Subcellular Compartmentalization of E2F Family Members Is Required for Maintenance of the Postmitotic State in Terminally Differentiated Muscle

R. Montgomery Gill and Paul A. Hamel

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Abstract. Maintenance of cells in a quiescent state after terminal differentiation occurs through a number of mechanisms that regulate the activity of the E2F family of transcription factors. We report here that changes in the subcellular compartmentalization of the E2F family proteins are required to prevent nuclei in terminally differentiated skeletal muscle from reentering S phase. In terminally differentiated L6 myotubes, E2F-1, E2F-3, and E2F-5 were primarily cytoplasmic, E2F-2 was nuclear, whereas E2F-4 became partitioned between both compartments. In these same cells, pRB family members, pRB, p107, and p130 were also nuclear. This compartmentalization of the E2F-1 and E2F-4 in differentiated muscle cells grown in vitro reflected their observed subcellular location in situ. We determined further that exogenous E2F-1 or E2F-4 expressed in myotubes at levels fourfold greater than endogenous proteins compartmentalized identically to their endogenous counterparts. Only when overexpressed at higher levels was inappropriate subcellular location for these proteins observed. At these levels, induction of the E2F-regulated genes, cyclins A and E, and suppression of factors associated with myogenesis, myogenin, and $p21^{Cip1}$ was observed. Only at these levels of E2F expression did nuclei in these terminally differentiated cells enter S phase. These data demonstrate that regulation of the subcellular compartmentalization of E2F-family members is required to maintain nuclei in a quiescent state in terminally differentiated cells.

Key words: E2F • muscle differentiation • cytoplasm • nucleus • cell cycle

Introduction

The transition from G_0 and G_1 into S phase is determined by a series of factors that regulate the activity of a family of transcription factors known collectively as E2F (for review see Dyson, 1998). The E2F family proteins consist of six members: E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and E2F-6. All but the latter have been shown to regulate the expression of genes involved in cell cycle progression, including cyclins A and E (DeGregori et al., 1995; Botz et al., 1996), *E2F-1* itself (Neuman et al., 1994; Smith et al., 1996), *DHFR* (Slansky et al., 1993; DeGregori et al., 1995), *cdc2* (DeGregori et al., 1995; Shimizu et al., 1995), thymidine kinase (Karlseder et al., 1996), *c-myc* (Hiebert et al., 1989, 1992; Thalmeier et al., 1989; Mudryj et al., 1990; Hamel et al., 1992), *HsOrc1* (Ohtani et al., 1996), *cdc6* (Leone et al., 1998; Ohtani et al., 1998), *pRB* (Shan et al., 1994), and p107 (Zhu et al., 1995b).

The pRB family proteins, pRB, p107, and p130, interact directly with and regulate the activity of the E2F family of transcription factors (Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991; Defeo-Jones et al., 1991; Mudryj et al., 1991; Cao et al., 1992; Cobrinik et al., 1993; Cress et al., 1993; Helin et al., 1993; Lees et al., 1993; Beijersbergen et al., 1994; Dynlacht et al., 1994; Ginsberg et al., 1994; Jiang et al., 1995; Smith and Nevins, 1995; Welch and Wang, 1995; Wolf et al., 1995; Zhu et al., 1995a). These interactions and their resultant growth-suppressive effects are antagonized by cyclin-dependent kinase-mediated phosphorylation of the pRB family proteins (Chellappan et al., 1991; Helin et al., 1992; Hinds et al., 1992; Kaelin et al., 1992; Beijersbergen et al., 1995; Suzuki-Takahashi et al., 1995; Mayol et al., 1996; Xiao et al., 1996). In addition to pRB family binding, regulation of E2F activity occurs by cell cycle–dependent fluctuation of pRB and E2F levels (Moberg et al., 1996; Sardet et al., 1995; Shan et al., 1994). Differences in the levels of these two cell cycle regulatory families of proteins, as well as the cyclins, are even more profound during embryogenesis, where tissue-specific expression of distinct members of these families has been documented (Aguzzi et al., 1996; Dagnino et al., 1997a,b; Jiang et al., 1997). This specificity is reflected by the tissue-specific effects of mice har-

Address correspondence to Paul A. Hamel, Department of Laboratory Medicine and Pathobiology, 6318 Medical Sciences Bldg., 1 King's College Circle, Toronto, Ontario, M5S 1A8. Tel.: (416) 978-8741. Fax: (416) 978-5959. E-mail: paul.hamel@utoronto.ca

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boring germline deletions of pRB, E2F, or D-cyclin family members (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Fantl et al., 1995; Sicinski et al., 1995, 1996; Cobrinik et al., 1996; Field et al., 1996; LeCouter et al., 1998a,b; Lindeman et al., 1998). The distinct expression patterns for these cell cycle regulatory proteins are reiterated in a variety of stem cells stimulated to differentiate in cell culture (Slack et al., 1993; Dobashi et al., 1995; Kiess et al., 1995; Kranenburg et al., 1995; Rao and Kohtz, 1995; Shin et al., 1995; Wang et al., 1995; LeCouter et al., 1996; Della et al., 1997; Gill et al., 1998). During neuronal differentiation of murine P19 embryonal carcinoma cells, for example, E2F-3 and E2F-4 levels decrease, E2F-2 levels remain constant, and E2F-1 is induced (Gill et al., 1994). Simultaneously, both pRB and p130 levels increase, as do the levels of their active hypophosphorylated forms. These changes in the pRB family protein activity are required for the strong reduction in free E2F transcription complexes (La Thangue and Rigby, 1987; La Thangue et al., 1990; Shivji and La Thangue, 1991; Corbeil et al., 1995; Gill et al., 1998), also supported in studies demonstrating that inactivation of pRB family proteins by viral oncoproteins inhibits terminal differentiation (Caruso et al., 1993; Bishopric et al., 1997; Slack et al., 1995). Analogous to neuronal differentiation, changes in the expression pattern and activity of these cell cycle regulatory proteins are required for differentiation of myoblasts into multinucleated myotubes (Corbeil et al., 1995; Kiess et al., 1995; Shin et al., 1995). Forced expression of E2F-1 before induction of differentiation inhibits formation of myotubes (Neuman et al., 1995; Wang et al., 1995; Guy et al., 1996; Strom et al., 1998) as does inactivation of pRB, due to mutation, loss of expression, or functional inactivation by viral oncoproteins (Weigel and Nevins, 1990; Weigel et al., 1990; Braun et al., 1992; Haider et al., 1994; Slack et al., 1995; Tedesco et al., 1995; Novitch et al., 1996; Tiainen et al., 1996; Zacksenhaus et al., 1996; Kobayashi et al., 1998).

Although the levels and phosphorylation state of both the E2F and pRB families fluctuate in a cell cycle and tissue-specific manner, additional mechanisms also regulate E2F transcriptional activity. Tissue culture fibroblasts synchronized by serum depletion showed cell cycle–dependent translocation of E2F-4, pRB, p107, and p130 (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). E2F-4 and E2F-5 are primarily cytoplasmic in cycling cells, due apparently to the lack of an NH_2-terminal nuclear localization signal (NLS),[1] this signal being present in E2F-1, E2F-2, and E2F-3 (Lindeman et al., 1997). However, E2F-4 can be shifted to the nucleus by coexpression of binding factors that encode NLS such as an alternatively spliced version of the heterodimerization partner, DP-3 (de la Luna et al., 1996; Puri et al., 1998), as well as p107 or p130 (Lindeman et al., 1997; Puri et al., 1998). The presence of E2F-1 or E2F-4 in the nucleus of undifferentiated myoblasts blocks cell cycle exit and promotes progression to S phase despite removal of mitogenic signals (Puri et al., 1998).

Previous studies have demonstrated that active E2F blocks the transition out of the cell cycle as cells terminally differentiate. We wished to determine whether sequestration of E2F family proteins in the cytoplasm was required to maintain cells in a quiescent state after terminal differentiation. Thus, we characterized the subcellular localization of E2F and pRB family members in terminally differentiated myotubes in cells grown in cell culture and in whole tissue. Our results demonstrate the distinct compartmentalization of these factors in terminally differentiated cells. Furthermore, significant increases in nuclear localization of either E2F-1 or E2F-4 causes the nuclei in terminally differentiated myotubes to enter S phase. This inappropriate S phase entry is mediated in part by altered expression of cell cycle regulatory factors, suggesting that terminally differentiated myotube nuclei are competent to respond to an E2F-mediated proliferation signal and that cytoplasmic compartmentalization of E2F is an important mechanism for maintaining the postmitotic state of these nuclei.

Materials and Methods

Antibodies

The anti-p107 (SC-318), anti-p130 (SC-317), anti–E2F-1 (SC-193), anti–E2F-2 (SC-633), anti–E2F-3 (SC-878), anti–E2F-4 (SC-866), anti–E2F-5 (SC-999), anti–cyclin E (SC-481), anti–cyclin A (SC-596), anti-cdk2 (SC-163), and anti-cdk4 (SC-749) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. The anti-pRB (14001A), anti-$p21^{Cip1}$ (65951A), anti-myogenin (65121A) mAbs were purchased from PharMingen. Anti-bromodeoxyuridine (BrdU) mAb was purchased as part of the BrdU labeling and detection kit I (Roche Diagnostics). Anti-hemagglutinin (HA) rat mAb (1867423) was purchased from Roche Diagnostics. IF8 anti-pRB mAb was provided by B. Gallie (Princess Margaret Hospital, Toronto, Ontario).

