Multiple Changes in E2F Function and Regulation Occur upon Muscle Differentiation

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We have examined regulation of the E2F transcription factor during differentiation of muscle cells. E2F regulates many genes involved in growth control and is also the target of regulation by diverse cellular signals, including the RB family of growth suppressors (e.g., the retinoblastoma protein [RB], p107, and p130). The following aspects of E2F function and regulation during muscle differentiation were investigated: (i) protein-protein interactions, (ii) protein levels, (iii) phosphorylation of the E2F protein, and (iv) transcriptional activity. A distinct E2F complex was present in differentiated cells but not in undifferentiated cells. The p130 protein was a prominent component of the E2F complex associated with differentiation. In contrast, in undifferentiated cells, the p107 protein was the prominent component in one of three E2F complexes. In addition, use of a differentiation-defective muscle line provided genetic and biochemical evidence that quiescence and differentiation are separable events. Exclusive formation of the E2F-p130 complex did not occur in this differentiation-defective line; however, E2F complexes diagnostic of quiescence were readily apparent. Thus, sole formation of the E2F-p130 complex is a necessary event in terminal differentiation. Other changes in E2F function and regulation upon differentiation include decreased phosphorylation and increased repression by E2F. These observations suggest that the regulation of E2F function during terminal differentiation may proceed through differential interaction within the RB family and/or phosphorylation.

In many cells, the trigger event in differentiation is withdrawal from the cell cycle with subsequent distinct morphological transitions. Muscle cells are an excellent system for examining molecular mechanisms of differentiation because they exhibit both permanent cell cycle withdrawal and a distinctive phenotype. Differentiation involves a transition from myoblasts to myotubes. Myoblasts are undifferentiated cells and are characterized by rapidly growing, mononucleated cells. In contrast, differentiated cells, or myotubes, are multinucleated and tubular. Upon differentiation, myotubes also express several muscle-specific markers. Because differentiated muscle cells (myotubes) are permanently withdrawn from the cell cycle, cellular mechanisms for the initiation and maintenance of the differentiated state must also exist (5).

Studies with viral oncogenes have indicated a critical role for the retinoblastoma family of growth suppressors (the retinoblastoma protein [RB], p107, and p130) in the differentiation pathway. The expression of E1A and polyomavirus large T antigen may block muscle differentiation (7, 49, 66). It is well established that these two divergent viral oncogenes can bind the RB family (69). Importantly, when the binding site for RB family members was mutated, both E1A and polyomavirus large T antigen mutants no longer blocked differentiation (7, 49). These studies suggest that RB family members function in the control of differentiation and that sequestration of RB family members by viral oncoproteins prevents differentiation. The targets of RB family members are usually transcription factors. One target is the MyoD family of transcription factors, which play a critical role in muscle determination and differentiation, but the molecular mechanism remains unclear (24, 59; for reviews, see references 54 and 67). Nonetheless, these divergent studies illustrate the interplay of general and muscle-specific differentiation pathways.

The E2F transcription factor is a potential target of differentiation signals because it is a well-known target of the RB family and has a critical role in cellular growth control. E2F regulates the expression of cell growth control genes, including MYC, MYB, the dihydrofolate reductase gene, the RB gene, and cdc-2 (14, 25, 33, 43, 52, 61, 65). Furthermore, the RB family of growth suppressor proteins are regulators of E2F function (for reviews, see references 28, 44, and 53). The interaction of RB and p107 has been localized to the C-terminal activation region of E2F (13, 30, 40), and high-level expression of RB or p107 can lead to inhibition of E2F function (21, 29, 60, 74, 75). A common feature of RB, p107, and p130 is the presence of pocket domains (19, 26, 36, 39, 47). In the case of RB, this pocket region is the site of tumorigenic inactivating mutations and is critical for the growth suppression function of RB. We and others have demonstrated that E2F binds to this region, providing a genetic correlation between E2F binding and growth suppression (1, 3, 8, 9, 32, 35, 56, 57; for additional reviews, see references 28, 44, and 53). Different E2F complexes with RB or p107 are found during the G1 and S phases of the cell cycle. The p107-E2F complex also has CDK2 and either cyclin A or E (6, 10, 16, 45, 51, 63). To add complexity, different E2Fs are associated with different RB family members, although the p130-interacting E2F(s) has not been described (4, 18, 22). Furthermore, the role of each RB family member in controlling E2F function is not yet clear.

