Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8028–8032, September 1993
Developmental Biology

Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination

(estrogen receptor/cycloheximide/hormone-withdrawal/myogenin)

STANLEY M. HOLLENBERG, PEI FENG CHENG, AND HAROLD WEINTRAUB

Howard Hughes Medical Institute Laboratory, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, A3-025, Seattle, WA 98104

Contributed by Harold Weintraub, June 3, 1993

ABSTRACT **DNA sequences encoding the hormone-binding domains of several steroid hormone receptors were fused in frame to the *MyoD* gene. When the gene for this chimeric protein was expressed in NIH 3T3 or 10T½ fibroblasts, these cells displayed hormone-dependent induction of myogenesis. Our experiments focused on cell lines expressing estrogen receptor–MyoD chimeras. Induction of these lines in the presence of estradiol and an inhibitor of protein synthesis, cycloheximide, resulted in the activation of the endogenous myogenin gene but did not activate the muscle-specific creatine kinase or cardiac α-actin gene. This result suggests that MyoD is not a "direct" activator of these downstream myogenic genes but must first activate myogenin as an intermediary. Once muscle is induced by estrogen receptor–MyoD the muscle phenotype is very stable and does not need the continued presence of estradiol for its maintenance.**

MyoD is a muscle-specific transcription factor that can activate downstream myogenic structural genes and myogenic conversion in many different cell types. MyoD also activates its own transcription, as well as the transcription of myogenin, another member of the *MyoD* gene family. The other members of the MyoD family—myogenin, *Myf-5*, and myogenic regulatory factor (MRF) 4/herculin—can, to some extent, also activate MyoD and cross-activate each other (refs. 1–6; for review, see refs. 7 and 8). Moreover, there is evidence that some myogenic family members can negatively regulate other members (9, 10). As a consequence of these auto- and cross-regulatory interactions, the exact role of each of these myogenic regulators can be confusing and difficult to decipher experimentally. Some insight has come from the fact that most muscle cell lines express either MyoD or Myf-5 in the committed state, but when induced to differentiation, all turn on the transcription of myogenin. An extreme interpretation of this result is that either MyoD or Myf-5 can define the committed myogenic state; either is required to activate myogenin, and myogenin is largely responsible for turning on most muscle-specific structural genes. Because mice with DNA-mediated mutations in either the *Myod-1* or *Myf-5* gene are born and express myogenin and muscle (9, 10), it is likely that for many of their functions, MyoD and Myf-5 proteins are redundant; however, because each mutation displays a phenotype of its own, this redundancy seems only to apply to a subset of their functions (e.g., activating muscle cell identity), and some additional unique function(s) seem not yet defined for each. This system is compatible with current thinking about the evolution of redundant gene pathways.

As an initial approach to exploring these problems, we have constructed an inducible MyoD protein by fusing MyoD to the hormone-binding regulatory domain of several steroid receptor proteins (11, 12). Under these conditions, MyoD and hence myogenesis, becomes hormone inducible. We show that in the absence of protein synthesis, hormone-activated MyoD directly induces myogenin expression but does not induce several downstream muscle structural genes, such as the genes encoding muscle creatine kinase or cardiac α-actin. This result supports the notion that muscle cell identity can be defined by MyoD, but the actual expression of this identity is largely fulfilled by myogenin. We also show that after induction of myogenesis by hormone, the myogenic state is stable, even when hormone is subsequently withdrawn. Thus, we assume that transient activation of MyoD leads to the activation of a downstream positive, autoregulatory circuit that preserves the myogenic phenotype.