Plasmids and Adenoviral Constructs

Adenoviral vectors expressing full-length human E2F-1 and E2F-4 under the control of the cytomegalovirus promoter were kindly provided by J. DeCaprio and G. Leone (Dana Farber Cancer Institute, Boston, MA). The pAdtrack and pAdeasy vectors (He et al., 1998), as well as BJ5183 bacterial cells were generously provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD). pEGFPC-1 (Clontech) was provided by J. Rutka (Hospital for Sick Children, Toronto, Ontario).

For the green fluorescent protein (GFP) fusion proteins, HA-tagged versions of human E2F-1 and E2F-4 cDNAs were subcloned in frame with GFP in pEGFPC-1. For GFP–E2F-1 or GFP–E2F-4 fusion proteins containing the simian virus 40 (SV40) NLS, oligos encoding the SV40 large T antigen (LgT) NLS (MVPKKKRKV) were subcloned into the polylinker between GFP and the E2F cDNA maintaining the reading frame. These GFP-tagged recombinant cDNAs were excised from pEGFPC-1 with Age1 (5′ of the GFP tag) and Hpa1 (in the SV40 polyA tail region) and cloned into the Age1 and Hpa1 sites of pAdtrack–cytomegalovirus. pAdtrack–cytomegalovirus constructs were linearized with PmeI and cotransformed with pAdeasy into BJ5183 bacterial cells by electroporation. Recombinant adenoviral constructs were isolated, prepared on cesium chloride gradient, and transfected into 293 cells by Hepes-buffered saline transfection. 10 d after transfection, virus was harvested by scraping and pelleting the transfected 293 cells, followed by resuspension in sterile PBS/10% glycerol, and three freeze–thaw cycles. The suspension was centrifuged at 8,000 *g* for 20 min at 4°C, and the supernatant was used to reinfect 1–10 100-mm plates of confluent 293 cells for 90 min at 37°C, in 3 ml serum-free media. Cells were then washed twice with PBS and incubated for 2–5 d at 37°C until 40–80% of cells detached from the plate. Virus was harvested as above, and the reinfection process repeated until viral titers as determined by plaque assay reached at least 10^8 pfu/μl.

[1]*Abbreviations used in this paper*: BrdU, bromodeoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; HA, hemagglutinin; LgT, large T antigen; NES, nuclear export signal(s); NLS, nuclear localization signal; SV40, simian virus 40.

Cell Culture

Rat L6 myoblasts (Yaffe, 1968) were cultured in H21 DME containing 10% FCS. Differentiation into myotubes was performed by switching confluent cells into H21 DME containing 2% FCS. Rat-12 D5 cells (kindly provided by Dr. Steve Reed, Scripps Research Institute, La Jolla, CA) (Resnitzky et al., 1994) were grown in DME containing 10% FCS and 2 μg/ml tetracycline, 150 μg/ml hygromycin, and 350 μg/ml G418. Induction of cyclin D1 was achieved by switching to DME containing 0% FCS and 2 μg/ml tetracycline for 40 h. Media were replaced with serum-free DME containing no tetracycline for 24 h. Then media with 10% FCS were added for the duration of the experiment. NIH 3T3 cells were cultured in H21 DME containing 10% FCS.

L6 cells to be infected with adenoviral vectors and analyzed for BrdU incorporation were first grown to confluence in 60-mm dishes and differentiated to multinucleated myotubes in 2% FCS for 2 d. Cells were then infected with adenovirus in 1.5 ml serum-free media for 90 min at 37°C, followed by two washes in PBS and incubation at 37°C for 24 h. BrdU was then added at a final concentration of 2 μM, and cells were incubated for a further 48 h.

Flow Cytometric Analysis

To determine the proportion of cells present in a particular cell cycle phase, FACS analysis of DNA content was performed as follows. Cells were harvested at the indicated timepoints after serum addition, and cells were fixed with 70% ice-cold ethanol for FACS analysis. The fixed cells were suspended in 500 μl PBS containing 0.6% NP-40 and 0.1 mg/ml propidium iodide, and treated with RNase at a concentration of 1 mg/ml for 30 min. DNA fluorescence was measured in a Becton Dickinson flow cytometer, and the percentages of cells in G_1, S, and G_2/M phases of the cell cycle were determined using the CellFIT software (version 2.01.2).

Immunofluorescence

L6 and Rat-12 D5 cells grown on coverslips were rinsed twice in PBS before fixation in ice-cold 100% methanol for 3 min. Coverslips were air-dried for ~1 h and stored at −20°C. P19 cells were grown on coverslips and rinsed twice in PBS before fixation in 4% paraformaldehyde, 0.2% picric acid for 30 min at room temperature. Without drying out, fixed cells were then washed twice in PBS and stored at 4°C in a moist chamber. Whole embryos from pregnant BALB C mice were removed at day 18.5 (detection of a vaginal plug following being day 0.5), snap-frozen in liquid nitrogen–cooled isopentane, and stored at −80°C. Cryosections (6–8 μm) were cut and collected on Superfrost Plus microscope slides (Fisher Scientific), air-dried, and stored at −80°C. Cells grown on coverslips to be analyzed for BrdU incorporation were washed three times in PBS and fixed for 20 min in ice-cold 70% ethanol, 50 mM glycine, pH 2.0, at −20°C. Coverslips were then washed three times in PBS and stored at 4°C.

Methanol-fixed cells and embryo sections were thawed and rehydrated for 30 min in PBS, whereas paraformaldehyde-fixed cells were used directly for immunostaining. Cells and sections were immersed in 0.1% BSA/PBS for 30 min to block nonspecific binding. Cells were then incubated with polyclonal antibody diluted 1:80 in 0.1% BSA/PBS, or 1:20 diluted mAb for 1 h at room temperature. Cells were washed three times with 0.1% BSA/PBS for 5 min at room temperature and subsequently incubated for 60 min at room temperature with FITC-conjugated goat anti–mouse or goat anti–rabbit IgG (1 mg/ml, 1:20 dilution, 0.1% BSA/PBS; Jackson ImmunoResearch). The cells were washed three times with 0.1% BSA/PBS for 5 min at room temperature and mounted. To image the signal, conventional fluorescence microscopy was carried out with a photomicroscope (Zeiss) equipped with an epifluorescence attachment.

BrdU incorporation was detected in ethanol-glycine–fixed BrdU-labeled cells using a BrdU labeling kit (Roche Diagnostics) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assays

Nuclear lysates were prepared as described previously (Schreiber et al., 1989). An end-labeled (^{32}P[γ-ATP]) double-stranded oligonucleotide containing a single E2F binding site (5′-GGATTTAAGTTTCGCGCC-CTTTCTCAA-3′) was used as template. 1.5 μg of nuclear lysates was used in 15-μl reactions containing 2×10^4 cpm of labeled DNA (~0.2 ng), 120 mM NaCl, 4% Ficoll, 20 mM Hepes, pH 7.9, 50 ng/μl salmon sperm DNA, 2.5 mM $MgCl_2$, 40 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 250 ng/μl BSA. For supershift electrophoretic mobility shift assays (EMSAs) or EMSAs with added glutathione *S*-transferase fusion proteins, reaction mixtures were incubated at room temperature for 10 min, followed by addition of 1 μl of mAb. These reactions were allowed to incubate for an additional 15–20 min. The DNA–protein complexes were then resolved on a 4.5% polyacrylamide gel in 0.25× Tris-buffered EDTA at 20 V/cm and the dried gel exposed to film for 8–48 h.

Western Blots

Whole cell or nuclear and cytoplasmic protein lysates were obtained from both L6 cells as described previously (Schreiber et al., 1989; Kiess et al., 1995). 20 μg of each lysate was run on SDS-PAGE gels and transferred to nitrocellulose membrane (Schleicher & Schuell). Blots were blocked overnight at 4°C in PBS, 0.1% Tween 20 containing 4% skim milk powder, followed by incubation at room temperature for 1.5 h in a 1:1,000 dilution of primary antibody. After a 45-min incubation with goat anti–rabbit or goat anti–mouse HRP-conjugated secondary antibody (1:8,000; BioRad), blots were developed with the ECL fluorescent detection kit according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Coimmunoprecipitation Assays

3T3 fibroblasts were infected with adenoviral vectors expressing GFP-tagged E2Fs at a multiplicity of infection of 300. 48 h after infection, whole cell lysates were prepared and 400 μg was immunoprecipitated for 16 h at 4°C with 0.5 μg polyclonal IgG. 20 μl protein A–Sepharose beads were then added and incubated for a further 2 h. Immunocomplexes were washed five times with ice-cold NP-40 lysis buffer and separated on 7% reducing SDS-PAGE gels. After transfer to nitrocellulose membrane, blots were probed as described above.

Results

Compartmentalization of E2F and pRB Family Proteins in Quiescent Cells during a Reversible Growth Arrest

As a starting point in our analysis of the localization of the pRB and E2F family proteins in terminally differentiated cells that irreversibly enter a quiescent state (G_0), we attempted to verify their cell cycle–dependent localization in both Rat-1 fibroblasts and L6 myoblasts. Previous reports using a number of cell lines, such as Rat-1 cells, demonstrated that E2F is nuclear in arrested cells and shifted to the cytoplasm as cells progressed into S phase (Muller et al., 1997; Verona et al., 1997). However, Fig. 1 demonstrates that in contrast to these previous reports, serum-starved cells arrested in G_0 will cause localization of the E2F and pRB family proteins to different compartments depending on the presence of even very weak mitogenic signals. Specifically, when Rat-1 fibroblasts are made quiescent by plating in the complete absence of serum (Fig. 1 A), pRB, p130, E2F-1, and E2F-4 are exclusively cytoplasmic. Serum stimulation and progression through the cell cycle drives these proteins into the nucleus. Similarly, L6 myoblasts arrested in 0% serum also show exclusive localization of E2F-1 and E2F-4 to the cytoplasm (Fig. 1 B, left panel). In contrast, the cytoplasmic localization of these factors is completely altered in quiescent cells arrested in 0.2% serum (Fig. 1 B, right panel). Specifically, E2F-1 and E2F-4 are primarily nuclear in undifferentiated myoblasts arrested in G_0/G_1 due to plating in 0.2% serum.

