Altered Shape and Cell Cycle Characteristics of Fibroblasts Expressing the E2F1 Transcription Factor

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To gain an understanding of the role the E2F1 transcription factor plays in cell physiology, the full length protein (E2F1) and an amino terminal deletion of 87 amino acids (E2F1d87) were constitutively expressed in NIH3T3 fibroblasts. Multiple cell lines were generated for each construct. These cells do not proliferate in media containing low serum and do not proliferate in soft agar, indicating that they are likely not transformed. However, both sets of cell lines show increased DNA synthesis and increased numbers of cells in S phase when cultured in media containing low serum, compared to the control cell lines. Cells expressing E2F1d87 (but not E2F1) have an extremely rounded morphology when cultured in 10% serum-containing media. These rounded cells lack detectable microfilaments, microtubules, and focal contacts. However, when these cells are cultured in low serum-containing media (0.5%), they attain the flattened morphology and cytoskeletal structure of normal NIH3T3 cells.

INTRODUCTION

The E2F transcription factor binds to elements within the promoters of a number of cellular genes in higher eukaryotic cells. The products of these genes play a very important role in controlling cell proliferation and include the *N-myc*, *c-myc*, and *c-myb* proto-oncogenes, DNA polymerase α , dihydrofolate reductase, thymidine kinase, *cdc2*, and the retinoblastoma susceptibility gene (Nevins, 1992; Farnham *et al.*, 1993; Hamel *et al.*, 1992). Evidence suggests that E2F regulates the transcription of these genes in part through its interaction with the retinoblastoma gene product (pRb) and with the pRb-related protein p107. The latter complex also includes cyclin A and cdk2 (Chellapan *et al.*, 1991; Mudryj *et al.*, 1991; Cao *et al.*, 1992; DeVoto *et al.*, 1992; Shirodkar *et al.*, 1992; Schwarz *et al.*, 1993). The E2F transcription factor appears to be the focal point of a diverse group of factors whose function is to control cell proliferation (Nevins, 1992). Therefore, to understand how normal cell proliferation is controlled, it is essential that the E2F factor be understood in detail.

E2F is recognized to be a multiprotein family (Girling *et al.*, 1993; Huber *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993). To date four members of the family have been cloned and are termed E2F1, E2F2, E2F3, and DP1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Girling *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993).

These genes produce proteins that bind an identical DNA sequence yet the proteins are distinct. E2F1 was the first clone to be isolated and contains a basic-helix-loop-helix (bHLH) domain with an adjacent leucine zipper (LZip) that is required for DNA binding (Kaelin *et al.*, 1992). However, the structure of the basic domain of E2F1 is unique among bHLH-containing proteins in that the center of the domain contains a turn instead of an α -helix (Jordan *et al.*, 1994). The transcriptional activation domain of E2F1 resides at the very carboxy terminus of the protein (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Flemington *et al.*, 1993). Interestingly, pRb binds E2F1 over its transcriptional activation domain, preventing the protein from activating transcription (Kaelin *et al.*, 1992; Helin *et al.*, 1992, 1993; Flemington *et al.*, 1993). E2F1 has been shown to activate transcription from the dihydrofolate reductase, thymidine kinase, and retinoblastoma promoters, indicating that the protein is a positive acting transcription factor for these genes (Johnson *et al.*, 1993; Slansky *et al.*, 1993; Shan *et al.*, 1994).

To gain a better understanding of the effect altered expression of E2F1 has on the cell, the full-length protein and an amino terminal deletion were constitutively expressed in NIH3T3 fibroblasts using a retroviral expression vector. Expression of both forms of the gene caused increased entry into S phase when cells were

serum-starved, yet the cells did not proliferate. Expression of the deletion mutant E2F1d87, but not the full length protein, also caused dramatic changes in cell shape when cells were cultured in 10% serum-containing media or in low serum media supplemented with platelet-derived growth factor (PDGF), yet the change in shape was reversible when cells were cultured only in low serum. A common mechanism regulating both cell shape and transit through the cell cycle will be discussed.

MATERIALS AND METHODS

Cell Culture and Generation of Stable Cell Lines

NIH3T3 fibroblasts (American Type Culture Collection, Rockville, MD) and ψ 2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS). All transfection experiments were initiated on 50% confluent monolayer cultures as in Ausubel *et al.* (1987). The cells were glycerol shocked 5–6 h after DNA addition. Plasmids (30 μ g) were transfected by the calcium phosphate procedure (Ausubel *et al.*, 1987). The E2F1 and E2F1d87 cDNA constructs were cloned into the *Eco*RI site of the pMV7 retroviral vector. When transfected into ψ 2 cells, this plasmid is converted to infectious retrovirus. The retrovirus also contains the neomycin resistance gene driven from the thymidine kinase promoter. This results in resistance to the drug G418 in retroviral-infected cells.

An epitope tag was linked in frame to the amino terminus of the protein encoded by the E2F1 or E2F1d87 cDNAs. A monoclonal antibody (M2, Kodak/IBI, Rochester, NY) directed toward this epitope was used in the electrophoretic mobility shift assays (EMSAs), immunoprecipitation, and immunoblotting experiments.

