After 24 hours, the cells were washed and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum plus antibiotics (during the first 2 days with 1 mg/ml of G418 alone; subsequently, with G418 plus 1 mg/ml of zeocin). Colonies resistant to both antibiotics were picked, amplified, and screened for Env-dependent fusion permissiveness in the vaccinia assay system (with the use of luciferase as the reporter).
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Requirement of p27^{Kip1} for Restriction Point Control of the Fibroblast Cell Cycle

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Cells deprived of serum mitogens will either undergo immediate cell cycle arrest or complete mitosis and arrest in the next cell cycle. The transition from mitogen dependence to mitogen independence occurs in the mid- to late G_1 phase of the cell cycle and is called the restriction point. Murine Balb/c-3T3 fibroblasts deprived of serum mitogens accumulated the cyclin-dependent kinase (CDK) inhibitor p27^{Kip1}. This was correlated with inactivation of essential G_1 cyclin-CDK complexes and with cell cycle arrest in G_1. The ability of specific mitogens to allow transit through the restriction point paralleled their ability to down-regulate p27, and antisense inhibition of p27 expression prevented cell cycle arrest in response to mitogen depletion. Therefore, p27 is an essential component of the pathway that connects mitogenic signals to the cell cycle at the restriction point.

Cells can respond to the absence of mitogenic signals and shift from a proliferating state to a quiescent state only during a brief window of time in the cell cycle. The transition from mitogen dependence to mitogen independence occurs during G_1 and has been called commitment (*1*). Many different antimitogenic signals cause the cell cycle to arrest at the same relative position in G_1, and cells become refractory to all of these signals at approximately the same time in mid- to late G_1 (*2*). This was named the restriction point, extending the original notion of commitment to include cellular responses to a variety of mitogenic cues. Time-lapse cinematography of mitotically proliferating single cells has confirmed that mitogen depletion causes cells in early G_1 to immediately exit the cell cycle, and that cell cycle commitment (autonomy from mitogenic signals) occurs in mid-G_1 (*3*). Thus, the proliferative effects of mitogenic signals are dependent on cell cycle position and are likely to be mediated by their action on proteins that control movement through the cell cycle.

Transit through G_1 and entry into the S phase requires the action of cyclin-dependent kinases (CDKs) (*4*), and CDKs are inactivated by growth inhibitory signals (*5*). The catalytic activity of CDKs is regulated by two general mechanisms, protein phosphorylation and association with regulatory subunits, including the cyclins and the CDK inhibitors (CKIs) (*6*). The CKI directly implicated in mitogen-dependent CDK regulation is p27^{Kip1} (*7*). Amounts of p27 increase in quiescent cells and rapidly decrease after stimulation with specific mitogens (*8*). Moreover, constitutive expression of p27 in cultured cells causes cell cycle arrest in G_1 (*7*). Thus, p27 regulation may be an essential step in the pathway that links mitogenic signals to cell cycle progression and may be a key molecular event in the physiological process of cell cycle commitment or passage through the restriction point.

Subconfluent, exponentially proliferating Balb/c-3T3 fibroblasts [retinoblastoma (Rb) wild type; p53 status unknown] transferred to medium containing low concentrations of serum mitogens arrest in G_1 within 24 hours, which is approximately the length of one cell cycle (Fig. 1A). This demonstrates that Balb/c-3T3 cells require a mitogenic signal to proceed through each division cycle. G_1 arrest correlated with a six- to eightfold increase in the amount of the p27^{Kip1} protein (Fig. 1B). Similar increases in p27 expression occur in primary human diploid fibroblasts deprived of serum mitogens and in primary human T lymphocytes after withdrawal of interleukin-2, indicating that this is a common pattern of p27 expression in nontransformed cells (*8*). In Balb/c-3T3 cells, the amount of p27 started to increase within 4 hours of serum withdrawal, reached 60% of maximal amounts within 12 hours, and peaked within 24 hours (*9*). The induction of p27 protein paralleled the accumulation of the initially asynchronous cell population in G_1 and was consistent with the hypothesis that it could play a critical role in the early events associated with exit from the cell cycle.

