p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription

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The nuclear phosphoprotein p300 is a new member of a family of 'co-activators' (which also includes the CREB binding protein CBP), that directly modulate transcription by interacting with components of the basal transcriptional machinery. Both p300 and CBP are targeted by the adenovirus E1A protein, and binding to p300 is required for E1A to inhibit terminal differentiation in both keratinocytes and myoblasts. Here we demonstrate that, in differentiating skeletal muscle cells, p300 physically interacts with the myogenic basic helix–loop–helix (bHLH) regulatory protein MyoD at its DNA binding sites. During muscle differentiation, MyoD plays a dual role: besides activating muscle-specific transcription, it induces permanent cell cycle arrest by up-regulating the cyclin-dependent kinase inhibitor p21. We show that p300 is involved in both these activities. Indeed, E1A mutants lacking the ability to bind p300 are greatly impaired in the repression of E-box-driven transcription, and p300 overexpression rescues the wild-type E1A-mediated repression. Moreover, p300 potentiates MyoD- and myogenin-dependent activation of transcription from E-box-containing reporter genes. We also provide evidence, obtained by microinjection of anti-p300 antibodies, that p300 is required for MyoD-dependent cell cycle arrest in either myogenic cells induced to differentiate or in MyoD-converted C3H10T1/2 fibroblasts, but is dispensable for maintenance of the post-mitotic state of myotubes.

Keywords: cell cycle arrest/MyoD/p300/skeletal muscle/E1A

Introduction

Biochemical and morphological differentiation of muscle cells require a coordinated sequence of events, including

arrest in the G₀ phase, irreversible exit from the cell cycle and a timely ordered expression of muscle-specific genes. This programme depends on the myogenic regulatory proteins, including the basic helix–loop–helix (bHLH) proteins MyoD, myogenin, Myf5, MRF4 (Lassar and Munsterberg, 1994; Olson and Klein, 1994) and the MADS domain transcription factor MEF2 (Gossett *et al.*, 1989; Yu *et al.*, 1992; reviewed by Olson *et al.*, 1995). As regards MyoD, a dual role during myogenesis has been demonstrated: it induces permanent cell cycle arrest by up-regulating the cyclin-dependent kinase (cdk) inhibitor p21 (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990; Guo *et al.*, 1995; Halevy *et al.*, 1995) and activates muscle-specific gene transcription (Weintraub *et al.*, 1991). MyoD transcriptional activity is mediated by binding to a common DNA sequence motif, the E-box, which is present in the regulatory region of many muscle-specific genes (Lassar and Munsterberg, 1994; Olson and Klein, 1994).

The viral oncoproteins E1A of adenovirus and large T antigens (TAg) of both SV40 and polyomavirus share the ability to inhibit myogenic differentiation (Fogel and Defendi, 1967; Webster *et al.*, 1988; Braun *et al.*, 1992; Maione *et al.*, 1992; Tedesco *et al.*, 1995). This property has been attributed to their binding to a common region of pRb, p107 and pRb2/p130, called the 'pocket domain'. The pocket proteins participate in the induction and maintenance of the post-mitotic state of differentiated myotubes (Gu *et al.*, 1993; Schneider *et al.*, 1994; Corbeil *et al.*, 1995; Kiess *et al.*, 1995; Shin *et al.*, 1995). They negatively regulate the transcriptional activity of E2F/DP family members (La Thangue, 1994), and pRb has been found to interact directly with MyoD (Gu *et al.*, 1993). However, the ability of E1A to prevent skeletal muscle cell differentiation has been shown to require sequences located at its N-terminus, which in turn bind the nuclear phosphoprotein p300 (Mymryk *et al.*, 1992; Caruso *et al.*, 1993).

p300 belongs to a new family of 'co-activators' (which also includes the CREB binding protein CBP) that are able to modulate transcription. Co-activators function as adaptor proteins for complex transcriptional regulatory elements by favouring the communication between certain transcription factors, including CREB, c-Jun, JunB, c-Fos, c-Myb, YY1 and nuclear receptors (Chrivia *et al.*, 1993; Arias *et al.*, 1994; Bannister *et al.*, 1995; Chakravarti *et al.*, 1996; Dai *et al.*, 1996; Kamei *et al.*, 1996; Lee *et al.*, 1996), and components of the basal transcriptional machinery. Indeed, both p300 and CBP have been shown to activate transcription when fused to a DNA binding domain (Chrivia *et al.*, 1993; Arany *et al.*, 1994) and both contain a 'bromodomain', a specialized protein structure present in several proteins implicated as global activators of transcription. The TATA binding protein (TBP)-associated 250 kDa factor (TAF_{II}250/CCG1) is a member of

this group (Sekiguchi *et al.*, 1991; Hisatake *et al.*, 1993; Kokubo *et al.*, 1993; Ruppert *et al.*, 1993; Weinzierl *et al.*, 1993). In addition, CBP can interact with the basal transcription factor TFIIB (Kwok *et al.*, 1994) and p300 is found in immune complexes with TBP (Abraham *et al.*, 1993). The protein sequence of p300 and CBP also predicts the presence of three cysteine/histidine-rich regions potentially involved in additional protein-protein interactions (Rikitake and Moran, 1992; Arany *et al.*, 1994, 1995; Eckner *et al.*, 1994; Lundblad *et al.*, 1995). Recently, a p300/CBP-associated factor (P/CAF) with intrinsic histone acetylase activity, which stimulates the transcriptional activity of p300/CBP-bound transcription factors, has been identified (Yang *et al.*, 1996). We have shown previously, by co-immunoprecipitation under low stringency salt conditions and by *in vitro* binding assays with *in vitro* synthesized p300 protein and purified bacterial GST-MyoD fusion protein, that p300 is able to bind the myogenic factor MyoD (Yuan *et al.*, 1996). This association occurs through the carboxy-terminal cysteine/histidine-rich domain of p300, which uses its two separate transactivation domains at the amino- and carboxy-terminus to communicate with the components of the basal transcriptional complex (Yuan *et al.*, 1996). Here, we demonstrate that p300-MyoD-containing complexes are recruited at specific MyoD DNA binding sites in differentiating muscle cells. This interaction results in the potentiation of MyoD-dependent transcription of the downstream skeletal muscle-specific genes myogenin and muscle creatine kinase (MCK). In addition, we also show that p300 is required for MyoD-dependent induction of cell cycle arrest in myogenic cells that have been induced to differentiate.

