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# Ectopic Expression of Cyclin D1 but Not Cyclin E Induces Anchorage-Independent Cell Cycle Progression

DALIA RESNITZKY*

*Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel*

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**Normal fibroblasts are dependent on adhesion to a substrate for cell cycle progression. Adhesion-deprived Rat1 cells arrest in the $G_1$ phase of the cell cycle, with low cyclin E-dependent kinase activity, low levels of cyclin D1 protein, and high levels of the cyclin-dependent kinase inhibitor $p27^{kip1}$. To understand the signal transduction pathway underlying adhesion-dependent growth, it is important to know whether prevention of any one of these down-regulation events under conditions of adhesion deprivation is sufficient to prevent the $G_1$ arrest. To that end, sublines of Rat1 fibroblasts capable of expressing cyclin E, cyclin D1, or both in an inducible manner were used. Ectopic expression of cyclin D1 was sufficient to allow cells to enter S phase in an adhesion-independent manner. In contrast, cells expressing exogenous cyclin E at a level high enough to overcome the $p27^{kip1}$-imposed inhibition of cyclin E-dependent kinase activity still arrested in $G_1$ when deprived of adhesion. Moreover, expression of both cyclins D1 and E in the same cells did not confer any additional growth advantage upon adhesion deprivation compared to the expression of cyclin D1 alone. Exogenously expressed cyclin D1 was down-regulated under conditions of adhesion deprivation, despite the fact that it was expressed from a heterologous promoter. The ability of cyclin D1-induced cells to enter S phase in an adhesion-independent manner disappears as soon as cyclin D1 proteins disappear. These results suggest that adhesion-dependent cell cycle progression is mediated through cyclin D1, at least in Rat1 fibroblasts.**

Growth of normal fibroblasts and epithelial cells in vitro requires not only growth factors but also adhesion to a solid surface coated with extracellular matrix proteins (reviewed in references 34, 37, and 42). Furthermore, transformation of cells by a variety of oncogenes and viral transforming proteins diminishes the cells' requirements not only for growth factors but also for anchorage (20, 41, 44). Anchorage-independent growth in vitro is thought to be correlated to invasiveness and metastasis in vivo (36). The requirement for adhesion has been shown to be cell cycle specific since nonadherent fibroblasts arrest in the $G_1$ phase of the cell cycle (10, 21, 27).

The progression of cells through the various cell cycle phases is regulated by a series of serine/threonine kinases termed cyclin-dependent kinases (CDKs) (reviewed in references 24 and 29). CDK activity is positively regulated by a family of positive regulatory subunits termed cyclins and negatively regulated by association with another family of CDK inhibitory proteins (reviewed in references 18, 23, and 40). The components that regulate the transition from $G_1$ into S phase in mammalian cells include three D-type cyclins (D1, D2, and D3) which assemble into holoenzymes with either CDK4 or CDK6; cyclin E, which combines later in $G_1$ with CDK2; and cyclin A, which combines with CDK2 at the beginning of S phase (reviewed in references 5, 16, and 39). One of the known substrates of $G_1$ CDKs is the product of the retinoblastoma susceptibility gene (pRb) (reviewed in references 14, 45, and 46). pRb is hypophosphorylated in $G_1$, when it binds to and negatively regulates the activities of transcription factors from the E2F family. Phosphorylation of pRb during mid-to-late $G_1$ by $G_1$ CDKs releases E2F from this inhibitory constraint, enabling free E2F to activate genes required for DNA replication (reviewed in references 1, 17, and 38).

The molecular pathway responsible for anchorage-dependent cell cycle progression in normal fibroblasts is of great interest, since any disruption of this pathway is expected to be associated with transformation and high metastasis. Some insight into this pathway was recently provided by several studies demonstrating that many components of the cell cycle machinery are down-regulated in adhesion-deprived, $G_1$-arrested fibroblasts. pRb was shown to be hypophosphorylated in these cells (35, 50). Cyclin E-associated kinase activity was shown to be down-regulated, probably due to an increase in the association of the cyclin-dependent kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$ with CDK2-cyclin E complexes (7, 35, 50). Cyclin D1 kinase activity was also reported to be down-regulated upon adhesion deprivation in some studies (35, 50), although other studies found it to be unchanged (7). In addition, contradictory results were reported concerning the regulation of cyclin D1 protein synthesis in adhesion-deprived, $G_1$-arrested fibroblasts (7, 35, 50). Cyclin A was also reported to be down-regulated at the transcriptional level upon adhesion deprivation (11, 35). This last observation is not surprising, since adhesion-deprived cells arrest at a point in $G_1$ known as the R point (4), a few hours before the onset of S phase (28) and before the initiation of cyclin A transcription (13).

To understand the signal transduction pathway underlying adhesion-dependent growth, it is important to know whether prevention of any one of these down-regulation events under conditions of adhesion deprivation is sufficient to prevent the $G_1$ arrest. To study the role of cyclins D1 and E in anchorage-dependent cell cycle progression, sublines of Rat1 fibroblasts capable of expressing cyclin E, cyclin D1, or both in an inducible manner (31, 33) were used to prevent the down-regulation of cyclin D1 and/or cyclin E in adhesion-deprived fibroblasts.

It is shown here that expression from a heterologous promoter (ectopic expression) of cyclin D1, and not of cyclin E, enabled cells to progress in the cell cycle in an anchorage-independent manner. Moreover, ectopic expression of both cyclins D1 and E in the same cell line conferred the same anchorage-independent growth ability as expression of cyclin D1 alone.

* Phone: 972-8-9342941. Fax: 972-8-9344125.

## MATERIALS AND METHODS

**Cells and culture conditions.** Rat1 clones E2 and D5, capable of expressing cyclins E and D1, respectively, in a tetracycline-repressible manner; clone DE5, capable of expressing both cyclins in the same cell line; and clone R12, which is a Rat1 clone containing the tetracycline transactivator (9), have been described previously (31, 33). These cells were maintained in Dulbecco's modified eagle medium with 10% fetal calf serum and 2 μg of tetracycline per ml. For adhesion conditions, regular tissue culture plates were used. For suspension conditions, the plates were coated with poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma) as previously described (8). Clone 6 was derived from rat embryo fibroblasts transformed by temperature-sensitive p53 (p53Val135) and *ras* and was previously described (22).