While there has been considerable effort to understand the regulation of E2F during the cell cycle, little is known about its regulation during cellular differentiation. Attractive possibilities for E2F regulatory mechanisms include the RB family of growth suppressors and/or phosphorylation. Because differentiation requires cell cycle withdrawal, our hypothesis was that the net result of E2F regulation was a decrease in E2F-dependent transcription. Using the muscle system, we focused on the following aspects of E2F transcription factor regulation during
cell differentiation: (i) protein-protein interactions, (ii) protein levels, (iii) phosphorylation of the E2F protein, and (iv) transcriptional activity. We have detected one major E2F complex that forms upon differentiation. The E2F complex associated with differentiation was not detected in variants of muscle cells blocked in differentiation but was detected as one of three complexes in quiescent muscle cells and fibroblasts. The p130 protein was detected as a major component of the differentiated complex. The p107 protein was detected as a minor component. In contrast, in undifferentiated cells, the p107 protein was a major component in one of three E2F complexes. While the level of E2F protein remained constant upon differentiation, the level of phosphorylation decreased upon muscle cell differentiation. There is also a decrease in E2F-dependent transcription upon differentiation. Taken together, these data provide evidence that both phosphorylation and differential protein-protein interactions that involve p130 may regulate E2F function during muscle differentiation. Furthermore, irreversible and exclusive formation of this p130-E2F complex is likely critical for differentiation.

MATERIALS AND METHODS

Plasmids. Glutathione S-transferase (GST)–E2F-1 was a generous gift of David Cobrinik and Robert Weinberg (11). Other RB antibodies were purchased from Pharmingen (245) or Oncogene Science. Antibodies for p130 were purchased from Santa Cruz Biotechnology (C-20). The control antibodies were anti-β-galactosidase (purified in Pichia pastoris), G. Corp., and monoclonal antibodies to polyomavirus large T antigen (tissue culture supernatant [PN11]).

In a direct comparison of reactivities, the p130 antibodies exhibited major reactivities with p130 and minor reactivities with p107. The p107 antibodies were absolutely specific for p107. Cell extracts were first immunoprecipitated with anti-p107 or anti-p30 and then analyzed by immunoblot with either p107 or p130 antibodies. Control antibodies were negative in all analyses. Similar results were obtained with untransfected and p107-transfected cell extracts. To locate immunoreactive bands, an immunoblot of a p107 immunoprecipitate (IP) with p107 antibodies revealed an ~115,000-molecular-weight band (~115 K), which is consistent with p107. An immunoblot of a p30 IP with p130 antibodies showed two 115K and 125K bands, which is consistent with both p107 and p30. To test cross-reactivity, the p107 IP was then immunoblotted with p130 antibodies, and the result was a 115K band. The p30 IP was then immunoblotted with p107 antibodies, and only the 115K, not the 125K band, reacted. Therefore, the p30 antibodies can recognize p107 in an immunoprecipitation and Western blot (immunoblot) with the p107 antibodies (data not shown).

Generation of polyclonal antibodies to E2F-1. Plasmids that encoded GST-E2F-1 protein were transformed into Escherichia coli JM109. Bacteria that expressed anti-polyomavirus large T antigen. These variant C2 cells were transferred to 2% FCS–DMEM. The inability to differentiate was confirmed by a lack of myosin heavy chain (MHC) immunofluorescence (data not shown).

For experiments to examine quiescence, either NFB or 10T1/2 (gift of Van Cherooting) cells were tested for 72 h in medium with 0.2% serum. This serum concentration is 10-fold lower than used in the previous experiment. This was necessary to obtain complete quiescence, which was scored as an absence of bromodeoxyuridine (BrdU) incorporation (data not shown).

Cell extracts. Extracts were prepared from undifferentiated and differentiated cell lines. Cells were lysed in buffer (150 mM NaCl, 1% NP-40 [w/vol], 50 mM Tris [pH 8.0]) that contained inhibitors (200 μM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 0.4 mM orthovanadate, 0.4 mM sodium fluoride, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid]). After brief centrifugation in a microcentrifuge to remove cellular debris, the lysate was spun at 100,000 × g and glycerol (to 20% [w/vol]) was added to the supernatant. Aliquots were stored at −70°C.

EMSA. Electrophoretic mobility shift assays (EMSA) were performed with undifferentiated and differentiated cell extracts. EMSAs were performed under standard conditions for E2F (72). The probe was derived from the adenovirus E2 promoter and consisted of two E2F sites. Competitor DNAs were added at 100-fold molar excess over the probe. Competition of two E2F sequences from the E2 promoter. Competitor A consists of a Ct-o-A mutation in each E2 site, and this mutant site exhibits reduced E2F binding (35). A typical E2F EMSA utilized 3 to 5 μg of muscle cell extract and 0.1 ng of 5′-labeled E2F probe.

Antibody shift EMSA. Antibody shift EMSA was performed exactly as described above for a normal EMSA, except that the indicated antibodies were included in the binding reaction. When a purified antibody was used (e.g., anti-p107 anti-serum), the control antiserum was a rabbit antiserum specific for p130-β-galactosidase and was used at an identical concentration. When tissue culture supernatants were used (e.g., anti-p107 SD6, SD9, and SD15), the control antibody was an identical amount of tissue culture supernatant from a monoclonal cell line that expressed anti-polyomavirus large T antigen. Addition of an excess (2- to 5-fold) of either control or test tissue culture supernatant resulted in complete nonspecific inhibition of E2F binding. In experiments with antibodies in the form of tissue culture supernatants (e.g., SD 15), 0.1 μl (or 1 μl of 1:10 dilution) was used. In experiments with purified antisera (anti-p130), 50 ng was used.

