G1/S Control of Anchorage-independent Growth in the Fibroblast Cell Cycle

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Abstract. We have developed methodology to identify the block to anchorage-independent growth and position it within the fibroblast cell cycle. Results with NRK fibroblasts show that mitogen stimulation of the G0/G1 transition and G1-associated increases in cell size are minimally affected by loss of cell anchorage. In contrast, the induction of G1/S cell cycle genes and DNA synthesis is markedly inhibited when anchorage is blocked. Moreover, we demonstrate that the anchorage-dependent transition maps to late G1 and shortly before activation of the G1/S $p34^{cdc2}$-like kinase. The G1/S block was also detectable in NIH-3T3 cells. Our results: (*a*) distinguish control of cell cycle progression by growth factors and anchorage; (*b*) indicate that anchorage mediates G1/S control in fibroblasts; and (*c*) identify a physiologic circumstance in which the phenotype of mammalian cell cycle arrest would closely resemble *Saccharomyces cerevisiae* START. The close correlation between anchorage independence in vitro and tumorigenicity in vivo emphasizes the key regulatory role for G1/S control in mammalian cells.

Growth factors, nutrients, and anchorage are the three physiologic mediators of fibroblast cell division (Benecke et al., 1978; DeLarco and Todaro, 1978; Moses et al., 1980; Pardee, 1989). Proliferating fibroblasts use growth factors to maintain cell cycle progression throughout most of G1 (Pledger et al., 1978; Pardee, 1989). Fibroblasts leave the cell cycle and enter a quiescent, G0 state when growth factors are removed from nutrient-containing medium (Stiles et al., 1979; Pardee, 1989). Reentry into the cell cycle requires stimulation of the G0/G1 transition, and these events are also growth factor mediated (Pardee, 1989). Fewer studies have addressed the role of nutrients in fibroblast proliferation, but it is clear that nutrient depletion arrests fibroblast cell cycle progression in early G1 and prevents G1-associated increases in cell size (Stiles et at., 1979; Moses et al., 1980).

In contrast to our long-standing knowledge of growth factor and nutrient control in the mammalian cell cycle, very little is known about the anchorage requirement for division of fibroblasts. It is well established that interactions between matrix proteins and cell surface integrins mediate anchorage (Yamada, 1983; Hynes, 1987; Ruoslahti, 1988; Vuorio and de Crombrugghe, 1990), and that proliferation of mitogen- and nutrient-treated fibroblasts can be blocked by suspending cells in soft agar or methylcellulose to preclude anchorage (Benecke et al., 1978). Fibroblast transformation often leads to decreased synthesis of matrix proteins and integrins as well as loss of the anchorage requirement for cell proliferation (DeLarco and Todaro, 1978); Adams et al., 1977; Olden and Yamada, 1977; Liotta et al., 1978; Wittelsberger et al., 1981; Oliver et al., 1983; Plantefaber and Hynes, 1989). In fact, induction of this anchorage-independent growth state is the best in vitro correlate to tumorigenicity in fibroblasts (Shin et al., 1975). Potential mechanisms associated with induction of anchorage independence have been extrapolated from studies with adherent cultures, but it is not clear that such extrapolations are of general validity.

Although poorly understood, anchorage control of fibroblast proliferation may be viewed as a cell-cycle regulator: non-adherent fibroblasts arrest before DNA synthesis (Otsuka and Moskowitz, 1975; Matsuhisa and Mori, 1981). Unfortunately, the inability to recover viable, non-adherent cells from soft agar and complications of growth factor diffusion in methylcellulose have severely limited the analysis of anchorage control of the fibroblast cell cycle with these systems. We have developed methodology that circumvents the limitations of soft agar and methylcellulose culture systems and allows a detailed analysis of controls associated with the anchorage requirement for fibroblast proliferation. In the experiments reported here, we define and localize the block to anchorage-independent growth within the fibroblast cell cycle.

Materials and Methods

Cell-Cycle Gene Expression

G0-synchronized cultures of early passage normal rat kidney (NRK)[1] fibro-

1. *Abbreviations used in this paper*: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRK, normal rat kidney; TK, thymidine kinase.