We hypothesized that the difference in localization in cells in 0.2% serum was due to the cell responding to very weak mitogenic signals in the absence of cell cycle progression. This hypothesis was tested using the Rat-1 D5 cell line (Resnitzky et al., 1994), where a cyclin D1 transgene was placed under the control of the tetracycline oper-

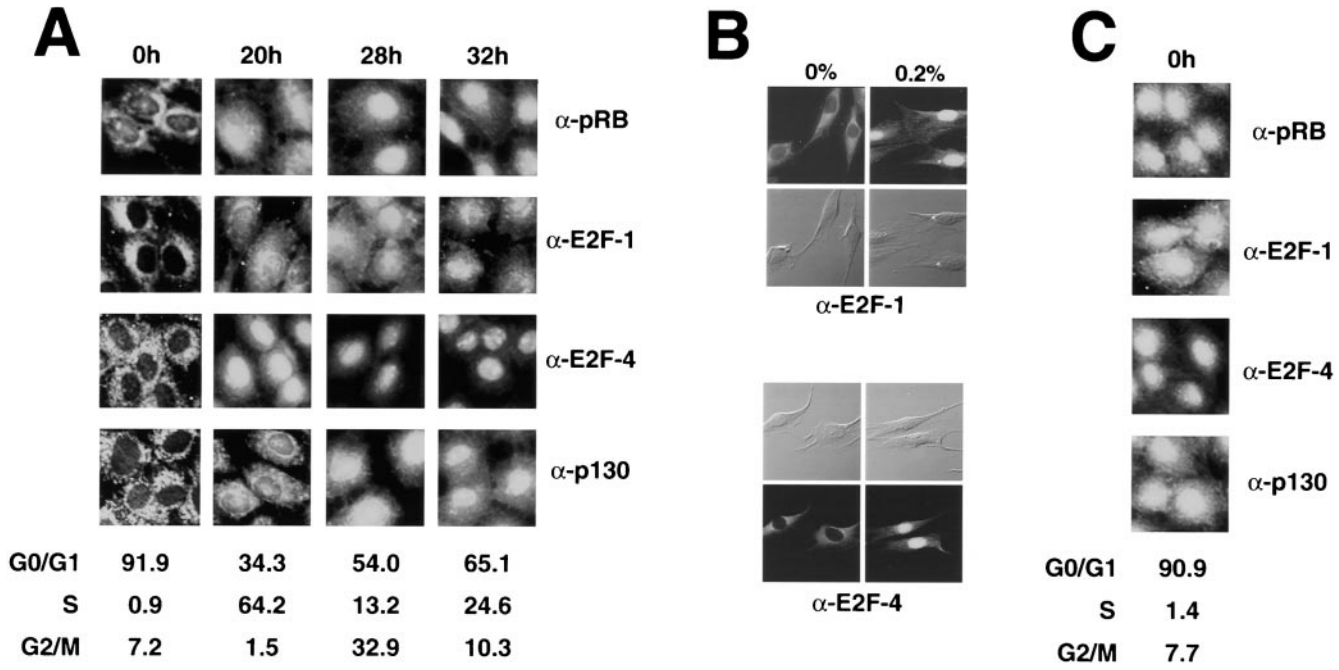

Figure 1. E2F and pRB family members are compartmentalized during the cell cycle. (A) Rat-1 cells were growth-arrested in 0% serum for 40 h, followed by addition of 10% serum. Cells were trypsinized and collected for FACS analysis at 4-h intervals after serum addition and proportions of cells in G_0/G_1, S phase, and G_2/M were determined. The subcellular localization of E2F and pRB members was determined by immunofluorescence as cells progress from G_0 back into the cell cycle. Cells grown on coverslips, were removed at G0, S phase, G2/M, and the subsequent G1, fixed in −20°C methanol, and immunostained with antibodies against E2F-1, E2F-4, pRB, and p130. (B) The effect of a growth stimulus provided by low levels of growth factors on E2F subcellular localization was determined by arresting L6 myoblasts in either 0% serum or in 0.2% serum for 40 h. Cells grown on coverslips were removed, fixed in −20°C methanol and immunostained with antibodies against E2F-1 and E2F-4. Fixed cells are also visualized by light microscopy using Nomarski optics. (C) The effect of an ectopic growth stimulus on subcellular localization during the cell cycle was examined by arresting cells in the presence of inducible cyclin D1. Cells grown on coverslips in the absence of tetracycline (induced cyclin D1) were removed, fixed in −20°C methanol, and immunostained with antibodies against E2F-1, E2F-4, pRB, and p130.

ator (Gossen and Bujard, 1992). Here, we expected cyclin D1 to provide a mitogenic signal insufficient to overcome the cell cycle arrest imposed on these fibroblasts in the absence (0%) of serum. Comparing the localization in Fig. 1 C to the same cells in the absence of cyclin D1 induction (Fig. 1 A, 0 h), it is evident that pRB, p130, E2F-1, and E2F-4 are primarily nuclear (some cytoplasmic localization is evident) despite the lack of cell cycle progression.

Thus, the dynamic regulation of pRB and E2F subcellular compartmentalization is clearly dependent on the state of the cells and the signals impinging on them, even in a quiescent, noncycling state. As will be made apparent below, localization of E2F in cells with no serum reflects their localization in terminally differentiated cells irreversibly arrested in G_0.

E2F Family Members Are Differentially Located in Terminally Differentiated Cells

We next determined the subcellular compartmentalization of E2F and pRB family members in differentiated multinucleated L6 myotubes that are in an irreversible quiescent (G_0) state (Fig. 2, A–H). By day 5 of differentiation, the fluorescence signal for the pRB family members, pRB, p107, and p130, coincided primarily with the signal for the 4,6-diamidino-2-phenylindole (DAPI)-stained nuclei in both multinucleated myotubes and undifferentiated myoblasts (Fig. 2, F–H). Only weak cytoplasmic localization for the pRB family proteins was observed using immunohistochemistry and confirmed by Western analysis of fractionated cell lysates (data not shown). In contrast to these negative cell cycle regulatory proteins, the different E2Fs exhibited distinct subcellular localization patterns in terminally differentiated myotubes (Fig. 2, A–E). We employed anti-E2F antibodies selected for their specificity, determined by Western analysis of L6 lysates (data not shown). In these cells, E2F-2 was primarily nuclear in myotubes, exhibiting very weak, albeit detectable cytoplasmic staining. E2F-4 showed little specificity for a given compartment, being present in both the cytoplasm and in the nucleus in myotubes. In contrast, E2F-1, E2F-3, and E2F-5 appeared exclusively cytoplasmic and were excluded from the nuclei of myotubes. For E2F-1 and E2F-5, this localization represents a major alteration, since these two factors were primarily nuclear in the exponentially growing myoblasts. For E2F-3, little change in compartmentalization was observed, this factor residing primarily in the cytoplasm throughout differentiation.

The studies previously examining the subcellular compartmentalization of the E2F family proteins have employed cells grown in culture. However, no data are published describing their localization in situ. Therefore, we

wished to determine if the localization observed for the E2Fs in differentiated cells grown in culture reflected their compartmentalization in situ. Day E18.5 murine embryos were sectioned and immunostained for E2F-1, E2F-4, or the nuclear myogenic transcription factor, E47 (Fig. 3). Skeletal muscle adjacent to the developing ribs revealed E2F-4 immunofluorescence localized to the cytoplasm. Areas within the cytoplasm that were devoid of E2F-4 signal (Fig. 3, arrowheads) coincided precisely to the DAPI-stained nuclei, confirming E2F-4 exclusion from the nuclei of muscle tissue. Likewise, E2F-1 staining was observed in compartments distinct from the DAPI-stained nuclei. In contrast to E2F-1 and E2F-4, immunostaining for the nuclear heterodimeric partner of the myogenic transcription factor, E47, was coincident with the DAPI signal. Thus, the localization observed for E2F-1 and E2F-4 in differentiated muscle cells (myotubes) grown in culture reflects their localization in embryonic muscle in situ.

LgT NLS Redirects E2F Localization in Differentiated Myotubes

We next determined that high levels of exogenous E2F-1 and E2F-4 localize similarly to their endogenous counterparts in differentiated myotubes. Adenoviruses, expressing fusion proteins between GFP (Cormack et al., 1996) and either E2F-1 or E2F-4, were used to infect multinucleated myotubes at 5×10^9 pfu (Fig. 4 A). 72 h after infection, GFP–E2F-1 was detected primarily in the cytoplasm of myotubes, whereas GFP-E2F-4 is both nuclear and cytoplasmic, reflecting immunostaining results of the endogenous proteins. However, cytoplasmic sequestering of these E2Fs could be overridden using the strong NLS from SV40 LgT (Yang and DeFranco, 1994; Makkerh et al., 1996; Xiao et al., 1997). The addition of this signal to either GFP-tagged E2F-1 or E2F-4 resulted in their exclusive nuclear localization. The subcellular localization of the direct GFP fluorescent signal was confirmed by Western analysis of fractionated lysates from infected myotubes (Fig. 4 B). Specifically, all of the expressed GFP–E2F-1 was detected in the cytoplasmic fraction, whereas GFP–E2F-1 (NLS) was exclusively nuclear. GFP–E2F-4 was found in both the cytoplasm and the nucleus, whereas addition of the NLS to this fusion protein caused exclusive nuclear compartmentalization.