Results

p300 in differentiating skeletal muscle cells

To study p300-MyoD interactions *in vivo*, we used C2C12 skeletal muscle cells (Yaffé and Saxel, 1977), which represent an amenable model for myogenic differentiation. In high serum [20% fetal calf serum (FCS)], C2C12 myoblasts proliferate until they reach confluence. Full differentiation into multinucleated myotubes is obtained by culturing confluent C2C12 cells in low serum (2% FCS). C2C12 myotubes do not incorporate bromodeoxyuridine (BrdU) and display several muscle-specific markers. Fusion into myotubes is an asynchronous process that starts within a few hours of culture under differentiation conditions (early myotubes) and lasts for up to 96 h (mature myotubes). Cycling C2C12 myoblasts, as well as early and mature myotubes, were labelled with [³⁵S]-methionine for 4 h and equal amounts of cell lysate were subjected to immunoprecipitation with either an anti-p300 polyclonal antiserum (Avantaggiati *et al.*, 1996) or a monoclonal anti-E1A antibody. The ability of this anti-p300 antiserum to immunoprecipitate authentic p300 has already been demonstrated by Western analysis and comparative peptide mapping experiments (Avantaggiati *et al.*, 1996). As shown in Figure 1A, lane 1, a 300 kDa protein is precipitated specifically by the p300 antiserum from C2C12 myoblasts; it co-migrates with both the p300 immunoprecipitated from 293 cells with the same anti-p300 antiserum (Figure 1A, lane 4) and the p300 co-immunoprecipitated with E1A from 293 cell extracts using

the anti-E1A-specific monoclonal antibody (Figure 1A, lane 6). The pre-immune serum (data not shown) as well as the monoclonal anti-E1A antibody (lane 5) failed to immunoprecipitate p300 from C2C12 cell extracts. Immunofluorescence staining revealed that p300, as expected, has a nuclear localization in C2C12 myoblasts (Figure 1B). Immunoprecipitation and indirect immunofluorescence with anti-p300 antibodies were also performed using C2C12 cells at different times during differentiation. We could not detect any change in either p300 levels or localization (Figure 1A, lanes 2 and 3, and Figure 1C) or p300 electrophoretic mobility (Figure 1A, lanes 1–3). Similar results in immunoprecipitation were obtained using a mouse monoclonal anti-p300 (clone NM11 from Pharmingen) (data not shown).

p300-MyoD interaction occurs at the specific MyoD DNA target sequences

We have shown previously that p300 and MyoD can be co-immunoprecipitated under low stringency salt conditions from C2C12 myoblasts and we confirmed their direct interaction by *in vitro* binding assays using *in vitro* translated p300 protein and purified bacterial GST-MyoD fusion protein (Yuan *et al.*, 1996). However, if the MyoD-p300 interaction plays an important role during muscle differentiation and in view of the putative role of p300 as a transcriptional co-activator, one should expect p300-MyoD complexes to be present on the specific MyoD DNA target sequences. To test this hypothesis, electrophoretic mobility shift assay (EMSA) experiments were performed using ³²P-labelled E-box binding sites. C2C12 cells cultured in differentiation medium display increasing levels of E-box DNA binding activity (Figure 2A, lanes 1–3). The binding specificity of this complex was confirmed by competition experiments with excess unlabelled wild-type and mutant E-box-specific oligonucleotides (Figure 2A, lanes 5 and 6). All the myogenic bHLH transcription factors bind these E-box sites together with the products of the E2A gene, E12 and E47, as heterodimers (Weintraub, 1993). Indeed, this E-box activity in C2C12 myotube extracts was supershifted specifically by antibodies directed against the bHLH proteins MyoD and myogenin (Figure 2B, lanes 6 and 7), as well as by anti-E12 antibodies (data not shown). In addition, our polyclonal anti-p300 antiserum (Figure 2B, lane 5) and the anti-p300 monoclonal antibody NM11 (data not shown) both partially supershifted this activity, demonstrating that p300 is part of this complex. The specificity of this supershift was confirmed using either the corresponding anti-p300 pre-immune antiserum (Figure 2B, lane 4) or by adding the antibodies to the probe directly in the absence of C2C12 cell extracts, which does not result in a detectable band (data not shown). Since p300 and CBP are both targeted by E1A (Arany *et al.*, 1995; Lundblad *et al.*, 1995) and share several partner proteins, including CREB and c-Jun (Arias *et al.*, 1994; Arany *et al.*, 1995; Lee *et al.*, 1995; Lundblad *et al.*, 1995), the presence of CBP in E-box DNA binding activity was tested. Using a specific anti-CBP antibody, we were unable to show any supershift of the E-box-bound complexes (Figure 2B). To confirm further the presence of MyoD-p300 complexes at the E-box sites, biotinylated oligonucleotides, containing the E-box consensus sequence, were also used to affinity

Fig. 1. p300 levels in differentiating C2C12 cells. **(A)** Immunoprecipitation of p300 from either semiconfluent C2C12 cycling myoblasts (20% FCS-containing medium) (lane 1) or from both early C2C12 myotubes (18 h in 2% FCS differentiation medium, DM) (lane 2) and mature C2C12 myotubes (72 h in 2% FCS DM) (lane 3), using a polyclonal anti-p300 antiserum. In lane 4, p300 was immunoprecipitated from 293 cells using the same polyclonal antibody. As a control, extracts from early C2C12 myotubes and 293 cells were immunoprecipitated with a monoclonal anti-E1A antibody (Oncogene Science) (lanes 5 and 6). As expected, p300 was co-immunoprecipitated by anti-E1A from 293 cells but not from C2C12 cells. Metabolic labelling, cell extract preparation and immunoprecipitation were performed as described in Materials and methods. **(B)** and **(C)** p300 immunofluorescence staining of C2C12 cells. Non-confluent cycling C2C12 cells growing in high serum (20% FCS) **(B)** and myotubes **(C)** were incubated with a polyclonal anti-p300 antiserum, followed by rhodamine-conjugated secondary antibody staining.