DOC release EMSA. DOC release EMSA proceeded essentially as described previously (63). Two hundred micromolar pseudotransfected and differentiated cell extracts was incubated with either 3 μl of p107-specific antisera (SD15) or 1 μl of p130-specific antisera (SD6) for 30 min on ice. Then the tissue culture supernatant with anti-polyomavirus large T antigen antibodies. Ten microfilters of beads was added to each tube. Tubes were incubated for a further 30 min on ice. Beads were washed three times with a HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-buffered solution (20 mM HEPES [pH 7.4], 40 mM KCl, 1 mM MgCl2, 0.1 mM EDTA). Beads were then incubated with 10 μl of 20 mM HEPES (pH 7.4) that contained 0.6% deoxycholate (DOC; wt/vol) for 10 min on ice. Next, 10 μl of 2.5% NP-40 (w/vol) was added to tubes to a final concentration of 1.25% (wt/vol) NP-40. After centrifugation, 7 μl was used for an E2F EMSA in the presence and absence of a 100-fold molar excess of unlabeled competitor (wild-type E2F DNA binding sites).

In vivo labeling and immunochemoical methods. Phosphorylation of E2F was measured by immunoprecipitation of C2 cells labeled with [32P] (1 mCi/ml; NEN) in phosphate-free media (Gibco-BRL) for 2 h. Cell extracts were normalized for protein concentrations and E2F levels (by immunoblot) prior to immunoprecipitations. Incorporation of 32P in undifferentiated C2 cells was approximately equal to that in differentiated C2 cells (data not shown). Equal amounts of protein were immunoprecipitated with 5 μl of anti-E2F-1 polyclonal serum and 40 μl of protein A sepharose beads. Beads were then washed several times with NETN, and bound proteins were boiled off the beads in the presence of 20 μl of solubilization buffer (50 mM triethanolamine [pH 7.4], 0.4% sodium dodecyl sulfate (SDS), and 2 mM β-mercaptoethanol). After boiling, this solution was added to 10 μl of 32P-labeled E2F, treated with 50 μl of 10% Triton X-100 (vol/vol) and reincubated with either anti-E2F-1 polyclonal antisera or preimmune antisera (12). IPs were analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE) and autoradiography. Immunoblots were performed essentially as described previously (27). Approximately 100 μg of protein extract was analyzed in each lane and transferred to nitrocellulose. E2F antisera was used at a dilution of 1:10,000. Immunoreactive species were visualized with an alkaline phosphatase secondary antibody complex (Jackson Labs). To establish the specificity of an immunoreactive
band, E2F antiserum was blocked by prior overnight incubation with purified GST-E2F or GST (100 µg/20 µl of serum). This blocked serum was then used in a standard immunoblot experiment.

**Transfections.** Transfections into C2 cells were done by the BES method (58), and expression in undifferentiated and differentiated C2 cells proceeded exactly as described by Olson and colleagues (64). Thirty micrograms of reporter plasmid and 3 µg of a reference luciferase plasmid (pGLC-luciferase [Promega] or SV-luciferase) were added per 100-mm-diameter plate. For expression in undifferentiated cells, cells were transfected at 20 to 40% confluence and the medium was changed after 16 h. After 48 h, cells were harvested for analysis as undifferentiated extracts. For expression in differentiated cells, cells were transfected at 70 to 80% confluence. After 16 h, the medium was changed to 20% FCS–DMEM for 24 h, and then cells were differentiated for 48 h in 2% FCS–DMEM prior to being harvested as differentiated extracts. In each case, cell pellets were lysed in 250 mM Tris (pH 7.8) by being freeze-thawed four times and sonicated twice for 30 s. Cell debris was removed by centrifugation for 10 min at 4°C in a microcentrifuge. Five microliters of each supernatant was assayed by the Promega luciferase assay system, and the luciferase amounts (in femtomoles of luciferase per sample) were determined from a standard curve with known amounts of luciferase. Chloramphenicol acetyltransferase (CAT) expression was assayed by CAT enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim), and the CAT amounts (in picograms of CAT per sample) were determined from a standard curve with known amounts of CAT protein. Relative CAT expression was expressed as picograms of CAT per femtomole of luciferase. All transfections were done in duplicate, and the values varied by less than 10%.

The E2F reporter construct contains four E2F binding sites from the adenovirus E2 promoter and is identical to the sites used in EMSA (72). The A10-CAT construct contains only the simian virus 40 (SV40) minimal promoter and served as a control.

