

A Cell Cycle and Mutational Analysis of Anchorage-independent Growth: Cell Adhesion and TGF- β 1 Control G1/S Transit Specifically

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Abstract. We have examined cell cycle control of anchorage-independent growth in nontransformed fibroblasts. In previous studies using G0-synchronized NRK and NIH-3T3 cells, we showed that anchorage-independent growth is regulated by an attachment-dependent transition at G1/S that resembles the START control point in the cell cycle of *Saccharomyces cerevisiae*. In the studies reported here, we have synchronized NRK and NIH-3T3 fibroblasts immediately after this attachment-dependent transition to determine if other portions of the fibroblast cell cycle are similarly regulated by adhesion. Our results show that S-, G2-, and M-phase progression proceed in the absence of attachment. Thus, we conclude that the adhesion requirement for proliferation of these cells can be explained in terms of the single START-like transition. In related studies, we show that TGF- β 1 overrides the attachment-dependent transition in NRK and AKR-2B fibroblasts (lines in which TGF- β 1 induces anchorage-independent growth), but not in NIH-3T3 or Balb/c 3T3 fibroblasts (lines in which TGF- β 1 fails to induce anchorage-independent growth). These results show

that (a) adhesion and TGF- β 1 can have similar effects in stimulating cell cycle progression from G1 to S and (b) the differential effects of TGF- β 1 on anchorage-independent growth of various fibroblast lines are directly reflected in the differential effects of the growth factor at G1/S. Finally, we have randomly mutagenized NRK fibroblasts to generate mutant lines that have lost their attachment/TGF- β 1 requirement for G1/S transit while retaining their normal mitogen requirements for proliferation. These clones, which readily proliferate in mitogen-supplemented soft agar, appear non-transformed in monolayer: they are well spread, nonrefractile, and contact inhibited. The existence of this new fibroblast phenotype demonstrates (a) that the growth factor and adhesion/TGF- β 1 requirements for cell cycle progression are genetically separable, (b) that the two major control points in the fibroblast cell cycle (G0/G1 and G1/S) are regulated by distinct extracellular signals, and (c) that the genes regulating anchorage-independent growth need not be involved in regulating contact inhibition, focus formation, or growth factor dependence.

CELL cycle progression in nontransformed fibroblasts is regulated by extracellular signals provided by peptide growth factors and cell adhesion. Mitogenic peptide growth factors, such as platelet-derived growth factor, insulin like growth factor-I, and EGF, are among the best studied examples of such external cell cycle regulators (Pardee, 1989; Sporn and Roberts, 1990). Although the exact roles of these growth factors seem to be somewhat variable in different fibroblast cell lines, it is clear that they are required to stimulate entry of quiescent (G0-synchronized) fibroblasts into the cell cycle and progression of the cells through most of G1 (Pledger et al., 1977; Pardee, 1989). Transit from late G1 into S is independent of mitogenic growth factor action (Pledger et al., 1977; Yen and Pardee, 1978; Rossow et al., 1979).

When compared to the growth factors discussed above, the effects of TGF- β 1 on fibroblast proliferation seem quite distinct. For example, TGF- β 1 does not directly stimulate DNA synthesis in adherent, G0-synchronized fibroblasts, and it can even inhibit the proliferation mediated by mitogens such as serum and EGF (Massagué, 1990; Moses et al., 1990; Sporn and Roberts, 1990). TGF- β 1 is also unusual in its ability to stimulate anchorage-independent growth of certain fibroblastic cell lines (Roberts et al., 1981; Assoian et al., 1983; Moses et al., 1985; Tucker et al., 1984). These distinct cellular effects of TGF- β 1 are consistent with recent studies showing that the TGF- β 1 receptor has a serine/threonine kinase activity rather than the tyrosine kinase activity typically associated with peptide growth factor receptors (Lin et al., 1992; Massagué, 1992).

In addition to growth factors, fibroblasts require extracellular signals provided by adhesion for proliferation (Benecke et al., 1978). This adhesion requirement is referred to as anchorage-dependence, and loss of this control (induction of

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anchorage-independence) is the best in vitro correlate to tumorigenicity in animals (Shin et al., 1975). Although the adhesion requirement for fibroblast proliferation is much less well understood than the growth factor requirement, it is clear that adhesion can also be viewed as a cell cycle regulator. For example, early studies showed that G0-synchronized, nonadherent fibroblasts arrest before DNA synthesis (Otsuka and Moshowitz, 1975; Matsuhisa and Mori, 1981), and more recent studies have shown that cell adhesion can influence growth factor-regulated events at G0/G1 (Dike and Farmer, 1988; Ingber et al., 1990).

We have recently extended these studies on attachment control of the fibroblast cell cycle and characterized a cell cycle block to anchorage-independent growth in detail (Guadagno and Assoian, 1991). In contrast to studies with standard soft agar cultures, our experiments are based on use of a preparative suspension culture system that allows for easy recovery and analysis of cells. Using this system, we prepared parallel cultures of adherent and nonadherent, G0-synchronized NRK or NIH-3T3 fibroblasts. After exposure to optimal concentrations of mitogens (serum and EGF), we monitored cell cycle progression by induction of cell cycle markers, flow cytometry, and [³H]thymidine incorporation into DNA. The results showed that the nonadherent cells arrest in late G1 and shortly before activation of G1/S histone H1 kinase activity: a marker for the action of G1 cyclins and G1/S p34^{cdc2}-like kinase(s). (Note that our use of "G1/S" refers to events associated with late G1- and S-phase progression and not the G1/S interface per se.) Cell size continued to increase despite cell cycle arrest. Importantly, growth factor responsiveness, as monitored by cell cycle progression from G0 through G1, was equivalent in the adherent and nonadherent fibroblasts.

Interestingly, the phenotype of cell cycle arrest observed upon loss of attachment in mitogen-treated NRK fibroblasts has several features that resemble the phenotype of *Saccharomyces cerevisiae* arrest at the G1/S control point called START. As we noted previously (Guadagno and Assoian, 1991), the shared features include: (a) blocked DNA synthesis despite normal cell cycle progression through most of G1, (b) selective failure to express G1/S cell cycle genes, (c) continued increases in cell size despite lack of cell cycle progression, and (d) arrest before activation of the cdc2-like kinase. Note that in *S. cerevisiae*, transit through START represents commitment to DNA synthesis and completion of the cell cycle (Cross et al., 1989).

In the studies reported here, we extend our previous studies on cell cycle control of anchorage-independent growth to demonstrate that transit from late G1 to S is the only portion of the cell cycle that requires adhesion in mitogen-treated NRK and NIH-3T3 fibroblasts. Similarly, we show that the stimulatory effect of TGF- β 1 on anchorage-independence can be explained completely by its ability to override this attachment requirement for G1/S transit. These results extend our previous studies and show that a START-like control is the principal regulator of anchorage-independent growth. Moreover, we report results from a random mutagenesis of NRK fibroblasts in which we have generated mutant lines that have lost adhesion/TGF- β 1 control of the cell cycle while retaining mitogen control of the cell cycle. The existence of this new fibroblast phenotype confirms the complementary actions of mitogens and adhe-

sion/TGF- β 1 on cell cycle progression and emphasizes that the two major control points in the fibroblast cell cycle (G0/G1 and G1/S) can be regulated by distinct extracellular signals.

Materials and Methods

Cell Culture

Early passage nontransformed NRK fibroblasts (clone 49F) were maintained in DME containing 5% newborn calf serum (NCS)¹ and gentamicin. NIH-3T3 cells (gift of M. Goldfarb, Regeneron) and Balb/c 3T3 cells (clone A31; gift of J. Pledger, Vanderbilt and C. Stiles, Harvard) were cultured similarly in calf serum rather than NCS. AKR-2B cells (Shipley et al., 1984; gift of H. Moses, Vanderbilt) were maintained in McCoy's 5A with 5% NCS. For experiments, cells were cultured in 5% FCS or 5% dialyzed FCS when noted. In some experiments, the cultures were also treated with 1 nM EGF and 100 pM TGF- β 1 unless noted otherwise in specific figure legends. These four nontransformed fibroblast lines were contact inhibited, well-spread, and nonrefractile when cultured in monolayer; none of the lines formed colonies efficiently when cultured in mitogen (5% FCS or 5% FCS/1 nM EGF)-supplemented soft agar.

