Journal of Cellular Biochemistry 65:83–94 (1997)

Altered Cell Shape Is Linked to Increased p34^{cdc2} Gene Expression in Fibroblasts Expressing a Mutant E2F-1 Transcription Factor

Thomas J. Logan, Kelly L. Jordan, Devon L. Evans, and David J. Hall*

Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract The E2F1 transcription factor or an amino terminal deletion mutant termed E2F1d87 was constitutively expressed in NIH3T3 fibroblasts. Cells expressing wild-type E2F1 display a morphology indistinguishable from that of normal fibroblasts. However, the E2F1d87-expressing cells exhibited a distinct rounding during culture in media containing 10% calf serum. The morphology change was most pronounced during S phase, which was considerably lengthened in the E2F1d87-expressing cells. Consistent with this rounded shape, the E2F1d87-expressing cells have significantly increased levels of both p34^{cdc2} mRNA and protein. Also observed was an increase in active p34^{cdc2} in immunoprecipitates from extracts of the E2F1d87 cell line, as assayed by histone H1 kinase assay. The upregulation of p34^{cdc2} expression occurs at the transcriptional level and requires ectopic E2F1d87 along with serum growth factor stimulation, since culture of these cells in low serum media results in a flattened shape and a drop in p34^{cdc2} expression compared to that of the control cells. J. Cell. Biochem. 65:83–94. © 1997 Wiley-Liss, Inc.

Key words: E2F1; E2F1d87; NIH3TH; fibroblasts; p34^{cdc2}

The E2F transcription factor is known to be a multiprotein family represented by E2F1-5, DP1, and DP2 [Girling et al., 1993; Helin et al., 1992; Ivey-Hoyle et al., 1993; Huber et al., 1993; Kaelin et al., 1992; Lees et al., 1993; Ginsberg et al., 1994; Sardet et al., 1995]. These proteins regulate expression of such genes such as dihydrofolate reductase, DNA polymerase alpha, p34^{cdc2}, thymidine kinase, and c-myc [Mudryj et al., 1990; Schwarz et al., 1993], which is consistent with the presence of E2F binding sites (GCGCGAAA) within these promoters [Nevins, 1992]. The targets of E2F code for proteins whose activities are needed for DNA synthesis [Nevins, 1992]. These E2F members are known to interact with the retinoblastoma susceptibility gene product, pRb, and the pRb-related proteins p107 and p130 as well as cyclin A/E and cdk 2 [Cao et al., 1992; Chellappan et al., 1991; Cress et al., 1993; Mudryj et al., 1991; Shirodkar et al., 1992]. These interactions appear to regulate the transcriptional and DNA binding activity of E2F.

The first member of the family to be cloned, E2F1, posesses multiple domains [Kaelin et al., 1992]. The bHLH domain is required for DNA binding; however, the structure of the basic domain of E2F1 appears unique among bHLH-containing proteins in that the center of the domain likely contains a turn instead of an α-helix [Jordan et al., 1994]. The transcriptional activation domain of E2F1 resides at the carboxy terminus and overlaps with the pRb binding domain [Helin et al., 1992; Kaelin et al., 1992; Flemington et al., 1993]. When the product of the retinoblastoma susceptibility gene, pRb, binds the C-terminus of E2F1 it blocks its ability to activate transcription [Helin et al., 1992, 1993; Kaelin et al., 1992; Flemington et al., 1993].

Constitutive expression of E2F1 in NIH3T3 fibroblasts promotes entry of the cells into S phase during serum starvation conditions, yet the cells are unable to complete S phase [Logan et al., 1994]. This is consistent with the effect of microinjection of E2F1 into serum-starved fibroblasts [Johnson et al., 1993]. The primary cell cycle phase affected by constitutive expression of E2F1 is G0, since this phase is dramatically shortened in serum-starved fibroblasts, while

Contract grant sponsor: NIH, contract grant number CA51170, CA67032; Contract grant sponsors: the Lucille P. Markey Charitable Trust, the Foerderer Foundation.

*Correspondence to: David J. Hall, Dept. of Biochemistry and Molecular Biology, Thomas Jefferson University, 208 BLSB, 233 South 10th St., Philadelphia, PA 19107.

Received 24 July 1996; accepted 25 November 1996

© 1997 Wiley-Liss, Inc.

the G1 phase is unaffected during the course of proliferation [Logan et al., 1995b].

Constitutive expression of a mutant E2F1 (an amino terminal deletion termed E2F1d87) has similar effects on G0/G1 phase transit and S phase entry as the cells expressing full-length E2F1, yet these cells demonstrate a lengthened S phase during proliferation [Logan et al., 1995a]. These cells are also more sensitive to S phase–specific toxins [Logan et al., 1995a]. In addition, the cells expressing E2F1d87 demonstrate a rounded morphology that is dependent on serum growth factors, such that cytoskeletal structures as microfilaments, micrutubules, and vinculin containing focal contacts are not detectable [Logan et al., 1994]. Since the morphology of the E2F1d87-expressing cells is similar to that of cells microinjected with $p34^{cdc2}$ [Lamb et al., 1990], a known target of E2F [Nevins, 1992; Dalton, 1992], we have analyzed in detail the effects of constitutive expression of E2F1 and E2F1d87 on $p34^{cdc2}$ gene expression in NIH3T3 fibroblasts.

MATERIALS AND METHODS

Cell Culture/Cell Cycle Analysis/Thymidine Block

NIH3T3 fibroblasts (ATCC, Rockville, MD) and the E2F1 and E2F1d87 cell lines [Logan et al., 1994] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum.

Cells to be processed for flow cytometry were rinsed once in chilled phosphate buffered saline (PBS) and then trypsanized and resuspended in 10 ml DMEM plus 10% serum. The cells were then pelleted and resuspended in 70% ethanol. The cells were kept on ice for 10 min, pelleted, and then treated with RNAase A (1.8 μg/ml) for 30 min at room temperature. Propidium iodide (Sigma, St. Louis, MO) was added to a final concentration of 2 μg/ml for an additional 15 min at room temperature. Cell cycle analysis was then performed on a Coulter (Hialeah, FL) Profile 2 flow cytometer.

To block cells at the G1/S boundary by double thymidine block, we treated proliferating cells with excess thymdine (2 mM) for 14 h. The cells were washed in PBS and fresh media added containing 10% serum. After 9 h, excess thymdine (2 mM) was again added for an additional 14 h. The cells were washed in PBS, fresh media containing 10% serum was added, and flow cytometry was performed.

Generation of Extracts

Nuclear extracts for Western blots and immunoprecipitations were generated by lysing the cells on ice in 0.1% NP-40, 10 mM Tris (pH 7.9), 10 mM $MgCl_2$, 15 mM NaCl, and the protease inhibitors PMSF (0.5 mM), pepstatin (2 μg/ml), and leupeptin (1 μg/ml). The nuclei were pelleted by centrifugation at 800*g* for 10 min and then were resuspended in extraction buffer consisting of 0.42 M NaCl, 20 mM Hepes (pH 7.9), 20% glycerol, PMSF (0.5 mM), pepstatin (2 μg/ml), and leupeptin (1 μg/ml) for 10 min on ice, and then were centrifuged at 14,000*g* for 8 min to pellet the residual nuclear material. This supernatant fraction was termed nuclear extract. For immunoprecipitations the nuclear extract was diluted with extraction buffer lacking NaCl to lower the salt concentration to 0.1 M.

