin·CDK2 complexes at a checkpoint following DNA damage or nucleotide pool perturbation (6–8). The function of p57^{Kip2} is unclear at this time.

Many observations suggest that p27 is a key regulator controlling entry into and exit from the cell cycle. Regardless of cell type or condition used to achieve growth arrest, an increase in the amount of p27·CDK2 complexes is a common feature of non-proliferating cells (9). In addition, ectopic expression of p27 in cells leads to G_0/G_1 arrest (10, 11), and antisense vectors that block p27 lead to an increase in the percentage of cells in S-phase (12). Furthermore, the phenotypes of *Kipl* mice suggest that the loss of p27 leads to an alteration in the balance between proliferating and non-proliferating cells (13–15). The stoichiometric nature of p27-mediated inhibition has led to the proposal that p27 functions to establish an inhibitory threshold above which CDKs accumulate before their activity can drive the cell cycle (16).

There are multiple mechanisms to regulate the amount of p27 available for interaction with cyclin·CDK2 complexes, dependent both on the cell type and the condition that leads to growth arrest. First, in proliferating cells, cyclin D·CDK complexes sequester the CDK2-inhibitory activity of p27. In proliferating mink lung epithelial cells (Mv1Lu) p27 associates with cyclin D2·CDK4 complexes (4). Exposure of Mv1Lu cells to transforming growth factor-β or growth to confluence leads to a displacement of p27 from cyclin D·CDK4 complexes, allowing it to associate with and inhibit the cyclin·CDK2 complex (17). In proliferating MANCA cells, p27 associates with cyclin D complexes in a non-inhibitory manner (5). Second, changing the amount of p27 mRNA can control accumulation of p27 protein; increased accumulation of p27 mRNA contributes to the increase in p27 protein following exposure of U937 cells to vitamin D3 (18). Third, ubiquitin-mediated degradation contributes to p27 accumulation in quiescent IMR-90 and MG-63 cells (19). We now demonstrate a fourth mechanism of p27 regulation that involves translation control, regulating association of p27 mRNA with polyribosomes.

EXPERIMENTAL PROCEDURES

Cell Culture, mRNA, and Protein Analysis—HL-60 cells were maintained at 2–5 × 10^5/ml in RPMI plus 10% fetal calf serum. Under these conditions, all cells incorporated bromodeoxyuridine during a 27-h labeling period (data not shown). To achieve growth arrest, cells were treated with 33 nM TPA. To obtain synchronized cell cycle fractions, 4 liters of cells were grown and elutriated as we described (20). DNA content was determined by propidium iodide staining as described (20). RNA was isolated from cells using RNA-STAT 60 following the recommendations of the manufacturer, and 20 μg of total RNA was used for Northern blotting as described (5). Proteins were isolated by sonicating cells in Tween lysis buffer as described (5). Immunoblot and kinase assays were performed as described previously (5, 20). To quantitatively determine the amount of protein using enhanced chemiluminescence (ECL), we included a standard protein curve in all experiments. This allowed us to determine a suitable range for quantitation based on the linear relationship between amount of protein and the intensity of signal. Signal quantitation was performed by spot densitometry using an IS1000 digital imaging system (Alpha-Innotech).

Accumulation of p27 in Cells Exposed to LLnL—Either following elutriation of an asynchronous population into cell cycle phase synchronized fractions or 48 h after TPA-treatment of an asynchronous population of cells, the G_0, G_1, or S-phase cells were exposed to either 2 μg/ml

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction.