In accord with this conclusion, cell cycle arrest of Balb/c-3T3 cells correlated with down-regulation of the cyclin E-Cdk2 and cyclin A-Cdk2 protein kinases (*9*), and this appeared to be related to induction of p27. First, both cyclin E-Cdk2 and cyclin A-Cdk2 were associated with increased amounts of p27 after mitogen withdrawal (Fig. 2C). Second, immune-depletion experiments showed that only a small portion of cyclin E in proliferating cells was bound to p27,

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whereas all of the cyclin E in arrested cells was bound to p27 (Fig. 1C). Similar results were obtained for cyclin A (*10*).

We examined the abilities of specific serum mitogens to both down-regulate p27 and induce cell proliferation. Subconfluent proliferating Balb/c-3T3 cells were transferred to medium containing low concentrations of serum supplemented with single mitogens: platelet-derived growth factor BB (PDGF-BB), epidermal growth factor (EGF), or insulin-like growth factor 1 (IGF-1). Only PDGF-BB was sufficient to prevent G_1 arrest and induction of p27 (Fig. 1, A and B; Table 1). When grown at high cell density, no single mitogen is sufficient to cause proliferation. Instead, PDGF initially stimulates the density-arrested quiescent cells to become competent to respond to progression factors IGF-1 and EGF (*11*). Thus, passage through the restriction point does not occur until cells have been exposed to all three mitogens. In density-arrested cells, PDGF-BB alone was insufficient to alter the abundance of p27; rather, p27 amounts declined once cells became committed to proliferate in response to the complete mitogenic signal provided by the combined action of PDGF-BB, EGF, and IGF-1 (*12*). Thus, under two different growth arrest conditions, the ability of specific mitogens to stimulate passage through the restriction point correlated with their ability to regulate p27. Decreased amounts of p27 reflected the integrated action of the collection of mitogens required for cell proliferation.

We used antisense oligonucleotides to block expression of the p27 protein and thereby determine whether regulation of