Generation of Ethyl Methane Sulfonate (EMS) NRK Cell Mutants

NRK cell mutants were generated using EMS similarly to the procedure described (Kao and Puck, 1974). Specifically, actively growing NRK cells were trypsinized, and 2×10^6 cells were plated into 100-mm dishes containing 10 ml of 10% FCS in DME. After 6 h, EMS (Sigma Chemical Co., St. Louis, MO) was added to the cultures at final concentrations of 8–20 mM. EMS was removed after 16 h, fresh medium was added to the cultures, and incubation proceeded for 3–4 d during which time the extent of cell death was monitored visually. EMS-induced cell death was typically complete within this time period. EMS concentrations <10 mM had no effect on cell death and concentrations >20 mM yielded essentially complete cell death. After 3–4 d, the medium was replaced, and the remaining cells were grown to confluence. Confluent cells arising from exposure to low (10 mM) and high (19 mM) concentrations of EMS were selected for analysis. They were cultured in soft agar to identify clones that had lost their attachment requirement for proliferation.

The analysis in soft agar was performed similarly to the procedure described (Assoian et al., 1989). Specifically, 100-mm dishes were coated with 4-ml of 0.5% agar (Difco, Noble Agar, Detroit, MI) and top layers contained 8 ml of 0.3% agar with 5×10^4 cells. Top and bottom layers contained 5% FCS and 1 nM EGF. The cultures were incubated for 10–14 d; colonies were picked and transferred to a 96-well plate containing 0.2 ml DME with 10% FCS. Explanted clones were expanded and the entire soft agar screening procedure was repeated for each explanted colony to eliminate false positives and genetically unstable clones. Finally, explanted colonies isolated from the second screen were replated in soft agar. Clones producing at least 75% colonies in this tertiary screen were considered to be true positives.

Cell Cycle Synchronization

Parental and EMS-mutated NRK fibroblasts were G0-synchronized by incubation of subconfluent monolayers in serum-free medium for 3 d as described (Guadagno and Assoian, 1991). NIH-3T3 and AKR-2B fibroblasts were G0-synchronized by a 2-d incubation in the same serum-free medium. To synchronize cells at G1/S, serum was added to the G0-synchronized fibroblasts (final concentrations: 5% FCS for NRK and 5% calf serum for NIH-3T3 cells) followed by addition of hydroxyurea (2 mM for NRK and 0.5 mM for NIH-3T3 cells final concentrations) 2 h later. Cultures were incubated with hydroxyurea for 16 h before use, and flow cytometry (not shown) confirmed that >95% of the hydroxyurea-treated cells had a G1 content of DNA.

1. Abbreviations used in this paper: EMS, ethyl methane sulfonate; NCS, newborn calf serum.

Characterization of Anchorage-independent Growth in Preparative Suspension Culture

Unless noted otherwise, G0- or G1/S-synchronized cells were trypsinized and placed in preparative suspension culture on agar-coated dishes (2×10^4 cells per 35-mm dish). After overnight incubation, FCS (5% final concentration unless noted otherwise) was added to the cultures. When appropriate, the cultures were also supplemented with 1 nM EGF or 1 nM EGF and 100 pM TGF- β 1. S-phase progression was assessed by incubating the serum-treated cultures with [3 H]thymidine (1 μ Ci/well for 24 h at days 0-1, 1-2, or 2-3) before collecting the cells and isolating the radiolabeled DNA (TCA-insoluble radioactivity). G2/M-phase progression, and cell proliferation in general, was monitored by direct counting of recovered cells. When applicable, mitogen-treated adherent cells, or TGF- β 1 treated nonadherent cells, were cultured in parallel as positive control. See Guadagno and Assoian (1991) for methodologic details.

Characterization of the Growth Factor Requirement for Proliferation of NRK/EMS Mutants in Monolayer

NRK/EMS mutants surviving the tertiary screening in soft agar were tested to assess their growth factor requirement for cell proliferation in monolayer. To measure increases in cell number, adherent cultures of wild-type and mutant NRK cells were G0-synchronized as described above. Trypsinized cells (6×10^4) were seeded in 35-mm dishes. The culture medium (2 ml) was DME with 2% dialyzed calf plasma-derived serum or DME with 2% dialyzed FCS (whole-blood derived serum) and 1 nM EGF. (Plasma-derived serum closely resembles whole blood-derived serum except that it lacks the mitogenic growth factors released from platelets.) After 1, 2, and 3 d, the dishes were washed three times with PBS and cell number was assessed by crystal violet staining (Sugarman et al., 1985). Specifically, washed cells were incubated 2-4 h in 1 ml crystal violet solution (0.5% crystal violet in 20% methanol). The stained cells were washed five times with water, and the dye was eluted (1 h at room temperature) into 1 ml of 0.1 M sodium citrate, pH 4.2, 50% in ethanol. Under these conditions, the absorbance at 540 nm is directly proportional to NRK cell number (Assoian et al., 1989, and unpublished data). To measure stimulation of DNA synthesis in the first cell cycle, NRK/EMS mutants were G0-synchronized by serum-starvation, exposed to [3 H]thymidine, and extracted for isolation of radiolabeled DNA (see the legend to Fig. 8 for details).

Results

Hydroxyurea Synchronization Establishes G1/S as the Sole Attachment-dependent Transition in the Fibroblast Cell Cycle

As discussed above, our previous studies on cell cycle control of anchorage-independent growth (Guadagno and Assoian, 1991) showed that nonadherent, G0-synchronized fibroblasts arrest in late G1 with a phenotype that resembles arrest at START in *S. cerevisiae*. To determine if there are additional attachment-dependent cell cycle transitions, we prepared parallel cultures of adherent and nonadherent NRK and NIH-3T3 fibroblasts that had been synchronized at the G1/S interface by exposure to hydroxyurea (Draetta and Beach, 1988; Pledger et al., 1977; DeCaprio et al., 1989). (Based on our previous studies, we reasoned that an hydroxyurea treatment would synchronize the cells immediately after the START-like block to anchorage-independent growth.) After removal of the drug, S- and G2/M-phase progression was monitored by [3 H]thymidine incorporation and changes in cell number, respectively. Results from these studies (Fig. 1), shown for both NRK fibroblasts (A) and NIH-3T3 fibroblasts (B), demonstrate that S-phase progression now occurs readily in the nonadherent cells and with kinetics similar to that observed with the adherent counterparts. Note that the time required for intracellular dilution of hydroxyurea somewhat prolonged S-phase in both the adherent and non-

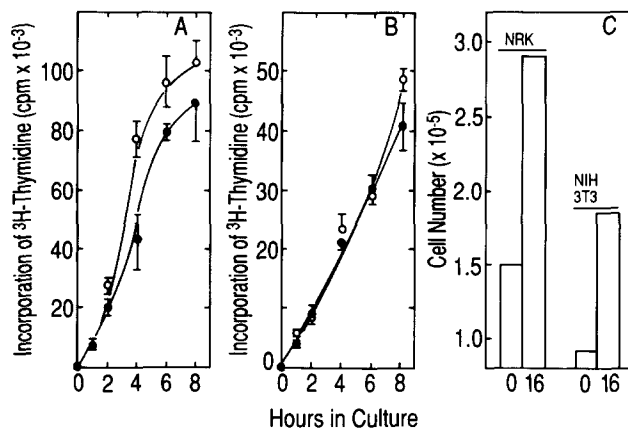


Figure 1. S- and G2/M-phase transit occurs in the absence of attachment. Cells were synchronized at the G1/S interface as described in Materials and Methods. The synchronized cells were detached by incubation with versene, and seeded on collagen (adherent cultures; open circles) or agar-coated (nonadherent cultures; closed circles) 35-mm dishes as described (Guadagno and Assoian, 1991). S-phase progression for NRK (A) and NIH-3T3 (B) fibroblasts was measured using standard preparative suspension culture procedures (see Materials and Methods) except that 5×10^4 cells were suspended in 1.5 ml of serum-supplemented medium with 1.5 μ Ci [3 H]thymidine. At 1, 2, 4, 6, and 8 h, both the adherent and nonadherent cells were processed for TCA-insoluble radioactivity as described (Guadagno and Assoian, 1991). G2/M-phase progression in non-adherent NRK and NIH-3T3 cells (C) was measured by direct counting of recovered cells. Specifically, G1/S synchronized fibroblasts (1×10^5 cells) were incubated as described for S-phase progression except that the medium lacked [3 H]thymidine. Cells were recovered by gentle centrifugation either immediately or after 16 h in culture. The results in A and B are shown as mean \pm range of duplicate determinations. For C, triplicate wells for each sample were pooled and counted before calculating the mean number of cells per dish.

