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An activated c-Ha-*ras* allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal

(oncogenes/myocytes/creatine kinase/transfection/transforming growth factors)

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ABSTRACT **During myogenesis, induction of muscle-specific genes is subject to negative control by polypeptide mitogens and type-β transforming growth factor. Since transduction of growth factor signals may require proteins encoded by cellular *ras* oncogenes, we have tested whether a mutationally altered Harvey *ras* expression vector, by itself, can prevent establishment of a differentiated phenotype in BC_3H1 mouse myoblasts. Transfection with the valine-12 allele of the human Harvey *ras* gene, under the control of its own promoter, was sufficient to prevent the induction of both muscle creatine kinase activity and the nicotinic acetylcholine receptor following mitogen withdrawal but did not inhibit withdrawal from the cell cycle. The loss of creatine kinase activity resulted from a corresponding block to induction of muscle creatine kinase mRNA. Similarly, mitogen withdrawal elicited little or no α-actin mRNA in *ras*-transfected cells. These results suggest that an activated *ras* allele can inhibit myogenesis through a mechanism independent of cell proliferation and can preclude activation of genes whose up-regulation normally accompanies mitogen withdrawal.**

Muscle differentiation is accompanied by induction of a set of tissue-specific gene products including the muscle isoenzyme of creatine kinase (mCK) and the nicotinic acetylcholine (AcCho) receptor, whose up-regulation can be suppressed by serum components (1–7), purified mitogens (8, 9), and transforming growth factors (10–12). The pathway for growth factor signals from cell membrane to nucleus is postulated to involve a cascade of proteins encoded by cellular oncogenes (13). For example, c-*sis* encodes a subunit of platelet-derived growth factor (14), c-*erbB* encodes the receptor for epidermal growth factor (15), and the ability of quiescent cells to reenter the cell cycle is attributed to intranuclear proteins encoded by c-*fos* and c-*myc* (e.g., refs. 16 and 17). Moreover, certain variant myocytes that cannot differentiate fail to down-regulate either c-*myc* (18) or the epidermal growth factor receptor encoded by *erbB* (19). The viral *myc* gene may inhibit myogenesis, but only indirectly, by maintaining myoblasts in a proliferative state (20). Furthermore, reinduction of c-*myc* in terminally differentiated myotubes does not impair the continued transcription of muscle-specific genes (6). Previously, we examined the possible functional role of c-*myc* in muscle differentiation by means of gene transfer (21) using the myogenic murine cell line BC_3H1 (22). We demonstrated that autonomous expression of c-*myc* did not prevent the establishment of a differentiated phenotype in BC_3H1 cells (7). Thus, induction of muscle-specific genes is not obligatorily coupled to down-regulation of c-*myc*.

In the present study, as a step toward defining the molecular mechanisms by which growth factors regulate myogenesis, we tested the potential contrasting role of cellular *ras* genes, by transfection of BC_3H1 cells with a *ras* expression vector activated by missense mutation (23). Transduction of growth signals has been ascribed to proteins encoded by three cellular *ras* genes, Harvey (Ha), Kirsten (Ki), and N (13), which share homology with guanine nucleotide-binding regulatory proteins such as transducin and the proteins that couple adrenergic receptors to adenylate cyclase (24, 25). At least some point mutations in *ras* impair hydrolysis of bound GTP and appear to activate the *ras* peptide constitutively (26, 27). Furthermore, activated *ras* proteins may themselves enable certain quiescent cells to traverse the cell cycle and replicate DNA (28). Conversely, monoclonal antibodies against *ras* proteins prevent DNA synthesis evoked by fetal serum (29). Finally, elevated *ras* expression can amplify growth factor effects without affecting receptor number or affinity (30). Thus, *ras* proteins might couple growth factor receptors to effector enzymes such as phospholipase C or A_2 and mediate the transduction of growth factor signals that lead to DNA synthesis and mitotic division. Whether down-regulation of muscle-specific genes by mitogens and transforming growth factors involves a *ras*-dependent cascade for transmembrane signal transduction is unknown. For example, in certain cells, activated *ras* proteins elicit differentiation, not cell division (31). Moreover, activated *ras* genes may not promote DNA replication in cells that are senescent (32). Thus, responses to *ras*-dependent signals reflect not only properties inherent in *ras* proteins but also properties intrinsic to a particular cell lineage or developmental stage. To test the prediction that activation of *ras* can block the induction of muscle-specific genes, we have investigated BC_3H1 cells that bear the valine-12 allele of the human Harvey *ras* gene (Val-12 *ras*). The missense *ras* mutant was found to prevent myogenic differentiation evoked by mitogen withdrawal.