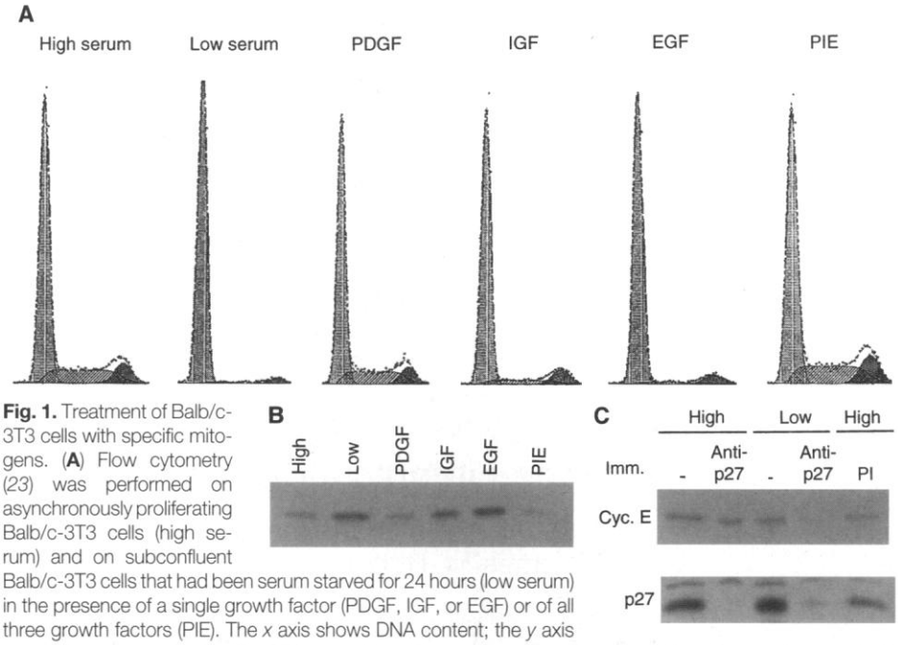

Fig. 1. Treatment of Balb/c-3T3 cells with specific mitogens. (**A**) Flow cytometry (*23*) was performed on asynchronously proliferating Balb/c-3T3 cells (high serum) and on subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours (low serum) in the presence of a single growth factor (PDGF, IGF, or EGF) or of all three growth factors (PIE). The *x* axis shows DNA content; the *y* axis shows the number of cells. (**B**) p27 immunoblots (*8*) were done on cell extracts (10 μg) from cells treated with growth factors as indicated. (**C**) Cell extracts from asynchronously proliferating Balb/c-3T3 cells (high) and from Balb/c-3T3 cells that had been serum starved (low) for 24 hours were depleted of p27. Extracts (100 μg) were incubated with antiserum to p27 (anti-p27) and protein A agarose for 1 hour at 4°C and collected by centrifugation. The supernatant was incubated twice more with p27 antiserum and protein A agarose. The immune-depleted (imm.) extracts (anti-p27) were analyzed by immunoblotting with antibodies to cyclin E (cyc. E) (*22*) or p27 and were compared with undepleted extracts (−) and with extracts depleted with p27 preimmune sera (PI).

Table 1. Cell cycle analysis. Balb/c-3T3 (*14, 16*) and SAOS-2 (*14*) cells were treated with antisense (AS) or mismatch (MSM) oligonucleotides as described. High and low designate cells grown in 10% or 0.1% serum, respectively. The cell cycle distribution of cells treated with single growth factors (PDGF-BB, IGF, or EGF) is also shown. The data are presented as the percentage of cells in each phase of the cell cycle as determined by flow cytometry (*23*).

Cell type and condition	G_1	S	G_2/M
	Balb/c-3T3		
High serum	63.7	27.4	8.9
Low serum	86.9	9.3*	3.9
MSM-low	81.7	11.6	6.7
AS-low	62.2	23.4	14.4
MSM-high	59.2	26.8	14.1
AS-high	42.3	35.1	22.6
PDGF-BB	69.4	21.4	9.2
IGF-1	83.2	7.7	9.1
EGF	90.5	3.4	6.1
PDGF–IGF-1–EGF	64.2	23.8	11.9
	SAOS-2		
High serum	54.3	25.8	19.9
Low serum	70.6	13.6	15.8
MSM-low	60.5	16.8	22.7
AS-low	44.2	27.9	27.9

*Flow cytometry analysis overestimated the percentage of cells in the S phase. BrdU staining demonstrated that under low serum conditions, 2 to 5% of the cells were in the S phase.

