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Uncoupling of p21 Induction and MyoD Activation Results in the Failure of Irreversible Cell Cycle Arrest in Doxorubicin-Treated Myocytes

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Abstract Doxorubicin (Dox, Adriamicin), a potent broad spectrum anthracycline anticancer drug, selectively inhibits muscle specific gene expression in cardiac cells in vivo and prevents terminal differentiation of skeletal muscle cells in vitro. By inducing the expression of the helix-loop-helix (HLH) transcriptional inhibitor Id2, Dox represses the myogenic function of the MyoD family of muscle regulatory factors (MRFs). In many cell types, terminal differentiation is coupled to an irreversible exit from the cell cycle and MyoD plays a critical role in the permanent cell cycle arrest of differentiating myocytes by upregulating the cyclin dependent kinase inhibitor (cdki) p21. Here, we correlate Dox effects on cell cycle with changes of E2F/DP complexes and activity in differentiating C2C12 myocytes. In Dox-treated quiescent myoblasts, which fail to differentiate into myotubes under permissive culture conditions, serum re-stimulation induces cyclin/cdk re-association on the E2F/DP complexes and this correlates with an evident increase in E2F/DP driven transcription and re-entry of myoblasts into the cell cycle. Despite Dox ability to activate the DNA-damage dependent p53/p21 pathway, when induced in the absence of MyoD or other MRFs, p21 fails to maintain the postmitotic state in Dox-treated myocytes induced to differentiate. Thus, uncoupling p21 induction and MyoD activity results in a serum-reversible cell cycle arrest, indicating that MRF specific activation of cdki(s) is required for permanent cell cycle arrest in differentiating muscle cells. J. Cell. Biochem. 66:27–36, 1997.

Key words: cell cycle; p21; MyoD; E2F; doxorubicin/adriamicin

Doxorubicin (Dox) is an anthracycline anticancer drug widely used in the treatment of many tumors, which causes a dilatative cardiomyopathy in vivo. Dox has been also reported to inhibit both biochemical and phenotypical skeletal muscle differentiation in vitro [Kurabayashi et al., 1993]. This effect has been related to Dox ability to induce Id2, an inhibitor of the myogenic differentiation [Kurabayashi et al., 1994]. Id2 exerts its inhibitory effect by sequestrating the E proteins, E12 and E47, which are the DNA binding partners of the basic-Helix-Loop-Helix (bHLH) muscle regulatory factors (MRFs) MyoD, myogenin, Myf5, and MRF/Myf6/Herculin [Benezra et al., 1990]. Upon appropriate in vitro conditions (i.e., cell confluence and low serum culture), MRFs bind specific DNA sequences, the E-box sites, thereby inducing the transcription of muscle specific genes [for review see Olson and Klein, 1994]. MRF activation in myoblasts undergoing myogenic differentiation is ensured by a complex regulatory interplay between these regulatory factors and is inhibited by high Id levels [see Lassar and Munsterberg, 1994].

Terminal differentiation of skeletal and cardiac myocytes, as well as of neurons, keratinocytes, and adipocytes, is coupled with an irreversible withdrawal from the cell cycle [Stockdale and Holtzer, 1961; Nadal-Ginard, 1978]. As regards MyoD, an overlap in the functions ensuring both biochemical/phenotypical differentia-

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tion and permanent cell cycle arrest has been described. Indeed, besides its role as activator of muscle-specific gene transcription, MyoD is able to suppress cell proliferation [Crescenzi et al., 1990; Sorrentino et al., 1990]. This property has been related to its specific ability to induce, during myogenic differentiation, the transcription of the universal cyclin-dependent kinase inhibitor (cdki) p21 [Halevy et al., 1995; Guo et al., 1995]. In addition, a physical interaction between MyoD and the growth suppressor factor pRb has been described and is thought to contribute to both cell cycle arrest and myogenic differentiation [Gu et al., 1993]. Upregulation of p21 occurs in several other circumstances. The paradigm for p21 induction is that observed following DNA damage-dependent p53 transcriptional activation after treatment with many chemiotherapic agents [see Ko and Prives, 1996].

In this study, we evaluate the effects of Dox, a chemiotherapic drug known to induce either directly or indirectly a DNA damage response, on the permanent withdrawal from the cell cycle of differentiating myocytes. We found that Dox-treated myoblasts, which fail to differentiate into myotubes upon conditions permissive for differentiation, undergo a serum-reversible cell cycle arrest despite a strong induction of p21. Since the MyoD downstream target p21 suppresses E2F/DP activity by inhibiting cyclin/cdk-dependent phosphorilation of the pRb-family members [see MacLachlan, et al., 1995] and prevents cyclin/cdk re-association on the E2F/DP complexes present at the G1/S phase boundary [Shiyanov et al., 1996; Puri et al., 1997b], we correlated Dox effects on cell cycle progression with changes of E2F/DP DNA bound complexes and activity in myocytes. We found that in Dox-treated myoblasts, p21 induction is uncoupled from MyoD (and other MRFs) activation and this results in the failure to induce a serum-irreversible cell cycle arrest. Thus, in skeletal muscle cells induced to differentiate, p21 requires the functional integrity of MyoD (or other MRFs) to induce a permanent exit from the cell cycle.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

C2C12 mouse cell line [Yaffe and Saxel, 1977] were grown in DMEM supplemented with 20% foetal bovine serum (cycling myoblasts) until they reached 80–90% confluence, when differentiation was induced by switching cell cultures to DMEM containing 2% foetal bovine serum (DM, differentiation medium). Quiescent C2C12 myoblasts are nonconfluent cells cultured in 0.1% foetal bovine serum for 48 h. They are negative for muscle-specific markers (myosin heavy chain and desmin), and for BrdU, but still able to incorporate BrdU following serum stimulation [Puri et al., 1995]. Dox treatment of C2C12 cells was performed according to Kurabayashi et al. [1994]. Briefly, cells were cultured for 24 h in 20% foetal bovine serum–enriched DMEM in the presence or absence of Dox (1 μM) and then switched to DM for 24 to 48 h.