METHODS

Cell Culture and Transfection. BC_3H1 cells (22) were cultured in Dulbecco's modification of Eagle's minimal essential medium/Ham's nutrient mixture F-12 (1:1) supplemented with 17 mM Hepes (pH 7.4), 3 mM $NaHCO_3$, 2 mM L-glutamine, 50 μg of gentamicin per ml, and 20% heat-inactivated fetal bovine serum (FBS). Cells were transfected with a 1:10 mixture of plasmids pSV2neo (33) and pEJ (23). pSV2neo provides mammalian cells with resistance to the

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Abbreviations: mCK, muscle creatine kinase; Val-12 *ras*, the valine-12 missense mutation of the human Harvey *ras* gene; FBS, fetal bovine serum; AcCho, acetylcholine.

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neomycin analogue G418. pEJ comprises the Val-12 *ras* gene under the control of its own promoter. Transfection was performed by the calcium phosphate method (21, 23). Colonies were selected in medium containing G418 at 400 μg/ml.

DNA Blot Hybridization. Southern analysis of restriction endonuclease-digested genomic DNA was done as described (34), using stringent conditions for hybridization and washing.

RNA Blot Hybridization. Total cellular RNA was isolated by the proteinase K/NaDodSO$_4$ method (35). Aliquots (15 μg per lane) were size-fractionated by formaldehyde/agarose gel electrophoresis and were transferred to nitrocellulose filters (36). cDNA hybridization probes (see Fig. 4 legend) were labeled to a specific activity of 1–4 × 10^9 dpm/μg (37). Following overnight incubation at 68°C (10^6 dpm/cm^2), the blots were washed twice in 2× SSC/0.5% NaDodSO$_4$ at room temperature and twice in 0.1× SSC/0.5% NaDodSO$_4$ at 68°C. (SSC, standard saline citrate, is 150 mM NaCl/15 mM sodium citrate.)

RESULTS

An Activated c-Ha-*ras* Gene Induces Morphological Alteration But Not Autonomous Proliferation in BC$_3$H1 Cells. Colonies resulting from transfection with the neomycin-resistance gene (pSV2neo) alone were morphologically indistinguishable from parental BC$_3$H1 cells (Fig. 1*A*). In contrast, colonies transfected with pSV2neo plus the Val-12 *ras* gene (pEJ) contained large, phase-bright, refractile cells (Fig. 1*B*), distinct from the effects of an activated c-*myc* vector (7). The normal Gly-12 c-Ha-*ras* protooncogene (23) produced no morphological change (data not shown). Southern hybridization confirmed the stable incorporation of the exogenous human Ha-*ras* gene (Fig. 1*C*). To determine whether an activated *ras* allele could block exit from the cell cycle under conditions used to induce myocyte differentiation (4, 7), confluent cultures were exposed to medium containing 0.5% FBS, cell number was determined with an electronic particle counter (Fig. 2*A*), and DNA synthesis was monitored by [^{3}H]thymidine autoradiography (Fig. 2*B*). BC$_3$H1 cells transfected with the Val-12 *ras* vector ceased DNA replication within 24 hr of mitogen withdrawal. In 20% FBS, 52.4% of the nuclei incorporated [^{3}H]thymidine (n = 2180), while DNA synthesis was found in only 1.33% of nuclei in 0.5% FBS (n = 5450). Thus, potential effects of the Val-12 *ras* gene on myogenic differentiation were independent of cell proliferation, since the *ras* expression vector was not itself mitogenic for BC$_3$H1 cells.

