Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts

Dawn E. Quelle,[1] Richard A. Ashmun,[1,2] Sheila A. Shurtleff,[1,3] Jun-ya Kato,[1] Dafna Bar-Sagi,[4] Martine F. Roussel,[1] and Charles J. Sherr[1,3,5]

[1]Departments of Tumor Cell Biology, [2]Experimental Oncology, and [3]Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, Tennessee 38105 USA; [4]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA

Mammalian D-type cyclins are growth factor-regulated, delayed early response genes that are presumed to control progression through the G_1 phase of the cell cycle by governing the activity of cyclin-dependent kinases (cdks). Overexpression of mouse cyclin D1 in serum-stimulated mouse NIH-3T3 and rat-2 fibroblasts increased their rates of G_0 to S- and G_1- to S-phase transit by several hours, leading to an equivalent contraction of their mean cell generation times. Although such cells remained contact inhibited and anchorage dependent, they manifested a reduced serum requirement for growth and were smaller in size than their normal counterparts. Ectopic expression of cyclin D2 in rodent fibroblasts, either alone or together with exogenous cdk4, shortened their G_0- to S-phase interval and reduced their serum dependency, but cyclin D2 alone did not alter cell size significantly. When cells were microinjected during the G_1 interval with a monoclonal antibody specifically reactive to cyclin D1, parental rodent fibroblasts and derivatives overexpressing this cyclin were inhibited from entering S phase, but cells injected near the G_1/S phase transition were refractory to antibody-induced growth suppression. Thus, cyclin D1, and most likely D2, are rate limiting for G_1progression.

[*Key Words*: D-type cyclins; cell cycle; G_1 progression; cdk4]

Received April 21, 1993; revised version accepted May 25, 1993.

The decision of eukaryotic cells to replicate their chromosomal DNA is made late in the first gap phase (G_1) of the cell cycle. In the budding yeast *Saccharomyces cerevisiae*, this transition is governed by the accumulation of G_1 cyclins encoded by three *CLN* genes and their catalytic subunit, the cyclin-dependent kinase (cdk) $p34^{CDC28}$ (or its equivalent, $p34^{cdc2}$, in the fission yeast, *Schizosaccharomyces pombe*). The G_1-specific activities of the holoenzymes are rate limiting for passage through Start (Hartwell 1974), a point late in G_1 at which environmental signals converge on the cell cycle clock to drive entry into S phase (for review, see Reed 1991). Cell size, presumed to be regulated by available nutrients, is critical for Start (Pringle and Hartwell 1981), and because both dominant and loss-of-function mutations in *CLN* genes reciprocally affect this parameter, *CLN* function likely determines when cell size is sufficient for Start to occur (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Hadwiger et al. 1989; Richardson et al. 1989). Conversely, the mating pheromone, α-factor, arrests G_1 progression in budding yeast by triggering signals that negatively regulate CLN function (Cross 1988; Nash et al. 1988; Chang and Herskowitz 1990; Elion et al. 1990; Wittenberg et al. 1990). The activity of any one of three *CLN* genes is sufficient for cell growth, but mutations in all three are necessary to induce G_1 arrest at the same position in the cell cycle as mutants defective in *CDC28* (Richardson et al. 1989). *CLN* genes are differentially regulated, and only the *CLN1* and *CLN2* RNAs, for example, vary in abundance during the cell cycle, peaking during G_1 (Wittenberg et al. 1990). Execution of Start occurs before the onset of DNA synthesis, and mutations in genes other than the *CLN*s or *CDC28* can arrest G_1 progression after Start but before the G_1/S transition (Pringle and Hartwell 1981; Hennessy and Botstein 1991; Johnston et al. 1991). It remains possible that a separate requirement for *CDC28*/*cdc2* at the G_1/S transition has not been observed in yeast because of the short time interval separating Start from the beginning of S phase.

In animal cells, passage through a late G_1 restriction point (R) analogous to Start defines the time at which cells no longer require growth factors to enter S phase, which occurs several hours later (Pardee 1989). An accumulation of unstable proteins is required to pass the R point, and it is reasonable to speculate that such proteins might be mammalian G_1 cyclins, which could regulate one or more cyclin-dependent kinases (cdks) to drive cell

[5]Corresponding author.

cycle progression in a rate-limiting manner. Cyclins identical to CLNs have not been isolated from mammalian cells, but several such proteins, including three D-type cyclins and cyclin E (Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991a; Motokura et al. 1991; Xiong et al. 1991), now appear likely to regulate events late in G_1. Cyclin E expression is periodic and peaks at the G_1/S transition (Lew et al. 1991), and it regulates the activity of cdk2 (Dulic et al. 1992; Koff et al. 1992), a cyclin-dependent kinase whose activity is necessary for S-phase entry (Pagano et al. 1993; Tsai et al. 1993). Fibroblasts engineered to overexpress cyclin E were recently found to exhibit a shortened G_1 interval, decreased cell size, and a reduced serum requirement for the G_1/S transition, providing the first direct evidence that this cyclin can be rate-limiting for G_1 progression in mammalian cells (Ohtsubo and Roberts 1993).

Data that D-type cyclins can regulate G_1 progression have so far been largely inferential. D-type cyclins generally appear earlier in G_1 than cyclin E, and they are differentially expressed in various cell lineages in a highly growth factor-dependent manner (Matsushime et al. 1991a,b; Cocks et al. 1992; Won et al. 1992; Ajchenbaum et al. 1993). The D-type cyclins interact with an atypical catalytic partner, cdk4 (Matsushime et al. 1992; Kato et al. 1993), but at least some of them can associate with other cdks, including cdk2 and cdk5 (Xiong et al. 1992; Ewen et al. 1993). Disruption of cyclin D1 function by microinjection of antisense plasmids or antibodies into serum-stimulated fibroblasts during mid-G_1 can prevent their entry into S phase, but microinjections near the G_1/S boundary are without effect (Baldin et al. 1993). Here, we show that overexpression of different D-type cyclins in rodent fibroblasts can shorten their G_1 interval, thereby providing direct evidence that they can positively regulate G_1 progression.

Results

Derivation of cell lines overexpressing D-type cyclins

Initially, we attempted to express exogenous murine cyclin D1 cDNA in rat-2 or mouse NIH-3T3 fibroblasts by use of retroviral vectors containing a selectable marker (*neo*) that confers resistance to the antibiotic, G418. Cyclin D1 cDNA was cloned 5′ to the *neo* gene immediately downstream of viral long terminal repeat (LTR) promoter/enhancers derived either from the Moloney murine leukemia virus (MuLV) [pZipSV(X)I vector] or the myeloproliferative sarcoma virus (pZEN–tk*neo* vector), respectively. Alternatively, we used a vector (pBC140) in which *neo* expression is driven by the retroviral 5′ LTR and cyclin D1 by an internal cytomegalovirus (CMV) promoter. Many fewer G418-resistant colonies arose in cultures transfected with the cyclin D1-containing vectors than with the naked control vectors (Table 1), and pooled surviving colonies failed to detectably overexpress cyclin D1 protein.

Cyclin D1 cDNA was then cloned into a Moloney murine sarcoma virus-based vector plasmid containing the SV40 replication origin (pCOS–MSV–tk*neo*), which enables its amplification in monkey cells expressing T antigen. Following transfection into a Cos packaging cell line engineered to express retroviral genes necessary for virion production, viruses harvested from the culture supernatants 48–72 hr later were titered on NIH-3T3 cells. In three independent transfection experiments using the vector alone, the average titer of released viruses able to confer G418-resistance in NIH-3T3 cells was 2.5×10^6 colony-forming units (cfu) per milliliter of culture supernatant, but cells transfected with the cyclin D1-containing vector yielded only 3.8×10^4 cfu per milliliter. Direct transfection of rat-2 cells with this cyclin D1 vector plasmid also generated many fewer G418-resistant colonies than cells transfected with the parental vector (Table 1), and again, infected or transfected G418-resistant fibroblasts did not detectably express elevated levels of cyclin D1 protein. Thus, there appeared to be a strong selection against the establishment of cell lines able to overexpress exogenous cyclin D1. Similar difficulties were encountered with MuLV-based vectors containing the cyclin D2 and D3 cDNAs.