Generation of Extracts and EMSAs

Nuclear extracts for EMSAs and for Western blots were generated by lysing the cells on ice in 0.1% NP-40, 10 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.9, 10 mM $MgCl_2$, 15 mM NaCl, and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (0.5 mM), pepstatin (2 μ g/ml), and leupeptin (1 μ g/ml). The nuclei were pelleted by centrifugation at $800 \times g$ for 10 min. The nuclei were resuspended in extraction buffer consisting of 0.42 M NaCl, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9, 20% glycerol, PMSF (0.5 mM), pepstatin (2 μ g/ml), and leupeptin (1 μ g/ml) for 10 min on ice and then centrifuged at $14\,000 \times g$ for 8 min to pellet the residual nuclear material. This supernatant fraction was termed nuclear extract. For EMSA and western blots, the nuclear extract was diluted with extraction buffer minus NaCl to lower the salt concentration to ~ 0.1 M.

EMSAs were performed essentially as described by Jordan *et al.* (1994). Briefly, 0.5 ng of a ^{32}P end-labeled, double-stranded oligonucleotide was incubated with nuclear extracts in the presence of 1 μ g of sheared salmon sperm DNA as a nonspecific competitor. The final buffer conditions for protein binding to the radiolabeled DNA were as follows: 16 mM HEPES pH 7.9, 16% glycerol, 80 mM KCl, 0.16 mM EDTA, 0.4 mM dithiothreitol, 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 used in the gel-shift assays is:

AAATCGCTTGGCGGGAAAC

CGGAACCGCCCTTTGTAA

This site reflects the E2F site in the c-myc P2 promoter, mapped to position -64 relative to P2 (Hall, 1990). It has been demonstrated

that E2F1 binds this site within the c-myc promoter (Jordan *et al.*, 1994).

Western Blot Hybridizations

Extracts were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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% BSA in TBST for 30 min at room temperature, and then incubated with the M2 anti-FLAG monoclonal antibody (10 ng/ml). The blot was incubated with a 1:7500 dilution of secondary antibody (goat anti-mouse, 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).

Immunofluorescence

For indirect immunofluorescence, cells were plated on 10-cm tissue culture dishes containing glass coverslips. The cells were washed once in phosphate-buffered saline (PBS), and the cells fixed with 4% paraformaldehyde in PBS for 20 min followed by an additional rinse in PBS. To permeabilize the cells, the coverslips were then treated with PBS plus 0.2% Triton X-100 for 15 min followed by three 5-min washes in PBS plus 0.2% gelatin. The anti- β -tubulin monoclonal antibody (Sigma) or phalloidin-TRITC (fluorescent conjugate) (Sigma) were diluted in PBS plus 0.2% gelatin. Fifty microliters of diluted antibody was added to a dish, and coverslips containing the fixed and permeabilized cells were placed cell side down on the drop of diluted antibody and incubated for 1.5 h at 37°C. The coverslips were washed three times in PBS plus 0.2% gelatin. Fluorescein-conjugated anti-mouse IgG (goat, Vector Laboratories) was diluted to 30 μ g/ml in PBS plus 0.2% gelatin. Again 50 μ l of diluted antibody were placed in a dish, and the coverslips were placed cell side down and incubated for 30 min at 37°C. The coverslips were washed in PBS plus 0.2% gelatin (10'), PBS plus 0.2% gelatin plus 0.05% Tween 20 (10'), and finally in PBS (10'). The coverslips were rinsed in deionized water, dried, mounted, and analyzed by fluorescence microscopy.

Cell-Cycle Analysis/ 3H -Thymidine Incorporation and Antisense Experiments

Cells were rinsed once in chilled PBS then trypsinized and resuspended in 10 ml DMEM plus 10% serum. The cells were pelleted and resuspended in 70% ethanol. The cells were kept on ice for 10 min, pelleted, and then treated with RNAase A (180 μ g) for 30 min at room temperature. Propidium iodide (Sigma) was added to a final concentration of 75 μ g/ml. Cell-cycle analysis was performed on a Coulter Profile 2, Flow Cytometer (Hialeah, FL).

For thymidine incorporation, cells were labeled for 2 h with 5 μ Ci/ml of 3H -thymidine at 20 Ci/mMol. The cells were washed once in PBS, trypsinized, and resuspended in 10% serum-containing DMEM; viable cells counted. Incorporation of 3H -thymidine into acid insoluble material was then assayed as in Hall *et al.* (1989).

E2F1d87 cells grown to low density in 10% serum were treated with unmodified sense or anti-sense oligodeoxynucleotides (80 μ M final concentration) for 4 d. The sequences used are as follows:

sense ATG-GAC-TAC-AAG-GAC-GAC

antisense GTC-GTC-CTT-GTA-GTC-CAT

The oligodeoxynucleotides reflect the sequence at the amino-terminus of E2F1 and E2F1d87 and correspond to the epitope recognized by the M2 antibody.