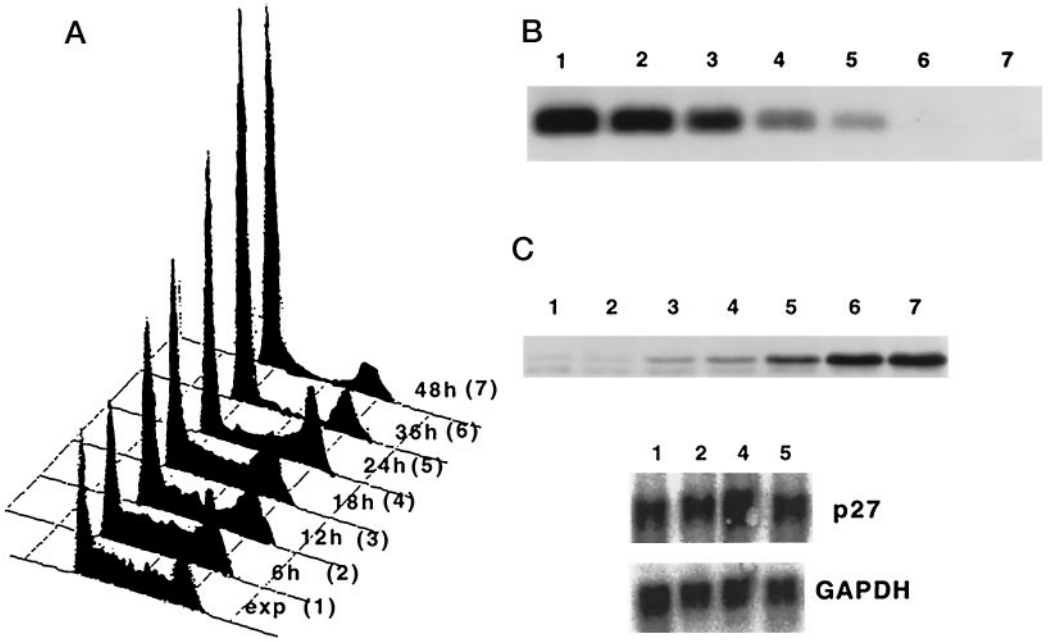


FIG. 1. **In HL-60 cells, activation of PKC leads to growth arrest and induction of p27.** *A*, flow cytometry. Asynchronous cultures of HL-60 cells were exposed to 33 nM TPA, and aliquots were removed at 6-h intervals as indicated on the *right* and processed for flow cytometry. *B*, CDK2 kinase activity is diminished in TPA-treated cells. Cell lysates were prepared, and the ability of CDK2 immunoprecipitates to phosphorylate histone H1 was measured. The *lanes* are labeled to correspond with *panel A*. *C*, p27 protein increases without a change in the amount of p27 mRNA. Protein and RNA were isolated from cell lysates, and the amount of p27 was determined by immunoblot (*top*) and Northern blotting (*bottom*). The *lanes* are labeled to correspond with *panel A*. p27 protein was detected with an affinity purified p27-specific antibody. Northern blots were probed with either p27 or GAPDH cDNA as indicated on the *right*.

LLnL (19) in Me_2SO or an equivalent volume of Me_2SO for 3 h. Extracts were prepared from treated and untreated cells, and proteins were either directly resolved by electrophoresis on a 10% SDS-polyacrylamide gel or immunoprecipitated using an excess amount of p27 antibody. The proteins were then transferred to Immobilon (Millipore Corp.), and p27 was detected by immunoblotting. Spot densitometry was performed, and the increase in p27 was measured.

Metabolic Labeling—G_0, G_1, and S-phase cells were isolated and treated with LLnL for 90 min in medium containing 1 mCi/ml Amersham Promix at a density of 4×10^6 cell/ml. Cells were washed twice in phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, pH 8.0) containing 10 μg/ml each aprotinin, leupeptin, soybean trypsin inhibitor, and pepstatin, and 1 mM phenylmethylsulfonyl fluoride. A small aliquot was removed, protein was precipitated with TCA, and the amount of radioactivity incorporated was quantitated in a scintillation counter. Immunoprecipitations were normalized for incorporated radioactivity, and proteins were resolved on a 12% SDS-polyacrylamide gel. After autoradiography, the p27 band was excised, and radioactivity was measured.

Differential Polysome Association—To examine polysome association of mRNA, we treated cells with 100 μg/ml cyclohexamide for 15 min prior to harvesting, collected 4×10^7 cells by centrifugation, and washed the pellet twice with ice-cold phosphate-buffered saline containing 100 μg/ml cyclohexamide. Cells were lysed in 1 ml of lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 150 μg/ml cyclohexamide, 20 mM dithiothreitol) containing 0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. Nuclei and cell debris were removed by centrifugation at $13,000 \times g$ for 5 min at 4 °C. We added 1.5 mg of heparin to the supernatant and applied this to the top of a 10-ml 15–40% continuous sucrose gradient. The gradient was prepared in lysis buffer containing 1.5 mg/ml heparin. Gradients were resolved by centrifugation at 38,000 rpm in an SW41 rotor for 1.5 h at 4 °C, and 1-ml fractions were collected. The fractionation of ribosomes was assessed by UV absorbance and ethidium bromide staining of agarose gels.

To quantitate the amount of p27 mRNA in each fraction, we blotted 400 μl (~40% of the total) to nitrocellulose and fixed the membrane in 10% formalin before UV cross-linking. Membranes were probed with a random primed human p27 cDNA. Quantitation was performed using a phosphoimager (Fuji MacBas 2000, Version 2.0).