purify E-box-bound complexes from C2C12 cells at different stages of differentiation. A band of the expected size of 300 kDa was detected using ^{35}S -metabolically labelled extracts from early and mature C2C12 myotubes but not from C2C12 myoblasts (Figure 2C). To confirm the efficiency of the affinity purification procedure, we subjected both the biotinylated oligonucleotide-bound material and the corresponding supernatants to E-box EMSA (Figure 2D). Further, the presence of p300 in the E-box DNA-bound complexes was confirmed by immunoblotting with either our polyclonal anti-p300 antiserum (Figure 2E, lane 2) or a monoclonal anti-p300 antibody (Figure 2E, lane 8). No CBP reactivity was detected by immunoblot in the purified E-box-bound complexes (data not shown). Taken together, these results clearly demonstrate that p300 interaction with MyoD (and possibly other myogenic bHLH transcription factors) occurs at E-box sites in differentiating muscle cells. Moreover, although it cannot be excluded that CBP-MyoD complexes, not bound to the MyoD DNA sites or bound to complex promoter elements, might be present in either myoblasts or differentiated myotubes, our results strongly suggest that p300 and not CBP is the preferential partner of MyoD at its DNA binding sites.

p300 potentiates MyoD-dependent activation of transcription

MyoD is involved in both the induction of permanent cell cycle arrest, which is required for terminal muscle differentiation, and in the activation of muscle-specific genes (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990; Weintraub *et al.*, 1991). Therefore, to assess the biological significance of the MyoD-p300 interaction, we next tested the ability of p300 to enhance the myogenic properties of MyoD. The role of p300 in MyoD-dependent activation of transcription was first studied using the synthetic E-box-containing CAT reporter plasmid p4R-tk-CAT. Since all the MyoD family bHLH proteins activate the E-box enhancer and, under the conditions of muscle differentiation, MyoD induces the expression of the other myogenic transcription factors in a variety of non-muscle cells (Weintraub, 1993), we used the Saos human osteosarcoma cell line, which is refractory to myogenic conversion by ectopic expression of muscle-specific bHLH transcription factors (Gu *et al.*, 1993). By doing so, it is possible to distinguish between the E-box activity mediated by MyoD and that mediated by other bHLH and MEF2 transactivators. In both human osteosarcoma Saos (Figure 3A) and U2-OS (data not shown) cells, the basal E-box reporter

Fig. 2. p300 localizes at E-box MyoD DNA binding sites. **(A)** E-box DNA binding activity in C2C12 cells during the cell cycle and differentiation. Extracts from semiconfluent C2C12 myoblasts (lane 1), from C2C12 early myotubes (cultured for 18 h in DM) (lane 2), and from mature C2C12 myotubes (cultured for 3 days in DM) (lane 3) were performed as described elsewhere (Gu *et al.*, 1993). Competition experiments were performed by adding a 100- to 200-fold molar excess of either wild-type (wt) (5'-CCCCAACACCTGCTGCCTGA3') or mutant (5'-CCCCAACACGGTAAC-CCTGA3') (mt) unlabelled E-box oligonucleotides (lanes 5 and 6, respectively). **(B)** Antibody supershift EMSAs of E-box complexes. A specific supershift was obtained with antibodies directed against the bHLH proteins MyoD (lane 6) and myogenin (lane 7), as well as with the anti-p300 polyclonal antibody (lane 5) but not with its pre-immune serum (lane 4) or a specific non-cross-reactive monoclonal antibody directed against the related transcriptional co-activator CBP (lane 3). **(C)** E-box-bound complexes were affinity purified from [³⁵S]methionine-labelled C2C12 myoblasts (lane 1), early C2C12 myotubes (lane 2) and mature myotubes (lane 3), using biotinylated double-stranded oligonucleotides containing the E-box consensus sequence (5'-biot-5'-CCCCAACACCTGCTGCCTGA3'). E-box-bound proteins were resolved on a 7.5% SDS-PAGE. **(D)** The affinity purification of E-box-bound complexes using the biotinylated E-box oligonucleotides was confirmed by EMSA. In the figure, Sup. is supernatant and Prec. are precipitated proteins after affinity purification. **(E)** Immunoblot with anti-p300 polyclonal antibody following affinity purification of E-box-bound proteins with biotinylated oligonucleotides from C2C12 early myotube (lane 2), C2C12 myoblast (lane 4) and 293 cell (lane 6) extracts. In lane 8, the immunoblot on the E-box-bound proteins from C2C12 early myotubes was performed using anti-p300 monoclonal antibody NM11. E-box mutant biotinylated oligonucleotides were used as controls to prepare similar samples run in lanes 1, 3, 5 and 7.

activity is almost undetectable (data not shown) and it is activated efficiently by MyoD expression (Figure 3A). Wild-type E1A 12S represses the MyoD-dependent E-box activity in a dose-dependent manner, while the E1A N-terminal deletion mutant dl2-36, which is defective for p300 binding but not for binding to the Rb family proteins (Giordano *et al.*, 1991), is much less active in transrepression (Figure 3A). Co-transfection of the p300 expression vector pCMV-βp300 overrides the E1A 12S

repression of the MyoD-dependent transcription and potentiates the MyoD-mediated transactivation (Figure 3A). This observation clearly supports the hypothesis that p300 plays an important role in MyoD-dependent activation of transcription during muscle differentiation. Indeed, co-transfection of a plasmid encoding the 1514–1922 p300 mutant, which is still able to bind MyoD but has no transcriptional activity (Yuan *et al.*, 1996), inhibits MyoD-dependent E-box activity on this promoter (data not

shown). Since we found that p300 is also effective in potentiating the E-box-dependent activity of myogenin on the same p4R-tk-CAT reporter (Figure 3A), it is likely that p300 also functions as co-activator for bHLH transcription factors other than MyoD. An elevated grade of redundancy has been demonstrated in the myogenic potential of the

