Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation

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Differentiation of skeletal muscle cells and B lymphocytes is regulated by basic helix–loop–helix (bHLH) proteins. Both differentiation programs are inhibited by the adenovirus E1A oncoprotein. Analysis of E1A mutants has implicated two of its cellular-binding proteins, p300 and CBP, in controlling certain aspects of differentiation. We find that p300 can cooperate with tissue-specific bHLH proteins in activating target genes and requires only the bHLH domain of such proteins to stimulate E box-directed transcription. Importantly, the ability of bHLH proteins to activate transcription correlates with the presence of p300/CBP in E box-dependent DNA-binding complexes, because both phenomena require at least two adjacent E-box motifs. Microinjection of p300/CBP antibodies into myoblasts blocks terminal differentiation, cell fusion, and transcriptional activity of myogenic bHLH proteins. These results suggest that the function of p300/CBP is essential for the execution of key aspects of cellular differentiation.

[Key Words: p300/CBP; myogenesis; B-cell differentiation]

Received June 17, 1996; revised version accepted August 16, 1996.

Members of the basic helix–loop–helix (bHLH) family of transcription factors have important roles in the differentiation of several cell types. In particular, the formation of skeletal muscle cells, neurons (for review, see Jan and Jan 1993; Weintraub 1993), B-lymphocytes (Bain et al. 1994; Zhuang et al. 1994) and erythrocytes (Shivdasani et al. 1995) requires the activity of various bHLH proteins.

The development of skeletal muscle cells is controlled by the four myogenic bHLH proteins—Myf-5, MyoD, myogenin, and MRF4—all of which are synthesized exclusively in skeletal muscle tissue. Forced expression of any of them in fibroblasts induces myogenic differentiation. Targeted inactivation of individual members of the myogenic bHLH proteins in mice has revealed functional differences between these regulatory factors (Weintraub 1993). During mouse embryogenesis, myogenin appears to be the major myogenic bHLH protein responsible for the myoblast to myotube transition. In its absence, essentially no myotubes are formed, and genes encoding contractile muscle proteins are not activated (Hasty et al. 1993; Nabeshima et al. 1993). Mice lacking myogenin die at birth because of their inability to breathe. In contrast, mice bearing inactivating mutations in either *myf-5* (Braun et al. 1992b), *myoD* (Rudnicki

et al. 1992), or *MRF4* (for review, see Olson et al. 1996) genes are capable of developing a functional skeletal muscle compartment. The role of Myf-5 and MyoD appears to lie in the specification and maintenance of myoblast identity, as mice without these two proteins do not form myoblasts (Rudnicki et al. 1993).

Mutational analysis of MyoD has shown that the bHLH region, by itself, is sufficient to direct conversion of fibroblasts into myocytes (Tapscott et al. 1988). Of particular importance for myogenesis and muscle-specific gene activation is the basic region of MyoD. This region likely undergoes a conformational change on DNA binding, leading to the exposure of a potent amino-terminal transcriptional activation domain (Ma et al. 1994). The bHLH region may also be the binding target of a cellular recognition factor (Weintraub et al. 1991). Indirect evidence suggests that this factor(s) regulates the activity of myogenic bHLH proteins in response to certain growth factors and oncogenes (Alema and Tato 1994; Kong et al. 1995 and references therein). Recent work has shown that MEF2 proteins (for review, see Olson et al. 1995) can physically interact with the bHLH domain and may therefore represent at least part of the postulated recognition factor activity (Kaushal et al. 1994; Molkenin et al. 1995). MEF2 proteins collaborate with myogenic bHLH gene products, and many muscle-specific transcriptional regulatory regions contain both E boxes (binding sites for bHLH proteins) and MEF2 sites (Olson et al. 1995). Inactivation of the single *MEF2* gene in *Drosophila* completely prevents differentiation of so-

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matic, cardiac, and visceral muscle cells (Bour et al. 1995; Lilly et al. 1995).

Similar to the role of MyoD family members in muscle differentiation, the products of the *E2A* gene have a key role in B-cell development. The two major proteins encoded by the *E2A* gene, E12 and E47, are expressed in most cell types and form heterodimers with tissue-restricted bHLH proteins. An exception are B lymphocytes, where E47 forms homodimers (Shen and Kadesch 1995). Gene targeting experiments have demonstrated the importance of these homodimers for B-cell formation. Mice lacking a functional *E2A* gene arrest B-cell development before immunoglobulin heavy chain rearrangement takes place (Bain et al. 1994; Zhuang et al. 1994). These animals do not display gross abnormalities in other cell types, suggesting that other widely expressed bHLH proteins (such as E2-2 or HEB) can substitute for *E2A* gene products as heterodimerization partners for tissue-specific E-box factors such as the myogenic bHLH proteins. Additional evidence for the involvement of *E2A* proteins in B-lymphocyte development was provided by studies using forced expression of E47. Overproduction of E47 induced germ-line immunoglobulin heavy chain gene transcription in pre-T cells (Schlissel et al. 1991) and activated the otherwise silent immunoglobulin heavy chain enhancer in fibroblasts (Ruezinsky et al. 1991).

The adenovirus E1A oncoprotein strongly interferes with cell differentiation and tissue-specific gene expression (Borrelli et al. 1984; Webster et al. 1988; Moran 1993; Bayley and Mymryk 1994). Analysis of the ability of E1A mutants to prevent skeletal muscle and neuronal differentiation has shown that maximally efficient inhibition of differentiation requires binding of E1A to both Rb family members and p300/CBP (Braun et al. 1992a; Boulukos and Ziff 1993). However, E1A mutants interacting with p300/CBP, but not with Rb-like proteins, were still capable of severely perturbing differentiation (Mymryk et al. 1992). Furthermore, such E1A mutants also retained the ability to repress tissue-specific genes (Stein et al. 1990). These results suggested an important role for p300 and CBP in cell differentiation and tissue-specific gene expression.

CBP was originally identified as a protein binding to the cAMP-responsive transcription factor CREB (Chrivia et al. 1993), whereas p300 was cloned by virtue of its ability to bind E1A (Eckner et al. 1994). p300 and CBP are large nuclear proteins of ~300 kD in size that are closely related in primary sequence and exhibit some of the properties of transcriptional adaptor molecules. Both proteins contain a bromodomain, an ~65-residue motif of unknown function that is found in several other proteins believed to function as transcriptional adaptors (Arany et al. 1994). When linked to a DNA-binding domain, both p300 and CBP revealed transcriptional activity (Chrivia et al. 1993; Arany et al. 1995).

Based on the above-noted indirect evidence implicating p300 and CBP in certain differentiation pathways, we have analyzed the behavior of these two proteins during myogenesis and B-cell specific transcription. Our results

show that p300/CBP interact, specifically with several bHLH proteins, at least in part, through their bHLH domains. p300/CBP also binds to MEF2 family members. In keeping with a key role of p300/CBP in differentiation, microinjection of p300/CBP monoclonal antibodies into myoblasts almost completely blocked myogenesis. We propose that the proper interaction of bHLH proteins and p300/CBP is essential for the execution of certain differentiation programs.