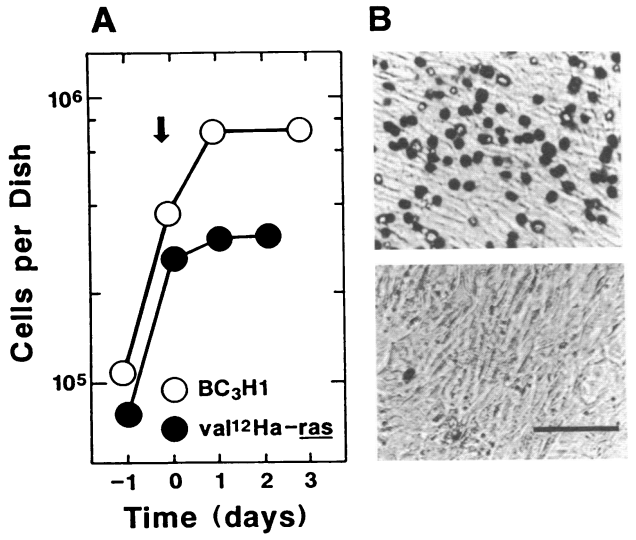

FIG. 2. The Val-12 *ras* gene does not induce autonomous proliferation or DNA synthesis in BC$_3$H1 cells. (*A*) Cell number was determined at intervals during mitogen withdrawal in 0.5% FBS, using a Coulter counter. (*B*) For thymidine autoradiography after mitogen withdrawal for 3 days, *ras*-transfected cells were incubated for 24 hr in medium containing [^{3}H]thymidine and 20% (*Upper*) or 0.5% (*Lower*) FBS. (Bar = 200 μm.)

An Activated c-Ha-*ras* Gene Prevents Induction of mCK and AcCho Receptor. To test the prediction that a mutationally activated *ras* gene could abolish the expression of tissue-specific gene products whose induction normally accompanies growth factor withdrawal, proliferative BC$_3$H1 cells and *ras*-transfectants were shifted from 20% to 0.5% FBS. As shown in Fig. 3*A*, mCK activity was induced within 2 days in control cells and was expressed at maximal levels within 7 days. Similar induction of mCK was confirmed in each of five replicate clones transfected with the Gly-12 *ras* protoonco-