Western Blot Hybridizations/Northern Blots/Transfections

Nuclear extracts were electrophoresed by SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose, washed in TBST buffer (10 mM Tris, pH 8; 150 mM NaCl; 0.05% Tween 20), blocked with 2.5% bovine serum albumin (BSA) in TBST for 30 min at room temperature, and then incubated with the primary antibodies for 30–60 min at room temperature in TBST. The primary antibodies used were anti-$p34^{cdc2}$ (Oncogene Science, directed against the C-terminal 8 amino acids; Ab-1, Manhasset, NY), anti-PCNA (proliferating cell nuclear antigen; Oncogene Science), and anti-cyclin A (UBI, Lake Placid, NY). The blots were then incubated with a 1:7,500 dilution of secondary antibody (goat antimouse; Vector Labs, Burlingame, CA) conjugated to alkaline phosphatase for 30 min at room temperature in TBST. The blot was then stained using the Protoblot system from Promega (Madison, WI).

For Northern blot hybridization, 20 μg of total RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto nitrocellulose. The blot was hybridized with 1×10^6 CPM/ml of ^{32}P-labelled $p34^{cdc2}$ cDNA probe.

Transient transfections were performed by the calcium phosphate method [Moberg et al., 1991]. The $p34^{cdc2}$ promoter-CAT construct encompasses 1,014 basepairs of the $p34^{cdc2}$ promoter [Ku et al., 1993] from position −941 to +73. CAT assays were performed and analyzed as in Moberg et al. [1991].

Immunoprecipitations/Histone H1 Kinase Assays

For immunoprecipitations, 50–100 μg of nuclear extract was incubated at 4°C with 1 μg of anti-$p34^{cdc2}$ polyclonal antibody (Ab-1; Onco-

gene Science) for 1 h. Two micrograms of Staphyloccocus protein-A acrylamide beads were added to the extracts for an additional 1.5 h at 4°C. The beads were pelleted and washed five times in NTEN (100 mM NaCl, 20 mM Tris, pH 8, 1 mM EDTA, 0.5% NP-40). The beads were boiled in SDS-PAGE sample buffer, and the soluble fraction was electrophoresed on SDS-PAGE, Western-blotted, and probed with and antiphosphotyrosine antibody (UBI).

For histone H1 kinase assays, immunoprecipitates on protein-A acrylamide beads were washed four times with 20 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40 (NETN) by pelleting in Eppendorf tubes. A final column wash was performed with kinase buffer (50 mM Tris (pH 7.6) 10 mM $MgCl_2$, and 1 mM DTT). Then 40 μl of kinase solution containing 10 μg histone H1 (GIBCO/BRL, Gaithersburg, MD), 75 μCi ^{32}P-γATP, and 10 μM ATP in kinase buffer was added to the immunoprecipitates for 8 min at 30°C. The supernatent was then analyzed for H1 kinase activity by SDS-PAGE and autoradiography.

Gel-Shift Assays

Gel-shift assays were performed as described by Moberg et al., [1991]. Briefly, 0.5 ng of a ^{32}P end-labelled, double-stranded oligonucleotide was incubated with nuclear extracts in the presence of 1 μg of sheared salmon sperm DNA as a nonspecific competitor. The final buffer conditions for protein binding to the radiolabelled DNA were 16 mM Hepes, pH 7.9, 16% glycerol, 80 mM KCl, 0.16 mM EDTA, 0.4 mM DTT, and 0.4 mM PMSF. The reactions were then electrophoresed in a low ionic strength (7 mM Tris, pH 7.9, 3.3 mM NaAcetate, 1 mM EDTA) 4% polyacrylamide gel and visualized by autoradiography. The double-stranded oligonucleotide representing the E2F element is

AATTCGCTTGGCGGGAAAAC

GCGAA<u>CCGCCCTTT</u>TGTTAA.

RESULTS

E2F1d87-Expressing Cell Lines Demonstrate Altered Morphology That Is Dependent on Serum Growth Factors and Is Most Distinct During S Phase

Cell lines were generated that constitutively express epitope-tagged full-length E2F1 (residues 1–437) protein or a tagged mutant E2F1 that spans amino acids 88–437 (termed E2F1d87) [Logan et al., 1994]. E2F1d87 lacks the proline/alanine-rich amino terminus [Helin et al., 1992; Kaelin et al., 1992] containing the cyclin A/cdk2 binding region [Krek et al., 1994; Xu et al., 1994]. The epitope tag is recognized by a specific monoclonal antibody (M2; VWR, Plainfield, NJ). Shown in Figure 1 is an immunoblot of the ectopically expressed E2F1 and E2F1d87 proteins in the cell lines growing in 10% calf serum containing media (Fig. 1A) or following serum starvation (0.5% serum) for 48 h (Fig. 1B). The blots show equivalent levels of expression of the full-length and mutant proteins (the slight difference in intensities is due to the fact that the blot in Fig. 1B has double the protein concentration compared to that in Fig. 1A). It has been previously demonstrated that constitutive expression of E2F1d87 produces a profound shape change (rounding) in NIH3T3 fibroblasts cultured in 10% calf serum [Logan et al., 1994]. However, culture of the E2F1d87-expressing cells in low serum (0.5%) causes them to revert to a normal fibroblast morphology [Logan et al., 1994]. This finding would suggest that the rounded morphology is dependent on movement through the cell cycle. Recently it has been shown that S phase is lengthened in the E2F1d87-expressing cells cultured in 10% serum [Logan et al., 1995a]. Therefore, experiments were performed to determine if morphology was a function of movement through S phase. The cells were synchronized at the G1/S boundary by a double thymidine block and then released. As seen in Figure 2A, about half of the E2F1d87-expressing cells have a very rounded morphology at the G1/S phase boundary, while the other half of the cells are spindle-shaped. The control and E2F1-expressing cells have the flattened morphology of normal fibroblasts. At 2 h after the release of the block, nearly 100% of the population of the E2F1d87 cells is spherical. This spherical morphology slowly begins to change, such that by 6–8 h following release from the block the cells adopt a more irregular rounded shape. The control and E2F1-expressing cells show little change in morphology during this time. By 24 h following the release, the E2F1d87-expressing cells display the original phenotype, reflected by a mix of rounded cells and some spindle-shaped cells. The data show that the E2F1d87 cells manifest the most spherical shape during the middle of S phase. Figure 2B shows the percent of cells in S phase, by flow cytometry, during the period between 0 and 8 h after