**Western blot analysis and kinase assays.** Cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer as previously described (43). Protein concentration was determined by the Bio-Rad protein assay.

For Western blot analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto an Immobilon membrane (Millipore), incubated for 1 h at room temperature with blocking solution (10% powdered milk, 0.2% Tween 20 in PBS), and reacted with specific antibodies. To detect exogenous human cyclin E protein, HE12 monoclonal antibodies (a gift from S. I. Reed) were used (hybridoma supernatant; dilution; 1:5). To detect rat and human cyclin D1 proteins, polyclonal antibodies against cyclin D1 (a gift from S. I. Reed) were used (complete serum; dilution, 1:500). To detect rat cyclin A protein, polyclonal antibodies against cyclin A (a gift from S. I. Reed) were used (complete serum; dilution, 1:500). To detect α-tubulin protein, monoclonal anti-α-tubulin antibody (clone DM1A; Sigma) was used. To detect rat $p27^{kip1}$, anti-α-p27 monoclonal antibody (Transduction Laboratories) was used. To detect rat $p21^{cip1}$, anti-α-p21 polyclonal antibody (C19; Santa Cruz) was used. Each antibody was followed by a secondary one, i.e., horseradish peroxidase-conjugated anti-mouse antibody for monoclonal antibodies and horseradish peroxidase-conjugated protein A for polyclonal antibodies (Jackson). Chemiluminescent signals were generated by incubation with ECL reagents (Amersham) in accordance with the manufacturer's instructions.

For cyclin E-associated kinase assay, cyclin E-associated complexes were immunoprecipitated by using a polyclonal antibody recognizing both rat and human cyclins E (a gift from S. I. Reed), washed, and incubated with histone H1 (Gibco BRL) and [γ-$^{32}$P]ATP as described previously (31). Following separation of the reaction products by SDS-PAGE, the gel was stained, dried, and exposed to an autoradiogram and the results were quantitated by counting gel pieces in a scintillation counter.

**Northern blot analysis.** RNA was extracted by using TRI REAGENT (Molecular Research Inc.) in accordance with the manufacturer's instructions. RNA (20 μg) was separated on a formamide-formaldehyde gel as previously described (49), blotted onto a nylon membrane (Hybond N; Amersham), fixed by UV cross-linking, and hybridized to the probes indicated in the legend to Fig. 3.

**Cell cycle analysis.** Cells were labeled for 30 min in the presence of 10 μM bromodeoxyuridine (BrdU; Sigma), collected, and fixed in 70% ethanol overnight at 4°C. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson) and propidium iodide (Sigma) as previously described (32). A total of 10,000 stained cells were analyzed in a fluorescence-activated cell sorter (FACSORT; Becton Dickinson).

**Flow cytometry analysis of cyclin-specific staining.** Cells were fixed in 100% methanol overnight at 4°C, centrifuged, resuspended in PBS, and allowed to rehydrate for 30 min on ice. Following a blocking step with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, cells were stained with cyclin-specific antibodies for 1 h at room temperature with occasional shaking. The HE12 monoclonal antibodies were used for cyclin E staining (hybridoma supernatant, 100 μl per cell pellet), and DCS-6 monoclonal antibodies (a gift from J. Lukas and J. Bartek) were used for cyclin D1 staining (hybridoma supernatant, 100 μl per cell pellet). The stained cells were washed once with 1% BSA in PBS and incubated with FITC-conjugated goat anti-mouse antibody (diluted 1:20 in PBS containing 0.5% Tween 20 and 1% BSA) for 30 min at room temperature. Following an additional wash, cells were resuspended in PBS containing 50 μg of RNase per ml and 25 μg of propidium iodide per ml and analyzed in a FACSORT fluorescence-activated cell sorter.

## RESULTS

All anchorage-dependent cell lines arrest in $G_1$ when forced to grow in suspension. However, some variation in the response of the cell cycle machinery to adhesion deprivation has been reported (see introduction). To study the response of Rat1 fibroblasts to adhesion deprivation, the cells were forced to grow in suspension by being seeded on poly-HEMA-coated plates (8). Cell cycle analysis indicated that after 24 h in suspension, Rat1 cells arrest in the $G_1$ phase of the cell cycle (suspension-induced $G_1$ arrest) and the percentage of cells in S phase decreases from 47% under adhesion conditions to 3% under suspension conditions (Fig. 1A). Analysis of the state of cyclins A, D1, and E in these arrested cells revealed down-regulation of the level of cyclin D1 and cyclin A proteins (Fig. 1B). Although cyclin E was not down-regulated at the protein level (data not shown), cyclin E-associated kinase activity was dramatically reduced (Fig. 1C), as has been reported in other cell lines (7, 35, 50).