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blasts (clone 49F; a non-transformed, anchorage-dependent line) were prepared by 3-d incubation of nearly confluent monolayers (four 150-mm dishes each containing 2.5×10^6 cells) in 30 ml of serum-free defined medium (1:1 DME: Hams F-10, 15 mM Hepes, pH 7.4, 1 mg/ml crystalline BSA [Sigma Chemical Co., St. Louis, MO], 25 nM sodium selenite, 5 μg/ml human transferrin [Sigma Chemical Co.], and 10 μg/ml bovine insulin [Boehringer-Mannheim Biochemicals, Indianapolis, IN]. Control studies showed that the G0-synchronized cells were >95% viable (by trypan blue exclusion) and that 90–95% of the cells had a 2n DNA content (assessed by flow cytometry). To prepare parallel non-adherent cultures, G0-synchronized monolayers were trypsinized and added to dishes (2.5×10^6 cells/150-mm dish in 30 ml serum-free defined medium) coated with agarose (18 ml 0.9% RNAse-free agarose in the serum-free medium). After overnight incubation, adherent and non-adherent cultures were treated with growth factors (5% dialyzed FCS, 3 nM EGF final concentrations) for selected times (see Figs. 2 and 3) before the collection of cells and isolation of poly A+ (Badley et al., 1988) or total (Wager and Assoian, 1990) RNA. The RNAs were fractionated on formaldehyde–agarose gels, transferred electrophoretically to Nytran, and hybridized to nick-translated DNA probes or riboprobes. Hybridization and washing of filters with nick-translated probes were performed as described (Wager and Assoian, 1990). For riboprobes, filters were prehybridized (2–4 h, 55°C) in a solution of 5× SSPE, 50% formamide, 5× Denhardt's reagent, 0.2% SDS, 0.2 mg *Escherichia coli* tRNA and 0.05 mg low molecular weight DNA/ml. Hybridization proceeded (20 h, 55°C) in 5× SSPE, 50% formamide, 1× Denhardt's reagent, 0.2 mg *E. coli* tRNA and 0.05 mg low molecular weight DNA/ml, 10% dextran sulfate, and 2×10^6 cpm/ml ^{32}P-UTP–labeled RNA probe. Filters hybridized to riboprobes were washed with 0.15× SSPE, 0.1% SDS at 80°C.

Flow Cytometry

Samples for flow cytometry were prepared similarly to the procedure described above except that cultures contained 10^6 cells (in 10 ml serum-free medium/100 mm dish). Adherent and non-adherent cultures were prepared in unmodified or agarose-coated dishes (6 ml agarose solution/dish), respectively. To collect cells, adherent cultures were washed with cold HBSS, trypsinized, diluted into HBSS, and recovered by centrifugation; non-adherent cells were collected by centrifugation directly and washed in HBSS. Non-adherent cells were resuspended in trypsin-EDTA (0.2 ml) for 3–5 min before dilution in HBSS. Samples were resuspended in 2 ml cold HBSS, fixed in 5 ml cold ethanol, collected by centrifugation, and resuspended in chromomycin A3 (50 μg/ml in 32 mM $MgCl_2$) before analysis for DNA content and cell size, by fluorescence and forward angle light scatter, respectively.

In Vitro Histone H1 Kinase Activity

Cultures of adherent and non-adherent, G0-synchronized NRK cells were prepared as described above for analysis of cell cycle genes. Collected cells (2.5×10^6 cells/time point) were washed with PBS and extracted by vortexing in 0.5 ml lysis buffer B (Morgan et al., 1989). The extracts were clarified by centrifugation (10,000 *g*, 10 min, 4°C). The protein concentration in each of the supernatants was determined by Coomassie binding (Bio-Rad Laboratories, Richmond, CA) and adjusted to 0.5 mg/ml by dilution with lysis buffer B. Phosphorylation of histone H1 (6 μg; Boehringer-Mannheim Biochemicals) was assessed in 10 μl reaction volumes containing 4 μl of the normalized extracts, 2 μl of kinase buffer (0.1 M Hepes, pH 7.4, 15 mM $MgCl_2$, 1 mM DTT), 20 μM ATP, 2 μCi [gamma-^{32}P]ATP (3,000 Ci/mmol), and 5 μM cAMP-dependent protein kinase inhibitor (Sigma Chemical Co.). Samples were incubated for 20 min at room temperature, and the reactions were stopped by addition of SDS-sample buffer containing 0.1 M DTT (25 μl/reaction). Samples were fractionated on SDS–polyacrylamide gels (10% acrylamide), the gels were dried, and the extent of H1 histone phosphorylation was assessed by autoradiography.