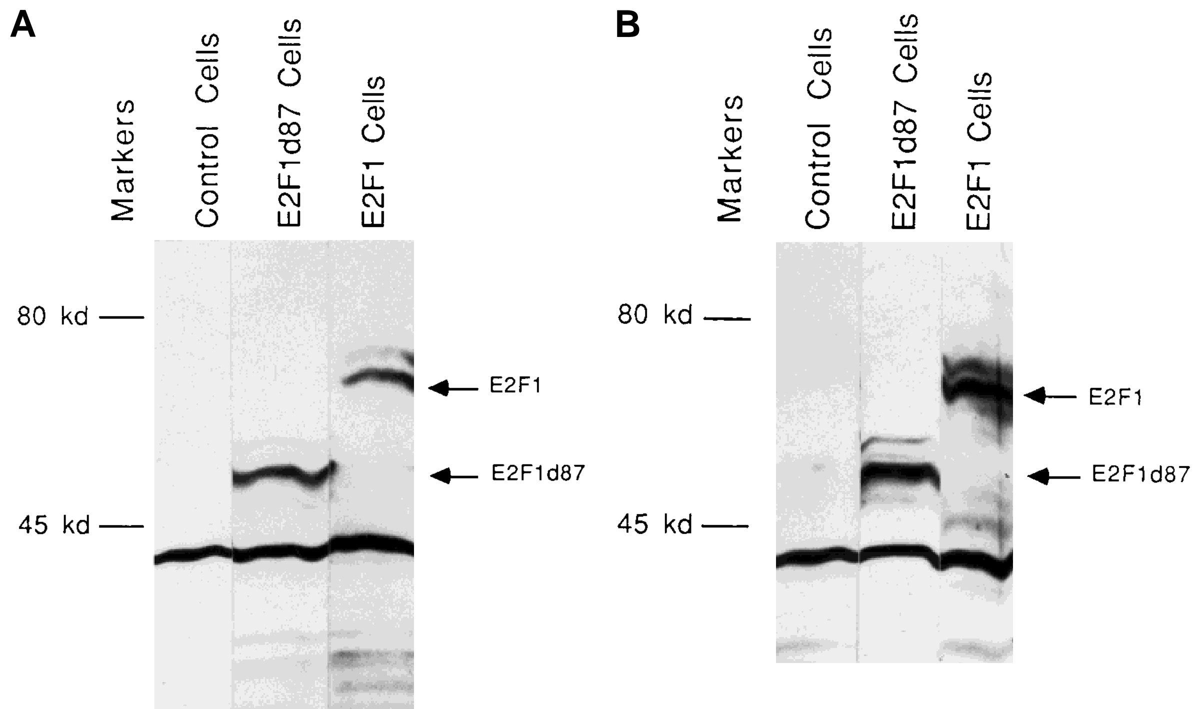


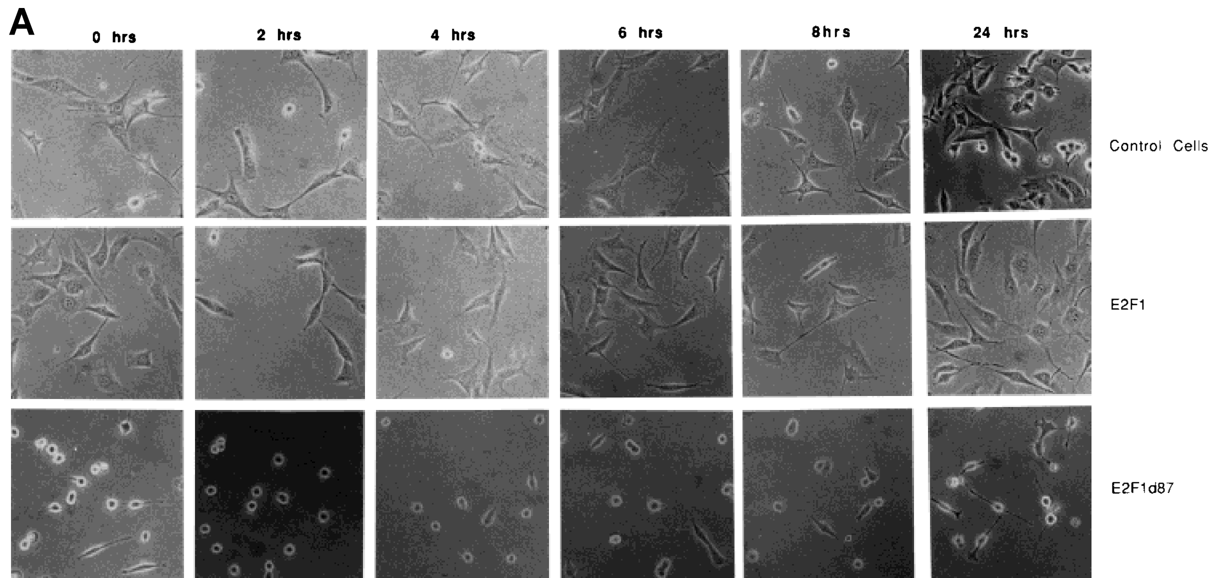
Fig. 1. Western analysis of E2F1- and E2F1d87-overexpressing cell lines. NIH3T3 fibroblasts infected with the E2F1- and E2F1d87-expressing retrovirus were grown in the presence of G418; colonies were selected and screened for E2F1 and E2F1d87 expression. The control cells were infected with the pMV7 virus alone. A Western blot of nuclear extracts (100 μg) from subconfluent cells growing in 10% calf serum containing media is shown in **A**, while a blot of nuclear extracts (200 μg) from cells serum starved for 48 h (0.5% serum) is shown in **B**. The M2 monoclonal antibody was used as a primary antibody followed by a secondary antibody conjugated to alkaline phosphatase. The position of ectopically expressed E2F1 and E2F1d87 is indicated by the *arrows*.

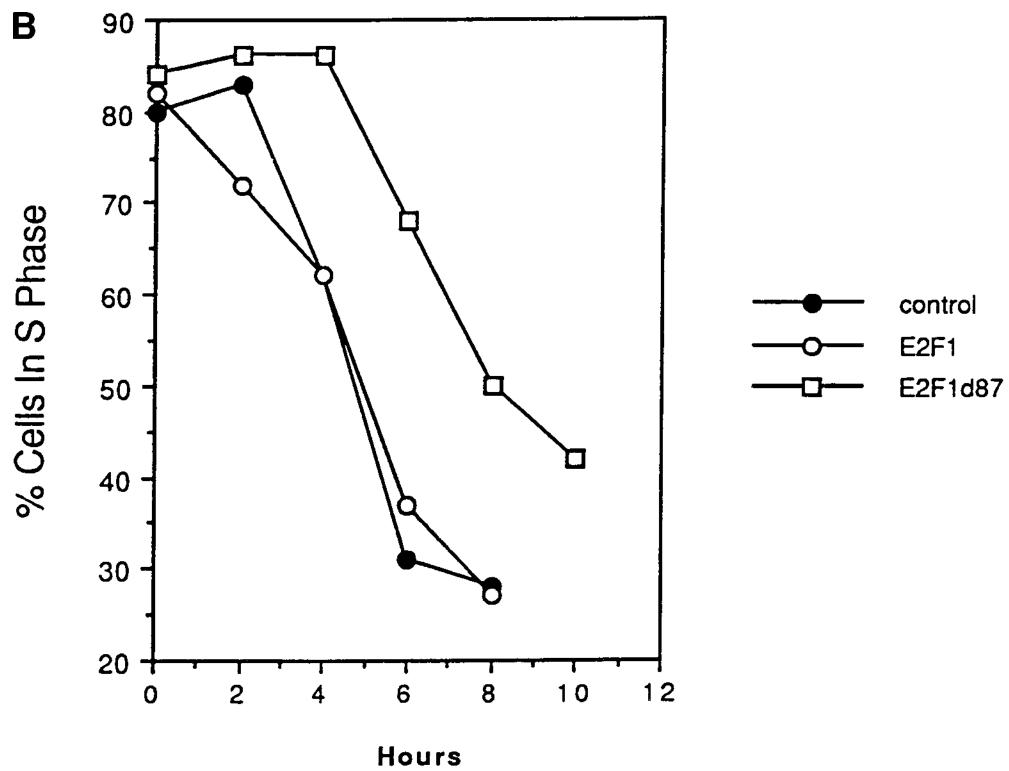
release from the block. The data show the slow progression of the E2F1d87 cell line through S phase compared to the control and E2F1-expressing cells, consistent with a recent report [Logan et al., 1995a]. Figure 2C shows a gel-shift assay, using extracts from the cells during the 0–8 h period after release from the thymidine block and a ^{32}P-labelled DNA fragment representing and E2F binding site. It is clear that the ectopic E2F1 and E2F1d87 proteins bind DNA through S phase. Addition of M2 antibodies to the gel-shift reactions results in a supershift of E2F1 and E2F1d87 (data not shown). The increase in DNA binding by E2F1d87 by 8 h may reflect insensitivity to the inhibitory effects of cyclin A/cdk2 phosphorylation, due to lack of the cyclin A/cdk2 binding domain on E2F1d87.