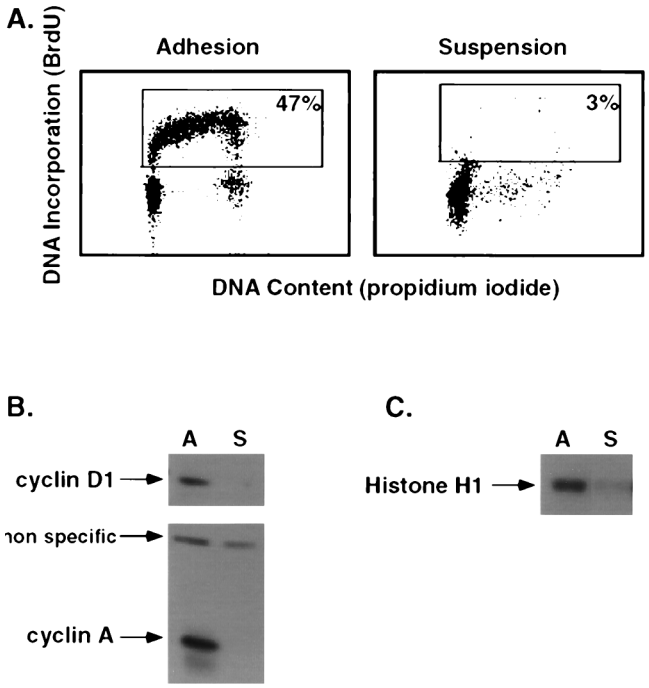


FIG. 1. Rat1 cells deprived of adhesion arrest in $G_1$ with low cyclin E-associated kinase activity and low cyclin D1 and cyclin A protein levels. Rat1 clone R12 cells were seeded on regular (adhesion conditions) or poly-HEMA-coated (suspension conditions) plates. After 24 h, cells were collected for cell cycle analysis (A) and protein extraction (B and C). (A) Cells were labeled with 10 μM BrdU for 30 min, collected, fixed, and analyzed by flow cytometry following double staining with FITC–anti-BrdU and propidium iodide. The small rectangles delineate the population of cells in S phase, and their percentage are indicated. (B) Western blot analysis following separation by SDS–12% PAGE was performed by using cyclin D1- and cyclin A-specific antibodies. Lane A represents adhesion conditions, and lane S represents suspension conditions. The nonspecific band cross-reacting with cyclin A antibodies served as a control for equivalent loading. (C) Cyclin E-associated kinase activity was determined following immunoprecipitation with cyclin E-specific antibodies by using histone H1 as the substrate. Lane A represents adhesion conditions, and lane S represents suspension conditions.

To determine whether ectopic expression of either cyclin D1 or cyclin E can prevent the suspension-induced $G_1$ arrest, Rat1 clone D5 and E2 cells capable of expressing cyclins D1 and E, respectively, in an inducible manner (31) were used. Cells were seeded on regular plastic plates (adhesion conditions) with or without cyclin induction and transferred to poly-HEMA-coated plates (suspension conditions) 24 h later. Cell cycle analysis after 24 h in suspension indicated that whereas noninduced cells arrested in $G_1$ as expected, induction of cyclin D1 led to an increase in the percentage of cells in the S, $G_2$, and M phases of the cell cycle (30% in induced cells compared to 11% in noninduced cells) (Fig. 2A). In contrast, induction of cyclin E did not change the percentage of cells in the S, $G_2$, and M phases significantly (10.2% in induced cells compared to 9.2% in noninduced cells). Cell cycle analysis at various time points after transfer to suspension conditions revealed that induction of cyclin D1 leads to an increase in the percentage of

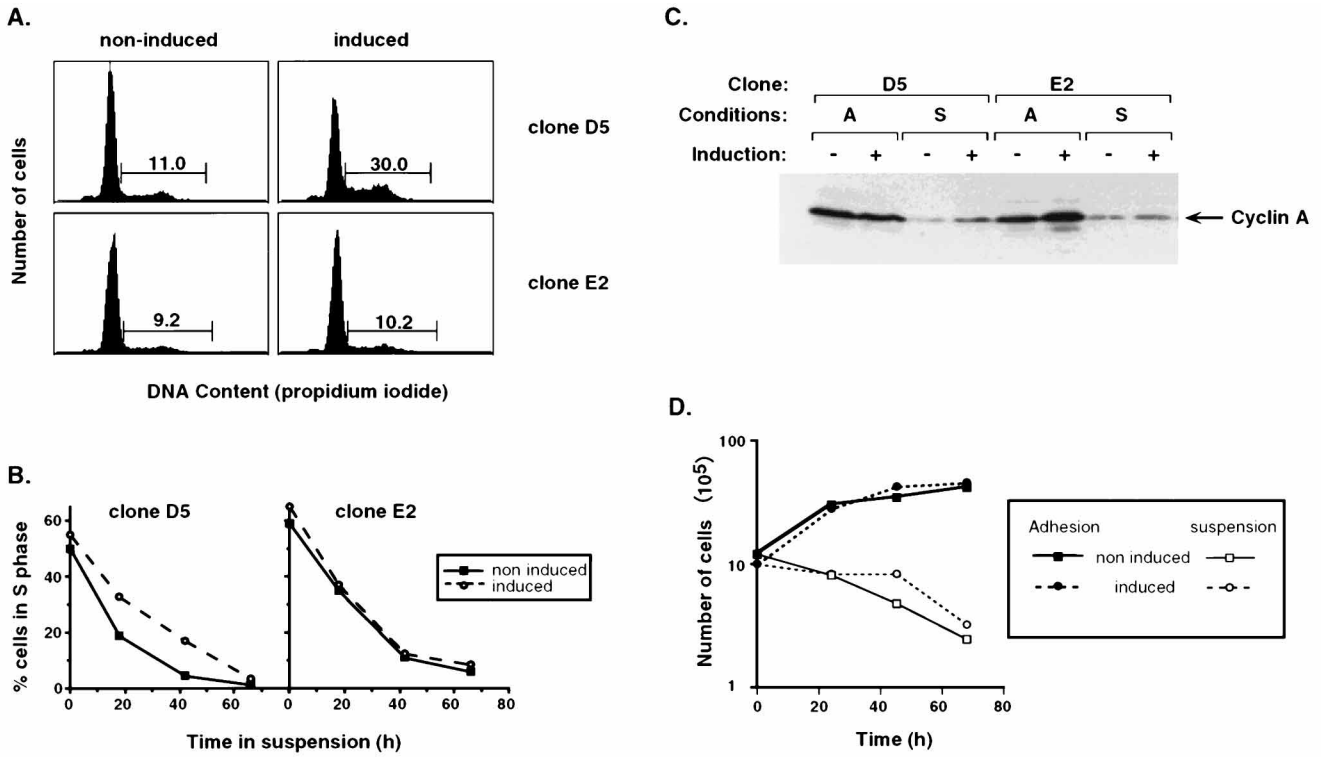


FIG. 2. Ectopic expression of cyclin D1, but not cyclin E, allows transient, adhesion-independent cell cycle progression. Rat1 clone D5 and E2 cells were seeded on regular tissue culture plates (adhesion conditions) with (noninduced) or without (induced) 2 μg of tetracycline per ml. After 24 h (time zero), cells were trypsinized and seeded on poly-HEMA-coated plates (suspension conditions) with or without tetracycline. At the indicated time points, cells were collected for cell cycle analysis (A and B), protein extraction (C), or counting (D). (A) Propidium iodide histograms of clones D5 (top) and E2 (bottom) after 24 h in suspension with (right) or without (left) cyclin induction. The percentages of cells in the S, $G_2$, and M phases of the cell cycle were calculated by using CELLQUEST and are shown above the histograms. (B) Cells were labeled with 10 μM BrdU for 30 min, collected, fixed, and analyzed by flow cytometry following double staining with FITC–anti-BrdU and propidium iodide. The percentages of cells in S phase at the various time points were calculated by using CELLQUEST and plotted versus the time in suspension. (C) Western blot analysis following separation by SDS–15% PAGE was performed by using cyclin A-specific antibodies. A, adhesion conditions; S, suspension conditions. (D) Number of cells per 10-cm-diameter plate under either adhesion or suspension conditions plotted versus time.