adherent cells. (See Guadagno and Assoian, 1991, for the S-phase period in hydroxyurea-free NRK fibroblasts.) Moreover, the hydroxyurea-synchronized, nonadherent cells complete G2 and M-phase as determined by a doubling of cell number (Fig. 1 C). We conclude that the attachment requirement for fibroblast proliferation is explainable in terms of the single START-like cell cycle transition. In fact, our results are consistent with early, albeit much less quantitative, studies examining attachment effects on 3T3 cell proliferation (see "Discussion").

TGF- β 1 Regulates Progression through the Attachment-dependent Transition in a Cell-Specific Fashion

Because the hydroxyurea experiments described above indicate that anchorage-independent growth in nontransformed fibroblasts is blocked by the attachment-dependent transition in late G1, we reasoned that the ability of TGF- β 1 to stimulate anchorage-independent growth must reflect its ability to stimulate G1/S transit. To address this possibility experimentally, G0-synchronized, nonadherent NRK fibroblasts were treated with serum, EGF, and TGF- β 1 in preparative suspension culture. We compared the time course for induction of DNA synthesis (G1/S transit) to the time course for induction of overall cell proliferation (anchorage-independent

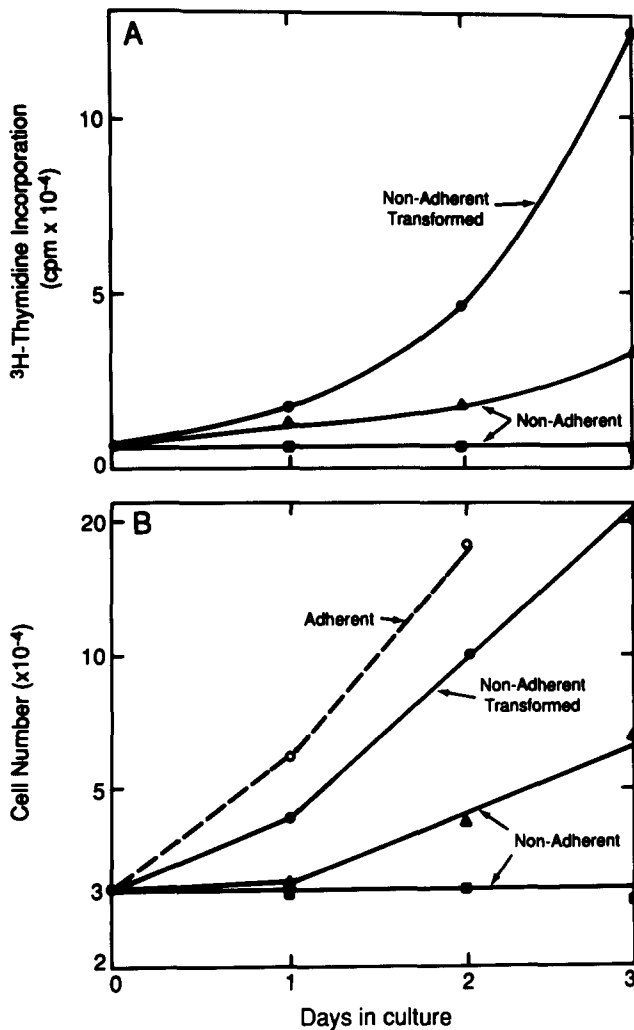


Figure 2. TGF- β 1 overrides G1/S control in nonadherent NRK fibroblasts. The experiment compares rates of DNA synthesis (A) and cell division (B) during induction of anchorage-independent growth by TGF- β 1. Anchorage-independent growth of G0-synchronized, NRK cells (nonadherent, transformed) was induced by addition of dialyzed 5% FCS, EGF, and TGF- β 1 (●). Nonadherent cells were also treated with either dialyzed FCS and EGF (▲) or dialyzed FCS and TGF- β 1 as controls (■). DNA synthesis (within each 24-h period) and cell proliferation was measured for adherent and nonadherent cultures as described in Materials and Methods. The dashed line in B shows the rate of cell proliferation in adherent cultures treated with 5% dialyzed FCS and EGF (determined by Coulter counting of recovered cells). Data shows means of duplicate determinations (range: \pm 6%).

growth). As shown in Fig. 2, treatment of the nonadherent cells with mitogens (FCS/EGF) and TGF- β 1 induces cell cycle progression as indicated by a systematic increase in DNA synthesis (A) and cell proliferation (B). After a 1-d lag, presumably required for TGF- β 1 to induce anchorage-independence fully, cell proliferation in the nonadherent transformed cells approaches the rate characteristic of the nontransformed adherent counterpart (dashed line, B); this behavior is maintained for at least two cell cycles (refer to "nonadherent, transformed," B). Thus, TGF- β 1 can override the attachment requirement for G1/S transit in mitogen-treated NRK cells. Control experiments showed that TGF- β 2 had

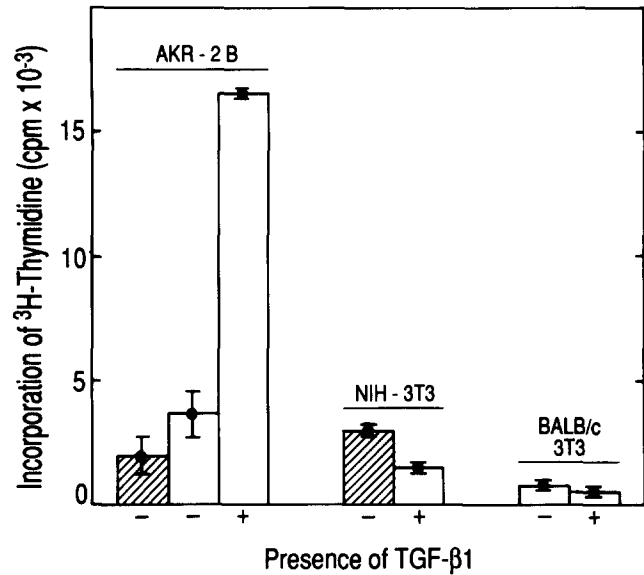


Figure 3. Cell-specific stimulation of G1/S transit by TGF- β 1. The figure compares the effect of TGF- β 1 on stimulation of [³H]thymidine incorporation in AKR-2B, NIH-3T3, and Balb/c 3T3 cells. Cultures of nonadherent G0-synchronized cells were prepared and placed in preparative suspension culture as described in Materials and Methods. To induce anchorage-independent growth, the cells were treated with mitogens (10% FCS, 1 nM EGF) and 100 pM TGF- β 1. The background level of [³H]thymidine incorporation (-) is represented either by 10% FCS (hatched bars) or 10% FCS, 1 nM EGF (open bars). Note that background [³H]thymidine incorporation is shown for both conditions with AKR-2B cells. The extent of cell cycling for each condition was assessed by measuring incorporation of [³H]thymidine into DNA between days 2 and 3. Data show the mean \pm range of duplicate determinations.

the same effect as TGF- β 1 (data not shown). Importantly, both the lag and subsequent increases in the onset of cell proliferation are accompanied by a parallel lag and increase in the onset of DNA synthesis. This result is consistent with our proposal that induction of G1/S transit is rate limiting for induction of anchorage-independence by TGF- β 1. Note that nonadherent NRK cells lacking either TGF- β 1 or the full complement of mitogens synthesize DNA (A) and proliferate (B) poorly in suspension.