$p34^{cdc2}$ Levels Are Significantly Enhanced in Extracts From the E2F1d87 Cell Line

In an attempt to understand how shape is altered by E2F1d87, we assayed E2F target gene expression. One such target is the $p34^{cdc2}$ protein kinase [Nevins, 1992; Dalton, 1992]. That altered $p34^{cdc2}$ expression may mediate the shape change of these cells is suggested by the work of Lamb et al. [1990], who showed that microinjection of $p34^{cdc2}$ caused significant rounding of fibroblasts. If $p34^{cdc2}$ mediates the shape change, then the level of its expression

Fig. 2. E2F1d87 cell lines display the greatest change in shape during S phase compared to control and E2F1 cell lines. **A:** Subconfluent, growing control cells and E2F1- and E2F1d87-expressing cells were treated with a double thymidine block to arrest them at the G1/S phase boundary. Fresh medium was then added to the cultures to release the cells from the block, and the morphology of the cells was monitored as they passed through S phase. Shown are the photomicrographs of the phase contrast microscopy after release from the thymdine block. ×10. **B:** Percent of cells in S phase. Aliquots of cells from A were processed for flow cytometry. Shown is the percent of cells in S phase following the release of the thymidine block. **C:** Nuclear extracts were generated from control cells and the E2F1 and E2F1d87 cell lines. Nuclear extracts were made from cells that were arrested at the G1/S phase boundary and then released as A above. Two micrograms of nuclear extract from each cell line under each condition was used in the gel-shift assay with a ^{32}P-labelled E2F element, except for the extracts from asynchronously growing E2F1d87 cells, where 8 μg was used. Shown is the autoradiograph of the gel; the free unbound probe was in excess and is not shown.





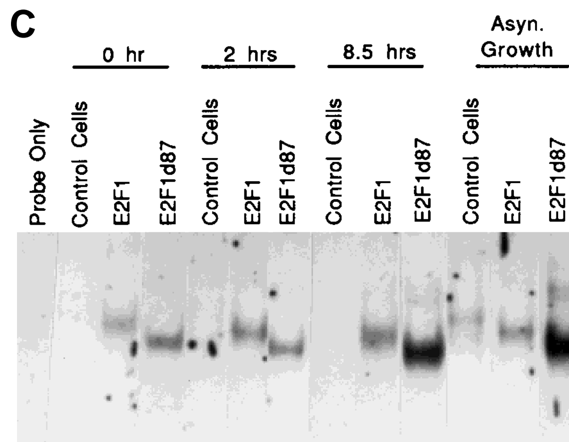


Figure 2

should be elevated in the E2F1d87 cell line. In particular, the level should be elevated during S phase since this is the time the cells display the most significant alteration in morphology. To determine if this is the case, we generated nuclear extracts from the cell lines following release from a block at the G1/S boundary with excess thymidine. These extracts were analyzed by Western blot using an antibody specific only for $p34^{cdc2}$ (the antibody recognizes the unique C-terminal domain of $p34^{cdc2}$). As can be seen in Figure 3A (left side), $p34^{cdc2}$ protein levels are significantly increased compared to the control cells or the E2F1 cell line. The slowly migrating species of $p34^{cdc2}$ is known to be doubly phosphorylated on thr14 and tyr15 [Norbury and Nurse, 1992]. Tyrosine phosphorylation is confirmed by the data in Figure 3A (right side) showing that the upper form is indeed a phosphotyrosine. Immunoprecipitations of $p34^{cdc2}$ from extracts followed by immunoblotting and hybridization with an antiphosphotyrosine antibody also demonstrate that the upper form in Figure 3A is phosphorylated on tyrosine (data not shown).

To determine if the effect of E2F1d87 was specific to $p34^{cdc2}$, other S phase–specific factors were also analyzed. One such factor is proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase δ that is required for DNA synthesis. Extracts were examined for PCNA levels during the transit of S phase by immunoblot using an anti-PCNA specific antibody. As seen in Figure 3B, PCNA levels were unchanged compared to the extracts from control cells and the E2F1-expressing cells. In addition, the level of cyclin A was the same in all three cell lines as assayed by immunoblot (data not shown). Thus, E2F1d87 does not exert a general effect on genes whose products are needed for DNA replication.

The levels of $p34^{cdc2}$ protein were quantitated by scanning the blots in Figure 3A. Graphs of the relative levels of $p34^{cdc2}$ protein were plotted over time after release from the G1/S block. As can be seen in Figure 3C, the levels of the upper form of $p34^{cdc2}$ are increased by fortyfold over that in the extracts from the other cell lines at the G1/S boundary and remain elevated over time. It should be noted that other E2F1d87 cell lines were examined and found to also have significantly elevated $p34^{cdc2}$ protein levels compared to control and E2F1 cell lines (data not shown), indicating that the effect is not due to clonal variation.

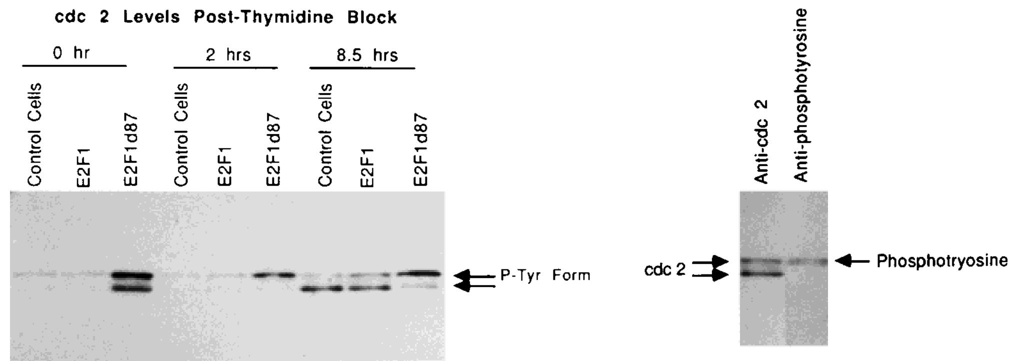
Northern blot assays were next performed to determine if the increase in $p34^{cdc2}$ was due to increases in the level of mRNA. As seen in Figure 4, there is a significant increase in the level of $p34^{cdc2}$ mRNA at the G1/S phase boundary. This increase is maintained through S phase and coincides with the increase in $p34^{cdc2}$ protein seen at this time. The levels of $p34^{cdc2}$ mRNA in the control and E2F1 cell lines increase from G1/S into G2/M, which is consistent the published behavior of $p34^{cdc2}$ gene expression during transit through S, G2, and M phases [Welch and Wang, 1992]. These data indicate that $p34^{cdc2}$ gene expression is significantly upregulated through S phase in the E2F1d87 cell line. By 2 h postrelease from the thymidine block, the H1 kinase activity in the extracts from the E2F1d87 cell line is roughly 2.5-fold higher than in the control and E2F1 cell line. This occurs at a time of greatest change in cell shape (from Fig. 2).

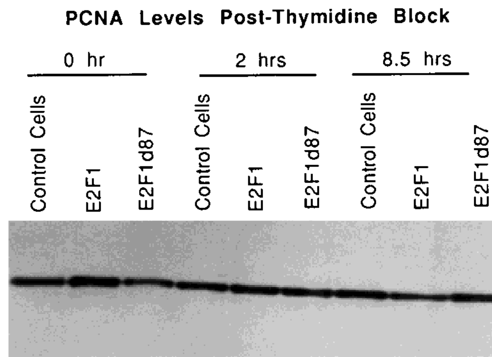
Increases in the Active Form of $p34^{cdc2}$ Are Evident in the E2F1d87 Cell Line

While $p34^{cdc2}$ levels are increased by E2F1d87 expression, it was important to determine if this corresponded with increased $p34^{cdc2}$ activity. The phosphotyrosine form of the protein (see Fig. 3A) is inactive as a kinase, while the non-tyrosine-phosphorylated form is active [Norbury and Nurse, 1992; Atherton-Fessler et al., 1994]. $p34^{cdc2}$ was therefore immunoprecipitated from extracts of cells blocked at the G1/S border and the immunoprecipitates used in a histone H1 kinase assay, a measure of active $p34^{cdc2}$ [Norbury and Nurse, 1992; Atherton-Fessler et al., 1994]. As seen in Figure 5, there was an increase in H1 kinase activity in $p34^{cdc2}$ immunoprecipitates from the E2F1d87 cells relative to the other cell lines. This indicates that the increased levels of the non-tyrosine-phosphorylated form of $p34^{cdc2}$ protein in S phase corresponds with an increase in the level $p34^{cdc2}$ kinase activity in the E2F1d87-expressing cells.