cells in S phase after 18 and 42 h in suspension. However, despite this initial elimination of the $G_1$ arrest, after 66 h in suspension, cyclin D1-induced cells arrested in $G_1$ similarly to noninduced cells (Fig. 2B). Cells of clone E2 withdraw from the cell cycle in suspension with somewhat slower kinetics (compare noninduced D5 cells to noninduced E2 cells in Fig. 2B). This difference is probably due to clonal variation, since different subclones of Rat1 cells withdraw from the cell cycle in suspension with slightly different kinetics (data not shown). However, induction of cyclin E did not change the cell cycle distribution of the cells significantly at any of the time points tested. Since cyclin A expression is a hallmark of S phase (13), it was of interest to know whether cyclin A levels were increased in either cyclin D1- or cyclin E-induced cells in suspension. Western blot analysis revealed that induction of cyclin D1 in suspension increased the level of cyclin A, while induction of cyclin E had no affect on the cyclin A level (Fig. 2C). However, the level of cyclin A in cyclin D1-induced cells in suspension was lower than its level in cells under adhesion conditions (compare lane 4 to lanes 1 and 2 in Fig. 2C). This is expected from the fact that the percentage of cells in S phase in cyclin D1-induced cells under suspension conditions is lower than the percentage of cells in S phase under adhesion conditions (Fig. 2B). The results in Fig. 2A to C suggest that induction of cyclin D1 but not cyclin E, leads to partial rescue of S-phase entry and cyclin A expression under suspension conditions.

To find out whether cells that entered S phase in suspension upon cyclin D1 induction are capable of proliferation in suspension, the number of clone D5 cells with and without cyclin induction grown under adhesion or suspension conditions for 3 days was determined. Figure 2D shows that while the number of cells under adhesion conditions increased with time as expected, the number of cells in suspension was reduced, probably due to loss of viability over time. Induction of cyclin D1 in suspension partially prevented the reduction in cell number but did not lead to an increase in the cell number as observed in cells grown under adhesion conditions (Fig. 2D). These results are hard to interpret. Since the effect of cyclin D1 induction on S-phase entry is partial (see above), it is hard to predict which increase in cell number is expected if, indeed, all of the cells that entered S phase as a result of cyclin D1 induction were able to proliferate (see Discussion).

Figure 2B suggests that the ability of cyclin D1 to induce anchorage-independent S-phase entry is transient, as cyclin D1-induced cells eventually arrest in $G_1$, similarly to noninduced cells. To understand the basis for this transient rescue, the cyclin D1 protein levels at various time points after transfer to suspension conditions were analyzed in induced and noninduced cells. Exogenously expressed cyclin D1 protein levels gradually decreased under suspension conditions (Fig. 3A), despite the fact that cyclin D1 was expressed from a heterologous promoter (31). A comparison of the rate of disappearance of cyclin D1 protein in induced cells and the rate of

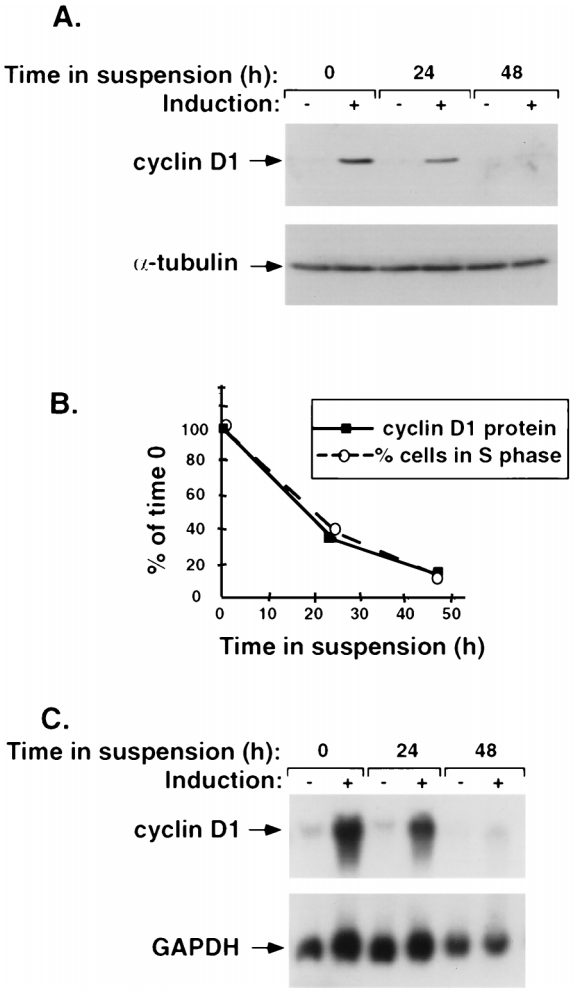


FIG. 3. Exogenously expressed cyclin D1 mRNA and protein are downregulated in suspension. Rat1 clone D5 cells were treated as described in the legend to Fig. 2. At the indicated time points, cells were labeled with BrdU for 30 min, collected, and used for cell cycle analysis and protein and mRNA extraction. (A) Western blot analysis following separation by SDS–12% PAGE was performed by using cyclin D1-specific polyclonal antibodies. The same blot was then reacted with α-tubulin-specific antibodies as a control for equivalent loading. (B) The percentage of cells in S phase at each time point was determined by flow cytometry following double staining with FITC–anti-BrdU and propidium iodide. The amount of cyclin D1 protein was quantitated by scanning the autoradiogram in A. The amount of cyclin D1 protein and the percentage of cells in S phase in cyclin D1-induced cells were plotted versus the time in suspension. (C) Northern blot analysis following separation on a formamide-formaldehyde gel was performed by using a fragment of human cyclin D1 as a probe. Exogenous cyclin D1 mRNA is shown. The same blot was then reacted with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a control for equivalent loading.

disappearance of cells in S phase in the same population revealed identical kinetics (Fig. 3B). These results suggest that the transient ability of exogenously expressed cyclin D1 to rescue cells from the suspension-induced $G_1$ arrest originates from its disappearance under suspension conditions.