DNA Synthesis

To measure DNA synthesis, G0-synchronized monolayers (see above) were trypsinized, suspended in defined medium (4×10^4 cell/ml), and 1-ml aliquots were added to 35-mm dishes coated with 100 μg type I collagen (adherent cultures) or agar (non-adherent cultures). Cells were incubated overnight; growth factors (5% dialyzed FCS, 1 nM EGF) and ^{3}[H]thymidine (0.5 μCi/ml; 90 Ci/mmol) were then added for selected times up to 20 h. Adherent cultures were fixed with 1–2 ml of 5% TCA, and processed for TCA-insoluble radioactivity as described (Assoian, 1985). Non-adherent cells were collected, diluted to 9 ml with PBS and lysed by addition of 50% TCA (1 ml). Samples were supplemented with 0.25 mg low molecular weight DNA as carrier; the precipitated DNA was collected, washed, and counted as described (Assoian et al., 1989).

Results

Fig. 1 shows our scheme for identifying the block to anchorage-independent growth and positioning it within the mammalian cell cycle. Non-transformed NRK fibroblasts were G0 synchronized by serum starvation of cells in monolayer. The quiescent cultures were trypsinized, added to agar-coated dishes (non-adherent, suspension cultures), and exposed to mitogens (serum and EGF) for analysis of cell cycle progression. G0-synchronized monolayers (adherent cultures) were mitogen treated in parallel and used as the positive control for efficient cell cycle progression. The full complement of nutrients was present in all cultures.

To determine whether loss of anchorage prevents cells from responding to mitogens and entering the cell cycle, we examined the time-dependent induction of the c-*fos* and c-*myc* genes as markers of mitogen-stimulated transit from G0 to G1 (Kelly et al., 1983; Greenberg and Ziff, 1984). Interestingly, results from this experiment (Fig. 2) showed that mitogen induction of c-*fos* (*upper panels*) and c-*myc* (*lower panels*) gene expression was unaffected by loss of anchorage in G0-synchronized NRK cells. In addition, we used flow cytometry (Table I, *Experiment I*) to examine the effect of anchorage on G1-associated growth; this experiment showed that mitogen treatment increases cell size similarly throughout G1 (G1 ends at 11–12 h in NRK cells; see Fig. 4 *B*) in both the adherent and non-adherent NRK cells. Thus, control of anchorage independence does not result from growth arrest in either G0 or early-mid G1. The data also emphasize that the G0/G1 transition and early G1 events are stimulatable by growth factors even when cell division is precluded.

In related experiments, we compared the expression of G1/S phase cell cycle genes in adherent and non-adherent cultures after mitogen treatment of G0-synchronized NRK cells. As shown in Fig. 3, histone H4 (*A*) and thymidine kinase (*TK*) (*B*) gene expression is strongly induced throughout S phase (S phase begins at 11–12 h; refer to Fig. 4, *B* and *C*) in the adherent cells; the induction is markedly reduced in mitogen-treated NRK cell suspensions. Other experi-

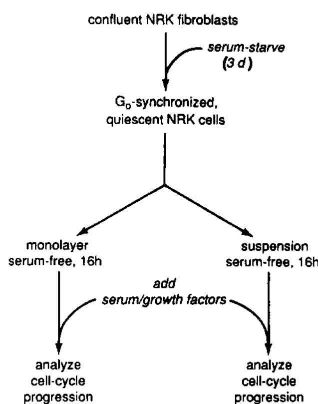

Figure 1. An experimental system for analysis of anchorage-dependent control of the fibroblast cell cycle. The figure shows our overall scheme for analysis of cell cycle progression in adherent and non-adherent NRK fibroblasts. Control studies showed that the G0-synchronized cells were >95% viable (by trypan blue exclusion), and that 90–95% of the cells had a 2n DNA content (by flow cytometry). Cell viability remained high (~90%) throughout all experiments shown.