four bHLH proteins of the MyoD family (Weintraub, 1993; Olson and Klein, 1994), and each family member can activate the programme for skeletal muscle differentiation when introduced into a variety of non-muscle cells (Davis *et al.*, 1987; Hollemberg *et al.*, 1993; Lassar and Mustenberg, 1994; Olson and Klein, 1994). In both skeletal muscle cells and in myogenic converted non-muscle cells, myogenin is a downstream effector of MyoD (Hollemberg *et al.*, 1993; Weintraub, 1993; Olson and Klein, 1994). More recently, MEF2 proteins have been shown to synergize with both MyoD and myogenin in the myogenic conversion of C3H10T1/2 fibroblasts and, for the MEF2C member of the MEF2 family, a direct interaction with myogenin has also been described (Molkentin *et al.*, 1995). We used C3H10T1/2 fibroblasts to evaluate the ability of p300 to potentiate MyoD transcriptional activation of the myogenin promoter. The pMyo84 CAT reporter plasmid contains both an E-box site at position -15 to -10, downstream of the TATA box, and a MEF2 site at position -66 to -58. The latter site has been shown to be essential for myogenin transcription both in cultured cells and *in vivo* in the mouse embryo (Edmonson *et al.*, 1992; Yee and Rigby, 1993). As shown in Figure 3B, the ability of MyoD to activate the pMyo84 CAT construct is stimulated >3-fold by co-transfection with the p300 expression vector. The ability of microinjected anti-p300 antibodies to suppress MyoD-dependent induction of myogenin expression in C3H10T1/2 cells (data not shown) further confirms the role of p300 in the activation of muscle-specific genes. When the MEF2 site is mutated in the pMyo84mutMEF2 reporter plasmid, activation by MyoD alone is reduced by a factor of two but the synergistic effect of p300 on MyoD-dependent activation is only slightly reduced. Although optimal induction of transcription by myogenic bHLH proteins requires multiple E-box/MEF2 sites (Molkentin *et al.*, 1995), p300 also cooperates with MyoD in inducing transcription

Fig. 3. p300 enhances E-box-dependent MyoD-induced transcriptional activation of the myogenin and MCK regulatory sequences. (A) MyoD and myogenin activity on E-box-driven transcription requires p300. Saos human osteosarcoma cells were co-transfected with 4 µg of the E-box CAT reporter p4R-tk-CAT and 2 µg of either the pCMV-MyoD expression vector (Yuan *et al.*, 1996) or the pEMSV-myogenin (a gift from F.Tatò). Co-transfection of increasing amounts of CMV wild-type E1A 12S expression vector (pE1A12S) blocks MyoD-dependent transcription, whilst the E1A deletion mutant lacking the N-terminal amino acids 2–36 (pE1A12SΔ2–36) is ineffective. Co-transfection of the p300 expression vector pCMV-βp300 (Eckner *et al.*, 1994) rescues the MyoD-dependent transcription in the presence of pE1A 12S and enhances both the MyoD- and myogenin-mediated transactivation. Co-transfection of a plasmid encoding the 1514–1922 p300 mutant, which is still able to bind MyoD but has no transcriptional activity, inhibits MyoD-dependent E-box activity on this promoter. (B) C3H10T1/2 fibroblasts were co-transfected with 2.5 µg of different myogenin-CAT reporter plasmids and increasing amounts of the MyoD expression vector pCMV-MyoD (Yuan *et al.*, 1996), in the presence or absence of 5 µg of pCMV-βp300. pMyo84 contains the myogenin promoter from nucleotide -84 upstream from the CAT gene. pMyo84mutMEF2 and pMyo84mutE1 are mutated in the MEF2 or the E-box site, respectively. pMyo84mutMEF2-E1 is mutated in both the MEF2 and the E-box sites. (C) C3H10T1/2 fibroblasts were co-transfected with 2.5 µg of the MCK CAT reporter plasmid pMCK1256 (containing the MCK regulatory sequences from -1256 to +7 upstream from the CAT gene) and various combinations of pCMV-MyoD and pCMV-βp300, as indicated. At 48 h after transfection, cells were harvested and then assayed for reporter gene expression.

Fig. 4. Injection of wild-type and mutant E1A 12S-encoding plasmids into C2C12 myoblasts. C2C12 cells were cultured for 6 h in 0.5% FCS-containing medium before nuclear microinjection of the different E1A, SV40 large T antigen and control plasmids. Myoblasts were maintained in 0.1% FCS-containing medium for 24 h and then assayed for E1A expression and BrdU incorporation. Serum-free medium containing BrdU was added 3 h before fixation. **(A)** Arrows indicate cells injected with either pE1A12S (a and b) or pE1A12Sdl2-36 (c and d) and stained for either E1A expression (a and c) or BrdU incorporation (b and d) with rhodamine- or fluorescein-conjugated antibodies, respectively. **(B)** Quantitative evaluation of E1A- and E1A mutant-induced DNA synthesis in serum-starved myoblasts. C2C12 cells were injected with the indicated plasmids (E1A wt = pE1A12S; dl 2-36 = pE1A12Sdl2-36; RG2 = pE1A12SRG2; TAG = pSV-TAG). Background BrdU incorporation of pCH110 mock-injected cells (designated as basal) was <7%. As a positive control, cells were maintained in 20% FCS-containing medium and mock-injected with the pCH110 tracer plasmid.

from promoters containing only one E-box site (i.e. the pMyo84mutMEF2 promoter, herein described, and the human cardiac α -actin promoter; V.Sartorelli, personal communication). This observation is also supported by the presence of p300 in protein complexes bound to one (Figure 2B) as well as to multiple (data not shown) E-box sites. Deletion of the E-box site in the pMyo84mutMEF2-E1 reporter completely abolishes p300 activity, thus confirming that p300 indeed acts as a co-activator of E-box-dependent MyoD transcriptional activity on the myogenin promoter. Surprisingly, we also found a synergistic effect of p300 on MyoD-dependent activation on the pMyo84mutE1 reporter, which only contains one MEF2 site. This would imply that p300 may also cooperate with member(s) of the MEF2 family, induced by MyoD during myogenic conversion/differentiation, either directly or via the interaction with myogenic bHLH proteins (Molkentin *et al.*, 1995). Indeed, both physical interaction and functional synergism between MEF2c and p300 was observed recently by Sartorelli *et al.* (personal communication). We also co-transfected C3H10T1/2 cells with the MCK CAT reporter plasmid pMCK1256, which contains the MCK regulatory sequences from -1256 to +7 upstream from the CAT gene, together with MyoD, myogenin (data not shown) and p300 expression vectors, either alone or in combination. Figure 3C shows that p300 potentiates both MyoD and myogenin activation of transcription from the MCK promoter. Taken together, these results support the thesis that p300 acts as co-factor for MyoD and other myogenic transcription factors in mediating biochemical muscle differentiation.

p300 and cell cycle arrest in differentiating skeletal myocytes

Cell proliferation and differentiation are usually mutually exclusive. Cell cycle withdrawal is a prerequisite for myoblast differentiation, representing an early event in terminal differentiation (Weintraub, 1993; Olson and Klein, 1994). Terminal differentiation in cultured myoblasts requires both serum deprivation and activation of the bHLH factors. C2C12 cells are prevented from cell cycle withdrawal and phenotypic differentiation by high concentrations of mitogens in the medium. Mitogens appear to block terminal differentiation by promoting the expression of Id, a dominant negative HLH factor that inhibits the binding of the myogenic bHLHs to their DNA target sequences. Once myotubes are formed, cells become unresponsive to further mitogen stimulation and are unable to re-enter the cell cycle. The mechanisms involved in the

Fig. 5. Effects of anti-p300 antibody injection into C2C12 myoblasts.

