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The Oncogenic Forms of N-*ras* or H-*ras* Prevent Skeletal Myoblast Differentiation

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Differentiation of skeletal muscle involves withdrawal of myoblasts from the cell cycle, fusion to form myotubes, and the coordinate expression of a variety of muscle-specific gene products. Fibroblast growth factor and type β transforming growth factor specifically inhibit myogenesis; however, the transmembrane signaling pathways responsible for suppression of differentiation by these growth factors remain elusive. Because *ras* proteins have been implicated in the transduction of growth factor signals across the plasma membrane, we used DNA-mediated gene transfer to investigate the potential involvement of this family of regulatory proteins in the control of myogenesis. Transfection of the mouse skeletal muscle cell line C2 with the oncogenic forms of H-*ras* or N-*ras* completely suppressed both myoblast fusion and induction of the muscle-specific gene products nicotinic acetylcholine receptor and creatine kinase. Inhibition of differentiation by activated *ras* genes occurred at the level of muscle-specific mRNA accumulation. In contrast, proto-oncogenic forms of N-*ras* or H-*ras* had no apparent effects on the ability of C2 cells to differentiate. Myoblasts transfected with activated *ras* genes exhibited normal growth properties and ceased proliferating in the absence of mitogens, indicating that *ras* inhibited differentiation through a mechanism independent of cell proliferation. These results demonstrate that activated *ras* gene products mimic the inhibitory effects of fibroblast growth factor and type β transforming growth factor on myogenic differentiation and suggest that each of these regulators of myogenesis may operate through a common intracellular pathway.

During terminal differentiation of skeletal muscle, proliferating undifferentiated myoblasts cease dividing and fuse to form multinucleated myotubes. The process of fusion is accompanied by coordinate induction of a battery of muscle-specific gene products which include several contractile proteins, the muscle isoenzyme of creatine kinase (MCK) and the nicotinic acetylcholine (ACh) receptor (32). Serum mitogens, fibroblast growth factor (FGF), and type β transforming growth factor (TGFβ) specifically inhibit myogenesis (17, 20, 26–29, 39, 51); however, the intracellular signaling pathways whereby these growth factors exert their effects remain elusive.

Recent studies suggest that the products of proto-oncogenes play a central role in the regulation of cellular proliferation and differentiation by functioning at distinct steps in intracellular growth factor cascades. For example, the c-*sis* gene encodes a polypeptide similar to the β-chain of platelet-derived growth factor (7), and the c-*fms*, c-*neu*, and c-*erbB* oncogenes encode growth factor receptor-like membrane proteins (8, 42, 45). The products of *ras* genes resemble the G proteins that modulate adenylate cyclase and other membrane-associated enzymes and ion channels (19), whereas c-*myc* and c-*fos* are DNA-binding proteins that have been postulated to function as intranuclear mediators of growth factor signals (2, 22). It is currently unknown whether one or more of these proto-oncogene products participates in the pathway for growth factor-mediated regulation of myogenesis.

To begin to define the pathways whereby growth factors regulate muscle-specific gene expression, we have examined the effects on myogenesis of a series of normal and mutationally activated *ras* genes. The mammalian *ras* family consists of the Harvey (H)-*ras*, Kirsten (K)-*ras*, and N-*ras* genes, each of which encodes GTP-binding proteins localized to the cytoplasmic surface of the plasma membrane (6, 40, 48). The oncogenic forms of the *ras* proteins differ from their normal homologs by single-amino-acid substitutions, generally at position 12 or 61, and are capable of morphologic and tumorigenic transformation of human and rodent cells (41, 49, 56). These subtle mutations do not influence the subcellular localization of the proteins or their ability to bind guanine nucleotides, but the mutations do result in a loss of intrinsic GTPase activity, a property of normal *ras* proteins (15, 30, 55). By analogy with other G proteins, in which the endogenous GTPase provides a mechanism for termination of transduced signals, the lack of GTPase activity in the oncogenic forms of *ras* has been suggested to cause constitutive activation of transmembrane signaling pathways modulated by *ras* (18, 19, 30, 55).