To control for the specificity of our p27 probe, we also performed RT-PCR. Each fraction was adjusted to 1% SDS and 10 mM EDTA and 150 ng of proteinase K was added. The fractions were subsequently incubated for 30 min at 37 °C, and then RNA was purified, following phenol/chloroform extraction, on Nensorb 20 nucleic acid purification cartridges as described by the manufacturer (Dupont NEN). RNA was reverse-transcribed using random 9-mer primers (Stratagene) and M-MLV reverse transcriptase. The RT product was subsequently amplified with specific p27 primers (5′-TTGCAGGAACCTCTTCGGCC-3′ and 5′-GGTCGCTTCCTTATTCCTGC-3′) for 40 to 45 cycles using Ampli*Taq* (Perkin-Elmer). PCR reactions were initiated with a 94 °C 5-min incubation and then cycled through 94 °C for 90 s, 55 °C for 60 s, and 72 °C for 120 s. PCR was completed with a 15-min extension at 72 °C. Following PCR, an aliquot of product was loaded onto an agarose gel, transferred to nylon membranes, and probed with random primer-labeled p27 cDNA probes.

For detection of GAPDH, one half of the RNA from each fraction was loaded directly onto agarose gels, and Northern blotting was performed with a random primer-labeled GAPDH probe. Data was collected directly either by autoradiography or by phosphoimager (Fuji MacBas2000, Version 2.0).

RESULTS

Cell Cycle-dependent and Growth-dependent Post-transcriptional Regulation of p27 in HL-60 Cells—The decision to either proliferate or withdraw from the cell cycle is made during G_1 phase and is affected by the relative amounts of cyclin·CDK complexes and CDK inhibitors (18, 21–25). We used HL-60 cells to study the mechanisms controlling cell cycle withdrawal during differentiation because we could isolate populations of cells in each phase of the cell cycle in sufficient quantities for biochemical analysis. HL-60 cells differentiate into monocytes in the presence of TPA (26). Proliferation in asynchronous cultures of HL-60 cells completely ceased within 36 h following exposure to TPA, and typically, greater than 90% of the treated cells arrested with a G_1 content of DNA (Fig. 1*A*). The significance of the 10% of cells arrested in G_2/M is unclear. TPA treatment of enriched G_1 or S-phase populations obtained by centrifugal elutriation suggested that cells arrested in a single cell cycle (Table I) at the next G_1 phase. The 15% reduction of G_1 cells following TPA treatment of elutriated G_1 cells is consistent with a TPA restriction point subdividing this phase of

the cell cycle. Concomitant with accumulation of cells with a G_1 content of DNA following exposure to TPA, there was a coordinated decrease in immunoprecipitable CDK2 kinase activity (Fig. 1*B*) and an induction of p27 protein (Fig. 1*C*). The amount of p27 mRNA remained the same (Fig. 1*C*). Similar results were obtained with the phase-enriched populations (data not shown).

Since TPA-treated cells arrest with a 2C DNA content, p27 might accumulate in these cells as an indirect consequence of G_1 arrest in a state where p27 protein is stabilized. To address this, we first determined whether p27 expression is cell cycle regulated. We obtained cell cycle phase-specific populations of HL-60 cells by centrifugal elutriation of asynchronous cultures and examined p27 protein expression (Fig. 2). This method does not induce perturbations in cell cycle-regulated protein expression often observed with drug or metabolic-induced cell cycle synchronization. The amount of p27 protein was maximal during the G_1 phase of the cell cycle (Fig. 2*B*). The amount of p27 protein detected was linearly dependent on the amount of cell extract subjected to immunoprecipitation (data not shown; but see Fig. 3*A*). The amount of p27 mRNA did not change during the cell cycle (data not shown). These data suggest that, during the unperturbed cell cycle, p27 levels are determined by a post-transcriptional mode of regulation. However, the amount of p27 in equal numbers of G_0 and G_1 cells was quite different (Fig. 3*A*). G_0 cells contained at least 3–4-fold more p27 than G_1 cells. We obtained similar results comparing the amount of p27 as a function of total protein rather than cell number (data not shown). The amount of p27 detected was linearly dependent on the amount of cell extract subjected to immunoprecipitation (Fig. 3*A*, compare *lanes 1* and *2*). Together, these data suggest that accumulation of p27 reflected post-transcriptional regulation during both the G_1/G_0 and G_1/S transitions.

