Ras Transformation Results in an Elevated Level of Cyclin D1 and Acceleration of G₁ Progression in NIH 3T3 cells

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Ectopic overexpression of v-H-Ras protein in NIH 3T3 cells resulted in cellular transformation and an acceleration of G₁ progression of these cells. A shortened G₁ phase was found to be associated with an increased level of cyclin D1 but not cyclin E protein. Using an antisense blocking method, reduced synthesis of cyclin D1 in v-H-Ras transformants resulted in a slower G₁ progression rate of these cells. Although constitutive overexpression of cyclin D1 in NIH 3T3 cells accelerated G₁ progression, cells remained untransformed. Furthermore, inhibition of cyclin D1 synthesis greatly impaired the soft-agar cloning efficiency of v-H-Ras transformants. These results suggest that increased expression of cyclin D1 is necessary but not sufficient for the transforming activity of v-H-Ras. Similar effect on cell cycle progression was also observed in Raf-transformed cells. In addition to cyclin D1, cyclin E protein was found to be elevated in Src transformants. This may account for the further shortening of the G₁ phase of these cells. Activation of an additional Ras-independent pathway was suggested to be responsible for the further acceleration of the G₁ phase in Src transformants.

The *ras* gene product, p21, is among those oncoproteins whose structure and function are most thoroughly characterized. By cycling between inactive GDP-bound and active GTP-bound forms, membrane-bound Ras protein has been found to be involved in transducing various extracellular signals into cells, for example, (i) growth factor stimulation of fibroblast cells; (ii) signaling pathway in T-cell activation; (iii) cytokine-induced proliferation and differentiation of hematopoietic cells; (iv) nerve growth factor (NGF)-induced, neuronal differentiation of PC12 cells; and (v) inhibition of epithelial cell proliferation by transforming growth factor β1 (TGF-β1) (reviewed in reference 34 and references therein). Although the immediate target of Ras protein is not clear, many cytoplasmic factors are known to function downstream of the Ras signaling pathway, such as Raf kinase, mitogen-activated protein (MAP) kinase kinase, and MAP kinase (reviewed in references 6 and 24). Recent reports by several groups suggest further that Ras and Raf may interact directly (25, 39–41, 44).

In addition to the activities of the above noted cytoplasmic proteins, the activity of c-Jun, one component of the nuclear transcription factor AP1 has also been shown to be modulated by the Ras signaling pathway (3). This suggests that Ras modulates cell growth by transducing signals from the outer membrane into the nucleus. Earlier studies (27) demonstrate that microinjection of Ras protein into quiescent cells induces DNA synthesis inside the nucleus. These findings suggest that the Ras signaling pathway is linked directly to the G₁/S phase transition of the cell cycle.

The purpose of this work was to identify those downstream targets of Ras whose activities are important for the G₁/S phase transition. In this report, we provide evidence that cyclin D1 is one of the major G₁/S transition control factors whose level is modulated by the Ras signaling pathway. We also demonstrate that increased expression of cyclin D1 accounts for the shortening of the G₁ phase associated with v-H-Ras transformation of NIH 3T3 cells.

MATERIALS AND METHODS

Plasmids. v-H-Ras (pZIP-v-H-*ras*) expression vectors carrying a *neo* selectable marker was kindly provided by Channing Der. cDNA-containing plasmids for cyclins A, D1, and E and *cdk4* were generous gifts from Christian Brechot, Andrew Arnold, James Roberts and Charles Sherr, respectively. Mammalian cyclin D1 expression vector, pCMVcycD1, was generated by inserting cyclin D1 cDNA (as an *Eco*RI fragment from pPL-8) into the *Bam*HI and *Xho*I sites of the pSLXCMV vector (35). pMEPASD1 plasmid was generated by inserting the cyclin D1 cDNA in a reverse orientation into the *Bam*HI and *Xho*I sites of pMEP4 vector (Invitrogen). This construct directs the synthesis of antisense mRNA of cyclin D1 under the control of an inducible metallothionein promoter.

Antibodies. Antibodies specific to cyclin D1, cyclin E, and cdc2 kinase were purchased from Upstate Biotechnology Incorporated. Anti-cdk4 antibody was a gift of Charles Sherr. Alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Zymed Immunochemicals.

Cell lines. NIH 3T3 cells were purchased from the American Type Culture Collection. 3T3v-H-Ras cells were generated by stably transfecting a v-H-*ras* expression vector (pZIP-v-H-*ras*) into NIH 3T3 cells by the calcium phosphate precipitation method. v-H-Ras overexpresser lines with transformed phenotype were used either as a single clone or pooled clones as described separately in the text. 3T3pZIP cells were generated in the same manner as 3T3v-H-Ras except that the control expression vector pZIP-NeoSV(X)1 (5) was used. These control cells mentioned in the text are a mixture of all G418-resistant clones. 3T3v-Src and 3T3v-Raf are v-Src- and v-Raf-transformed NIH 3T3 cells, respectively, and were generously provided by U. Rapp of the National Cancer Institute-Frederick Cancer Research and Development Center. Another v-Src-transformed line, 3T3v-SrcL was generated in our laboratory by cotransfection of NIH 3T3 cells with a plasmid carrying the neomycin-resistant gene (pSV2*neo*) and a v-*src* expression vector, pMv-*src* (17). The NIH 3T3 cell line transfected with pSV2*neo* alone was designated 3T3Neo. 3T3CYCD is a stable cell line that overexpresses cyclin D1, and 3T3pCMV is a control cell line that was stably transfected only with control expression vector (pSLXCMV). Neo and IV5 are murine C3H10T1/2 fibroblasts stably transfected by pSV2*neo* or pSV2*neo* plus pMv-*src*, respectively (20). These two cell lines were generously provided by Sarah J. Parsons. RAD-2 and RAD-5 cell lines were generated by stably transfecting 3T3v-H-Ras cells with plasmid pMEPASD1 as described above. 3T3v-H-Ras transfected with the control expression vector pMEP4 was used as a control in

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some related experiments as described in the text. These latter three cell lines were selected in medium containing 100 μg of hygromycin per ml and 400 μg of G418 per ml.

Cell culture. Parental and all oncogene-transformed NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum at 37°C in a 5% CO_2-containing incubator. In the case of cells that were selected in medium containing G418 and/or hygromycin, these antibiotics were routinely included in their growth medium until 1 to 2 days before all analyses were carried out. Neo and IV5 were cultured under the same conditions as 3T3v-H-Ras except 10% fetal calf serum was used. For isolation of phase-specific cells, cells were treated with 0.06 μg of nocodazole per ml for 13 to 14 h to enrich for cells in mitosis. Mitotic cells were harvested by gently shaking the culture dishes, washed twice in phosphate-buffered saline (PBS), and replated into fresh growth medium. At various times after replating, cells were harvested and their DNA content was determined by flow cytometry or they were processed further for other analyses.

DNA transfections. For the stable clones, NIH 3T3 cells were transfected with the indicated plasmid by either the calcium phosphate precipitation (10) or the liposome-mediated gene transfer method as recommended by the manufacturer (lipofectamine, GIBCO-BRL). After 2 to 4 weeks of selection with 400 μg of G418 per ml, v-H-*ras* or v-*src* overexpressers were screened by their transformed phenotypes and confirmed by immunoprecipitation or Western blotting (immunoblotting) by using their respective antibodies (Oncogene Science). Clones overexpressing cyclin D1 (3T3CYCD) were screened by analysis of cell lysates by the immunoblotting method with anti-cyclin D1 specific antibody.

Flow cytometry. Cells to be analyzed for DNA content were harvested following trypsinization, washed twice in PBS containing 0.1% glucose, and fixed overnight in 70% ethanol at 4°C. Fixed and permeabilized cells were then stained with propidium iodide and analyzed by flow cytometry with a Becton Dickinson FACScan according to the manufacturer's procedure.