**RESULTS**

**Multiple changes in E2F complexes are associated with muscle differentiation.** The initial goal was the identification of an E2F complex associated with differentiation. For these studies, the C2 muscle cell line was selected because differentiation proceeded efficiently and completely in culture (71). The standard conditions for growth were 20% FCS with subsequent exposure to 2% FCS to initiate differentiation (64). Full differentiation is observed within 48 h and is characterized by the appearance of multinucleated myotubes and expression of several muscle-specific markers. For these experiments, a C2 subclone that had been selected for efficient myotube fusion was used (gift of Zach Hall).

A critical parameter in the identification of an E2F complex associated with differentiation is the degree of differentiation of the cell population. A strength of this muscle system is its ability to score differentiation unambiguously. A common marker for differentiation is MHC, and excellent antibodies are available (F59; gift of Frank Stockdale). To ensure that a given extract contained completely undifferentiated or differentiated muscle cells, we used immunofluorescence to determine the percentage of MHC-positive cells in any population. Undifferentiated cells typically exhibit <1% MHC-positive myoblasts, but differentiated cells display 100% MHC-positive myotubes. In prepared extracts, cells were cultured in petri dishes in which several coverslips were placed for later quantitation of cell differentiation by MHC immunofluorescence.

The E2F complexes in undifferentiated and differentiated muscle cells were then characterized by a standard E2F EMSA. Unless indicated otherwise, E2F refers to a specific binding activity for an E2F-specific DNA probe which contains two sites and is derived from the E2 promoter (72, 73). Recent evidence indicates that in human cells, E2F represents a transcription factor family (at least E2F-1 to E2F-4 and DP-1) (4, 22, 23, 30, 31, 37, 40, 42, 46, 62). The E2F and DP families form heterodimeric complexes that are implicated in enhanced E2F-dependent transcriptional activation (2, 31, 42). There has been some characterization of murine E2Fs, but the list is probably not complete (23, 48). Therefore, we hereafter refer to our complexes from murine C2 cells simply as E2F.

![FIG. 1. E2F EMSA of undifferentiated and differentiated C2 muscle cells. E2F for E2F were performed with undifferentiated (U) and differentiated (D) wild-type C2 cell extracts. Cell culture, differentiation conditions, and extract preparation were carried out as described in Materials and Methods. The probe was derived from the adenovirus E2 promoter and consisted of two E2F sites. Competitor DNAs were added at a 100-fold molar excess. Competitor wt consisted of the E2F sequences from the E2 promoter. Competitor A consisted of a C-to-A mutation in each E2F site, and this mutant site gave reduced binding by E2F. The wild-type competitor, but not the mutant competitor, should abolish binding to the 32P-labelled E2F-specific probe. The competitor used in each lane is indicated, and 5 µg of each extract was used for each reaction. E2F complexes are indicated by arrowheads.](http://mcb.asm.org/).
plexes were not simply a result of the tissue culture conditions used to induce differentiation. An E2F complex formed in response to reduced-serum medium conditions, but not to full differentiation, would be present in both normal and variant C2 lines. However, an E2F complex associated with differentiation should be evident in normal C2 differentiated cells but not in C2 variants.

The two nondifferentiating variants were NFB and WT-LT C2 cells. The disruption of differentiation occurs by genetic mutation and the expression of a virus oncogene, respectively. The NFB line was originally isolated by mutagenesis of a C2 line and selection for the inability to differentiate under normal conditions. The genetic locus that becomes mutated in the NFB line has not yet been defined, but levels of myogenic regulators were markedly reduced. The hypothesis, supported by heterokaryon fusion experiments, is that the mutation leads to production of a dominant repressor of myogenic regulators (55). A second nondifferentiating variant is WT-LT, a C2 line in which the expression of polyomavirus large T antigen blocks muscle cell differentiation (49, 50). Analogous studies with adenovirus E1A (66), which also blocks muscle differentiation, have suggested that the mechanism may involve RB family members.

E2F EMSA from NFB and WT-LT C2 cell extracts are shown in Fig. 2. Differentiated extracts refer to cells treated under differentiation conditions exactly as described for normal C2 cells. MHC immunofluorescence confirmed that differentiation did not occur in NFB or WT-LT cells (data not shown). In contrast to normal C2 cells, only E2F complexes characteristic of undifferentiated cells were observed in all extracts (Fig. 2A, lanes 1 to 6, and B, lanes 13 to 18). In these distinct differentiation-defective cell lines, the E2F complexes associated with differentiation did not form under conditions in which normal C2 cells efficiently undergo differentiation (compare lanes 10 to 12 in Fig. 2 with lanes 4 to 6 [NFB] and 16 to 18 [WT-LT]). Therefore, the presumptive E2F complex associated with differentiation that we have identified in normal C2 cells could be correlated with terminal differentiation of muscle cells.