Phase-dependent Changes in Synthesis of p27—The accumulation of p27 represents the sum of protein synthesis and protein degradation. Precedence exists for the regulation of protein half-life as a function of cell cycle phase during both the G_1/S transition (27) and at the metaphase/anaphase transition (28). Determination of protein half-life during specific phases of the cell cycle is problematic because traversal of the cell cycle phases during the chase period will obscure the measured value. Consequently, to define the post-transcriptional mechanisms regulating p27, we measured the accumulation of p27 and the actual synthesis of p27 in G_0, G_1, and S-phase cells treated with LLnL (*N*-acetyl-leucinyl-leucinyl-norleucinal-H), an inhibitor that binds to the chymotryptic site on the proteasome. To measure accumulation of p27, we titrated each extract against recombinant p27 over a 16-fold range using 2-fold serial dilution. The resulting autoradiograph was analyzed by spot densitometry using an IS-1000 digital imaging system (Alpha Innotech), and we compared only those points that fell within the linear range (established by comparing signal intensity to amount of recombinant protein). Although the amount of p27 increased in all populations treated with LLnL, consistent with detection of newly synthesized p27 (shown below), the magnitude of the increase was cell cycle phase-specific (Fig. 3*B*). The increase in p27 levels was greatest in S-phase cells (about 160%) and more moderate in G_1 and G_0 cells (both about 50%).

To measure the amount of p27 synthesis in each population treated with LLnL, we labeled cells with ^{35}S-methionine and -cysteine for 90 min and immunoprecipitated p27. The short labeling period ensured that synchronized cells did not progress into the next phase of the cell cycle and inclusion of LLnL would prevent protein degradation; however, cells within G_1 phase might traverse the restriction point during this period.[2] After normalizing lysates for total protein synthesis, we isolated p27 with affinity purified p27-specific antibodies. We quantitated the amount of p27 synthesis by scintillation counting of excised bands, normalizing incorporation to 100% in G_1 cells. We found that the amount of newly synthesized p27 was greatest in G_0 cells and lower in G_1 and S-phase cells (Fig. 3*C*). We were unable to demonstrate if LLnL affected synthesis of p27 in a cell cycle phase-specific manner; however, similar increases in translation of p27 have been reported in other systems where LLnL was not included (29, 30). This suggested that the actual rate of p27 synthesis increased in G_0 cells.

Increased Synthesis of p27 Occurs as a Consequence of an Increase in the Amount of p27 mRNA in Polyribosomes—To determine the mechanism responsible for accumulation of p27 in G_0 cells, we compared the association of p27 mRNA with polysomes in proliferating and TPA-treated cells. To accomplish this, we fractionated RNA on continuous sucrose gradients and monitored the UV absorbance of the ribonucleoprotein complexes at A_{254}. In this assay, RNA equilibrates within the gradient as a function of the associated proteins; polysomal RNA equilibrates in the densest regions of the gradient. The

TABLE I
Percent of population with a G_0/G_1 DNA content
Numbers represent the mean of two independent experiments.

Time	S-phase fraction		G_1 phase fraction	
	+TPA	−TPA	+TPA	−TPA
h				
0	34.3		91.0	
6	30.7	36.2	86.8	87.5
12	18.8	56.5	79.7	74.6
18	30.7	56.5	75.0	62.0
24	61.4	44.4	74.2	40.3
30	70.8		87.2	
36	78.4		92.0	

[2] S. S. Millard, J. S. Yan, H. Nguyen, M. Pagano, H. Kiyokawa, and A. Koff, unpublished data.

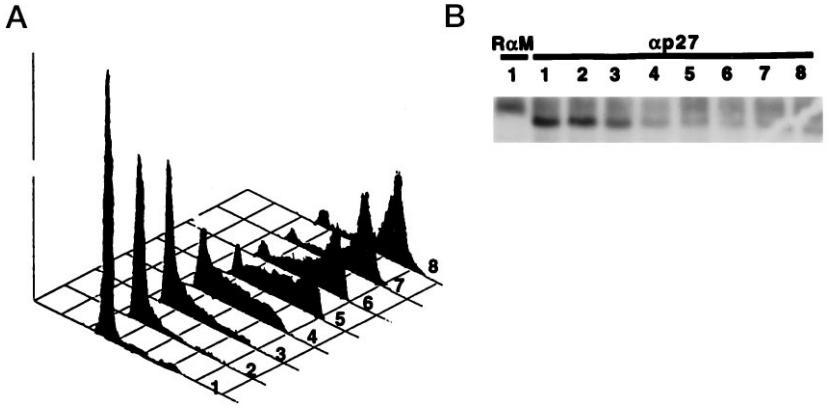


FIG. 2. **The expression of p27 is regulated during the cell cycle.** *A*, Flow cytometry. Asynchronous cultures of HL-60 cells were elutriated into cell cycle phase-specific fractions. DNA content was determined by propidium iodide. Fractions are numbered on the *right*. *B*, accumulation of p27 protein is maximal during G_1 phase of the cell cycle. p27 was immunoprecipitated from each fraction of cells indicated at the *top* of each lane. The *lanes* are numbered to match *panel A*. A mock immunoprecipitation with a rabbit-anti mouse antibody (*RαM*) is shown.