Estimation of the duration of G$_1$ and S phases. Cells at mitosis were isolated and replated into fresh medium as described above. At various times after plating, cells were harvested and their cell cycle distribution was determined by flow cytometry. The percentage of cells in each phase was plotted against time after release from mitotic block (see Fig. 11 as one example). The length of G$_1$ is estimated as the number of hours required to reach the first time point at which G$_1$ and S phase distribution curves intersect each other. The length of S phase is estimated as the time interval between the first and second time points at which the G$_1$ and S phase distribution curves intersect each other.

Determination of the cell doubling time. Cells to be counted were seeded at low density in culture dishes (60 by 15 mm). Total cell number was determined periodically by the trypan blue exclusion method. For each time point, three plates of cells were counted, and each plate was only counted once. The cell doubling time was determined as the number of hours required for the cells to double during their exponential growth phase.

Immunoblotting. Cells to be analyzed were lysed with RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholate, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 5 ng of leupeptin per ml, and 50 ng of aprotinin per ml), and lysates were electrophoretically resolved on SDS-containing 7% polyacrylamide gels. Separated proteins were then transferred to polyvinylidene difluoride nylon membranes (Millipore), and blots were probed with their respective primary antibodies. After extensive washing, the blots were reprobed with appropriate alkaline phosphatase-conjugated secondary antibodies, and specific protein bands were visualized by the Bio-RAD NBT-BCIP color development system. Alternatively, after the primary antibody binding, the blot was reprobed with ^{125}I-labeled protein A and the reacted bands were then quantitated by a phosphoimager (Molecular Dynamics).

Northern (RNA) blotting. Total RNA was isolated from cultured cells using the method previously described (43). Twenty micrograms of total RNA was resolved on a 1% formaldehyde–agarose gel and blotted onto a nitrocellulose filter using the standard method. The blot was then probed sequentially with various [α-^{32}P]dCTP-labeled DNA fragments labeled to a specific activity of 10^9 cpm/μg by the random-priming method. After overnight hybridization at 42°C with standard buffer containing 50% formamide, the blot was washed once in 2× SSC–0.1% SDS and twice in 0.2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C and subjected to either autoradiography or direct quantitation with a phosphoimager.

Soft-agar cloning assay. Three thousand cells were seeded onto each 60-mm culture dish in Dulbecco's modified Eagle's medium containing 10% calf serum and 0.33% low-melting-point agarose over a base of 0.5% agarose. Two weeks after seeding, colonies were stained with 0.05% *p*-iodonitrotetrazolium violet (Sigma) before they were counted and photographed. All conditions were done in triplicate and repeated at least three times. In the case of cells requiring cadmium treatment, cells were treated overnight with 2 μM cadmium before they were seeded onto the soft-agar cloning dishes in which the same concentration of cadmium was included.

RNA stability assay. Cells to be analyzed were treated with 5 μg of actinomycin D (Sigma) per ml. At various times after treatment, cells were harvested and lysed and their total RNA was analyzed by Northern blotting as described above. The level of cyclin D1 mRNA at each time point, normalized for the level of
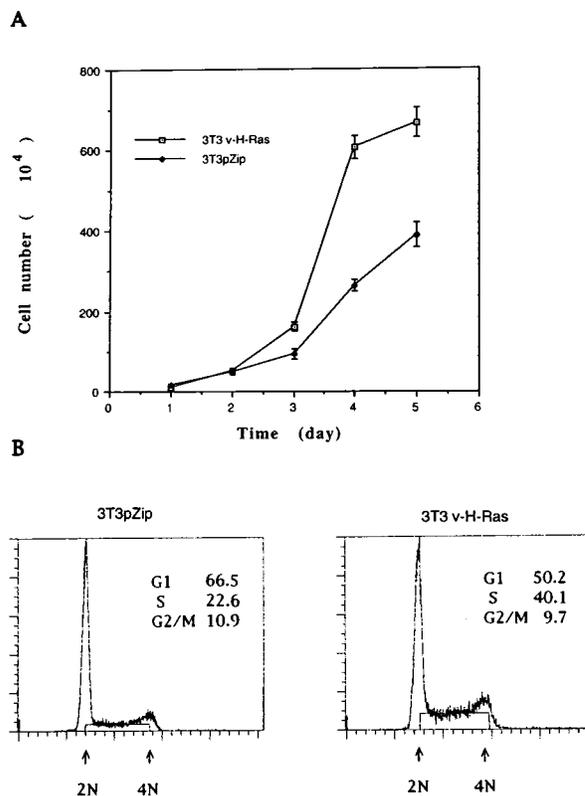

FIG. 1. (A) Growth curves of 3T3pZIP and 3T3v-H-Ras cells. About 5×10^4 cells were seeded into culture dishes (60 by 15 mm) with medium containing 10% calf serum. Growth medium was changed every other day. Total cell numbers in each dish were determined every day 24 h after seeding. Very similar growth curves were obtained from eight independent experiments. Shown here is the result of one representative assay done in triplicate. (B) Cell cycle distribution of day 3 cells from Fig. 1A (see text for details). Control (3T3pZIP) or 3T3v-H-Ras cells harvested at day 3 were fixed and stained with propidium iodide, and their DNA contents were analyzed by flow cytometry. The result of one representative assay from three similar independent experiments is shown. X and Y axes denote DNA content and cell number, respectively. The percentages of cells in G$_1$, S, and G$_2$/M are also shown as indicated.

G3PDH, was converted to the percentage of cyclin D1 mRNA at time zero and plotted against time (hours) of treatment to determine the half-lives.