We reasoned that if high levels of exogenous cyclin D synthesis were incompatible with cell growth or viability, the use of weak or inducible promoters might enable us to circumvent this problem. After transfection of rat-2 cells with a vector in which cyclin D1 gene expression was driven by the avian Rous sarcoma virus promoter (pRc/RSV–*neo*), no significant reduction in the number of G418-resistant colonies was observed as compared with cells transfected with a control vector (Table 1). Following metabolic labeling with [^{35}S]methionine and quantitative immunoprecipitation with rabbit antise-

Table 1. *Transfection efficiency of cyclin D expression vectors in rat-2 fibroblasts*

Vector plasmid	Transfection efficiency (*neo*-resistant colonies/μg DNA)
pZIPSV(X)I	1.1×10^4
pZIPSV(X)I–D1	1.0×10^2
pZEN	1.9×10^4
pZEN–D1	4.4×10^2
pBC140	9.0×10^3
pBC140–D1	1.6×10^2
pCOS–MSV–tk*neo*	9.1×10^3
pCOS–MSV–tk*neo*–D1	6.0×10^1
pRc/RSV–*neo*	9.1×10^3
pRc/RSV–*neo*–D1 (rat-D1^{lo})	9.0×10^3
pRc/RSV–*neo*	6.0×10^4
pRc/RSV–*neo*–D1 (rat-D1^{hi})	2.6×10^4

Cells transfected with the indicated vector plasmids containing the neomycin resistance gene were selected in G418, and colonies of resistant cells were enumerated 3 weeks later. (–D1) Vectors containing cyclin D1 cDNA. The transfection experiments giving rise to pooled populations expressing relatively low (rat–D1^{lo}) or high (rat–D1^{hi}) levels of cyclin D1 are indicated in parentheses.

rum to cyclin D1, the pooled *neo*-resistant populations (designated rat-D1^{lo}) were found to synthesize approximately threefold more of the 36-kD cyclin D1 protein (p36^{D1}) than that normally produced in parental rat-2 cells (Fig. 1A, cf. lanes 8 and 2). Cyclin D1 mRNA and protein were not detected in serum-starved parental fibroblasts (also see Won et al. 1992; Baldin et al. 1993); but when quiescent cells were stimulated to re-enter the cell cycle, cyclin D1 protein was induced by 3 hr and accumulated to maximal levels by 7 hr after serum addition. In contrast, cells transfected with the cyclin D1 gene constitutively synthesized elevated levels of the protein throughout the cell cycle, as determined by sequential immunoprecipitation and immunoblotting (data not shown).

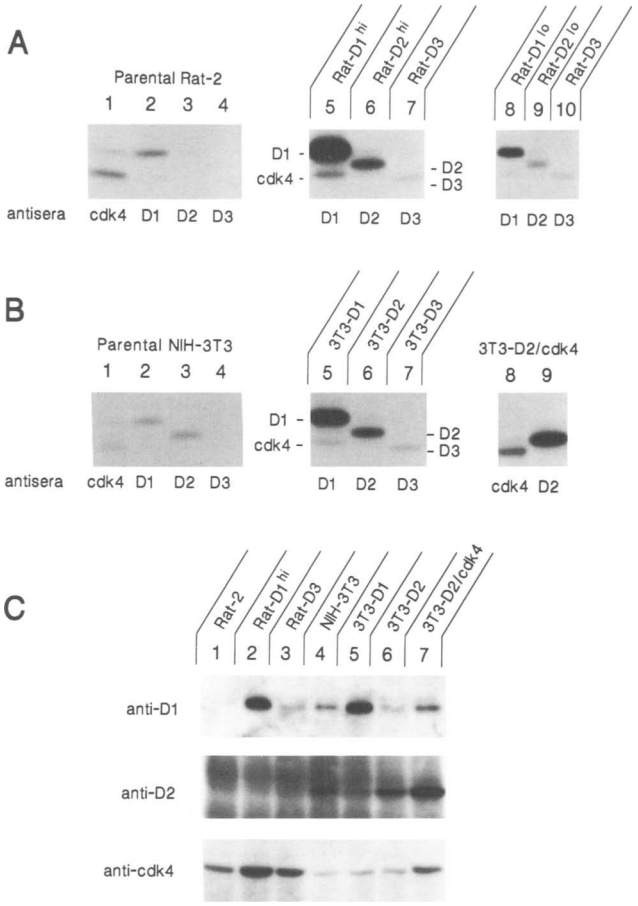

Figure 1. Expression of D-type cyclins in transfected cell lines. Rat-2 (*A*) and NIH-3T3 (*B*) cell lines were metabolically labeled with [^{35}S]methionine, and 125 μg of protein per lysate was precipitated with the antisera indicated below the panels, denatured, and separated on polyacrylamide gels. In *A* and *B*, lanes *1–4* illustrate the relative levels of synthesis of cdk4 and D-type cyclins in parental, untransfected cell lines. Lanes *5–10* in *A* show results with six independently derived rat-2 cell lines transfected with the cyclin D genes indicated above each lane and immunoprecipitated with the cognate antisera. Lanes *5–7* in *B* show similar results for three NIH-3T3 derivatives, whereas lanes *8* and *9* show results of immunoprecipitations performed with anti-cdk4 or anti-cyclin D2 with a single cell line cotransfected with both genes. All autoradiographic exposure times were 2 days. Immunoblotting analysis (*C*) was used to quantitate steady-state levels of cyclin D and cdk4 in the transfected cell lines. Equal amounts of protein from cell lysates prepared from the cell lines designated above the panels were precipitated with antisera to cyclin D1 (*top*), cyclin D2 (*middle*), or cdk4 (*bottom*). Immune complexes were separated on denaturing gels, transferred to nitrocellulose, and immunoblotted with cognate antisera. The autoradiographic exposure times were 2 days for the cyclins and 7 days for cdk4.

Parental rat-2 cells normally synthesize no detectable cyclin D2 and very low levels of cyclin D3 (Fig. 1A, lanes 3,4), but enforced expression of each was also achieved by use of pRc/RSV–*neo* vectors containing these cDNAs (Fig 1A, lanes 9,10). The three D-type cyclins can be distinguished by their electrophoretic mobilities on denaturing gels (lanes 8–10), as predicted from small differences in their molecular masses (Matsushime et al. 1991b). Although the rabbit antisera to bacterially produced cyclins D1 and D2 reciprocally cross-react with one another, the antiserum to cyclin D2 reacts preferentially with the cognate protein and did not quantitatively coprecipitate endogenous cyclin D1 under the conditions used (lane 9). In contrast, the antiserum to cyclin D3 does not cross-react with cyclins D1 or D2 (Matsushime et al. 1992). With optimized transfection conditions, 5–10-fold higher levels of synthesis of the exogenous mouse cyclins D1 and D2 (rat-D1^{hi} and rat-D2^{hi}) were realized (Fig. 1A, lanes 5,6). Even under the best circumstances, however, the levels of enforced cyclin D3 synthesis remained relatively low (lane 7).

Rat-2 cells express relatively high levels of cdk4 (Fig. 1A, lane 1), the major catalytic partner of cyclin D1, and most p34^{cdk4} coprecipitated in immune complexes with cyclin D1 when the latter was highly overexpressed (lane 5). Cyclin D2 also functionally interacts with cdk4 (Matsushime et al. 1992; Kato et al. 1993), and although it was not readily observed in anti-D2 precipitates from metabolically labeled cells (lane 6), immunoblotting of the denatured D2 immunoprecipitates with antiserum to cdk4 revealed its presence (data not shown). The latter method is more sensitive in detecting p34^{cdk4} in such complexes, because unlike the cyclins, which have a short half-life ($t_{1/2}$ = 20 min), p34^{cdk4} is relatively stable ($t_{1/2} > 4$ hr), and only a fraction of the pool incorporates [^{35}S]methionine during a 1-hr labeling period (Matsushime et al. 1992). Nonetheless, we do not know whether the observed differences in the amounts of cdk4 detected in complexes with cyclins D1 and D2 were attributable to the higher levels of ectopically expressed D1 versus D2 (cf. lanes 5 and 6) or reflect physiologic differences in their ability to stably interact with this catalytic subunit.

By use of the same vector, we were also able to confer increased expression of the three D-type cyclins in NIH-3T3 cells (Fig. 1B, lanes 5–7), which normally express cyclins D1 and D2, but not D3 (lanes 2–4). As for rat-2 cells, enforced synthesis of ectopic cyclin D3 was nominal, whereas 5- to 10-fold increases in cyclin D1 and D2 production were observed. NIH-3T3 cells express lower

levels of cdk4 than rat-2 fibroblasts (Fig. 1, cf. A and B, lanes 1), but cotransfection of NIH-3T3 cells with pRc/RSV–*neo* vectors encoding cyclin D2 and cdk4 gave rise to cells overexpressing both proteins (Fig. 1B, lanes 8,9). The latter cells also expressed approximately two- to threefold more cyclin D2 (lane 9) than those transfected with the cyclin D2 gene alone (lane 6). In parallel transfections, we did not derive stable NIH-3T3 transformants significantly overexpressing only cdk4. As for the rat-2 derivatives, anti-D1 precipitates contained more cdk4 than those prepared with anti-D2 (cf. lane 5 with lanes 6 and 9).

Because D-type cyclins are highly unstable proteins (Matsushime et al. 1991a, 1992), immunoblotting was used to estimate their relative steady-state levels in asynchronously dividing fibroblasts (Fig. 1C). In agreement with metabolic labeling studies, the levels of cyclins D1 and D2 in cell lines transfected with the corresponding genes were increased 5- to 10-fold over those in their parental counterparts. These experiments also confirmed that rat-2 cells express more $p34^{cdk4}$ than mouse NIH-3T3 fibroblasts and that cotransfection of cyclin D2 and cdk4 into the latter cells led to a three- to fourfold increase in their cdk4 levels. NIH-3T3 cells overexpressing cyclin D2 paradoxically exhibited a somewhat reduced level of endogenous D1 than that in parental controls [Fig. 1C, lanes 4,6 (top)], but cells transfected with both cyclin D2 and cdk4 showed no reduction in their D1 levels [lane 7, (top)]. Consistent with our inability to efficiently enforce cyclin D3 expression in rodent fibroblasts (Fig. 1A,B), we were unable to document its overexpression in the cognate transfectants by immunoblotting techniques (data not shown). Subsequent studies therefore focused on cell lines overexpressing cyclins D1 and D2.