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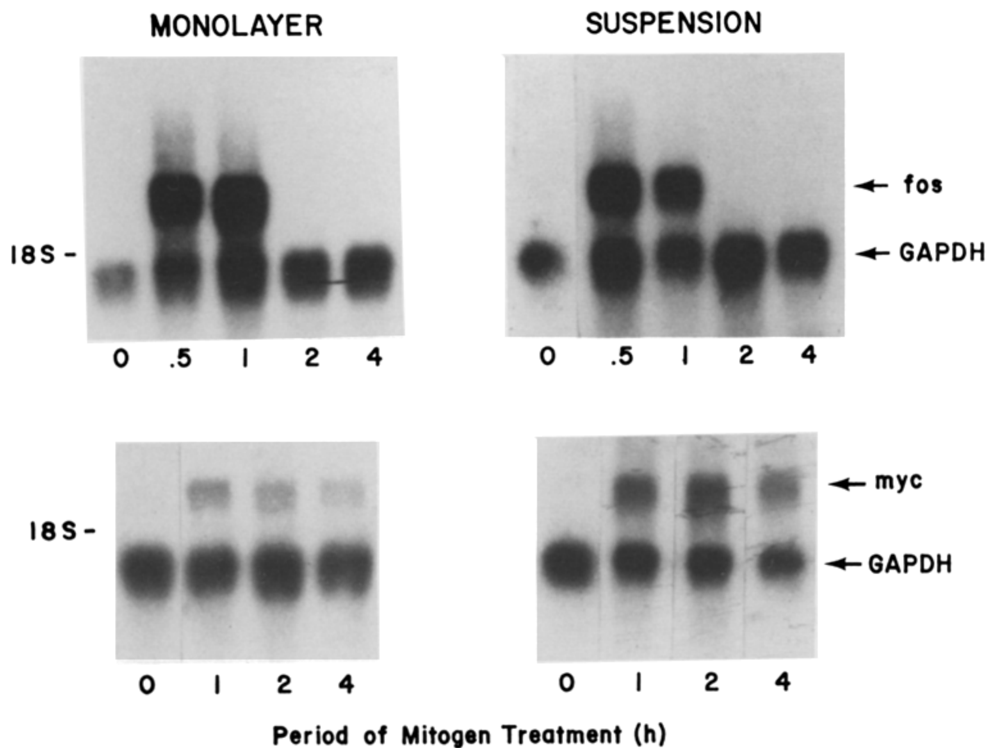

Figure 2. The block to anchorage-independent growth is subsequent to G0/G1 and G1-growth events. Adherent (*MONOLAYER*) and non-adherent (*SUSPENSION*) cultures of G0-synchronized NRK fibroblasts were treated with 5% FCS, 3 nM EGF for times indicated in the figure. mRNA was isolated from 10^7 cells, and ~25% of the mRNA was fractionated on denaturing agarose gels, transferred to Nytran, and hybridized to a GAPDH cDNA probe to normalize mRNA loading. Filters with normalized mRNA were hybridized to c-*fos* (*upper panels*) or c-*myc* (*lower panels*) riboprobes.

ments also showed a tendency for delayed induction of these transcripts in the non-adherent cells (not shown). Controls on this experiment demonstrated that ^{3}[H]thymidine incorporation into DNA was reduced ninefold in the non-adherent cells relative to the adherent counterpart (see Fig. 3 legend). We note that TK gene expression in NRK fibroblasts yields two transcripts of 2.5 and 1.4 kb, and that only the 2.5-kb transcript is regulated by anchorage. In mouse cells a 2.5-kb transcript is one of several precursors (Gudas et al., 1988); we have been unable to find data on the number and sizes of functional TK mRNAs in rat cells.

Table I. Cell Size Increases in G1 after Mitogen Treatment of Adherent and Non-adherent, G0-synchronized NRK Fibroblasts

		Relative cell size	
	Incubation	Adherent	Non-adherent
Experiment 1	0 h	1.00	1.00
	9 h	1.15	1.13
	12 h	1.26	1.20
Experiment 2	0 h	NA	1.00
	12 h	NA	1.13
	16 h	NA	1.18
	20 h	NA	1.23

Changes in cell size in G1 are shown for adherent and non-adherent cells (relative to the G0-synchronized cells) as measured by forward angle laser light scatter. Note that relative changes in cell size do not reflect cell mass or volume directly: G1 and G2/M NRK cell subpopulations show a 35% difference in relative cell size by forward angle light scatter. For Experiment 1, controls performed at 12 h confirmed that the large majority of cells were still in G1: 80–90% of the cells had a 2n DNA content. For Experiment 2, results presented exclude any cells that had >2n DNA content. Controls using parallel cultures showed an 11-fold decrease in DNA synthesis relative to adherent cells at 20 h. *NA*, not applicable.