Results

p300 and MyoD can cooperate in transcriptional activation

Because p300 exhibits properties of a transcriptional adaptor protein and because experiments with E1A have implicated p300 in myogenesis, we tested whether p300 can enhance MyoD-dependent transcription. A minimal, MyoD-dependent reporter plasmid harboring four tandem E boxes upstream of a thymidine kinase promoter (4R-tk-CAT) was transfected into 10T1/2 cells together with expression vectors encoding MyoD, p300, or a p300–VP16 fusion protein containing all of p300 fused to the strong VP16 transactivation domain. We anticipated that p300–VP16 may activate transcription to a greater extent than p300, thereby serving as a sensitive indicator for the recruitment of p300 to a particular promoter. A small, but highly reproducible, increase in reporter activity was observed after cotransfecting MyoD and p300 (Fig. 1A). Coexpression of p300–VP16 and MyoD further enhanced transcription. These stimulatory effects depended on the presence of MyoD because the basal activity of the reporter plasmid was not affected by either cotransfected p300 or p300–VP16 alone (Fig. 1A). Similarly, the activity of a MyoD mutant (MyoD–E12b) was not increased by p300 or p300–VP16 (Fig. 1B). This MyoD mutant is known to bind DNA but fails to induce muscle-specific gene expression attributable to an altered basic region (Weintraub et al. 1991). Therefore, there is a correlation between the responsiveness of MyoD toward p300-mediated transcriptional stimulation and its ability to induce myogenic gene expression.

The bHLH domain of MyoD is sufficient to mediate a twofold increase in transcription upon cotransfection of p300 and a 10-fold increase upon introduction of p300–VP16 (Figure 1C). We conclude that p300 and MyoD can synergize in the activation of transcription and that this synergism depends on a specific domain of MyoD, the bHLH region, which was shown previously to be central for the myogenic functions of this protein (Tapscott et al. 1988).

Previous work in H. Weintraub's laboratory showed that complex muscle-specific transcriptional regulatory regions respond differently to MyoD than simplified reporter plasmids like 4R-tk-CAT (Weintraub et al. 1991). We therefore asked whether myogenic bHLH proteins and p300 can also cooperate in activating a complex promoter/enhancer, derived from a myosin light chain (MLC) gene. As shown in Figure 1D, cotransfection of

Figure 1. Synergistic and specific activation of transcription by p300 and myogenic bHLH proteins. 10T1/2 cells were transiently transfected [see Materials and Methods for details] with either a minimal MyoD-responsive reporter plasmid, 4R-tk-CAT, which carries 4 tandem E-box motifs upstream of a tk promoter/enhancer (A–C) or with a plasmid where a myosin light chain promoter drives expression of CAT (MLC-CAT; D). Expression plasmids coding for the proteins indicated underneath each bar were cotransfected with the CAT reporter. A dash (–) below a bar indicates cotransfection of the vector alone (without insert). The values shown represent the average of four independent experiments that were normalized to β -galactosidase activity (internal standard).

MyoD (left panel) or myogenin (right panel) together with wild-type p300 led to an enhancement of MLC reporter gene expression in the range of three- to fivefold compared with that observed with either myogenic bHLH protein alone. Therefore, myogenic bHLH proteins and p300 can synergize to activate both a simple and a more complex transcriptional unit.

Collaboration of p300 and E47 in activation of the immunoglobulin heavy chain enhancer

The immunoglobulin heavy chain (IgH) enhancer directs B cell-specific gene expression (for review, see Ernst and

Smale 1995) and contains three E boxes that are recognized by homodimeric products of the *E2A* gene. Because the IgH enhancer is strongly repressed by E1A (Borrelli et al. 1984), we investigated whether p300 can stimulate the activity of this enhancer. These assays were carried out in NIH3T3 cells because these cells were shown previously to depend on exogenously expressed E47 protein for activation of IgH reporter plasmids (Ruezinsky et al. 1991). Figure 2A illustrates that the combination of p300 and E47 synergistically activated the reporter plasmid. As with muscle-specific reporters, p300, by itself, failed to augment the basal activity of the IgH enhancer.

We next assayed the ability of p300 to cooperate with E47 alone by using the 4R-tk-CAT reporter plasmid. Cotransfection of E47 did not appreciably activate transcription (Fig. 2B). Similarly, wild-type p300 was unable to increase gene expression over basal levels, either alone

Figure 2. E47 and p300 cooperate in activating transcription directed by the immunoglobulin heavy chain enhancer. NIH3T3 cells were cotransfected with the reporter plasmid indicated above each panel and expression plasmids encoding the proteins listed below each bar (see Materials and Methods for details). The data are expressed as fold activation of CAT activity above that observed with the vector alone (represented by the first bar in each panel, marked with –). Values represent the average of four independent experiments.

or in combination with E47 (data not shown). However, the chimeric p300–VP16 protein was capable of strongly stimulating transcription in an E47-dependent manner, indicating that E47 is able to recruit p300–VP16 to this promoter. As in the case of MyoD, the bHLH domain of E47 was sufficient to direct wild-type p300 to an E box-containing reporter DNA, because coexpression of this domain of E47 and p300 resulted in enhanced transcription (Fig. 2C). This result again points to the importance of the DNA-binding domain of E box-binding proteins for cooperation with p300.

p300/CBP form complexes with tissue-specific E box proteins

To investigate whether p300/CBP and myogenic bHLH proteins can be detected in a common protein complex, we performed electrophoretic mobility shift assays. For these experiments, we used oligonucleotides bearing one or two E boxes. The presence of p300/CBP in gel shift complexes was monitored by the ability of antibodies reacting with both p300 and CBP to further retard (supershift) complexes. When incubated with extracts of differentiated C2C12 myotubes, both oligonucleotide probes gave rise to several bands of intermediate mobility (bands marked myo/E in Fig. 3A). These bands were supershifted by antibodies directed against MyoD or myogenin (data not shown) and, most likely, represent heteromeric complexes between myogenic and ubiquitous bHLH proteins.

Significantly, a complex of low mobility, migrating above the complexes with intermediate mobility, was only observed with the DNA fragment containing two E boxes. No such complex was visible with the single E-box probe (Fig. 3A, cf. lanes 1 and 7). The low mobility complex was supershifted quantitatively by two different p300/CBP monoclonal antibodies (Fig. 3A, lanes 5 and 6). A third p300 antibody (lane 4) failed to supershift this complex, likely because its epitope was not exposed. In addition, an unrelated control antibody (lane 3) also failed to supershift this band. Binding of the intermediate and low mobility complexes to the labeled probe was competed successfully by an excess of unlabeled, 2× E-box oligonucleotide (lane 2), but not by an unrelated oligonucleotide (data not shown). Moreover, none of the antibodies supershifted any of the complexes formed with the single E-box probe (Fig. 3A, lanes 7–11), or with a 2× E-box probe bearing two point mutations in one of the two E boxes (data not shown). From these results, we conclude that p300/CBP constitute part of a specific, DNA-bound complex that appears to depend on the presence of two adjacent E-box motifs.