Although TGF- β 1 efficiently induces anchorage-independence in mitogen-treated NRK fibroblasts, this effect is specific to certain fibroblast cell lines (refer to Rizzino, 1987). For example, TGF- β 1 also induces anchorage-independent growth (assessed by colony formation in soft agar) in mitogen-treated AKR-2B fibroblasts whereas NIH-3T3 and Balb/c 3T3 cells are poorly responsive. If the stimulatory effect of TGF- β 1 on anchorage-independent growth results from induction of G1/S transit alone, then DNA synthesis should only be stimulated in the cell lines that respond to TGF- β 1 by undergoing anchorage-independent growth. To test this prediction, AKR-2B, NIH-3T3, and Balb/c 3T3 fibroblasts were G0-synchronized, placed in preparative suspension culture, and treated with mitogens and TGF- β 1 (see Materials and Methods for details). Stimulation of G1/S transit was assessed by incorporation of [³H]thymidine into DNA (see figure legend for details).

As expected from their behavior in soft agar (Moses et al.,

1985), TGF- β 1 stimulated DNA synthesis in preparative suspension cultures of AKR-2B cells (Fig. 3); these cells also increased 3.5-fold in number during the 3-d period (not shown). In contrast, TGF- β 1 failed to stimulate DNA synthesis in either NIH-3T3 or Balb/c 3T3 cells (Fig. 3); these cells showed no increase in number (not shown). Note that NIH-3T3 and Balb/c 3T3 cells have TGF- β 1 receptors as determined by ligand binding (Massagué et al., 1990; Segarini, 1990) and respond to 100 pM TGF- β 1 as evidenced by stimulation of fibronectin or α 5 β 1 integrin levels (Ignatz and Massagué, 1986; Blatti et al., 1988; and Dalton and Assoian, unpublished data). Moreover, the lack of colony formation in TGF- β 1-treated NIH-3T3 cells and Balb/c 3T3 cells is not likely due to insufficient mitogenic stimulation because the concentrations of mitogens (10% FCS, 1 nM EGF, and 10 μ g/ml insulin) that were present in the cultures were highly growth stimulatory for the cells in monolayer.

The results of Fig. 3 document the extent of [3 H]thymidine incorporation between days 2 and 3 (the optimal time period for TGF- β 1-mediated DNA synthesis in NRK cells; refer to Fig. 2) and deliberately show the different mitogens we tested (10% serum with or without EGF). However, other studies (not shown) characterized the TGF- β 1 effects on NIH-3T3, Balb/c 3T3, and AKR-2B cells in further detail. Results from these experiments showed that (a) the same cell-specific effects shown in Fig. 3 were obtained when we measured [3 H]thymidine incorporation between days 0–1 or days 1–2, (b) the presence or absence of EGF did not alter the response of non-adherent NIH-3T3 or Balb/c 3T3 cells to TGF- β 1, and (c) the effect of TGF- β 1 on AKR-2B cells was decreased threefold by deleting EGF from culture medium. The enhancing effect of EGF that we observe in preparative suspension culture differs somewhat from studies with AKR-2B cells in soft agar (Moses et al., 1985) which showed that EGF had no effect on TGF- β 1-stimulated colony formation. Nevertheless, our data fundamentally agree with these initial studies because we find that TGF- β 1 does stimulate anchorage-independent growth of AKR-2B cells in the absence of exogenous EGF. Overall, the results of Figs. 2 and 3 allow us to propose (a) that the ability of TGF- β 1 to stimulate anchorage-independent growth is directly correlated with its ability to stimulate G1/S transit, and (b) that stimulation of G1/S transit is both necessary and sufficient for induction of anchorage-independent growth by TGF- β 1.

Isolation of NRK Fibroblast Mutants That Have Lost the Attachment/TGF- β 1 Requirement for Cell Cycle Progression

The results described previously (Guadagno and Assoian, 1991; Pardee, 1989) and above suggest the following model for control of the fibroblast cell cycle: cell cycle progression from G0 through early-mid G1 is regulated by mitogenic growth factors (and perhaps modulated by cell adhesion; Ingber et al., 1990) and progression from late G1 to S is regulated by cell adhesion or TGF- β 1. Interestingly, this model predicts that at least a portion of the subcellular events regulated by mitogenic growth factors and adhesion/TGF- β 1 should be distinct and separable.

In an attempt to demonstrate that mitogenic growth factors and adhesion/TGF- β 1 have separable roles in fibroblast cell cycle control, we randomly mutagenized nontransformed

NRK fibroblasts by exposure to EMS and asked whether we could identify clones that had lost only their adhesion requirement for proliferation. To identify these potential clones, the EMS-mutagenized cells were first cultured in mitogen (FCS/EGF)-supplemented soft agar; cells giving rise to colonies were recovered, expanded in monolayer, and subjected to secondary and tertiary screens in soft agar to ensure fidelity of the phenotype. The parental cell line was cultured in parallel.

From 2×10^5 cells (10^5 cells from cultures mutagenized with 10 and 19 mM EMS), we isolated 40 clones that formed colonies in the first soft agar screen (20 each from the 10 and 19 mM EMS-treated monolayers). Three of these clones, (all derived from exposure to 10 mM EMS) scored positive in the secondary and tertiary screens and were named NRK/EMS clones B, F, and J. Consistent with previous studies (Roberts et al., 1981; Assoian et al., 1983), we observed that a few colonies occasionally formed when the non-mutagenized NRK cell parent was cultured with serum and EGF alone. However, the colonies recovered from this culture condition never yielded clones that maintained high efficiency colony formation (>75% of cells seeded; refer to Materials and Methods) in secondary or tertiary screens. Thus, in all probability, the high efficiency colony formation we observed with NRK/EMS clones B, F, and J reflect the EMS-induced mutation rather than the existence of a mutated subpopulation in the parental line.

Fig. 4 shows the phase contrast micrographs obtained when the proliferation of parental NRK fibroblasts and the three final NRK/EMS clones was examined in soft agar. As

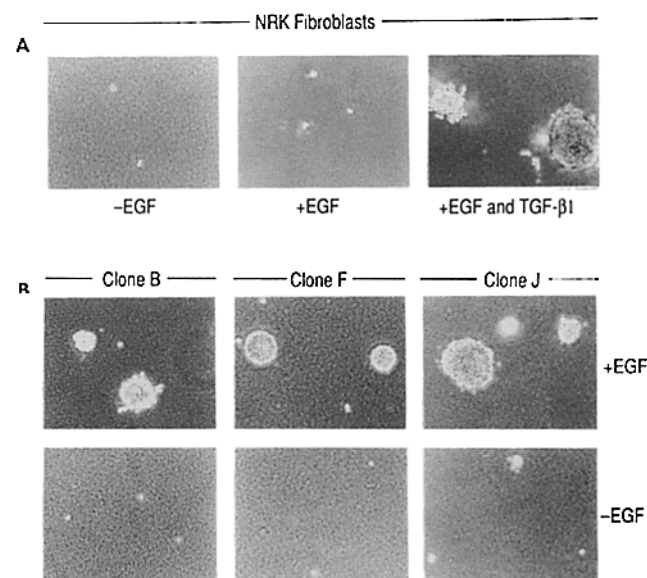


Figure 4. Colony formation of NRK fibroblasts and the NRK/EMS mutants. The figure shows phase contrast micrographs (10 \times magnification) of representative colonies resulting from 10-d incubation of NRK fibroblasts and the NRK/EMS mutants in soft agar. A shows, from left to right, results obtained when the parental NRK cell line was cultured in soft agar containing 5% FCS, 5% FCS/EGF, and 5% FCS/EGF supplemented with 100 pM TGF- β 1. B shows results obtained when each NRK/EMS mutant was cultured in soft agar containing 5% FCS, with (top panels) and without (bottom panels) EGF.

expected, parental NRK fibroblasts (*A*) proliferate very poorly when cultured in soft agar containing serum and EGF. In contrast, identically treated NRK/EMS cell lines (*top of B*) are able to undergo multiple rounds of cell division and form colonies in soft agar. In fact, the colonies induced in the NRK cell parent by the combined action of serum, EGF, and TGF- β 1 (*right-hand side of A*) were only slightly larger than the colonies induced in the EMS mutants in the absence of TGF- β 1. Interestingly, the EMS clones retained the EGF requirement for colony formation (a hallmark of parental NRK fibroblasts; Roberts et al., 1981): colony formation was minimal in each of the EMS mutants when the culture medium contained serum alone (*bottom of B*). When cultured with serum and EGF, at least 75% of the cells in each EMS clone formed colonies in soft agar.