RESULTS

Generation of E2F1 Overexpressing Cell Lines

To determine the effect E2F1 expression has on cell physiology, it was ectopically expressed in nontransformed

NIH3T3 fibroblasts using the retroviral expression vector pMV7 (containing the neomycin resistance gene). Two human E2F1 gene constructs were used for expression in these cells (Figure 1A). Both had an epitope tag engineered into the amino terminus. One construct was the full length cDNA (termed E2F1) and the other was deleted for the first 87 amino acids (termed E2F1d87). E2F1d87 still contains the DNA-binding domain and the transcription activation domain (Helin *et al.*, 1992, 1993; Kaelin *et al.*, 1992; Flemington *et al.*, 1993). The amino terminus has been shown to have no effect on transcriptional activation when used as part of a GAL4 fusion protein in yeast (Helin *et al.*, 1992) or when the E2F1 protein alone is used to activate transcription from an artificial promoter containing multiple E2F1 sites (Cress *et al.*, 1993). Yet, the amino terminus is a proline- and alanine-rich region, which is similar to the regulatory domains in some transcription

factors (Mermod *et al.*, 1989; Licht *et al.*, 1990; Pyrc *et al.*, 1992; Han and Manley, 1993). It is possible that this region has a specific function but only in context of a specific target promoter.

The epitope tag at the amino terminus allowed identification of the ectopically expressed protein, using a monoclonal antibody (M2, Kodak/IBI) that specifically recognizes the epitope. To generate cell lines in which either E2F1 or E2F1d87 is overexpressed, the cDNA was cloned into the pMV7 retroviral expression vector and transfected into ψ 2 cells; the produced infectious retrovirus was used to infect nontransformed NIH3T3 fibroblasts. The infected cells were grown in the neomycin analogue G418, and the resulting colonies were isolated and expanded.

Cell lines were assayed for expression of the full length E2F1 protein. Five out of twelve expressed E2F1,

Figure 1. Western blot analysis of E2F1- and E2F1d87-overexpressing cell lines. (A) Diagram of the E2F1 constructs used. The E2F1 gene is shown alone with the various domains. NLS, nuclear localization sequence; bHLH, basic helix-loop-helix domain; LZip, the lucine zipper; Pro/Ala-rich. The proline- and alanine-rich amino terminus and the pRb- and p107-binding domain at the carboxy terminus are shown. An epitope tag (FLAG epitope, Kodak/IBI) was engineered into the amino terminus of both the E2F1 and E2F1d87 expression constructs. E2F1d87 refers to the construct in which the amino terminal 87 amino acids are deleted. These E2F1 constructs were cloned into the pMV7 retroviral vector. (B) Western of E2F1-overexpressing cell lines. NIH3T3 fibroblasts infected with the E2F1-expressing retrovirus were grown in G418; colonies were selected and screened for E2F1 expression. A Western blot of nuclear extracts from two expressing cell lines (#3 and #4) is shown using the M2 monoclonal antibody as a probe. The control cells were infected with the pMV7 virus alone. In vitro-transcribed and -translated E2F1 was electrophoresed on the same gel and blotted. The blot was exposed to film and shown as an alignment of the labeled E2F1 with the E2F1 protein produced by the two cell lines. No RNA refers to the control where no RNA was used in the in vitro transcription translation reaction. The position of ectopically expressed E2F1 is shown. (C) Western of E2F1d87-overexpressing cell lines. NIH3T3 fibroblasts infected with the E2F1d87-expressing retrovirus were grown in G418; colonies were selected and screened for E2F1d87 expression. A Western blot of nuclear extracts from three expressing cell lines (#1, #2, and #8) is shown, using the M2 monoclonal antibody as a probe. The control cells were infected with the pMV7 virus alone. In vitro-transcribed and -translated E2F1d87 was electrophoresed on the same gel and blotted. The blot was exposed to film and shown as an alignment of the labeled E2F1d87 with the E2F1d87 protein produced by the three cell lines. No RNA refers to the control where no RNA was used in the in vitro transcription translation reaction. The position of ectopically expressed E2F1d87 is shown.

and two of these expressing cell lines (#3 and #4) were chosen for subsequent analysis. Figure 1B shows the Western of nuclear extracts of these two cell lines along with *in vitro*-transcribed and -translated E2F1. It is clear that ectopically expressed E2F1 is produced in these cells, resulting in a novel band of ~60 kDa recognized by the M2 antibody that migrates along with *in vitro*-transcribed and -translated E2F1 in SDS-PAGE.

When cell lines were assayed for E2F1d87 expression, 11 out of 19 showed expression. Three of these expressing cell lines were chosen for further analysis (#1, #2, and #8). When nuclear extracts from these cell lines were assayed by Western blot with the M2 antibody, a band of ~50 kDa was seen that was not present in the extracts from the control cells (Figure 1C). This band corresponds to the size of E2F1d87 because of the fact that it migrates with the *in vitro*-transcribed and -translated E2F1d87 in the SDS-PAGE (Figure 1C). Also seen in the Western blot of Figure 1C are additional higher molecular weight bands, in lanes 4, 5, and 6, that are recognized by the M2 antibody. These may be modified forms of E2F1d87. We have found that E2F1d87 can be efficiently phosphorylated *in vitro* at a number of sites throughout the protein (Jordan and Hall, unpublished data).