Here we report that transfection of the mouse muscle cell line C2 with oncogenic N-*ras* or H-*ras* alleles completely suppresses expression of muscle-specific genes after withdrawal of myoblasts from the cell cycle in medium without mitogens. In contrast, proto-oncogenic *ras* genes exhibit no apparent effects on the ability of C2 cells to differentiate. Activated *ras* genes did not release C2 cells from their dependence on exogenous growth factors to proliferate, indicating that the inhibitory effects of these genes were not a secondary consequence of cell proliferation. Thus, the developmental program controlling myogenic differentiation can be regulated in a negative manner by *ras* proteins, possibly through activation of pathways normally controlled by FGF or TGFβ.

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MATERIALS AND METHODS

Cell culture. The mouse skeletal muscle cell line C2 (58) was grown in Dulbecco modified Eagle medium (DME) containing 20% fetal calf serum, as described by Inestrosa et al. (23). To initiate differentiation, cultures at approximately 80% confluence were transferred to DME containing 10% horse serum. Cell numbers were determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Calcium phosphate transfection. Cultures of C2 cells containing approximately 5×10^6 cells were transfected by calcium phosphate precipitation (21) with the oncogenic or proto-oncogenic forms of N-*ras* and H-*ras* genes. Each *ras* gene was contained within the vector pSV2 *neo*, which confers resistance to G418 as a selectable marker (50). The details of the constructs have been described elsewhere (40, 57). At 48 h after transfection, cultures were transferred to growth medium containing 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml, and individual colonies were isolated after 14 days and propagated into stable cell lines.

CK and ACh receptor assays. Creatine kinase (CK) was assayed, as described previously (35). ACh receptors were assayed by the specific binding of ^{125}I-labeled-α-bungarotoxin to cell monolayers, as described previously (36, 37).

Northern blot hybridization analysis. Total cellular RNA was prepared from cells by the 8 M guanidine hydrochloride procedure (35), and the relative abundance of individual mRNAs was determined by Northern blot hybridization, as described previously (51). Plasmids used for measurement of individual mRNAs were as follows: MCK, a 775-base-pair *Sma*I-*Pvu*I fragment from a canine MCK cDNA (43); GAPDH, a 1,650-base-pair *Hha*I fragment from a chicken GAPDH cDNA (9); c-*myc*, a 2,500-base-pair *Hin*dIII-*Xba*I fragment from pSVc-*myc*-1, containing exons 2 and 3 of mouse c-*myc* (25). DNA probes were labeled with ^{32}P by the method of Feinberg and Vogelstein (12).

RESULTS

Activated *ras* genes inhibit fusion and expression of MCK and ACh receptors. Undifferentiated C2 myoblasts exhibit a flattened stellate shape and proliferate with a doubling time of ~12 h in DME containing 20% fetal calf serum (23, 39, 58). Transfer of cultures at 80% confluence to DME with 10% horse serum results in cessation of cell division and the onset of fusion within 24 to 48 h. Fusion is accompanied by coordinate induction of a battery of muscle-specific gene products, which includes CK and ACh receptors (3, 5, 23, 39, 58).

To investigate the potential involvement of *ras* in myogenesis, C2 cells were transfected with a series of oncogenic and proto-oncogenic *ras* genes contained within the vector pSV2 *neo*, which confers resistance to the neomycin analog G418 as a selectable marker (50). *ras* genes contained within these constructs were under transcriptional control of their normal cellular promoters. The oncogenic *ras* genes used in these experiments differed from their proto-oncogenic counterparts by single-amino-acid substitutions at position 12, resulting in their conversion to activated forms. The activated N-*ras* gene was isolated from a human teratocarcinoma cell line, PA1, after late passage (57). The activated H-*ras* gene pEJ comprised the valine 12 mutation of human c-H-*ras* (40). For transfections with the proto-oncogenic form of N-*ras* and both forms of H-*ras*, several neomycin-resistant colonies were isolated, propagated as stable cell lines, and characterized with respect to their abilities to differentiate. Results shown are of representative clones from each oncogene transfection. For the oncogenic form of N-*ras*, several colonies obtained from a single transfection were pooled before analysis. Because the neomycin phosphotranferase gene, which confers resistance to G418, was contained within the same vector as the *ras* genes, colonies that survived the selection procedure also contained exogenous *ras*. Stable integration of *ras* genes was confirmed by Southern blot analysis (data not shown).