(A) Anti-p300 antibodies block the exit from the cell cycle by serum-starved C2C12 myoblasts. Semiconfluent C2C12 cells were cultured for 12 h in 0.5% FCS-containing medium before microinjection into the cytoplasm of either ammonium sulfate-concentrated polyclonal anti-p300 antibodies (a) or the corresponding ammonium sulfate-concentrated pre-immune antiserum (b). After an additional 24 h in 0.5% FCS, DNA synthesis was measured by BrdU incorporation. Serum-free medium containing BrdU was added 3 h before fixation of cells. Cells are double-stained for either anti-p300 and pre-immune serum (rhodamine) or BrdU incorporation (fluorescein).

(B) Quantitative evaluation of multiple experiments. Background BrdU incorporation of uninjected cells (indicated as basal) was <7%. As a positive control, cells were re-exposed to 20% FCS immediately after mock injection with the pCH110 tracer plasmid.

maintenance of terminal cell cycle arrest in myotubes under conditions of mitogen stimulation remain a matter for discussion. A role in this process has been attributed to pRb and, indeed, cultures of myotubes from Rb-deficient mice maintain the competence for DNA synthesis (Schneider *et al.*, 1994). In addition, MyoD overexpression in both muscle and non-muscle cells induces cell cycle

arrest, which has been correlated with the ability of MyoD to up-regulate, in a p53-independent manner, the expression of the cell cycle inhibitor p21 (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990; Hollemberg *et al.*, 1993; Guo *et al.*, 1995; Halevy *et al.*, 1995). To evaluate whether p300 is involved in the induction of cell cycle arrest which occurs in differentiating myocytes, we first microinjected

Fig. 6. Effects of p300 blockade in MyoD-dependent inhibition of cell proliferation. C3H10T1/2 cells cultured in 20% FCS-containing medium and then injected with MyoD and p21 expression vectors either alone or in combination with wild-type and mutant E1A plasmids, anti-p300 antibodies and p300 vectors. (A) Arrows indicate cells injected with pCMV-MyoD alone (a and b) or in combination with anti-p300 antibodies (c and d) or the corresponding pre-immune serum (e and f) (see legend to Figure 5). The pCH110 expression vector (encoding β -galactosidase) was used as a marker for productively injected cells. After 18 h in 20% FCS-containing medium, cells were fixed and stained for β -galactosidase (rhodamine) and BrdU incorporation (fluorescein). BrdU was added 3 h before fixation of cells. The a, c and e fields show double β -gal and BrdU staining. (B) Arrows indicate cells injected with pCMV-MyoD alone (a, b and c) or in combination with an expression plasmid encoding the p300 negative dominant protein 1514–1933 (see text) (d, e and f). After 18 h culture in 20% FCS, cells were fixed and stained for MyoD expression (rhodamine) and BrdU incorporation (fluorescein). BrdU was added 3 h before fixation. Fields b and e show the double staining for MyoD and BrdU. (C) Quantitative evaluation of multiple experiments (p21 = pCMV-p21, E1A wt = pE1A12S; dl 2–36 = pE1A12Sdl2-36; p300 DN = pCMV- β p3001514-1922, p300 wt = pCMV- β p300). BrdU incorporation of pCH110 mock-injected cells (designated as basal) was >80%.

into the nuclei of serum-starved C2C12 myoblasts expression plasmids encoding either SV40 large TAG or the wild-type 12S E1A protein. Double immunofluorescence staining for either TAG (data not shown) or E1A expression and BrdU incorporation (Figure 4A, panels a and b) demonstrated that DNA synthesis still occurred after 24 h of serum withdrawal in a high proportion of injected myoblasts (>45%), as compared with the surrounding uninjected cells (<7%) (Figure 4B). The E1A p300 binding mutants RG2 (pE1A12SRG2), which includes an

Arg→Gly substitution at the second position and which interacts poorly with p300 (Wang *et al.*, 1993) (Figure 4B), and dl 2-36 (pE1A12Sdl2-36) (Figure 4A, panels c and d; Figure 4B), show a reduced ability to impair the G₀ arrest in serum-starved myoblasts. These results imply that p300 is involved in the induction of cell cycle withdrawal. Similar results in the modulation of DNA synthesis have been obtained using a panel of E1A mutants in cardiac myocytes (Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996). To test more directly the role of

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Fig. 7. Injection of wild-type and mutant E1A 12S-encoding plasmids and anti-p300 antibodies into C2C12 myotubes. **(A)** C2C12 3-day-old myotubes were injected into the nuclei with different E1A and control plasmids. Immediately after injection, 20% FCS-enriched medium containing BrdU was added. After an additional 18 h, cells were fixed and stained for nuclear expression of injected plasmids and BrdU incorporation. Arrows indicate myotubes injected with pE1A12S (a and b), pE1A12Sdl2-36 (c and d), pE1A12Sdl922-947 (e and f) and pRSV-cJun (g and h) (used as negative control). Myotubes were stained for either E1A (a, c and e) or c-Jun (g) expression (rhodamine) and BrdU incorporation (b, d, f and h) (fluorescein). Uninjected nuclei in a, c, e and h are visualized by DAPI counterstaining. In g and h, the open arrow indicates the nucleus of an uninjected myoblast which incorporates BrdU in response to serum. **(B)** C2C12 3-day-old myotubes were injected at multiple sites into the cytoplasm and occasionally into the nuclei with anti-p300 antibodies, switched to 20% FCS for 18 h and stained for accumulation of injected antibodies (rhodamine) and BrdU incorporation (fluorescein). The a field shows the double staining for BrdU incorporation and cytoplasmic anti-p300. In b, only cytoplasmic anti-p300 localization is represented.