RESULTS

Ras transformants have a shorter G$_1$ phase. To study the downstream signaling events of Ras which are closely related to cell cycle control, our approach was to constitutively over-express v-H-Ras protein in NIH 3T3 cells and look for any growth changes that are associated with Ras transformation. This was first accomplished by stably transfecting NIH 3T3 cells with a retroviral expression vector carrying the v-H-*ras* coding region, pZIP-v-H-*ras*. All positive clones (G418 resistant and with transformed phenotype) were pooled, and their growth properties in medium containing 10% serum were analyzed. From eight independent experiments, we consistently observed that the v-H-Ras-transformed cells (3T3v-H-Ras) grew faster than the vector-transfected control (3T3pZIP). A representative growth curve of cells grown under these conditions is shown in Fig. 1A. The cell doubling time of 3T3v-H-Ras cells during the exponential growth phase was 15.0 ± 1.0 (mean ± standard deviation [SD]) hours, while that of the control cells was 17.2 ± 1.1 h. 3T3pZIP cells seeded at the density of ~5×10^4 per dish (60 by 15 mm) reached confluence
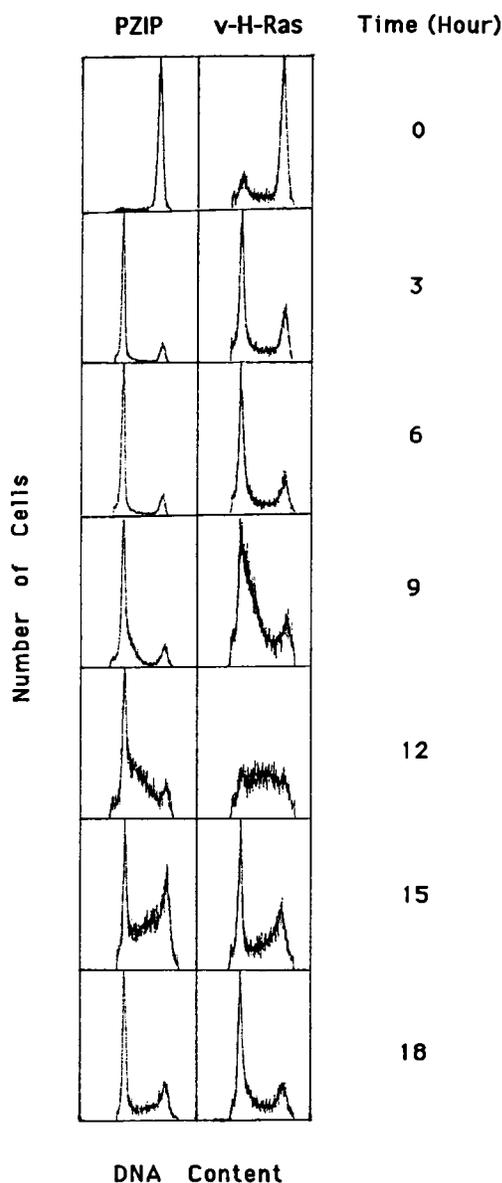
FIG. 2. Ras transformation results in a shortened G_1 phase. Mitotically arrested cells were isolated, washed, and replated into cell culture dishes as described in Materials and Methods. At different times as indicated, cells were harvested, fixed, and stained with propidium iodide. The DNA content of these cells was analyzed by flow cytometry. PZIP, 3T3pZIP cells; v-H-Ras, 3T3v-H-Ras cells.
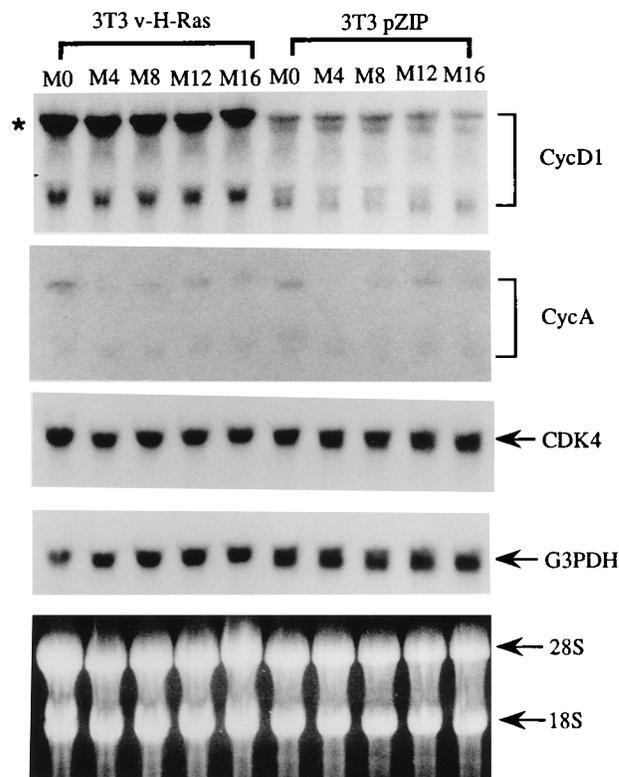

FIG. 3. Northern blot analysis of cyclin mRNA during M-G_1-S progression in parental (3T3pZIP) and v-H-Ras-transformed (3T3v-H-Ras2) cells. Twenty micrograms of total RNA isolated from cells released for various hours as indicated (M0 through M16) from mitotic block was separated on a 1% formaldehyde-agarose gel, blotted onto nitrocellulose, and probed sequentially with ^{32}P-labeled cyclin D1-, cdk4-, cyclin A-, or G3PDH-specific cDNA probe. Bottom panel shows approximately equal amounts of total RNA loaded as judged by equal intensity of their ethidium bromide-staining patterns. The 4.5- and 3.8 kb-long cyclin D1 mRNAs were indicated by an asterisk.

at about day 5, with a saturation density of $\sim 1 \times 10^5$ cells per cm^2. After day 5, some dead cells started to appear. On the other hand, 3T3v-H-Ras, seeded and grown under the same conditions, reached a cell density of $\sim 3 \times 10^5$ cells per cm^2 at day 5. These cells could continue to grow for 1 to 2 days as long as the medium was frequently replaced with fresh medium. The cell cycle distribution of the day 3 cells (i.e., when cells are still at the exponential growth phase) were analyzed by flow cytometry. Among the three independent experiments performed, 3T3v-H-Ras consistently showed more cells in S phase than the control cells did. One representative result of these experiments is shown in Fig. 1B. While the percentage of cells in G_2/M phase of these two cell lines was comparable, 3T3v-H-Ras had $\sim 17\%$ more cells in S phase than that of the control

cells. The G_1 percentage of these transformed cells was accordingly reduced.

These results suggested that the v-H-Ras-transformed cells might have a shorter G_1 phase. To examine this possibility, measurement of the G_1 length of both cell lines was carried out. As shown in Fig. 2, under the experimental conditions used, a significant amount of control cells in S phase could not be detected until ~ 12 h after cells were released from the M phase. However, it only took the Ras transformants about 9 h to progress from M to S phase. These results indicated that constitutive overexpression of v-H-Ras protein in NIH 3T3 cells shortened the cell cycle time by 2 to 3 h, and the shortened period fell in the G_1 phase of the cell cycle.

Ras transformants have higher levels of cyclin D1. We then checked whether activation of the Ras signaling pathway would lead to the activation of any factors that play important roles in G_1/S transition control. Since G_1 cyclins had been shown to be important regulatory factors that control cell cycle progression from the G_1 to the S phase (15, 36), we examined first whether the mRNA levels of the G_1 cyclins in the Ras transformants were affected. To address this, Northern blot analysis was carried out with total RNA prepared from control and a single isolated clone of 3T3v-H-Ras cells (3T3v-H-Ras2) at various stages after release from mitotic block (0 to 16 h, indicated as M0 to M16 in Fig. 3). At the time points analyzed, neither the 4.5- nor the 3.8-kb cyclin D1 mRNA (marked by an asterisk) in

either cell lines had a pronounced oscillation, as in other reported cases (23, 26). There was only a slight decrease at the M16 time point (around late G_2/M or early G_1 phase). After correction for slightly different loading of RNA as reflected from the blot probed with internal control, G3PDH, cyclin D1 message was found to be consistently expressed at a two- to threefold higher level in the v-H-Ras-transformed cells than in control cells at all time points examined (Fig. 3). In contrast, the mRNA levels of the *cdk4* gene, which encodes the catalytic subunit of the D type-cyclin dependent kinase, were not affected in the 3T3v-H-Ras cells. The same blot in Fig. 3 was also examined with a cyclin A-specific cDNA probe. Similarly, no significant difference could be identified between these two cell lines. For some unknown reasons, cyclin E mRNA in this blot was not detectable even after prolonged exposure (data not shown). However, immunoblot analysis (see below) excluded the possibility that the shortened G_1 phase of 3T3v-H-Ras cells resulted from increased expression of cyclin E protein.

To examine whether increased message level of cyclin D1 in 3T3v-H-Ras cells also resulted in its increased expression at the protein level, immunoblot analysis was done with proteins extracted from control or v-H-Ras-transformed cells at various stages after release from mitotic block. As shown in Fig. 4, no significantly different patterns or levels of protein expression could be seen for either cyclin E or cdc2 in both cell types, while cyclin D1 protein was constitutively expressed at two- to threefold higher levels in v-H-Ras-transformed cells than in control cells (compare lanes 1 to 5 to lanes 6 to 10). Similar results were also obtained with two other clones of Ras transformants (data not shown). These results suggested that the shortened G_1 phase associated with v-H-Ras transformation of cells might have resulted from increased expression of cyclin D1 protein in these cells. To examine this possibility, the following few experiments were carried out.