MATERIALS AND METHODS

Hormone Inducible MyoD. Hybrid expression constructs are derivatives of the MyoD expression vector EMC11S (13). To construct MyoD–glucocorticoid receptor (GR) and –thyroid hormone receptor (TR), the *Nar* I site of EMC11S (amino acids 173–174) was converted to *Bgl* II with the adapter 5′-CGCCGAGATCTTGCC-3′ (sense) and 5′-CGGGCAAGATCTCGG-3′ (antisense) to yield EMC11S-B. This technique allowed use of existing primers. The hormone-binding domain of the human GR (ref. 14; aa 512–777) was amplified by PCR with primers 5′-AGGAGTCTCACGGATCCCCTCTGAAAAT-3′ (sense) and 5′-ATCAAAAGTGGATCCCTTAATAAG-3′ (antisense), digested with *Bam*HI and inserted into the *Bgl* II site of EMC11S-B. In a similar fashion, sequences encoding the hormone-binding domain of the rat thyroid hormone receptor α (15) were amplified with primers 5′-CCAGAGCCCAGGATCCAAGAGTGGGA-3′ (sense) and 5′-GGCCGCCTGAGATCTTGACTTCCTG-3′ (antisense) and introduced into EMC11S-B to yield MyoD–TR. The hormone-binding domain of the human estrogen receptor (ER) (refs. 16 and 17; aa 282–595) was directly inserted into the *Nar* I site of EMC11S after PCR amplification and *Cla* I digestion. The ER primers used were 5′-ACGGCATCGATTCTGCTGGAGACATGAGAGCT-3′ (sense) and 5′-GCGCCATCGATGACTGTGGCAGGGAAACCCT-3′ (antisense).

Stable lines in 10T½ fibroblasts were generated by coelectroporation with RSVneo (*Bam*HI-digested) and a 10-fold excess of *Kpn* I-linearized MyoD–fusion plasmid. Cells were maintained for 2 weeks in G418 at 500 μg/ml; then resistant clones were picked to duplicate multi-well dishes. Cells were kept in double-stripped serum (18) throughout this period. Clones that exhibited morphological differentiation after 48–72 hr in the presence of hormone and low serum (19) were expanded from the undifferentiated duplicate plate. Therefore, all clones are hormone-naive.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TGF-β, transforming growth factor β; ER, estrogen receptor; TR, thyroid hormone receptor; GR, glucocorticoid receptor; RT, reverse transcriptase; RPL7, ribosomal protein L7.

Reverse Transcriptase (RT)-PCR Analysis. RT-PCR was done as described (20). To enable quantitation, a cycle number was chosen for each primer pair that maintained approximately exponential amplification with the most enriched sample. Primer pairs and their nucleotide positions, the size of amplified product, and typical number of cycles wereasfollows:myogenin(5),5′-CTGGGGACCCCTGAGCATTG-3′ (nt 283–302) and 5′-ATCGCGCTCCTCCTGGTTGA-3′ (nt 555–536), 273 nt, 22 cycles; cardiac α-actin (21), 5′-GATGGCCACAGCTGCATCTT-3′ (nt 678–697) and 5′-GCACAATACGGTCATCCTGA-3′ (nt 1176–1157), 499 nt, 28 cycles; endogenous MyoD (ref. 13; S.M.H. & H.W., unpublished results), 5′-AGGACACGACTGCTTTCTTC-3′ (nt −23 to −4) and 5′-GCACCGCAGTAGAGAAGTGT-3′ (nt 367–348), 390 nt, 28 cycles; muscle creatine kinase (22), 5′-CAATAAGCTTCGCGATAAGGAG-3′ (nt 189–210) and 5′-GATGGGATCAAACAGGTCCTTG-3′ (nt 348–327), 160 nt, 26 cycles. Elongation factor EF1α primers were designed for detection in *Xenopus* (20) and amplify the mouse homolog at reduced efficiency (28 cycles). Finally, for ribosomal protein L7 (RPL7) (23) primers were 5′-GAAGCTCATCTATGAGAAGGC-3′ (nt 231–251) and 5′-AAGACGAAGGAGCTGCAGAAC-3′ (nt 432–412), 202 nt, 25 cycles.

RESULTS AND DISCUSSION

Hormone-inducible MyoD vectors were made by cloning the hormone-responsive domains from the ER, TR, and GR downstream from the helix–loop–helix domain of MyoD (Fig. 1*A*). In transient assays using NIH 3T3 cells, these constructs activated myogenic reporter genes, as well as myotube formation, only when the corresponding hormone was supplied (data not shown). Stable lines were generated from both 10T½ and 3T3 cells under hormone-free conditions; representative clones were chosen by the criteria that they gave copious, hormone-dependent induction of muscle (Fig. 1*B*); for each clone, cell division ceased in the presence of hormone, and characteristic muscle-specific marker genes were activated (Fig. 2*A*). We chose to focus on the MyoD–ER lines because endogenous ER is absent from these cells.