Given our initial difficulties in achieving stable ectopic D-type cyclin expression from what we assume are strong mammalian viral promoters (Table 1), it is formally possible that the cells transfected with the D-type cyclin-containing pRc/RSV–*neo* vectors had sustained secondary mutations that facilitated cyclin overexpression. This seems unlikely for several reasons. First, no significant selective pressure against exogenous D-type cyclin expression was observed when pRc/RSV–*neo* vectors were used (Table 1). Second, because numerous G418-resistant colonies were pooled from each transfection experiment, each cell population represented a product of multiple vector–cell interactions, as confirmed by Southern blotting with a *neo* probe (see Materials and methods). Third, results similar to those using pRc/RSV–*neo* were obtained following transfection of rat-2 cells with an expression vector containing an artificial promoter composed of reiterated heavy metal response elements (Kato et al. 1989). In the absence of added heavy metals, cyclin D1 was modestly overexpressed and was further induced twofold by addition of zinc and cadmium to the culture medium (data not shown). Although the latter manipulations failed to provide a nonleaky system for studying conditional cyclin D expression, the results argue that pRc/RSV–*neo* is not unique in facilitating ectopic D-type cyclin expression in rodent fibroblasts.

Overexpression of cyclin D1 in fibroblasts shortens their G_1 interval

To determine whether cyclin D1 could accelerate G_1 progression, parental rat-2 cells, as well as transfected populations expressing low (Fig. 1A, lane 8) or high (Fig. 1A, lane 5) levels of cyclin D1, were arrested in metaphase with the microtubule inhibitor, nocodazole, and their rates of transit through the cell cycle were measured after reversal of the mitotic block. To obtain a high degree of synchrony and reduce potential drug toxicity, subconfluent cells starved for 36 hr in medium containing 0.1% fetal calf serum (FCS) were released from G_0 in medium containing 10% FCS, and after 15–17 hr of serum stimulation, cells that had progressed into the late S and early G_2 phases were treated with a minimum effective dose of nocodazole for an additional 5 hr. Arrested cells exhibited a 4N DNA content (Fig. 2; Table 2, 0 hr), and the vast majority synchronously entered G_1 after nocodazole was removed (Fig. 2, 4.5 hr). However, ~10% of the cells did not revert to a 2N DNA content after nocodazole release (Table 2), reflecting some toxicity of the drug. Moreover, not all 2N cells were able to traverse G_1 after release, and some cell death was observed during the course of the experiment.

In spite of these difficulties, >60% of parental rat-2 cells and cyclin D1 overexpressers progressed through the cell cycle. Parental rat-2 cells entered S phase between 10 and 12 hr after nocodazole release, whereas those expressing high levels of cyclin D1 (rat-$D1^{hi}$) exhibited a significantly shortened G_1 interval and entered S phase after only a 6-to 8-hr delay (Fig. 2; Table 2). Cells expressing lower levels of cyclin D1 (rat-$D1^{lo}$) yielded an intermediate result, entering S phase 8–10 hr after reversal of the nocodazole blockade. Parental rat-2 cells completed the cell cycle and were maximally mitotic ~20 hr later (as determined by microscopic enumeration of metaphase chromosome spreads), but the generation time of cells overexpressing cyclin D1 was shortened by 2 (rat-$D1^{lo}$) to 3 (rat-$D1^{hi}$) hr, reflecting their respectively abbreviated G_1 intervals. Therefore, premature entry of the cells into S phase did not result in a compensatory prolongation of the DNA replicative phase. G418-resistent rat-2 cells transfected with a vector expressing *neo* alone yielded results indistinguishable from those of untransfected cells. A similar 2.5-hr contraction of the G_1 interval relative to vector-transfected controls was observed using NIH-3T3 cells overexpressing cyclin D1 (data not shown).

Although Figure 2 and Table 2 illustrate only a single representative data set for three cell lines, the comparative results were highly reproducible and significant (see Analysis of cell cycle kinetics, Materials and methods). That is, rat-2 or NIH-3T3 cells overexpressing high levels of cyclin D1 always exhibited equivalently faster rates of entry into S phase (2.4 ± 0.6 hr) and mitosis than

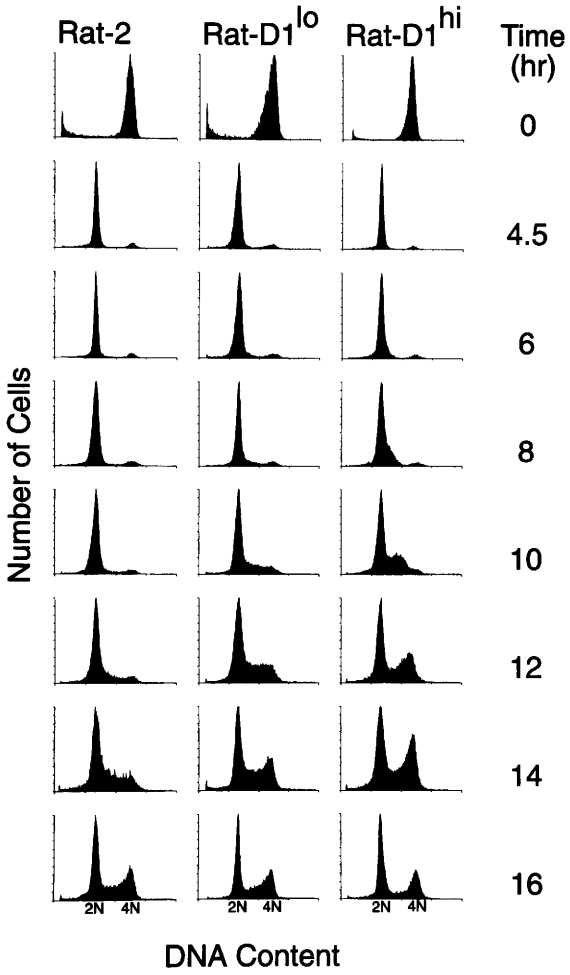

Figure 2. Kinetics of cell cycle progression after reversal of a mitotic blockade. Parental rat-2 cells (*left*) or derivatives expressing relatively low (*middle*) or high (*right*) levels of cyclin D1 were collected at metaphase (time 0) and released from a nocodazole-induced arrest. Cultures harvested at the indicated times after release of the block were analyzed by flow cytometry for DNA content. The channel positions for 2N (G_1) and 4N (G_2/M) cells are noted at the bottom of each column. Cells with DNA content between 2N and 4N represent the S-phase population. The numerical distribution of G_1, S, and G_2/M phase fractions for time intervals through 20 hr after release of the block is given in Table 2. Peak mitotic indices (determined by enumeration of metaphase spreads) for the rat-2, rat-D1^{lo}, and rat-D1^{hi} populations were obtained after 20, 18, and 17 hr, respectively, indicating that the shortened G_1 intervals observed for cyclin D1 overexpressers translated to a shorter overall generation time. Rat-2 cells transfected with the naked *neo* vector were indistinguishable from untransfected parental cells.

parental cells in six independent determinations, even though the exact transit times varied slightly in different experiments. Consistent kinetic data were obtained when incorporation of BrdU into replicating DNA was used to determine the onset of DNA replication (data not shown, but see below). The probability that these differences were attributable to chance alone is low ($P \leq 0.05$). In three experiments, cyclin D2 also shortened G_1- to S-phase transit times by ~1 hr, but the observed differences may not be significant.

Cyclins D1 and D2 accelerate G_0- to S-phase transit in rodent fibroblasts

We then studied the interval from G_0 to S phase. Confluent serum-starved cells in G_0 were trypsinized and replated in medium containing 10% FCS, and their entry into S phase was monitored both by incorporation of BrdU into replicating DNA or by flow cytometric analysis of DNA content. As calculated from flow cytometric analyses (Fig. 3A), parental NIH-3T3 cells began to enter S phase 12 hr after serum restimulation, whereas the derivative cell line overexpressing cyclin D1 initiated DNA synthesis ~2.5 hr faster. Similar data were obtained by measurements of BrdU uptake in independent experiments (e.g., see Fig. 4A, below); and in multiple determinations, the differences observed between overexpressers and vector-transfected control cells were highly significant ($P \leq 0.05$). As judged by enumerating metaphases later in the cell cycle, NIH-3T3 cells overexpressing cyclin D1 again exhibited a 3-hr shorter mean generation time, corresponding to their contracted G_1 intervals.

Rat-2 cells overexpressing cyclin D1 (rat-D1^{hi}) also exhibited a 2- to 3-hr shortening of their G_0- to S-phase transit time (Fig. 3B) and peak mitosis relative to parental cells or to those transfected with the control *neo* vector. Here, however, the measurements were complicated by the fact that rat-2 cells have a faster generation time than NIH-3T3 cells (e.g., cf. Figs. 3B and 4A) and lose synchrony more rapidly, so that the maximum percentage of cells in S phase 15.5 hr after serum restimulation was only 65%. Even with the parental rat-2 cell line, it was evident that as a many as 20% of cells remained in S phase at the time (22 hr) when peak mitosis was observed. An additional problem was that as many as 15% of the cyclin D1 overexpressers remained in cycle when deprived of serum, as opposed to <5% of the parental rat-2 cells, and these had a DNA content of >2N at the start of the experiment. Nonetheless, a comparison of the results here (Fig. 3B) with those shown for nocodazole arrest (Fig. 2) indicates that the G_0- to S-phase intervals for cells overexpressing cyclin D1 were ~3 hr longer than their respective G_1 to S transit times, reflecting the additional time required for the cells to reenter the cell cycle from quiescence. In multiple determinations, cyclin D1 overexpression contracted both the G_0 to S (2.2 ± 0.8 hr) and G_1 to S (2.4 ± 0.6 hr) intervals by similar extents, suggesting that it did not affect the G_0 to G_1 transition.