Cyclin D1 overexpressers have a shorter G_1 phase. First, NIH 3T3 cells were transfected with the cyclin D1-overexpressing vector. Stable transformants expressing increased level of cyclin D1 proteins were selected and characterized. The property of one of these clones, designated 3T3CYCD, was reported here. Unlike that of 3T3v-H-Ras cells, the morphology of 3T3CYCD showed no characteristics of the transformed phenotype, although both cell lines seemed to grow at the same rate, a rate greater than that of the parental cells (see below). As shown in Fig. 5, cyclin D1 was constitutively expressed at a two- to threefold higher level in 3T3CYCD cells than in vector-transfected cells (3T3pCMV). Cell doubling time of 3T3CYCD was 12.7 ± 0.6 (mean $\pm$ SD; $n = 5$) hours in contrast to 14.6 ± 0.5 (mean $\pm$ SD; $n = 5$) hours for the control cells. This difference is statistically significant ($0.001 < P < 0.01$). When cells were seeded and cultured under the same conditions described in the legend to Fig. 1A, an interesting phenomenon was observed (Fig. 6A). Although for the first 3 days 3T3CYCD grew at about the same rate as 3T3v-H-Ras, the former but not the latter cells reached confluence at day 4 and many dead cells appeared on day 5. The saturation density of 3T3CYCD was $\sim 10^5/\text{cm}^2$, which was very similar to that of the parental or vector-transfected NIH 3T3 cells.

To show that the faster growth rate of 3T3CYCD cells was due to their shorter G_1 phase, the cell cycle progression of these two cell lines analyzed at various points after release from mitotic block was compared (Fig. 6B). Vector-transfected control cells grew slightly faster than the wild-type NIH 3T3 cells (data not shown). However, only two- to threefold more cyclin D1 in the 3T3CYCD cells was enough to shorten their G_1 phase further by about 2 h (Fig. 6B). Similar results were also observed with two other isolated clones that overexpress
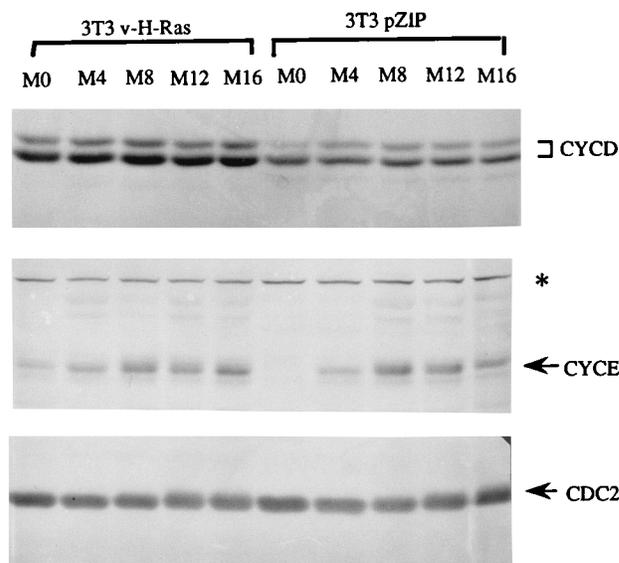
FIG. 4. Increased expression of cyclin D1 protein in 3T3v-H-Ras cells. Equal amounts (70 μg) of proteins isolated from cells released for 0 to 16 h from mitotic arrest were separated on SDS–10% polyacrylamide gels, transferred to nylon membranes, and immunoblotted with anti-cyclin D1 (CYCD), anti-cyclin E (CYCE), or anti-cdc2 (CDC2) antibody. *, an unknown protein recognized by anti-cyclin E antibody.

similar levels of cyclin D1 (data not shown). These results were very similar to those obtained from 3T3v-H-Ras cells, indicating that in these cells a shortened G_1 phase associated with v-H-Ras transformation probably resulted from increased expression of cyclin D1.

Reduced synthesis of cyclin D1 reverses the shorter G_1 phase of Ras transformants. To prove that it is the increase of cyclin D1 in the v-H-Ras transformed cells that accounts for the shortening of G_1 phase, 3T3v-H-Ras cells stably transfected with pMEPASD1, a construct which expresses the cyclin
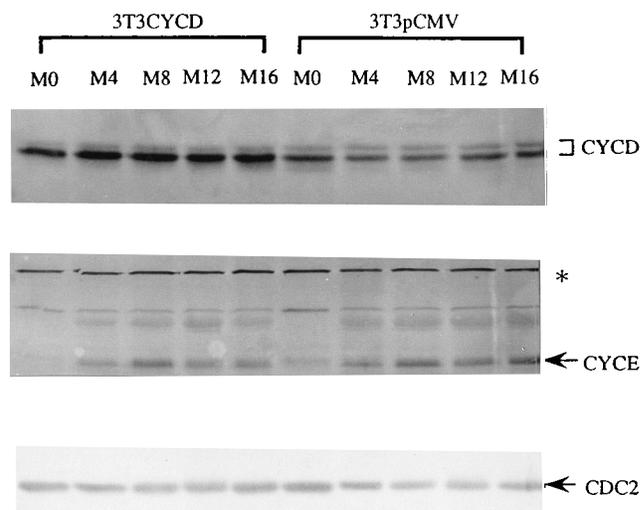
FIG. 5. Cyclin D1 levels in overexpresser (3T3CYCD) and control (3T3pCMV) cells. Equal amounts (70 μg) of proteins isolated from each cell line treated in the same way described in the legend to Fig. 4 were separated on SDS–10% polyacrylamide gels, transferred to nylon membranes, and immunoblotted with anti-cyclin D1 (CYCD), anti-cyclin E (CYCE), or anti-cdc2 (CDC2) antibody. *, an unknown protein recognized by anti-cyclin E antibody.
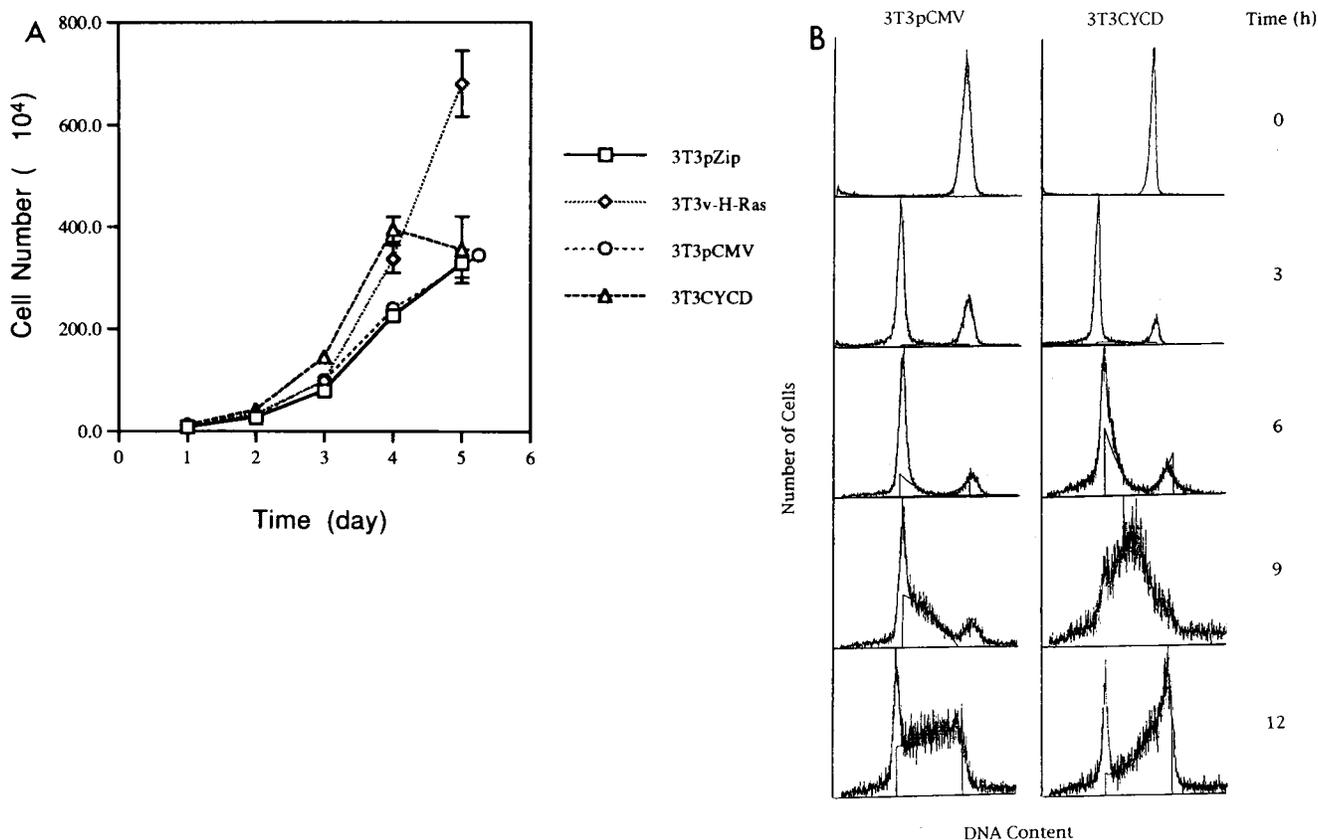
FIG. 6. (A) Growth curves of control (3T3pCMV, 3T3pZip), 3T3v-H-Ras, and 3T3CYCD cells. Cells were seeded and counted as described for Fig. 1A. Very similar results were obtained from five independent experiments. The result shown is one representative of these experiments done in triplicate. (B) Cell cycle progression of 3T3pCMV and 3T3CYCD cells at various time points after release from mitotic block. Cells were treated, harvested, and analyzed as described in the legend to Fig. 2.