p300 in the induction of cell cycle arrest in differentiating myoblasts, we microinjected anti-p300 antibodies into the cytoplasm of C2C12 cells. A significant delay in G_0/G_1 entry following serum starvation, as demonstrated by persistent BrdU incorporation, was observed (Figure 5A

and B). We next investigated whether p300 is involved specifically in the MyoD-dependent induction of cell cycle arrest (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990). Injection of C3H10T1/2 fibroblasts, growing in high serum, with a MyoD expression vector (pCMV-MyoD)

led, as expected (Sorrentino *et al.*, 1990), to inhibition of cell proliferation (Figure 6A, panels a and b; Figure 6B, panels a–c; Figure 6C). This effect was significantly reduced by co-injection of pE1A12S but not of pE1A12Sdl2-36 (Figure 6C). Co-injection of pCMV-MyoD with either anti-p300 antibodies (Figure 6A, panels c and d; Figure 6C) or a plasmid encoding the 1514–1922 p300 mutant (Yuan *et al.*, 1996), which is still able to bind MyoD but has no transcriptional activity and has been described previously to inhibit MyoD-dependent transactivation (Yuan *et al.*, 1996) (Figure 6B, panels d and f; Figure 6C), resulted in an increase of BrdU-positive cells. Taken together, these results support the thesis that p300 is required for MyoD-induced permanent cell cycle withdrawal in both muscle and MyoD-converted non-muscle cells.

To test whether p300 is also involved in the maintenance of cell cycle exit in differentiated myotubes, we injected C2C12 myotubes with plasmids encoding the wild-type E1A (pE1A12S), the p300 binding-defective mutant dl2-36 (pE1A12Sdl2-36) and the mutant dl922-947 (pE1A12Sdl922-947), which is unable to bind the 'pocket proteins' (Caruso *et al.*, 1993). In agreement with independent observations (Crescenzi *et al.*, 1995; M.Crescenzi, personal communication), wild-type E1A is able to re-induce the expression of cyclins (data not shown) and DNA synthesis in C2C12 myotubes with (Figure 7A, panels a and b) or without concomitant serum stimulation (data not shown). This property is retained by the E1A p300 binding mutant dl2-36 (Figure 7A, panels c and d) and it is lost by the E1A mutant dl922-947 which is unable to bind the 'pocket proteins' (Figure 7A, panels e and f). The injection of anti-p300 antibodies into differentiated myotubes does not re-induce DNA synthesis, even upon serum stimulation (Figure 7B). Thus, p300 does not play an essential role in the maintenance of the post-mitotic state of fully differentiated multinucleated myotubes. Other factors besides p300 (e.g. pRb) induced progressively during differentiation (Martelli *et al.*, 1994; Corbeil *et al.*, 1995; P.L.Puri, unpublished observation) might ensure myotube unresponsiveness to mitogenic stimuli.

Discussion

We have shown here that p300/MyoD-containing complexes are recruited at specific MyoD DNA binding sites in differentiating muscle cells and we have investigated the biological relevance of this interaction in the differentiation of skeletal muscle cells. Myogenic differentiation involves at least three major distinct steps: (i) commitment, in which proliferating myoblasts withdraw irreversibly from the cell cycle; (ii) induction of muscle-specific genes; and (iii) fusion of committed myoblasts into multinucleated myotubes (Weintraub, 1993; Olson and Klein, 1994). We first demonstrated that p300–MyoD interaction results in the potentiation of MyoD-dependent activation of downstream myogenic factors and muscle-specific gene transcription. This p300 activity is not restricted to MyoD, but may include other myogenic bHLH and/or MEF2 proteins. Next, we provided evidence that p300 participates in the induction of cell cycle arrest in myogenic cells induced to differentiate. Thus, our results indicate that

p300 plays an important role in two distinct differentiation-related phenomena.

The involvement of p300 in differentiation was first suggested by experiments which used the adenovirus E1A oncoprotein to interfere with key cellular proteins involved in the control of transcription, DNA synthesis, cell cycle and differentiation. These studies showed that the amino-terminal region of E1A is required for transformation (Whyte *et al.*, 1989) and is responsible for inhibition of a number of viral enhancers, as well as of enhancers and promoters of tissue-specific genes induced during differentiation (Moran, 1993, and references therein). Sequences in this region are involved in E1A binding to p300 (Moran, 1993; Eckner *et al.*, 1994); E1A mutants which have lost the ability to interact with p300 are also unable to inhibit terminal differentiation in many cell types, including skeletal muscle cells (Mymryk *et al.*, 1992; Caruso *et al.*, 1993), neuroblasts (Kalman *et al.*, 1993) and keratinocytes (Missero *et al.*, 1995). The demonstration that the induction of p21 expression in keratinocyte differentiation is dependent on p300 (Missero *et al.*, 1995) is the first direct evidence that p300 is involved in the transmission of a differentiation signal linked to growth arrest. p300 involvement in the induction of p21 during myogenic differentiation is also supported by the observation that microinjection of C2C12 differentiating cells with an E1A mutant defective for p300 binding is unable to interfere with both p21 up-regulation and myoblast fusion into myotubes, in contrast to wild-type E1A (P.L.Puri, unpublished observation). However, p300 alone has been shown to be unable to stimulate p21 promoter activity to any significant extent in keratinocytes, under either growing or differentiating conditions (Missero *et al.*, 1995). Thus, p300 protein by itself is not limiting, but is required for other differentiation-related factors to function. In agreement with this observation, we show that p300 synergizes with the myogenic factor MyoD in skeletal muscle cells undergoing differentiation, but displays only a limited activation of E-box-dependent transcription when expressed alone.