Given the exclusive nuclear localization of the NLS-modified GFP–E2F-1 and GFP–E2F-4 fusion proteins, we next sought to determine if nuclei of these infected cells entered S phase. Differentiated cells were treated with BrdU and simultaneously infected with either the GFP–E2F-1 (NLS) or GFP–E2F-4 (NLS) viruses. 72 h later, cells were fixed and BrdU incorporation determined. As Fig. 5 indicates, despite the presence of high levels of the GFP fusion proteins in the nuclei of these myotubes, very rare nuclei stained positive for BrdU incorporation. Quantitation of GFP-expressing nuclei revealed, in fact, that $<0.01\%$ of nuclei expressing the strictly nuclear GFP–E2F-1 (NLS) or GFP–E2F-4 (NLS) proteins stained for BrdU.

However, the failure to drive nuclei into S phase was not due to the inability of these fusion proteins to complex the pRB family proteins or to an inability to bind DNA. Fig. 6 A demonstrates that immunoprecipitation of pRB resulted in coimmunoprecipitation of GFP–E2F-1 and GFP–E2F-1 (NLS). Furthermore, EMSAs demonstrated that these fusion proteins could bind specifically to a double stranded oligonucleotide encoding an E2F consensus sequence (Fig. 6 B). Addition of the antibody directed against the HA tag on the exogenous GFP-tagged proteins (Fig. 6 B, middle panel) confirmed the presence of these fusion proteins in complexes binding the E2F site. In the case of GFP–E2F-1 and GFP–E2F-1 (NLS), supershift assays using an antibody directed against pRB demonstrated its presence in the GFP–E2F-1–containing complexes (Fig. 6 B, right panel).

Finally, we determined the expression pattern of known E2F-1 target genes in myotubes infected with the various GFP-tagged E2F fusion proteins. Using cyclin E as one specific target, we determined that its expression was unaltered in the presence of the GFP-tagged E2Fs (Fig. 6 C). Specifically, cyclin E is expressed at low but detectable levels in myotubes. We compared cyclin E levels in GFP–E2F expressing cells to its level in cells expressing the dominant-negative cell cycle protein, pRBΔp34/HA (levels for the latter are identical to uninfected cells; data not shown). In all cases, cyclin E levels were unchanged. Thus, although the GFP–E2F fusion proteins bind pRB family members, this activity is not sufficient to alter expression of cell cycle regulatory factors or drive nuclei in postmitotic myotubes into S phase.

Nuclear Localization of Ectopic Wild-Type E2F-1 and E2F-4 Causes S Phase Entry of Nuclei in Terminally Differentiated Myotubes

Cytoplasmic sequestration of the E2Fs in terminally differentiated myotubes suggests that this localization is one mechanism preventing multinucleated myotubes from undergoing additional rounds of DNA replication. Therefore, we attempted to overcome the nuclear exclusion of E2F-1 and E2F-4 in multinucleated myotubes by expressing increasing levels of these factors. L6 myotubes were infected at 0.8×10^8 (not shown), 4×10^8, 2×10^9, or 1×10^{10} pfu with adenoviruses expressing wild-type E2F-1 or E2F-4. Localization of E2F-1 and E2F-4 was determined by indirect immunoflourescence using antibodies directed against the specific E2Fs, whereas entry into S phase was determined by BrdU incorporation (Fig. 7). In uninfected myotubes, endogenous E2F-1 is cytoplasmic, whereas E2F-4 is primarily cytoplasmic but with significant nuclear localization. Infection with virus up to 4×10^8 pfu resulted in significant overexpression of ectopic E2F-1 and E2F-4 (approximately fourfold over endogenous by Western analysis; Fig. 8). At this level of expression, the E2Fs localized similarly to their endogenous counterparts in uninfected cells. Under these conditions, no nuclei in these myotubes incorporated BrdU, indicating that they had not entered S phase. However, infection at 2×10^9 pfu or greater caused the appearance of E2F-1 in the nucleus as well as an increase in E2F-4 in this compartment. For both E2Fs, the majority of nuclei in the terminally differentiated myotubes had undergone DNA synthesis. In the case of E2F-1, the occasional nuclei that did not stain for BrdU were also devoid of signal for this E2F family member (see

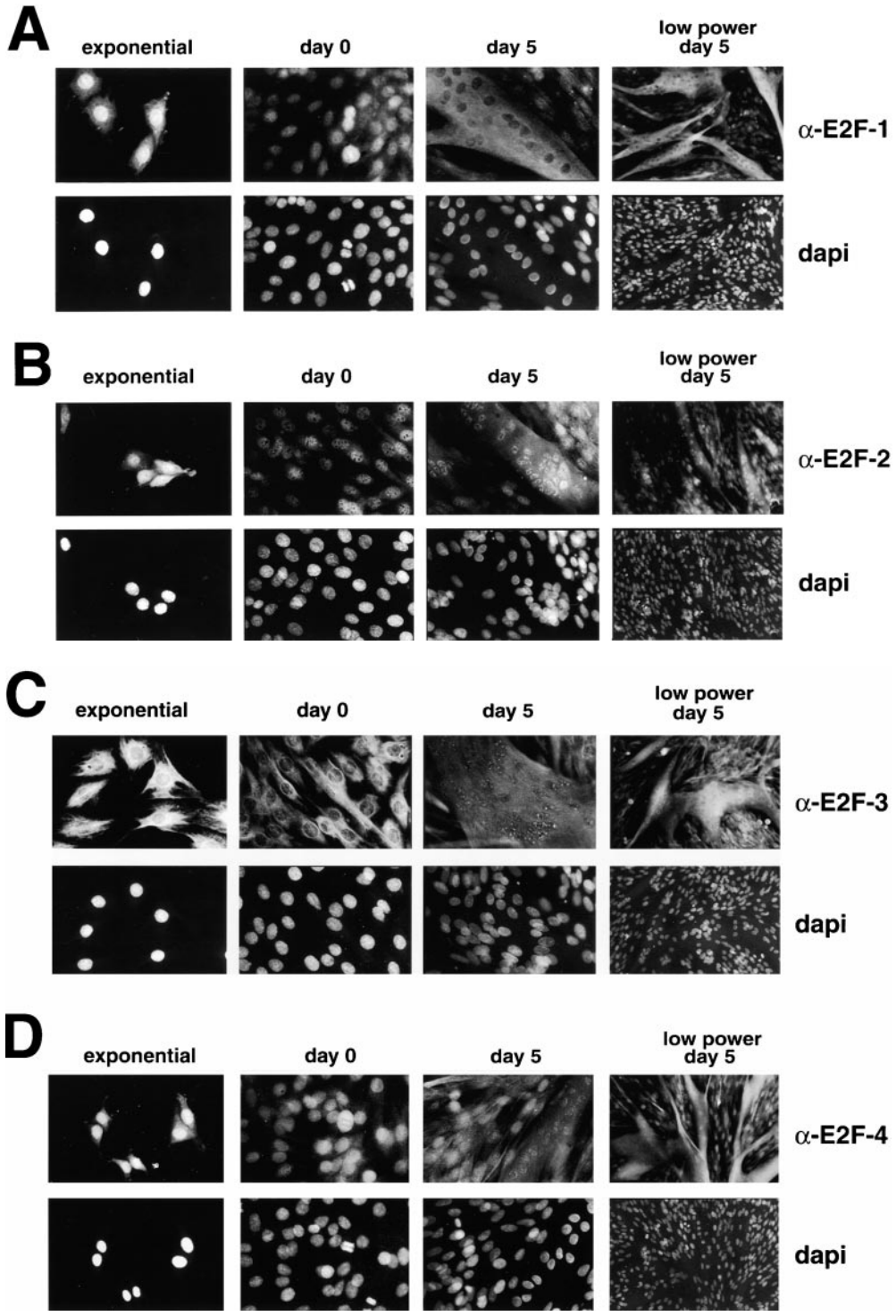

Figure 2 (continues on facing page).

Fig. 7, arrows in cells infected with 2×10^9 pfu). Since these nuclei were directly adjacent to nuclei that had undergone DNA synthesis, it is apparent that entry into S phase for individual nuclei was dependent on the presence of E2F-1 in that nucleus and not due merely to expression of other positive cell cycle regulatory factors (see below) in the myotube. However, a block preventing mitosis is apparent in these cells, since mitotic figures were never observed in the BrdU-positive E2F-expressing cells.