The Northern blots used in Fig. 3, *A* and *B* were also hybridized with probes for genes that are not cell cycle regulated. The results show that expression of gamma-actin (Fig. 3 *C*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (Fig. 3 *D*) is not inhibited by loss of anchorage for at least 20 h. Thus, the decreased expression of histone H4 and TK genes observed in non-adherent cells at 12–20 h does not reflect non-specific inhibition of macromolecular synthesis. The same conclusion was drawn from flow cytometry experiments showing a persistent increase in the size of non-adherent NRK cells (Table I, *Experiment 2*) despite continued cell cycle arrest as indicated by blocked DNA synthesis (see Table I legend). Similar results were obtained with detachment-arrested NIH-3T3 cells (not shown). Thus, the block to anchorage-independent growth leads to selective inhibition of G1/S cell cycle gene expression.

Although control of the mammalian cell cycle has focused on G0/G1, a major regulatory point in the yeast cell cycle maps to late G1 at a site termed START (Pringle and Hartwell, 1981; Cross et al., 1989). Mechanistically, START arrest in yeast is linked to inactivity of the CDC28/cdc2$^+$ gene product, a serine–threonine kinase required for both the G1/S and G2/M transitions (Pringle and Hartwell, 1981; Cross et al., 1989; Broek et al., 1991). Activation of this kinase is detectable by phosphorylation of histone H1 in vitro (Brizuela et al., 1989; Pines and Hunter, 1990). We prepared total cell extracts from adherent and non-adherent, mitogen-stimulated NRK fibroblasts and tested their ability to phosphorylate histone H1 (Fig. 4 *A*; also see Fig. 4 legend). A low background level of histone H1 phosphorylation, with little cell cycle periodicity, was detected in all samples and likely results from the action of non-p34^{cdc2}–like kinases in the total cell extracts. However, a clear, biphasic induction of histone H1 phosphorylation was observed in the

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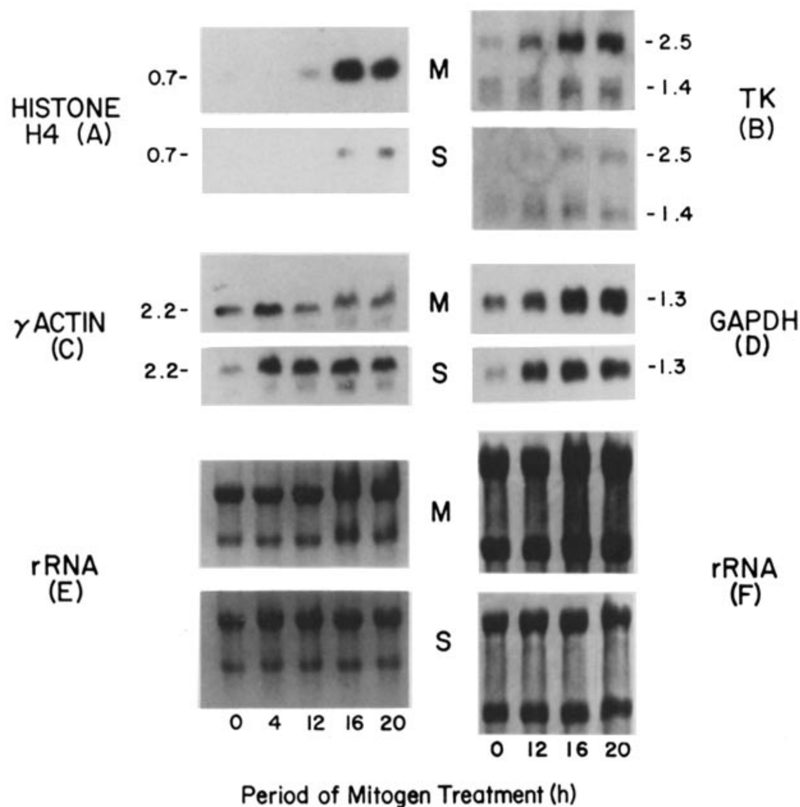