Cells transfected with pSV2 *neo* alone were morphologically indistinguishable from untransfected C2 cells and fused normally after transfer to medium with 10% horse serum (Fig. 1a and b). After fusion, CK activity and ACh receptors also were induced in a manner identical to that in untransfected C2 cells (Fig. 2). Myoblasts bearing activated *ras* genes were readily distinguishable from normal myoblasts by a refractile, phase-bright appearance. Moreover, cells transfected with either an activated N-*ras* or H-*ras* gene neither fused (Fig. 1c and e) nor expressed CK activity or ACh receptors (Fig. 2) under the appropriate conditions. Of 11 clonal cell lines containing activated H-*ras* oncogenes, only one was found to undergo limited differentiation. More than 50 colonies resulting from transfection with activated N-*ras* oncogenes were pooled before detailed analysis. No evidence of differentiation was observed in this mixed population, suggesting that the frequency of inhibition of differentiation by this activated oncogene may be 100%. The lack of differentiation of these *ras*-transfected cell lines cannot be attributed to a simple delay in the onset of differentiation, because cultures maintained under fusing conditions for longer than 3 weeks failed to fuse or to express muscle proteins (data not shown).

To determine whether the ability to suppress differentiation was common to all transfected *ras* genes or was unique to the oncogenic forms of *ras*, the proto-oncogenic forms of N-*ras* and H-*ras* were introduced into C2 cells. As shown in Fig. 1 and 2, neither of the proto-oncogenic forms of *ras* was able to inhibit either fusion or induction of muscle-specific gene products. Of eight separate proto-oncogene H-*ras* clones and five proto-oncogene N-*ras* clones examined, all were found to fuse and to express muscle markers under appropriate conditions. Thus, a single point mutation in either N-*ras* or H-*ras* is sufficient to completely suppress expression of the muscle phenotype.

Myoblasts bearing activated *ras* genes require exogenous growth factors to proliferate. We considered the possibility that the failure of activated *ras*-transfectants to differentiate might be due to the ability of these cell lines to proliferate in the presence of low levels of exogenous growth factors, as has been reported for some *ras*-transfected fibroblasts (44). Because myogenic differentiation depends absolutely on withdrawal of myoblasts from the cell cycle, an increased responsiveness to growth factors could lead indirectly to inhibition of differentiation. To examine the dependence of *ras*-transfectants on growth factors, cultures at low densities were exposed to DME containing either 20 or 0.5% fetal calf serum, and cells were counted on consecutive days. As shown in Fig. 3, cells transfected with either proto-oncogenic or activated *ras* genes exhibited doubling times equivalent to those of *neo* transfectants in medium with 20% fetal calf serum and ceased dividing at subconfluent densities when transferred to medium with 0.5% serum. Cells bearing activated *ras* genes also exhibited contact inhibition of cell growth at confluent densities in medium containing either