Oligonucleotides

The wt E2F oligonucleotide probe sequences were derived from the adenovirus E2 promoter and consisted of one E2F site. The mutant E2F oligonucleotide contains a C-to-A mutation, which determines a strongly reduced E2F binding activity. The E-box oligonucleotide probe sequence derived from the MCK enhancer was 5′CCCCAACACCTGCTGCCTGA3′.

DNA, Transfections, and CAT Assay

C2C12 cells were plated in 10% serum-enriched medium in the presence or absence of Dox for 24 h and transfected by the calcium phosphate method. After 24 h of incubation with the DNA precipitate, cells were washed and maintained for 72 h in medium containing 2% serum, and harvested and assayed for reporter gene expression as previously described [Natoli et al., 1994]. For these experiments we used a CAT reporter plasmid containing 3 E2F sites derived from the c-Myc P2 promoter (a gift from D. Hall).

Western Blot

Total cell lysates were loaded and then resolved on SDS-PAGE. After overnight electrophoresis, gels were equilibrated for 30 min in transfer buffer (25 mM Tris, 200 mM Glycine) and transferred to PDVF membranes (Millipore, Bedford, MA) at 0.25 milliAmpere for 5 h at 4°C. Membranes were first incubated in 1 × TBS (20 mM Tris, pH 6.5, 0.5M NaCl) with 5% BSA for 1 h, then overnight with a polyclonal anti-21 antibody (M-19 from Santa Cruz Biotechnology, Santa Cruz, CA) at the dilution of 1:1,000 in 0.5 × TBS with 2.5% BSA and, finally, with a secondary antibody conjugated to

horseradish peroxidase for 1 h. The antigen-antibody interaction was visualized by incubation for 30 s in a chemilluminant reagent (ECL Western blotting detection from Amersham, Arlington Heights, IL) and exposure to an X-ray film.

Electrophoretic Mobility Shift Assay (EMSA) and Antibody Shift EMSA

Electrophoretic mobility shift assays (EMSAs) for E2F were performed using 5 µg of whole cell extracts prepared as previously described [Pagano et al., 1992], mixed with 2 µl of reaction buffer (final concentration: NaCl 80 mM, Hepes 4 mM, MgCl 2 mM, glycerol 10%, DTT 0.5 mM, EDTA 0.2 mM), 1 µl of poly(dAdT), and a large excess of ^{32}P-end labelled double-strand E2F oligonucleotide derived from the adenovirus E2 promoter. After 20 min at room temperature, samples were subjected to electrophoresis in 4% nondenaturing polyacrylamide gel, as described [Puri et al., 1997b]. For competition studies, a 100- to 200-fold molar excess of either unlabeled E2F wild type or E2F mutant oligonucleotide as well as of NFkB control oligonucleotide were included in the reaction before specific labelled probe was added. For E-box EMSAs, cell extracts were prepared as previously described by Puri et al. [1997b]. Then 20 µg of cell extract were combined in 30 µl of binding reaction containing 1 µg of poly(dIdC) in 20 mM HEPES (pH 7.6), 50 mM KCl, 1 mM dithioreitol, 1 mM EDTA, 5% glycerol, and ^{32}P-end labelled E-box probe derived from the muscle creatine kinase promoter.

For antibody shift EMSAs, antibodies were added to extracts at either room temperature or in ice for 30 min to 1 h (depending on the antibody or antiserum used) before the DNA binding reaction was started. Antibodies against cFos (sc52, Santa Cruz), p107 (SD9, Santa Cruz) and pRb2/p130 (C-20, Santa Cruz) were used.

Immunohistochemistry and DNA Synthesis Assay

For DNA synthesis assay BrdU (10 mM) was added to cells 3 h before cell fixation. After washing, cells were fixed in paraformaldeide 1% for 5 min at room temperature, rinsed in TBS (Tris Buffer/NaCl 0.05 M, pH 7.6), treated with TRITON-X 100 0.1% in TBS and incubated in 2N HCl for 1 h at 37°C. After neutralization in 0.1 M borate buffer, pH 8.5, cells were washed in TBS and processed for the immunohistochemical staining with anti-BrdU monoclonal antibody (Dako, Carpinteria, CA) according to the APAAP complexes method [Cordell et al., 1984]. Nuclei were counterstained with hematoxilin.

Immunofluorescence

Cells were washed in PBS, fixed in a methanol/acetone 1:2 v/v solution, dried and incubated with anti-MHC monoclonal antiserum MF20 (a gift from G. Cossu), a monoclonal anti-cyclin A antibody (clone BF 683, Pharmigen (San Diego, CA), or a polyclonal anti-p53 antibody (PAb 421, Oncogene Science, Manhasset, NY), at 37°C for 30 min. Specifically bound antibody was visualized by incubation with rhodaminate second-step antibody against immunoglobulin of the relevant species and observed by using a fluorescent microscope. Immunofluorescence for the detection of 5-bromo-2′-deoxyuridine (BrdU), as an indicator of DNA synthesis, was performed using the BrdU labelling and detection Kit (Boehringer, Indianapolis, IN). For double staining for MHC and BrdU, the anti-MHC antibody MF-20 was used first, before cells were incubated with the HCl containing buffer and a separate immunofluorescence assay was performed.