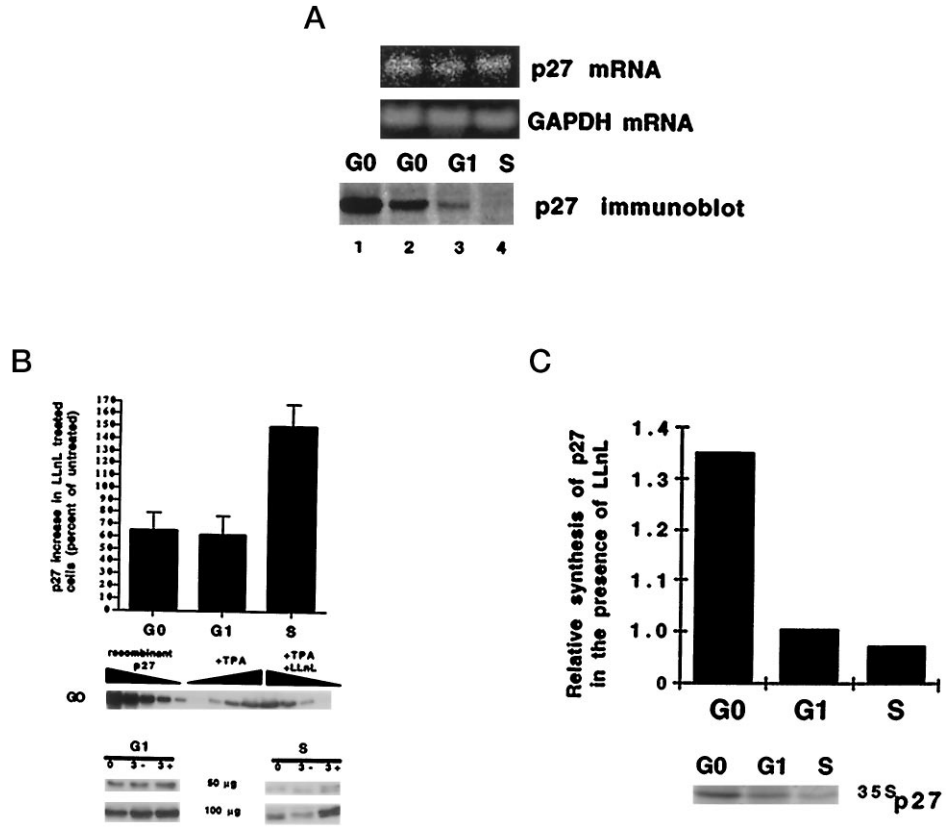


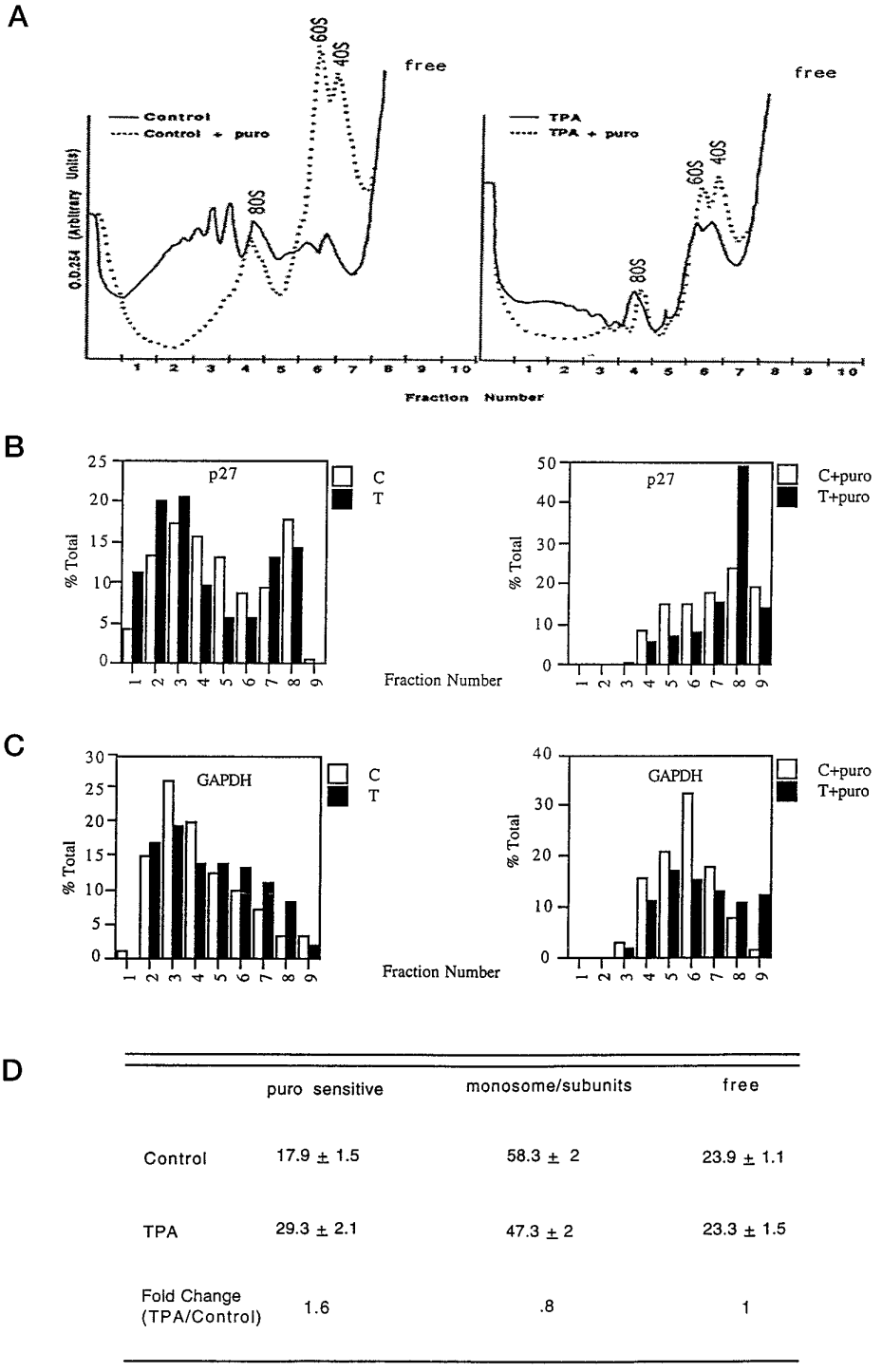
FIG. 3. **Cell cycle phase-specific post-transcriptional regulation of p27.** *A*, p27 protein accumulates in G_0 cells. G_0 cells were obtained following 48 h of treatment with TPA. G_1 and S-phase cells were obtained by centrifugal elutriation of asynchronous cultures. p27 and GAPDH mRNA were detected by Northern blotting and visualized on a Fuji phosphoimager using MacBas 2000 software. The amount of p27 mRNA is constant during these phases of the cell cycle (*top panels, lanes 2–4*). In the *bottom panel*, p27 was immunoprecipitated from 1 mg of each extract (*lanes 2–4*) and detected by immunoblotting. To ensure that excess antibody was used in the immunoprecipitation, 3 mg of G_0 extract was immunoprecipitated under the same conditons (*lane 1*). The amount of p27 is greatest in G_0 cells, minimal in S-phase cells, and intermediate in G_1 cells. *B*, accumulation of p27 in the presence of LLnL. Cells were treated with LLnL for 3 h prior to lysis. p27 was quantitated by immunoblotting. On the *ordinate*, we plotted the LLnL-dependent increase in p27. This experiment was repeated four times with comparable results, and the data presented is from a single representative experiment. Linearity was confirmed by including a titration of known amounts of recombinant p27 in the immunoblot. For quantiation of p27 accumulation in G_1 and S-phase cells, we compared the accumulation at 3 h in the presence and absence of LLnL. p27 accumulates to the greatest extent in G_0 phase cells. *C*, synthesis of p27 is maximal in cells treated with TPA. The p27 bands were excised from an SDS-polyacrylamide gel (shown below the *graph*), and the amount of radioactivity incorporated was measured by scintillation counting. Background was determined individually for each *lane* in a similar manner. On the *ordinate*, we plotted incorporation of radioactivity into p27 relative to the amount in G_1 cells.

extent of polysomal association is a reflection of the rate of protein synthesis; increased association reflects either an increase in the rate of initiation or an increase in the rate of elongation. To quantitate the p27 mRNA, we dot-blotted each fraction of the gradient and probed the membranes with a p27 cDNA probe. In untreated cells, p27 mRNA distributed in the free mRNA fraction of the gradient and in fractions containing monosomes and small polysomes (Fig. 4*B*). In TPA-treated cells, p27 mRNA distributes throughout the gradient, with the greatest differences occurring in the densest region of the gradients.