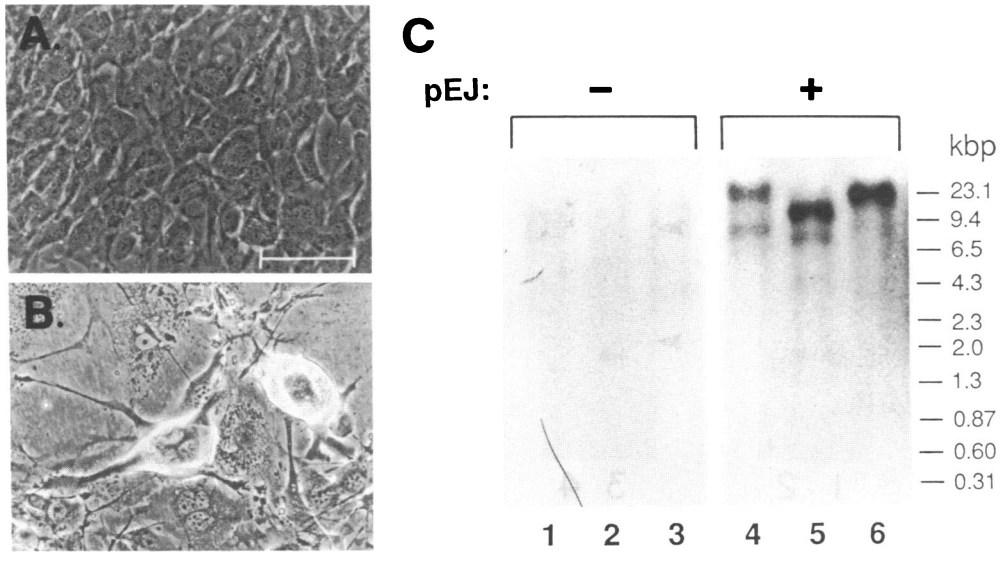

FIG. 1. (*A* and *B*) Morphology of transfected BC$_3$H1 myocytes. Phase-contrast microscopy is shown for representative G418-resistant colonies resulting from transfection with pSV2neo (*A*) or with pSV2neo plus the Val-12 *ras* vector pEJ (*B*). (Bar = 100 μm.) (*C*) Southern blot analysis of BC$_3$H1 (lanes 1–3) and the clonal *ras*-transfectant shown in *B* (lanes 4–6). DNA (10 μg per lane) was digested with restriction endonuclease *Hin*dIII (lanes 1 and 4), *Bam*HI (lanes 2 and 5), or *Eco*RI (lanes 3 and 6). The 6.3-kilobase-pair (kbp) insert of pEJ was used as hybridization probe. Markers at right show positions of *Hin*dIII-digested phage λ DNA electrophoresed in parallel.

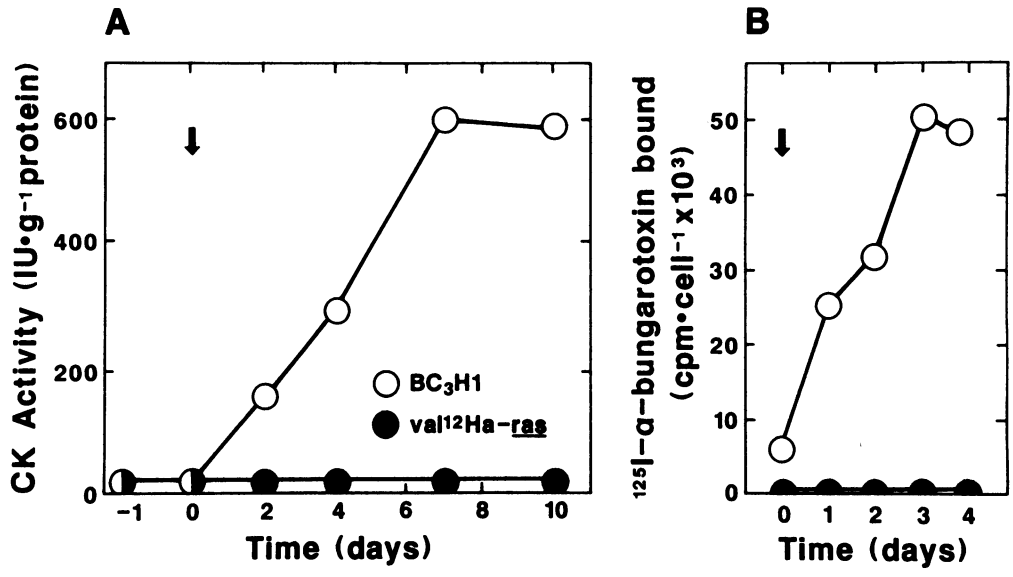

FIG. 3. Expression of CK activity and AcCho receptors is abolished in BC_3H1 myocytes transfected with the Val-12 *ras* allele. At the indicated times after mitogen withdrawal, CK activity (units/g of protein) was determined enzymatically (2) (*A*) and AcCho receptors were quantitated by specific binding of ^{125}I-labeled α-bungarotoxin (3) (*B*). Results comparable to those shown for BC_3H1 cells were obtained using cells modified by pSV2neo alone or with the Gly-12 *ras* protooncogene. Arrowheads denote withdrawal of 20% FBS.

gene. In contrast, transfection with the Val-12 *ras* allele prevented the expression of mCK activity in each of four independent clones, even after 5 days of growth factor withdrawal. For BC_3H1 cells, mCK activity evoked by serum-free medium (5) was ≈1.8-fold higher than in 0.5% FBS; no mCK was elicited in *ras*-transfected cells by either medium. Furthermore, the Val-12 *ras* allele also prevented expression of the nicotinic AcCho receptor (Fig. 3*B*). Together, these observations suggest that an activated *ras* gene can block the induction of representative and unlinked muscle proteins in BC_3H1 cells, through a mechanism independent of cell proliferation. These results contrast with those we obtained previously using the neomycin-resistance gene alone (7), an autonomous c-*myc* vector (7), or a viral *erbB* gene (unpublished results) or in the present study using the c-Ha-*ras* protooncogene.