Serum Growth Factors and Ectopic E2F1d87 Expression Are Needed for Upregulation of $p34^{cdc2}$

It has been previously shown that the change in morphology of the E2F1d87-expressing cells was due to both growth factor signals and ectopic expression of E2F1d87 [Logan et al., 1994]. If the rounded morphology is linked to increased expression of $p34^{cdc2}$, then culture of





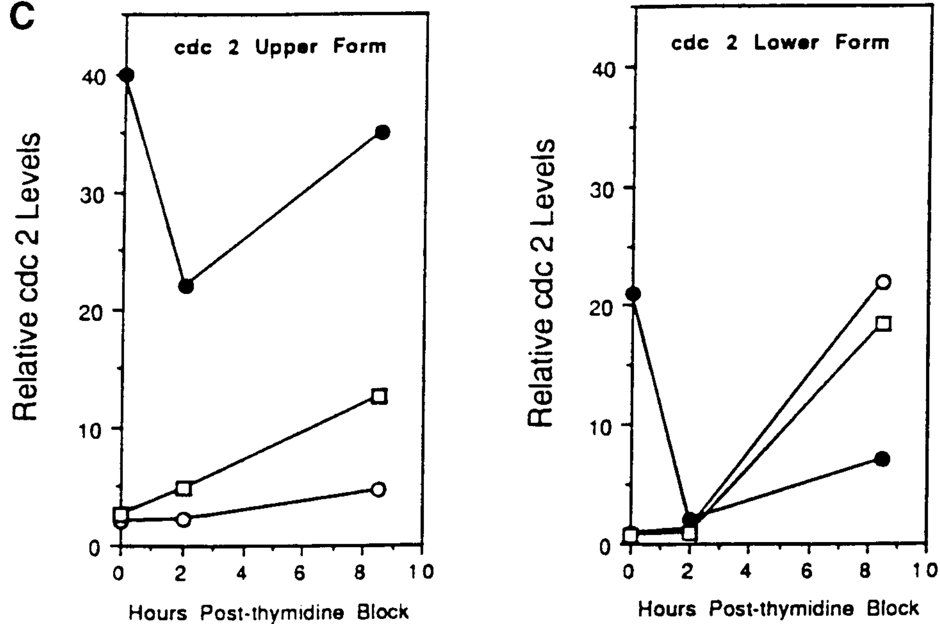


Fig. 3. Western blots reveal elevated p34^{cdc2} levels in extracts from the E2F1d87 cell line. **A, left panel:** Control cells, E2F1-expressing cells, and E2F1d87-expressing cells were arrested at the G1/S phase boundary with a double thymidine block and then released as in Fig. 2. Nuclear protein extracts were generated and 25 µg of protein was immunoblotted and probed with an anti-p34^{cdc2}–specific antibody (Oncogene Science). The *arrows* point to the phosphotyrosine-containing p34^{cdc2} band and the underphosphorylated form. **Right panel:** Protein extracts (25 µg) from G1/S arrested E2F1d87 cells were electrophoresed in adjacent lanes of SDS-PAGE. The gel was immunoblotted, the blot containing the lanes was separated, and one lane was probed with anti-p34^{cdc2} antibody and the other lane with anti-phosphotyrosine-specific antibody. **B:** Protein extracts (25 µg) as in A were immunoblotted and probed with an anti-PCNA–specific antibody. **C:** Immunoblots as in A were processed for image analysis to determine the levels of increase of the forms of p34^{cdc2}. Shown are the levels of the upper form (phosphotyrosine-containing) and the lower form of p34^{cdc2} in extracts of control cells (○) and E2F1d87 (●) and E2F1 (□) cell lines. The levels are relative to the lower form in the control cells at 0 time (set equal to 1).

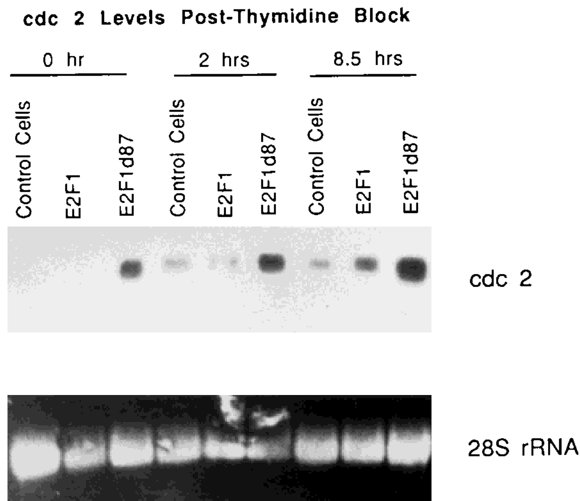


Fig. 4. Northern blots reveal elevated p34^{cdc2} mRNA levels in the E2F1d87 cell line. Total RNA (25 μg) from control cells and E2F1 and E2F1d87 cell lines were electrophoresed on formaldehyde-agarose gels, blotted onto nitrocellulose, and probed with an ^{32}P-labelled p34^{cdc2} cDNA. The cells were arrested at the G1/S phase boundary with a double thymidine block and then released as in Fig. 2. Shown is an autoradiogram of the blot. Also shown is the ethidium bromide–stained gel, after blotting, indicating the remaining 28S rRNA which reflects equally loaded lanes.

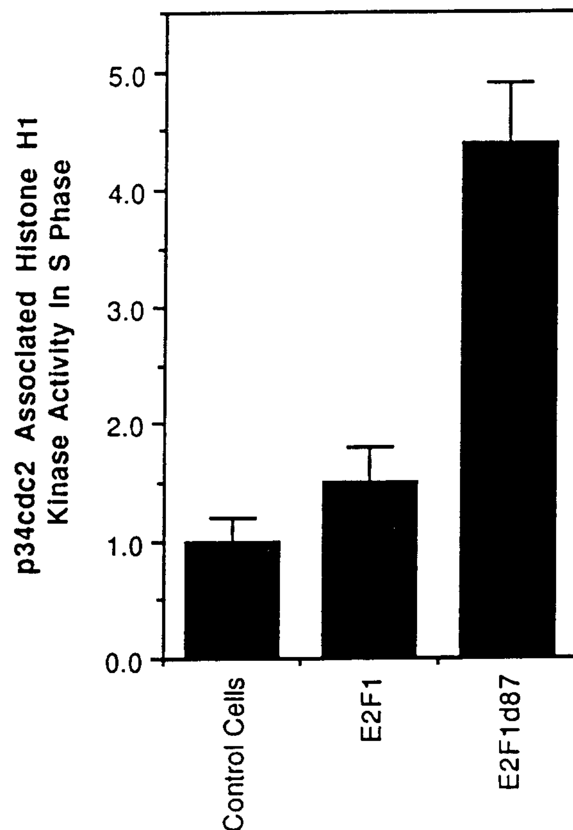


Fig. 5. p34^{cdc2} associated histone H1 kinase activity is increased in extracts from the E2F1d87 cell line. Nuclear protein extracts (25 μg) from control cells and E2F1 and E2F1d87 cell lines were immunoprecipitated with an anti-p34^{cdc2}–specific antibody. The extracts were generated from cells that had been arrested at the G1/S phase boundary with a double thymidine block and then released as in Fig. 2. The extracts were generated at the time of release. The immunoprecipitates were used in a histone H1 kinase assay. Shown are the relative levels of phosphorylated histone H1 generated by the immunoprecipitates.