To determine if the ectopically produced E2F1 and E2F1d87 were able to bind DNA, electrophoretic mobility shift assays were performed using a radiolabeled element representing the E2F1 site present in the *c-myc* promoter (Jordan *et al.*, 1994). Figure 2 shows an EMSA with nuclear extracts generated from control cells and the E2F1 and E2F1d87 cell lines. In all cases, extracts were made from growing cells. In the lane containing the control extracts, two bands are evident. The lower is E2F alone, whereas the upper is a multiprotein complex of E2F (Mudryj *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Moberg *et al.*, 1992). It is evident that extracts from the E2F1 cell lines produce a predominant single band, whereas extracts from the E2F1d87 cell lines produce two bands. The two bands in the lanes of the E2F1d87 cell lines migrate faster than the two bands present in the extracts from control cells. This may be expected given that the E2F1 protein is truncated and should therefore migrate faster in the EMSA.

To determine if the binding pattern seen in Figure 2 is because of ectopic E2F1 and E2F1d87 expression, the M2 antibody was included in the binding reactions. As seen in Figure 2, the inclusion of the antibody altered the migration of the binding complexes. Importantly, there was no effect of the antibody on migration of the complexes in the control cells. The fact that the M2 antibody altered the migration indicates that ectopic E2F1 and E2F1d87 is a part of the DNA:protein complex. As an additional step to determine if binding of the ectopically produced proteins to DNA was specific, 100-fold molar excess of unlabeled E2F element was included in the binding reactions (+ Comp). This completely eliminates all binding as seen in Figure 2, indi-

Figure 2. The overexpressing cell lines produce E2F1 and E2F1d87 proteins that are able to bind DNA. Nuclear extracts from E2F1 (#3 and #4) and E2F1d87 (#1, #2, and #8) cell lines were used in the EMSA with extracts from control cells (pMV7 only). A 32 P-labeled E2F element from the *c-myc* promoter was used as a target in the binding reaction. Extracts were made from subconfluent growing cells. (Top) Extracts from control cells (pMV7 only), E2F1 (#3), and E2F1 (#4) cell lines were used in the EMSA either alone, with 2 μ l of the M2 antibody, or with 100-fold molar excess of the unlabeled E2F element as a specific competitor (Comp). The position of the E2F1 complex is shown. The position of the E2F1 complex without and with M2 antibody is also shown. Free probe was in excess but was not shown in the figure. (Bottom) Extracts from control cells (pMV7 only), E2F1d87 (#1), E2F1d87 (#2), and E2F1d87 (#8) cell lines were used in the EMSA either alone, with 2 μ l of the M2 antibody, or with 100-fold molar excess of the unlabeled E2F element as a specific competitor (Comp). The positions of the E2F1d87 complexes without and with M2 antibody are also shown. Free probe was in excess but was not shown in the figure.

cating that binding was specific. As an additional test for specificity, a mutant E2F site (GGCTTGAAA) was used as a competitor in the binding reaction against the wild-type probe (GGCGGGAAA). It did not compete for binding.

Throughout the experiments described below, multiple control cell lines were used (infected with pMV7 only, no cDNA), whereas only one is shown in the figures. In all experiments described below, the data from the control cell lines were always comparable. In addition uninfected NIH3T3 cells were also used as a control to ensure that the cell lines did not become spontaneously transformed.

Growth Characteristics of the E2F1- and E2F1d87-expressing Cell Lines

The cell lines were next analyzed for any alterations in growth characteristics. The cells were plated out at low density (250 000 cells/10 cm plate), and then after attachment to the substratum, the cells were cultured in either high (10%) or low (0.5%, also referred to as serum

starved) bovine calf serum-containing media. Growth was then assayed by viable cell counts on day 7 (fresh media with 10% or 0.5% serum was added to the cells on day 3). As shown in Figure 3A, none of these cell lines, E2F1 or E2F1d87, grow in low serum.

When cell density is monitored at postconfluence (day 11), it is clear that only the E2F1d87 cell lines reach densities four- to sixfold higher than the control cells (Figure 3B), indicating that when cultured in high serum these cells have lost their contact inhibition of growth, yet the control cells and the E2F1 cell lines have not.

Lack of Growth in Soft Agar

The high cell density at postconfluence of the E2F1d87 cell lines suggests that these cells may be transformed because they have lost their contact inhibition. Yet, the lack of growth in low serum suggests that they are not. To determine if these cell lines are transformed, growth was monitored in soft agar. The ability to grow in soft agar demonstrates anchorage independent growth, a characteristic of transformation. None of these cell lines, E2F1 or E2F1d87, were able to grow in soft agar over a period of 9 d, indicating that these cells do not share this feature of oncogenic transformation. As a positive control, C6 cells (glioblastoma) showed significant growth in soft agar over the same time period, generating colonies 0.5–1 mm in diameter.