The Val-12 *ras* Allele Can Block Activation of Muscle-Specific Genes. The induction of mCK activity by mitogen withdrawal in myogenic cells is contingent on accumulation of mCK transcripts and is mediated by increased transcription of the mCK gene (38), as shown for diverse sarcomeric proteins (39). To determine whether suppression of mCK activity by the Val-12 *ras* gene depends, conversely, on a block to expression of mCK mRNA, blot hybridization of electrophoretically fractionated RNA was performed. Levels of c-Ha-*ras* mRNA were ≈3-fold higher after c-Ha-*ras* transfection than in the control cells (Fig. 4, lanes 1 and 2). In agreement with the absence of mCK activity, no detectable mCK mRNA was evoked by mitogen withdrawal after transfection with the Val-12 *ras* gene (lanes 3 and 4), even in a 10-fold longer exposure of the autoradiogram than that shown here.

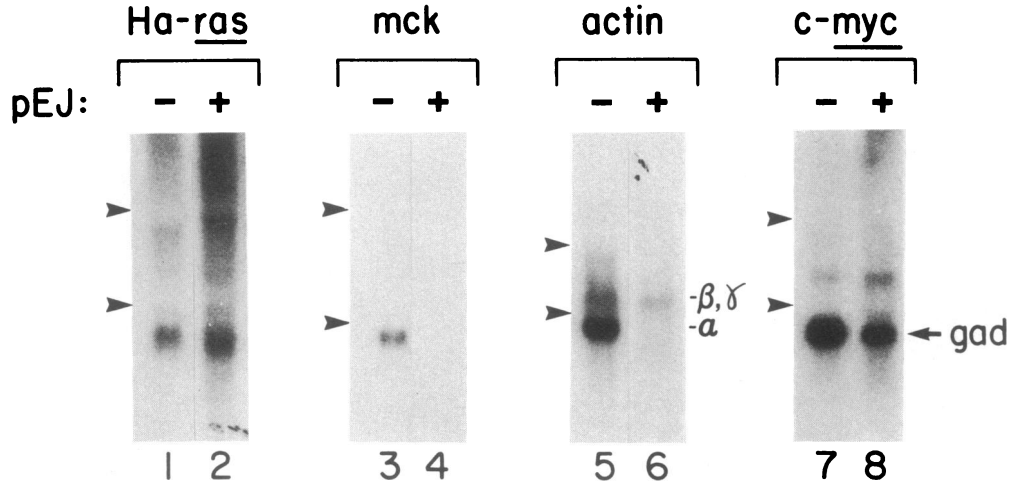

FIG. 4. Val-12 *ras* blocks the induction of mCK and α-actin mRNAs. For blot hybridization (7), total RNA was isolated from BC_3H1 cells (lanes 1, 3, 5, and 7) and *ras* transfectants (lanes 2, 4, 6, and 8) after mitogen withdrawal for 4 days. Arrowheads denote 28S and 18S ribosomal RNA. Hybridization probes were as follows. Lanes 1 and 2: Ha-*ras*, the 1.0-kbp *Bam*HI fragment of Harvey murine sarcoma virus clone HB-11 (40). Lanes 3 and 4: mCK, the 0.85-kbp *Pst* I fragment of pHMCKIa (41). Lanes 5 and 6: actin, the 0.93-kbp *Pst* I fragment of pJ, a skeletal muscle α-actin clone isolated from a differentiated BC_3H1 cDNA library (provided by S. Sharp, R. LaPolla, and N. Davidson). Lanes 7 and 8: glyceraldehyde-3-phosphate dehydrogenase (gad), the 1.65-kbp *Hha* I fragment of pGAD-28 (42), and c-*myc*, the 2.5-kbp *Xba* I–*Hin*dIII fragment of pSVc-myc-1 (23).