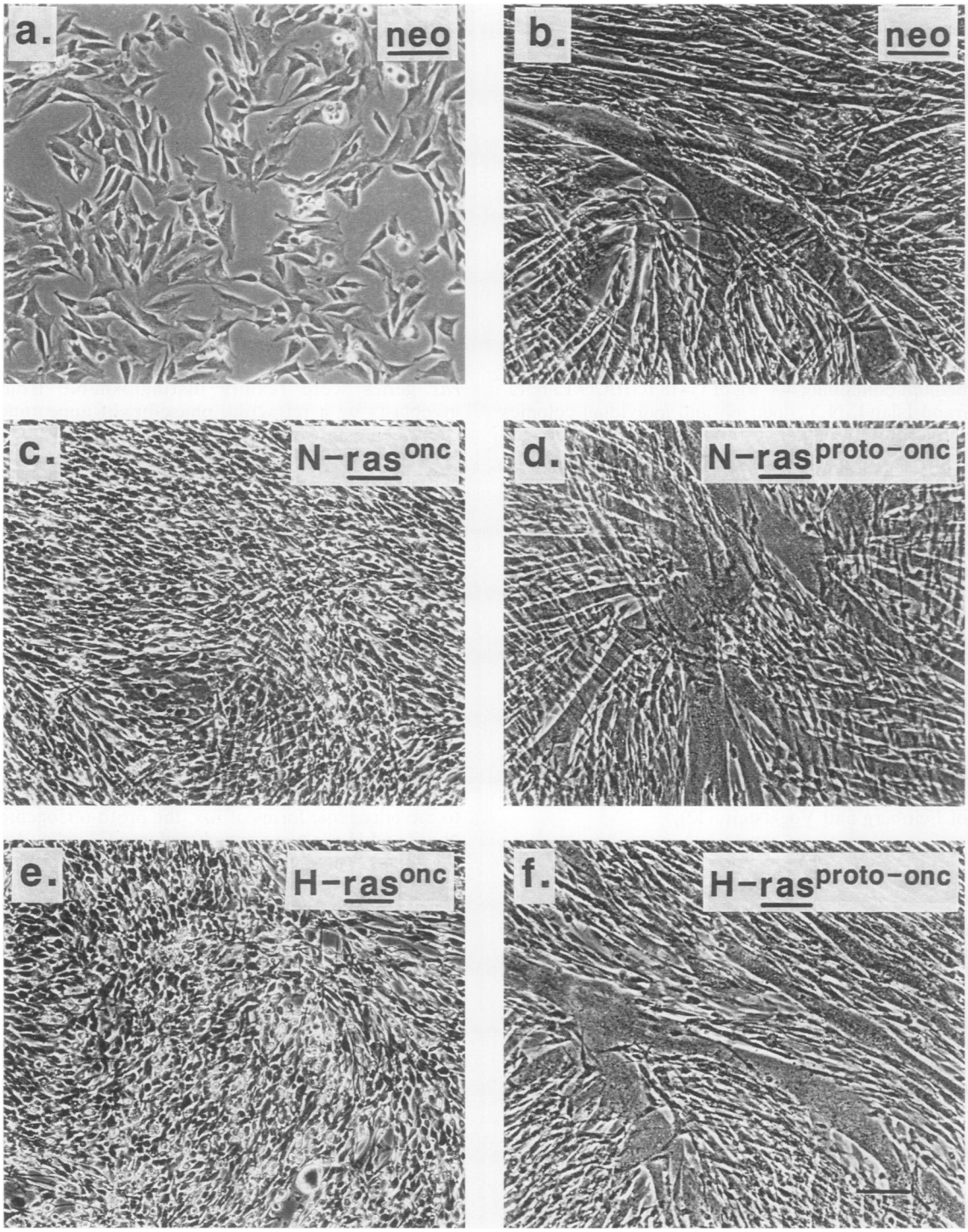

FIG. 1. Morphology of *ras*-transfected myocytes. To initiate differentiation, cultures at 80% confluence were transferred from DME containing 20% fetal calf serum to DME with 10% horse serum. (a and b) *neo* transfectants in DME with 20% fetal calf serum or after 5 days in 10% horse serum, respectively. (c and d) N-ras^{onc} and N-$ras^{proto\text{-}onc}$ transfectants, respectively, after 5 days in 10% horse serum. (e and f) H-ras^{onc} and H-$ras^{proto\text{-}onc}$ transfectants, respectively, after 5 days in 10% horse serum. Note that both activated *ras* genes inhibited fusion, whereas proto-oncogenic *ras* genes had no apparent effects. Bar, 100 μm.

10% horse serum or 20% fetal calf serum (Fig. 3) and ceased incorporating [^{3}H]thymidine into DNA (data not shown). Thus, the requirement of C2 cells for exogenous growth factors to proliferate is not abrogated by oncogenic *ras* genes. The inhibition of differentiation by *ras*, therefore, appears to be due to interference with the differentiation program rather than being a secondary consequence of cell proliferation.