Because quantitation of anchorage-independent growth by colony formation in soft agar is intrinsically subjective, we prepared preparative suspension cultures of non-mutated NRK cells, NRK/EMS clone B, and NRK/EMS clone F and quantitated the extent of anchorage-independent growth by measuring incorporation of [3 H]thymidine into DNA. We have previously shown that this methodology results in an objective assessment of anchorage-independence and gives appropriate "weight" to all size colonies (Assoian et al., 1989). All of the cultures contained mitogens (serum/EGF), and all four cell lines were tested in the absence and presence of TGF- β 1.

As shown in Fig. 5, the parental NRK cell line in preparative suspension cultures showed minimal cell cycling in the absence of TGF- β 1. In contrast, each of NRK/EMS clones was able to cycle in the presence of mitogens (serum/EGF) alone although there was a noticeable difference in the efficiency of cycling between the different clones. Specifically, the proliferation of clone B was enhanced by the addition of TGF- β 1 (although to a much smaller degree than observed with the NRK cell parent) whereas TGF- β 1 did not enhance the proliferation of clones F or J. The results from three separate experiments showed that the extent of cell cycling in serum/EGF-treated clone B was somewhat variable (30–80% of maximal) but was always significantly greater than that observed with the NRK parent within any one experiment (the data shown is the most representative). Thus, random mutagenesis has allowed us to isolate NRK fibroblast mutant lines that have lost, at least in large part, their attachment/TGF- β 1 requirement for cell proliferation.

The results in Fig. 5 raised the possibility that EMS-induced mutagenesis had affected cell cycle genes that are normally regulated by cell adhesion or TGF- β . However, it remained possible that these mutant lines were proliferating in soft agar because they were secreting active TGF- β , they had a constitutively active TGF- β receptor, or they were activating the latent TGF- β in serum or produced by the cells. Several control experiments were designed to address these concerns.

It is well-established that many cell types express low levels of TGF- β 1 mRNA (Derynck et al., 1985) and therefore have the potential to secrete the growth factor. TGF- β 1 is typically secreted in latent form, as a result of its association with its prodomain or α 2-macroglobulin (Massagué, 1990), but EMS-induced mutations (e.g., affecting the association of the mature growth factor with its prodomain or increasing plasminogen activator levels; refer to Massagué, 1990)

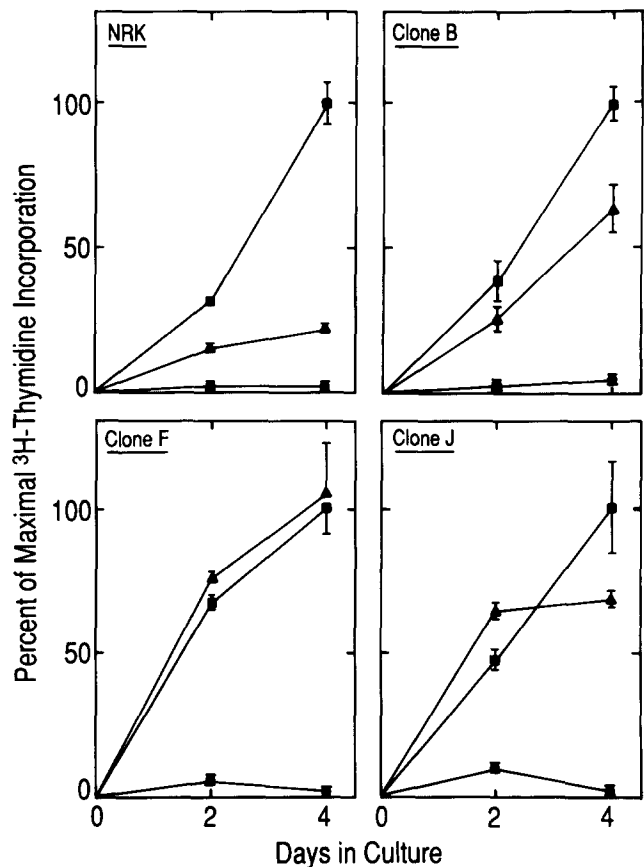


Figure 5. Quantitation of anchorage-independent growth in NRK fibroblasts and the NRK/EMS mutants using preparative suspension cultures. NRK fibroblasts and the three NRK/EMS mutants were placed in preparative suspension culture. The cultures were pulsed with [3 H]thymidine for 24 h at days 1–2 and 3–4; cell proliferation was then assessed by measuring the incorporation of radiolabel into TCA-insoluble products. The figure shows results obtained when the cultures were supplemented with 5% FCS (●); 5% FCS, EGF (▲); or 5% FCS, EGF, and TGF- β 1 (■). The data are presented as means and ranges of duplicate determinations and are plotted as percent of maximal [3 H]thymidine incorporation (defined as the level of DNA synthesis at day 4 in the TGF- β 1-supplemented cultures). For clarity, overlapping range bars are shown for a single direction only.

might result in secretion of the active dimer. To determine if active TGF- β -like activity was being secreted from the NRK/EMS mutants, we prepared conditioned medium from suspension cultures of NRK fibroblasts and each of the three NRK/EMS mutant cell lines. Conditioned media was prepared from suspension cultures to ensure that it would be obtained from conditions resulting in anchorage-independent growth. Preparative suspension cultures were prepared using these distinct conditioned media, and we asked if any of the media could stimulate anchorage-independent growth (measured as [3 H]thymidine incorporation into DNA) of parental NRK fibroblasts (Fig. 6 *A*). As expected, addition of purified EGF and TGF- β 1 to each of the conditioned media resulted in anchorage-independent growth (condition 3 for each cell type). In the absence of exogenous TGF- β 1, the conditioned media from clones B and F (tested in the ab-

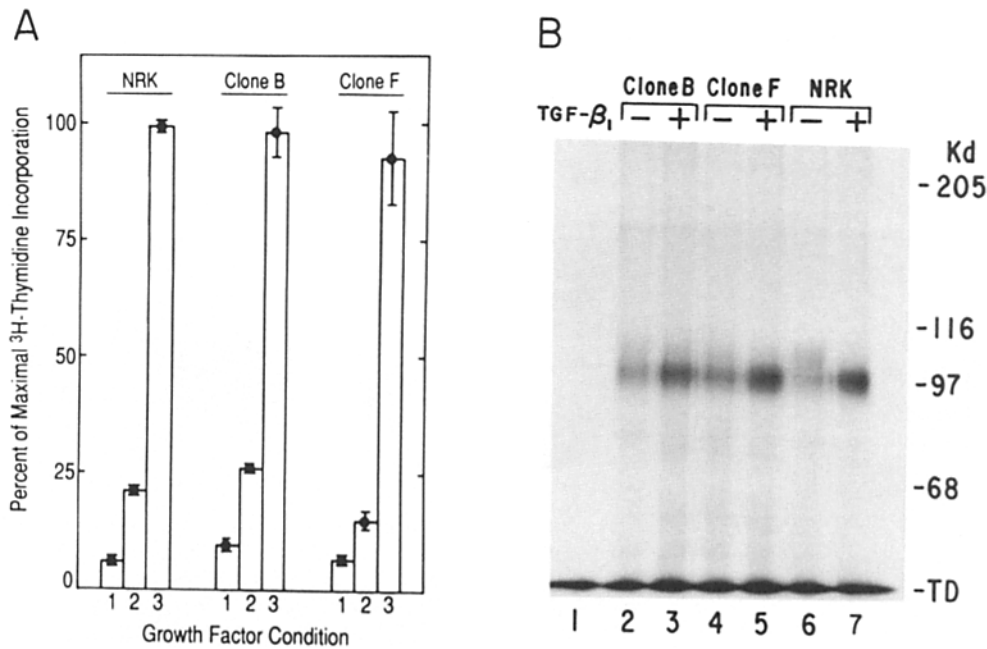


Figure 6. NRK/EMS mutants B and F neither secrete TGF-β₁-like activity nor have constitutively active TGF-β receptors. For the experiment shown in A, NRK fibroblasts and each NRK/EMS mutant were placed in preparative suspension culture using two 100-mm dishes per cell type. Cell culture dishes were coated with 5 ml of 1% agar in 5% FCS-DME, and 2 × 10⁶ cells (in 10 ml of 5% FCS-DME) were added to each dish. After 3 d in culture, the cells and their corresponding conditioned media were collected and subjected to gentle centrifugation. The conditioned medium was carefully removed. These different conditioned media were used to prepare standard preparative suspension cultures in 35-