RESULTS AND DISCUSSION

We first tested whether Dox treatment interferes with cell cycle regulation in myoblasts. A 24 h Dox treatment of C2C12 skeletal muscle cells growing in high serum, results in an altered cell shape (data not shown) and determines a slight but reproducible reduction in the number of BrdU positive cells, as compared to mock-treated cells (Fig. 1A). Similarly to what has been shown in other cell types [Waldmann et al., 1996], a large proportion of Dox-treated myocytes accumulate in G2/M phase (data not shown). One explanation for this suppression of cell proliferation may reside in Dox ability to cause DNA damage, to up-regulate p53 expression, and to induce p53 cell growth suppressive activity [see Ko and Prives, 1996]. Indeed, upon Dox treatment, at the same dosage used to inhibit differentiation, C2C12 myoblasts display, as expected, p53 nuclear accumulation (Fig. 1B) without undergoing apoptosis (data not shown). After removal of Dox containing medium, C2C12 cells cultured in differentiation medium fail to fuse into multinucleated myotubes (Fig. 2B). Although a small propor-

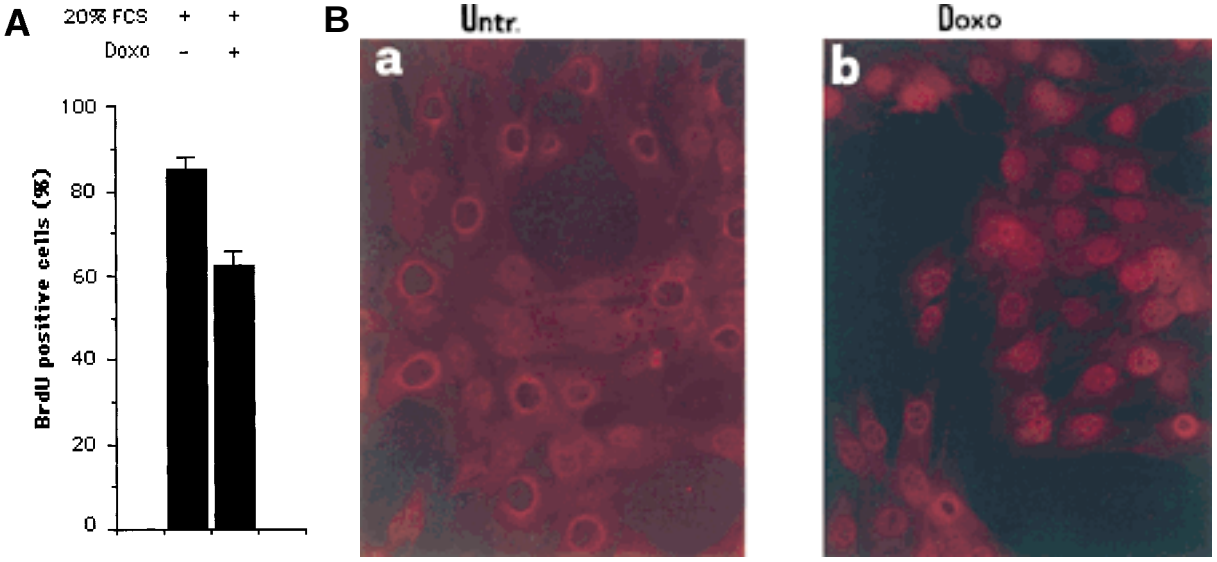

Fig. 1. Effect of Dox treatment on DNA synthesis and p53 expression in C2C12 myoblasts. C2C12 cells were cultured in growth medium (GM, 20% serum containing medium) in the presence or absence of Dox. **A:** DNA synthesis was measured by immunostaining for BrdU incorporation performed as described in Materials and Methods. **B:** Untreated **(a)** and Dox treated **(b)** C2C12 cells were fixed and stained for the nuclear p53 expression as described in Materials and Methods.