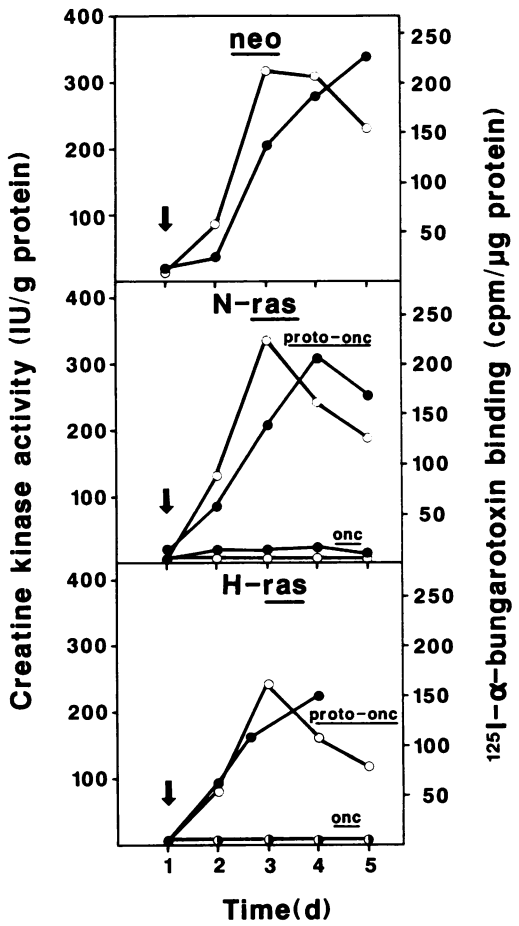

FIG. 2. Effects of *ras* genes on induction of CK activity and ACh receptors. C2 cells, transfected with pSV2 *neo* alone or in combination with the oncogenic or proto-oncogenic forms of N-*ras* or H-*ras*, as specified, were cultured in DME with 20% fetal calf serum. On day 1, cultures were transferred to DME with 10% horse serum, and ACh receptors (○) and CK acitivity (●) were measured each day. Note that both activated *ras* genes inhibited induction of ACh receptors and CK, whereas these muscle markers were induced in cells transfected with proto-oncogenic *ras* genes.

In some cell types, activated *ras* genes have been reported to induce synthesis of transforming growth factors (1, 53). Because TGFβ is a potent inhibitor of myogenic differentiation (17, 29, 38), we tested culture medium conditioned by cells transfected with activated *ras* genes for the ability to inhibit differentiation of normal C2 myoblasts. Nontransfected C2 cells differentiated normally in the presence of conditioned medium from *ras* transfectants, indicating that *ras* does not suppress differentiation solely by an autocrine mechanism (data not shown).

Activated *ras* genes inhibit MCK mRNA expression. During myogenesis, muscle-specific proteins accumulate in parallel with their corresponding mRNAs. To determine whether activated *ras* genes inhibited differentiation by preventing expression of muscle-specific mRNAs, steady-state levels of MCK mRNA were measured by Northern blot analysis, using an MCK cDNA probe. Differentiation of cells transfected with pSV2 *neo* alone was accompanied by a greater than 10-fold increase in the level of MCK mRNA (Fig. 4), which is equivalent to that in untransfected cells. As predicted from the normal induction of CK activity in cells transfected with the proto-oncogenic forms of N-*ras* or H-*ras*, these cell lines also expressed MCK mRNA at levels similar to that for *neo* transfectants. In contrast, MCK mRNA was undetectable in cells transfected with either an N-*ras*- or an H-*ras*-activated oncogene. The absence of MCK mRNA in cells transfected by activated *ras* genes cannot be attributed to a generalized abnormality in mRNA expression because the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which is not muscle specific, was unchanged in all cell lines examined. These results indicate that activated *ras* genes inhibit an early step in the transition of myoblasts to the differentiated state.

c-*myc* mRNA fails to be down regulated after withdrawal from the cell cycle of myoblasts containing activated *ras* genes. Recently, Endo and Nadal-Ginard (10) reported that terminal differentiation of rat L6E9 myoblasts was accompanied by a decline in steady-state levels of c-*myc* mRNA. Similar results have been obtained with the nonfusing mouse muscle cell line BC_3H1 (46). Sejersen et al. (47) also showed that c-*myc* was down regulated during differentiation of primary myoblast cultures and established muscle cell lines. In