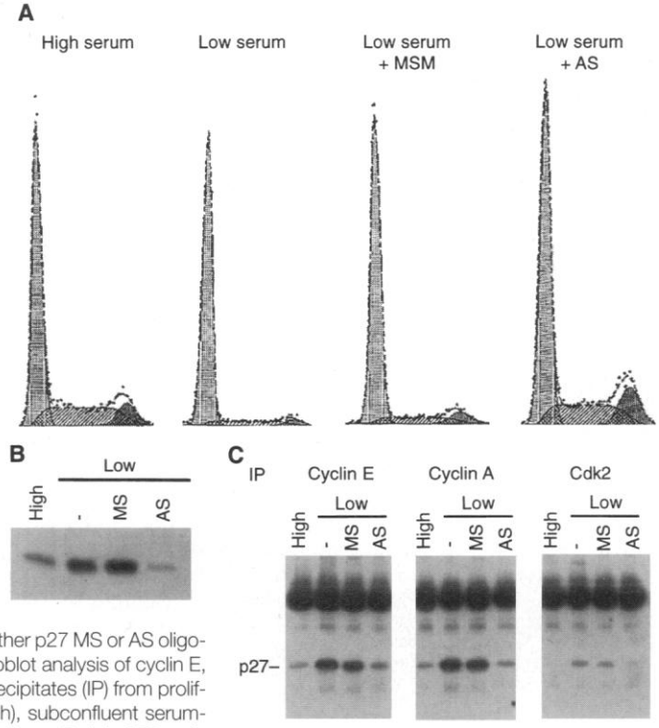

Fig. 2. Requirement of p27 for cell cycle withdrawal. (**A**) Flow cytometry analysis of proliferating Balb/c-3T3 cells (high serum), subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours (low serum), or subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either p27 MSM or p27 AS oligonucleotides (*14*). *x* axis, DNA content; *y* axis, number of cells. (**B**) p27 immunoblot analysis of extracts (10 μg) from control proliferating Balb/c-3T3 cells (high), subconfluent serum-starved Balb/c-3T3 cells (low), and subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either p27 MS or AS oligonucleotides. (**C**) p27 immunoblot analysis of cyclin E, cyclin A, or Cdk2 immunoprecipitates (IP) from proliferating Balb/c-3T3 cells (high), subconfluent serum-starved Balb/c-3T3 cells (low), or Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either MS or p27 AS oligonucleotides.

p27 was necessary for cell cycle control by serum mitogens. Phosphorothioate oligonucleotides were modified by the addition of a propynl group to the pyrimidine bases, which is thought to enhance base stacking and facilitate the sense-antisense interaction (*13*). Oligonucleotides were efficiently delivered to cells by association with a cationic lipid, GS2888 cytofectin. We used fluorescein isothiocyanate–labeled oligonucleotides to show that 90 to 95% of the cells took up and concentrated the oligonucleotides in the cell nucleus (*14*). We used two different 15-base antisense oligonucleotides directed against murine p27, and two different mismatch control oligonucleotides, which had the same base composition as the antisense oligonucleotides but a scrambled nucleotide sequence (*14*).

Balb/c-3T3 fibroblasts were exposed to p27 antisense and mismatch control oligonucleotides and then transferred for 24 hours to medium lacking serum mitogens. Identical results were obtained with both antisense or with both control oligonucleotides. The expression of p27 protein was substantially decreased in the antisense-treated cells (Fig. 2B), whereas the mismatch control had no effect on accumulation of p27 after serum withdrawal. Treatment of cells with antisense oligonucleotides to p27 did not decrease expression of the related CKI, $p21^{CIP1}$ (*15*). A decrease in the association of p27 with cyclin A and cyclin E corresponded to the decrease in overall amount of p27 in the antisense-treated cells (Fig. 2C). This was associated with restoration of cyclin E– and cyclin A–associated kinase activities in serum-starved cells (*9*).

In a proliferating population of Balb/c-3T3 fibroblasts, 27% of the cells were in S phase, and this fell to about 9% of cells within 24 hours after serum withdrawal (Fig. 2A, Table 1). Cells exposed to the mismatch oligonucleotide behaved identically to control cells. However, cells exposed to p27 antisense oligonucleotides did not undergo G_1 arrest after serum withdrawal; 23% of the cells remained in S phase (Fig. 2A, Table 1). The p27 antisense oligonucleotides also prevented the osteosarcoma cell line SAOS-2 (Rb mutated; p53 mutated) from exiting the cell cycle in response to serum withdrawal (Table 1). Thus, p27 is required for mitogen responsiveness in more than one cell type, and the requirement is independent of the Rb status of the cell.

Incorporation of bromodeoxyuridine (BrdU) or tritiated thymidine into nuclear DNA was used as an independent measure of the effect of p27 antisense oligonucleotides on cell cycle progression (*16*). Both techniques showed that cells exposed to p27 antisense oligonucleotides continued to synthesize DNA for at least 24 hours after serum withdrawal, whereas cells treated with mismatch control oligonucleotides did not (Fig. 3C) (*16*). Although the duration of antisense p27 inhibition is limited, cells treated with p27 antisense oligonucleotides express low amounts of p27 protein and continue to proliferate for at least 48 hours without serum mitogens.