In preliminary characterization of the MyoD–ER lines, cells were placed into inducing medium in the presence of hormone for increased periods of time, then the hormone was removed, and the average number of muscle cells per field was determined by cell morphology 2 days later. We found

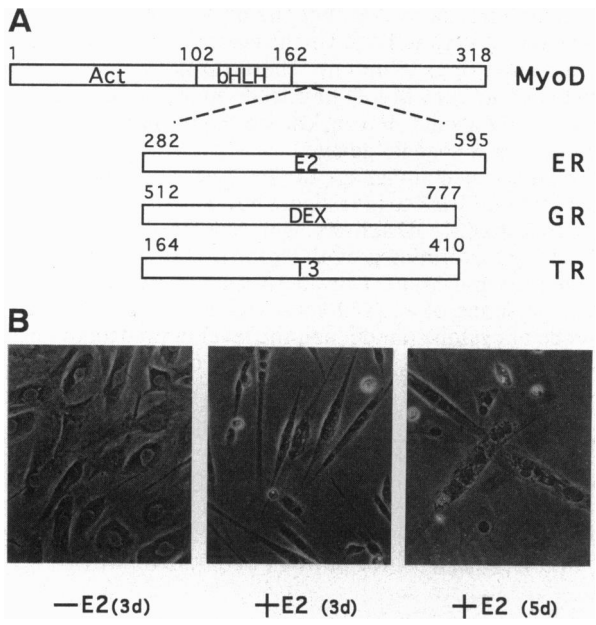

FIG. 1. Structure and function of hormone-dependent MyoD chimeras. (*A*) Full-length MyoD is shown with the amino-terminal activation domain (Act) and the basic helix–loop–helix region (bHLH) indicated. Regions encoding the hormone-binding domains from the human ER, human GR, and rat TR α were introduced in-frame with MyoD between codons 173–174 (13). The reading frame was maintained such that the translated hybrid protein includes full-length MyoD. Numbers indicate amino acid positions. Activating hormones are estradiol (E2), dexamethasone (DEX), and thyroid hormone (T3). (*B*) Hormone-dependent myogenesis of a 10T½ stable line expressing the MyoD–ER fusion protein is shown. Cells were grown to near-confluence in Dulbecco's modified Eagle's medium (minus phenol red)/10% double-stripped calf serum and then allowed to differentiate without serum (19) in the presence or absence of 10^{-7} M estradiol for 3 or 5 days (d).