Increased DNA Synthesis in the E2F1 and E2F1d87 Cells when Grown in Serum-deficient Media

It has been recently demonstrated that microinjection of E2F1 into serum-starved fibroblasts will cause them to leave G0/G1 phase and enter S phase (Johnson *et al.*, 1993). As shown above, cells expressing E2F1 or E2F1d87 do not grow in media containing low serum (cells cultured in 0.5% bovine calf serum for 48 h). Given the data of Johnson *et al.* (1993), it may be expected that these E2F1 and E2F1d87 cell lines may enter S phase when cultured in low serum, even though they don't proliferate. To determine if this was the case, ^3H -thymidine incorporation was assayed in these cells after culture in low serum for 48 h. As can be seen in Figure 4, the E2F1-expressing cell lines show a dramatic increase in the level of ^3H -thymidine incorporation over the control cells. The magnitude of the difference was ~8- to 10-fold. The E2F1d87 cell lines also show an increase in ^3H -thymidine incorporation over the control cells. As shown in Figure 4, the increase is three- to fourfold. In all cases ^3H -thymidine incorporation was normalized for differences in cell number.

To determine if these cells were progressing into S phase, cell-cycle analysis was performed on cells after culture in 0.5% calf serum for 48 h. As can be seen in Figure 5, the E2F1 and E2F1d87 cell lines showed increased numbers of cells in S phase. The level ranges

Figure 3. Growth of the E2F1 and E2F1d87 cell lines in low (0.5%) and high (10%) serum-containing media. (A) Control cells (pMV7 only) the E2F1, and the E2F1d87 cell lines were plated out at 250 000 cells per 10-cm plate. After attachment to the substratum, the cells were cultured in media containing either 10% or 0.5% bovine calf serum. Viable cell numbers were assayed at 7 d after plating. The numbers given are viable cells (1×10^6) $\pm$ SD. The media was changed at day 3. Nontransformed NIH3T3 fibroblasts typically reach about 5×10^6 cells per 10-cm plate by days 4–5. The error bars indicate the SD. (B) Cell numbers were assayed at postconfluence of cells grown in 10% serum and are given as 1×10^6 . Cells were plated out at 250 000 cells per 10-cm plate. Cell numbers were assayed at day 11 of culture. The error bars indicate SD.

Figure 4. ³H-thymidine incorporation is elevated in the E2F1 and E2F1d87 cells cultured in low serum. The control cells (pMV7 only) the E2F1, and the E2F1d87 cell lines were grown to a density of $\sim 4 \times 10^6$ per plate, near confluence, then placed in low serum (0.5%) for 48 h. ³H-thymidine incorporation into insoluble material was then monitored by pulsing the cells for 2 h (5 μ Ci/ml). Cell numbers were assayed just before determining the ³H-thymidine incorporation to ensure that comparisons were made over equal numbers of cells. The error bars indicate SD.

from 2- to 4.5-fold over the control cells depending on the cell line. To determine if these cells were actually traversing the cell cycle in low serum conditions, cells were analyzed for G2/M DNA content. As seen in Table 1, the percentage of cells in G2/M was also increased slightly over that of the control cells, by two- to threefold depending on the cell line. Because it is clear that the cells do not proliferate in low serum media, it is likely that the cells are arrested within the various phases of the cell cycle or move through the cell cycle very slowly.

The cell-cycle distribution of these cell lines during exponential growth was also analyzed. As seen in Table 1, the distribution of G1, S, and G2/M phases in all these growing cells is roughly similar, including the controls. However, the cell-cycle distribution of serum-starved and proliferating E2F1 and E2F1d87 cell lines is distinct. In total, these data indicate that the cells expressing E2F1 or E2F1d87 are able to enter S phase when cultured in low serum but are unable to complete the cell cycle.

Altered Morphology of E2F1d87 Cell Line Is Reversible and Is Dependent on Serum Growth Factors

Morphology is a critical feature of cellular phenotype and can reflect oncogenic transformation. When these

cell lines were analyzed for any changes in morphology, it was apparent that the E2F1-expressing cell lines were indistinguishable from the control cells (Figure 6A), whether grown in the presence of high (10%) or low (0.5%) serum. However, the E2F1d87 cell lines showed a dramatic change in morphology when grown in high serum (Figure 6B). This change was manifested by a round morphology; the E2F1d87 cells completely lack the normal flattened phenotype characteristic of normal nontransformed NIH3T3 fibroblasts. The E2F1d87 expressing cells cover only ~ 10 – 15% of the surface area of the substratum compared to the control or E2F1-expressing cells. However, when the cells were placed in low serum-containing media for 48 h, they reverted to a more flattened phenotype (Figure 6B). This change in morphology was completely reversible in that cells could be shifted back and forth between high (10%) and low (0.5%) serum-containing media, and the cells would round up or flatten out after each change. The minimum time required for the change in morphology to be noticeable, after a change from 10 to 0.5% serum, is 12 h. By 24 h all of the cells have acquired the new morphology.