mm dishes (see Materials and Methods). Freshly trypsinized, G₀-synchronized NRK cells (2 × 10⁴; the parental line only) were suspended in 2 ml of each different conditioned medium and seeded on the corresponding bottom layer. After 48 h, [³H]thymidine (1 μCi) was added to each well, and the extent of DNA synthesis was determined for the next 24-h period. For each cell type, columns 1–3, respectively, show results obtained with conditioned media, conditioned medium supplemented with EGF, and conditioned medium supplemented with EGF and TGF-β₁. The data show means and ranges of duplicate determinations and are plotted as percent of maximal [³H]thymidine incorporation (defined as the TGF-β₁ response obtained when NRK cells were tested in autologous conditioned medium). B shows the effect of TGF-β₁ on synthesis of the β₁ integrin subunit in NRK/EMS clone B (lanes 2 and 3), NRK/EMS clone F (lanes 4 and 5), and the nonmutagenized NRK parent (lanes 6 and 7). Freshly trypsinized cells (5 × 10⁵ cells/in 10 ml 5% FCS-DME and 1 nM EGF) were added to 100-mm dishes and incubated in the absence (–) or presence (+) of 100 pM TGF-β₁ for 2 d. The resulting monolayers were washed once in methionine-free minimum essential medium and incubated in methionine-free MEM with 5% dialyzed FCS, 1 nM EGF, and [³⁵S]methionine (1 mCi/dish; Translabel, ICN Biomedicals) for 2 h in the absence or presence of TGF-β₁. The biosynthetically labeled cells were extracted in buffer A, and equivalent amounts of TCA-precipitable radioactivity (4 × 10⁶ cpm/sample) were incubated with an antibody to the β₁ integrin subunit under conditions that immunoprecipitate the individual β₁ subunit. Lane 1 shows that the integrin subunit was not immunoprecipitated by normal rabbit serum. See Dalton et al. (1992) for details regarding the extraction and immunoprecipitation. The relative migration of molecular weight standards (kD) are indicated on the right hand side of the figure. TD, tracking dye.

sence [condition 1] or presence [condition 2] of EGF) failed to stimulate anchorage-independent growth and, in general, behaved identically to conditioned medium prepared from the NRK cell parent. In contrast, the EGF-supplemented conditioned medium from clone J showed variable, but often elevated, incorporation of [³H]thymidine (not shown). Because this result raises the possibility that NRK/EMS clone J secretes active TGF-β₁, or a biological activity resembling TGF-β₁, further studies were focused on clones B and F.

To determine whether the mutation(s) in NRK/EMS clones B and F had resulted in a constitutively active TGF-β receptor, we incubated NRK/EMS clones B and F with [³⁵S]methionine and asked whether exogenous TGF-β₁ retained its well-established ability to stimulate synthesis of the β₁-integrin subunit (Massagué, 1990). The NRK parental line was tested in parallel and served as the positive control. As shown in Fig. 6 B, synthesis of the β₁ integrin subunit was stimulated to a similar degree by TGF-β₁ in the two mutant lines and in the NRK cell parent. This similar responsiveness indicates that the TGF-β receptor has not been constitutively activated in the NRK/EMS clones B and F.

Finally, we wanted to exclude the possibility that anchorage-independent growth of NRK/EMS clones B and F was not due to activation of the latent TGF-β in 5% serum. To address this issue, we designed an experiment similar to that shown in Fig. 5, but we stimulated anchorage-independent growth by adding EGF and TGF-β₁ to preparative suspension cultures of cells (the NRK parent, NRK/EMS clone B, and NRK/EMS clone F) maintained in defined medium lacking serum. Proliferation was assessed by determining the extent of [³H]thymidine incorporation occurring between days 1 and 2. (Serum is beneficial, but not obligatory, during the first two days of anchorage-independent growth with the defined medium we use.) The results from this control experiment (not shown) resembled those shown in Fig. 5; efficient DNA synthesis in the NRK parent required EGF and TGF-β₁ whereas clones B and F required EGF alone. Together, these data and the results of Fig. 6, A and B indicate that the gene(s) mutated in clones B and F regulate the attachment/TGF-β₁ requirement for cell cycle progression rather than secretion of TGF-β-like activity, basal activity of the TGF-β receptor, or the conversion of serum-derived TGF-β from a latent to active form.

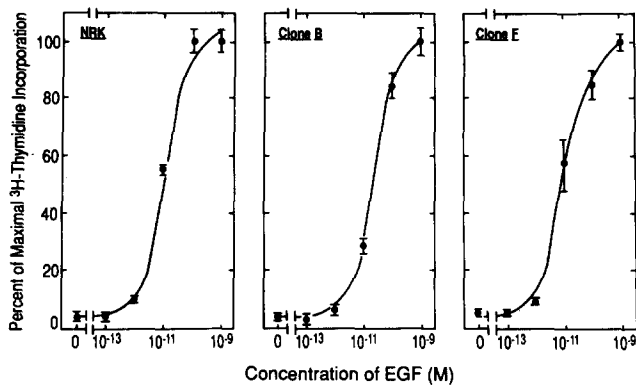


Figure 7. NRK fibroblasts and the NRK/EMS mutants have similar dose-dependent responses to EGF in preparative suspension culture. NRK fibroblasts, NRK/EMS clone B, and NRK/EMS clone F were incubated in preparative suspension culture (see Materials and Methods) in medium containing 5% FCS and selected concentrations of EGF. The NRK cell parent line was also treated with 100 pM TGF- β 1. The EGF dose-response curve for anchorage-independent growth was determined between days 3 and 4 by incubating the cultures with [3 H]thymidine (1 μ Ci/well) and counting the radiolabeled DNA. Basal and maximal [3 H]thymidine incorporation (mean \pm SD) was: 5,000 \pm 500 and 135,400 \pm 1,500, respectively, for NRK fibroblasts; 3,350 \pm 450 and 82,670 \pm 4,150, respectively, for clone B; and 8,070 \pm 150 and 148,000 \pm 5,100, respectively, for clone F.

The Attachment/TGF- β 1 Requirement for Cell Cycle Progression Is Separable from the Growth Factor Requirement

We next performed a series of experiments to determine if EMS mutants B and F had lost their growth factor requirement (or showed a decreased growth factor requirement) as well as their attachment requirement for proliferation. We have already shown that the EGF requirement for proliferation in soft agar suspension is retained in each of the mutant lines (refer to Fig. 4), but to extend these qualitative results we used our preparative suspension culture system and compared dose-dependent EGF effects in the NRK parent, clone B and clone F. As shown in Fig. 7, the EGF dose-response curve is very similar for the three lines: 50% maximal stimulation is observed with 10–20 pM EGF. Although clone B typically required slightly more EGF than the parent or clone F, neither EMS mutant showed a reduced dependency on EGF. Thus, quantitative analysis of proliferation under actual conditions of anchorage-independent growth shows that the EGF requirement is retained in NRK/EMS mutants B and F.