Several different antibodies directed against either MyoD or myogenin did not supershift the low mobility complex possibly because their epitopes are masked in the high molecular weight complex (data not shown). In an effort to further ascertain whether MyoD was present in this complex, we performed immunodepletion experiments. For this approach, extracts of undifferentiated C2C12 myoblasts, which contain only MyoD as the sole

myogenic bHLH protein, were used. As shown in Figure 3B (lanes 1 and 2), a low mobility complex that was successfully competed by an excess of unlabeled 2× E box-oligonucleotide was also present in proliferating myoblasts. This band was supershifted by three different p300/CBP monoclonal antibodies (data not shown). Importantly, immunodepletion of the extract with a combination of a MyoD monoclonal antibody and a polyclonal E47 antiserum (that is also reactive with other ubiquitous E box-binding proteins) abolished both the low mobility complex and the heteromeric MyoD/E intermediate mobility complex (Fig. 3B, cf. lanes 3 and 1). A p300/CBP monoclonal antibody only eliminated the low mobility complex (lane 4), and none of the complexes was eliminated by an unrelated antibody (lane 5). From these findings, it appears that the low mobility complex contains, at a minimum, MyoD, an ubiquitous E box-binding protein, and p300/CBP. It should be noted that, under the conditions employed, none of the p300/CBP monoclonal antibodies discriminated between p300 and CBP.

The ability of p300 to interact with MyoD was also investigated by an *in vitro* binding assay using Gst–myoD protein produced in bacteria and *in vitro*-translated p300 protein. Figure 3C shows that *in vitro*-translated p300 or E12 proteins were specifically retained on beads loaded with Gst–myoD protein (Fig. 3C, lanes 3,4). These interactions depended on the MyoD moiety of the Gst–myoD protein because neither E12 nor p300 bound to Gst itself (Fig. 3C, lanes 5,6). Importantly, a Gst fusion protein composed of the bHLH domain of myoD also retained both p300 and E12 (Fig. 3C, lanes 7,8). The results of this experiment suggest that p300 exhibits considerable affinity for, and may actually contact, MyoD and its bHLH domain. These findings are consistent with our previous result showing that the bHLH region of MyoD is sufficient to confer a strong activation by p300–VP16 (Fig. 1C). Additional *in vitro* binding experiments with carboxy-terminal p300 fragments indicate that MyoD binding requires residues 1752–1869 (which are located in the C/H3 region) of p300, because removal of these residues abolished binding (data not shown).

Figure 3D shows that extracts of human B lymphocytes (BJAB cells) can also form a low mobility complex that can be quantitatively supershifted by three different p300/CBP antibodies. As was the case with muscle cell extracts, only an oligonucleotide carrying two E boxes led to the formation of this p300/CBP-containing complex. Immunodepletion experiments suggest that this B-cell complex contains, at a minimum, E47 and p300/CBP (data not shown). Therefore, two different cell types (muscle and B cells), whose differentiation depends on bHLH proteins, exhibited high molecular weight complexes composed of bHLH proteins and p300/CBP. It should be noted that the presence of p300/CBP in DNA-binding complexes generated on two E boxes correlates well with the ability of bHLH proteins to activate transcription, which also requires the presence of at least two E box-binding sites (Weintraub et al. 1990; Ruezinsky et al. 1991).

Figure 3. p300/CBP are components of low mobility complexes formed on DNA fragments carrying two adjacent E-box motifs. (A) Electrophoretic mobility shift assay using whole-cell extracts from differentiated C2C12 myotubes. (Lanes 1–6) A gel shift probe containing two E boxes was employed. For lanes 7–11, a probe with one E box was used. Lanes 1 and 7 show the gel shift pattern obtained with extract alone. In lane 2, a 200-fold excess of cold 2× E-box oligonucleotide was added to the gel shift reaction. For the other lanes, the antibodies indicated at the top were added to the binding reaction. Note the complete supershift of the low mobility complex (marked p300 + myo/E) by the RW 144 and AC 238 monoclonal p300/CBP antibodies in lanes 6 and 7. Complexes with intermediate mobility are labeled a–d and are collectively referred to myo/E on the left. Supershift experiments suggested that these complexes contain heterodimeric complexes between myogenic bHLH proteins and ubiquitous E box-binding proteins (data not shown). (B) The low mobility complex contains MyoD and p300/CBP. Extracts immunodepleted by either a combination of a myoD and E47 antibodies (lane 3), a p300/CBP monoclonal antibody (AC 238, lane 4), or a control antibody (PAb419, lane 5) were assayed for their ability to form a low mobility complex. (C) In vitro-translated p300 specifically binds to Gst–myoD and Gst–myoD–bHLH. In vitro-translated E12 or p300 proteins (see lanes 1,2 for input) were tested for their ability to bind to Gst–myoD (lanes 3,4), Gst alone (lanes 5,6), or Gst–myoD–bHLH (lanes 7,8). (D) Nuclear extracts of BJAB cells were assayed in a mobility shift assay with either a probe harboring two (lanes 1–6) or one E-box motif (lanes 7–11). Lanes 1 and 7 show the pattern resulting from the presence of extract, alone. Three p300/CBP antibodies (lanes 4–6) supershifted the low mobility complex (marked p300 + E47). The position of E47 homodimers is indicated on the left and right.

Interaction of MEF2 proteins with p300/CBP

The activity of many muscle-specific promoters and enhancers depends on both E boxes and binding sites for MEF2 family members. For cardiac myocytes and, to a lesser extent, for smooth muscle cells, MEF2 sites appear to be essential for the expression of several contractile protein genes (Olson et al. 1995; Kuisk et al. 1996). Given the importance of MEF2 function in myogenesis, we asked whether p300 can also bind MEF2 proteins. Figure 4 shows the result of an electrophoretic mobility

shift assay with a DNA probe bearing a single MEF2-binding site. Extracts of differentiated C2C12 myotubes revealed a specific MEF2 DNA-binding complex (Fig. 4, lanes 1–3) that was supershifted by a p300/CBP antibody (Fig. 4, lane 5). Moreover, in vitro-translated MEF2D protein appeared to associate quantitatively with p300/CBP, as a gel shift complex containing MEF2D was completely supershifted by a p300/CBP antibody (AC 238; Fig. 4, lane 11). In the absence of a MEF2 oligonucleotide, this antibody does not react with in vitro-translated MEF2 proteins, therefore ruling out cross reactivity of

Figure 4. MEF2 proteins bind p300/CBP. Electrophoretic mobility shift assay using an oligonucleotide bearing a single MEF2 site. In lanes 1–6, whole-cell extract of differentiated C2C12 cells were incubated with either a cold competitor oligonucleotide (lanes 2,3) or with the p300/CBP antibody noted at top (lanes 4–6). The DRE (differentiation responsive element) oligonucleotide served as competitor unrelated to a MEF2 site. For lane 7, AC 238 was added to the probe alone. Binding reactions in lanes 8 and 12 contained unprogrammed reticulocyte lysate and in lanes 9–11, reactions included in vitro-translated MEF2D protein. The migration position of the major MEF2 DNA-binding complex is indicated on the left. Unbound probe was run off these gels.

AC 238 with MEF2 proteins (data not shown). We conclude that p300 can interact physically with members of the myogenic bHLH and MEF2 protein families, the two major regulatory activities driving skeletal myogenesis.