It is important to point out that while these E2F1d87 cell lines show an extremely rounded phenotype they are nonetheless attached to the substratum; they do not float in suspension (trypsin/EDTA treatment is required to detach them). It is also evident that the round morphology is not simply a function of arrest in mitosis

Figure 5. The percentage cells in S phase is increased in the E2F1 and E2F1d87 cell lines grown in low (0.5%) serum. Cells were cultured as in Figure 3 and were assayed for percentage cells in S phase by flow cytometry. The error bars indicate SD.

Table 1. Distribution of cells in the cell cycle after culture in low (0.5%) or high (10%) serum

	G0/G1 phase	S phase	G2/M phase
Culture in low serum			
E2F1 (#3)	72%	24%	4%
E2F1 (#4)	73	21	6
E2F1d87 (#1)	84	12	4
E2F1d87 (#2)	71	22	7
E2F1d87 (#8)	70	23	7
Control cells (pMV7 only)	93	5	2
Growing cells in high serum			
E2F1 (#3)	48	38	14
E2F1 (#4)	53	30	17
E2F1d87 (#1)	43	43	14
E2F1d87 (#2)	44	39	17
E2F1d87 (#8)	38	50	12
Control cells (pMV7 only)	39	46	15

Subconfluent growing cells (cultured in 10% serum) or confluent cells cultured for 48 hours in 0.5% serum-containing media were processed for cell-cycle analysis by flow cytometry. Given is the percentage of cells in each cell-cycle phase. The SD for the values varied between 5 and 10%.

(cells blocked in mitosis show a rounded morphology). The cell-cycle data from growing cells, presented in Table 1, indicates that the cells expressing E2F1d87 have a similar cell-cycle distribution as the control or E2F1-expressing cells. Also, to ensure that this is not simply a function of clonal variation, a large number of G418 resistant colonies were examined that were infected with the E2F1d87-expressing retrovirus. Eleven of the colonies that showed expression of E2F1d87 had a round morphology in 10% serum, whereas eight colonies that did not express E2F1d87 had a normal flattened morphology. This strongly suggests that it is the expression of E2F1d87 that affects cell shape and not simply clonal variation.

In an attempt to reverse the round morphology of the E2F1d87 cells, unmodified antisense oligodeoxynucleotides (ODNs) representing the DNA sequence over the translation initiation region, which encompasses the epitope tag, were added to the culture medium (containing 10% serum) of the E2F1d87 (#2) cell line. Over a period of 4 d it was found that the morphology change could be reversed with antisense treatment but not by treatment with sense ODNs (Figure 7). It is important to note that treatment with either sense

Figure 6. Morphology of subconfluent E2F1 and E2F1d87 cell lines grown in low (0.5%) and high (10%) serum. E2F1 cell lines (A), E2F1d87 cell lines (B), and control cells (pMV7 only) were plated out at 250 000 cells per 10-cm plate. After 1–2 d the morphology of the cells grown in 10% serum was monitored by phase contrast microscopy. Cells were also cultured for an additional 48 h in low serum (0.5%) and then monitored by phase contrast microscopy. Shown are the photomicrographs of the phase contrast microscopy. The magnification is 10 $\times$.

or antisense oligonucleotides was not toxic to the cells, as determined by viable cell counts. This data indicates that the morphology change is in part due to ectopic E2F1d87 expression.

It is possible that the reversion to normal morphology by the E2F1d87 cell lines when the serum concentration was lowered was because of a change in ectopic E2F1d87 expression, even though expression is under control of the viral LTR and should therefore be constitutive. To determine if this was the case, a gel-shift assay was performed with extracts from control cells, E2F1 (#3) cells, and E2F1d87 (#2) cells cultured in high (10%) or low (0.5%) serum. E2F binding dropped significantly in the control cell extracts cultured in low serum compared to control cells cultured in 10% serum, 14-fold (Logan and Hall, unpublished observations). This is consistent with the data of Mudryj *et al.* (1990). However, there was no effect of serum concentration on binding in the extracts from the E2F1d87 (#2) or the E2F1 (#3) cell lines. The level of binding was the same in the E2F1d87 and E2F1 cells, whether they were cultured in high or low serum. This indicates that there is no effect of serum concentration on the ectopic expression of the E2F1d87 protein. Taken together with the data from Figure 7, this indicates that serum growth factors and ectopic E2F1d87 both contribute to the change in cell shape.

The effect of serum concentration on the shape of E2F1d87-expressing cell lines is likely because of specific growth factors present in the serum. One of the predominant growth factors for NIH3T3 fibroblasts in serum is PDGF. To determine if PDGF could affect the shape of the E2F1d87 cell line, the cells were cultured in high serum so that they were rounded, and then the media was changed to low serum (0.5%) with the addition of pure PDGF to 5 ng/ml (Table 2). This level of PDGF is sufficient to initiate proliferation. It was found that the cells maintained their rounded morphology in the presence of PDGF. Conversely, if cells were cultured in low serum for 48 h so that they attained a flattened shape and then PDGF alone was added, the cells reverted to a rounded morphology within 24 h. TGF- β addition to cells cultured in 0.5% serum had no effect on morphology. These data indicate that a single growth factor is capable of affecting the morphology change in these cells in conjunction with ectopic expression of E2F1d87.