To find out whether the reduction in exogenously expressed cyclin D1 under suspension conditions is regulated at the transcriptional or translational level, cyclin D1 mRNA levels were analyzed. Cyclin D1 mRNA levels were reduced under suspension conditions, with kinetics very similar to those of the reduction in cyclin D1 protein (Fig. 3C). These results suggest that cyclin D1 protein levels are reduced in suspension due to a reduction in cyclin D1 mRNA levels. The basis for the reduction in exogenously expressed cyclin D1 mRNA levels is unknown (see Discussion).

The fact that the percentage of cells in S phase in cyclin D1-rescued cells in suspension is lower than in cells growing under adhesion conditions (see above) suggests that cyclin D1 is expressed at different levels in individual cells. Indeed, immunostaining experiments indicated that although clones D5 and E2 are isolated from single cells (31), only 15 to 30% of the cells stained positive for cyclin expression following induction (Fig. 4A and B). If ectopic expression of any cyclin can, indeed, rescue cells from the suspension-induced $G_1$ arrest, one would expect the subpopulation capable of expressing this cyclin to a high level to have a higher percentage of cells in S phase under suspension conditions than the subpopulation in which this cyclin is not expressed or is expressed at a low level. Therefore, induced and noninduced clone D5 and E2 cells growing under suspension conditions were stained with cyclin-specific antibodies and the cell cycle distribution of cells expressing high cyclin levels and cells expressing low cyclin levels within the cyclin-induced population was analyzed. As shown in Fig. 4, the percentage of cells in the S, $G_2$, and M phases was much higher in cells that expressed high cyclin D1 levels than in those that expressed low levels (Fig. 4, compare C to D). Furthermore, the cells expressing high levels of cyclin D1 in suspension exhibited a cell cycle distribution very similar to that of cells

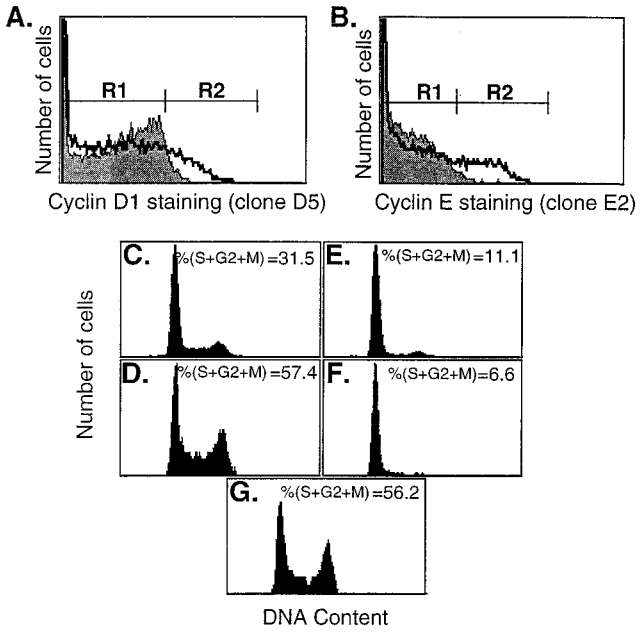


FIG. 4. Cell cycle distribution of cells with high-level cyclin D1 expression in suspension is very similar to that of adherent cells. Rat1 clone D5 and E2 cells were treated as described in the legend to Fig. 2. Twenty-eight hours after transfer to suspension conditions, induced and noninduced cells were collected, fixed, double stained with cyclin-specific antibodies and propidium iodide, and analyzed by flow cytometry. (A and B) FITC staining of cyclin D1-specific (A) and cyclin E-specific (B) antibodies (logarithmic scale). Shaded areas represent noninduced cells, and bold lines represent induced cells. In each histogram, two regions were defined: R1, representing negatively stained cells (low-level expression), and R2, representing positively stained cells (high-level expression). (C to G) Cell cycle histograms (propidium iodide staining, linear scale). Cell cycle distribution of cells with high-level (D and F) and low-level (C and E) expression of cyclin D1 (C and D) or cyclin E (E and F) within cyclin-induced cells after 28 h in suspension and cell cycle distribution of cells with high-level cyclin D1 expression within cyclin D1-induced cells under regular adhesion conditions (G) are shown.

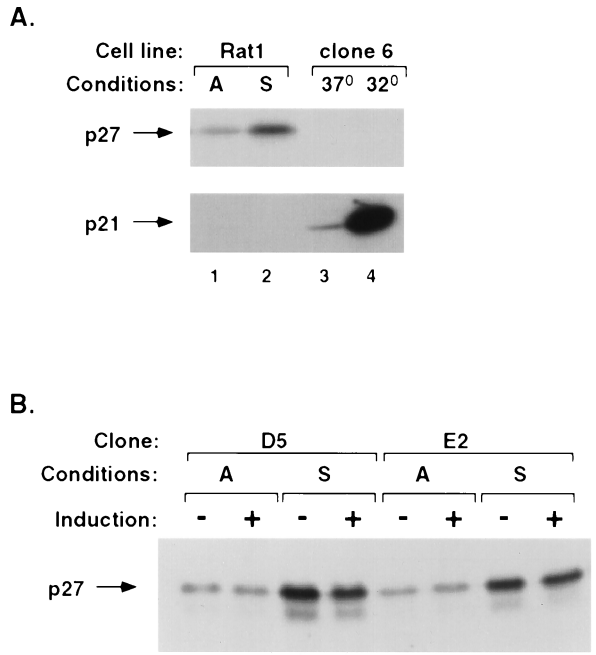


FIG. 5. The level of $p27^{kip1}$ is increased in suspension. (A) Rat1 cells were seeded on regular (A, adhesion conditions) or poly-HEMA-coated (S, suspension conditions) plates. After 24 h, cells were collected for protein extraction. Clone 6 cells were grown at either 37 or 32°C for 24 h and collected for protein extraction. Western blot analysis following separation by SDS–15% PAGE was performed by using p27-specific monoclonal and p21-specific polyclonal antibodies. (B) Rat1 clone D5 and E2 cells were treated as described in the legend to Fig. 2. Lanes: A, cells collected for protein extraction after 24 h under adhesion conditions; S, cells collected for protein extraction 40 h after transfer to suspension conditions. Western blot analysis following separation by SDS–15% PAGE was performed by using p27-specific monoclonal antibodies.

growing under adhesion conditions (Fig. 4, compare D to G). In contrast, cells that expressed low and high levels of cyclin E did not vary in cell cycle distribution (Fig. 4, compare E to F). These results further support the conclusion that ectopic expression of cyclin D1, but not cyclin E, can rescue cells from suspension-induced $G_1$ arrest in Rat1 fibroblasts.