Rodent fibroblasts overexpressing cyclin D2 also manifested a significantly shortened G_0- to S-phase interval (1.6 ± 0.6 hr) with earlier entry into mitosis (~2 hr) relative to the parental cells (Fig. 3). The impression that cyclin D2 was less potent than D1 in shortening transit from G_0 to S may simply reflect the levels of the overexpressed proteins in these cell lines, rather than a biological difference between them. The apparently more

Table 2. *Cell cycle progression after mitotic arrest*

Time (hr)	Rat-2			Rat-D1^{lo}			Rat-D1^{hi}		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
0	0.3	12.0	87.7	0.4	37.7	61.9	0.1	23.6	76.2
4.5	80.5	10.9	8.6	85.0	8.8	6.2	85.5	8.5	5.9
6	81.3	10.9	7.9	84.9	7.5	7.6	79.6	14.5	5.8
8	86.1	8.5	5.4	76.2	16.5	7.3	70.3	26.2	3.5
10	83.3	10.8	5.9	67.3	27.7	5.0	56.3	41.7	2.0
12	71.4	22.8	5.8	55.3	40.8	3.8	47.5	42.8	9.7
14	52.9	42.4	4.7	38.8	42.5	18.7	35.5	48.9	15.5
16	35.7	45.2	19.1	44.7	34.1	21.3	38.3	40.7	21.0
18	40.0	37.0	23.0	48.5	24.8	26.7	50.5	30.9	18.6
20	63.3	14.7	21.8	63.9	15.9	20.2	71.7	12.8	15.5

Cells released from nocodazole arrest were allowed to reenter G_1, and the fractions of cells in different phases of the cell cycle were computed from flow cytometric histograms of DNA content (Fig. 2) using the computer program PEAK (Dean 1980a, b). Untransfected rat-2 cells initiated DNA synthesis between 10 and 12 hr after release of the block. Rat-D1^{lo} cells began to enter S phase by 8 hr, and the rat-D1^{hi} population initiated DNA replication by 6–8 hr. As expected, peak mitotic indices (determined by enumeration of metaphase spreads) for the rat-2, rat-D1^{lo}, and rat-D1^{hi} populations were obtained after 20, 18, and 17 hr, respectively, slightly after the observed G_2/M maxima calculated from flow cytometric analysis.

limited effect of cyclin D2 on the G_0 to S interval was consistent with results obtained after release of a nocodazole block, where its effect versus that of D1 was also less obvious.

Because overexpression of D-type cyclins in fibroblasts shortened both their G_1 intervals and generation times, their effects on the cell cycle distribution of asynchronously dividing cells are predicted to be relatively small. Even in the best case using rat-D1^{hi} cells, as much as a 25% contraction of the G_1 interval coupled with a proportionate decrease in total cell generation time (15% overall, as confirmed by growth curves) would reduce the G_1 fraction of asynchronously dividing cyclin D overexpressers only by ~8%. In agreement, an extended series of independent flow cytometric measurements of DNA content for asynchronously growing rat-D1^{hi} overexpressers revealed that their average G_1 fraction was reduced by 6 ± 3% relative to their parental counterparts. It would be anticipated that cells exhibiting a less evident contraction in their G_1 interval would be virtually indistinguishable from their parental counterparts in this regard when examined as asynchronous populations.

Cells overexpressing cyclins D1 and D2 manifest a reduced serum dependency

Although rodent fibroblasts overexpressing cyclins D1 and D2 required serum for G_1 progression, it was more difficult to arrest cells overexpressing D-type cyclins in G_0 by lowering their serum concentration (see above). To study their serum dependence, subconfluent NIH-3T3 cells were growth arrested for 36 hr in medium containing 0.1% FCS and then restimulated with medium containing different serum concentrations. Using BrdU labeling to quantify DNA synthesis, we observed that parental NIH-3T3 cells, as well as those transfected with the control *neo* vector, entered S phase ~10.5 hr after

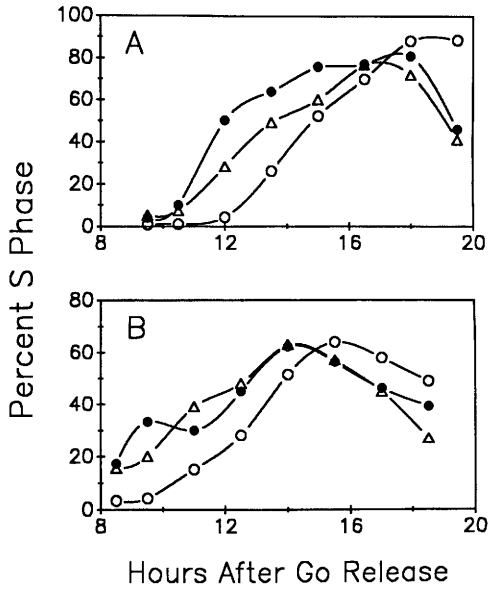

Figure 3. Cell cycle progression after serum stimulation of G_0-arrested cells. NIH-3T3 (*A*) and rat-2 (*B*) cell lines and their derivatives expressing cyclins D1 or D2 were rendered quiescent by serum starvation and then restimulated to enter the cell cycle. The percentages of cells in S phase for each cell line at various times after serum stimulation were calculated from flow cytometric measurements of DNA content during the ensuing cycle. (*A*) (○) Parental NIH-3T3 cells; (●) 3T3-D1; (△) 3T3-D2. (*B*) (○) Parental rat-2 cells; (●) rat-D1^{hi}; (△) rat-D2^{hi}. Entry into mitosis (as determined from enumeration of metaphase spreads) for NIH-3T3, 3T3-D1, and 3T3-D2 were observed 21, 18, and 19.5 hr, respectively, after serum stimulation. Maximal mitoses were observed for rat-2, rat-D1^{hi}, and rat-D2^{hi} populations after 22, 19, and 19 hr, respectively. Cells transfected with the control *neo* vector behaved indistinguishably from the parental cell lines.

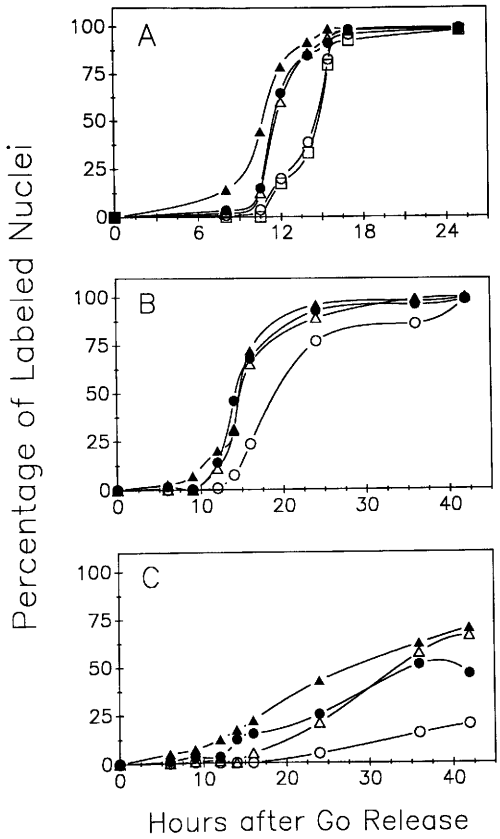

Figure 4. Serum dependence for S-phase entry. NIH-3T3 cells and derivative populations overexpressing D-type cyclins were rendered quiescent by starvation in 0.1% serum for 36 hr and then restimulated with fresh media containing either 10% (*A*), 2% (*B*), or 0.2% (*C*) FCS. Entry of cells into S phase was determined by incorporation of BrdU, which was added to the cultures immediately after release from G_0. (○) NIH-3T3 cells transfected with the control *neo* vector; (□) parental NIH-3T3 cells; (●) 3T3-D1; (△) 3T3-D2; (▲), NIH-3T3 cells cotransfected with cyclin D2 plus cdk4. Note the different scales for the abscissa in *B* and *C* vs. *A*. Although all cells manifested delayed entry into S phase after stimulation with 2% serum (*B*), the relative rates of entry for different cell lines were generally maintained (cf. *B* and *A*). Not all cells stimulated with 0.2% FCS entered S phase, but a much higher proportion of cyclin D overexpressers than control cells were replicating DNA 35–40 hr after stimulation.

stimulation with 10% serum (Fig. 4A). Incorporation of BrdU into replicating DNA unambiguously identifies cells in early S phase by virtue of their nuclear labeling; and because this method is somewhat more sensitive than measurements of DNA content in scoring the onset of DNA replication, it yields reduced values for G_0 to S transit times (e.g., cf. Figs. 4A and 3A). Nonetheless, in this experiment, cells overexpressing cyclins D1 and D2 again entered S phase ~3 hr earlier than parental cells or those transfected with the control *neo* vector. Cells coexpressing exogenous cyclin D2 and cdk4 exhibited an even faster onset of DNA replication (Fig. 4A), but here, we could not distinguish whether the added impetus was attributable to ectopic expression of cdk4 or, rather, to the synthesis of even higher levels of cyclin D2 (Fig. 1B, lane 9) than those in cells transfected with the cyclin gene alone (Fig. 1B, lane 6).