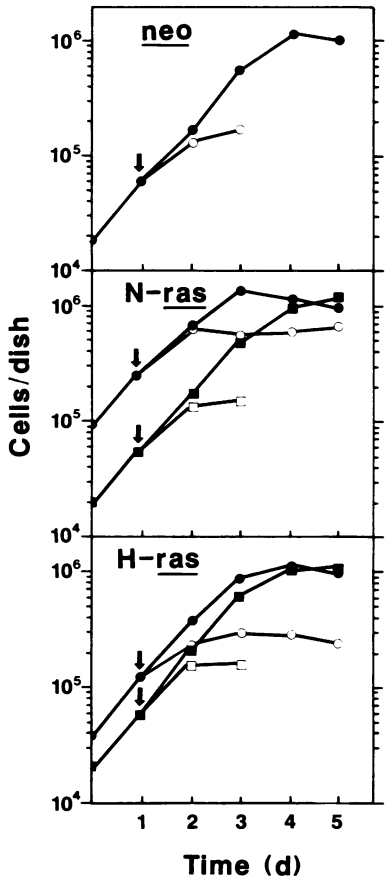

FIG. 3. Effects of *ras* genes on cell proliferation. C2 cells, transfected with pSV2 *neo* alone or in combination with the oncogenic or proto-oncogenic forms of N-*ras* or H-*ras*, as specified, were cultured in DME with 20% fetal calf serum. On day 1, cultures were exposed to fresh medium containing either 20% serum (closed symbols) or 0.5% serum (open symbols). Cell numbers were determined on consecutive days by using a Coulter Counter. Circles, *neo* or ras^{onc} transfectants, as specified. Squares, $ras^{proto\text{-}onc}$ transfectants. Note that all cell lines exhibited similar doubling times, withdrew from the cell cycle in 0.5% serum, and achieved similar saturation densities in 20% serum.

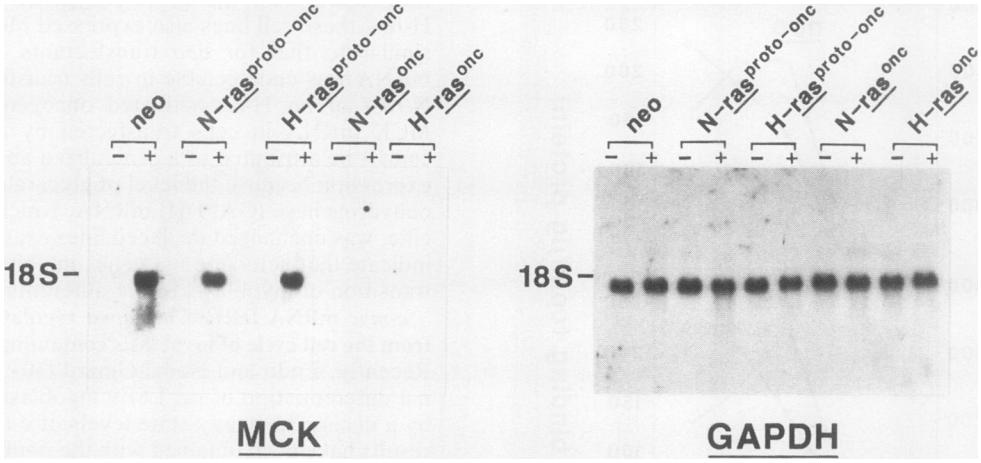

FIG. 4. Effects of *ras* genes on MCK mRNA and GAPDH mRNA expression. C2 cells, transfected with pSV2 *neo* alone or in combination with the oncogenic or proto-oncogenic forms of N-*ras* and H-*ras*, as specified, were cultured in DME with 20% fetal calf serum (−). To initiate fusion, cultures were transferred to DME with 10% horse serum for 5 days (+). Expression of MCK and GAPDH mRNAs was measured by Northern blot hybridization, as described in Materials and Methods. The position of 18S ribosomal RNA is indicated.

differentiation-defective myoblasts, however, c-*myc* remained at a high level after withdrawal from the cell cycle, suggesting that a decline in c-*myc* may be coupled to myogenic differentiation.