Microinjection of p300/CBP antibodies prevents transactivation by MyoD

To address, more directly, the importance of p300/CBP function for MyoD-dependent transactivation, we performed microinjection experiments with monoclonal antibodies raised against p300/CBP. 10T1/2 mouse embryo fibroblasts served as recipient cells. CAT reporter plasmids driven either by the myogenin promoter or by the enhancer/promoter of the muscle creatine kinase (MCK) gene were injected into 10T1/2 cells together with a MyoD expression vector and either p300/CBP antibodies or control mouse immunoglobulin G. Twenty-four hours later, cells were stained with antibodies against CAT protein to score for induction of the reporter gene. Figure 5A shows that the coinjected p300/CBP antibodies prevented, almost completely, the activation of MCK–CAT (Fig. 5, bottom two panels). In con-

trast, no inhibition was observed when mouse IgG were coinjected (Fig. 5, top two panels). In parallel experiments, injected p300/CBP antibodies did not interfere with the activity of a CAT reporter driven by the cytomegalovirus (CMV) enhancer/promoter (data not shown). We also tested the myogenin promoter in the same type of assay and observed a block in expression comparable to that of MCK–CAT. These results are quantitated in Figure 5B. They indicate that p300/CBP function is required for the ability of MyoD to transactivate differentiation-specific genes.

Figure 5. Injection of p300/CBP antibodies inhibits transactivation by MyoD. (A) The ability of MyoD to activate an MCK–CAT reporter plasmid was examined in microinjected 10T1/2 cells. The top two panels show a field of cells into which mouse IgG were introduced, whereas the bottom two panels show cells microinjected with p300/CBP antibodies. Cells in the two panels on the left (green signals) were stained for the presence of mouse IgG (top) or p300/CBP antibodies (bottom), cells in the two panels on the right (red signal) were incubated with an antibody against CAT. Injection of p300/CBP antibodies prevented activation of the MCK–CAT reporter plasmid. (B) Graphic representation of the results of injecting 10T1/2 cells with anti-p300/CBP and murine IgG. Injected cells were scored for reporter gene activation as described in A. The numbers of cells exhibiting activation of MCK–CAT were: 54.5% (305 out of 559 cells) of cells injected with control mouse IgG (mIgG) and 0.6% (5 out of 807) for cells receiving p300/CBP antibodies. Numbers for the myogenin promoter–CAT were: 48.2% (306 out of 635 injected cells) receiving mouse IgG and 1.5% (9 out of 601 cells) of cells receiving p300/CBP Ab activated the reporter.

Interference with the function of p300/CBP prevents differentiation of skeletal myoblasts

To evaluate the role of p300/CBP during the myoblast to myotube transition, exponentially growing C2C12 myoblasts were microinjected with either p300/CBP monoclonal antibodies or control mouse IgG. Subsequently, cells were incubated in differentiation medium and processed for immunofluorescence 3 days later. Distinct steps in the differentiation process, each taking place at a different time point, were monitored. Cell cycle withdrawal was analyzed by staining for the cyclin-dependent kinase (cdk) inhibitor, p21, which is upregulated in C2C12 cells as a prelude to differentiation (Halevy et al. 1995). Myogenin served as a marker for the beginning of the actual differentiation process, and the appearance of troponin T provided a marker for terminal differentiation.

Fig. 6A illustrates the inability of cells microinjected with p300/CBP antibodies (Fig. 6A, green cells in left panel in bottom row) to display troponin T synthesis following serum deprivation (Fig. 6A, see middle panel, arrowhead indicates a representative cell). A neighboring non-injected myotube displayed clear troponin T immunofluorescence (Fig. 6A, see bottom of middle panel). In parallel injections, control antibodies did not interfere with troponin T induction (Fig. 6A, top three panels).

In a second experiment, myogenin expression was analyzed. Cells injected with p300/CBP antibodies failed to express myogenin (Fig. 6B, bottom three panels), whereas control antibodies did not inhibit myogenin induction (Fig. 6B, top three panels). Strikingly, p300/CBP antibodies also prevented induction of p21 (Fig. 6C, bottom three panels). The percentage of cells exhibiting a p21 signal (relative to the total number of cells injected) is summarized in Figure 6D. Importantly, injection of p300/CBP antibodies, but not control IgG, also inhibited cell fusion as can be seen by the absence of multinucleated myotubes in these cell samples (see Fig. 6E for a quantification of myotube formation). In a negative control experiment, inhibition of differentiation was abolished when, before injection, p300/CBP antibodies were preabsorbed with the Gst–CBP protein fragment against which they were raised (data not shown). In contrast, preabsorption with unfused Gst protein did not eliminate inhibition of differentiation.

Taken together, these results indicate that the unperturbed activity of p300/CBP is required for the execution of key steps during the differentiation of myoblasts into myotubes.

Discussion

The results of this study establish a fundamental role for p300/CBP function in two cellular differentiation pathways. We show that these proteins are a component of large DNA-binding complexes that assemble on transcriptional regulatory regions containing at least two E boxes. In skeletal muscle cells, the high molecular weight complex is composed, at a minimum, of MyoD/E

heterodimers and p300/CBP. The B-cell complex, most likely, contains homodimers of E47 and p300/CBP. Because in both cell types the presence of two E-box motifs is necessary for effective promoter function (Weintraub et al. 1990; Ruezinsky et al. 1991), we conclude that the physical presence of p300/CBP in these complexes correlates with the ability of tandem E boxes to activate transcription. Apart from myogenic bHLH proteins, p300/CBP can also interact with members of the other major transcription factor family pivotal for muscle cell formation, the MEF2 proteins. Finally, in agreement with the above-noted findings, microinjection experiments strongly suggest that the activity of p300/CBP is essential for several aspects of skeletal myogenesis.

A common characteristic of many muscle-specific promoters and enhancers is the presence of two or more E boxes. Examples include the MLC1/3 locus (Wentworth et al. 1991), the promoter for the acetylcholine receptor α subunit (Piette et al. 1990), and the enhancers regulating expression of the genes encoding MCK (Buskin and Hauschka 1989; Lassar et al. 1989), and myoD (Goldhamer et al. 1995). In most of these cases, the presence of at least two E boxes is essential for muscle-specific transcriptional activity (e.g., see Lassar et al. 1989). Similarly, in B cells, immunoglobulin light and heavy chain enhancers contain two and three E-box motifs, respectively, and these elements are required for full activity of these control regions (Ernst and Smale 1995). Given these observations, our finding that p300/CBP preferentially interact with myogenic or B-lymphoid-specific bHLH proteins occupying two E-box motifs is of physiological relevance. The ability of p300/CBP to participate in such higher order complexes provides a clue to the biological functions of these two large proteins. In such complexes, p300 and CBP may be in an ideal position to act as molecular matchmakers or integrators (see below) that assist in coordinating or bringing together individual protein components, which together effect a biologically important process, such as tissue-specific transcription. The presence of multiple, evolutionarily conserved segments in p300/CBP, a *Drosophila* (N. Modjtahedi and D.M. Livingston, unpubl.) and a *Caenorhabditis elegans* homolog (Arany et al. 1994) is consistent with such an idea.