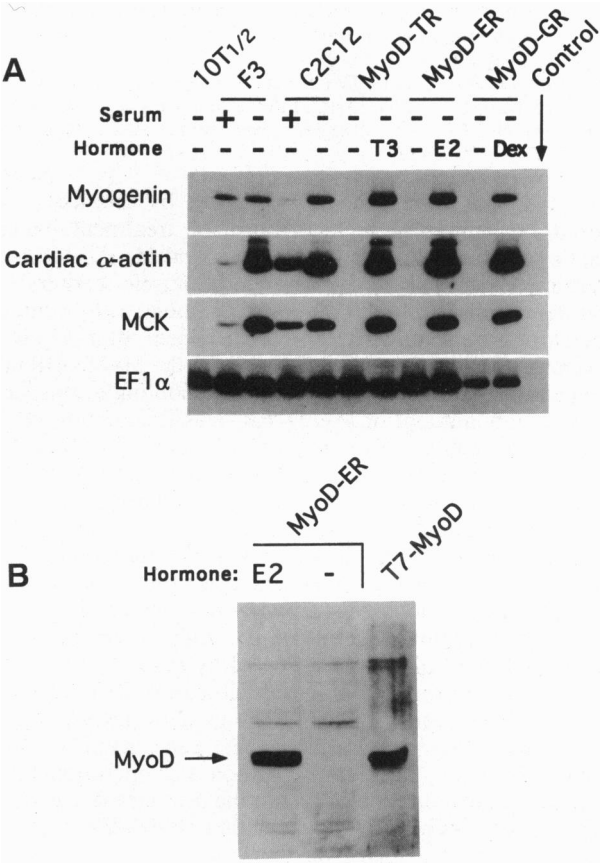

FIG. 2. Characterization of RNA and protein levels in hormone-dependent MyoD stable lines. (*A*) RT-PCR was used to quantitate the levels of RNA encoding myogenic proteins. 10T½ fibroblasts, myogenic lines F3 (13) and C2C12, and 10T½ stable lines expressing hormone-dependent MyoD hybrids were harvested for RNA after 2 days in the absence of serum. The RNA was converted to cDNA by reverse transcription with random primers. Primers specific for the myogenin, cardiac α-actin, and muscle creatine kinase (MCK) were used for PCR, and the resultant amplified fragments were analyzed by PAGE. Primers for elongation factor 1 α (EF1α) were used as a control for the amount of RNA, reverse transcription efficiency, and amplification variability. Control lanes were amplified without added cDNA. Each sample was tested for the absence of amplification product without reverse transcription (data not shown). E2, estradiol (10^{-7} M); T3, thyroid hormone (10^{-7} M); Dex, dexamethasone (10^{-6} M). (*B*) Level of wild-type MyoD protein expressed in the 10T½-MyoD–ER stable line was analyzed by immunoblot. Cells were treated as in *A* but were harvested for protein. Extracts were resolved by SDS/PAGE and blotted to nitrocellulose; MyoD was detected by monoclonal antibody 5.8A (24). The position of full-length MyoD was determined by loading bacterially expressed MyoD (T7-MyoD). The MyoD–ER fusion protein is not detectable and, therefore, we assume it to be well below the level of the induced, endogenous MyoD.

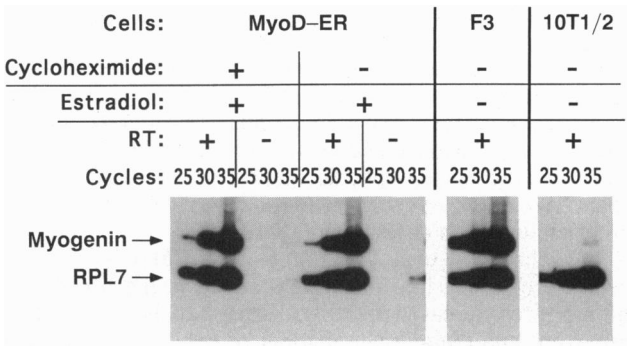

FIG. 3. Direct activation of myogenin by MyoD–ER. MyoD–ER cells were induced with estradiol (10^{-7} M) in the presence or absence of cycloheximide (50 μg/ml) for 10 hr; then RNA was isolated and analyzed by quantitative RT-PCR for 25, 30, or 35 cycles by using myogenin-specific primers. F3 cells are an azacytidine-induced myogenic line from 10T½ cells (13), and 10T½ cells are a negative control. RPL7, encoded by the control gene, was used to assess input RNA and RT-PCR efficiency.

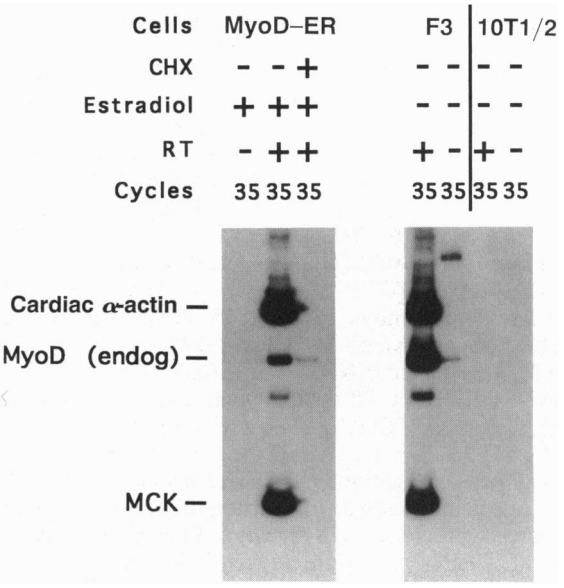

FIG. 4. Failure to directly activate downstream myogenic structural genes by MyoD–ER. Conditions were the same as those described in Fig. 3. The indicated sequences were amplified separately and then loaded as a mixture. CHX, cycloheximide; endog, endogenous; MCK, muscle creatine kinase.