the E2F1d87 cell line in low serum should cause a drop in the p34^{cdc2} levels. Therefore, cells were cultured for 48 h in low serum (0.5%), extracts were generated, and p34^{cdc2} levels were analyzed by immunoblot. The levels of p34^{cdc2} were found to be roughly equivalent among the three cell lines (Fig. 6A). However, an additional band appeared in the lane of the E2F1d87 extracts (Fig. 6A, asterisk), migrating at the position of a phosphorylated form of p34^{cdc2} [Draetta et al., 1988; Atherton-Fessler et al., 1994].

p34^{cdc2} was then immunoprecipitated from these extracts of serum-deprived cells. The immunoprecipitates were used in histone H1 kinase assays. The level of activity between the cell lines was found to be equal (Fig. 6B). This result is consistent with the similar levels of p34^{cdc2} protein found in the three cell lines following culture in low serum (Fig. 6A). These data indicate that during serum starvation the levels of p34^{cdc2} protein and activity are approximately equivalent to that in the control cells and E2F1 cell lines.

Induction of p34^{cdc2} Gene Expression Occurs at the Transcriptional Level

To determine if the elevated p34^{cdc2} expression in the E2F1d87 cell line was due to transcriptional induction, we transfected a p34^{cdc2} promoter-CAT reporter construct into the three cell lines. An SV40 promoter–β galactosidase reporter construct was cotransfected to correct for differences in transfection efficiency among the three cell lines. As seen in Figure 7A, proliferating E2F1d87 cells demonstrate increased transcriptional activity from the p34^{cdc2} promoter compared to the other cell lines. When the cells were synchronized in S phase following transfection by a double thymidine block, transcription from the p34^{cdc2} promoter was further increased over that of the other cell lines. When similar transfection experiments were performed with a cyclin D1 promoter–CAT construct, responsive to E2F1 [Sala et al., 1994], there was no enhanced effect on tran-

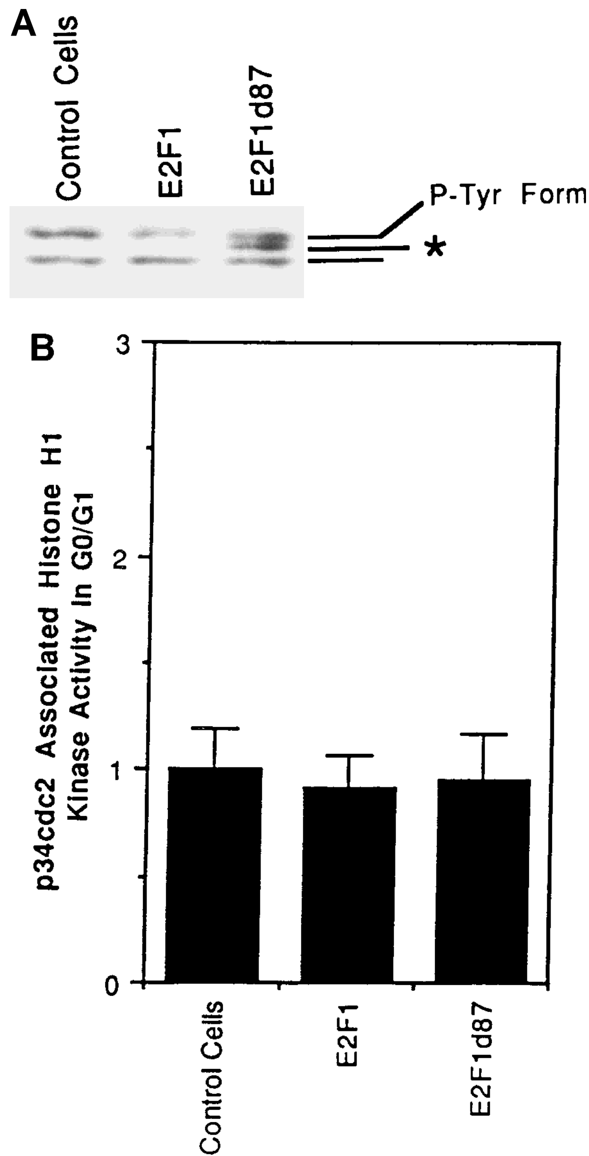


Fig. 6. Following serum starvation, Western blots reveal similar p34^{cdc2} protein levels and activity in extracts of the three cell lines. **A:** Control cells and E2F1- and E2F1d87-expressing cells were cultured in 0.5% calf serum for 48 h to arrest them in the G0/G1 phase. Nuclear protein extracts were generated, and 25 µg was immunoblotted and probed with an anti-p34^{cdc2}–specific antibody. The *arrows* point to the phosphotyrosine-containg p34^{cdc2} band and the underphosphorylated form (the faster migrating species). In addition, a new band in the extracts from the E2F1d87 cells is marked (*). **B:** Nuclear protein extracts (25 µg) from A were immunoprecipitated with an anti-p34^{cdc2}–specific antibody. The immunoprecipitates were used in a histone H1 kinase assay. The phosphorylated H1 was electrophoresed on SDS-PAGE and exposed to X-ray film, and the films were scanned with a densitometer. Shown are the relative levels of phosphorylated histone H1 generated by the immunoprecipitates.

scription following transfection into the E2F1d87 cell line over that seen in the E2F1 cell line (Fig. 7B). This data indicates that the effect of E2F1d87 may be specific to a subset of E2F1-responsive promoters.

When the cells were cultured in low serum, following the transfection, the level of transcription from the p34^{cdc2} promoter was identical to that of the other cell lines (Fig. 7C). This indicates that p34^{cdc2} promoter activity is due to the dual action of ectopic E2F1d87 expression and serum growth factor stimulation.

DISCUSSION

In this report we have analyzed the effect of constitutive expression of a mutant form of E2F1 on both cell shape and p34^{cdc2} gene expression in NIH3T3 fibroblasts. The data presented here show that levels of p34^{cdc2} mRNA, protein, and activity are elevated during S phase in the E2F1d87-expressing cells. This occurs at a time when these cells demonstrate the greatest changes in morphology, reflected by a significant rounding. That increased p34^{cdc2} levels correspond with the morphology change in the E2F1d87-expressing cells is consistent with the results of a previous study by Lamb et al. [1990] demonstrating that microinjection of p34^{cdc2} into fibroblasts results in a similar type of rounding phenomena. However, the change in shape and cytoskeletal structure appears much more significant in the E2F1d87-expressing cells (results presented here and Logan et al. [1994]) than in cells injected with p34^{cdc2} [Lamb et al., 1990]. Since p34^{cdc2} plays a major role in regulation of transit through mitosis, primarily in conjunction with cyclin B [Norbury and Nurse, 1992], it was previously proposed that inappropriate p34^{cdc2} expression drives nonmitotic cells into a mitotic phenotype [Lamb et al., 1990]. This may be occuring during S phase in cells expressing E2F1d87. Yet, while this may be detrimental to the cells, because S phase is lengthened, it is not lethal because the cells do proliferate.