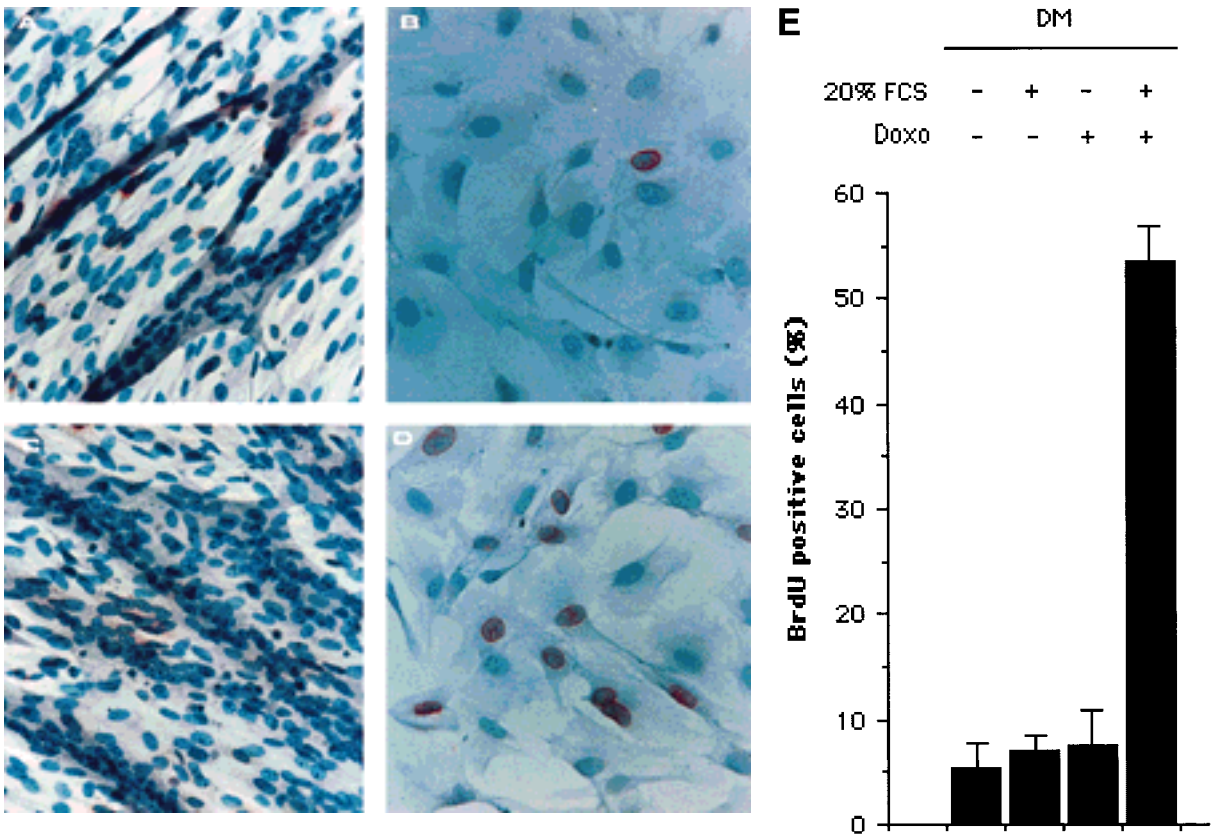

Fig. 2. Effects of Dox treatment on morphological differentiation and DNA synthesis in response to serum stimulation. C2C12 cells were cultured for 36 h in differentiation medium (DM, 2% serum containing medium) (**A**) and restimulated with 20% serum for 12 h (**C**). C2C12 myotubes do not respond to serum stimulation with BrdU incorporation, but with an increase in size (C). Dox-treated C2C12 cells cultured in DM for 36 h do not display any morphological feature of differentiation (**B**) and reincorporate BrdU after serum stimulation (**D**). **E:** Quantitative evaluation of the percentages of nuclei from untreated differentiated myotubes and Dox-treated C2C12 cells that incorporate BrdU before and after serum stimulation. Immunoistochemical stainings and BrdU incorporation were performed as described in Materials and Methods.

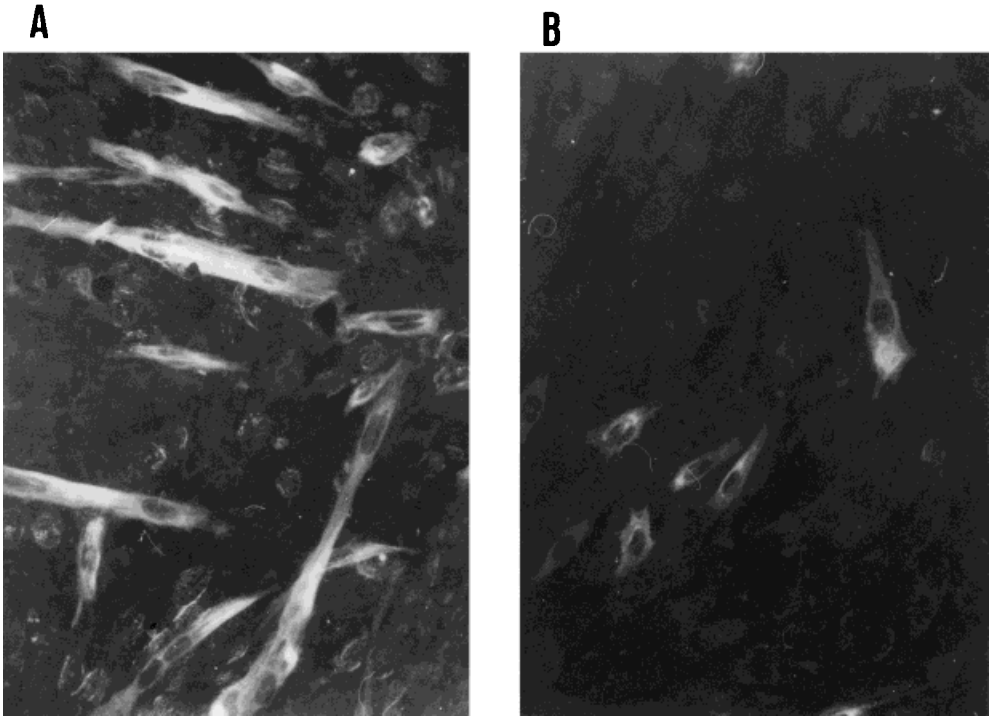

Fig. 3. Effects of Dox on biochemical differentiation of C2C12 cells. Immunostaining for myosin heavy chain (MHC) expression in the cytoplasm of untreated (**A**) or Dox-exposed (**B**) C2C12 cells induced to differentiate was performed using the anti-MHC monoclonal antibody, MF20, as described in Materials and Methods.

tion of these mononucleated cells is positive for the specific muscle differentiation marker myosin heavy chain (MHC) (Fig. 3) and features of terminal differentiation have been described to occur also in mononucleated myocytes [Andrés and Walsh, 1996], Dox-treated cells re-enter into the cell cycle and synthesize DNA in response to mitogens, as demonstrated by the increased nuclear cyclin A expression (Fig. 4) and by the induction of BrdU incorporation (Fig. 2D and E).