S phase entry of E2F-1–expressing cells predicted that alteration in expression of a number of cell cycle regulatory proteins and myogenic differentiation factors would be observed. Thus, cell lysates prepared from the terminally differentiated L6 cells infected with increasing levels

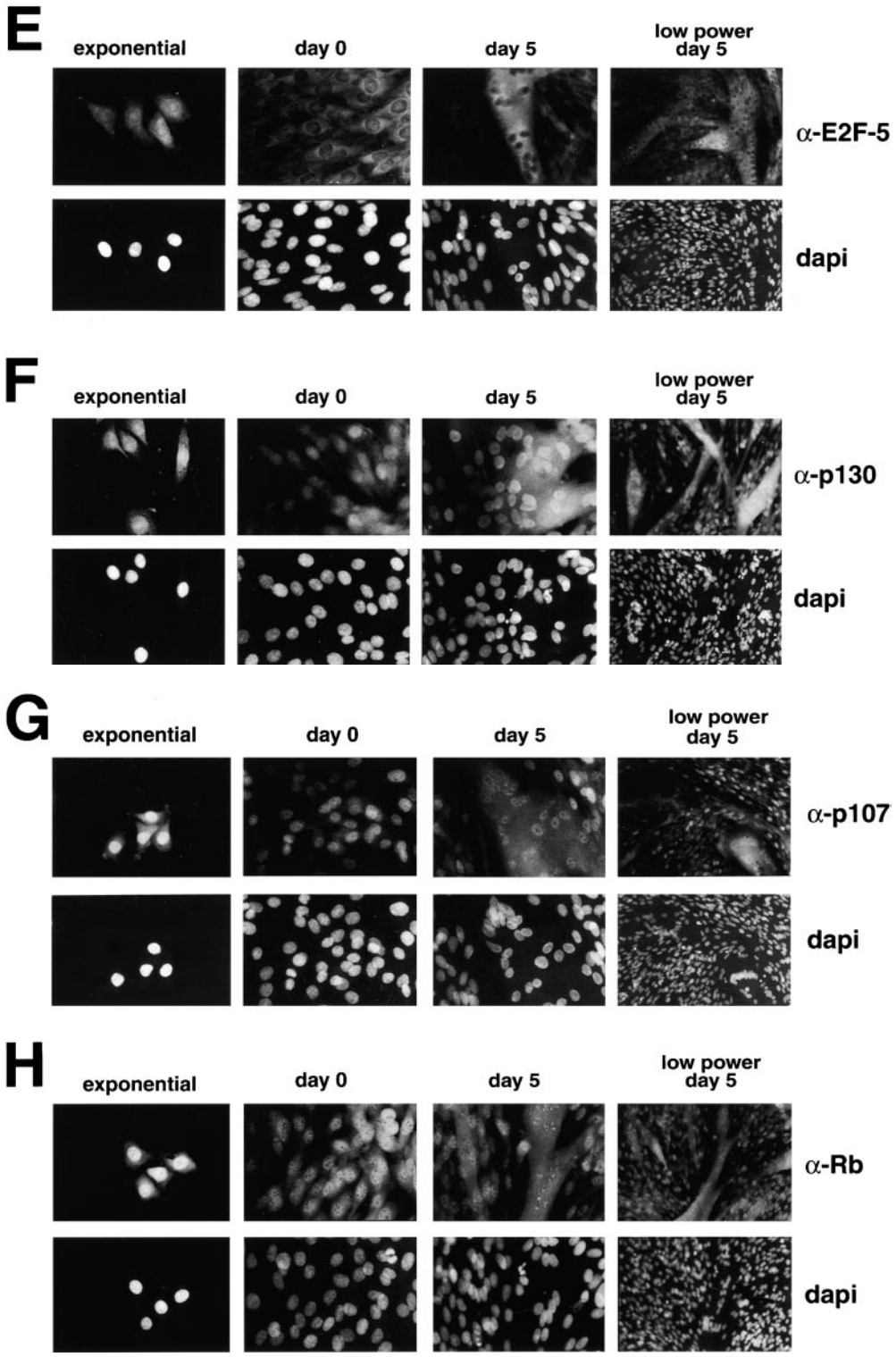

Figure 2. E2F family members are differentially sequestered in the cytoplasm of terminally differentiated myotubes. The subcellular localization of E2F and pRB family members was examined by immunofluorescence as L6 myoblasts exit the cell cycle and terminally differentiate. Exponentially growing L6 cells were plated at 20% density and allowed to reach confluence, when serum was reduced to 2% (day 0) to induce differentiation to multinucleated postmitotic myotubes. Coverslips were removed from exponentially growing cultures, as well as at day 0, day 2, and day 5 after serum reduction. (A–E) Undifferentiated, day 0, day 2, and day 5 cells were immunostained with primary antibodies against E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5 followed by FITC-conjugated secondary antibody. Nuclei are visualized by DAPI staining and the corresponding panels are presented below the appropriate FITC-labeled immunostained panel. (F–H) Undifferentiated, day 0, day 2, and day 5 cells were also immunostained with primary antibodies against pRB, p107, and p130 followed by FITC-conjugated secondary antibody. Undifferentiated cells are presented in the left-hand panels, and terminally differentiated multinucleated myotubes in the right-hand panels.

of adenovirus expressing E2F-1 or E2F-4 were probed for expression of a number of genes involved in mediating either the G_1–S transition or myogenic differentiation by Western analysis (Fig. 8). Expression of either exogenous E2F-1 or E2F-4 caused increased levels of pRB, the promoter region of which encodes an E2F binding site (Shan et al., 1994). pRB was also converted to its hyperphosphorylated state at the highest level of multiplicity of infection, as would be expected for cells entering S phase. Consistent with this change in the phosphorylation status of pRB, cyclin A and cyclin E levels increased and the faster migrating active form of cdk2 appeared. Furthermore, two factors involved in the terminal differentiation of myotubes, $p21^{Cip1}$ and myogenin, are downregulated by ec-

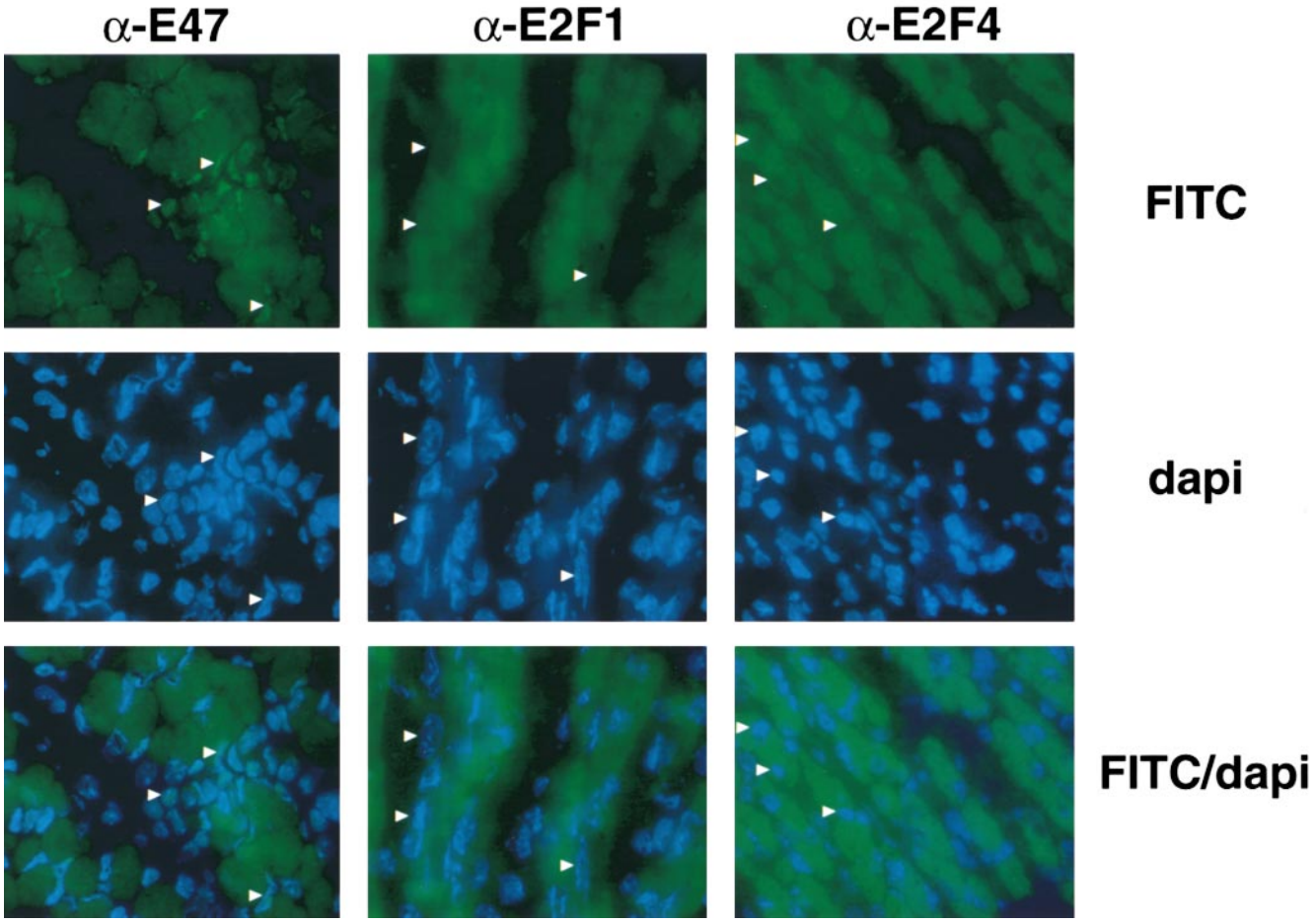

Figure 3. E2F family members are localized in the cytoplasm of myotubes in the developing embryo. To determine whether cytoplasmic compartmentalization of the E2Fs occurs in vivo, sagittal sections of a murine day E18.5 embryo were immunostained against E2F-1, E2F-4, and as a control, the nuclear myogenic transcription factor, E47. Skeletal muscle sections adjacent to the developing ribs were probed with primary antibodies against E47, E2F-1, and E2F-4, followed by FITC-labeled secondary antibody. Nuclei are visualized by DAPI staining. FITC and DAPI signals were determined by fluorescent microscopy and are presented individually and as a combined FITC–DAPI image. Specific nuclei demonstrating nuclear immunostaining of E47 or nuclear exclusion of E2F-1 and E2F-4 are indicated by arrowheads.

topic E2F-1 and E2F-4 expression, supporting the observation that E2F promotes cell cycle reentry of myotubes while opposing the differentiation program.