D1 mRNA in an antisense orientation, were established. Since the metallothionein promoter used to drive the antisense construct was leaky, among 12 clones isolated only 2 had cyclin D1 levels repressed in a Cd-dependent manner (Fig. 7A and data not shown). These two clones, designated RAD-2 and RAD-5, were further characterized. As shown in Fig. 7B, while the progression rates of control, RAD-2, and RAD-5 cell lines from M to S phase (M0 to M10) in the absence of cadmium were very similar (− panels), those of RAD-2 and RAD-5 cells in the presence of cadmium were significantly slower than that of control cells (+ panels). These results further support the notion that increased expression of cyclin D1 in v-H-Ras transformants is responsible for the shorter G_1 phase of these cells.

Increased expression of cyclin D1 contributes to some but not all of the transforming activity of Ras. As mentioned above, except for their faster growth rate, 3T3CYCD behaved very much like their parental or vector-transfected control cells. That is, they all grew as a monolayer and reached confluence with a very similar saturation density. Their nontransformed phenotype was further supported by the soft-agar cloning assay. As shown in Fig. 8, 3T3CYCD cells, like parental and either control vector-transfected NIH 3T3 cells, were unable to form colonies in this assay. We then asked whether elevated level of cyclin D1 in the Ras transformants could contribute to the transforming activity of these cells. To address this issue, we examined whether reduced synthesis of cyclin D1 in v-H-Ras transformants would affect the soft-agar cloning efficiency of these cells. As shown in Fig. 9, the num-

bers of colonies formed by control and RAD-2 cells in the absence of cadmium were comparable (1,269 ± 30 [mean ± SD] versus 1,164 ± 51). Under the same conditions, RAD-5 had a lower efficiency in forming soft-agar colonies (687 ± 108). This difference is most likely either due to clonal variations between original Ras-transformed cells or to different integration sites of the antisense construct. Although cadmium decreased the soft-agar cloning efficiency of control cells by ~50%, it further reduced that of RAD-2 and RAD-5 cells to ~2 and ~16%, respectively. The reduction in the latter two cell lines is statistically significant ($P \ll 0.001$) and is most likely due to cadmium-induced expression of antisense cyclin D1 mRNA in these cells which in turn reduces their endogenous cyclin D1 protein levels. Taken together, these results suggest that elevation of the level of cyclin D1 by v-H-Ras contributes to some but not all of the transforming ability of this oncogene.

Ras transformation stabilizes cyclin D1 mRNA. Increased cyclin D1 message in the v-H-Ras transformed cells could be the result of transcriptional or posttranscriptional regulation or to the combination of both regulations. Using the actinomycin D blocking method (see Materials and Methods), the stability of the cyclin D1 mRNA in control and v-H-Ras-transformed cells was compared. In four independent experiments, we observed one time that its stability in both cell lines was very similar (half-lives in both cases were ~14 h), while the other three experiments cyclin D1 mRNA was slightly more stable in v-H-Ras transformants than in the control cells (Fig. 10). Its estimated half-life in the control cells was 13.9 ± 0.8 h (mean
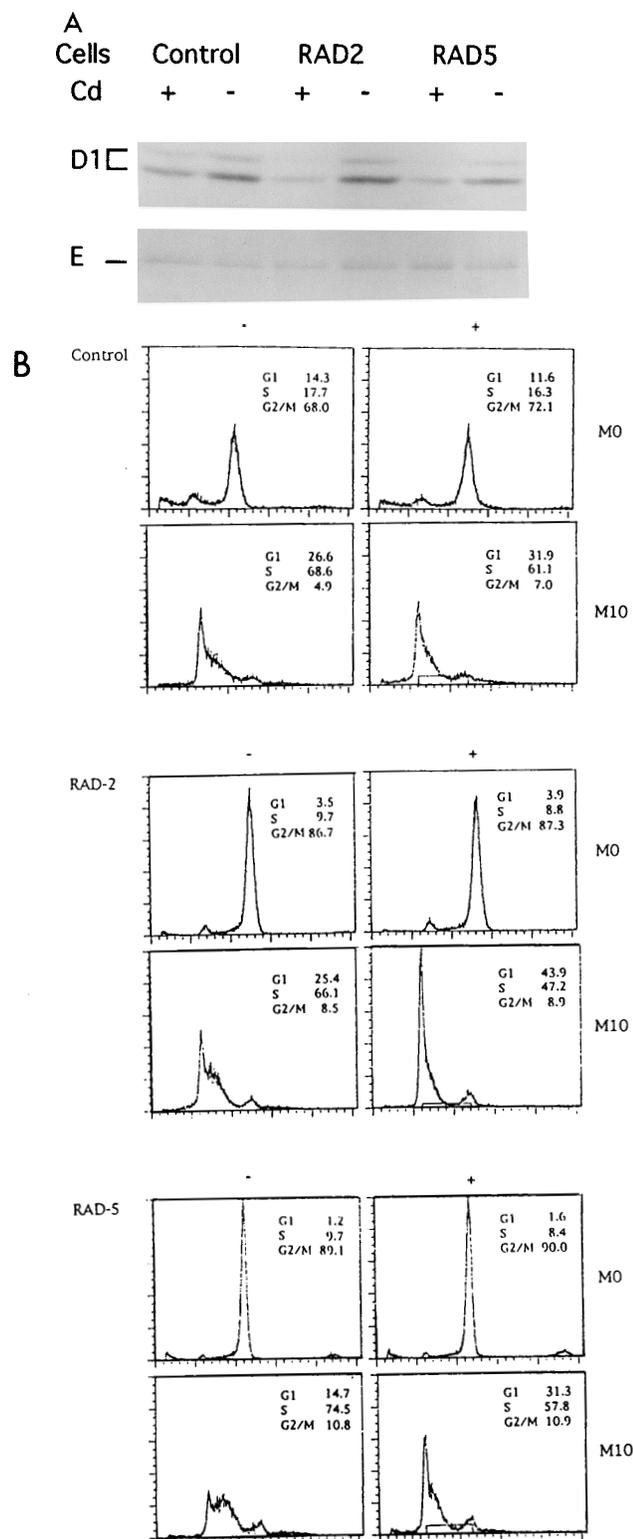
FIG. 7. Reduced synthesis of cyclin D1 slowed down the G$_1$ progression rate of ras transformants. (A) 3T3v-H-Ras cells stably transfected with control vector (control) or a construct directing the synthesis of antisense cyclin D1 mRNA (RAD2 and RAD5) were cultured in medium with (+) or without (−) 2 μM of cadmium for 18 h before they were lysed and their total proteins were analyzed by Western blotting. (B) Cells to be analyzed were enriched in M phase by treatment of these cells with nocodazole (see Materials and Methods). During the 12-h nocodazole treatment, cells were cultured in medium with (+) or without (−) 2 μM of cadmium. Cells in mitotic phase were shaken off and released into medium containing (+) or not containing (−) cadmium. At 0 and 10 h after release from mitotic block, cells were harvested and their DNA contents were subjected to analysis by flow cytometry. X and Y axes denote DNA content and cell number, respectively. The percentages of cells in each phase were as indicated in each panel.