that myogenic commitment, as defined by this assay, required a minimum of 12 hr of hormone treatment. Similar results were seen with the MyoD–GR and MyoD–TR lines. The time course for the appearance of muscle markers was also determined under continuous treatment with hormone. The earliest muscle markers begin to appear after ≈10 hr of hormone treatment; this is also seen with the MyoD–GR line. We believe that at least one factor contributing to this lag is the very low amount of MyoD–ER, less than one-tenth the level of induced MyoD, as assayed by immunoblots (Fig. 2*B*). In contrast, when MyoD-ER is transiently cotransfected into 10T½ cells with a muscle reporter gene and hormone is added a day later, activation of the reporter shows a lag of <2 hr (data not shown). Thus, the high levels of expression usually attributed to transiently transfected vectors result in a significant decrease in the lag for hormone-induced expression.

We used the MyoD–ER lines to determine which myogenic genes could be "directly" activated by inducing the MyoD chimera in the presence of an inhibitor of protein synthesis (cycloheximide) and monitoring the level of mRNA expression of a number of myogenic marker genes using a quantitative PCR assay. ("Direct" activation is an operational term indicating that activation occurs in the presence of cycloheximide; it does not necessarily mean that MyoD–ER is, in fact, the immediate molecular species mediating the observed activation.) The genes encoding muscle creatine kinase and cardiac α-actin were not activated in the presence of cycloheximide and estradiol; the endogenous *Myod-1* gene was only marginally activated, but the myogenin-encoding gene was quantitatively activated in the presence of cycloheximide and estradiol (Figs. 3 and 4). These results suggest that the initial activation of MyoD first leads to myogenin transcription, and myogenin, in turn, can be the primary activator of downstream myogenic genes.

We have taken advantage of the direct activation of myogenin by MyoD–ER to investigate how a number of myogenic inhibitors alter MyoD activity. Butyrate (25, 26), okadaic acid (27), and transforming growth factor β TGF-β (28) all inhibit myogenesis and MyoD trans-activation. Cells were induced in the presence of each of these agents in the presence or absence of cycloheximide, and the level of myogenin mRNA was determined (Fig. 5). In the absence of cycloheximide, all

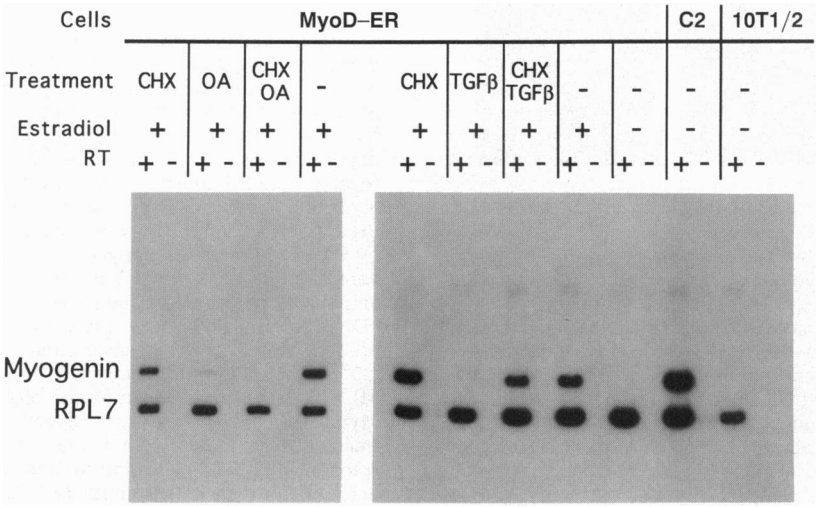

FIG. 5. Myogenic inhibition: Requirement of protein synthesis for TGF-β effect, but not for okadaic acid effect. Conditions were the same as for Fig. 3. However, one plate of cells also received only okadaic acid (OA) (50 ng/ml), and another received okadaic acid plus cycloheximide (CHX) (at left) or TGF-β (0.2 nM) and TGF-β plus cycloheximide (at right). C2, C2C12 myogenic cells.