Cell cycle progression is coordinated by an ordered sequence of events including cyclin/cdk complexes activation and cdk-dependent phosphorilation of nuclear substrates. Among these, the Rb-family members pRb/p105, p107, and pRb2/p130 (also termed *pocket proteins*) play a critical role in the regulation of the G1/S phase progression, by binding to and negatively regulating E2F/DP transcription factor [see MacLachan et al., 1995]. E2F/DP consists of a family of different members (E2Fs from 1 to 5 and DP from 1 to 3), which heterodimerize and bind to specific DNA sites contained in the promoters of genes involved in the control of the G1/S phase progression (i.e., c-myc, DHFR, etc.) [La Thangue, 1996]. During the G1 to S phase transition, cyclin E and cyclin A are found in

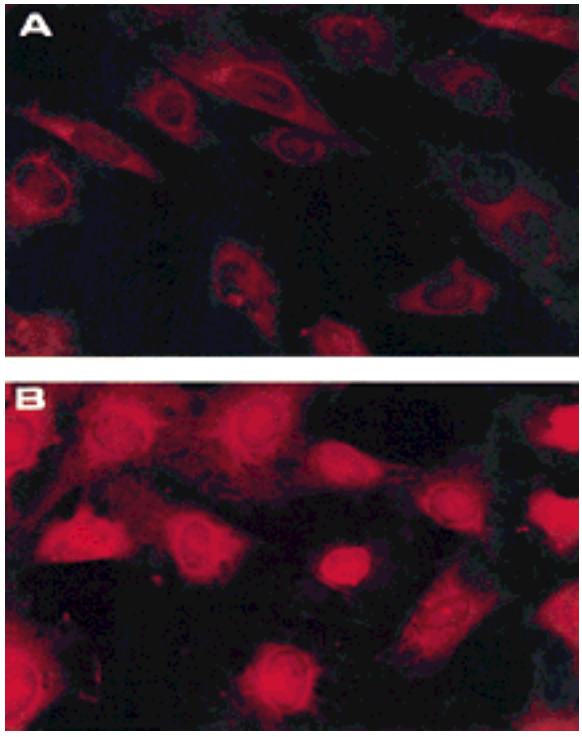

Fig. 4. Serum-induced nuclear accumulation of cyclin A in Dox-treated C2C12 cells. Staining for nuclear expression of cyclin A in Dox-treated C2C12 cells before (**A**) and following (**B**) serum stimulation was performed as described in Materials and Methods.

p107- and pRb2/p130-containing E2F/DP complexes [Pagano et al., 1992; Lees et al., 1992]. As cells enter G0/G1, an E2F4-pRB2/p130 complex is detectable in a variety of cell types [Chittenden et al., 1993; Cobrinick et al., 1993]. To correlate cell cycle effect with E2F/DP complex changes, we analyzed E2F/DP complexes in both cycling and differentiating C2C12 skeletal muscle cells in the presence or absence of Dox.

Changes in the pattern of E2F/DP complexes have been described in C2C12 myocytes when cells are shifted from high to low serum containing medium [Shin et al., 1995]. Upon serum removal the slower migrating band found in cycling cells (complex A, Fig. 5A, lanes 1 to 3), which contains cyclin A and cdk2 [Pagano et al., 1992; Puri et al., 1997b], is replaced by a faster migrating complex characteristic of both quiescent myoblasts and myotubes (complex B, Fig. 5A, lanes 4 to 6). This latter complex contains the growth suppressor protein pRb2/p130, as demonstrated by supershift analysis using a specific anti-pRb2/p130 antibody (Fig. 5C). A similar complex also forms in quiescent non-muscle cells where it is associated with the inhibition of E2F transcriptional activity and proliferative growth [Chittenden et al., 1993; Cobrinick et al., 1993; Claudio et al., 1994; Tommasi and Pfeiffer, 1996]. E2F/DP EMSAs of extracts from Dox-treated C2C12 cells cultured in high serum (GM, growth medium), reveals the coexistence of complexes A and B (Fig. 5B, lane 1). However, in Dox-treated C2C12 cells