Gel shift experiments suggest that p300/CBP can also interact with MEF2 proteins. Given the importance of MEF2 sites for the transcription of many muscle-specific genes (Olson et al. 1995), this finding is relevant for the differentiation of all three muscle cell types (skeletal, cardiac, and smooth muscle cells). In the case of skeletal muscle differentiation, the two major transcription factor families regulating this process, myogenic bHLH and MEF2 proteins, both associate with p300/CBP. Because a single MEF2-binding site is sufficient to recruit p300/CBP to DNA, MEF2 may facilitate the interaction of a myogenic bHLH protein bound to an adjacent single E-box motif (which, alone, is unable to recruit p300/CBP) with these two large proteins. Such a facilitated interaction might be important for the strong, cooperative action of myogenic bHLH and MEF2 proteins in in-

Figure 6. Injection of p300/CBP antibodies blocks differentiation of C2C12 myoblasts. (A) Cell fusion and troponin T expression are blocked in p300/CBP injected cells. The three panels in the *top* row show a field of cells injected with inert mouse IgG (mIgG, marker antibody), and the *bottom* three panels show a field of cells into which p300/CBP antibodies were introduced. Injected cells were identified by staining them with an antibody to mouse IgG (green signal). The *middle* panels of both rows were stained with a troponin T antibody (red signal) and the *right* panels show the location of the nuclei (DAPI staining). Note the large myotube (top row, marked with arrowhead) that formed from cells injected with mIgG (left panel) and that expresses the terminal differentiation marker troponin T (middle panel). In the *bottom* row, cells injected with p300/CBP mouse monoclonal antibody remained unfused (left panel) and did not express TNT (middle panel). The nucleus of one of the injected cells is marked by an arrowhead. A myotube formed from uninjected cells can be seen at the *bottom* of the middle panel. (B,C) The early muscle differentiation markers, p21 and myogenin, are not induced in cells injected with p300/CBP antibodies. The *top* row of B and C each show a field of cells injected with control mouse IgG, whereas the *bottom* row of each of the two figures displays a field of cells that received p300/CBP antibodies. The *middle* panels in B and C were stained with a myogenin and a p21 antibody, respectively. The *right* panels (marked double) show a superimposed image of the first two panels. Cells injected with inert mIgG formed myotubes and expressed high levels of nuclear myogenin or p21. Note that some of the cells injected with mIgG failed to fuse but, nevertheless, produced p21 or myogenin. Cells into which p300/CBP antibodies had been introduced did not form multinucleated myotubes and failed to express either p21 or myogenin (a representative cell nucleus is indicated by an arrowhead). Note that surrounding, uninjected cells formed myotubes and expressed p21 or myogenin. (D) Percentage of injected cells exhibiting nuclear p21 staining. In a summary of three independent experiments, 69.4% (148 out of 213 injected cells) and 0.5% (1 out of 201 injected cells) p21 positive cells were observed for mIgG and p300/CBP antibody-injected cells, respectively. Multinucleated myotubes were scored as one positive cell. (E) Number of myotubes derived from injected cells. In a summary of six, independent experiments, 135 and 1 myotubes were observed in cells injected with mouse IgG and p300/CBP antibodies, respectively.

ducing myogenic differentiation (Molkentin et al. 1995). If true, p300/CBP may again behave as a coordinator or integrator of the functions of these two classes of transcriptional regulators.

Which are the structural features of bHLH proteins recognized by p300/CBP? Our transfection results indicate that the bHLH domain of MyoD or E47, each of which is, most likely, bound to its endogenous dimerization partner, is sufficient to mediate interaction with p300/CBP. Previous reports have shown that MEF2 proteins also bind to this part of myogenic bHLH proteins (Kaushal et al. 1994; Molkentin et al. 1995). However, unlike p300/CBP, MEF2 proteins preferentially bind to myogenic bHLH proteins and, likely, do not interact with dimers formed by *E2A* gene products (Molkentin et al. 1995). Because p300/CBP binds to bHLH domains derived from both myogenic and B cell-specific bHLH proteins, we assume that p300/CBP recognize determinants of structure or sequence common to both sets of bHLH proteins. These determinants may be located in either the basic or the HLH region, or both.

MyoD harbors a strong transactivation domain in its amino terminus (Weintraub et al. 1991). Our data demonstrate that this region of MyoD is dispensable for p300 binding, and therefore separate the segment of MyoD carrying out transactivation from that directing p300 binding. However, these two domains of MyoD do not operate completely independently from each other. Results from mutagenesis experiments (Weintraub et al. 1991) and the crystal structure of MyoD (Ma et al. 1994) indicate that the bHLH region influences the exposure of the transactivation domain. Upon DNA binding, a conformational change in myogenic bHLH proteins is likely to trigger exposure of the transactivation domain. This conformational change in the DNA-binding domain may also be important for high-affinity binding of p300/CBP to myogenic bHLH proteins because we have not detected such complexes after direct immunoprecipitation from cellular extracts (data not shown). Under physiological MyoD protein levels, stable complexes containing, at a minimum, MyoD and p300/CBP were only observed in the presence of a DNA fragment harboring two adjacent E-box motifs. It is conceivable that, by virtue of their close proximity to the transactivation domain of myogenic bHLH proteins, p300/CBP influence the functional exposure of the MyoD transactivation domain, therefore providing another level of control for the activity of this class of bHLH proteins.

Such a regulatory mechanism may be relevant to an understanding of how signal transduction events influence muscle and B cell-specific transcription. Several specific agents like cyclic AMP (Li et al. 1992; Winter et al. 1993), activated ras (Kong et al. 1995) or Notch (Kopan et al. 1994), and high concentrations of serum have been shown to interfere efficiently with myogenic differentiation. In most of these cases, the activity of myogenic bHLH proteins was affected indirectly, that is, neither heterodimer formation, DNA-binding activity, nor the activation potential of the transactivation domains of myogenic bHLH proteins, linked to Gal4, were impaired.

Instead, it has been postulated that a coactivator-like activity may represent the direct target of these inhibitory agents. p300/CBP are attractive candidate molecules for mediating this type of regulation because they have been implicated previously in transcriptional pathways responsive to signal transduction pathways (Arias et al. 1994) and because, in many cases, this response is independent of new protein synthesis. Our results show that both myoblasts (grown in high serum) and myotubes (grown under low serum conditions) exhibit complexes containing p300/CBP and myogenic bHLH proteins, and therefore p300/CBP appears to interact constitutively with these myogenic factors. This raises the possibility of an additional regulatory step in which p300/CBP influence the activity of bHLH proteins. Because p300/CBP are known to be differentially phosphorylated in response to certain signaling events (e.g., see Kitabayashi et al. 1995), it is conceivable that the phosphorylation status of p300/CBP may regulate the functional exposure of the transactivation domain of myogenic bHLH proteins. Consistent with such a finely tuned interplay is the finding that a MyoD–VP16 fusion protein is resistant to negative regulation by Notch (Kopan et al. 1994).

A third function of p300/CBP may be to participate in determining the pronounced specificity of target gene activation by individual myogenic bHLH proteins. For example, only myogenin, and not MyoD or Myf-5, is capable of activating the terminal skeletal muscle differentiation program (Hasty et al. 1993; Hollenberg et al. 1993; Nabeshima et al. 1993). Functional differences also exist between MyoD and Myf-5. Each of these two proteins is expressed in a distinct muscle cell lineage, and, in vivo, the *myoD* gene promoter can only be activated by MyoD but not by Myf-5 (Braun and Arnold 1996). Because p300/CBP can interact with several transcription factors involved in myogenesis (bHLH and MEF2 proteins), these two large proteins may be part of a complex monitoring mechanism that assures that a given promoter is activated by the correct transcription factor.