In BC_3H1 cells as in embryonic skeletal muscle, the β and γ-cytoplasmic isoforms of actin are down-regulated upon differentiation, and α-actin is induced (39, 43). At 4 days of mitogen withdrawal, BC_3H1 cells expressed predominantly α-actin transcripts, together with lesser amounts of β- and γ-actin mRNA (lane 5). In contrast, expression of α-actin was extinguished in the *ras*-transfected cells (lane 6). As shown by a 2- to 3-fold increase in c-*myc* expression and by the constitutive expression of glyceraldehyde-3-phosphate dehydrogenase mRNA (lanes 7 and 8), transfection with the Val-12 *ras* allele did not result in a parallel reduction of all cellular transcripts. Taken together, our data suggest that an activated *ras* allele introduced into BC_3H1 cells can selectively suppress the induction of muscle-specific genes whose expression normally is coupled to release from exogenous growth factors.

DISCUSSION

In the present study, we have demonstrated that a single altered Harvey *ras* allele inserted into BC_3H1 myoblasts can interfere with the activation of muscle-specific genes. Moreover, we have shown that the molecular mechanisms that preclude myogenic differentiation do not require continued, autonomous proliferation. These observations are in agreement with effects exerted by polypeptides such as fibroblast growth factor and type-β transforming growth factor, which directly inhibit myogenic differentiation but do not themselves provoke DNA replication or mitotic division (4, 10–12). The BC_3H1 myogenic cell line, widely studied as a model of muscle differentiation and its modulation by growth factors, was selected for these studies because differentiation in this system is accompanied by induction of mCK, nicotinic AcCho receptor subunits and α-actin; is essentially synchronous; and remains susceptible to inhibition by mitogens or transforming growth factors (2–4, 7, 10, 22). Furthermore, we previously showed that the establishment of a biochemically differentiated state in these cells is not prevented by transfection with the neomycin-resistance gene alone or with a transcriptionally activated c-*myc* vector (7). Thus, the results reported here are specifically due to the Val-12 *ras* gene that was used. Corroborative findings have been reported for a complementary muscle cell line (44).

Caffrey *et al.* (45) recently obtained evidence that differentiated BC_3H1 myocytes express voltage-gated Ca^{2+} channels that are distinct from those in smooth muscle cells and largely correspond to Ca^{2+} channels formed in the transverse (T) tubules of skeletal muscle. During logarithmic growth, neither functional Ca^{2+} channels assayed by whole-cell clamp techniques nor the associated dihydropyridine-binding protein could be detected (cf. refs. 46 and 47). Furthermore, transfection with the Val-12 *ras* gene (but not an autonomous c-*myc* gene) prevented the induction of Ca^{2+} channels (45). The formation of "skeletal muscle" Ca^{2+} channels agrees with the expression of nicotinic AcCho receptors, α-skeletal actin, and mCK in this line and supports the inference that BC_3H1 cells can be useful as a model to investigate mechanisms of gene activation during skeletal muscle differentiation. Since BC_3H1 cells do not fuse or commit to terminal differentiation, alternative methods are necessary to study these later transitions (6). Because Ca^{2+} channels could not be detected in Val-12 *ras*-transfected cells even after 40 days in serum-free medium, our results may indicate that cell differentiation induced by mitogen withdrawal was prevented, not merely delayed, by this missense mutation of the Ha-*ras* gene.

Several possible mechanisms exist to account for the concomitant regulation of diverse muscle-specific genes by an exogenous *ras* expression vector. In view of extensive evidence that this mutation may up-regulate a number of autocrine factors including type-β transforming growth factor (48), it is plausible to surmise that suppression of muscle-specific genes in *ras*-transfected BC_3H1 cells might depend, at least in part, on increased synthesis and release of type-β transforming growth factor or other autocrine peptides. Alternatively, or in addition, an activated *ras* protein might exert negative regulation principally through its own ability to serve as a surrogate for growth factors and initiate inositol-phospholipid hydrolysis. The results we present here do not allow us to distinguish between these intriguing possibilities.