Next we determined if the E2F complex correlated with differentiation was also found in quiescent cells. Both differentiated and quiescent cells are characterized by cell cycle exit, although differentiated cells can never reenter the cell cycle. The most common system for studies of quiescent cells is serum starvation of fibroblasts. The C3H 10T1/2 fibroblast line was used since this line can eventually form muscle upon the expression of myogenic regulators (15). However, a caveat of this analysis is that a fibroblast is not a muscle cell. A second line analyzed was the NFB muscle cell line used in the previous experiment. In normal C2 muscle cells, quiescence and differentiation cannot be uncoupled. For the differentiation-defective NFB cell line, we hypothesized that these cells were rendered quiescent without exhibiting full differentiation. In contrast to Fig. 2, complete quiescence was achieved only with much lower serum conditions than those used for differentiation. It should be also noted that both NFB and C3H 10T1/2 cells are derived by chemical mutagenesis.

Analyses of E2F complexes in quiescent muscle cells and fibroblasts revealed three distinct E2F complexes (B, C, and D). Complexes C and D were similar in both growing and quiescent cells, indicating that these complexes are growth independent (Fig. 3; compare lanes 1 and 3 and lanes 5 and 6). Complex B comigrated with the E2F complex from differentiated cells. The same three complexes were also obtained in quiescent NIH 3T3 cells, another fibroblast line. The percentage of cycling cells was assayed by immunofluorescence following a pulse of BrdU. Only cells in S phase should incorporate BrdU. Growing and quiescent cells exhibit about 30 and 0% BrdU immunofluorescence, respectively (data not shown). In NFB cells, there is apparently some residual complex A, although the appearance of complex B is more striking. Thus, quiescence resulted in an intermediate complement of E2F complexes between undifferentiated and differentiated cells. However, these studies underscore the importance of complex B, which was present in both quiescent and terminally differentiated cells.

In summary, undifferentiated muscle cells and growing fibroblasts result in three E2F complexes (A, C, and D). Complex A appeared to be primarily cell cycle dependent. The trigger event in differentiation is cell cycle withdrawal; this characteristic is shared by quiescence. However, a major difference between differentiated and quiescent cells is the reversibility of cell cycle exit; it is irreversible in differentiated
cells but reversible in quiescent cells. During differentiation, quiescence cannot usually be distinguished from terminal differentiation. However, in the NFB muscle cell line, quiescence and differentiation can be distinguished since terminal differentiation is blocked by mutation. Quiescence of this differentiation-defective muscle cell line or two fibroblast lines resulted predominantly in the formation of three E2F complexes (B, C, and D). Complexes C and D appeared to be cell cycle independent and were present in both growing and quiescent cells. These are likely the presumptive free forms of E2F since RB family members added in vitro bound to these complexes (data not shown). Complex B appeared to be formed in response to cell cycle exit. In contrast to quiescence, differentiation resulted in the appearance of a single E2F complex (complex B) and in the disappearance of cell cycle-independent E2F complexes C and D. Furthermore, its irreversible and exclusive formation of E2F complex B could account for the permanent cell cycle withdrawal that is characteristic of terminal differentiation.

**p130 protein is a component of the predominant E2F complex associated with differentiation.** Because a single E2F complex appeared with terminal differentiation, our next efforts focused on the identification of regulatory components. The likely candidates are the RB family of growth suppressors since they are known inhibitors of E2F function and may participate in cell cycle exit. Thus, we performed complete analyses of the known RB family members, p107, RB, and p130. Three immunological approaches were used to probe the presence of RB family members in association with E2F during differentiation. Thus, the critical reagents were antibodies against each RB family member. By immunoprecipitation, monoclonal p107 antibodies are specific for p107 and do not cross-react with RB or p130 (generous gift of Ed Harlow and Nick Dyson) (11, 18) (data not shown). The RB antibodies used (21C9 and 245) have been demonstrated to react with murine RB and not to react with p107 or p130 (11). By immunoblot with cell extracts, p130 antibodies appeared to be specific for p130 and did not exhibit cross-reactivity with either RB or p107. However, in a more direct test of cross-reactivity between p107 and p130 by immunoprecipitation-immunoblot assay with p107-transfected cells, this anti-p130 antibody exhibited major reactivity with p130 and weak reactivity with p107 (data not shown).

Our first approach tested for the presence of RB family members in the E2F complex associated with differentiation by antibody shift EMSA. Antibodies to each RB family member were added to an EMSA reaction, and the effects on various DNA-binding complexes were assessed. For comparison, identical experiments were performed on complexes from undifferentiated cells. As seen in Fig. 4, a supershifted complex was observed in differentiated cells when p130 antibody was added (Fig. 4, lane 12). Because p130 antibody has cross-reactivity with p107, this supershift could be due to reactivity with p130 and/or p107. However, p107 antibodies gave no supershifted complex in differentiated cells (Fig. 4, lane 11). The lack of a supershift with p107 antibodies and the complete supershift of the differentiated E2F complex with p130 antibodies suggest that p130 is the major component. Two control antibodies were also employed (in purified form [Fig. 4, lane 13] and as a tissue culture supernatant [Fig. 4, lane 14]). No RB antibody (21C9, 245, or RB AB 1, RB AB 2, and RB AB 3 [Oncogene Science]) had any effect on the differentiated complex (Fig. 4, lane 10, and data not shown).