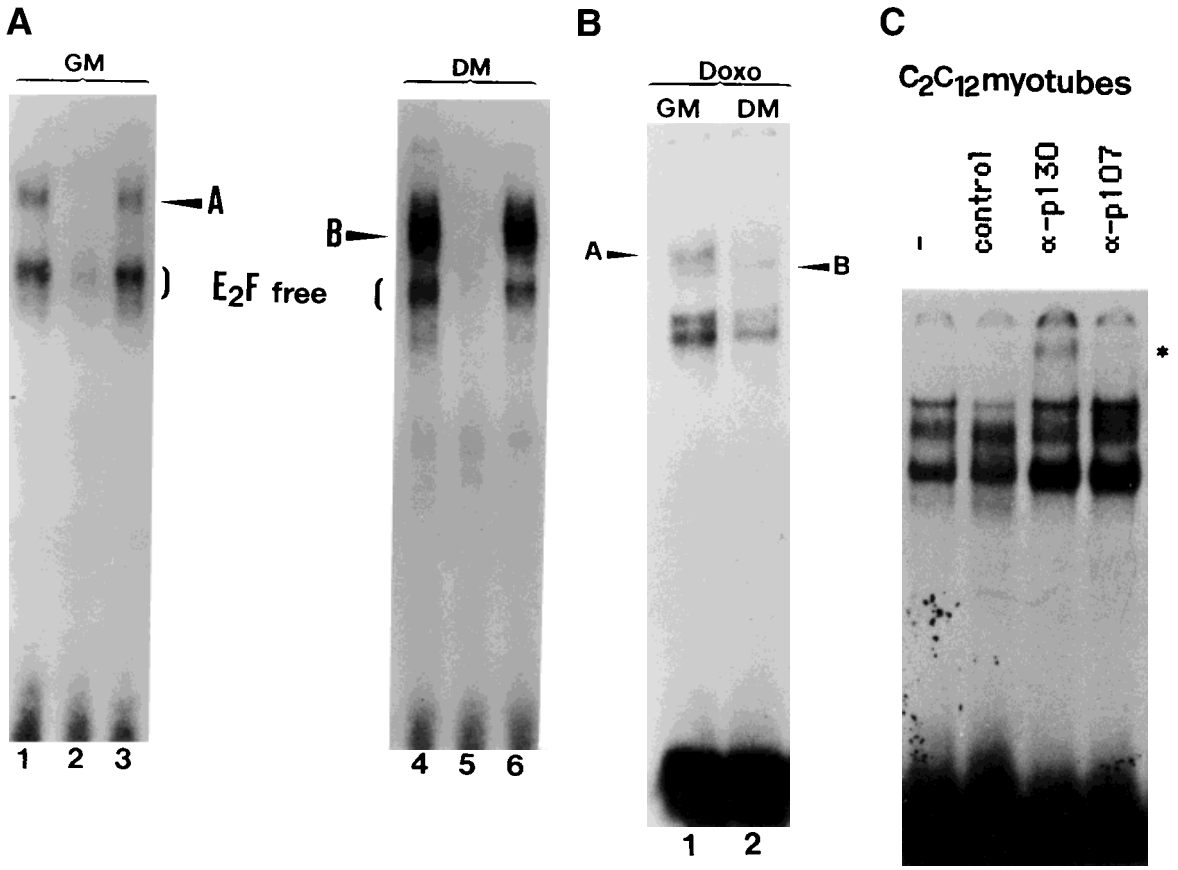

Fig. 5. E2F/DP complexes in untreated and Dox-treated C2C12 cells. **A:** Extracts from C2C12 cells cultured in GM and in differentiation medium DM were analyzed for E2F DNA binding activity by EMSA (**lanes 1 and 4**). To assess the binding specificity of the different E2F complexes, competition experiments were performed by adding 100-fold molar excess of wild type (**lanes 2** and **5**) and mutant (**lanes 3** and **6**) E2F DNA binding sites. Competitor wild type probe consists of unlabelled E2F sequences from the E2 adenovirus promoter. Mutant competitor-consisted of unlabelled mutated sequences from the same E2 promoter. The wild type, but not the mutant competitor, abolishes binding to ^{32}P-labelled E2F specific probe. **B:** E2F EMSA was performed using extracts from C2C12 cells cultured in GM containing Dox (lane 1) or in DM for 12 h (lane 2) after a 24-h period of exposure to Dox. **C:** Antibody shift EMSA of E2F complexes from C2C12 myotubes indicates that pRb2/p130 is a component of complex B. As a control, an anti-cFos antibody was used.

cultured in differentiation medium (DM), only complex B is present (Fig. 5B, lane 2). Mitogen stimulation of quiescent myoblasts results in the re-induction of DNA synthesis [Puri et al., 1995], and correlates with the re-formation of the cyclinA/cdk2 containing complex A [Puri et al., 1997b]. In serum-stimulated myotubes, cyclin/cdk re-association to E2F/DP complexes is prevented (Fig. 6, lane 2), DNA synthesis cannot be reactivated (Fig. 2E), and E2F transcriptional activity remains low (Fig. 7). Treatment with Dox of myocytes cultured in DM, results in re-formation of the cyclinA/cdk2 containing complexes in response to serum (Fig. 6, lane 4), followed by an evident increase in E2F driven CAT activity (Fig. 7) and DNA replication (Fig. 2E). S phase re-entry can be induced in quiescent myoblasts, as well as in myotubes, by oncoproteins such as the adenovirus E1A [Crescenzi et al., 1995; Puri et al., 1997a]. Interestingly, differently from the E1A-induced cell cycle re-entry, which is followed by cell death instead of mitosis, Dox-treated cells undergo DNA synthesis without any induction of apoptosis (data not shown).

To characterize the mechanism through which Dox might influence E2F/DP complexes and cell cycle in muscle cells, we monitored the DNA-binding activity of the myogenic specific growth suppressor, MyoD, and the levels of its downstream effector, p21. We expected that Id2 protein, induced by Dox, would downregulate MyoD DNA binding activity, by acting as a bHLH dominant negative, in the presence of culture conditions permissive for differentiation. As a consequence, MyoD-dependent cell responses, including permanent cell cycle arrest, should be affected. Indeed, Dox treatment determines a strong reduction of E-box DNA binding activity in myocytes induced to differentiate, as compared to untreated cells (Fig. 8).