In support of the view that p300/CBP are important for determining target gene specificity of transcription factors are results of P. Chambon's laboratory. These investigators analyzed the response of the IgH enhancer to expression of the adenovirus E1A oncoprotein in both B lymphocytes and fibroblasts. In B cells, E1A strongly downregulated IgH enhancer activity (Borrelli et al. 1984), whereas in fibroblasts, where this enhancer is normally inactive, E1A actually induced IgH enhancer activity to some extent (Borrelli et al. 1986). Although the regions of E1A required for these effects were not determined, it was concluded that E1A relieves negative regulation imposed on the IgH enhancer in fibroblasts. In this way, E1A enabled ubiquitous transcription factors to bypass repression and activate the IgH enhancer in cells where it is normally not active. The results discussed here, though not yet wholly conclusive, are consistent with a model in which p300/CBP has a dual role in controlling gene expression. These two proteins may

be required for both activation (in B cells) and repression (in non-B cells) of the IgH enhancer.

In the last two years, p300 and CBP have been shown to interact with a variety of transcriptional regulators, including CREB, YY1, *c-fos*, *c-jun*, *c-myb*, and certain members of the steroid hormone receptor family (Hanstain et al. 1996; Kamei et al. 1996; Yao et al. 1996). In addition, we have observed recently that p300/CBP binds to a transcription factor (HIF-1) that is induced specifically on oxygen deprivation. A common theme underlying all of these interactions is that they involve transcription factors participating in regulated gene expression. Based on these observations, we believe that p300/CBP participates in a broad spectrum of cellular processes requiring changes in transcription rates. Such p300/CBP-dependent processes are likely to include cellular differentiation, signal transduction, cell cycle regulation and stress responses.

Materials and methods

Plasmids

p300—VP16 was generated by ligating a PCR fragment encompassing residues 413–490 of the herpes simplex virus (HSV) VP16 protein in frame to the *NheI* site near the carboxy-terminus of p300. The chimeric cDNA was expressed from CMVβ (Eckner et al. 1994). For all transient transfection studies, expression of myogenic bHLH proteins, E47, and their derivatives was driven by the CMV promoter (contained in either pCSA or pcDNA3). DNA fragments comprising the bHLH domains of Myf-5 and E47 (including a translation initiation signal) were amplified by PCR using the following primers: 5′-GCGGAATTCACCATGGCCTGCAAAGCCTGCAAG-3′ and 5′-CGTCAAGCTTCTAGTTCTCCACCTGCTCTC-3′ for myf-5; and 5′-GCGGAATTCACCATGGCCCGGACCAGTACGGAC-3′ and 5′-CGTCCAAGCTTCTAATTCAGGTTCCGCTCTCGCA-3′ for E47. The reporter plasmids 4R-tk-CAT and MLC-CAT have been described (Weintraub et al. 1990; Wentworth et al. 1991), respectively. The MLC-CAT reporter was composed of a 173-bp fragment of the rat MLC1/3 gene 3′ enhancer linked to the MLC1 promoter. The plasmid referred to as MCK-CAT in this study was originally called –1256MCK-CAT (Jaynes et al. 1988); the myogenin promoter-CAT construct used for microinjections was originally designated Myf41-CAT (Salminen et al. 1991). IgH-CAT contained fragment 18 of the murine IgH enhancer (Kiledjian et al. 1988).

Cells and transfections

C2C12 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal calf serum, and B-JAB cells were grown in RPMI 1640 supplemented with 10% Fetalclone I serum (Hyclone). 10T1/2 were grown in DMEM containing 10% Fetalclone I serum, and NIH3T3 cells were cultured in DMEM plus 10% iron-supplemented bovine calf serum (Hyclone). The latter two lines were maintained at low density and were transfected by the CaPO₄-method (Ausubel et al. 1989). In general, transfections contained 5 μg of the indicated reporter plasmid. When 4R-tk-CAT or IgH-CAT were used as reporter plasmids, transfection mixes contained, where indicated, 2 μg of an expression vector encoding either a myogenic bHLH protein, E47, or derivatives thereof. If MLC-CAT was employed, transfection mixes contained, where indicated, 1 μg of either

CMV-myod or CMV-myogenin. To investigate the effect of p300 expression on reporter activity, 6 μg of CMV-p300 or CMV-p300-VP16 were used. All transfections contained 2 μg of CMV-lacZ as an internal standard. Twenty-four hours after removal of the DNA-precipitate, 10T1/2 and NIH 3T3 cells were switched to DMEM supplemented with 2% horse serum and cultured for another 36–40 hr (10T1/2) or 24 hr (NIH 3T3). Cells were lysed and processed for lacZ and CAT assays as described (Ausubel et al. 1989).

Gel mobility shift assays and immunodepletion

Whole-cell extracts of exponentially growing or differentiated C2C12 cells were prepared according to Lassar et al. (1991). Nuclear extracts of BJAB cells were prepared essentially as described (Schreiber et al. 1989), except that the nuclear extraction buffer was modified to contain 15% glycerol. Two microliters of such extracts (containing 10–14 μg of protein) were added to 13 μl of gel shift buffer (20 mM Tris-HCl at pH 7.5, 120 mM NaCl, 5% Ficoll, 1 μg poly(dI-dC), 0.1–0.3 ng of ³²P-labeled probe). Where applicable, 1 μl of ascites fluid containing p300/CBP monoclonal antibody was added, and after a 10-min incubation at room temperature, reactions were loaded on 4.5% acrylamide gels. In this concentrated form, the p300/CBP antibodies used react with both p300 and CBP. Gels were electrophoresed in the cold (–8°C) with a Tris/glycine buffer (25 mM Tris, 190 mM glycine, 0.1 mM EDTA) for 3 hr. The 1× E-box oligonucleotide probe corresponds to the right E-box motif in the MCK enhancer and had the sequence 5′-GATCCCCCAACACCTGCTGCCTGG-3′ (upper strand, E box is underlined). The sequence of the upper strand of the 2× E-box probe was 5′-GATCCCCGAGCAGGTGTTGGGAGGCAGGTCGAGAC-3′ and that of the 2× E-box oligonucleotide carrying a mutation in the second E box was 5′-GATCCCCGAGCAGGTGTTGGGAGGCAGCGGGAGGAGAC-3′. The upper strand of the MEF2 probe reads: 5′-GATCCTTGGCTATATTTATCTCTGG-3′.

To immunodeplete C2C12 extracts of MyoD/E heterodimers or p300/CBP, two successive rounds of immunoprecipitations were performed. Twenty micrograms of antibody were bound to 15 μl of protein A sepharose [1:1 slurry of beads in NET-N (Harlow and Lane 1988)]. Antibody-loaded beads were washed three times with EBC (50 mM Tris-HCl at pH 8.0, 180 mM NaCl, 0.5% NP-40) followed by one washing step with extraction buffer (Lassar et al. 1991). Ten microliters of extract was added to the drained beads and incubated for 1 hr in the cold with occasional flicking of the tube. Beads were pelleted and the supernatant was subjected to another, identical round of immunoprecipitation. The following antibodies were used: 5.8A MyoD monoclonal antibody (Novocastro), affinity-purified rabbit antiserum against E47 (Santa Cruz), one of two p300/CBP monoclonal antibodies, AC 238 or AC 240 (Eckner et al. 1996), and PAb419 (Harlow et al. 1981) as a control antibody. AC 238 and AC 240 were used in the form of ascites. Immunodepletion of E2A gene products from BJAB extracts was achieved by using monoclonal antibody Yae (Santa Cruz).