Discussion

The levels of free E2F transcription complexes, unbound by pRB family members, are strongly reduced as cells leave the cell cycle during cellular differentiation (La Thangue and Rigby, 1987; La Thangue et al., 1990; Shivji and La Thangue, 1991; Corbeil et al., 1995; Kiess et al., 1995; Shin et al., 1995; Gill et al., 1998), whereas expression of exogenous E2F-1 before differentiation blocks this process (Wang et al., 1995, 1996; Guy et al., 1996; Guo and Walsh, 1997; Strom et al., 1998). Subcellular compartmentalization is one of a number of overlapping mechanisms involved in regulating E2F-dependent transcription (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). This mechanism is analogous to the regulation of activity of transcription factors such as TFIIIA (Fridell et al., 1996), engrailed (Maizel et al., 1999), IκBα (Fritz and Green, 1996), and p53 (Stommel et al., 1999), which also occur in part due to dynamic changes in their location within a cell.

We demonstrated here that E2F-1 is localized in the cytoplasm of terminally differentiated myotubes, both in vitro and in situ. In myotubes grown in culture, E2F-4 is partitioned between the nucleus and the cytoplasm. However, at the stage of muscle development examined in E18.5 embryos, E2F-4 appeared to be excluded from the nucleus. E2F-3 and E2F-5 are also primarily cytoplasmic in differentiated myotubes. p130 and pRB, which are induced during myoblast differentiation (Kiess et al., 1995; Yee et al., 1998), are detected primarily in the nuclei of terminally differentiated myotubes. This localization for the pRB family proteins in myotubes may reflect their interaction with myogenic factors, which together promote muscle differentiation (Gu et al., 1993; Novitch et al., 1996; Skapek et al., 1996; Zacksenhaus et al., 1996; Sellers et al., 1998). In contrast, our preliminary data examining their

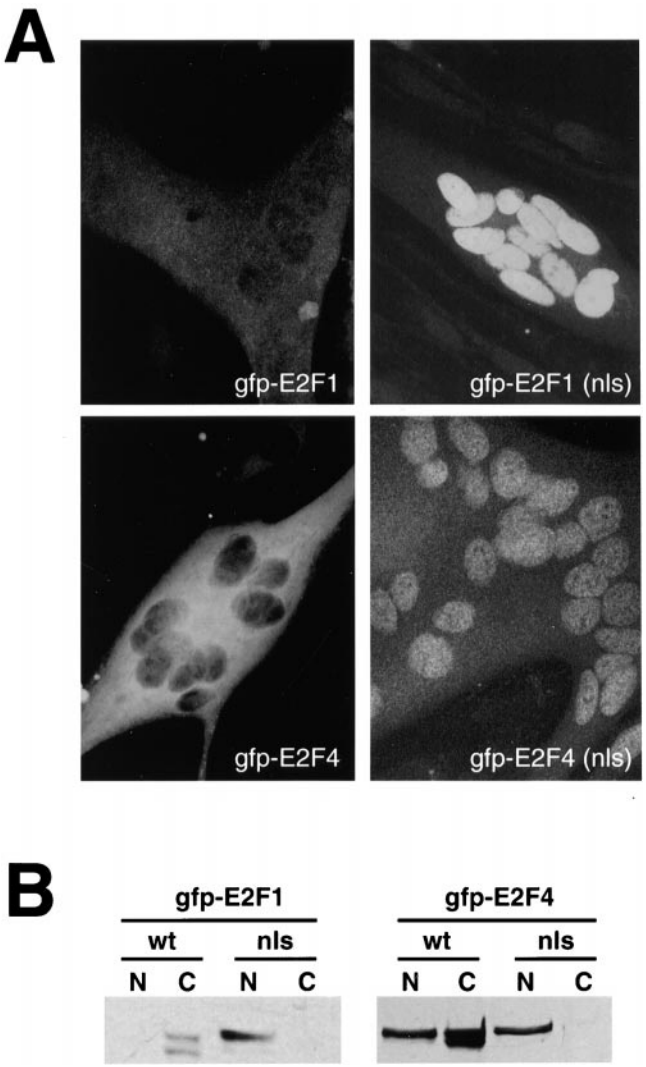

Figure 4. Ectopic GFP-tagged E2F-1 and E2F-4 localize to the cytoplasm of myotubes and an ectopic NLS overcomes this compartmentalization. L6 cells were differentiated to multinucleated myotubes in 2% serum for 72 h. Myotubes were then infected with 5×10^9 pfu of adenovirus expressing GFP–E2F-1 or GFP–E2F-4 fusion proteins, or GFP–E2F fusion proteins containing an ectopic SV40 LgT NLS. GFP signals were determined by fluorescent microscopy using the FITC stimulation wavelength. (A) Immunofluorescence of GFP-tagged E2F-1 (left side) or E2F-4 (right side) proteins without (top) or with (bottom) the SV40 NLS were visualized by fluorescence microscopy. (B) Localization of the GFP–E2F fusion proteins was also determined by Western analysis of fractionated lysates from infected cells. Nuclear and cytoplasmic lysates from terminally differentiated L6 myotube cultures infected with adenoviral vectors expressing GFP-tagged wild-type, or LgT NLS-tagged E2F-1 and E2F-4 were run on an SDS-PAGE gel and Western blotted with anti–E2F-1 (left panel) and anti–E2F-4 (right panel) polyclonal antibodies. Ectopic proteins are distinct from endogenous E2F due to the 25-kD GFP moiety.

localization during neuronal differentiation, indicate that p130 and pRB reside in the cytoplasm of these terminally differentiated cells (R.M. Gill, unpublished observation).

E2F-1, E2F-2, and E2F-3 encode an NH_2-terminal NLS, whereas E2F-4 and E2F-5 are deficient for this sequence. These latter two E2F family proteins appear to rely on interactions with DP family members or pRB family proteins for their nuclear translocation (Lindeman et al., 1997; Muller et al., 1997; Puri et al., 1998; Verona et al., 1997). Although distinct in harbouring an NLS, all members of the E2F family proteins encode a conserved domain that resembles the leucine-rich nuclear export signals (NES) of HIV-1 Rev protein (Fischer et al., 1995; R.M. Gill, unpublished data). Interestingly, pRB, p107, and p130 also contain potential NES consensus sequences in their NH_2-terminal domains (R.M. Gill, unpublished observation), a region of p107 and p130 that is not otherwise well conserved with pRB (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993; Sidle et al., 1996). The Rev NES, which is similar to those of *Xenopus* TFIIA (Fridell et al., 1996), rat protein kinase I (Fischer et al., 1995; Wen et al., 1995), and murine IκBα (Arenzana-Seisdedos et al., 1997), is bound by CRM1 protein and transported to the cytoplasm (Fornerod et al., 1997). The presence of putative NES supports the notion that localization of the E2F and pRB family proteins to the cytoplasm during terminal differentiation may be due to an active export mechanism. Recently, RanBP1, a factor that is involved in nuclear import and export has been reported to be regulated by E2F during the cell cycle (Di Fiore et al., 1999). It contains two E2F binding sites in its promoter, a proximal site responsible for repression of transcription during G_0, and a distal site that controls upregulation during S phase (Di Fiore et al., 1999). RanBP1 contributes to nuclear import by stabilizing the interaction of Ran with importin-β (Chi et al., 1996; Lounsbury and Macara, 1997). It may also be involved in transport from the nucleus to the cytoplasm by releasing CRM1 from the nuclear pore complex, allowing the terminal step in nuclear export to occur (Kehlenbach et al., 1999). It is interesting to speculate that the cell cycle–dependent compartmentalization of E2F and pRB family members may be regulated through a feedback mechanism involving RanBP1.

We determined that differentiation-dependent cytoplasmic partitioning of the E2Fs could be overridden by cloning the SV40 LgT NLS at the NH_2 terminus of GFP-tagged E2F-1 and E2F-4. These recombinant molecules, when expressed in terminally differentiated myotubes using an adenoviral vector, localized exclusively to the nucleus. In the absence of the LgT NLS, exogenous GFP-tagged E2F-1 and E2F-4 were primarily cytoplasmic, identical to the distribution of endogenous protein as determined by immunostaining. When targeted to the nuclei of terminally differentiated myotubes, these GFP-tagged proteins did not promote S phase entry, despite being capable of complexing DNA, pRB, and cdk2. However, these GFP fusion proteins were not active in inducing E2F target genes, suggesting that the presence of either the large GFP moiety and/or the LgT NLS at the NH_2 termini of E2F-1 and E2F-4 was interfering with E2F-dependent activated transcription. These results indicate that E2F-1 and E2F-4 do not drive cells into S phase merely as a consequence of nuclear translocation and sequestration of all the pRB family proteins. Rather, progression into S appears to also require activated transcription of E2F target genes.