When restimulated with 2% FCS, each of the cell lines entered S phase more slowly, but the temporal relationships between them were maintained (Fig. 4B). As the serum concentration was lowered to 0.2%, most of the parental cells no longer entered S phase, but a much higher proportion of cells overexpressing D-type cyclins continued to replicate DNA (Fig. 4C). Analysis of growth curves indicated that as the serum concentration was decreased, the doubling time for control cells and cyclin D overexpressers declined in parallel (data not shown), consistent with the data shown in Figure 4. Cells overexpressing cyclin D1 or D2 are therefore not oncogenically transformed. They remain dependent on serum for proliferation, do not form colonies in semisolid medium (data not shown), and are contact inhibited, even though they exhibit a reduced serum dependency.

Cells overexpressing cyclins are reduced in size

Microscopic analysis of confluent cultures suggested that cells overexpressing cyclin D1 were smaller than parental cells or those transfected with the control *neo* vector, and their apparent saturation density appeared to be somewhat higher. When quiescent, contact-inhibited cells were trypsinized and single cell suspensions were analyzed in a Coulter counter, the modal cell volumes of fibroblasts overexpressing cyclin D1 were reduced compared with their parental counterparts, with rat-2 derivatives showing greater differences (Fig. 5). Only minimal modal size differences between rat-D2 and parental control populations were seen, and the corresponding NIH-3T3 counterparts were indistinguishable from one another (for modal cell volumes, see the legend to Fig. 5). NIH-3T3 cells overexpressing higher levels of D2 together with cdk4 were smaller than controls, but this result is again interpreted less easily, given the enforced coexpression of the catalytic subunit. Parallel measurements of asynchronously growing cells yielded similar percentage differences in modal size between overexpressers and parents, although the overall volumes of growing cells were uniformly larger. Thus, although both cyclin D1 and D2 overexpressers exhibited contracted G_0- to S-phase intervals and a reduced serum dependency, the effects of D2 alone on these parameters and on cell size were less dramatic.

Inhibition of cyclin D1 function inhibits S-phase entry

Baldin et al. (1993) recently demonstrated that microinjection of antibodies to cyclin D1 into human lung fibroblasts prevented their entry into S phase. Progression of cells injected during mid-G_1 was inhibited, whereas those injected just prior to G_1/S were unaffected and initiated DNA synthesis. To verify that rodent fibroblasts or derivatives overexpressing cyclin D1 require the protein for G_1 progression, cells were made quiescent by serum starvation; restimulated with medium con-

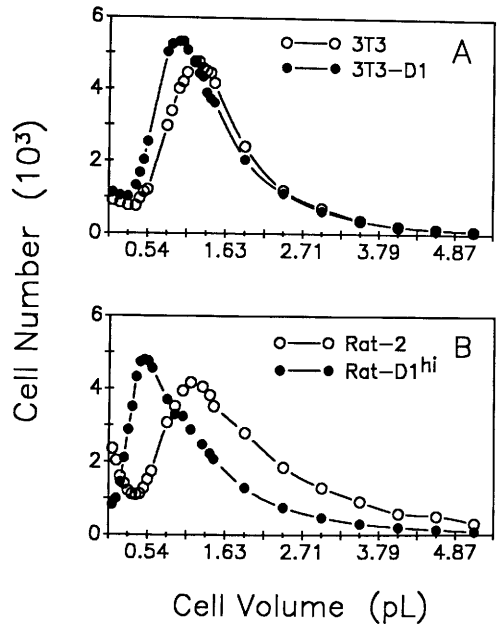

Figure 5. Volume of confluent cells. Cell lines were grown to confluence, trypsinized, and analyzed on a Coulter counter. The modal cell volumes (10^{-12} liters) were NIH-3T3 (1.3 ± 0.14); NIH-3T3, *neo*-resistant (1.17 ± 0.06); 3T3-D1 (1.04 ± 0.09); 3T3-D2 (1.27 ± 0.17); 3T3-D2/cdk4 (0.95 ± 0.07); rat-2 (1.08 ± 0.14); rat-2, *neo*-resistant (1.24 ± 0.14); rat-D1^{hi} (0.51 ± 0.08); and rat-D2^{hi} (0.98 ± 0.15).

taining 10% FCS; and at different times thereafter, were microinjected with a purified monoclonal antibody to mouse cyclin D1 that does not cross-react with cyclins D2 or D3. As a control, parallel cultures were injected with an isotype-matched antibody to a T lymphocyte cell-surface antigen (CD3) that is not expressed in fibroblasts. BrdU was added to the cells at the time of serum stimulation, and its incorporation into DNA was measured 20 hr later. Microinjections of cyclin D1 antibody into NIH-3T3 cells (Fig. 6A) or rat-2 cells (Fig. 6B) either 3 or 6 hr after serum stimulation prevented the majority from entering S phase, but injections performed near the G_1/S-phase boundary were no longer inhibitory. Similar results were obtained with cell lines overexpressing cyclin D1, indicating that its G_1 accelerating effect could be overcome and that S-phase entry remained dependent on its function (Fig. 6). Therefore, cyclin D1 overexpressers could not have sustained secondary mutations that were themselves responsible for the observed alterations in cell cycle phenotype. Because the G_0 to G_1 transition in these cells lasts no longer than 3–4 hr (cf. Figs. 2 and 3), the ability of the cyclin D1 antibody to inhibit S-phase entry when injected as late as 6 hr after serum stimulation confirms that this cyclin must play a rate-limiting function during G_1.

Discussion

Until recently, evidence to support the putative role of D-type cyclins in regulating G_1-specific cell cycle transitions has been indirect. First, D-type cyclins are normally synthesized during the delayed early response to growth factor stimulation in many cell types (Matsushime et al. 1991a,b; Cocks et al. 1992; Won et al. 1992; Ajchenbaum et al. 1993). In macrophages, cyclin D1 synthesis is nonperiodic and persists throughout the cell cycle as long as colony-stimulating factor 1 is continuously present; but because both D1 mRNA and protein are unstable, its level declines rapidly following premature growth factor withdrawal during G_1, correlating with the failure of cells to enter S phase (Matsushime et al. 1991a). Cyclin D1 protein synthesis is regulated similarly in response to serum in fibroblasts. The D-type cyclins therefore appear to act as growth factor sensors rather than cell cycle-regulated gene products, and on this basis, we proposed that they would function during the G_1 interval. Second, cyclins D1 and D2 are targets of chromosomal translocations, gene amplification, and retroviral insertional activation in a variety of rodent and human tumors (Lammie et al. 1991, 1992; Motokura et al. 1991; Rosenberg et al. 1991; Withers et al. 1991; Hanna et al. 1993; Jiang et al. 1992; Bianchi et al. 1993), suggesting that they act as positive growth regulators. Third, the D-type cyclins can physically associate with hypophosphorylated forms of the retinoblastoma gene product (pRb) (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993), a tumor suppressor protein that can prevent

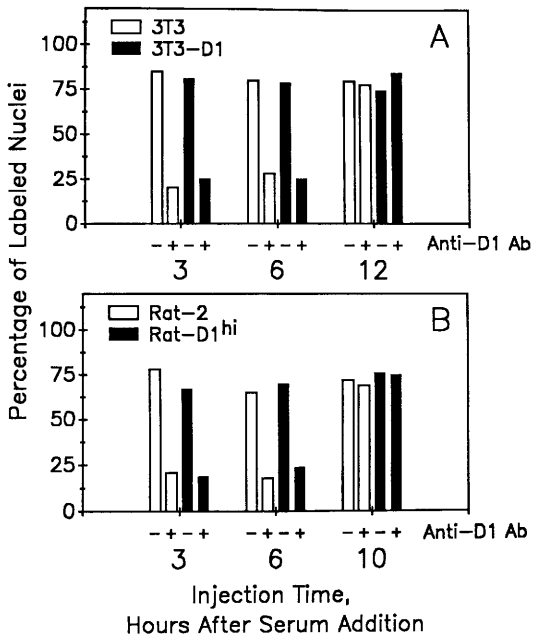

Figure 6. Microinjection of antibodies to cyclin D1 arrests S-phase entry. Parental fibroblasts and derivatives overexpressing cyclin D1 were arrested by serum starvation and restimulated to enter the cell cycle with medium containing 10% serum. Cells were microinjected in their cytoplasm either with a control antibody to CD3 antigen (−) or with antibody to cyclin D1 (+) at 3, 6, and 10 or 12 hr after serum stimulation. BrdU was added to the cells with serum, and its incorporation into replicating DNA was measured after 20 hr.