In vitro translations and Gst-binding assay

E12 and p300 were translated in vitro in the presence of [³⁵]methionine using a TNT kit (Promega). In vitro-translated products were incubated with glutathione-Sepharose beads loaded with either Gst-myod, Gst-myod-bHLH (Lassar et al. 1989) or Gst proteins in EBC for 1 hr in the cold. Beads were washed four times in EBC, resuspended in SDS sample buffer, and the released proteins were separated in an SDS-protein gel.

Microinjection and immunostaining

All antibodies and plasmids were injected in a buffer containing 50 mM HEPES at pH 7.2, 100 mM KCl, and 5 mM NaPO₄, pH 7.2. Nuclei were injected with a Femtotip (Eppendorf) with the help of a microinjector (Nikon). C2C12 cells were injected with either a mixture of the two p300/CBP monoclonal antibodies, AC 217 (2 mg/ml) and AC 240 (5 mg/ml), or mouse IgG (control). We have found recently that injection of AC 217 is sufficient for blocking C2C12 differentiation. Following injection, cells were transferred to DMEM supplemented with 2% horse serum and cultured for 3 days. Cells were then fixed and processed for immunostaining with different antibodies (see below).

For MyoD reporter assays, C3H 10T1/2 fibroblasts were starved, before injection, for ~24 hr in DMEM containing 2% horse serum. Plasmids were injected at the following concentrations: CMV–myoD at 100 ng/μl, MCK–CAT and myogenin promoter–CAT at 180 ng/μl. Together with these plasmids, either a mixture of AC 217 (1 mg/ml) and AC 240 (2.5 mg/ml), or a control antibody (3.5 mg/ml of mouse IgG) was introduced. The injected cells were processed and stained with a CAT antibody 24 hr later. For immunostaining, cells were fixed in 3% paraformaldehyde, permeabilized by a short treatment with 0.2% Triton X-100 in PBS and then incubated with primary antibody at room temperature for 1–2 hr, or at 4°C overnight. Antibodies were diluted in the following way: CAT: 1:500 (5' → 3'), p21, and myogenin 1:500 (Santa Cruz), troponin T: 1:5000. Secondary antibody (Jackson) conjugated to FITC or rhodamine was then applied at a dilution of 1:200. Nuclei were visualized by staining with Hoechst 33258 (Sigma).

Acknowledgments

We thank H.-H. Arnold, J. Buskin, P. Houghton, T. Kadesch, D. Krainc, B. Novitsch and A. Lassar, J. Schwarz and E. Olson, H. Weintraub, and W. Wright for providing plasmids and antibodies enabling us to perform this study. R.E. was supported by fellowships from the Swiss National Science Foundation and Ciba-Geigy Jubilaeums-Stiftung. T.-P.Y. acknowledges a fellowship from Damon Runyon-Walter Winchell Cancer Foundation (DRG 1359). Research in the laboratory of D.M.L. is supported by grants from the National Institutes of Health and by the Sandoz/Dana-Farber Cancer Institute drug discovery program.

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References

- Alema, S. and F. Tatro. 1994. Oncogenes and muscle differentiation: Multiple mechanisms of interference. *Cancer Biol.* **5**: 147–156.
- Arany, Z., W.R. Sellers, D.M. Livingston, and R. Eckner. 1994. E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. *Cell* **77**: 799–800.
- Arany, Z., D. Newsome, E. Oldread, D.M. Livingston, and R. Eckner. 1995. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. *Nature* **374**: 81–84.
- Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smeal, M. Karin, J. Feramisco, and M. Montminy. 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**: 226–229.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, and K. Struhl. 1989. *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York, NY.
- Bain, G., E.C. Robanus Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintraub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, M. van der Falk, H.P.J. te Riele, A. Berns, and C. Murre. 1994. E2A proteins are required for proper B cell development and initiation of Immunoglobulin gene rearrangements. *Cell* **79**: 885–892.
- Bayley, S.T. and J.S. Mymryk. 1994. Adenovirus E1A proteins and transformation. *Int. J. Oncol.* **5**: 425–444.
- Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* **312**: 608–612.
- Borrelli, E., R. Hen, C. Waslylyk, and P. Chambon. 1986. The immunoglobulin heavy chain enhancer is stimulated by the adenovirus type 2 E1A products in mouse fibroblasts. *Proc. Natl. Acad. Sci.* **83**: 2846–2849.
- Boulukos, K.E. and E.B. Ziff. 1993. Adenovirus 5 E1A proteins disrupt the neuronal phenotype and growth factor responsiveness of PC12 cells by a conserved region 1-dependent mechanism. *Oncogene* **8**: 237–248.
- Bour, B.A., M.A. O'Brien, W.L. Lockwood, E.S. Goldstein, R. Bodmer, P.H. Taghert, S. Abmayr, and H.T. Nguyen. 1995. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes & Dev.* **9**: 730–741.
- Braun, T. and H.-H. Arnold. 1996. myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. *EMBO J.* **15**: 310–318.
- Braun, T., E. Bober, and H.H. Arnold. 1992a. Inhibition of muscle differentiation by the adenovirus E1a protein: Repression of the transcriptional activating function of the HLH protein Myf-5. *Genes & Dev.* **6**: 888–902.
- Braun, T., M.A. Rudnicki, H.-H. Arnold, and R. Jaenisch. 1992b. Targeted Inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71**: 369–382.
- Buskin, J.N. and S.D. Hauschka. 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9**: 2627–2640.
- Chrivia, J.C., R.P.S. Kwok, N. Lamb, M. Hagiwara, M.R. Montminy, and R.H. Goodman. 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**: 855–859.
- Eckner, R., M.E. Ewen, D. Newsome, M. Gerdes, J.A. DeCaprio, J.B. Lawrence, and D.M. Livingston. 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes & Dev.* **8**: 869–884.
- Eckner, R., J.W. Ludlow, N. Lill, E. Oldread, Z. Arany, N. Modjtahedi, J.A. DeCaprio, D.M. Livingston, and J.A. Morgan. 1996. Association of p300 and CBP with SV40 large T antigen. *Mol. Cell. Biol.* **16**: 3454–3464.
- Ernst, P. and S.T. Smale. 1995. Combinatorial regulation of transcription II: The immunoglobulin μ heavy chain gene. *Immunity* **2**: 427–438.
- Goldhamer, D., B. Brunk, A. Faerman, A. King, M. Shani, and C.P. Emerson. 1995. Embryonic activation of myoD is regulated by a highly conserved distal control element. *Development* **121**: 637–649.
- Halevy, O., B.G. Novitsch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, and A.B. Lassar. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**: 1018–1021.
- Hanstein, B., R. Eckner, J. DiRenzo, S. Halachmi, H. Liu, B. Searcy, R. Kurokawa, and M. Brown. 1996. p300 is a component of an estrogen receptor co-activator complex. *Proc.*