When untagged versions of E2F-1 and E2F-4 were expressed in differentiated myotubes at levels consider-

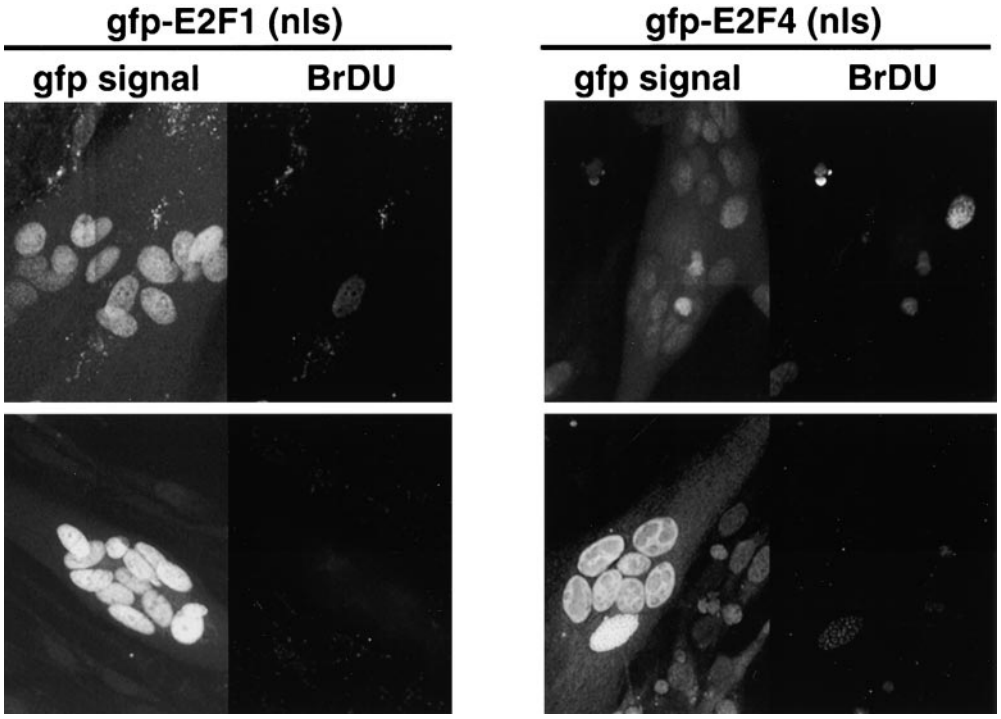

Figure 5. Nuclear targeted GFP–E2F-1 and GFP–E2F-4 do not induce S phase in myotubes. L6 cells were differentiated to multinucleated myotubes in 2% serum for 72 h. Myotubes were then infected with 5×10^9 pfu of adenovirus expressing GFP–E2F-1 (NLS) or GFP–E2F-4 (NLS) fusion proteins. 24 h after infection, BrDU labeling reagent was added and the cells were incubated for a further 48 h, at which point cells grown on coverslips were fixed, and nuclear and cytoplasmic lysates were prepared. Immunofluorescence of GFP-tagged E2F-1 (NLS) (left panel) or E2F-4 (NLS) (right panel) proteins was visualized by fluorescence microscopy using the FITC stimulation wavelength. S phase entry of GFP–E2F-expressing cells was determined by anti-BrdU immunohistochemistry using a Texas red–labeled anti–mouse secondary antibody.

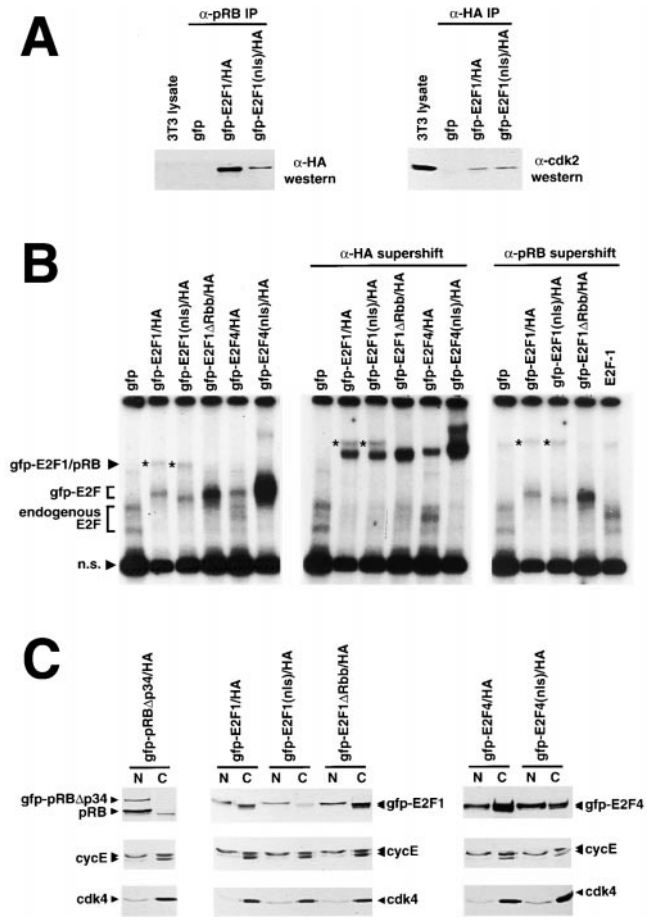

Figure 6. GFP-tagged E2F molecules bind DNA and pRB family proteins but have altered transactivation properties. (A) To determine if endogenous pRB is capable of binding the GFP-tagged E2F-1 fusion proteins, 3T3 cells were infected with 1×10^{10} pfu of adenoviral vectors expressing GFP alone as well as GFP–E2F-1/HA and GFP–E2F-1 (NLS)/HA. 400 μg of whole cell lysate was immunoprecipitated with anti-pRB polyclonal antibody, and coimmunoprecipitating GFP-tagged E2F was determined by Western blotting with an anti-HA mAb. To determine whether the cyclin A binding domain is accessible, ectopic GFP-tagged E2F-1/HA and E2F-1 (NLS)/HA proteins, as well as a GFP control, were immunoprecipitated from adenoviral infected 3T3 lysates with monoclonal anti-HA antibody, and coimmunoprecipitating cdk2 protein was determined by anti-cdk2 Western blot (right panel). (B) Nuclear lysate from 3T3 cells infected with adenoviral vectors expressing either untagged E2F-1, or GFP-tagged wild-type, or NLS-tagged E2F-1 and E2F-4 was incubated at room temperature for 10 min with a ^{32}P end-labeled double-stranded oligonucleotide probe containing a single E2F binding site, before resolving on a 4.5% nondenaturing acrylamide gel. The middle panel contains reactions in which 1 μl of monoclonal anti-HA was added for an additional 10 min, whereas the right panel contains reactions with anti-pRB monoclonal added. Endogenous E2F complexes are indicated, as are those containing GFP-tagged E2F. A larger complex containing pRB as well as ectopic GFP-tagged E2F-1 is indicated by an asterisk. Note that a mutant E2F molecule, which is deleted in the pRB-binding domain GFP–E2F-1ΔRbb/HA, does not form this pRB-containing complex. (C) 3T3 cells were infected with 1×10^{10} pfu of adenoviral vectors expressing either untagged E2F-1, or GFP-tagged wild-type, or NLS-tagged E2F-1 and E2F-4, as well as the pRB-binding mutant GFP–E2F-1ΔRbb/HA, and 72 h after infection, nuclear and cytoplasmic lysates were harvested and separated by SDS-PAGE. Levels of endogenous cyclin E protein were determined by Western blot. Exogenous GFP-tagged proteins are detected by an anti-HA monoclonal, and cdk4 is Western blotted as a control for protein loading.