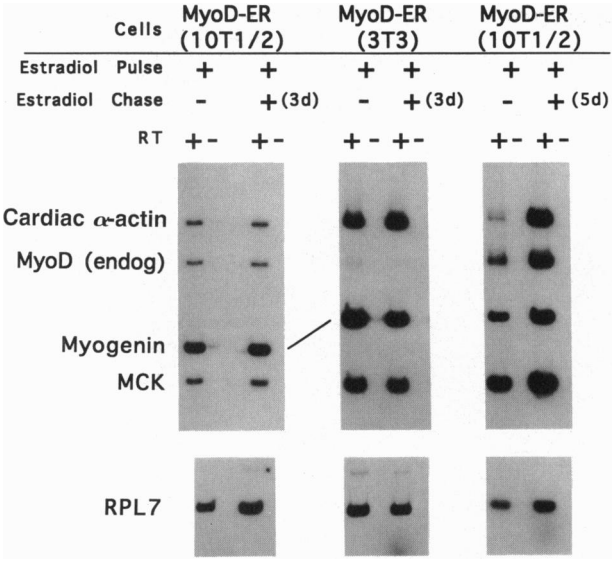

FIG. 6. Stability of myogenic activation in the absence of continued exposure to hormone. MyoD–ER cell lines (derived from NIH 3T3 or 10T½ cells) were treated with estradiol for 3 days in the absence of serum, and then the cells were washed extensively and withdrawn for 3 or 5 days (d) in the presence of serum. RNA was assayed by RT-PCR. endog, Endogenous.

of these compounds inhibited induced myogenin transcription. However, in the presence of cycloheximide, butyrate (data not shown) and okadaic acid continued to inhibit, whereas TGF-β did not continue to inhibit myogenin transcription. These results establish a classification for these inhibitors and suggest that butyrate and okadaic acid are direct inhibitors of myogenic activation, whereas TGF-β requires new protein synthesis. In separate experiments, induction of muscle in the presence of estradiol does not require DNA synthesis, as assayed by using the inhibitors hydroxyurea and aphidicolin and can even occur when cells are arrested in G_0 after incubation for up to 8 days in low serum (data not shown).

Use of a conditional MyoD also allows us to ask whether induction of myogenesis is stable even after hormone is removed. Cells were induced with estradiol for 3 days, and then the estradiol was continued for another 3 days or removed for 3 days, either with or without serum. Cells retained their myogenic phenotype, whether chased in the presence or absence of hormone, and PCR analysis of a variety of myogenic markers showed that their levels were similar (Fig. 6). The same conclusion is reached when cells are assayed for muscle creatine kinase, myosin heavy chain, and myogenin using antibody staining (data not shown). After 6 days of hormone withdrawal in the presence of serum a small decrease in some myogenic markers is seen; however, interpretation is difficult because some nondifferentiating cells in the population begin dividing in the absence of hormone, and these take over the culture and often prevent the myotubes from attaching to the substrate.

Because the MyoD–ER 10T½ cells activate the endogenous MyoD, which, in turn, can autoactivate its own expression, the observed maintenance of the myogenic state is anticipated on the basis of previous results (29). In contrast, 3T3-MyoD–ER cells do not activate the endogenous MyoD, and neither the 10T½ nor the 3T3 lines activate the gene for myogenic regulatory factor (MRF) 4 or *Myf-5* (data not shown). Surprisingly, the 3T3-MyoD–ER line also retained its myogenic memory as assayed by the same protocol. Possibly, the transient expression of MyoD–ER permanently activated an endogenous positive autoregulatory loop. We would guess that this might involve the myogenin gene (30) in the case of the 3T3 parent and the MyoD gene or the myogenin gene in the case of the 10T½ parent. Alternatively, the stability might reflect the assembly of a stable transcription complex or a stable, active chromatin structure.

Our results with hormone-inducible MyoD are complicated by the fact that the fusion protein could have myogenic properties not intrinsic to the endogenous MyoD; nevertheless, the results allow us to draw several tentative conclusions not easily approached otherwise: MyoD is a "direct" activator of myogenin but is not a direct activator, under these conditions, of muscle creatine kinase or cardiac α-actin. Once activated, the myogenic program is unusually stable even when hormone is removed, suggesting that MyoD induces a stable downstream regulatory loop, presumably involving myogenin, that can be independent of MyoD itself. The use of hormone-inducible, chimeric transcription factors in conjunction with cycloheximide should prove a useful adjunct technology in dissecting out complex regulatory circuits.