The mechanism by which p34^{cdc2} could affect cellular morphology in S phase is not clear. p34^{cdc2} normally forms an active complex with cyclin B during mitosis, with relatively little cyclin B present during S phase [Norbury and Nurse, 1992]. Since cyclin A/p33^{cdk2} is the primary S phase cyclin kinase complex [Norbury and Nurse, 1992], it is possible that elevated p34^{cdc2} associates with cyclin A, resulting in an

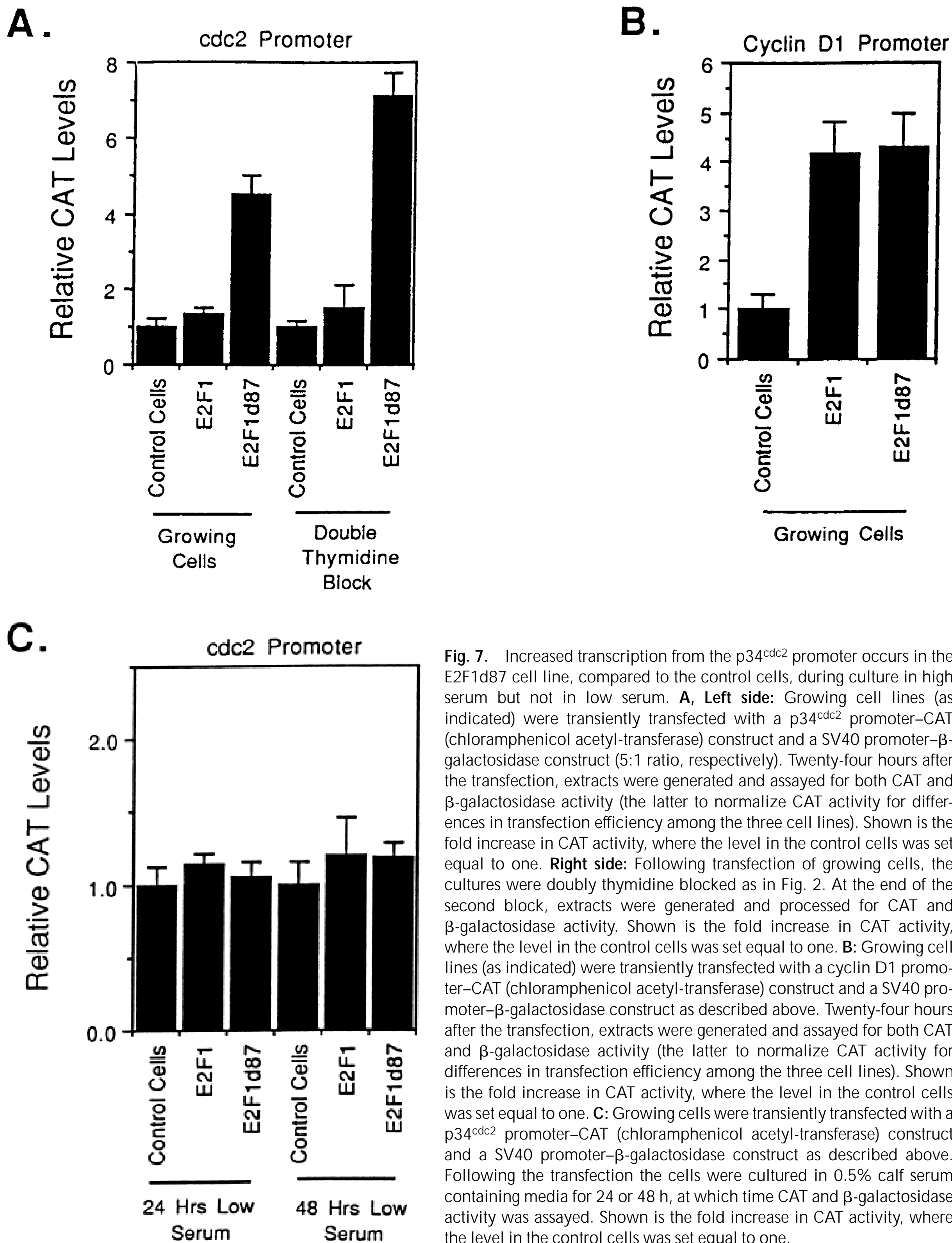


Fig. 7. Increased transcription from the p34^{cdc2} promoter occurs in the E2F1d87 cell line, compared to the control cells, during culture in high serum but not in low serum. **A, Left side:** Growing cell lines (as indicated) were transiently transfected with a p34^{cdc2} promoter–CAT (chloramphenicol acetyl-transferase) construct and a SV40 promoter–β-galactosidase construct (5:1 ratio, respectively). Twenty-four hours after the transfection, extracts were generated and assayed for both CAT and β-galactosidase activity (the latter to normalize CAT activity for differences in transfection efficiency among the three cell lines). Shown is the fold increase in CAT activity, where the level in the control cells was set equal to one. **Right side:** Following transfection of growing cells, the cultures were doubly thymidine blocked as in Fig. 2. At the end of the second block, extracts were generated and processed for CAT and β-galactosidase activity. Shown is the fold increase in CAT activity, where the level in the control cells was set equal to one. **B:** Growing cell lines (as indicated) were transiently transfected with a cyclin D1 promoter–CAT (chloramphenicol acetyl-transferase) construct and a SV40 promoter–β-galactosidase construct as described above. Twenty-four hours after the transfection, extracts were generated and assayed for both CAT and β-galactosidase activity (the latter to normalize CAT activity for differences in transfection efficiency among the three cell lines). Shown is the fold increase in CAT activity, where the level in the control cells was set equal to one. **C:** Growing cells were transiently transfected with a p34^{cdc2} promoter–CAT (chloramphenicol acetyl-transferase) construct and a SV40 promoter–β-galactosidase construct as described above. Following the transfection the cells were cultured in 0.5% calf serum containing media for 24 or 48 h, at which time CAT and β-galactosidase activity was assayed. Shown is the fold increase in CAT activity, where the level in the control cells was set equal to one.

inactive or only partially dimer with cyclin A, especially since much of the $p34^{cdc2}$ is in an inactive phosphotyrosine form (Fig. 3A). Thus, $p34^{cdc2}$ may effectively compete with $p33^{cdk2}$ for binding to cyclin A, or $p34^{cdc2}$ may inhibit optimal binding of $p33^{cdk2}$ to cyclin A. Alternatively, a small amount of active cyclin B/$p34^{cdc2}$ may form in S phase, resulting in the inappropriate phosphorylation of critical substrates, possibly the known mediators of cell shape, such as components of the cytoskeleton or extracellular matrix. Additionally, since it appears that $p34^{cdc2}$ plays a role in transit S phase, possibly through a novel mechanism [Furukawa et al., 1990; Mercer et al., 1992; D'Urso et al., 1990; Marraccino et al., 1992], it may be that the elevation of $p34^{cdc2}$ alters this normal function leading to changes in substrate phosphorylation as just mentioned.

In keeping with the notion that increased $p34^{cdc2}$ expression is linked to changes in cell shape, it was found that culture of the E2F1d87 cells in low-serum-containing media caused them to adopt the morphology and cytoskeletal structure of normal fibroblasts [Logan et al., 1994]. Under these culture conditions, it was shown here that the levels of $p34^{cdc2}$ protein and activity, as well as transcription from the $p34^{cdc2}$ promoter, approach that of the control cells. Thus, two signals appear to be needed for the elevation of $p34^{cdc2}$, ectopic E2F1d87 expression and serum growth factor stimulation.

That the E2F1d87 protein has a profound effect on cellular physiology is consistent with the fact that the amino terminus of E2F1 is the site of cyclin A/cdk2 association [Krek et al., 1994; Xu et al., 1994]. Binding of cyclin A/cdk2 results in the phosphorylation of both E2F1 and DP1, the heterodimer partner of E2F1. These phosphorylations affect the binding of the E2F1:DP1 heterodimer to DNA [Krek et al., 1994; Xu et al., 1994]. Importantly, E2F1d87 lacks the cyclin A/cdk2 binding domain and is therefore not under regulation by cyclin A/cdk2. In keeping with this fact, the cells expressing E2F1d87 have a lengthened S phase [Logan et al., 1995a], presumably in part because E2F1d87 remains bound to DNA and induces a partial arrest at the S phase checkpoint [Krek et al., 1995]. The mechanism by which E2F1d87, in conjunction with serum stimulation, leads to increased expression of $p34^{cdc2}$ is not known at this time, although attempts are being made to better understand this regulation.