To demonstrate the specificity of the antisense oligonucleotides (*17*), we showed that enforced expression of p27 in antisense-treated cells restored serum responsiveness. The degeneracy of the genetic code was exploited to construct a p27 expression plasmid that could not be inhibited by the antisense oligonucleotides but nevertheless encoded wild-type p27 protein (the p27 "wobble" plasmid) (Fig. 3A). A "tagged" version of the p27 wobble plasmid also was constructed, which encoded an electrophoretic variant of p27 resulting from a single amino acid change outside of the domain targeted by the antisense oligonucleotide. The tagged p27 could be separated and thereby distinguished from endogenous p27, enabling us to simultaneously test the effects of p27 antisense oligonucleotides on expression from the genes encoding the wild-type and wobble p27 in the same cell. The p27 antisense oligonucleotides effectively inhibited expression from both an exogenous wild-type gene for p27, and from the endogenous gene for p27, but did not inhibit p27 protein expression from the p27 wobble plasmid (Fig. 3B). Balb/c-3T3 cells were lipofected with mismatch or p27 antisense oligonucleotides and then microinjected with a plasmid encoding β-galactosidase (β-Gal) (to mark the injected cells) and the p27 wobble plasmid. Cells were then serum-starved for 24 hours, and the percentage of cells in S phase was measured by exposure to BrdU. Lipofection of cells with p27 antisense oligonucleotides decreased the percentage of cells that withdrew from the cell cycle after mitogen depletion, and this was reversed by microinjection with the p27 wobble plasmid (Fig. 3C). These results showed that the inability of p27 antisense-treated cells to exit the cell cycle after mitogen depletion is specifically caused by the loss of p27 expression.

It has been suggested that the basal amount of p27 expressed in proliferating cells may contribute to an inhibitory threshold imposed on CDK activation during G_1 (*18*). At one extreme, large amounts of p27 prevent CDK activation and arrest the cell cycle in G_1 (*7*), whereas decreased p27 expression might allow premature CDK activation and a shortened G_1 (*19*). To test this idea, exponentially proliferating Balb/c-3T3 cells were lipofected with p27 antisense or mismatch control oligonucleotides and allowed to proliferate in medium containing a high concentration of serum for an additional 24 hours. The p27 antisense treatment decreased p27 protein expression in proliferating cells to well below the normal basal amount. This markedly decreased the percentage of cells in G_1 (Table 1), showing that the length of G_1 was shortened relative to other phases of the cell cycle. No effect on p27 expression or cell cycle distribution was seen in the mismatch control (*9*). This is consistent with the hypothesis that the amount of p27 expressed in proliferating cells contributes to the length of G_1.

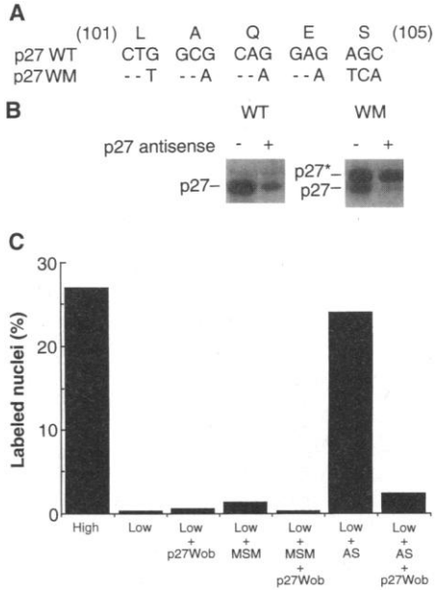

Fig. 3. Restoration of serum responsiveness by enforced expression of p27. (**A**) Depiction of bases that were changed in the p27 wild-type (WT) sequence to create the p27 wobble mutant (WM) plasmid (*28, 29*). These are the bases that hybridize to p27 antisense oligonucleotides. (**B**) p27 immunoblot analysis of proliferating Balb/c-3T3 cells 24 hours after lipofection in the presence (+) or absence (−) of p27 AS oligonucleotides with plasmid encoding either WT or the tagged (p27*) p27 wobble mutant (WM). (**C**) Proliferating Balb/c-3T3 fibroblasts (high) were lipofected with p27 MSM or AS oligonucleotides for 6 hours in a high concentration of serum. Cells were then microinjected with plasmids encoding β-Gal (*14*) and untagged p27 wobble mutant plasmid (p27Wob) as indicated. Cells were rinsed once with serum-free medium and incubated for 24 hours in medium containing 0.1% serum (low). Cells were labeled for the last 3 hours of the experiment with BrdU. Cells were stained for β-Gal expression (*14*) and immunostained for BrdU incorporation. The percentage of β-Gal–positive cells that incorporated BrdU was determined. Data represent the average of three independent experiments.