- Natl. Acad. Sci.* **93**: (in press).
- Harlow, E. and D. Lane. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Harlow, E., L.V. Crawford, D.C. Pim, and N.M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**: 861–869.
- Hasty, P., A. Bradley, J.H. Horris, D.G. Edmondson, J.M. Venuti, E.N. Olson, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**: 501–506.
- Hollenberg, S.M., P.F. Cheng, and H. Weintraub. 1993. Use of a conditional myoD transcription factor in studies of myoD trans-activation and muscle determination. *Proc. Natl. Acad. Sci.* **90**: 8028–8032.
- Jan, Y.N. and L.Y. Jan. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75**: 827–830.
- Jaynes, J.B., J.E. Johnson, J.N. Buskin, C.L. Gartside, and S.D. Hauschka. 1988. The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. *Mol. Cell. Biol.* **8**: 62–70.
- Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass, and M.G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**: 403–414.
- Kaushal, S., J.W. Schneider, B. Nadal-Ginard, and V. Mahdavi. 1994. Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with myoD. *Science* **266**: 1236–1240.
- Kiledjian, M., L.-K. Su, and T. Kadesch. 1988. Identification and characterization of two functional domains within the murine heavy chain enhancer. *Mol. Cell. Biol.* **8**: 145–152.
- Kitabayashi, I., R. Eckner, Z. Arany, R. Chiu, G. Gachelin, D.M. Livingston, and K. Yokoyama. 1995. Phosphorylation of the adenovirus E1A-associated 300-kDa protein in response to retinoic acid and E1A during the differentiation of F9 cells. *EMBO J.* **14**: 3496–3509.
- Kong, Y., S.E. Johnson, E.J. Taparowsky, and S.F. Konieczny. 1995. Ras p21^{Val} inhibits myogenesis without altering the DNA binding or transcriptional activities of the myogenic basic helix-loop-helix factors. *Mol. Cell. Biol.* **15**: 5205–5213.
- Kopan, R., J.S. Nye, and H. Weintraub. 1994. The intracellular domain of mouse Notch: A constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**: 2385–2396.
- Kuisk, I.R., H. Li, D. Tran, and Y. Capetanaki. 1996. A single MEF2 site governs desmin transcription in both heart and skeletal muscle during mouse embryogenesis. *Dev. Biol.* **174**: 1–13.
- Lassar, A.B., J.N. Buskin, D. Lockshon, R.L. Davis, S. Apone, S.D. Hauschka, and H. Weintraub. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58**: 823–831.
- Lassar, A.B., R.L. Davis, W.E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**: 305–315.
- Li, L., R. Heller-Harrison, M. Czech, and E.N. Olson. 1992. Cyclic AMP-dependent protein kinase inhibits the activity of myogenic helix-loop-helix proteins. *Mol. Cell. Biol.* **12**: 4478–4485.
- Lilly, B., B. Zhao, G. Ranganayakulu, B.M. Paterson, R.A. Schulz, and E.N. Olson. 1995. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science* **267**: 688–693.
- Ma, P.C.M., M.A. Rould, H. Weintraub, and C.O. Pabo. 1994. Crystal structure of MyoD bHLH domain-DNA complex: Perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**: 451–459.
- Molkentin, J.D., B.L. Black, J.F. Martin, and E.N. Olson. 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**: 1125–1136.
- Moran, E. 1993. DNA tumor virus transforming proteins and the cell cycles. *Curr. Opin. Genet. Dev.* **3**: 63–70.
- Mymryk, J.S., R.W.H. Lee, and S.T. Bayley. 1992. Ability of adenovirus 5 E1A proteins to suppress differentiation of BC₃H1 myoblasts correlates with their binding to a 300 kDa cellular protein. *Mol. Biol. Cell* **3**: 1107–1115.
- Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, L. Nonaka, and Y. Nabeshima. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**: 532–535.
- Olson, E.N., M. Perry, and R.A. Schulz. 1995. Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev. Biol.* **172**: 2–14.
- Olson, E.N., H.-H. Arnold, P.W.J. Rigby, and B.J. Wold. 1996. Know your neighbors: Three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* **85**: 1–4.
- Piette, J., J.-L. Bessereau, M. Huchet, and J.-P. Changeux. 1990. Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α -subunit gene. *Nature* **345**: 353–355.
- Rudnicki, M.A., T. Braun, S. Hinuma, and R. Jaenisch. 1992. Inactivation of myoD in mice leads to up-regulation of the myogenic HLH gene myf-5 and results in apparently normal muscle development. *Cell* **71**: 383–390.
- Rudnicki, M.A., P.N.J. Schnegelsberg, R.H. Stead, T. Braun, H.H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**: 1351–1359.
- Ruezinsky, D., H. Beckmann, and T. Kadesch. 1991. Modulation of the IgH enhancer's cell type specificity through a genetic switch. *Genes & Dev.* **5**: 29–37.
- Salminen, A., T. Braun, A. Buchberger, S. Jürs, B. Winter, and H.-H. Arnold. 1991. Transcription of the muscle regulatory gene Myf4 is regulated by serum components, peptide growth factors and signaling pathways involving G proteins. *J. Cell Biol.* **115**: 905–917.
- Schlissel, M., A. Voronova, and D. Baltimore. 1991. Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes & Dev.* **5**: 1367–1376.
- Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. *Nucleic Acids Res.* **17**: 6419.
- Shen, C.-P. and T. Kadesch. 1995. B-cell-specific DNA binding by an E47 homodimer. *Mol. Cell. Biol.* **15**: 4518–4524.
- Shivdasani, R.A., E.L. Mayer, and S.H. Orkin. 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein lal-1/SCL. *Nature* **373**: 432–434.
- Stein, R., M. Corrigan, P. Yaciuk, J. Whelan, and E. Moran. 1990. Analysis of E1A-mediated growth regulation functions: Binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. *J. Virol.* **64**: 4421–4427.
- Tapscott, S.J., R.L. Davis, M.J. Thayer, P.-F. Cheng, H. Weintraub, and A.B. Lassar. 1988. MyoD1: A nuclear phosphoprotein requiring a myc homology region to convert fibroblasts

Eckner et al.

- to myoblasts. *Science* **242**: 405–411.
- Webster, K.A., G.E.O. Muscat, and L. Kedes. 1988. Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. *Nature* **332**: 553–557.
- Weintraub, H. 1993. The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell* **75**: 1241–1244.
- Weintraub, H., R. Davis, D. Lockshon, and A. Lassar. 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proc. Natl. Acad. Sci.* **87**: 5623–5627.
- Weintraub, H., V.J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S.J. Tapscott. 1991. Muscle-specific transcriptional activation by MyoD. *Genes & Dev.* **5**: 1377–1386.
- Wentworth, B.M., M. Donoghue, J.C. Engert, E.B. Berglund, and N. Rosenthal. 1991. Paired myoD-binding sites regulate myosin light chain gene expression. *Proc. Natl. Acad. Sci.* **88**: 1242–1246.
- Winter, B., T. Braun, and H.H. Arnold. 1993. cAMP-dependent protein kinase represses myogenic differentiation and the activity of the muscle-specific helix-loop-helix transcription factors myf-5 and myoD. *J. Biol. Chem.* **268**: 9869–9878.
- Yao, T.-P., G. Ku, N. Zhou, R. Scully, and D.M. Livingston. 1996. The nuclear hormone receptor co-activator SRC-1 is a specific target of p300. *Proc. Natl. Acad. Sci.* **93**: [in press].
- Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell* **79**: 875–884.

Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation.

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Genes Dev. 1996, **10**:

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