p300 and the closely related protein CBP have been shown to exhibit the properties of transcriptional adaptors between the basal transcription machinery and transcription factors that are bound to their target sequences (Arany *et al.*, 1994; Eckner *et al.*, 1994). The activity of both p300 and CBP is down-regulated directly and specifically by E1A (Arany *et al.*, 1995; Lundblad *et al.*, 1995). E1A binds to CBP through a domain conserved with p300 and inhibits the CREB-dependent co-activator functions of both CBP and p300 (Arany *et al.*, 1995; Lundblad *et al.*, 1995). Moreover, both CBP and p300 have a similar binding activity for the protein kinase A-phosphorylated form of CREB (Lundblad *et al.*, 1995) and p300 can substitute for CBP in potentiating CREB-activated gene expression (Arany *et al.*, 1995; Lundblad *et al.*, 1995). CBP stimulates c-Jun/c-Fos-dependent transcription in F9 cells (Bannister and Kouzarides, 1995) and binds both c-Jun and c-Fos (Arias *et al.*, 1994; Bannister and Kouzarides, 1995). p300 also binds c-Jun (Lee *et al.*, 1995; M.L.Avantaggiati, unpublished observations), but its ability to interact physically with c-Fos is at present unknown. Despite these observations, the degree of redundancy between these proteins *in vivo* remains largely

unexplored. Indeed, while CBP is readily demonstrable in the CRE binding activity of U2-OS cell extracts, p300 is not (Arany *et al.*, 1995). Moreover, p300, but not CBP, has been shown to be part of the DRF complexes that bind to the DRE element in the *c-jun* promoter and is required for both retinoid acid and E1A-induced expression of the *c-jun* gene in F9 cells (Kitabayashi *et al.*, 1995). In agreement with these observations, we did not detect CBP in E-box-bound complexes. Thus, although we did not test directly the ability of CBP to replace p300 in the potentiation of MyoD-dependent transcription and we did not exclude formally the existence of MyoD–CBP complexes, it appears that p300 is the preferential partner of MyoD at its DNA binding sites during differentiation of skeletal muscle cells.

As regards the role of p300 in cell growth arrest, we show for the first time that p300 is involved directly in the induction of terminal cell cycle arrest during muscle differentiation. MyoD-dependent induction of p21 is a likely target of p300. In fact, p300 cooperates with MyoD in the induction of p21 transcription (P.L.Puri and M.Levrero, manuscript in preparation). Although the results we obtained in MyoD-converted C3H10T1/2 cells would define a simple model where p300 would act as an essential co-factor for MyoD-dependent inhibition of cell proliferation, it is likely that in the more complex scenario of myogenic differentiation p300 might interact either positively or negatively with other factors to regulate myocyte cell cycle withdrawal. This would be in agreement with the more recently reported function of CBP/p300 proteins. Indeed, it has been observed that activation of transcription by nuclear receptors requires their binding to CBP, and inhibition of AP1 activity by nuclear receptors apparently results from competition for limited amounts of p300/CBP in cells (Kamei *et al.*, 1996). Thus, the p300/CBP family would act as an integrator at the nuclear level for different transcription factors activated by multiple signal transduction pathways. AP1 activation and muscle terminal differentiation are mutually exclusive phenomena, and functional antagonism between Jun/Fos and MyoD has been described (Bengal *et al.*, 1992; Li *et al.*, 1992). In this view, p300 association with antagonist transcription factors, i.e. c-Jun and MyoD, might dictate the choice between two mutually exclusive processes, such as proliferation and quiescence, during terminal differentiation. We also explored whether p300 plays a role in the maintenance of the post-mitotic state of myotubes. The results obtained by injection of E1A mutants and anti-p300 antibodies into myotubes argue against a role for p300 in preventing the cell cycle re-entry of myotubes upon serum stimulation. E1A mutants defective for 'pocket protein' binding lose the ability to stimulate DNA synthesis (this study and M.Crescenzi, personal communication). Since p107 is not able to complement pRb in preventing DNA synthesis upon mitogen stimulation in pRb^{-/-} myotubes (Schneider *et al.*, 1994), it is likely that pRb and/or pRb2/p130 are important in this process.

The observation that p300 levels remain substantially unchanged in cycling versus arrested cells (Moran, 1993), as well as during differentiation, suggests that post-translational modifications of p300 might be important in determining shifts of p300 partner(s) and function. A recent report demonstrated that changes in the phosphoryl-

ation status of p300 correlate with both retinoid acid- and E1A-induced terminal differentiation of embryonal carcinoma (EC) F9 cells (Kitabayashi *et al.*, 1995). Kitabayashi and colleagues found that the p300/DRF-containing complexes exist in F9 cells and bind their target DNA sequences before differentiation takes place. They speculate that differently phosphorylated forms of p300 might reflect a dual role for this protein in positive and negative regulation of transcription, with unphosphorylated p300 being correlated with a repressive activity of the protein towards the *c-jun* promoter in undifferentiated cells. In this model, upon stimulation with retinoic acid or expression of E1A, p300 would be hyperphosphorylated and would become transcriptionally active. p300 hyperphosphorylation also occurs in calcium-induced keratinocyte differentiation (Missero *et al.*, 1995). However, in this case, the p300 partner is unknown and p300-dependent differentiation-related functions are blocked and not stimulated by E1A. The potential role of the p300 phosphorylation status and skeletal muscle differentiation remains to be elucidated and might reflect a more complex scenario. p300 interaction with MyoD can be detected in undifferentiated C2C12 myoblasts (Yuan *et al.*, 1996) when E-box DNA binding activity is absent or very low. Further experiments are needed to define the role of p300 modifications, which occur after binding of p300–MyoD complexes to the DNA, for p300 function. We have shown recently that SV40 large T antigen forms a specific complex with p300 (Avantaggiati *et al.*, 1996). SV40 TAG binding to p300 is restricted to a newly identified unphosphorylated and ubiquitinated form of the protein and these differences are reflected *in vivo* as differences between the two oncoproteins in modulating the expression of CRE-containing genes (Avantaggiati *et al.*, 1996). Both E1A and TAG have been shown to inhibit muscle cell differentiation and both wild-type E1A and a temperature-sensitive mutant of SV40 TAG have been shown to re-induce the cell cycle in differentiated myotubes (Cardoso *et al.*, 1993; Crescenzi *et al.*, 1995). We also observed that E1A and TAG do not differ in their capacity to re-induce DNA synthesis upon microinjection into G₀-arrested myoblasts. However, some differences do exist between the two oncoproteins in their ability to interfere with discrete steps of myogenic differentiation. In fact, SV40 TAG inhibits muscle cell differentiation without affecting the induction of myogenin expression which occurs early in the process (Tedesco *et al.*, 1995). It will be interesting to investigate whether these differences between E1A and TAG can be explained by their different ability to bind various forms of p300.