Lack of Actin Stress Fibers, Microtubules, and Vinculin-containing Focal Contacts in the E2F1d87 Cell Lines

The altered shape seen in the E2F1d87 cell lines likely results from a number of perturbations of the cytoskeleton, the main determinant of cell shape. These perturbations should affect elements of microfilaments, microtubules, and focal contacts. To determine if microfilament structure was altered, the control cells

Figure 7. Antisense oligodeoxynucleotides against E2F1d87 reverse the shape of E2F1d87 cells. Phase contrast microscopy of the E2F1d87 (#2) cell line cultured in 10% serum, at subconfluence, and treated for 4 d with unmodified antisense or sense oligodeoxynucleotides (80 μ M). Also shown are untreated cells. By cell counts there was no toxic effect of these oligodeoxynucleotides on cell viability.

(pMV7 only), E2F1d87 (#2), and E2F1 (#3) cell lines were fixed and stained with phalloidin conjugated with rhodamine. As seen in Figure 8, the control cells and the E2F1-expressing cells demonstrate stress fibers, regardless of the culture conditions. The E2F1d87 cell line does not contain any detectable microfilaments when the cells are grown in 10% serum. When the cells are cultured in 0.5% serum, microfilaments are present, but they appear more disorganized compared to the other cell lines. They appear to lack the ordered array that is characteristic of actin stress fibers, as seen in the control and E2F1 (#3) cell lines.

To determine if microtubules were affected in the same way as microfilaments, control cells, E2F1d87 (#2), and E2F1 (#3) cell lines were fixed and stained with a β -tubulin specific antibody followed by a second antibody conjugated with fluorescein. Figure 9 shows the results of the indirect immunofluorescence. The E2F1d87 cell line contains no detectable microtubules when cultured in 10% serum. However, after culturing

Table 2. PDGF can substitute for 10% serum in causing the morphology change in E2F1d87-expressing cells

Culture conditions	Morphology of E2F1d87 (#2) cells	Morphology of control cells
10% Serum	Round	Flat
0.5% Serum	Flat	Flat
10% Serum	Round	Flat
↓	↓	↓
0.5% Serum + 5 ng/ml PDGF ^a	Round	Flat
0.5% Serum for 96 h	Flat	Flat
↓	↓	↓
5 ng/ml PDGF ^b	Round	Flat

Cells at 250 000/10 cm plate were cultured in 10% serum-containing media for 1 d or 0.5% serum-containing media for 48 h. Cells were examined by phase contrast microscopy and categorized as flat or round if they conformed to the morphology shown in Figures 6, A and B. The E2F1d87 cells in 10% serum therefore appear round, whereas control and E2F1-expressing cells appear flat.

^a Cells were cultured in 10% serum-containing media for 48 h then the media changed to 0.5% serum plus 5 ng/ml purified PDGF.

^b Cells were cultured in 0.5% serum-containing media for 96 h. Then purified PDGF was added to 5 ng/ml.

in 0.5% serum, these cells acquire microtubules that are indistinguishable from the other two cell lines.

Finally, an anti-vinculin specific antibody was used to identify focal contacts, as shown in Figure 10. Cells were processed for indirect immunofluorescence and stained with an α -vinculin specific antibody followed by a second antibody conjugated with fluorescein. The E2F1d87 cell line, when cultured in 10% serum, contains no detectable vinculin-containing focal contacts. However, after culturing in 0.5% serum, these cells acquire

focal contacts that are indistinguishable from the other two cell lines.

DISCUSSION

In the experiments outlined here, full length E2F1 and an amino terminal deletion of E2F1 (E2F1d87) were constitutively expressed in nontransformed NIH3T3 fibroblasts. Multiple cell lines were generated for each construct. These cell lines did not appear to be transformed, because they did not grow in low serum-containing media and they did not grow in soft agar. However, these cell lines did show increased DNA synthesis when cultured in low serum, compared to the control cells. The E2F1 cell lines, as opposed to the E2F1d87 cell lines, show the greatest amount of DNA synthesis over the control cells. By cell-cycle analysis, both the E2F1 and E2F1d87 cell lines had entered S phase when cultured in low serum. This data is consistent with that of Johnson *et al.* (1993), who showed that when E2F1 is transiently transfected or microinjected into serum-starved fibroblasts, DNA synthesis is increased. Because the E2F1 and E2F1d87 cell lines that we have generated do not proliferate when cultured in low serum, it is clear that they do not complete the cell cycle, even though they have entered S phase and have initiated DNA synthesis. The data presented here suggest that expression of E2F1 provides only part of what is needed for completion of S phase and that additional signals from serum growth factors provide the remainder. These signals may affect other members of the E2F family that could dimerize with E2F1. It is now important to 1) determine how far into S phase the E2F1 and E2F1d87 cell lines have progressed when cultured in low serum and 2) identify the components that are needed to allow for completion of S phase.