In contrast, in undifferentiated cells, the addition of p107 antibody resulted in complete conversion of the upper complex to a supershifted complex (Fig. 4, lane 4). Furthermore, addition of p130 antibody also partially supershifted the upper undifferentiated E2F complex (Fig. 4, lane 5). Again, neither control antibodies nor RB antibodies exhibited significant effects (Fig. 4, lanes 3, 6, and 7). Because only one of these three complexes was affected, these antibody reactions are likely to be specific. The apparent absence of p107 and p130 in these faster-migrating E2F complexes from undifferentiated cells suggests that these may represent free E2F forms. Indeed, the addition of GST-p107 did result in efficient binding to these faster-migrating E2F complexes (data not shown). The results in Fig. 4 are consistent with the presence of p107 for two reasons. The p130 antibody does recognize p130 better than it does p107 and would be predicted to give a partial supershift of a p107-containing complex, but this alone does not rule out the presence of p130. However, p107 antibody, which is absolutely

![FIG. 3.](image) E2F EMSA of quiescent muscle and fibroblast cell lines. NFB muscle cells and 10T1/2 fibroblasts were either grown in 15% serum or rendered quiescent by treatment in 0.2% serum for 72 h. Quiescence was quantitated by BrdU incorporation and immunofluorescence. Growing cells exhibited about 30% staining; quiescent cells exhibited 0% staining (data not shown). Please note that these NFB cells were treated with 10-fold-less serum than those used for the experiment shown in Fig. 2. Lanes 1 to 4, NFB cells; lanes 5 to 7, 10T1/2 cells. Lanes 1 and 2, E2F complexes from growing NFB cells; lanes 3 and 4, complexes from quiescent NFB cells. Competitor DNAs were included in lanes 2 and 4 to establish specificity. Lane 5, E2F complexes from growing 10T1/2 fibroblasts; lanes 6 and 7, E2F complexes from quiescent 10T1/2 cells. Competitor DNA was included in lane 7 to establish specificity. For comparison with previous figures, E2F complexes A, C, and D comigrate with those from undifferentiated cells in Fig. 1 and 2. E2F complex B comigrates with the single complex observed in differentiated cells in Fig. 1 and 2.
specific for p107, completely supershifted this undifferentiated E2F complex. Thus, the data argue that p107 is the predominant component in the upper undifferentiated E2F complex.

To probe for the presence of p130 and p107 in all E2F complexes, DOC release EMSA from p107 and p130 IPs were utilized. DOC releases proteins, e.g., E2F bound to p107, but does not significantly perturb the antibody-p107 interaction (63). The released E2F activity can be detected by EMSA.

Because protein-protein interactions were largely disrupted, the mobilities of the E2F complex from undifferentiated and differentiated cell extracts were equal. In both cases, specificity was established by competition with excess plasmid that contained wild-type E2F sites.

In Fig. 5, the results of DOC release EMSA with anti-p107 and anti-p130 antibodies are shown. E2F-DNA binding activity was detected in differentiated cells by both p130 and p107 immunoprecipitations (Fig. 5, lanes 9 to 12). This confirms the presence of p130 in association with E2F during differentiation and further suggests that a p107-E2F complex is present at a minor level. No p107-E2F DNA-binding complex was detected by the supershift assay upon differentiation. In agreement with the data in Fig. 4, the levels of the p107-E2F complexes do
appear to decline with differentiation. In addition, E2F activity was also detected in undifferentiated cells by assays with p130 and p107 antisera (Fig. 5, lanes 3 to 6). The DOC EMSA results for p130 (Fig. 5, lane 3) are likely due to cross-reactivity with p107. However, the presence of p130 cannot be ruled out unambiguously. This antisera binds with high affinity to both p130 and p107, although there is reactivity selectivity for p130. While it is apparent that the levels of p107-E2F complexes decline with differentiation, no comparison of p130-E2F complexes during differentiation is possible because of antisera cross-reactivity. Immunoprecipitation with control antibodies gave no E2F signal (Fig. 5, lanes 1, 2, 7, and 8). Thus, during differentiation, the association of p107 and p130 with E2F parallels their presence in active E2F DNA-binding complexes.