Materials and methods

Cells, DNA transfections and CAT assays

C2C12 mouse cells (Yaffé and Saxel, 1977) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FCS (cycling myoblasts) until they reached confluence, when differentiation was induced by switching cell cultures to DMEM containing 2% FCS (DM, differentiation medium). Saos human osteosarcoma cells and C3H10T1/2 fibroblasts were grown in DMEM supplemented with 10% FCS. Cells were transfected by the calcium phosphate precipitation method. Total amounts of transfected DNA were equalized by pUC19 DNA. After 12–18 h incubation in medium containing the precipitated DNA, the cells were washed and cultured in fresh medium containing either 2% FCS (C3H10T1/2 cells) or 10% FCS (Saos cells) for 36 h before harvesting.

Cell lysates were prepared and CAT assays were performed as described (Puri *et al.*, 1995). The quantities of cell extracts used for CAT assays were normalized to β -galactosidase activity by co-transfection of 1 μ g of the β -gal expression vector pON260. The results were quantitated with a phosphorimager and expressed either as percentage conversion or fold induction, which is the ratio of the percentage of conversion obtained in the co-transfection experiment with the expression vector, to the percentage of conversion obtained with the pUC19 plasmid. The results are the mean of three different experiments $\pm$ standard error (SEM).

DNA binding assays

Extracts for E-box EMSAs were prepared as previously described (Gu, *et al.*, 1993). Thirty μ g of cell extract were combined in 30 μ l of binding reaction buffer containing 3 μ g of poly(dI-dC) in 10 mM Tris pH 7.5, KCl 40 mM, 10% glycerol, 1 mM dithiothreitol (DTT) and 32 P-end-labelled E-box probe derived from the muscle creatine kinase promoter (5'CCCCAACACCTGCTGCCTGA3'). After 20 min at room temperature, samples were subjected to electrophoresis in a 4% non-denaturing polyacrylamide gel, as described elsewhere (Puri *et al.*, 1995). For antibody shift EMSAs, concentrated antibodies were added to extracts either at room temperature or on ice for 30 min to 1 h (depending on the antibody or antiserum used) before the DNA binding reaction was started. Polyclonal antibodies against CBP (A-22 from Santa Cruz), myogenin (M-225 from Santa Cruz), MyoD (a gift from M.Crescenzi and S.Alemà), E12 (H208 from Santa Cruz) and p300 (Avantaggiati, *et al.*, 1996) and monoclonal antibody against p300 (NM11 from Pharmingen) were used. For affinity purification of E-box-bound complexes, 100 ng of a biotinylated E-box probe were immobilized to streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin). After several washes, the beads were resuspended with 500 μ g of [35 S]methionine-labelled cellular extracts, prepared as described above, in the presence of poly(dI-dC). After 1 h incubation at 4°C, the magnetic beads were removed using a magnet and the E-box-bound protein complexes were resuspended in SDS sample buffer and loaded on a 7.5% SDS-PAGE. Depletion of E-box binding activity in the supernatants was confirmed by gel retardation assay using the E-box probe as described.

Immunoprecipitations

Cells were pre-incubated with methionine-free DMEM for 2 h and then labelled with 0.5–3 mCi of [35 S]methionine (ICN) for 3 h in methionine-free DMEM, supplemented with 5% FCS. After labelling, cells were washed extensively with ice-cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (20 mM NaPO₄, 250 mM NaCl, 5 mM MgCl₂, 1% NP-40, 0.01% SDS 1 mM DTT), supplemented with freshly prepared protease and phosphatase inhibitors (10 mM sodium fluoride, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and leupeptin, aprotinin and pepstatin at 10 μ g/ml each). After 30 min of incubation on ice, cell extracts were clarified by centrifugation at 12 000 r.p.m. and the supernatants were pre-cleared using either the rabbit pre-immune serum (for anti-p300 immunoprecipitation) or a control isotype-matched monoclonal antibody (for anti-E1A immunoprecipitation) and protein A immobilized on Trisacril (Pierce) for 2 h at 4°C. After centrifugation at 12 000 r.p.m., the supernatants were immunoprecipitated with the different specific antibodies and protein A immobilized on Trisacril for 3–12 h at 4°C. Immune complexes were then washed 6–10 times in lysis buffer, eluted in 2 $\times$ SDS sample buffer and then loaded on 7.5% SDS-polyacrylamide gels, which typically were run overnight.

Western blot

After overnight electrophoresis, gels were equilibrated for 30 min in transfer buffer (25 mM Tris, 200 mM glycine). E-box-bound proteins were transferred to PDVF membranes (Millipore) at 0.25 A for 9 h at 4°C. Membranes were first incubated in 1 $\times$ Tris-buffered saline (TBS; 20 mM Tris, pH 6.5, 0.5 M NaCl) with 5% bovine serum albumin (BSA) for 6 h, then overnight with the specific anti-p300 antibody (dilution 1:1000) in 0.5 $\times$ 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 chemiluminant reagent (ECL Western blotting detection from Amersham) and exposure to an X-ray film.

Immunofluorescence

For immunofluorescence, cells were washed in PBS, fixed in a 1:2 methanol/acetone solution, dried, pre-incubated with 5% BSA/PBS and incubated for 30 min at 37°C with an ammonium sulfate concentrated preparation of the rabbit polyclonal anti-p300 antiserum at the final

dilution of 1:20. Specifically bound antibody was visualized by incubation with rhodamine-conjugated second-step anti-rabbit immunoglobulin antibody and observed using a fluorescence microscope. Immunofluorescence for the detection of BrdU, as DNA synthesis indicator, was performed using the BrdU Labeling and Detection Kit (Boehringer).

Microinjection

For microinjection experiments, cells were grown on small glass slides subdivided into numbered squares of 2 mm $\times$ 2 mm. Cells were micro-injected with either 100 molecules of DNA per nucleus or concentrated antibodies, as previously described (Graessman and Graessmann, 1983). Plasmid pCH110, which encodes β -galactosidase under the control of the SV40 early enhancer-promoter, was used as a marker for micro-injected cells, and its cytoplasmic expression was visualized by a specific anti- β -galactosidase antibody followed by a secondary rhodamine-conjugated antibody.

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