Figure 3. Decreased expression of G1/S phase genes in non-adherent fibroblasts. Expression of G1/S phase genes was compared in monolayer (*M*) and suspension (*S*) cultures of G0-synchronized NRK fibroblasts as described in the legend to Fig. 2 except that total RNA was isolated from cells at the times indicated, and 10-μg aliquots were fractionated on denaturing agarose gels before Northern blot hybridization with probes for histone H4 (*A*), thymidine kinase (*TK*, *B*), gamma-actin (*C*), or GAPDH (*D*). *E* and *F* show ethidium-bromide stained rRNAs. *A*, *C*, *E*, and *B*, *D*, *F* show results from duplicate filters of the same experiment. Numbers on *A–D* identify the size of each transcript in Kb. Faint signals below the 2.2-Kb actin mRNA represent residual GAPDH signals from previous hybridizations. ^{3}[H]thymidine incorporation into DNA (at 20 h) of adherent and non-adherent cells was 2.2×10^6 and 2.4×10^5 cpm, respectively. Cell viability in all cultures was 90% (by trypan blue exclusion) throughout the experiment.

adherent cells after 11 and 15 h of mitogen treatment. Consistent with results of others on mammalian cells (Brizuela et al., 1989; Pines and Hunter, 1990), control studies with ^{3}[H]thymidine incorporation into DNA and flow cytometry showed that these early and late peaks of H1 histone kinase activity from total cell extracts map to G1/S (Fig. 4, *B* and *C*) and G2/M (Fig. 4, *D* and *E*), respectively. Importantly, cell cycle–dependent induction of histone H1 kinase activity was strongly inhibited in the non-adherent cells (Fig. 4 *A*); these cultures also failed to synthesize DNA (Fig. 4 *B*). We conclude that loss of anchorage arrests fibroblast cell cycle progression prior to activation of G1/S p34^{cdc2}-like kinase. Note that our use of "G1/S" refers to events associated with late G1 and S phase progression and not the G1/S transition per se.

To map the block to anchorage-independent growth relative to the cell cycle markers described above (c-*myc* and p34^{cdc2}-like kinase in particular), we measured the time period required for induction of DNA synthesis when non-adherent, cycle-arrested NRK cells were allowed to reattach and resume cell cycle progression. As shown in Fig. 5, DNA synthesis was induced 2–3 h after reattachment whereas the complete G0 to S transition required 12 h in this experiment (see Fig. 5 legend). Similar results were obtained with NIH-3T3 cells (data not shown). Since cell attachment, and most likely spreading (Folkman and Moscona, 1978), must occur before cell cycle progression can resume, the block to anchorage-independent growth is imposed somewhat later in G1 than this experiment indicates. (Attachment and spreading is complete in approximately 60 min with NRK cells; not shown.) Importantly, the comparison of results from this experiment and Fig. 4 position the block to anchorage-independent growth in late G1 and shortly before activation of G1/S p34-like kinase.

Discussion

This report defines the anchorage requirement for fibroblast proliferation by identifying a discrete anchorage-dependent transition in the fibroblast cell cycle. Our data on expression of cell cycle genes, p34^{cdc2}-like kinase activity, DNA synthesis, and cell cycle progression all support the identification of an anchorage-dependent growth control in late G1. We also show that loss of anchorage does not block the G0/G1 transition or G1-associated growth events. These data emphasize that anchorage control of the fibroblast cell cycle is discrete rather than non-specifically permissive. Previous studies examining mammalian cell cycle control have used adherent cultures, transformed cells, or hematopoietic cells (reviewed in Cross et al., 1989; and Pardee, 1989). None of these systems would be expected to reveal the G1/S block to anchorage independence.

The effect of cell anchorage on cell growth has also been examined by others. Studies showing that c-*myc* expression is stimulated by serum in non-adherent 3T3 cells (Dhawan and Farmer, 1990), are in complete agreement with the data presented here. Interestingly, Dike and Farmer (1988) have shown that c-*fos* and c-*myc* gene expression is induced upon reattachment of non-adherent 3T3 cells, indicating that anchorage can stimulate the G0/G1 transition in the absence of growth factors. In light of these latter results, our data suggest that the key distinguishing feature between growth fac-