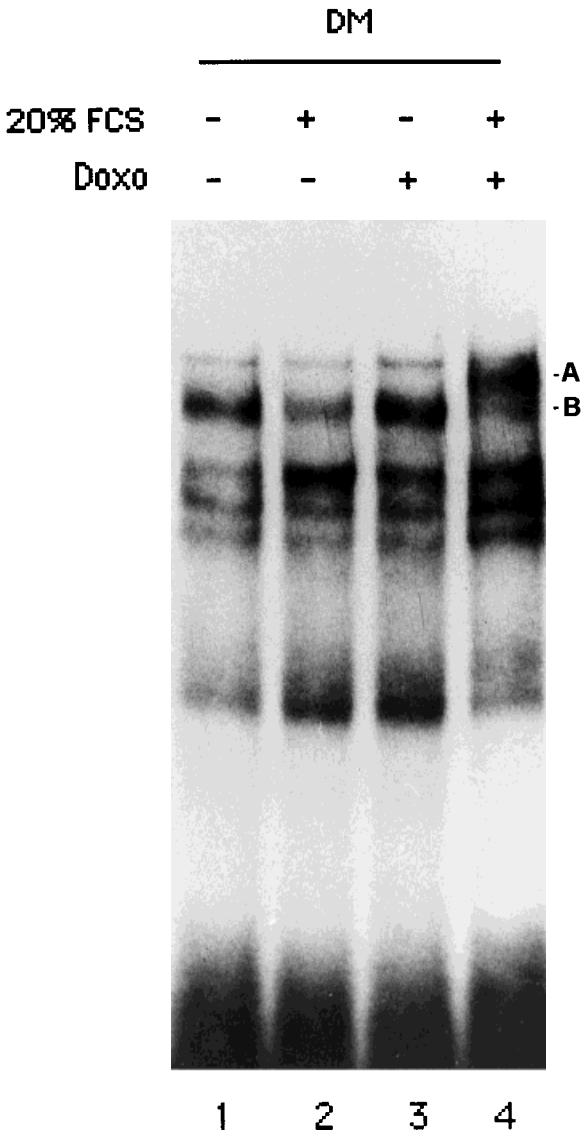

Fig. 6. Effects of Dox treatment on E2F/DP complexes. Complex B in extracts from C2C12 myotubes (**lane 1**) is not affected by Dox (**lane 3**), but is replaced by the complex A in extracts from C2C12 cells treated with Dox and then cultured in DM (**lane 4**). No modification of E2F complex B was observed in untreated C2C12 cells (**lane 2**), after serum stimulation (20% FCS).

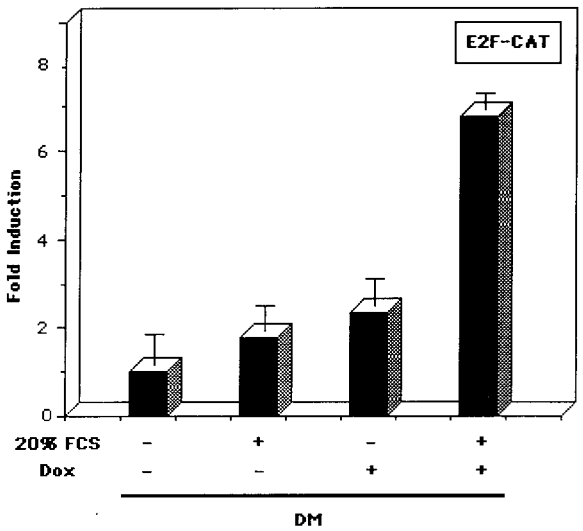

Fig. 7. E2F/DP transcriptional activity in Dox-treated C2C12 cells. C2C12 cells were transiently transfected with increasing amounts of a CAT reporter plasmid containing three E2F sites. Transcriptional activity was expressed as relative fold induction of CAT expression. Cell culture and transfection conditions are described in Materials and Methods.

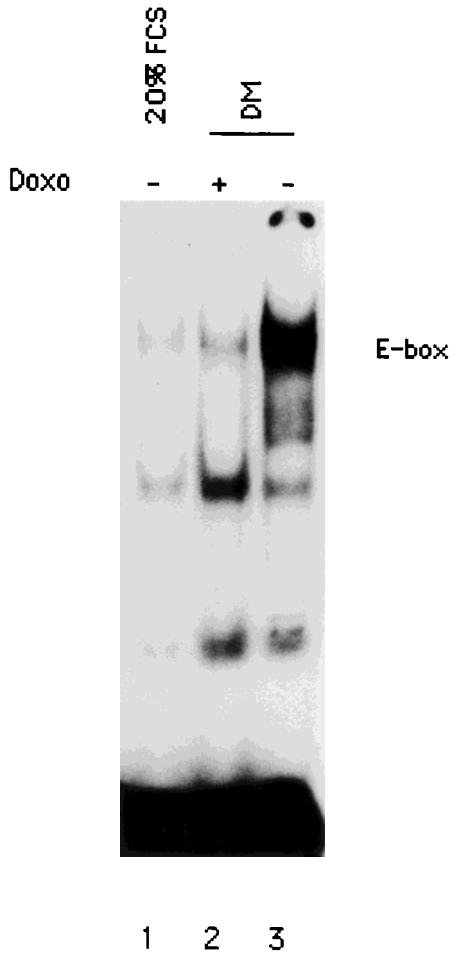

Fig. 8. Effects of Dox treatment on E-box binding activity in C2C12 cells. Dox inhibits E-box binding activity in C2C12 confluent cells cultured 48 h in DM (**lane 2**) as compared to untreated C2C12 cells growing in the same culture conditions (**lane 3**).