Treatment of cells with puromycin will disrupt polysomes and lead to an accumulation of mRNA in monosome and subunit fractions of the gradient. When we exposed TPA-treated cells to puromycin, the p27 mRNA shift was eliminated, confirming polysome association (Fig. 4*B*). We found approximately 60% more p27 mRNA associated with polysomes in TPA-treated cells (Fig. 4*D*). This increase appears to occur at the expense of the monosome and small polysome associated mRNA (Fig. 4*D*), suggesting that p27 synthesis might be regulated at the level of translation elongation. We were unable to detect a significant change in the distribution of p27 mRNA in polyribosomes isolated from G_1 and S-phase cells, consistent with our observation that p27 synthesis, in the presence of the proteasome inhibitor LLnL, was approximately equal in these populations (data not shown).

We confirmed the specificity of the dot-blot by isolating RNA from sucrose gradients and performing RT-PCR with p27-specific oligonucleotide primers. In this experiment, we assessed polysomal fractionation by ethidium bromide staining and detection of 28 and 18 S rRNA following agarose gel electrophoresis (Fig. 5). Consistent with our dot blot results, we found a shift in the distribution of p27 mRNA with a substantial amount fractionating with heavier polysomes in TPA-treated cells (Fig. 5). We confirmed that the PCR product was p27 by restriction endonuclease mapping (data not shown). Furthermore, addition of a control DNA encoding p27 but missing 100 base pairs of the sequence between the primer binding sites was specifically amplified (data not shown).

To ensure that the change of p27 migration into heavier polysomes did not represent a nonspecific change in bulk translation, we directly probed fractionated RNA with GAPDH. There was little change in the migration of GAPDH mRNA following TPA treatment, either in the RT-PCR analysis (Fig. 5) or dot-blotting procedure (Fig. 4*C*). This suggests that the changes in polysome distribution of p27 mRNA were specific and did not reflect general changes that cells might undergo during alternative developmental pathways. Together, these experiments demonstrate that increased expression of p27 pro-



	puro sensitive	monosome/subunits	free
Control	17.9 ± 1.5	58.3 ± 2	23.9 ± 1.1
TPA	29.3 ± 2.1	47.3 ± 2	23.3 ± 1.5
Fold Change (TPA/Control)	1.6	.8	1

FIG. 4. **p27 mRNA is distributed to heavier polysomes in TPA-treated cells.** *A*, sucrose gradient tracings. Cells were cultured with (*right panel*) or without TPA (*left panel*) as described under "Experimental Procedures." Puromycin (150 μg/ml) was added 45 min prior to harvesting. Fractions were collected from the bottom of the gradient, and the A_{254} was measured during fractionation. *Solid lines* represent the absorbance in the absence of puromycin, and *dashed lines* represent the absorbance in the presence of puromycin. *B*, quantitation of p27 mRNA in fractions of a representative sucrose gradient. The amount of mRNA in each fraction was measured by determining the amount of probe hybridized to dot-blots of each fraction on a phosphoimager and plotting the value as a percent of total p27 mRNA signal. In the *left panel*, control (*white*) and TPA-treated (*black*) gradients were compared. In the *right panel*, cells were treated with puromycin before collection and comparison of the p27 mRNA amounts. Results are representative of seven separate experiments. *C*, TPA increases the polysomal associated p27 mRNA 60%. Shown is the quantitation of GAPDH mRNA in each fraction of the sucrose gradients. Labeling is as described in *panel B*. *D*, the increase in polysomal mRNA occurs as a consequence of more efficient conversion of monosome and subunit-associated mRNA into polysomes. We compared the total amount of puromycin-sensitive p27 mRNA, the free mRNA (determined on ethidium bromide-stained cells by absence of both 28 and 18 S rRNA species in the fraction), and the remaining fractions. These values are tabulated (mean ± S.E.).

tein in cells exposed to TPA could be attributed to an increase in the density of ribosomes associated with p27 mRNA.

DISCUSSION

To study how the p27-mediated CDK inhibitory threshold is modulated during G_1, we explored the mechanisms regulating p27 as HL-60 cells withdraw from the cell cycle or enter S-phase. We have analyzed synchronous cell populations in G_0, G_1, or S-phase. We have shown that the translation of p27 changes in a growth-dependent manner, the association of p27 mRNA with polysomes is a determinant of the accumulation of p27 protein. Furthermore, degradation of p27 is a major determinant of accumulation of p27 during the G_1/S transition.