The simplest interpretation of these results is that p130-E2F is a major complex and that p107-E2F is a minor complex in differentiated cells. p130 antibody supershifted the entire E2F complex associated with differentiation (Fig. 4, lane 12). p130 antibody exhibited good reactivity with p130 and weaker cross-reactivity with p107. Because p107 antibody was clearly functional in a supershift assay (Fig. 4, lane 4), the lack of reactivity for anti-p107 with the E2F complex associated with differentiation (Fig. 4, lane 11) was consistent with the hypothesis that p130, not p107, is the major component. DOC EMSA (Fig. 5) also confirmed the results of supershift assays and provided further evidence for a major E2F interaction with p130. p107-E2F interaction was also detected by this assay. Consistent with other work (6, 51, 60, 63, 74), p107 is a prominent component in at least one E2F complex from undifferentiated cells. The data in Fig. 4 and 5 are consistent with the interpretation that there are three E2F complexes in undifferentiated cells, one of which contains p107. Upon differentiation, there is a conversion to one E2F complex in which p130 is the predominant component.

Phosphorylation of E2F decreases with differentiation. Regulation of E2F during differentiation may proceed through changes at several levels: protein-protein interactions, amounts of E2F protein, and/or E2F phosphorylation. Work by our laboratory and others has indicated that phosphorylation of E2F is complex and may result in regulation of function (17, 41, 70). Changes in protein-protein interactions have been demonstrated above. As a first step to determining whether there are other differentiation-specific changes in E2F protein, we measured E2F levels and phosphorylation changes upon differentiation.

Antibodies were generated to GST–human E2F-1, and cross-reactivity in mouse muscle cells was tested. Probing antibodies were raised against human E2F-1 but reacted specifically in mouse muscle cell extracts with an ~60K band, a molecular weight similar to that of human E2F-1 (Fig. 6A). Preincubation of antibodies with excess GST–E2F-1 blocked the signal in mouse extracts (Fig. 6B), confirming specificity for E2F. However, assignment of this band to mouse E2F-1 cannot be made, so this band is hereafter referred to as E2F. Like E2F-1 antibodies generated in other laboratories, these antibodies failed to affect most of the E2F DNA binding activity observed (40).

After the E2F antibody had been characterized, E2F protein and phosphorylation levels were examined during differentiation. In Fig. 6, when equal amounts of extract protein were loaded, the levels of E2F were unchanged by differentiation. E2F phosphorylation levels were assessed by immunoprecipitation from 32P-labelled undifferentiated and differentiated C2 cells. In Fig. 7, extracts from undifferentiated and differentiated cells were analyzed by immunoblotting and immunoprecipitation from 32P-labelled cells (12, 27). Incorporation of 32P in undifferentiated cells was similar to that in differentiated cells (data not shown). A preimmune serum was used as a control. No signals were detected in immunoprecipitations of identical samples with preimmune sera, which establishes the specificity of any E2F signal (Fig. 7B, lanes 1 and 3). However, there was a marked decrease (~fivefold) in phosphorylation of E2F protein upon differentiation (compare lanes 2 and 4 in Fig. 7B). Immunoblotting of the identical extracts again indicated no changes in E2F levels and ensured that equal amounts were used in immunoprecipitation reactions (Fig. 7B).
7A). Therefore, while E2F protein levels did not decline with differentiation, the extent of phosphorylated E2F was markedly decreased.

**Differentiation regulates E2F-dependent transcription.** Because protein-protein interactions and phosphorylation of E2F changed markedly with differentiation, we next determined whether E2F-dependent transcriptional activity also varied with differentiation and whether any changes correlated with the appearance of a specific E2F complex.

The transcriptional activity of E2F in undifferentiated and differentiated cells was measured by transient transfection of an E2F reporter gene (72). E2F sites identical to those used for all previous EMSA were also used in the reporter construct, which consists of four E2F sites fused upstream of a minimal SV40 promoter (A10-E2F-CAT). Furthermore, this reporter construct had previously been used to demonstrate that E1A-dependent activation proceeded through E2F sites (72). The control construct contained only a minimal SV40 promoter and lacked E2F sites (A10-CAT). The reference plasmid SV-luciferase was used to normalize for transfection efficiency. SV40 promoter activity does not vary with C2 cell differentiation (64). Relative CAT expression was expressed as a CAT protein/luciferase ratio (in picograms per femtomole). Transfection efficiency was determined by the expression of SV-luciferase. Both CAT protein and luciferase were measured by quantitative ELISA and enzymatic assays, respectively. The values were determined with standard curves of known CAT protein and luciferase enzyme amounts.

**DISCUSSION**

In this paper, we have reported on experiments that examined regulation of E2F protein in response to terminal differentiation of muscle cells (summarized in Fig. 9). Because of their ability to clearly exhibit the differentiated phenotype, muscle cells provide an excellent system for determining the molecular mechanisms of initiation and maintenance of differentiation. In C2 muscle cells, we have demonstrated that the E2F transcription factor forms a single complex during differentiation. This E2F complex comigrated with one of the three complexes identified in three quiescent cell lines, suggesting that it is indicative of cell cycle exit. In our studies, quiescence represents an intermediate state between growing and differentiated cells.