Our results show that restriction point control of nontransformed, immortalized Balb/c-3T3 cells requires the CDK inhibitor $p27^{Kip1}$. In cells deprived of mitogens, p27 amounts are elevated, essential cyclin-CDK complexes are inactivated, and the cell cycle stops in G_1. If p27 amounts do not increase, then the cell remains committed to the cell cycle independently of serum mitogens. As in the cells studied here, p27 expression is also mitogen dependent in primary cells and in other established cell lines. However, it remains possible that some regulatory pathways that contribute to restriction point control in vivo are no longer evident in some established cell lines.

Mitogenic signals have other effects on cell cycle regulators such as the Rb protein, whose phosphorylation by CDKs temporally coincides with the restriction point in G_1 (*20*). p27 is a global regulator of CDKs in G_1, so the control of Rb phosphorylation by mitogens could reflect the intermediary action of p27. Some aspects of the restriction point are altered in cells lacking Rb, such as the ability of the cell cycle to arrest after partial inhibition of protein synthesis (*21*), but $Rb^{-/-}$ mouse embryo fibroblasts remain responsive to mitogens. Rb phosphorylation may represent just one of a set of CDK-dependent events that occur at the restriction point. G_1 cyclin expression is also mitogen-dependent (*22*), and constitutive cyclin overexpression in cultured fibroblasts partially overcomes the antiproliferative effect of mitogen depletion (*19*). Thus, mitogens have complementary effects on CDK activation—they both increase expression of cyclins, the CDK-activating subunits, and decrease expression of p27, a CDK-inhibitory subunit. The concerted regulation of cyclins and CDK inhibitors allows transit through G_1 and is required to link mitogenic signals with cell cycle progression at the restriction point. How these pathways become independent of mitogens in post–restriction point cells remains to be determined.