Thus, the data presented here show that the amino terminus of E2F1 provides a regulatory domain for the protein. A mutant E2F1 lacking this domain is able to significantly upregulate $p34^{cdc2}$ gene expression, in conjunction with serum growth factor stimulation. This is in keeping with the fact that E2F1 is known to transactivate $p34^{cdc2}$ [DeGregori et al., 1995]. However, it extends their findings by showing that, at low level of expression from the retroviral vector, the mutant E2F1d87 has a much more profound affect on transactivation than wild-type E2F1.

ACKNOWLEDGMENTS

This work was supported in part by grants from the NIH to D.J.H. (CA51170 and CA67032) and from the Lucille P. Markey Charitable Trust. K.L.J. and T.J.L. were supported by the Foerderer Foundation.

REFERENCES

Atherton-Fessler S, Liu F, Gabrielli B, Lee MS, Peng CY, Piwnica-Worms H (1994): Cell cycle regulation of the p34cdc2 inhibitory kinases. Mol Biol Cell 5:989–1001.

Cao L, Faha B, Dembski M, Tsai LH, Harlow E, Dyson N (1992): Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature 355:176–179.

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR (1991): The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053–1061.

Cress WD, Johnson DG, Nevins JR (1993): A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol Cell Biol 13:6314–6325.

Dalton S (1992): Cell cycle regulation of the human cdc2 gene. EMBO J 11:1797–1804.

DeGregori J, Kowalik T, Nevins JR (1995): Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol Cell Biol 15:4215–4224.

Draetta G, Piwnica-Worms H, Morrison D, Druker B, Roberts T, Beach D (1988): Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. Nature 336:738–744.

D'Urso G, Marraccino RL, Marshak DR, Roberts JM (1990): Cell cycle control of DNA replication by a homologue from human cells of the p34cdc2 protein kinase. Science 250: 786–791.

Flemington EK, Speck SH, Kaelin WG (1993): E2F1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci U S A 90:6914–6918.

Furukawa Y, Piwnica-Worms H, Ernst TJ, Kanakura Y, Griffin JD (1990): cdc 2 gene expression at the G1 to S transition in human T lymphocytes. Science 250:805–808.

Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, DeCaprio JA, Lawrence JB, Livingston DM (1994): E2F4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev 8:2665–2679.

Girling R, Partridge JF, Bandara LR, Burden N, Totty NF, Hsuan JJ, La Thangue NB (1993): A new component of the transcription factor DRTF1/E2F. Nature 362:83–87.
Helin K, Lees JA, Vidal M, Dyson N, Harlow E, Fattaey A (1992): A cDNA encoding a pRb-binding protein with properties of the transcription factor E2F. Cell 70:337–350.
Helin K, Harlow E, Fattaey A (1993): Inhibition of E2F1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 13:6501–6508.
Huber HE, Edwards G, Goodhart PJ, Patrick DR, Huang PS, Ivey-Hoyle M, Barnett SF, Oliff A, Heimbrook DC (1993): Transcriptiona factor E2F binds DNA as a heterodimer. Proc Natl Acad Sci U S A 90:3525–3529.
Ivey-Hoyle M, Conroy R, Huber HE, Goodhart PJ, Oliff A, Heimbrook DC (1993): Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol Cell Biol 13:7802–7812.
Johnson DG, Schwarz JK, Cress WD, Nevins JR (1993): Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature 365:349–352.
Jordan KL, Haas AR, Logan RJ, Hall DJ (1994): Detailed analysis of the basic domain of the E2F1 transcription factor indicates that it is unique among bHLH proteins. Oncogene 9:1177–1185.
Kaelin WG, Krek W, Sellers WR, DeCaprio JA, Ajchenbaum F, Fuchs CS, Chittenden T, Li Y, Farnham PJ, Blanar MA, Livingston DM, Flemington EK (1992): Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell 70:351–364.
Krek W, Ewen ME, Shirodkar S, Arany Z, Kaelin WG, Livingston DM (1994): Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A–dependent protein kinase. Cell 78:161–172.
Krek W, Xu G, Livingston DM (1995): Cyclin A–kinase regulation of E2F1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83:1149–1158.
Ku EH, Wen SC, Engelhard A, Nicolaides NC, Lipson KE, Marino TA, Calabretta B (1993): c-myb transactivates cdc2 expression via myb binding sites in the 5′-flanking region of the human cdc2 gene. J Biol Chem 268:2255–2259.
Lamb NJ, Fernandez A, Watrin A, Labbe JC, Cavadore JC (1990): Microinjection of p34cdc2 kinase induces marked changes in cell shape, cytoskeletal organization, and chromatin structure in mammalian fibroblasts. Cell 60: 151–165.
Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N, Helin K (1993): The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13:7813–7825.
Logan TJ, Jordan KL, Hall DJ (1994): Altered shape and cell cycle characteristics of fibroblasts expressing the E2F1 transcription factor. Mol Biol Cell 5:667–678.
Logan TJ, Evans DL, Mercer WE, Bjornsti MA, Hall DJ (1995a): Expression of a deletion mutant of the E2F1 transcription factor in fibroblasts lengthens S phase and increases sensitivity to S phase specific toxins. Cancer Res 55:2883–2891.
Logan TJ, Jordan KL, Hall DJ (1995b): Constitutive expression of the E2F-1 transcription factor in fibroblasts alters G0 and S phase transit following serum stimulation. Biochem Cell Biol 74:21–28.
Marraccino RL, Firpo EJ, and Roberts JM (1992): Activation of the p34 cdc2 protein kinase at the start of S phase in the human cell cycle. Mol Biol Cell 3:389–401.
Mercer WE, Ullrich SJ, Shields M, Lin D, Alder H (1992): Cell cycle effects of microinjected antisense oligonucleotides to p34cdc2 kinase. Ann N Y Acad Sci 660:209–218.
Moberg KH, Tyndall WA, Pyrc J, Hall DJ (1991): Analysis of the c-myc P2 promoter. J Cell Physiol 148:75–84.
Mudryj M, Hiebert SW, Nevins JR (1990): A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. EMBO J 9:2179–2184.
Mudryj M, Devoto SH, Hiebert SW, Hunter T, Pines J, Nevins JR (1991): Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell 65:1243–1253.
Nevins JR (1992): E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. Science 258: 424–429.
Norbury C, Nurse P (1992): Animal cell cycles and their control. Annu Rev Biochem 61:441–470.
Sala A, Nicolaides NC, Engelhard A, Bellon T, Lawe DC, Arnold A, Grana X, Giordano A, Calabretta B (1994): Correlation between E2F1 and requirement in the S phase and E2F1 transactivation of cell cycle–related genes in human cells. Cancer Res 54:1402–1406.
Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A, Weinberg RA (1995): E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc Natl Acad Sci U S A 92:2403–2407.
Schwarz JK, Devoto SH, Smith EJ, Chellappan SP, Jakoi L, Nevins JR (1993): Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J 12:1013–1020.
Shirodkar S, Ewen M, DeCaprio JA, Morgan J, Livingston DM, Chittenden T (1992): The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle–regulated manner. Cell 68:157–166.
Welch PJ, Wang JEJ (1992): Coordinated synthesis and degradation of cdc2 in the mammalian cell cycle. Proc Natl Acad Sci U S A 89:3093–3097.
Xu M, Sheppard KA, Peng CY, Yee AS, Piwnica-Worms H (1994): Cyclin A/cdk2 binds directly to E2F1 and inhibits the DNA-binding activity of E2F1/DP1 by phosphorylation. Mol Cell Biol 14:8420–8431.