We also examined the growth factor requirements of clones B and F when cultured in monolayer. When we tested the response of these clones to FCS using standard mitogen assays (Fig. 8), we found that the dose-dependent FCS effect in the NRK cell parent, NRK/EMS clone B, and NRK/EMS clone F were similar. For the experiment shown, half-maximal stimulation of [3 H]thymidine incorporation was observed with 0.5, 0.7, and 0.9% FCS for the NRK parent, clone B, and clone F, respectively. Clones B and F never showed a reduced requirement for FCS relative to the NRK cell parent. The background levels of [3 H]thymidine incor-

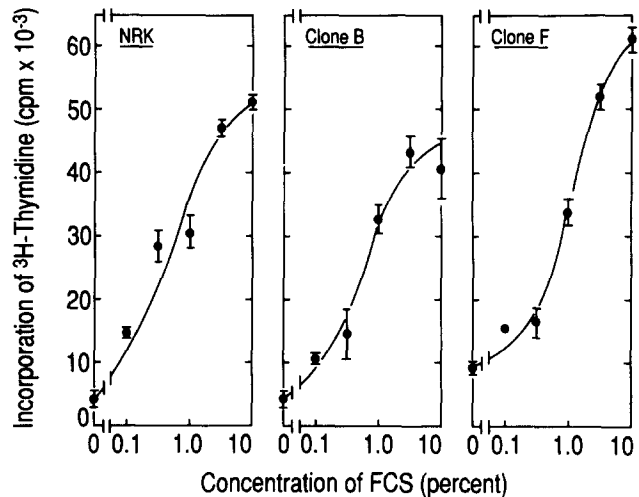


Figure 8. Similar mitogenic responses in serum-starved monolayer cultures of NRK fibroblasts and the NRK/EMS mutants. The figure shows results obtained from assays measuring the effect of serum on stimulation of [3 H]thymidine incorporation into DNA with NRK fibroblasts, NRK/EMS clone B, and NRK/EMS clone F. The cells were plated, serum starved, exposed to FCS and [3 H]thymidine, and extracted for TCA-insoluble radioactivity. This experiment was performed similarly to the method described (Assoian et al., 1983) except that the serum was added in a volume of 25 μ l (diluted into DME, 1 mg/ml BSA as necessary) and radiolabel was added 10 h after serum. The data show means and ranges of duplicate determinations.

poration, and the maximal fold stimulations, were also similar for the parent and these two EMS mutant lines when tested in monolayer (Fig. 8).

Finally, we prepared subconfluent monolayers of G0-synchronized parental NRK cells, NRK/EMS clone B, and NRK/EMS clone F in growth factor-deficient (DME with 2% dialyzed plasma-derived serum) and growth factor-enriched (DME with 2% FCS and EGF) media. Cell proliferation was monitored over a 3-d period. The results from this experiment (Fig. 9) show that both clones B and F failed to increase in number when the culture media was deficient in exogenous mitogenic growth factors. When three separate experiments were compared, proliferation of the NRK cell parent and the mutants in medium supplemented with plasma-derived serum was indistinguishable. Moreover, all three NRK lines (the parent and the two mutants), increased in number at similar rates when cultured in the mitogen-enriched medium. Thus, three distinct experimental designs (Figs. 7–9), using both adherent and nonadherent cells, indicate that clones B and F have retained their normal mitogen requirements for proliferation.

The top panels of Fig. 10 show phase contrast micrographs of the parent and mutant cell lines cultured in monolayer. Remarkably, the EMS mutants are well-spread and nonrefractile. We never observe foci-formation with clones B and F even when kept at confluence for several days. In all these regards, the EMS mutant lines have a phenotype in monolayer that resembles the parental NRK fibroblast line despite the fact that they are anchorage-independent for proliferation. In contrast and as expected, when we cultured NRK fibroblasts expressing activated mos or K-ras on-

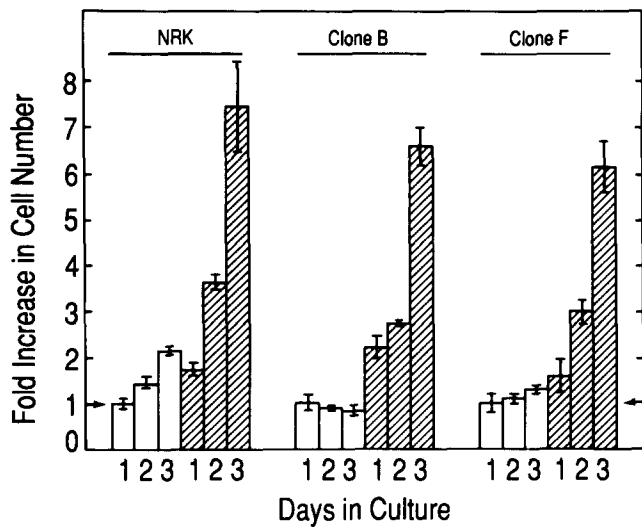


Figure 9. Growth factor-dependent proliferation of NRK fibroblasts and NRK/EMS mutants in monolayer. The figure shows results from an experiment measuring proliferation of parental and mutant NRK cell monolayers in growth factor-deficient (*open bars*) and growth factor-enriched (*hatched bars*) media over a 3 d period. See Materials and Methods for details of the culture media and method for quantitating changes in cell number. The data show means and ranges of duplicate determination plotted as fold increase in cell number (defined as the cell number determined after 1 d incubation in growth factor-deficient medium; *horizontal arrows*).

cogenes (not shown), the cells were highly refractile and poorly spread in monolayer. These two cell lines were also able to proliferate in plasma-derived, as well as, whole-blood derived serum. Similar results have been reported for NRK cells expressing the src oncogene (Chen et al., 1977; Durkin and Whitfield, 1984).

To demonstrate further that NRK/EMS clones B and F maintain nontransformed growth properties in monolayer,

we compared the two mutant lines and the NRK parent with respect to their rates of cell proliferation, the onset of contact inhibition, and their cell densities at confluence. As shown in the bottom panels of Fig. 10, parental NRK cells and the two EMS mutants proliferate at similar rates in response to mitogens (FCS and EGF) and become contact-inhibited at similar times. Moreover, the three lines reach final cell densities that differ by less than a factor of 1.5 despite the fact that final cell numbers have increased ~ 20 -fold during the 7-d incubation period.

Discussion

As a general issue, the role of cell adhesion in controlling cell cycle progression was first raised many years ago. Otsuka and Moskowitz (1975) showed that 3T3 cells in S phase could be trypsinized and transferred to a methylcellulose suspension culture without affecting their ability to continue DNA synthesis. Although poorly quantitated, the authors also showed that at least some of these cells gave rise to mitotic figures. In contrast, DNA synthesis did not occur if the cells were in G1 at the time of trypsinization and transfer to the methylcellulose suspension. Matsuhisa and Mori (1981) obtained similar results with Balb/c 3T3; their method for assessing cell cycle progression was based primarily on visual distinctions between single cells and cell doublets (taken as evidence of interface and M-phase cells, respectively).