We thank Geoffrey Greene and Catherine Thompson for providing plasmids and Jeffrey Miner for NIH 3T3 cells. S.M.H. was supported by a fellowship through the National Institutes of Health. This work was supported, in part, by funds awarded to H.W. from the National Cancer Institute and the Howard Hughes Medical Institute.

1. Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. & Weintraub, H. (1989) *Cell* **58**, 241–248.
2. Braun, T., Bober, E., Buschhausen-Denker, G., Kotz, S., Grzeschik, K. H. & Arnold, H. H. (1989) *EMBO J.* **8**, 3617–3625.
3. Miner, J. H. & Wold, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1089–1093.
4. Rhodes, S. J. & Konieczny, S. F. (1989) *Genes Dev.* **3**, 2050–2061.
5. Edmondson, D. G. & Olson, E. N. (1989) *Genes Dev.* **3**, 628–640.
6. Wright, W. E., Sassoon, D. A. & Lin, V. K. (1989) *Cell* **56**, 607–617.
7. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. & Lassar, A. (1991) *Science* **251**, 761–766.
8. Olson, E. (1990) *Genes Dev.* **4**, 1454–1461.
9. Rudnicki, M. A., Braun, T., Hinuma, S. & Jaenisch, R. (1992) *Cell* **71**, 383–390.
10. Braun, T., Rudnicki, M. A., Arnold, H. H. & Jaenisch, R. (1992) *Cell* **71**, 369–382.
11. Picard, D., Salser, S. J. & Yamamoto, K. R. (1988) *Cell* **54**, 1073–1080.
12. Hollenberg, S. M. & Evans, R. M. (1988) *Cell* **55**, 899–906.
13. Davis, R. L., Weintraub, H. & Lassar, A. B. (1987) *Cell* **51**, 987–1000.
14. Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G. & Evans, R. M. (1985) *Nature (London)* **318**, 635–641.
15. Thompson, C. C., Weinberger, C., Lebo, R. & Evans, R. M. (1987) *Science* **237**, 1610–1614.
16. Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. & Shine, J. (1986) *Science* **231**, 1150–1154.
17. Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J. M. & Chambon, P. (1986) *EMBO J.* **5**, 891–897.
18. Samuels, H. H., Stanley, F. & Casanova, J. (1979) *Endocrinology* **105**, 80–85.
19. Lassar, A. B., Thayer, M. J., Overell, R. W. & Weintraub, H. (1989) *Cell* **58**, 659–667.
20. Rupp, R. A. & Weintraub, H. (1991) *Cell* **65**, 927–937.
21. Leader, D. P., Gall, I. & Campbell, P. C. (1986) *Biosci. Rep.* **6**, 741–747.
22. Buskin, J. N., Jaynes, J. B., Chamberlain, J. S. & Hauschka, S. D. (1985) *J. Mol. Evol.* **22**, 334–341.
23. Meyuhas, O. & Klein, A. (1990) *J. Biol. Chem.* **265**, 11465–11473.
24. Shaknovich, R., Shue, G. & Kohtz, D. S. (1992) *Mol. Cell. Biol.* **12**, 5059–5068.
25. Endo, T. & Nadal-Ginard, B. (1987) *Cell* **49**, 515–526.

26. Johnston, L. A., Tapscott, S. J. & Eisen, H. (1992) *Mol. Cell. Biol.* **12**, 5123–5130.
27. Kim, S.-J., Kim, K. Y., Tapscott, S. J., Winokur, T. S., Park, K., Fujiki, H., Weintraub, H. & Roberts, A. B. (1992) *J. Biol. Chem.* **267**, 15140–15145.
28. Massague, J., Cheifetz, S., Endo, T. & Nadal-Ginard, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8206–8210.
29. Thayer, M. J. & Weintraub, H. (1990) *Cell* **63**, 23–32.
30. Edmondson, D. G., Cheng, T.-C., Cserjesi, P., Chakraborty, T. & Olson, E. N. (1992) *Mol. Cell. Biol.* **12**, 3665–3677.