Differentiation can be divided into two stages, initiation and maintenance. Generally, withdrawal from the cell cycle is the trigger step for initiation of differentiation. This step may be reversible and share some characteristics with quiescent, non-differentiating cells. The maintenance step is characterized by irreversible cell cycle exit and morphological transitions. The hallmark of a terminally differentiated cell is its inability to reenter the cell cycle. Thus, entry into G0 is necessary but is not sufficient for terminal differentiation. Mechanisms to maintain permanent cell cycle exit must exist.

Analyses of E2F complexes suggest an intriguing model in which full differentiation requires permanent formation of the E2F-p130 complex associated with quiescent fibroblasts and fully differentiated muscle cells. Quiescent cells can be viewed as being in a metastable state in which cell cycle exit is revers-
ible. Correspondingly, the E2F complexes exhibited during quiescence are composites of those in undifferentiated and differentiated cells. In contrast to previous studies (11), we consistently observed three E2F complexes in three separate cell lines that had been rendered quiescent. The reason for this disparity is not clear, but it may be the result of our more sensitive assay conditions. From studies discussed in this paper and others, it is likely that the E2F-p130 complex is indicative of cell cycle exit. Irreversible and exclusive formation of the singular E2F-p130 complex in differentiated cells would explain the established inability of differentiated cells to reenter the cell cycle.

This notion was supported by experiments with the differentiation-defective NFB cell line and wild-type C2 muscle cell line in which quiescence and differentiation were separated. Full quiescence of NFB muscle cells resulted in the appearance of three E2F complexes, but full differentiation in wild-type C2 muscle cells resulted in formation of one E2F-p130 complex. These experiments also indicate that NFB cells are blocked in the differentiation pathway downstream of cell cycle exit, allowing the uncoupling of quiescence and differentiation within a muscle cell. Another notable difference between quiescence and differentiation is the disappearance of cell cycle-independent forms of E2F (complexes C and D). These experiments indicate that a specific transition in E2F complexes occurs with simple quiescence and that it likely involves the disappearance of the p107-containing E2F complex and replacement with a p130-E2F complex. There are probably no changes in cell cycle-independent E2F complexes with quiescence. In the transition to terminal differentiation, the appearance of the E2F-p130 complex is exclusive. Either the cell cycle-independent forms are degraded or they are completely bound by p130. These possibilities cannot yet be distinguished. The net result is a predominant E2F-p130 complex in the terminally differentiated cell.

These studies indicate the importance of conversions within the RB family during differentiation. In undifferentiated muscle cells, there are three E2F complexes, one of which contains p107. This is consistent with the diversity found in other cells (3, 6, 10, 16, 45, 51, 63, 70). Net transcriptional output is presumably a composite of both activating and repressive E2F complexes. There is already evidence that E2F may function as a positive or negative regulator of transcription (34, 38, 43, 68). Our previous data for cyclin A-CDK2 regulation of E2F-1 as a positive or negative regulator of transcription (34, 38, 43, 68).

Upstream of the transcription output are a number of regulatory events that converge during differentiation. These events include the uncoupling of quiescence and differentiation. In the transition to terminal differentiation, the appearance of the E2F-p130 complex is exclusive. Either the cell cycle-independent forms are degraded or they are completely bound by p130. These possibilities cannot yet be distinguished. The net result is a predominant E2F-p130 complex in the terminally differentiated cell.

Upon cell cycle exit and subsequent differentiation, there is eventual conversion to an E2F complex that contains p130 and an increase in E2F-mediated repression of transcription. The transcriptional data in Fig. 8 are intriguing and suggest that E2F can function as a repressor upon differentiation. The appearance of this predominant p130-E2F complex is consistent with a role in the repression of E2F-dependent transcription. Because differentiation requires cell cycle withdrawal and E2F is known to activate genes in a cell cycle-dependent manner, altering the function of E2F from activator to repressor may be one mechanism by which differentiation is initiated and maintained. These studies indicate that p130 protein is a critical player in initiation of cell cycle withdrawal and maintenance of differentiation.

Upon full muscle differentiation, there is also a decline in the phosphorylation of E2F protein, with no decrease in the E2F protein level. A recent study suggested that phosphorylation modulates E2F interaction with RB (20). The hypothesis that differential phosphorylation modulates interactions with different RB family members during differentiation is attractive. Then p130 would be predicted to bind to the unphosphorylated form of E2F. This interaction would also lead to repression of E2F-dependent transcription. Although this is an attractive model, a direct test requires definitive identification of the E2F family member in C2 cells. A direct test of this model also requires analyses of numerous phosphorylation sites, and only the CDK sites of E2F-1 have been analyzed (70). Nonetheless, the studies described in this paper document multiple regulatory changes that ultimately result in decreased transcription or increased repression by E2F. Because E2F is only one component that must be regulated, these studies provide a glimpse of the complexity of pathways that must converge during differentiation in muscle cells.

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