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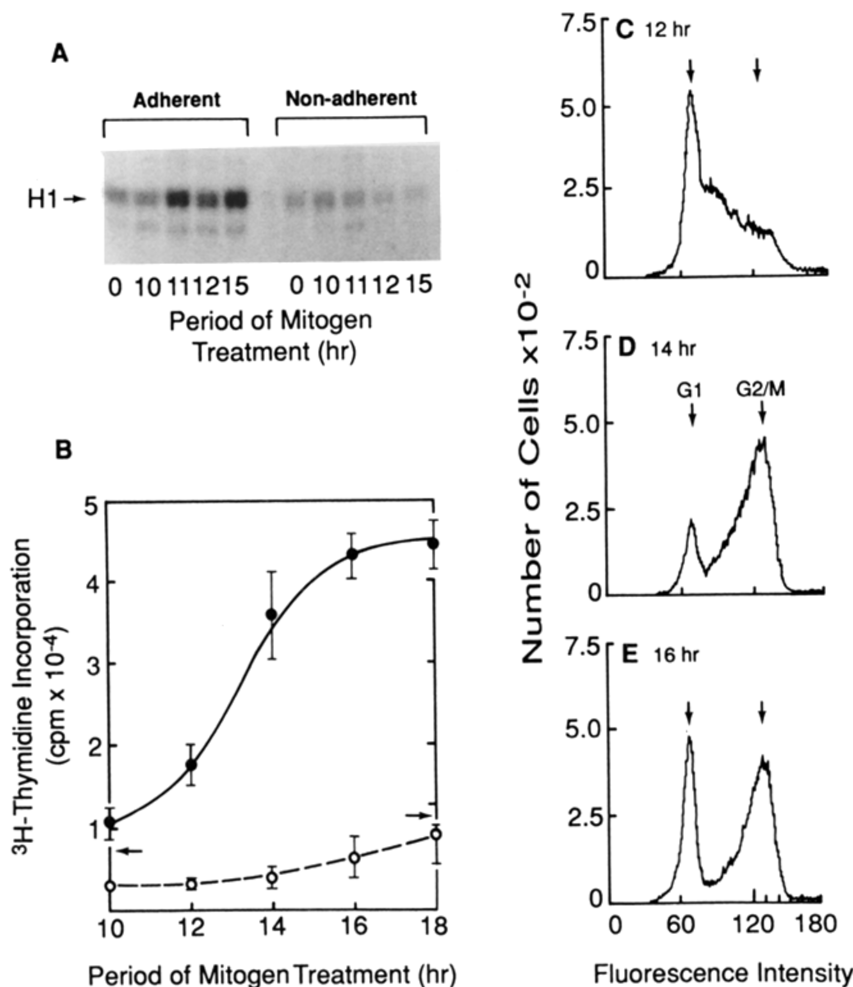

Figure 4. Cell cycle–dependent histone H1 kinase activity is blocked by loss of attachment. Total cell extracts from mitogen-treated NRK fibroblasts were tested for histone H1 kinase activity. *A* shows an autoradiograph of histone H1 phosphorylation by extracts from adherent and non-adherent, G0-synchronized NRK cells prepared at selected times after exposure to mitogens. Other results with in vitro phosphorylation of histone H1 in adherent NRK cells (not shown) demonstrated that: (*a*) the early peak is transient but reproducibly present in G1/S; (*b*) no cell cycle–dependent histone H1 phosphorylation was detected before 11 h; and (*c*) the late peak persists throughout G2/M. *B* shows results of a parallel experiment examining the time-dependent induction of DNA synthesis in adherent (●) and non-adherent (○) cells. The arrows in *B* show the extent of ^{3}[H]thymidine incorporation observed at 10 and 18 h in growth factor-free monolayers. *C–E* show flow cytometric analysis of the adherent cells at selected times after mitogen treatment. The increased amplitude of the G1 peak in *E*, relative to *D*, represents cells that completed mitosis between 14 and 16 h in monolayer.

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tor and anchorage control on the fibroblast cell cycle may be the ability to mediate G1/S transit.

Our results also indicate that cell cycle arrest associated with control of anchorage-independent growth is notably similar to START arrest in yeast (Pringle and Hartwell, 1981; Cross et al., 1989). Shared phenotypes include: (*a*) a block to DNA synthesis despite normal entry into the cell cycle and progression through most of G1 (Fig. 2); (*b*) selective failure to express G1/S cell cycle genes (Fig. 3); (*c*) continued increase in cell size despite lack of cell cycle progression (Table I); and (*d*) arrest shortly before activation of the $p34^{cdc2}$-like kinase (Figs. 4 and 5). Activation of G1/S histone H1 kinase in yeast results from an interaction between G1 cyclins and the CDC28/$cdc2^+$ kinase (Wittenberg et al., 1990). Although greatly complicated by the many $p34^{cdc2}$ family members (Lee and Nurse, 1987; Draetta et al., 1988; Riabowol et al., 1989; Pines and Hunter, 1990; Ben-David et al., 1991; Hamaguchi et al., 1991) and G1 cyclins (Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991) present in mammalian cells, we are currently trying to identify anchorage effects on components of G1/S histone H1 kinase in our system.