Figure 8. E2F1d87 cells cultured in high serum lack actin stress fibers. Cells were plated out on glass cover slips. After culture in high (10%) or low (0.5%) serum, the cells were fixed and processed for staining of actin filaments with phalloidin-TRITC. Shown are the photomicrographs of the fluorescent cells: control cells (pMV7 only), E2F1 (#3), and the E2F1d87 (#2) cell lines. The magnification is 40X.

Figure 9. E2F1d87 cells cultured in high serum lack microtubules. Cells were plated out as in Figure 8 on glass cover slips. After culture in high (10%) or low (0.5%) serum, the cells were fixed and processed for staining for β -tubulin with anti-tubulin antibody followed by fluorescein-conjugated second antibody. Shown are the photomicrographs of the stained cells: control cells (pMV7 only), E2F1 (#3), and the E2F1d87 (#2) cell lines. The magnification is 40 $\times$.

An interesting aspect of the present study is that the E2F1d87 cell lines undergo a reversible and significant shape change that depends on ectopic expression of E2F1d87 and on serum growth factors, such as PDGF. This change is reflected by rounding of the cells when they are cultured in 10% serum-containing media and is not because of arrest in mitosis (Table 1). At least one component of serum that can cause this effect is PDGF. This rounding phenomena appears extreme even when compared to the changes in morphology seen during oncogenic transformation (Cooper, 1990). It is known that cell shape and morphology, an aspect of phenotype, is a function of diverse effectors (Ben-Ze'ev, 1991). These include growth factor stimulation of quiescent cells and oncogenic transformation (Bockus and Stiles, 1984; Herman and Pledger, 1985; Cooper, 1990). In addition, overexpression of specific genes in nontransformed cells can cause changes in cell shape. These genes include *cdc2*, *rho*, and *GAP* and like E2F1 are associated with the control of cell proliferation (Lamb *et al.*, 1990; Paterson *et al.*, 1990; McGlade *et al.*, 1993). The data presented here indicate that E2F1 is a member of the family of genes whose products affect cell shape, however, it is a mutant form of E2F1 that affects this change.

The work described here is the first demonstration of a role for the amino-terminus of E2F1 in cell physiology, in that the lack of this domain affects aspects of both growth (i.e., loss of contact inhibition) and morphology. Because this region is proline and alanine rich, it is possible that it plays a role in repressing transcription of specific target genes, as is the case for the proline and alanine rich regions in the *even-skipped* and *Kruppel* transcription factors (Licht *et al.*, 1990; Han and Manley, 1993). Because E2F1d87 has the components of a tran-

scription factor, in that it contains DNA binding and transcription activation domains, it should be possible to use these cell lines to determine which genes it regulates and what role the amino terminus plays in this regulation.

When normal NIH3T3 cells are made quiescent by serum starvation and then stimulated with PDGF, changes can be seen in components of the cytoskeleton (Bockus and Stiles, 1984; Herman and Pledger, 1985), i.e. rearrangements of actin microfilaments. It is possible that overexpression of E2F1d87 causes the cells to be acutely sensitive to growth factor action because of elevation of critical signaling components. Therefore, the changes in shape due to cytoskeletal reorganization that normally take place upon PDGF stimulation are now amplified many fold in these E2F1d87 cell lines, resulting in lack of the structural components needed for spreading on the substratum. This lack of spreading is reflected by a loss of actin stress fibers, microtubules, and vinculin-containing focal contacts. Potential downstream targets of E2F1d87 that would affect changes in cell shape may include the genes for cytoskeletal regulatory proteins such as gelsolin (Vanderkerhove *et al.*, 1990) or *rho* (Paterson *et al.*, 1990). At present it is not known whether there are E2F sites in the promoters of these cytoskeletal regulatory genes.

Comparison of the E2F1 and E2F1d87 cell lines indicate that they have distinct phenotypes. In addition to the effect on cell shape by E2F1d87, there is a loss of contact inhibition of growth. These E2F1d87 cell lines therefore appear to bridge the gap between normal growth and oncogenic transformation, in that the cells have lost contact inhibition of growth when cultured in high serum and they show increased DNA synthesis in low serum (both aspects of transformation). Yet, they

Figure 10. E2F1d87 cells cultured in high serum lack focal contacts. Cells were plated out as in Figure 8 on glass cover slips. After culture in high (10%) or low (0.5%) serum, the cells were fixed and processed for staining for focal contacts with anti-vinculin antibody followed by fluorescein-conjugated second antibody. Shown are the photomicrographs of the stained cells: control cells (pMV7 only), E2F1 (#3), and the E2F1d87 (#2) cell lines. The magnification is 100 $\times$.

do not proliferate in low serum or in soft agar (aspects of normal growth).

In conclusion, the cell lines generated here should provide a resource for studying the effect of altered E2F1 expression (mutants and wild-type) on the cell cycle and on cellular morphology. The fact that a mutant E2F1 produces such a profound change in cell shape indicates that this transcription factor may target genes that regulate the cytoskeleton. The analysis of this factor should lead to a better understanding of the mechanisms controlling both cell proliferation and cell shape, two aspects of cellular physiology known to be linked.

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