We found that an increase in the rate of p27 protein synthesis modulates the amount of p27 as HL-60 cells withdraw from the cell cycle. First, the amount of p27 is higher in growth-arrested cells than in G_1 cells although the amount of mRNA remained the same. Second, the p27 synthesis rate is increased in G_0 cells compared with either G_1 or S-phase cells when proteolysis was prevented by treating cells with LLnL. Third, the polysome distribution of p27 mRNA shifts to heavier fractions in G_0 cells, representing a greater density of actively translating ribosomes on each mRNA. This type of polysome distribution might represent more efficient utilization of the p27 mRNA template either by altering the initiation or elongation phases of translation. Translation control is not unique to either TPA or differentiating HL-60 cells. Hengst and Reed

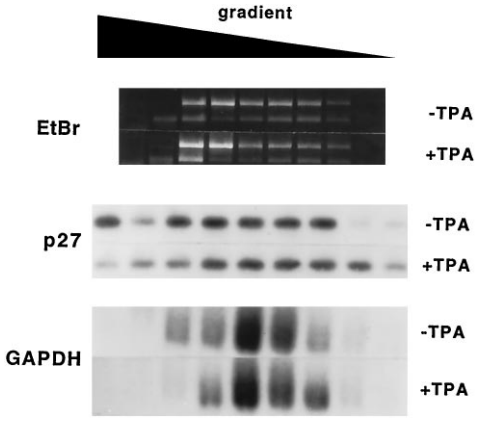


FIG. 5. **Specificity of p27 mRNA detection and distribution in the gradients.** The sucrose gradient is represented by the *triangle*. At the *top*, ethidium bromide-stained agarose gels indicate the migration of the 18 and 28 S rRNA species after centrifugation of untreated and treated cells as indicated on the *right*. The *middle panel*, shows the Southern blot of the amplified p27 product in untreated and TPA-treated cells as indicated on the *right*. The *bottom panel* shows direct Northern blotting of GAPDH from untreated and TPA-treated cells indicated on the *right*.

(29) reported translation regulation of p27 in both lovastatin-arrested HeLa cells and confluent human diploid fibroblasts, and Agrawal *et al.* (30) showed that platelet-derived growth factor treatment of BALB/c 3T3 cells represses translation of p27 mRNA. Our data expands this observation to events following the programmed withdrawal of a cell from the cell cycle into a terminally differentiated state.

There are many examples where mRNA interaction with polysomes occurs as cells re-enter the cell cycle from a non-proliferative state (31, 32). In contrast, p27 mRNA appears to be a member of a small group of messages that interact more efficiently with the translation apparatus as cells withdraw from the cell cycle. The mechanism regulating association is unclear. Factors might either promote ribosome association with p27 mRNA in G_0 cells or antagonize the association in non-G_0 cells. There is precedence for factors operating at sequences located in non-translated regions that might control ribosome association (32–36) or subcellular localization (37, 38). Alternatively, ribosomal proteins specific to non-proliferating cells might eliminate secondary structures in the p27 mRNA, allowing it to interact efficiently with ribosomes. The eIF-4E helicase facilitates unwinding and ribosomal association of mRNAs required to enter the cell cycle (38, 39). A presumptive factor regulating p27 mRNA association might act in a similar fashion, but with opposite biologic effect, and selectively recruit G_0-specific mRNA. However, we favor a model in which an inefficient repressor of translation is alleviated as cells withdraw from the cell cycle because there is translation occurring in proliferating cells, albeit less efficiently than in non-proliferating cells. Future experiments are underway to explore the mechanism regulating p27 translation.

The degradation of proteins is a key regulatory mechanism controlling protein expression as cells traverse the cell cycle (40). Substantial evidence has accumulated to support the crucial role of proteolysis at the metaphase/anaphase transition. Likewise, the involvement of a proteasome at the G_1/S transition has been implicated in *Saccharomyces cerevisiae* (27, 41, 42). We have shown that the amount of p27 decreases precipitously at the G_1/S transition although the synthesis of p27 remained the same. Furthermore, p27 accumulates to a similar extent when LLnL is added to either G_1 or S-phase cells. These observations suggest that protein degradation controls p27 accumulation during the cell cycle, and the amount of LLnL used in our experiments is sufficient to inhibit that proteasome activity. Confirmation of this hypothesis, however, awaits the ability to measure the half-life of p27 in cell cycle phase-specific extracts capable of degrading p27.

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