± standard error), whereas that in the Ras transformants was at least 32 h and could not be accurately estimated because either no or a very low level of degradation (less than 8%) was observed after a 4-h treatment of cells with actinomycin D. These results suggest that RNA stabilization may contribute to most, if not all, of the observed ~threefold increase of cyclin D1 mRNA in the Ras-transformed cells. Furthermore, they suggest that if transcriptional regulation would play any role in this case, its contribution would be only minimal. Because of the limited sensitivity of available assays, we could not examine whether transcriptional regulation played any role in this case.

Cyclin D1 levels in other oncogene-transformed cells. The next important issue arising from the above findings was whether the phenomenon of shortened G$_1$ phase was confined to Ras-transformed cells. To address this issue, a pilot experiment using a single isolated clone of v-Src- and v-Raf-transformed cells was carried out, since these two oncogenes' signaling pathways were known to be connected to that of Ras. As shown in Fig. 11, the S-phase lengths for all cells examined were very similar (i.e., ~7 h) except for that of 3T3vSrc (~8.5 h). While the estimated G$_1$ length of the parental cells was about 9 to 10 h, that of v-Raf- and v-H-Ras-transformed NIH 3T3 cells was shortened to approximately 7.5 h. The G$_1$ length of vSrc-transformed cells was further shortened to about 4.5 h. The cell doubling time of these cells was, accordingly, adjusted. This reflected a change from 17 ± 1 (mean ± standard error) hours for control cells to 15 ± 1 h for 3T3v-Raf and 3T3v-H-Ras and down further to 13 ± 1 h for 3T3v-Src cells. Immunoblot analysis using extracts made from cells 4 h after release from mitotic block showed that the cyclin D1 levels in these oncogene-transformed cells were all increased compared with that of parental or vector-transfected NIH 3T3 cells (Fig. 12A). Interestingly, increased expression of cyclin E was only evident in v-Src transformants. To rule out the possibility that this phenomenon is an artifact of this particular cell line, additional lines of v-Src-transformed cells generated in our laboratory were examined. As shown in Fig. 12B, another v-Src-transformed line, 3T3v-SrcL, had increased expression of cyclins D1 and E compared with that of the vector-transfected control cells (3T3Neo). To further examine whether the effect of Src on the G1 cyclins was just confined to the NIH 3T3 cell type, another murine fibroblast line (C3H10T1/2) transformed by v-Src (IV5) was examined. Consistent with that observed in v-Src-transformed NIH 3T3 cells, other than their faster growth rate (data not shown), IV5 also showed increased levels of both cyclins D1 and E compared with that of the control cells (Neo) (Fig. 12B).

DISCUSSION

Using ectopic overexpression of v-H-Ras protein, we found that oncogenic Ras could accelerate cell growth by shortening the G$_1$ phase of the cell cycle. This phenomenon was correlated with the increased expression of endogenous cyclin D1 in these transformed cells. Using an antisense construct, we further demonstrated that the shortened G$_1$ phase in the Ras transformants could be specifically reversed by decreased expression of endogenous cyclin D1. These results suggest that elevated level of cyclin D1 in these cells was indeed responsible

3T3 v-H-Ras 3T3CYCD 3T3pCMV 3T3pZip NIH3T3
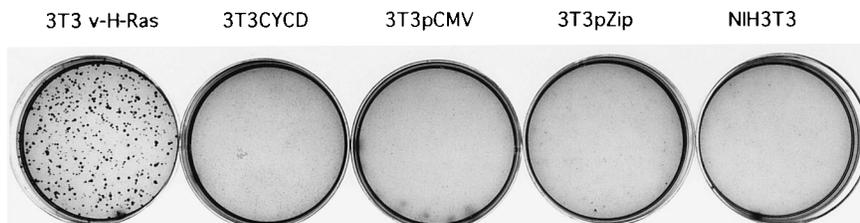
FIG. 8. Overexpression of cyclin D1 does not confer anchorage-independent growth on NIH 3T3 cells. Cells of each type (3×10^3) as indicated were seeded onto soft-agar culture dishes (60 by 15 mm). Two weeks after seeding, colonies were stained and photographed. Data shown here were from one representative assay done in triplicate. Very similar results were obtained with two other independent experiments.

for their shorter G_1 phase phenotype. This phenomenon is reminiscent of the case in *Saccharomyces cerevisiae*, where triple *CLN* mutations result in cells arrested at G_1 phase (32) while overexpression of yeast G_1 cyclins shortens the G_1 phase of the cell cycle (7, 11, 28).

Overexpression of cyclin D1 has been shown to be able to accelerate G_1 progression in some (16, 30, 31) but not all (4) cell types. Interestingly, various reported cyclin D1 overproducers with contracted G_1 phases do not always have shorter cell generation times. Our results are consistent with that reported in one study (30) but different from those in other two cases (16, 31). This discrepancy could result from differences in the cell types used, differences in systems used to overexpress the protein, or differences in the levels of overexpressed cyclin D1. In budding yeast cells, depending on the growth condition of this organism, activation of the Ras-cyclic AMP pathway could either activate or repress the expression of their G_1 cyclins. In the former case, this process is rapid and is independent of protein synthesis (14). In the latter case, activation of the same pathway would affect the critical size of cells before they divide (2, 38). Our finding that Ras could increase the expression of cyclin D1 is not surprising, since expression of the latter protein is affected by the growth factors. However, this result provides a molecular mechanism for the faster growth rate of Ras transformants which may contribute to some of their transforming activities. In this study, we also find that, except for their faster growth rate, the morphology and saturation density of NIH 3T3 cells overexpressing cyclin D1 are very similar to those of the parental cells. In addition, these cells cannot grow in soft agar, suggesting that they are not

transforming. These results are consistent with that reported by Quelle et al. (30) but different from that observed in another study (16). The difference in the latter case could be due to either different cell types or the level of overexpressed cyclin D1.

Although cyclin D1 overproducers characterized here cannot form colonies in soft agar, our data clearly show that an elevated level of cyclin D1 is important for the anchorage-independent growth of Ras transformants. These results suggest that increased expression of cyclin D1 is necessary but not sufficient for the transforming activity of Ras. In other words, there must exist other events which lie downstream of Ras, and activation of all of these events are required for the observed transforming activity of oncogenic Ras. The importance of cyclin D1 in the transforming activity of Ras is also revealed in two recent reports. One is that cyclin D1 can contribute to cell transformation by Ras by complementing a defective adenovirus E1A (12), and the other is that it can cooperate with Ha-*ras* in inducing foci in primary rat embryo fibroblasts (19).