G_1 exit (Weinberg 1991). Introduction of cyclin D2 together with *RB* into *RB*-negative human Saos-2 cells results in pRb hyperphosphorylation and abrogates its ability to prevent the onset of DNA synthesis (Ewen et al. 1993), whereas cyclin D1, which overrides pRb function less efficiently (Hinds et al. 1992), appears to do so via another mechanism (Dowdy et al. 1993). Fourth, disruption of cyclin D1 function by microinjection of specific antibodies or antisense plasmids into serum-stimulated human fibroblasts prevents their entry into S phase (Baldin et al. 1993). Because injections performed during mid-G_1 phase were inhibitory but those at G_1/S were not, cyclin D1 function must be required after the G_0 to G_1 transition and before the onset of DNA synthesis.

As shown here, overexpression of cyclin D1 in rodent fibroblasts can accelerate G_1 progression by several hours, and these effects could also be reversed by microinjection of specific antibodies to the protein. A contraction of the G_1 interval was observed in cells exiting mitosis from a nocodazole-induced block similar to quiescent cells entering G_1 phase after serum restimulation, consistent with the notion that overexpression of cyclin D1 affected G_1 to S phase, but not G_0 to G_1 phase, progression. In the case where independently derived rat fibroblast populations expressing relatively high or low levels of cyclin D1 were compared, those expressing more of the protein manifested shorter G_1 transit and correspondingly reduced mean generation times. Because overexpressers move more rapidly through the cell cycle, only minimal decreases in the G_1 fraction were observed in asynchronously proliferating populations. These cells manifested a reduced serum requirement for S-phase entry and were smaller in size. By use of an inducible system, others have observed an identical phenotype in rat-1 cells engineered to overexpress cyclin D1 (D. Resnitzky and S. Reed, pers. comm.).

Cells overexpressing cyclin D2 also showed a significantly reduced G_0- to S-phase interval following serum stimulation and were less serum dependent, but changes in cell size were not as evident. NIH-3T3 cells expressing exogenous cyclin D2 plus cdk4 entered S phase more rapidly from quiescence than those expressing cyclin D2 alone, but these effects might either be attributed to collaboration between cyclin D2 and the overexpressed catalytic subunit or to the increased cyclin D2 levels observed in the doubly transfected cells. Normally, the D-type cyclins are differentially expressed in various cell lineages, implying that they are not strictly redundant. Cyclins D1 and D2 can exert independent effects on pRb function (Dowdy et al. 1993; Ewen et al. 1993) and interact combinatorially with various cdk partners (Matsushime et al. 1992; Xiong et al. 1992; Ewen et al. 1993; Kato et al. 1993). Although it is unlikely that they function equivalently, cyclin D1 and most likely D2 are rate limiting in fibroblasts during G_1.

Several caveats in the interpretation of these findings need to be emphasized. First, we were initially unable to obtain detectable ectopic overexpression of cyclin D1 in rodent fibroblasts using a series of mammalian retroviral vectors that have been widely used to express other genes. In these cases, the numbers of G418-resistant colonies obtained after transfection were greatly reduced relative to cells transfected with the corresponding *neo*-containing control vectors. Therefore, expression of cyclin D1 from what we assume are stronger promoters than the avian RSV LTR might have been toxic to transfected rodent fibroblasts. pRc/RSV–*neo*, however, is not unique in its ability to confer ectopic cyclin D expression, because we have been able to overexpress moderate levels of D-type cyclins in rat-2 fibroblasts, mouse myeloid 32Dcl3 cells, and BAC1.2F5 mouse macrophages using an artificial heavy metal-responsive promoter. Transfections utilizing the pRc/RSV–*neo* vector did not reduce the frequency of G418-resistant colonies and gave rise to thousands of transformants that were pooled before analysis. We reasoned that such polyclonal cell populations, independently derived from multiple transfection experiments with two parental cell lines, would be unlikely to have consistently sustained complementing mutations that might facilitate cyclin expression or contribute directly to the phenotypes observed. The ability of microinjected antibodies to cyclin D1 to prevent S-phase entry in cells overexpressing the protein provides further evidence that secondary mutations that bypassed cyclin function had not occurred.

Another concern is that the ability of D-type cyclins to alter G_1 progression might reflect functional substitution for another cyclin that normally plays this role. In budding yeast, for example, the enforced expression of several mammalian cyclins can complement G_1 cyclin (CLN) deficiency, and in this system, mammalian (but not yeast) mitotic cyclins paradoxically proved more effective than D-type cyclins in rescue, indicating that cyclin overexpression can result in a loss of specificity (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991). Overexpression of cyclin E in rat-1 or human foreskin fibroblasts also shortens their G_1 interval, decreases cell size, and reduces their serum requirement for the G_1- to S-phase transition (Ohtsubo and Roberts 1993), leaving open the possibility that D-type cyclins might override a function that is physiologically regulated by cyclin E, or vice versa. Whereas the generation time of cyclin E overexpressers was unchanged because of a compensatory lengthening of S phase (Ohtsubo and Roberts 1993), cells overexpressing cyclins D1 and D2 entered mitosis somewhat faster. These apparent differences in phenotype are probably significant, but cyclins D and E need to be tested in parallel with similar vectors and cell lines.

Overexpression of cyclin D or E does not lead to fibroblast transformation in the sense that the cells remain serum dependent, contact inhibited, and unable to form colonies in semisolid medium. Preliminary results (D.E. Quelle and M.F. Roussel, unpubl.) indicate that cells overexpressing cyclin D1 do not form tumors in nude mice. Why, then, is cyclin D1, observed to be a frequent target of specific gene rearrangements in tumor cells? Although the shortening of cell generation time and diminished serum dependency should provide overexpressers with a modest growth advantage, this in itself would be unlikely to induce tumor formation in vivo,

and other collaborating changes are therefore likely to contribute to frank neoplasia. In a different biologic setting, the propensity of D-type cyclins to promote G_1 progression might override programs for cell cycle arrest and differentiation. Results with growth factor-dependent myeloid precursors have now indicated that overexpression of cyclins D2 and D3, but not D1, prevent granulocyte differentiation (J-Y. Kato and C.J. Sherr, in prep.).

If both cyclins D and E independently control the rate of G_1 progression through the mammalian cell cycle, they presumably regulate different G_1 transitions. In general, D-type cyclins appear earlier in the cell cycle than cyclin E, and the timing of appearance of the latter is periodic and corresponds most closely to the G_1/S transition (Lew et al. 1991; Dulic et al. 1992; Koff et al. 1992). Passage through the late G_1 restriction point results in a reduced growth factor requirement (Pardee 1989) and, ultimately, the onset of S phase, which occurs several hours later. Possibly, the functions of D- and E-type cyclins are mechanistically linked in controlling sequential events. For example, restriction point control and the initiation of DNA synthesis should involve discrete decisions, each of which could be rate limiting for G_1 progression or exit. Perhaps, D-type cyclins act upstream of cyclin E as targets of growth factor-induced signals, whereas cyclin E might govern the actual onset of DNA replication. Whatever the interpretation, both classes of genes appear to be bona fide mammalian G_1 cyclins that control rate-limiting events during this phase of the cell cycle.

Materials and methods

Expression vectors and transfection procedures

Complete coding sequences of cyclins D1, D2, and D3 and cdk4 (Matsushime et al. 1991a,b, 1992) were excised from plasmids, blunt ended with the Klenow fragment of DNA polymerase I, ligated to oligonucleotide adaptors, and recloned into mammalian expression vectors. In pRc/RSV–*neo* (Invitrogen, San Diego, CA), inserted cDNAs are driven by the RSV LTR and the *neo* gene by the SV40 early promoter. In pZEN–tk*neo* (Johnson et al. 1989) and COS–MSV–tk*neo* (Muller et al. 1991), the inserted cDNAs are transcribed from the myeloproliferative sarcoma virus or MuLV LTRs, respectively, whereas *neo* is driven by the thymidine kinase (tk) promoter; the plasmid backbone of COS–MSV–tk*neo* contains an SV40 origin, which enables its amplification in T antigen-positive COS monkey cells. In pBC140 (constructed and provided by Drs. Thomas von Ruden and Erwin Wagner, Institute of Molecular Pathology, Vienna, Austria), the orientation of the two transcription units is reversed with *neo* transcription being controlled by the MuLV LTR and the inserted gene being driven by an internal CMV promoter. In pZIP*neo*SV(X)I (Cepko et al. 1984), all transcripts arise from the MuLV LTR, with the inserted gene encoded by the full genome length mRNA and *neo* specified by a spliced subgenomic transcript. The latter four vector plasmids can generate infectious virions when transfected into retroviral packaging cell lines that encode viral structural proteins in *trans*.

Cells were transfected by use of a modified calcium phosphate precipitation technique (Chen and Okayama 1987) with 20 μg of input plasmid DNA per dish and were selected for 3 weeks in complete culture medium with 0.8 mg/ml of G418 (Geneticin; GIBCO, Grand Island, NY). Several thousand drug-resistant colonies from each independent transfection were pooled, expanded, and frozen as seed stocks. After 2 months in continuous culture, cells were replaced from seed stocks to avoid clonal selection.