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9. Proliferating Balb/c-3T3 fibroblasts were rinsed in serum-free medium and refed with medium containing 0.1% serum. p27 protein immunoblots (ECL, Amersham) were performed on cells harvested at 4, 8, 12, 16, and 24 hours after refeeding. Histone H1 kinase assays (*8*) were done on cyclin A, cyclin E, and Cdk2 (*23*) immunoprecipitated from Balb/c-3T3 extracts made from proliferating and serum-starved cells. Serum-starved cells lipofected with p27 antisense oligonucleotides contained increased amounts of cyclin E– and cyclin A–associated histone H1 kinase activity as compared with serum-starved cells. For experiments done with proliferating cells, proliferating cells were lipofected with either p27 mismatch or p27 antisense oligonucleotides and analyzed 24 hours later by flow cytometry and p27 immunoblots. The amount of p27 was three to five times less in cells treated with p27 antisense oligonucleotides than in proliferating cells and proliferating cells lipofected with p27 mismatch oligonucleotides.
10. Experiments were done as described (Fig. 1C), with the exception that cyclin A and p27 immunoblots were done on extracts depleted in p27. All of the cyclin A was bound to p27 in extracts from serum-starved cells, whereas only a small fraction (5%) of cyclin A was associated with p27 in proliferating cells.
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12. Density-arrested Balb/c-3T3 fibroblasts were rinsed in serum-free medium and refed with medium containing 0.1% serum and 10 ng per milliliter of medium of PDGF-BB, IGF-1, EGF, or IGF-1 and EGF, or all three growth factors. Cells were harvested 24 hours later and analyzed by flow cytometry for DNA content and p27 immunoblots. A combination of all three growth factors was required to stimulate 70% of the cells to enter the cell cycle and decrease p27 amounts by 10 times.
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14. The oligonucleotides were synthesized on an automated synthesizer (model 8750, Milligen Bioresearch, Bedford, MA) as described (*24*). The antisense oligonucleotide sequence used in these experiments was 5′-UGG CUC UCC UGC GCC-3′ (targets base pairs 306 to 320 of murine Kip1) and the mismatch sequence was 5′-UCC CUU UGG CGC GCC-3′. For the lipofection procedure, 30 nM oligonucleotides were mixed with GS2888 cytofectin (2.5 μg/ml) (*25*) (Gilead Scientific, Foster City, CA) in serum-free medium and incubated for 10 min at 37°C. Proliferating Balb/c-3T3 fibroblasts were rinsed once in serum-free medium and refed with oligonucleotide-cytofectin solution in medium containing 0.1% serum. Cells were then incubated for 24 hours in humidified incubators at 37°C with 5% CO_2. The percentage of cells that were positive for uptake of fluorescein isothiocyanate–labeled oligonucleotides was determined by ultraviolet fluorescence microscopy. Microinjection, immunofluorescence staining, and fluorescence microscopy were carried out as described (*26*). For costaining of β-Gal and BrdU, the cells were fixed and stained as previously described (*26, 27*).
15. Proliferating Balb/c-3T3 fibroblasts were lipofected with antisense and mismatch oligonucleotides (*14*). The amount of p21 was increased in proliferating cells as compared with that in serum-starved cells (*23*). Cells lipofected with either p27 mismatch or antisense oligonucleotides expressed slightly larger amounts of p21 as compared with amounts in serum-starved control cells.
16. Proliferating Balb/c-3T3 fibroblasts were lipofected with p27 mismatch or antisense oligonucleotides (*14*). For BrdU experiments, Balb/c-3T3 cells were labeled for the last 3 hours of the experiment with BrdU and immunostained with monoclonal antibodies to BrdU as described (*22*). The percentage of total cells (on a 1-mm cover slip) that stained positive for BrdU incorporation (percent of labeled nuclei) was determined. Thirty-five percent of serum-starved cells treated with p27 antisense oligonucleotides incorporated BrdU into nuclear DNA, whereas only 2 to 3% of the cells treated with mismatch control oligonucleotides did so. For tritiated thymidine experiments, cells were labeled for the last 3 hours of the experiment with [^{3}H]thymidine (1 μCi/ml). The amount of [^{3}H]thymidine incorporated into serum-deprived cells treated with antisense or mismatch oligonucleotides was compared with the amount of [^{3}H]thymidine incorporated into asynchronously proliferating cells.
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28. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; L, Leu; Q, Gln; and S, Ser.
29. To construct the p27 wobble expression plasmid used in Fig. 3C, a megaprimer was generated by polymerase chain reaction (PCR) amplification with the use of a primer to plasmid sequences (T7 primer) and a primer that contained mutations at the wobble positions for the amino acid sequence LAQESQ (*28*) (amino acids 102 to 108) of murine p27. (5′-TAA AGG CAC CGC CTG GCG ACT ACC GCT GAC GTC CTG TGA TTC TTG TGC AAG CAC CTT GCA GGC GCT C-3′). The megaprimer was subsequently used with a primer to plasmid sequences (T3 primer) at the 3′ end to PCR-amplify a full-length clone, which was subcloned into the expression vector pCS2+. These mutations created a p27 sequence with seven bases unmatched to the p27 antisense oligonucleotide and created a unique Aat II site. In addition to the base changes listed above for amino acids 102 to 108, the tagged p27 wobble mutant used in Fig. 3B also fortuitously contained mutations at Ser^{111} and Arg^{112}. These amino acids had been converted to Thr and Ser, respectively. Electrophoretically, the tagged p27 wobble mutant migrates slightly more slowly than do endogenous murine p27 and exogenous wild-type p27.

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