Here, we demonstrated that activated Ras specifically increased the mRNA and protein levels of the regulatory subunit of a cdk family kinase, cyclin D1, but not that of its catalytic subunit, cdk4. Similar observations has also been made with epithelial cells derived from normal rat intestine and mouse mammary gland (9). In contrast to our results and those of Filmus et al. (9), NRK cells transfected by a transforming allele of H-*ras* do not overexpress cyclin D1 protein (33). This discrepancy may result from experiments with different cell lines.

Expression of Ras has been shown to affect the stability of several mRNA species (13, 18). Stabilization of cyclin D1
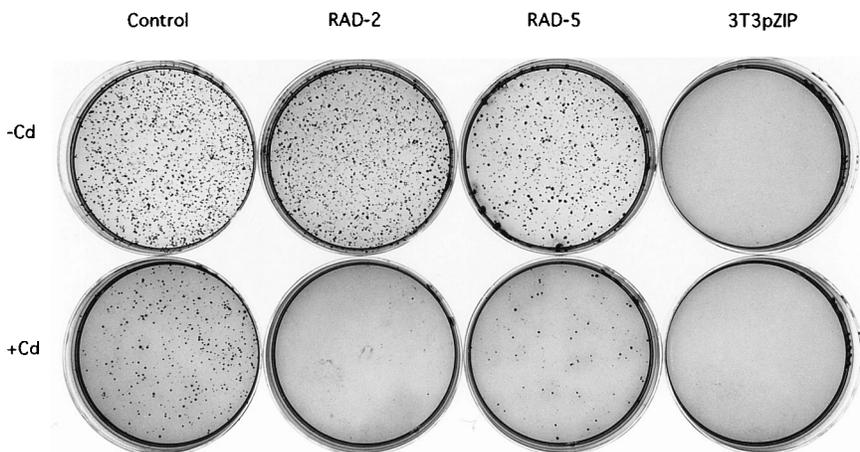
Control RAD-2 RAD-5 3T3pZIP

FIG. 9. Reduced synthesis of cyclin D1 decreased soft-agar cloning efficiency of Ras-transformed cells. Cells of each type (3×10^3) were seeded onto each soft-agar dish (60 by 15 mm) containing or not containing cadmium. Cells requiring cadmium treatment were pretreated with cadmium for 12 h prior to plating into soft-agar dishes. Two weeks after seeding, colonies were stained, counted, and photographed.
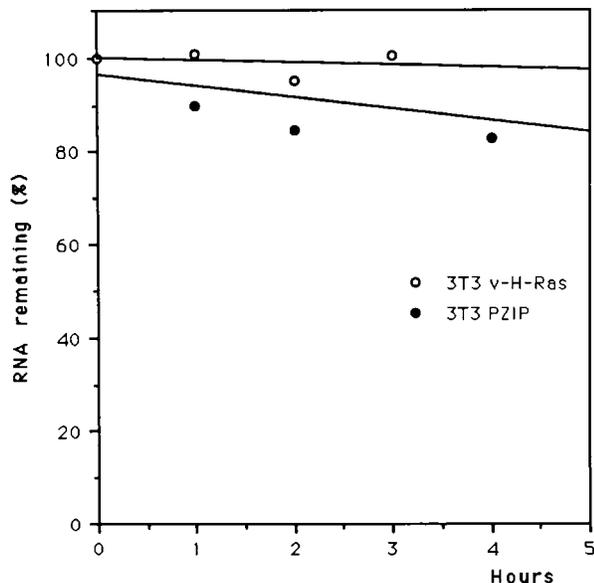
FIG. 10. Cyclin D1 mRNA is slightly more stable in ras transformants than in control cells. 3T3pZIP (solid circle) or 3T3v-H-Ras (open circle) cells were treated with 5 μg of actinomycin D per ml for various times before their total RNAs were harvested and analyzed by Northern blotting. The level of cyclin D1 RNA at each time point was normalized for that of G3PDH mRNA, converted to the percentage of cyclin D1 RNA at time zero, and plotted against time.

mRNA identified in this study represents another example of Ras-modulated, posttranscriptional events. The half-life of cyclin D1 mRNA in the parental NIH 3T3 cells determined here is much more stable than that reported in reference 23 (~13 versus <2.5 h). The exact reason for this discrepancy is unclear. However, it could be because that the latter study was done with colony-stimulating factor-1-deprived macrophages. To be able to directly compare the RNA stability in these two cases, the same methodology needs to be used in continuously proliferating cells. Unlike that reported for HeLa S3 cells (26), cyclin D1 mRNA in both control and Ras-transformed cells oscillated only minimally. This is, however, consistent with the report by Sherr (36) that cyclin D1 levels in continuously proliferating cells fluctuate only minimally. This variation could be due to cell type-specific difference. After all, the expression of cyclin D1 is known to have tissue specificity (23).

In addition, we showed that there was no apparent change of cyclin D1 at the protein levels during cell cycle progression (Fig. 4). This also contradicts the results obtained in two other studies (19a, 23). Again, this could be due to cell type variation. The fluctuation of cyclin D1 levels in some cells may be important to their cell cycle progression. However, in other cell types with a constant amount of cyclin D1 protein, such as the NIH 3T3 cells studied here, other ways of regulation may be involved. If the latter case is true, how would Ras then be able to promote G_1 progression by constitutively elevating the total amount of cyclin D1 protein? Several possibilities might explain this phenomenon. On the basis of present knowledge about cyclin D1, the following two are the most likely cases. First, the cyclin D1 protein needs to be modified prior to being functional, and the total number of modified cyclin D1 molecules would determine the G_1 progression rate of that cell. The activity of this hypothetical cyclin D1 modifier must be also regulated in a cell cycle-dependent manner. It must be activated immediately before or at the same time as cyclin D1 is activated. This activity may or may not be affected by the Ras

signaling pathway. Since overexpression of cyclin D1 in the parental NIH 3T3 cells alone was sufficient to accelerate G_1 progression in these cells, we believe that this activity is not further up-regulated by the Ras signaling pathway. Second, in addition to the absolute level of cyclin D1 protein, its subcellular localization is also pivotal to the G_1/S transition. Both Baldin et al. (1) and Lukas et al. (19a) have shown that cyclin D1 is localized to the nucleus during the G_1 phase of the cell cycle. Upon progression into S phase, the nuclear staining of cyclin D1 disappears. It was suggested that the exclusion of cyclin D1 from the nucleus and/or its degradation are required for progression into S phase (1). If the latter possibility is correct, the absolute amount of cyclin D1 that is localized to the nucleus during the G_1 phase of the cell cycle or the amount of proteins that could be removed from the nucleus would determine the G_1/S transition rate of that cell. Whatever mechanism is responsible for this subcellular localization of cyclin D1 protein, its activity should also oscillate in the cell cycle and most likely peak at the G_1/S transition point. Again, this hypothetical activity may or may not be affected by the Ras signaling pathway. These two possibilities are not mutually exclusive, since protein modification may be required before they could be localized to another subcellular compartment or they cannot be modified until they are in the right locus. Since the Ras-transformed cells have a larger pool of cyclin D1 proteins, during the G_1/S transition there should be more cyclin D1 protein modified or relocalized to the right subcellular compartment assuming that the amount of the hypothetical modifier described above is not limited. If this hypothesis is correct, it would explain our observation that Ras-transformed NIH 3T3 cells have a shorter G_1 phase than their parental cells
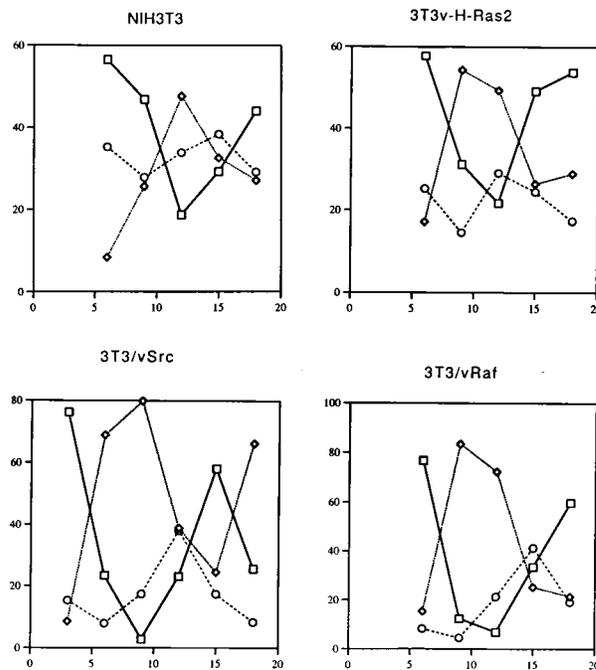