Cells and culture conditions

Mouse NIH-3T3 and rat-2 fibroblasts and their transfected derivatives were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. To assess the rate of cell cycle progression following G_0 arrest, cells grown to confluence were starved in medium containing 0.1% FCS for 30–36 hr, and quiescent cells were trypsinized and replated in complete medium at subconfluent densities. To enrich for mitotically arrested cells, cultures seeded at 1×10^6 cells per 150-mm-diam. dish were first starved in medium containing 0.1% FCS for 30–36 hr to induce quiescence. Complete medium containing 10% FCS was added to bring the cells synchronously into cycle, and incubation was continued for 15–17 hr, enabling their entry into late S and early G_2 phase (as confirmed by flow cytometric analysis of DNA content). Nocodazole (0.07 μg/ml for rat-2 and 0.4 μg/ml for NIH-3T3 cells) was then added for 5 hr, and rounded cells arrested in mitosis were detached by gentle pipeting. Cells were washed free of the drug with phosphate-buffered saline (PBS) and replated at subconfluence in complete medium. Cells progressing through the cell cycle after reversal of G_0 or mitotic blockades were harvested at the intervals shown in the figures, and their positions in the cell cycle were determined.

Analysis of cell cycle kinetics

For flow cytometric analysis of DNA content, $\sim 1 \times 10^6$ cells were detached from culture dishes by trypsinization, diluted 1 : 1 in complete medium, and collected by low speed centrifugation at 23°C. The cell pellet was resuspended in a 1-ml solution of 0.1% sodium citrate and 0.1% Triton X-100 containing 50 μg/ml of propidium iodide and treated with 1 μg/ml of RNase (Calbiochem, San Diego, CA) for 30 min at 23°C. Samples were filtered through 44-μm nylon mesh immediately before analysis by flow cytometry. Fluorescence >570 nm emitted from the propidium iodide–DNA complex in each cell nucleus was measured with a Coulter EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL), with laser output adjusted to deliver 500 mW at 488 nm. Approximately 50,000 cells were examined for each sample with a flow rate of 300–500 cells per second. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the computer program PEAK (Dean 1980a,b).

Cell cycle progression into S phase was also monitored by incorporation of BrdU into newly synthesized DNA. Synchronized cells were stimulated to enter the cell cycle in complete medium supplemented with 100 μM BrdU (Sigma Chemicals, St. Louis, MO) and plated into 8-well slides at 1×10^5 cells per ml. For detection of BrdU incorporation, cells were washed once with PBS, fixed for 5 min at 4°C with 100% methanol, and permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. After two more washes with PBS, cells were incubated for 10 min at room temperature in 1.5 M HCl and then washed four more times with PBS. Cells were incubated for 30 min at 37°C in a humidified chamber with mouse monoclonal antibodies to BrdU (1 : 3 dilution, Oncogene Sciences, Manhasset, NY) and, after three PBS washes, with fluorescein-conjugated antiserum to mouse immunoglobulin (1 : 50 dilu-

tion; Sigma Chemicals). After washing three times with PBS, cells were wet mounted with Permafluor (Lipshaw), and the labeled nuclear fractions were enumerated under a fluorescence microscope.

To determine when synchronized cell populations reinitiated mitosis, metaphase chromosome spreads were prepared from cells harvested at different intervals after release from a G_0 or mitotic block. Approximately 5×10^4 cells were collected by centrifugation, swollen for 15 min in 1 ml of 0.075 M KCl, and 0.2 ml of ice-cold Carnoy's solution [3 parts methanol, 1 part glacial acetic acid (vol/vol)] was added. Cells were centrifuged, resuspended in 0.2 ml of Carnoy's solution on ice, and samples were pipeted onto a glass slide, blown dry, and stained with Giemsa solution. Metaphases per total number of nuclei were counted under a light microscope. As expected, maximal mitotic indices in these experiments were achieved ~1 hr later than peak G_2/M levels determined by flow cytometry.

Cyclin D1 (six experiments) and D2 (six experiments) overexpressers always exhibited accelerated entry into S phase from G_0 as compared with parental or vector-transfected control cells. The contraction of the G_0- to S-phase interval was 2.2 ± 0.8 hr for cyclin D1 and 1.6 ± 0.6 hr for cyclin D2. Although the exact times of G_1 transit varied slightly between individual experiments, the probability that the observed differences were attributable to chance alone is ≤ 0.05 (Wilcoxon's test and Sign test). In a similar series, cyclin D1 overexpressers also showed a reduced G_1 to S interval relative to control cells (2.4 ± 0.6 hr, $P \leq 0.05$), which was not significantly different from the contraction of their G_0 to S interval. Therefore, we have no evidence for a role of cyclin D1 in G_0 to G_1 progression. Although cyclin D2 shortened G_1 to S (from a nocodazole block) by ~1 hr, the number of experiments (three) was insufficient to conclude that these populations differed significantly from parental control cells.

Other analytical procedures

The clonality of pooled, *neo*-resistant populations was assessed by Southern blotting analyses of cellular DNAs performed with a *neo* probe. In brief, DNAs were either digested with restriction endonucleases that recognize vector DNA sites flanking the *neo* gene or with an enzyme that cuts 5′ to *neo* and releases the gene linked to host cellular DNA sequences. In the first instance, a single *neo*-containing fragment was observed, whereas in the second case, a smear of fragments was detected, which hybridized at lower intensity. Therefore, each transfected population contained multiple vector integrations and was polyclonal.

For biosynthetic studies, 1×10^6 exponentially growing cells were labeled for 1 hr with 0.2 mCi/ml of [^{35}S]methionine (Amersham, 800 Ci/mmole) in 1 ml of methionine-free medium supplemented with 10% FCS. Labeled cells were lysed for 30 min on ice in 1 ml of EBC buffer (50 mM Tris-HCl at pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 5 μg/ml of aprotinin, 5 μg/ml of leupeptin, and 1 mM PMSF (protease inhibitors from Sigma Chemicals). Lysates were clarified by centrifugation, and the cleared supernatants were incubated overnight at 4°C with the indicated antibodies. After incubation for 1 hr at 4°C with protein A–Sepharose beads, immunoprecipitates were collected by centrifugation, washed three times in EBC buffer at 4°C, and denatured in sample buffer for electrophoresis on polyacrylamide slab gels containing SDS as described (Anderson et al. 1984). Samples separated on 12.5% gels were visualized by autoradiography. Immunoblotting analyses were performed with rabbit antisera prepared against full-length cyclins or cdk4 produced in bacteria (Matsushime et al. 1991a, 1992). Immunoprecipitated proteins separated on 10% polyacrylamide gels containing SDS were transferred to nitrocellulose, and blocked filters were immunoblotted with the same antisera, followed by detection with ^{125}I-labeled protein A (Downing et al. 1988).

Microinjection studies and monoclonal antibody

Rat-2 or NIH-3T3 cells plated at subconfluence onto gridded coverslips (Belloc Inc.) were starved for 30 hr in medium containing 0.1% FCS and then restimulated with complete medium containing 10% FCS and BrdU. At the indicated times thereafter (Fig. 6), cells were microinjected with glass capillaries drawn to a tip diameter of <1 μm as described (Wang et al. 1982). A control antibody (OKT3) to the T lymphocyte antigen CD3 and a purified mouse monoclonal antibody specific for cyclin D1 were suspended at 3 mg/ml in 0.5× PBS, and $\sim 10^{-14}$ liters of either solution was introduced per cell. Each injected area contained 30–50 cells per time point per experiment and was identified by grid number. At 20 hr after serum stimulation, cells were washed twice with PBS, fixed with acid alcohol (ethanol/acetic acid, 95 : 5) for 20 min at 20°C, and BrdU incorporation into DNA was measured immunochemically (Amersham Cell Proliferation Kit) by use of a monoclonal antibody to BrdU and peroxidase-conjugated goat anti-mouse antibodies. The peroxidase substrate consisted of diaminobenzidine, which in the presence of cobalt and nickel gave blue–black staining at sites of BrdU incorporation. The results shown represent the averages of three independent experiments with each cell line.

The antibody used here was generated and purified from serum-free hybridoma culture supernatants using methods similar to those described previously (Anderson et al. 1982). The monoclonal immunoglobulin G to cyclin D1 reacts specifically with the murine protein but not with its human cognate or with mouse cyclins D2 and D3. The antibody is active in immunoprecipitation and immunoblotting and is useful for detecting cyclin D1 in fixed permeabilized nuclei of mouse cells synthesizing the protein.

Acknowledgments

We thank Dr. Horng-Mo Lee for his help in deriving the mouse monoclonal antibody to cyclin D1, Carol Bockhold and Shawn Hawkins for assistance with experiments, and the other members of our laboratory for helpful suggestions and criticisms. This work was supported in part by National Institutes of Health grants CA-47064 (C.J.S.) and CA-55360 (D.B.); by Cancer Center core grant CA-21765; and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Ajchenbaum, F., K. Ando, J.A. DeCaprio, and J.D. Griffin. 1993. Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. *J. Biol. Chem.* **268:** 4113–4119.

Anderson, S.J., M. Furth, L. Wolff, S.K. Ruscetti, and C.J. Sherr. 1982. Monoclonal antibodies to the transformation-specific glycoprotein encoded by the feline retroviral oncogene v-*fms*. *J. Virol.* **44:** 696–702.

Anderson, S.J., M.A. Gonda, C.W. Rettenmier, and C.J. Sherr.

1984. Subcellular localization of glycoproteins encoded by the viral oncogene v-*fms*. *J. Virol.* **51:** 730–741.

Baldin, V., J. Likas, M.J. Marcote, M. Pagano, J. Bartek, and G. Draetta. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. *Genes & Dev.* **7:** 812–821.