The cip/kip family consists of three proteins, $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$, which bind and inhibit cyclin-CDK complexes (reviewed in reference 40). Three independent studies indicate that the level of $p21^{cip1}$ and/or $p27^{kip1}$ increases under conditions of adhesion deprivation in various cell lines (7, 35, 50). To find out if this is also the case in Rat1 fibroblasts, the levels of the $p21^{cip1}$ and $p27^{kip1}$ proteins in Rat1 cells under adhesion and suspension conditions were analyzed. Figure 5A shows that in Rat1 cells in suspension, the level of $p27^{kip1}$ is elevated while $p21^{cip1}$ is not detected. To verify that the antibodies used in this experiment are able to recognize rat $p21^{cip1}$, clone 6 cells derived from rat embryo fibroblasts transformed by temperature-sensitive p53 (p53Val135) and *ras* (22) were used. In this cell line a temperature shift to 32°C leads to activation of p53 and an increase in $p21^{cip1}$, whose gene is a target of p53 (6). Figure 5B further shows that a $p27^{kip1}$ increase can be detected in clones D5 and E2 in suspension and that it is not significantly affected by induction of cyclin D1 or cyclin E.

The fact that ectopic expression of cyclin D1, but not of cyclin E, induces anchorage-independent cell cycle progression (Fig. 2 and 4) might result from the different abilities of these two cyclins to overcome the inhibitory threshold imposed by $p27^{kip1}$. It is possible that the levels of exogenous cyclin D1 expression are enough to overcome the $p27^{kip1}$ induced inhibition and to give rise to active cyclin D1-dependent kinase, while the levels of exogenous cyclin E are not enough to overcome that inhibition, and cyclin E-dependent kinase remains nonactive. It was therefore important to measure the kinase activity associated with cyclin E in suspension in cells of clone E2 with and without cyclin E induction. Cyclin E-associated kinase activity in noninduced cells was reduced after 30 h in suspension (Fig. 6), as expected (Fig. 1C). However, following induction of cyclin E, the kinase activity after 30 h in suspension was as high as it was in noninduced cells under adhesion conditions (Fig. 6). The results of Fig. 2, 4, and 6 therefore suggest that even though ectopic expression of cyclin E leads to high cyclin E-associated kinase activity in suspension, it is unable to rescue cells from the suspension-induced $G_1$ arrest when expressed alone.

Cyclin E-associated kinase has been previously suggested to recognize its substrate only after it is phosphorylated by cyclin D1-associated kinase (12). It is therefore possible that active cyclin E-associated kinase is capable of contributing to growth in suspension but requires active cyclin D1-associated kinase.

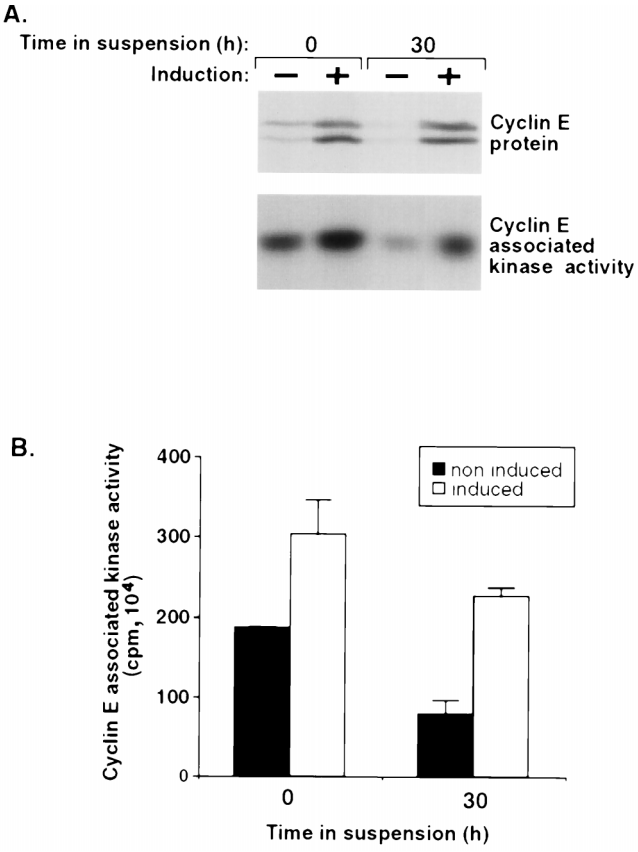


FIG. 6. Ectopic expression of cyclin E leads to high cyclin E-associated kinase activity in suspension. Rat1 clone E2 cells were treated as described in the legend to Fig. 2. At the indicated time points, cells were collected and lysed in lysis buffer. (A) Cyclin E protein level analyzed by Western blot analysis, following separation by SDS–12% PAGE, by using specific HE12 monoclonal antibodies which recognize exogenous human cyclin E but not endogenous rat cyclin E. Cyclin E-associated kinase activity was determined following immunoprecipitation with polyclonal antibodies which recognize both exogenous and endogenous cyclin E by using histone H1 as the substrate. (B) Determination of cyclin E-associated kinase activity (see A) was performed in duplicate, and the radioactivity incorporated into histone H1 was determined by scintillation counting. Standard deviations are shown.