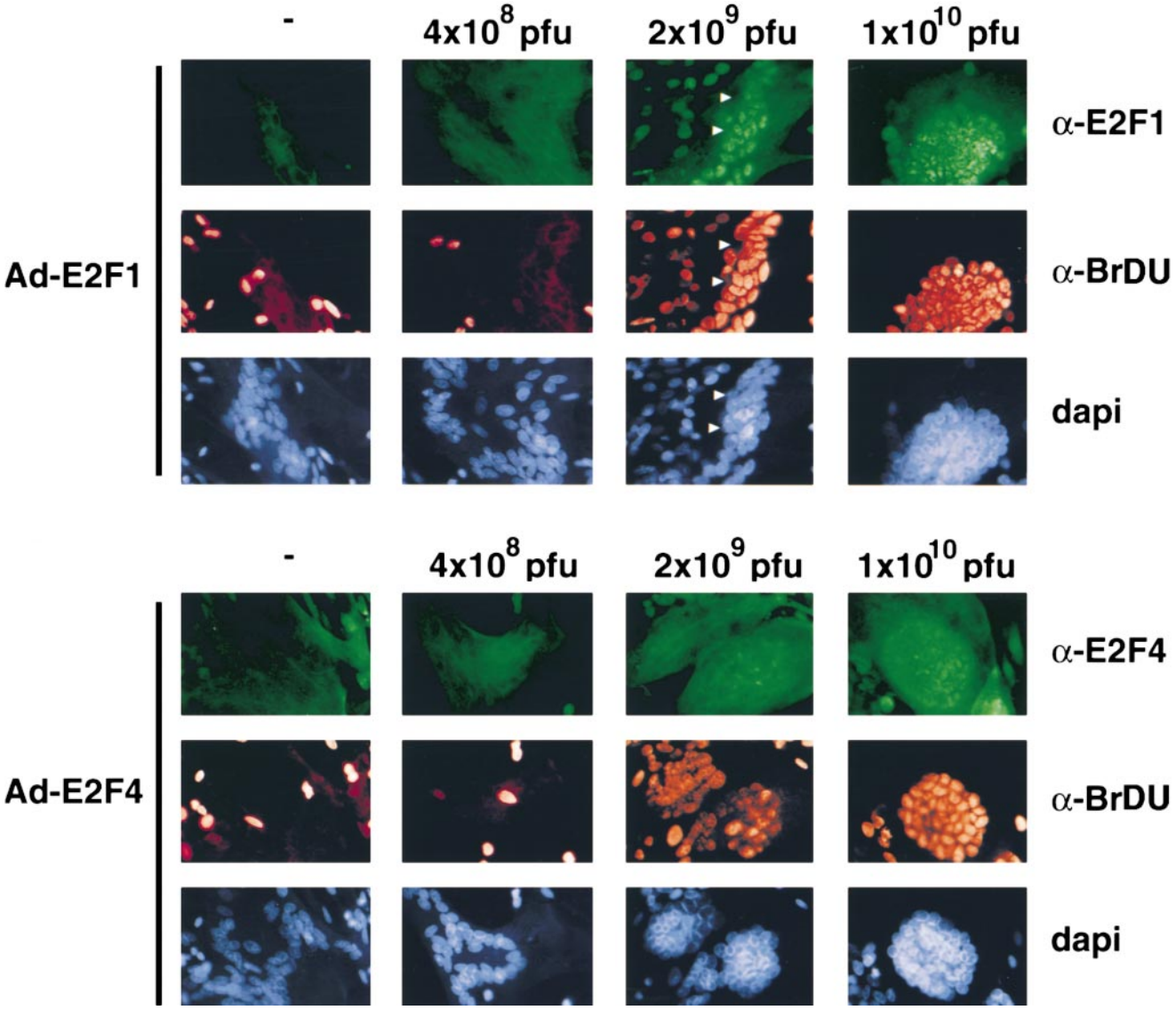

Figure 7. Overexpression of ectopic E2F-1 and E2F-4 can overcome cytoplasmic sequestering and forces postmitotic myotube nuclei to enter S phase. L6 cells were differentiated to multinucleated myotubes in 2% serum for 48 h. Myotubes were then infected with the E2F-1 or E2F-4–expressing adenoviruses at either 0.8×10^8, 4×10^8, 2×10^9, or 1×10^{10} pfu, and 48 h after infection DNA synthesis was determined by adding BrdU labeling reagent and incubating a further 24 h. Cells on coverslips were fixed and immunostained with mAb against BrdU and a Texas red–labeled anti–mouse secondary antibody, as well as polyclonal antibodies against E2F-1, or E2F-4, and FITC-labeled anti–rabbit secondary antibody. Nuclei were then stained with DAPI and each signal was captured by fluorescence microscopy. FITC, Texas red, and DAPI signals for myotubes infected with adenovirus expressing E2F-1 are shown in the top panel, whereas cells infected with E2F-4 adenovirus are shown in the bottom panel. Nuclei in Ad-E2F-1 infected cells (2×10^9 pfu), which do not stain for BrdU or E2F-1, are indicated by arrowheads.

ably higher than that of their endogenous counterparts, both E2F-1 and E2F-4 remain compartmentalized appropriately. There is a limit in the ability of the cell to maintain these transcription factors in the cytoplasm, however, since very high levels of E2F-1 or E2F-4 become partitioned between the cytoplasm and the nucleus. However, it is clear that the mechanism(s) maintaining the E2F family proteins in the cytoplasm in differentiated cells are very robust and can tolerate a considerable increase in the levels of these transcription factors.

It was interesting to note that both E2F-1 and E2F-4 were capable of driving myotube nuclei into S phase when present in the nucleus at significant levels. For E2F-1, these data are consistent with its ability to promote cell cycle reentry of cardiac myocytes and cortical neurons (Suda et al., 1994; Kirshenbaum et al., 1996) and that E2F-1 is important for the transition from G_0 to G_1, but not for subsequent cell cycles, as cells continue to proliferate (Leone et al., 1998). Little data from differentiated cells exist for the effect of E2F-4 on cell cycle progression. Here we demonstrate that similar to E2F-1, E2F-4 induces expression of the positive cell cycle regulatory factors, cyclin A, cyclin E, and cdk2 (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994, 1995; Wimmel et al., 1994; Duronio and O'Far-

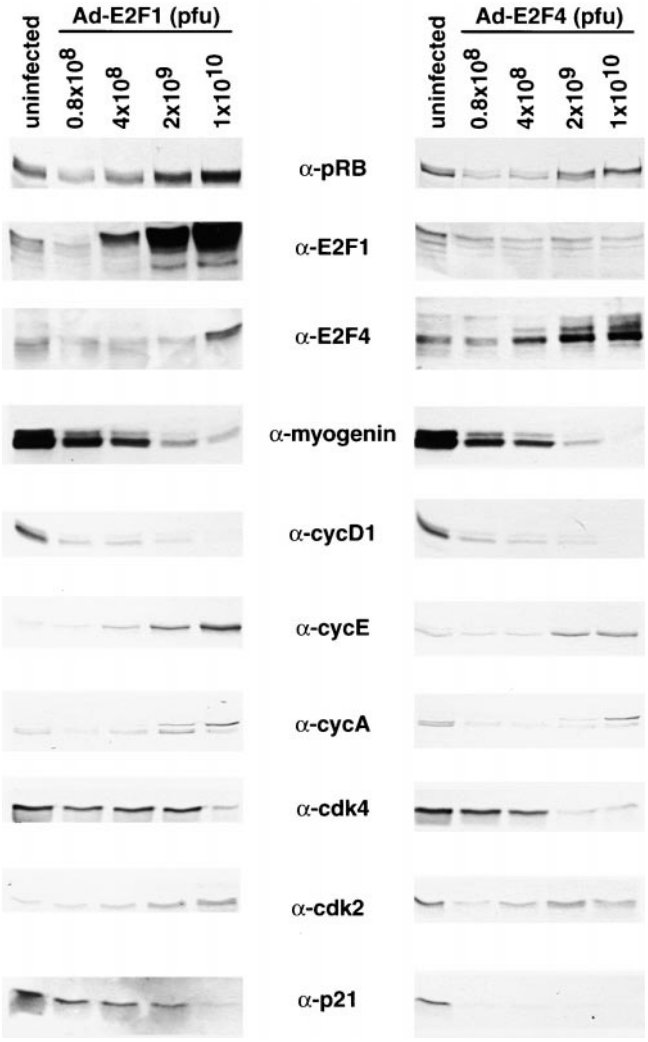

Figure 8. DNA synthesis caused by ectopic E2F-1 or E2F-4 is accompanied by changes in expression of cell cycle and differentiation-specific factors. L6 cells were differentiated to multinucleated myotubes in 2% serum for 48 h. Myotubes were then infected with the E2F-1 or E2F-4–expressing adenoviruses at either 4×10^8, 2×10^9, or 1×10^{10} pfu and 72 h after infection total lysates were isolated. 20 μg of lysate was run on an SDS-PAGE gel and Western blotted with antibodies against E2F-1, E2F-4, pRB, myogenin, $p21^{Cip1}$, cdk2, cdk4, and cyclins D1, A, and E.

rell, 1995; Ohtsubo et al., 1995; Resnitzky and Reed, 1995; Rosenberg et al., 1995; Chen et al., 1996; Duronio et al., 1996), represses factors required for muscle differentiation, such as myogenin and p21 (Edmondson and Olson, 1989; Wright et al., 1989; Guo et al., 1995; Missero et al., 1995), and promotes S phase entry. However, although postmitotic nuclei in myotubes are capable of responding to an E2F-1 and E2F-4–mediated proliferative stimulus, a block preventing mitosis is evident in these nuclei since mitotic figures are never observed in the infected myotubes. These data for E2F-1 and E2F-4 are reminiscent of the effect of deregulated E2F activity in pRB-deficient animals. Specifically, expression of a weak allele of pRB on the pRB-null background resulted in progression of muscle differentiation (Zacksenhaus et al., 1996). However, in these muscles, abundant large nuclei were present that had undergone several rounds of endoreduplication, whereas mitosis was not observed. The nature of this block in mitotic entry is currently under investigation.

The ability of exogenous nuclear-localized E2F-1 and E2F-4 to induce S phase appears to be linked to their ability to properly regulate the transcription of E2F target genes. GFP-tagged E2F, even if targeted to the nucleus by an ectopic NLS, was unable to cause postmitotic myotube nuclei to enter S phase. These GFP-tagged molecules were able to bind DNA and pRB family members, but have altered transactivation properties. This would suggest that it is their failure to appropriately regulate expression of E2F target genes that is the cause of their defect in S phase induction.

It has been reported previously that E2F-4 is nuclear during a reversible G_0 arrest of Rat-1 fibroblasts and shifts to the cytoplasm as cells pass into S phase (Muller et al., 1997). However, we detected E2F-4 in the cytoplasm of serum-starved Rat-1 fibroblasts, followed by nuclear compartmentalization as cells reenter the cell cycle. The principle discrepancy in these data occurs in cells arrested in low serum. We observed that cytoplasmic compartmentalization of E2F occurs when both fibroblasts, as well as undifferentiated myoblasts, are arrested in 0.0% serum. However, when the same cells were arrested using 0.2% serum, we observed nuclear staining for the E2Fs. We hypothesized that even a very weak mitogenic stimulus (e.g., 0.2% serum) was sufficient to alter the subcellular localization of the cell cycle regulatory factors. This hypothesis was supported by our observation that tetracycline-dependent expression of cyclin D1 in serum-starved Rat-1 cells caused nuclear localization of E2F-4 and E2F-1. It is interesting to speculate that cytoplasmic localization of E2F in the complete absence of growth factors is analogous to their cytoplasmic localization in terminally differentiated myotubes, which are incapable of responding to growth stimuli.

In conclusion, we have shown that E2F family members, E2F-1, E2F-3, E2F-5, and to a lesser extent E2F-4, are sequestered in the cytoplasm during the G_0 arrest associated with terminal differentiation of muscle. These postmitotic nuclei are still capable of reentering the cell cycle upon an E2F-1– or E2F-4–mediated proliferation stimulus, indicating that cytoplasmic compartmentalization of E2F-1 and E2F-4 is necessary in order to maintain nuclei in a quiescent state in terminally differentiated cells.

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