FIG. 11. Accelerated progression of G_1 phase is also found in v-Src- and v-Raf-transformed cells. Mitotically arrested cells were isolated from control (NIH 3T3), 3T3v-H-Ras2, 3T3v-Src, and 3T3v-Raf. At various time points after release from mitotic block, cells were trypsinized, fixed, and subjected to DNA content analysis using flow cytometry. X and Y axes denote the time after release from mitotic block (in hours) and percentage of cells in each phase of the cell cycle, respectively. Cells in G_1, S, or G_2/M phase were plotted as open square, diamond, or circle symbols, respectively.
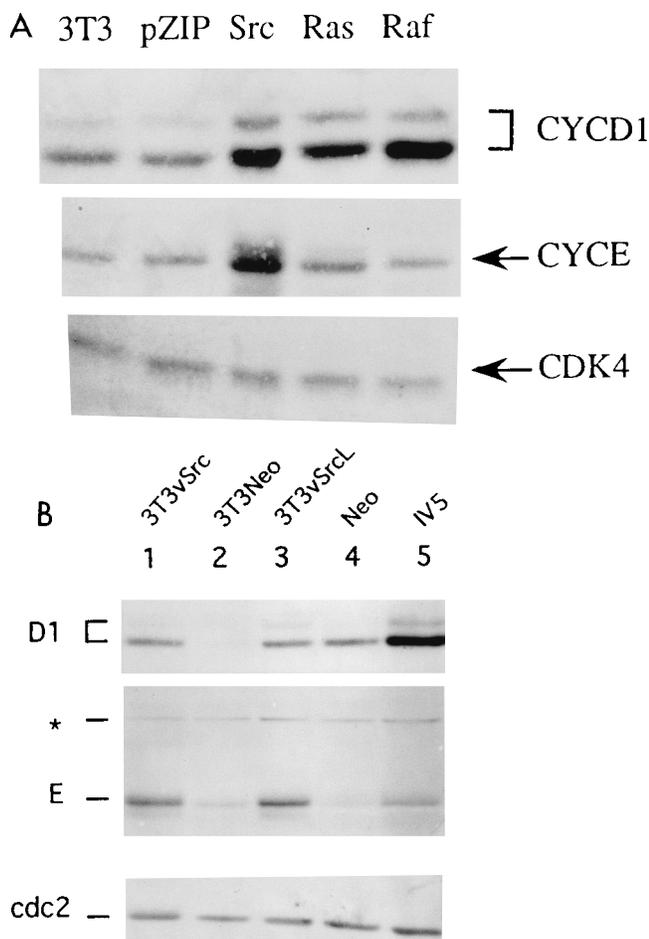
FIG. 12. Elevated level of cyclin D1 is also observed in v-Src- and v-Raf-transformed cells. (A) Whole-cell extracts were prepared from control and various oncogene-transformed NIH 3T3 cells as indicated at 4 h after release from mitotic block (M4 extracts). Sixty ug of protein extracts from each sample were resolved on an SDS–8% polyacrylamide gel and transferred to nylon membrane, and immunoblot analysis was carried out with antibodies specific to cyclin D1 (CYCD1), cyclin E (CYCE), and cdk4 (CDK4), respectively. 3T3, NIH 3T3; pZIP, 3T3pZIP; Src, 3T3v-Src; Ras, 3T3v-H-Ras2; and Raf, 3T3v-Raf. (B) M4 extracts were prepared from various cell lines as indicated and analyzed as described for panel A.

even though cyclin D1 protein levels in both cell lines do not oscillate in the cell cycle. Further analysis will be required to uncover the exact mechanism responsible for this phenomenon.

Matsushime et al. (22) reported that rat-2 cells overproducing five- to eightfold more D1 than control counterparts also expressed much higher levels of cyclin D1-associated kinase activity. It is possible that in this case, the cdk4 kinase activity of the Ras transformants is also increased. Furthermore, it would be interesting to see whether the levels of two other D-type cyclins, D2 and D3, and their associated kinase activities are also affected by the Ras signaling pathway.

The finding that cells transformed by other oncogenes such as v-*src* and v-*raf* also have a shorter G_1 phase is not surprising, since these oncogene products are known to be connected in the Ras signaling pathway (6). In the case of Raf, its effect on cell cycle progression is very similar to that of Ras, since it functions downstream of Ras. The activation of each protein not only shortens G_1 phase to the same extent but also results

in the increased expression of cyclin D1 but not cyclin E protein. The increased expression of cyclin D1 is also observed in some pre-B cell lines expressing v-H-Ras or v-Raf proteins (4). It would be interesting to examine whether these cells also have a shorter G_1 phase. v-Src-transformed cells gave a more complicated but novel result. These cells not only have an even shorter G_1 phase (~3 h shorter than that of Ras-transformed cells) but also have an elevated level of another G_1 cyclin, cyclin E, in addition to increased expression of cyclin D1. Overexpression of cyclin E has been reported to shorten G_1 phase (29, 31). However, these overexpresser cells have a compensated longer S phase. As a result of the compensation, the cell doubling time of cyclin E-overexpressing cells is the same as that of the parental cells. The cell doubling time of Src-transformed cells is only about 1 to 2 h shorter than that of Ras transformants despite their near 3-h shorter G_1 phase. This is further reflected in the slightly longer duration of S phase in the Src transformants (Fig. 11). Activation of another Ras-independent pathway that leads to additional cyclin E overexpression may account for Src's net effect on cell cycle progression. Since MAP kinase functions downstream of Src, Ras, and Raf, and since MAP kinase is activated in G_1 and around the M phases of the cell cycle (37), it is possible that the accelerated G_1 progression in these three transformed cells is through constitutive activation of the MAP kinase activity in the G_1 phase which then sends signals into the nucleus that trigger the activation of a G_1/S control factor such as cyclin D1.

Studies carried out by other laboratories (21, 23, 42) suggest that cyclin D may play an important role in controlling G_1/S transition, as it is induced in G_1 and can complex with a few CDK proteins and proteins involved in DNA replication and repair. Cyclin D1 (PRAD1) is overexpressed in parathyroid tumors (26), while the negative growth effect of TGF-β is exerted through its inhibition in the amount and activity of cdk4, the major catalytic partner of D-type cyclins (8). This suggests that cyclin D-cdk4 kinase is an important target for many growth factor-signaling pathways. The results presented here show another good example of these pathways.

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