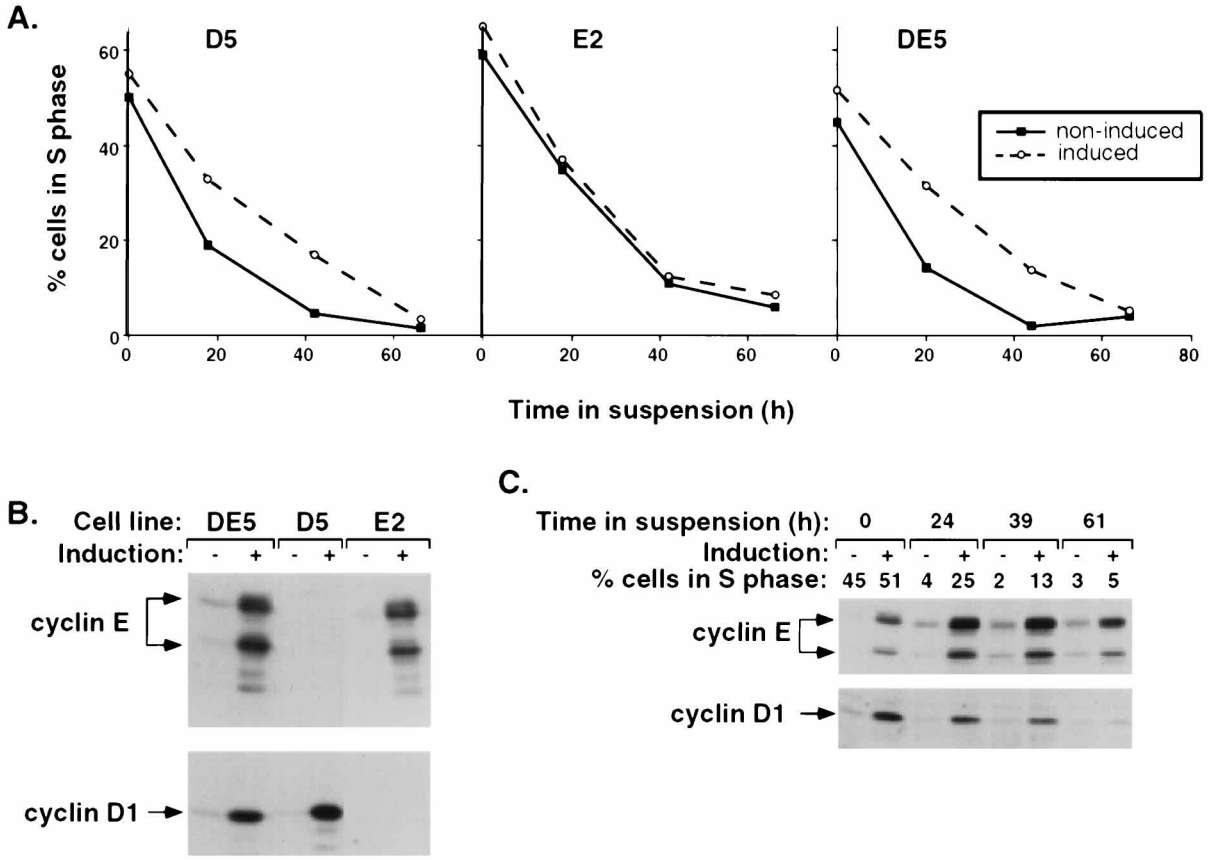


FIG. 7. Expression of both cyclins E and D1 in the same cells has the same effect on growth in suspension as expression of cyclin D1 alone. Rat1 clone DE5, D5, and E2 cells were treated as described in the legend to Fig. 2. At the indicated time points, cells were labeled with BrdU for 30 min, collected, and used for cell cycle analysis and protein extraction. (A) The percentage of cells in S phase at each time point was determined by flow cytometry following double staining with FITC–anti-BrdU and propidium iodide. (B and C) Western blot analysis was performed, following separation by SDS–12% PAGE, by using specific antibodies against cyclin D1 and cyclin E. Cell lysates were prepared after 18 h in suspension (B) or at the indicated time points (C).

To study this possibility, the ability of clone DE5 (capable of expressing both cyclin D1 and cyclin E in an inducible manner [33]) to grow in suspension was compared to that of clones D5 and E2. The ability of cyclin D1 to rescue cells from suspension-induced $G_1$ arrest was not enhanced when it was expressed together with cyclin E (Fig. 7A). Western blot analysis indicated that clone DE5 expressed the same amount of cyclin D1 as did clone D5 and the same amount of cyclin E as did clone E2 in this experiment (Fig. 7B). The expression pattern of cyclin D1 and cyclin E in clone DE5 in suspension was then studied. Cyclin D1 protein in clone DE5 was down-regulated in suspension (Fig. 7C), following an expression pattern similar to that in clone D5 (compare Fig. 7C and 3A). In contrast, cyclin E protein was not reduced and was even enhanced to some extent in suspension (Fig. 7C). However, the ability of DE5 cells to enter S phase seems to be dependent solely on the presence of cyclin D1 protein because the percentage of cells in S phase decreased with the amount of cyclin D1 protein (Fig. 7C).

## DISCUSSION

**Causal relationships between adhesion-dependent cell cycle progression and the cell cycle machinery.** Adhesion-deprived Rat1 cells arrest in the $G_1$ phase of the cell cycle with low cyclin E-dependent kinase activity, low levels of the cyclin D1 and cyclin A proteins (Fig. 1), and high levels of the CDK inhibitor $p27^{kip1}$ (Fig. 5), similarly to other normal fibroblasts (7, 35, 50). The experiments described here were aimed to study which of these events actually cause(s) $G_1$ arrest, based on the assumption that abrogation of the key regulatory event(s) which causes $G_1$ arrest would enable cells to grow in an anchorage-independent manner. It is shown here that ectopic expression of cyclin D1 was sufficient in Rat1 cells to induce S-phase entry in an adhesion-independent manner (Fig. 2). Moreover, complete rescue of cells from the suspension-induced $G_1$ arrest was observed in cells expressing high levels of cyclin D1 (Fig. 4). In contrast, ectopic expression of cyclin E at a level high enough to overcome $p27^{kip1}$ inhibition and give rise to high kinase activity in suspension (Fig. 6) did not rescue cells from the suspension-induced $G_1$ arrest (Fig. 2 and 4). Moreover, expression of both cyclins D1 and E in the same cells did not confer any additional growth advantage in suspension, compared to overexpression of cyclin D1 alone (Fig. 7).