Bianchi, A.B., S.M. Fischer, A.I. Robles, E.M. Rinchik, and C.J. Conti. 1993. Overexpression of cyclin D1 in mouse skin carcinogenesis. *Oncogene* **8:** 1127–1133.

Cepko, C.L., B.E. Roberts, and R.C. Mulligan. 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* **37:** 1053–1062.

Chang, F. and I. Herskowitz. 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63:** 999–1011.

Chen, C. and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:** 2745–2752.

Cocks, B.G., G. Vairo, S.E. Bodrug, and J.A. Hamilton. 1992. Suppression of growth factor-induced CYL1 cyclin gene expression by antiproliferative agents. *J. Biol. Chem.* **267:** 12307–12309.

Cross, F. 1988. DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevesiae*. *Mol. Cell. Biol.* **8:** 4675–4684.

Dean, P.N. 1980a. A simplified method of DNA distribution analysis. *Cell Tissue Kinet.* **13:** 299–308.

———. 1980b. Simplified methods of analyzing DNA distributions from perturbed cell populations. Proceedings of the Cell Kinetics Society fourth annual meeting. *Cell Tissue Kinet.* **13:** 672–681.

Dowdy, S.F., P.W. Hinds, K. Louis, S.I. Reed, A. Arnold, and R.A. Weinberg. 1993. Physical interactions of the retinoblastoma protein with human cyclins. *Cell* **73:** 499–511.

Downing, J.R., C.W. Rettenmier, and C.J. Sherr. 1988. Ligand-induced tyrosine kinase activity of the colony stimulating factor-1 receptor in a murine macrophage cell line. *Mol. Cell. Biol.* **8:** 1795–1799.

Dulic, V., E. Lees, and S.I. Reed. 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* **257:** 1958–1961.

Elion, E.A., P.L. Grisafi, and G.R. Fink. 1990. FUS3 encodes a $cdc2^+$/CDC28-related kinase required for the transition from mitosis into conjugation. *Cell* **60:** 649–664.

Ewen, M.E., H.K. Sluss, C.J. Sherr, H. Matsushime, J.-Y. Kato, and D.M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73:** 487–497.

Hadwiger, J.A., C. Wittenberg, H.E. Richardson, M. de Barros Lopes, and S.I. Reed. 1989. A novel family of cyclin homologs that control G1 in yeast. *Proc. Natl. Acad. Sci.* **86:** 6255–6259.

Hanna, Z., M. Jankowski, P. Tremblay, J. Xiaoyan, A. Milatovich, U. Francke, and P. Jolicoeur. 1993. The *vin*-1 gene, identified by provirus insertional mutagenesis, corresponds to the G1-phase cyclin D2. *Oncogene* (in press).

Hartwell, L.H. 1974. *Saccharomyces cerevisiae* cell cycle. *Bacteriol. Rev.* **38:** 164–198.

Hennessy, K.M. and D. Botstein. 1991. Regulation of DNA replication during the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* **56:** 279–284.

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70:** 993–1006.

Jiang, W., S.M. Kahn, N. Tomita, Y-J. Zhang, S-H. Lu, and I.B. Weinstein. 1992. Amplification and expression of the human cyclin D gene in esophageal cancer. *Cancer Res.* **52:** 2980–2983.

Johnson, G.R., T.J. Gonda, D. Metcalf, I.K. Hariharan, and S. Cory. 1989. A lethal myeloproliferative syndrome in mice transplanted with bone marrow cells infected with a retrovirus expressing granulocyte-macrophage colony stimulating factor. *EMBO J.* **8:** 441–448.

Johnston, L.H., N.F. Lowndes, A.L. Johnson, and A. Sugino. 1991. A cell-cycle-regulated trans-factor, DSC1, controls expression of DNA synthesis genes in yeast. *Cold Spring Harbor Symp. Quant. Biol.* **56:** 169–176.

Kato, J-Y., M.F. Roussel, R.A. Ashmun, and C.J. Sherr. 1989. Transduction of human colony stimulating factor-1 receptor into interleukin-3-dependent mouse myeloid cells induces both CSF-1-dependent and factor-independent growth. *Mol. Cell. Biol.* **9:** 4069–4073.

Kato, J-Y., H. Matsushime, S.W. Hiebert, M.E. Ewen, and C.J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase, CDK4. *Genes & Dev.* **7:** 331–342.

Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J.M. Roberts. 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* **66:** 1217–1228.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J.W. Harper, S. Elledge, T. Nishimoto, D.O. Morgan, B.R. Franza, and J.M. Roberts. 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257:** 1689–1694.

Lammie, G.A., V. Fantl, R. Smith, E. Schuuring, S. Brookes, R. Michalides, C. Dickson, A. Arnold, and G. Peters. 1991. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and is linked to BCL-1. *Oncogene* **6:** 439–444.

Lammie, G.A., R. Smith, J. Silver, S. Brookes, C. Dickson, and G. Peters. 1992. Proviral insertions near cyclin D1 in mouse lymphomas: A parallel for BCL1 translocations in human B-cell neoplasms. *Oncogene* **7:** 2381–2387.

Lew, D.J., V. Dulic, and S.I. Reed. 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (CLN) function in yeast. *Cell* **66:** 1197–1206.

Matsushime, H., M.F. Roussel, R.A. Ashmun, and C.J. Sherr. 1991a. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65:** 701–713.

Matsushime, H., M.F. Roussel, and C.J. Sherr. 1991b. Novel mammalian cyclin (CYL) genes expressed during G_1. *Cold Spring Harbor Symp. Quant. Biol.* **54:** 69–74.

Matsushime, H., M.E. Ewen, D.K. Strom, J-Y. Kato, S.K. Hanks, M.F. Roussel, and C.J. Sherr. 1992. Identification and properties of an atypical catalytic subunit ($p34^{PSKJ3}$/CDK4) for mammalian D-type G1 cyclins. *Cell* **71:** 323–334.

Motokura, T., T. Bloom, H.G. Kim, H. Juppner, J.V. Ruderman, H.M. Kronenberg, and A. Arnold. 1991. A novel cyclin encoded by a *bcl*1-linked candidate oncogene. *Nature* **350:** 512–515.

Muller, A.J., J.C. Young, A-M. Pendergast, M. Pondel, N.R. Landau, D.R. Littman, and O.N. Witte. 1991. *BCR* first exon sequences specifically activate the *BCR/ABL* tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. *Mol. Cell. Biol.* **11:** 1785–1792.

Nash, R., G. Tokiwa, S. Anand, K. Erickson, and A.B. Futcher. 1988. The $WHi1^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7:** 4335–4346.

Ohtsubo, M. and J.M. Roberts. 1993. Cyclin-dependent regula-

tion of G1 in mammalian fibroblasts. *Science* **259:** 1908–1912.

Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek, and G. Draetta. 1993. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J. Cell. Biol.* **121:** 101–111.

Pardee, A.B. 1989. G1 events and regulation of cell proliferation. *Science* **246:** 603–608.

Pringle, J.R. and L.H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In, *The Molecular biology of the yeast* Saccharomyces: *Life cycle and inheritance* (ed. J.N. Strathern, E.W. Jones, and J.R. Broach), pp. 97–142. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Reed, S.I. 1991. G1-specific cyclins: In search of an S-phase promoting factor. *Trends Genet.* **7:** 95–99.

Richardson, H.E., C. Wittenberg, F. Cross, and S.I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. *Cell* **59:** 1127–1133.

Rosenberg, C.L., E. Wong, E.M. Petty, A.E. Bale, Y. Tsujimoto, N.L. Harris, and A. Arnold. 1991. PRAD1, a candidate BCL1 oncogene: Mapping and expression in centrocytic lymphoma. *Proc. Natl. Acad. Sci.* **88:** 9638–9642.

Sudbery, P.E., A.R. Goodey, and B.L.A. Carter. 1980. Genes which control cell division in the yeast *Saccharomyces cerevisiae*. *Nature* **288:** 401–404.

Tsai, L-H., E. Lees, B. Faha, E. Harlow, and K. Riabowol. 1993. The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* (in press).

Wang, K., J.R. Feramisco, and J.F. Ash. 1982. Fluorescent localization of contractile proteins in tissue culture cells. *Methods Enzymol.* **85:** 514–562.

Weinberg, R.A. 1991. Tumor suppressor genes. *Science* **254:** 1138–1146.

Withers, D.A., R.C. Harvey, J.B. Faust, O. Melnyk, K. Carey, and T.C. Meeker. 1991. Characterization of a candidate *bcl*-1 gene. *Mol. Cell. Biol.* **11:** 4846–4853.

Wittenberg, C., K. Sugimoto, and S.I. Reed. 1990. G1-specific cyclins of *S. cerevisiae*: Cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{CDC28}$ protein kinase. *Cell* **62:** 225–237.

Won, K-A., Y. Xiong, D. Beach, and M. Gilman. 1992. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. *Proc. Nat. Acad. Sci.* **89:** 9910–9914.

Xiong, Y., T. Connolly, B. Futcher, and D. Beach. 1991. Human D-type cyclin. *Cell* **65:** 691–699.

Xiong, Y., H. Zhang, and D. Beach. 1992. D-type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71:** 505–514.

Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts.

D E Quelle, R A Ashmun, S A Shurtleff, et al.

Genes Dev. 1993, **7:**
Access the most recent version at doi:10.1101/gad.7.8.1559