Thus, upon Dox treatment, myocytes normally exit from the cell cycle, but retain their proliferative potential and this correlates with changes in both E2F/DP complexes composition and induction of E2F transcriptional activity. According to the currently available data, the mechanism through which MyoD suppresses cell cycle progression and interferes with E2F/DP complex formation and activity involves the induction of the cdk inhibitor p21 [Halevy et al., 1995]. Surprisingly, we found that p21 is induced in Dox-treated myocytes cultured in differentiation medium, even in the absence of MyoD DNA binding activation, at higher levels than in untreated cells (Fig. 9). This p21 induction might represent a cellular response to Dox-induced DNA damage mediated by p53 activation. However a p53-independent induction of p21 in leukemic cells following Dox treatment has been described [Gartenhaus et al., 1996]. In any case, differently from MyoD-dependent p21 induction, the increase of p21 that takes place in Dox-treated myocytes is not followed by an irreversible cell cycle withdrawal. It seems that, in the absence of MyoD (or other MRFs) activation, muscle cells are unable to differentiate biochemically and morphologically and also fail to undergo permanent cell cycle arrest. It is noteworthy that cis-platinum, a chemiotherapic drug that induces DNA damage and p53/p21 activation, but does not impair MRFs function, when used at non-apoptotic doses, was found to enhance rather than prevent myogenic differentiation even in the presence of high serum (data not shown). Myotubes derived from cis-platinum-treated myoblasts do not re-enter the cell cycle, strongly supporting the hypothesis that MRFs activation is required to obtain the irreversible cell cycle arrest during myogenic differentiation. Failure of p21 to ensure a permanent block in G0 when expressed independently from MyoD could simply reflect the inability to reach levels high enough to buffer the cyclin/cdk function. Indeed, the balance between cyclin/cdk activity and their block by cdki(s) inhibitors has been demonstrated to depend on their stechiometric ratio [Zhang et al., 1994]. However, since Dox-induced p21 upregulation is even stronger than that observed in both untreated and cis-platinum-treated differentiating myocytes (Fig. 9), we can rule out this possibility. In the absence of MyoD and other MRFs, p21 transcription in Dox-treated myoblasts is likely stimulated by p53, or eventually other p21 inducers, to obtain a reversible cell cycle arrest as part of a cellular response different from differentiation (i.e., DNA repair). Alternatively, one might

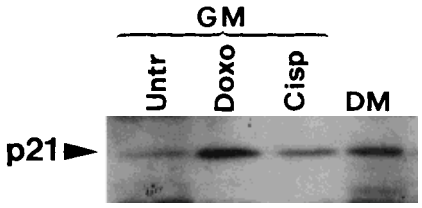

Fig. 9. Effects of Dox treatment on p21 levels in C2C12 cells. Dox treatment increases p21 level in C2C12 cells growing in GM, as compared to untreated myoblasts cultured in the same conditions (compare Dox to GM). Dox effect on p21 levels is higher than those observed following Cis-platinum treatment (Cisp) and the switch of culture conditions from GM to DM (DM).

speculate that, even in the presence of p21 levels capable to buffer cdk function, muscle cells require additional MyoD-induced activities to enter and stably remain in a postmitotic state. Clearly, p21 and MyoD potentiate each other through an autoregulatory positive loop: MyoD induces p21 transcription and p21 prevents cyclin/cdk dependent phosphorilation of specific target proteins, like pRb, p107, or pRb2/p130, whose function is essential for MyoD activity [Gu et al., 1993] and for regulating proteins involved in cell cycle progression, such as E2F/DP [Shin et al., 1995; Corbeil et al., 1995; Puri et al., 1997b]. High levels of p21 also prevent re-formation of cdk containing E2F/DP complexes specific of S phase [Shiyanov et al., 1996; Puri et al., 1997b]. Interestingly, a large proportion of Dox-treated myocytes are rather arrested in G2/M phase and it is likely that at this stage myoblasts are refractory to irreversibly withdraw from the cell cycle despite the induction of p21. However, we were not able to define whether the E2F4-pRb2/p130 complex also forms in the fraction of Dox-treated cells arrested in G2/M. These findings implicate that permanent cell cycle arrest in myocytes undergoing terminal differentiation, involves a temporally ordered sequence of events triggered by MyoD (or other bHLH myogenic proteins) and that the aberrant activation of downstream effectors of this process would result in the failure of the program.

If p21 upregulation is unable, in the absence of MyoD activity, to impose an irreversible cell cycle withdrawal, it can be reasoned that MyoD, in addition to induce p21 transcription, should exert other anti-proliferative activities required for terminal cell cycle arrest of differentiating myocytes. In agreement with this hypothesis, we observed that MyoD ectopic expression, by microinjection, in fibroblasts growing in high serum, induces growth arrest in a small number of cells after only 12 h, well before any detectable nuclear accumulation of p21 (Puri, P.L., unpublished observation). An antiproliferative activity of MyoD independent from its ability to induce p21 is also suggested by the normal terminal differentiation of skeletal muscle cells in mice lacking p21 [Deng et al., 1995].

In conclusion, our results support a model of terminal cell cycle arrest in differentiating myocytes as a coordinated program involving multiple and timely ordered processes highly dependent on MyoD (or other MRF) function.

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