Mitogenic control of muscle-specific genes has been shown to involve regulation at the level of gene transcription possibly involving the conformation or methylation of specific flanking sequences (39), as well as posttranscriptional mechanisms (49). The coordinate suspension of multiple muscle-specific genes by the introduction of a single mutation suggests that control of muscle-specific gene induction might occur via recognition of cis-acting DNA sequences shared by these genes (38, 39). It is unknown whether an activated *ras* gene can alter mCK-gene chromatin structure and transcription rates, corresponding to the effects of growth factors themselves. Since BC_3H1 myocytes first express smooth muscle α-actin after mitogen withdrawal, followed by the sarcomeric isoform (50), it would be of interest to establish whether *ras* might impinge on the consecutive induction of these two contrasting actin genes. Modifying cells by gene transfer with mutationally altered *ras* alleles thus can provide a useful model not only for studies of the cascade leading to cell proliferation but also for understanding the molecular mechanisms employed by factors that regulate cell differentiation.

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1. Linkhart, T. A., Clegg, C. H. & Hauschka, S. D. (1980) *J. Supramol. Struct.* **14,** 483–492.
2. Olson, E. N., Caldwell, K. C., Gordon, J. I. & Glaser, L. (1983) *J. Biol. Chem.* **259,** 3330–3336.
3. Olson, E. N., Glaser, L., Merlie, J. P., Sebbane, R. & Lindstrom, J. (1983) *J. Biol. Chem.* **258,** 13946–13952.
4. Spizz, G., Roman, D., Strauss, A. & Olson, E. N. (1986) *J. Biol. Chem.* **261,** 9483–9488.
5. Schneider, M. D., Payne, P. A., Ueno, H., Perryman, M. B. & Roberts, R. (1986) *Mol. Cell. Biol.* **6,** 4140–4143.
6. Endo, T. & Nadal-Ginard, B. (1986) *Mol. Cell. Biol.* **6,** 1412–1421.
7. Schneider, M. D., Perryman, M. B., Payne, P. A., Roberts, R. & Olson, E. N. (1987) *Mol. Cell. Biol.* **7,** 1973–1977.
8. Lathrop, B. K., Olson, E. N. & Glaser, L. (1984) *J. Cell. Biol.* **100,** 1540–1547.
9. Gospodarowicz, D., Weseman, J., Moran, J. S. & Lindstrom, J. (1976) *J. Cell Biol.* **70,** 395–401.
10. Olson, E. N., Sternberg, E., Hu, J. S., Spizz, G. & Wilcox, C. (1986) *J. Cell Biol.* **103,** 1799–1805.
11. Massague, J., Cheiftez, S., Endo, T. & Nadal-Ginard, B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8206–8210.
12. Florini, J. R., Roberts, A. B., Ewton, D. Z., Falen, S. L., Flanders, K. C. & Sporn, M. B. (1986) *J. Biol. Chem.* **261,** 16509–16513.
13. Bishop, J. M. (1987) *Science* **235,** 305–311.
14. Waterfield, M., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J. S. & Deuel, T. F. (1983) *Nature (London)* **304,** 35–39.

15. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. & Seeburg, P. H. (1984) *Nature (London)* **309,** 418–425.
16. Studzinski, G. P., Brelvi, Z. S., Feldman, S. C. & Watt, R. A. (1986) *Science* **234,** 467–470.
17. Nishikura, K. & Murray, J. M. (1987) *Mol. Cell. Biol.* **7,** 639–649.
18. Sejersen, T., Suemegi, J. & Ringertz, N. R. (1985) *J. Cell. Physiol.* **125,** 465–470.
19. Lim, R. W. & Hauschka, S. D. (1982) *Cold Spring Harbor Conf. Cell Proliferation* **9,** 877–884.
20. Falcone, G., Tatò, F. & Alemà, S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 426–430.
21. Wigler, M., Sweet, R., Kim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Cell* **16,** 777–785.
22. Schubert, D., Harris, A. J., Devine, C. E. & Heinemann, S. (1974) *J. Cell Biol.* **61,** 398–413.
23. Land, H., Parada, L. F. & Weinberg, R. A. (1983) *Nature (London)* **304,** 596–602.
24. Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. I. & Gilman, A. G. (1984) *Science* **226,** 860–862.
25. Tanabe, T., Nukada, T., Nishikawa, Y., Sugimoto, K., Suzuki, H., Takahashi, H., Noda, M., Haga, T., Ichiyama, A., Kangawa, K., Minamino, M., Matsuo, H. & Numa, S. (1985) *Nature (London)* **315,** 242–245.
26. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1984) *Nature (London)* **312,** 71–75.
27. Lacal, J. C., Srivastava, S. K., Anderson, P. S. & Aaronson, S. A. (1986) *Cell* **44,** 609–617.
28. Feramisco, J. R., Gross, M., Kamata, T., Rosenberg, M. & Sweet, R. W. (1984) *Cell* **38,** 109–117.
29. Mulcahy, L. S., Smith, M. R. & Stacey, D. W. (1985) *Nature (London)* **313,** 241–243.
30. Wakeham, M. J. D., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J. & Hall, A. (1986) *Nature (London)* **325,** 73–176.
31. Bar-Sagi, D. & Feramisco, J. R. (1985) *Cell* **42,** 841–848.
32. Lumpkin, C. K., Knepper, J. E., Butel, J. S., Smith, J. R. & Pereira-Smith, D. M. (1986) *Mol. Cell. Biol.* **6,** 2990–2993.
33. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1,** 327–341.
34. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
35. Dillmann, W. H., Barrieux, A., Neeley, W. E. & Contreras, P. (1983) *J. Biol. Chem.* **258,** 7738–7745.
36. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
37. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
38. Jaynes, J. B., Chamberlain, J. S., Buskin, J. N., Johnson, J. E. & Hauschka, S. D. (1986) *Mol. Cell. Biol.* **6,** 2855–2864.
39. Emerson, C., Fischman, D., Nadal-Ginard, B. & Siddiqui, M. A. Q., eds. (1986) *Molecular Biology of Muscle Development* (Liss, NY).
40. Ellis, R. W., DeFeo, D., Maryak, J. M., Young, H. A., Shih, T. Y., Chang, E. H., Lowy, D. R. & Scolnick, E. M. (1980) *J. Virol.* **361,** 408–420.
41. Perryman, M. B., Kerner, S. A., Bohlmeyer, T. J. & Roberts, R. (1986) *Biochem. Biophys. Res. Commun.* **140,** 981–989.
42. Dugaiczyk, A., Maron, J. A. & Scolnick, E. M. (1980) *J. Virol.* **36,** 408–420.
43. Strauch, A. R., Offord, J. D., Chalkey, R. & Rubinstein, P. A. (1986) *J. Biol. Chem.* **261,** 849–855.
44. Olson, E. N., Spizz, G. & Tainsky, M. T. (1987) *Mol. Cell. Biol.* **7,** 2104–2111.
45. Caffrey, J. M., Brown, A. M. & Schneider, M. D. (1987) *Science* **236,** 570–574.
46. Schmidt, A., Renaud, J.-F., Fosset, M., Meaux, J.-P. & Lazdunski, M. (1984) *J. Biol. Chem.* **259,** 11366–11372.
47. Nirenberg, M., Wilson, S. P., Higashida, H., Rotter, A., Krueger, K., Busis, N., Ray, R., Kenimer, J. G. & Adler, M. (1983) *Science* **225,** 794–799.
48. Stern, D. F., Roberts, A. B., Roche, N. S., Sporn, M. B. & Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6,** 870–877.
49. Endo, T. & Nadal-Ginard, B. (1987) *Cell* **49,** 515–526.
50. Strauch, A. R. & Reeser, J. C. (1986) *J. Cell Biol.* **103,** 399a (abstr.).