It was recently suggested that modification of cyclin E kinase activity might be sufficient to induce anchorage-independent growth (7). This suggestion was based on the observation that cells of the chemically transformed cell line HUT12 (derived from KD human diploid fibroblasts) had high levels of cyclin E and CDK2 and high cyclin E-dependent kinase activity in suspension and were able to grow in an anchorage-independent manner (7). However, it is possible that the ability of HUT12 cells to grow in suspension results from other, unknown ge-

netic changes induced by chemical transformation rather than from the increase in cyclin E and CDK2 expression. Moreover, KD cells were shown to arrest in suspension with high levels of D-type cyclins and high cyclin D1-dependent kinase activity (7), in contrast to primary human skin fibroblasts, NIH 3T3 cells, and Rat1 cells in which cyclin D1 protein was down-regulated in suspension (50; Fig. 1). It remains possible that activation of cyclin E-dependent kinase in cells that arrest in suspension with high cyclin D1 (like KD cells) will be sufficient to induce anchorage-independent cell cycle progression. However, it is clearly shown here that in Rat1 cells which arrest in suspension with low cyclin D1, activation of cyclin E is not sufficient to induce anchorage-independent cell cycle progression while activation of cyclin D1 is. The ability of cyclin D1 expression to induce anchorage-independent cell cycle progression was also shown for NIH 3T3 cells by using transient transfection of cyclin D1 (35) and retroviral infection of a cyclin D1 expression vector (50). The results shown here confirm these observations and further extend them. Furthermore, this is the first study in which the relative contribution of cyclin D1 and cyclin E expression has been studied in the same cell system.

It was previously shown that expression of exogenous cyclin A in NRK cells conferred anchorage-independent growth (11), similarly to what was shown here for cyclin D1. NRK cells respond to adhesion deprivation differently than all other studied normal fibroblasts, since they arrest with phosphorylated pRb and active cyclin E and D kinases (50). It is not clear whether ectopic expression of cyclin A in other normal fibroblasts will have the same effect as in NRK cells. An attempt to approach this question was made by using Rat1 cell lines capable of expressing cyclin A in an inducible manner (32). However, exogenously expressed cyclin A protein was dramatically reduced as soon as the cells were transferred to suspension conditions (data not shown). The basis for this reduction is not known; however, it is clear that these cell lines cannot be used to study the role of cyclin A in anchorage-dependent growth in Rat1 cells.

**Downstream targets of cyclin D1.** Cyclin E- and cyclin D1-dependent kinases are the two major CDKs responsible for progression of fibroblasts through $G_1$ and entry into S phase. It has previously been shown that under normal growth conditions (in the presence of adhesion and growth factors), each of these two cyclins is required for entry into S phase (2, 26). Moreover, each of these cyclins is rate limiting for entry into S phase, since their ectopic expression accelerates the $G_1$-to-S transition (25, 30, 31). Phosphorylation of pRb has been shown to be the main downstream event controlled by cyclin D1-associated kinase (19, 33), although some studies have suggested that it controls phosphorylation of p107 as well (3, 47). The downstream events controlled by cyclin E-associated kinase are not fully understood. Some studies suggest that it is also involved in phosphorylation of pRb (12, 15). However, it must have other targets as well, since it is still required for S-phase entry in Rb-negative cells (26).

The results shown here suggest that the pathway controlled by cyclin D1 is necessary and sufficient for S-phase entry under suspension conditions. As discussed earlier, pRb phosphorylation and the consequent release of E2F have been suggested to be the main functions of cyclin D1. In agreement with this, induction of cyclin D1 in Rat1 cells in suspension led to the appearance of hyperphosphorylated pRb (data not shown), whereas without cyclin D1 induction, Rat1 cells arrested in $G_1$ with hypophosphorylated pRb (data not shown), as has been observed in other cell lines (35, 50). Based on these data, it is reasonable to suggest that down-regulation of pRb phosphorylation, mediated through down-regulation of cyclin D1 synthesis, pushes adhesion-deprived cells into $G_1$ arrest. If this interpretation is correct, one would expect Rb-negative cells to be able to grow in the absence of adhesion. This assumption is currently being investigated. Recent results showing that E2F overexpression enables cells to grow in soft agar (48) are in agreement with this model.

**Down-regulation of exogenously expressed cyclin D1 in suspension.** It was shown here that exogenously expressed cyclin D1 was down-regulated in suspension, despite the fact that it is expressed from a heterologous promoter (Fig. 3). Furthermore, the ability of cyclin D1-induced cells to enter S phase in suspension disappeared as soon as the cyclin D1 protein disappeared. The down-regulation of exogenous cyclin D1 in suspension was shown to result from a reduction in the level of exogenously expressed cyclin D1 mRNA (Fig. 3), the basis for which is unknown. It could result from down-regulation of transcription, probably due to nonspecific inhibition of the tetracycline-repressible promoter in suspension. This is unlikely, however, since cyclin E protein, expressed from the same promoter, is not down-regulated in suspension (Fig. 7C). Alternatively, it could result from reduced stability of cyclin D1 mRNA in suspension, which might be a specific effect of adhesion deprivation on cyclin D1 mRNA. Such a mechanism, if it exists, may be responsible for the down-regulation of endogenous cyclin D1 mRNA in suspension (50; unpublished data) and may be a crucial element in the signal transduction pathway responsible for down-regulation of cyclin D1, and subsequently $G_1$ arrest, upon adhesion deprivation. Additional experiments are required to discriminate between these two possibilities.

**Is S-phase entry all that is required for anchorage-independent proliferation?** It was clearly demonstrated here that expression of cyclin D1 in suspension enabled Rat1 cells to enter S phase in an anchorage-independent manner. However, it is not clear whether cyclin D1 expression is sufficient for long-term, anchorage-independent growth. Unfortunately, this important issue cannot be studied in the cyclin D1-inducible Rat1 cell lines, due to down-regulation of the exogenous cyclin D1 in suspension, which only allow studies of cyclin D1 effects in short-term assays. Understanding of the basis for cyclin D1 reduction in suspension will make it possible to create cell lines in which cyclin D1 is constitutively expressed in suspension. Such cell lines will make it possible to address the important issue of whether S-phase entry is all that is required for anchorage-independent growth or whether additional signals are required. Such putative signals, if they really exist, are expected to be perturbed in the transformation process.

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