A G1/S commitment point, *R*, has also been detected in mammalian fibroblasts by monitoring cell cycle progression of cycloheximide-treated 3T3 cells (Pardee, 1989). *R* corresponds to the point at which cell division in adherent 3T3 fibroblasts becomes independent of growth factors and protein synthesis (Rossow et al., 1979), and it reflects induction of a labile protein (Croy and Pardee, 1983) that may be a G1 cyclin (Lewin, 1990). These studies, together with the identification of human p34-like kinases and G1 cyclins (referenced above), predict that START control should exist in mammalian cells. Our report identifies the physiologic circumstance that would result in a START phenotype. Moreover, our results allow us to complete the picture of fibroblast cell cycle control with respect to its three physiologic growth regulators: the absence of growth factors, nutrients, and anchorage leads to cell cycle arrest at G0, early G1, and late G1, respectively. Finally, our ability to identify this START-like block on anchorage-independent growth, in particular, is in notable agreement with studies showing that the pRb (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989) and p53 (Mercer et al., 1984; Finlay et al., 1988) tumor suppressors act at G1/S.

We thank Fred Alt, Arthur Bank, Tom Curran, and Nat Heintz for cDNA probes, and Mitchell Goldfarb and Aaron Mitchell for criticizing the manuscript. We thank Thomas Delohery (Columbia University Cancer Center, New York) for performing the flow cytometry.

These studies were supported by National Institutes of Health grants

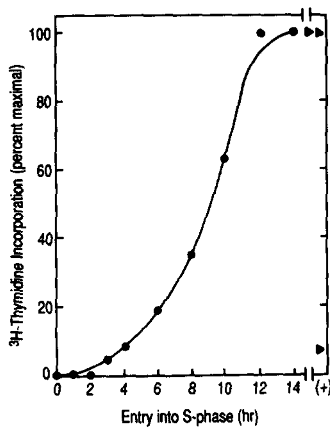

Figure 5. The block on anchorage-independent growth maps to late G1 in fibroblasts. Nonadherent, cycle-arrested NRK cells (see below) were reattached to collagen-coated dishes and the induction of DNA synthesis was determined by ^{3}[H]thymidine incorporation into DNA at the times shown in the figure. Background and maximal ^{3}[H]thymidine incorporation was 2,500 and 24,000 cpm, respectively. Data are plotted as percent maximal incorporation (minus background), and show the mean of triplicate determinations (ranges were 1-7% of the means). Flow cytometry confirmed that the non-adherent, cycle-arrested cells remained in G1 before reattachment and had entered S phase 3 hr after reattachment (data not shown). Controls (+) showed that parallel cultures of adherent, G0-synchronized NRK cells treated with mitogens enter (*single arrowhead*) and complete (*double arrowhead*) S phase at 12 and 22 h, respectively. To measure induction of DNA synthesis upon reattachment of cycle-arrested cells, a pool of non-adherent, G0-synchronized NRK fibroblasts (1.5 × 10^6 cells in 30 ml) was incubated with growth factors (5% dialyzed FCS, 1 nM EGF final concentrations), and ^{3}[H]thymidine (1 μCi/ml; 90 Ci/mmol) for 12 h. The resulting non-adherent, cycle-arrested cells were collected with the original medium, diluted 1:1 with fresh growth factor–supplemented medium, and 2-ml aliquots (5 × 10^4 cells) were added to collagen-coated 35-mm dishes. DNA synthesis was monitored as described for adherent cells in Materials and Methods.

HD05077, HL38884, and Basil O'Connor award 5-714 from the March of Dimes (to R. K. Assoian). T. M. Guadagno was supported by a predoctoral fellowship from the National Cancer Center. R. K. Assoian is supported by an Irma T. Hirschl Career Scientist Award.

Received for publication 5 September 1991.

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