The results presented in this report confirm and extend these early studies with 3T3 cells. Our experiments with hydroxyurea show that the extent of S-phase progression in adherent and nonadherent NRK and NIH-3T3 fibroblasts is identical if the cells are synchronized after the START-like attachment-dependent transition in late G1. Moreover, these nonadherent cells progress through G2 and M as determined by a doubling of cell number. The analytical methods we used are both direct and quantitative, and similar results were obtained with two distinct fibroblastic lines. We con-

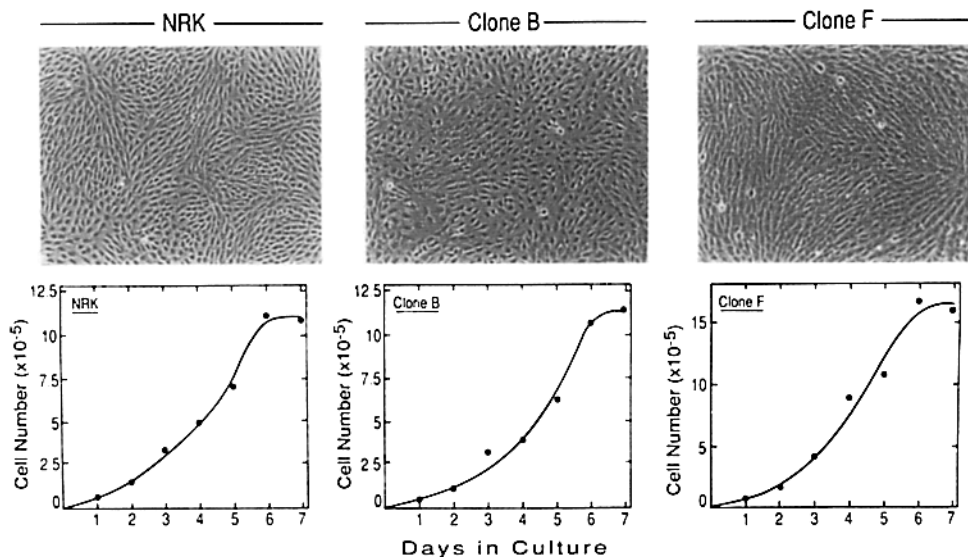


Figure 10. Normal phenotype of NRK/EMS clones B and F in monolayer. The panels show results obtained when NRK fibroblasts (*left*) and NRK/EMS clones B (*middle*) and F (*right*) are cultured in monolayer. The top panels show phase contrast micrographs ($10\times$ magnification) of each line as a confluent monolayer in 5% FCS-DME. The bottom panels show the rate of cell proliferation and the onset of contact inhibition for each line seeded as a subconfluent monolayer (6×10^4 G0-synchronized cells/35-mm well) and brought to confluence with 2% dialyzed FCS and 1 nM EGF. The culture medium was replaced every

other day to avoid potential effects of nutrient or growth factor depletion. To obtain the results shown, triplicate samples were trypsinized, pooled, and counted in a hemocytometer before calculating the mean cell number per dish.

clude that the attachment requirement for proliferation of these mitogen-treated fibroblasts can be explained in terms of a single cell cycle transition imposed at G1/S.

Our data also show that treatment with TGF- β 1 mimics the effect of attachment and stimulates G1/S transit in nonadherent NRK and AKR-2B fibroblasts. This result suggests that the subcellular mechanisms underlying attachment and TGF- β 1 action may be quite similar despite the fact that the proliferating cells have very different phenotypes (adherent and nonadherent, respectively) in culture. Importantly, TGF- β 1 fails to mimic the attachment effect in Balb/c and NIH-3T3 cells: two fibroblast lines that failed to undergo anchorage-independent growth in response to the growth factor. These results do not exclude the possibility that other experimental conditions (e.g., involving addition of other cytokines) might allow TGF- β 1 to override the adhesion requirement for G1/S transit in NIH-3T3 or Balb/c 3T3 cells, but they do show that there is a direct correlation between the distinct cellular effects of TGF- β 1 on anchorage-independent growth and the distinct subcellular effects of TGF- β 1 on stimulation of G1/S transit. Although we have not examined the mechanism by which TGF- β 1 *inhibits* proliferation of cells, it is interesting to note that the inhibitory effect of TGF- β 1 on proliferation of mink lung epithelial cells and skin keratinocytes is specifically associated with decreased G1/S transit (Laiho et al., 1990; Pietenpol et al., 1990a,b; Howe et al., 1991). Thus, the bifunctional effects of TGF- β 1 on cell proliferation may be the consequence of its bifunctional effects on G1/S transit.

Growth factors and adhesion both act in the G1 portion of the fibroblast cell cycle, and our previous studies (Guadagno and Assoian, 1991) suggest that an attachment-dependent transition maps subsequent to the large majority of growth-factor dependent transitions. Thus, we speculated that the combined requirement for growth factors and attachment/TGF- β 1 in fibroblast proliferation might reflect the fact that these extracellular growth regulators have complementary roles in guiding the cell from G0 to S. To test this hypothesis, we asked if we could obtain fibroblast mutants that had lost only their attachment/TGF- β 1 requirement for cell cycle progression. Our ability to demonstrate this mutant phenotype by EMS-induced mutagenesis of NRK cells demonstrates genetically that the growth factor and attachment/TGF- β 1 requirements are separable. Thus, there must be genes that specifically mediate the attachment requirement for cell cycle progression at G1/S. In fact, we believe that the mutations in clones B and F have affected distinct genes because their proliferative rates in preparative suspension culture (relative to the maximal rate observed with TGF- β 1) consistently differ. The cdc2-like kinases and G1-cyclins, retinoblastoma protein, p107, E2F, and p53 all have established effects in regulating cell cycle progression from late G1 to S (Hunter and Pines, 1991; Nevins, 1992; Levine et al., 1991; Bischoff et al., 1990), and we are currently trying to determine if the genes for any of these proteins are controlled by attachment or TGF- β 1. Interestingly, Newman et al. (1986) have isolated a spontaneous transformant of NRK fibroblasts that has also lost its adhesion/TGF- β 1 requirement for proliferation. The relationship between this spontaneous transformant and the EMS-mutants described here remains to be determined.

Recent studies have shown that activation of α β 1 integrins

stimulates tyrosine phosphorylation of cytosolic proteins in fibroblastic (Guan et al., 1991) and epithelial cell lines (Kornberg et al., 1991). Thus, surface integrins, as well as surface growth factor receptors, may be involved in transmitting growth-regulatory signals to the cell. We have previously shown that nonadherent fibroblasts lose the ability to maintain α 5 β 1 integrin (the major fibronectin receptor) on their cell surface (Dalton et al., 1992). Because this loss of surface α 5 β 1 integrin is associated with loss of cell cycle progression (discussed in Dalton et al., 1992), attachment-dependent expression of surface integrins may be an important mechanism involved in preventing anchorage-independent growth of nontransformed fibroblasts.

TGF- β 1 increases the steady state expression levels of several attachment factors and integrins (reviewed in Massagué, 1990), and others (Ignotz and Massagué, 1986) have proposed that upregulated adhesion may underlie the stimulatory effect of TGF- β 1 on anchorage-independent growth. Our data, showing that adhesion and TGF- β 1 share the ability to stimulate transit through the same START-like portion of the cell cycle, is consistent with this proposal. However, extracellular fibronectin and cell-surface α 5 β 1 integrin levels are dramatically reduced upon loss of attachment (Dalton et al., 1992), and we do not yet know the extent to which these effects can be reversed by TGF- β 1. We are currently trying to use our preparative suspension culture system to measure the effects of TGF- β 1 on these downregulated adhesion systems and correlate the effects to induction of anchorage independence.

Anchorage-dependence, requirements for exogenous growth factors, and contact-inhibition comprise a group of properties that jointly reflect the phenotype of nontransformed fibroblasts. Similarly, most transformed cells are anchorage-independent, have a greatly reduced requirement for exogenous growth factors, and lose contact inhibition. For example, these three properties are lost in parallel when NRK fibroblasts are transformed by K-ras (see above), v-mos (see above), or v-src (Chen et al., 1977; Durkin and Whitfield, 1984). The reduced requirement for exogenous growth factors likely results, at least in part, from the fact that many transformed cells secrete mitogenic growth factors that stimulate cell proliferation in an autocrine fashion (reviewed in Pardee, 1989). Consistent with this result, we note that NRK/EMS clone J seems to have an altered responsiveness to growth factors, perhaps as a consequence of autocrine growth factor secretion (see Results), as well as a decreased requirement for cell adhesion. In contrast, NRK/EMS clones B and F have lost only their adhesion requirement for proliferation. Despite the fact that clones B and F are anchorage-independent, they do not show phenotype of typically transformed cells when cultured in monolayer. Mutants B and F also failed to form detectable tumors in nude mice under conditions in which ras-transformed NRK cells formed very large (2 cm \times 3 cm) tumors (not shown). Existence of this new anchorage-independent, growth factor-dependent phenotype demonstrates that neither loss of contact inhibition nor reduced growth factor dependence need accompany anchorage-independent growth.

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