In light of the inverse relationship between expression of c-*myc* and muscle-specific gene products, it was of interest to investigate whether *ras*-dependent inhibition of myogenic differentiation was accompanied by a disruption in normal c-*myc* expression. The pattern of c-*myc* mRNA expression in myoblasts transfected with pSV2 *neo* alone or in combination with activated *ras* alleles is shown in Fig. 5. In agreement with previous reports in other systems, myoblast fusion was accompanied by a dramatic decline in levels of c-*myc* mRNA. Myoblasts containing proto-oncogenic *ras* genes showed patterns of expression of c-*myc* identical to those of *neo* transfectants (data not shown). In contrast, cells bearing activated *ras* genes failed to down regulate

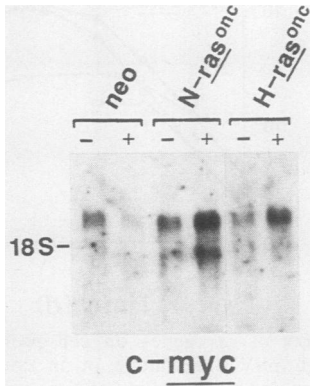

FIG. 5. Expression of c-*myc* mRNA in *neo* or activated *ras* tranfectants. C2 cells, transfected with pSV2 *neo* alone or in combination with the oncogenic forms of N-*ras* or H-*ras*, as specified, were cultured in DME with 20% fetal calf serum (−). To initiate fusion, cultures were transferred to DME with 10% horse serum for 5 days (+). Expression of c-*myc* mRNA was measured by Northern blot analysis, as described in Materials and Methods. The position of 18S ribosomal RNA is indicated.

c-*myc* mRNA and actually exhibited a two- to threefold increase in levels of c-*myc* under fusion-promoting conditions. Whether this failure of c-*myc* to be appropriately down regulated in oncogenic *ras* transfectants is a cause, or instead a consequence, of their inability to differentiate remains to be determined.

DISCUSSION

The coordinate induction of a series of unlinked muscle-specific genes during myogenesis is consistent with the notion that these genes respond to common developmental signals and are governed by the same regulatory mechanism(s). Although the precise mechanisms responsible for their coordinate expression remain elusive, it is clear that muscle-specific genes share the properties of being activated only in quiescent, G_0-arrested myocytes (34) and of being suppressed by serum mitogens, FGF or TGFβ (17, 20, 26–29, 39, 51). The results of this study demonstrate that activated *ras* genes uncouple cell cycle withdrawal from muscle-specific gene expression and completely suppress the ability of myoblasts to differentiate. In contrast, proto-oncogenic *ras* genes had no apparent consequences on expression of the muscle phenotype. Because myocytes transfected with activated *ras* genes exhibited normal growth properties and withdrew from the cell cycle after exposure to medium lacking mitogens, it can be concluded that *ras* oncogene proteins do not inhibit myogenesis simply by preventing myoblasts from exiting the cell cycle. Activated *ras* proteins appear instead to interfere more directly with the mechanism(s) responsible for fusion and coordinate expression of muscle-specific mRNAs and proteins in quiescent myoblasts. Recently, FGF and TGFβ also were shown to prevent myogenic differentiation through a mechanism independent of cell proliferation (17, 29, 39, 51). Thus, activated *ras* proteins mimic the inhibitory effects of these growth factors on expression of the differentiated phenotype.

In addition to preventing the accumulation of muscle-specific gene products in quiescent myoblasts, activated *ras* genes appeared to prevent the down regulation of c-*myc*, an early molecular event in the pathway of myoblasts toward

the differentiated state (10, 46, 47). Whether the aberrant pattern of c-*myc* regulation in oncogenic *ras* transfectants reflects a subtle alteration in growth factor responsiveness or a partial loss of the normal controls governing c-*myc* expression remains to be determined. It will be particularly interesting to examine whether other gene products, such as ornithine decarboxylase (38) and β and γ actin (3), which have also been reported to be down regulated during myogenesis, continue to be expressed at elevated levels following growth arrest of myoblasts bearing oncogenic *ras* genes.

Several recent studies suggest that *ras* proteins participate in the transduction of growth factor signals by interacting, either directly or indirectly, with cell surface growth factor receptors. Epidermal growth factor, for example, stimulates the GTP-dependent phosphorylation of v-H-*ras* protein and enhances the guanine nucleotide-binding activity of activated c-H-*ras* or v-H-*ras* proteins (24). Microinjection of antibodies against proto-oncogenic *ras* proteins into fibroblasts inhibits serum-stiumlated DNA synthesis, and microinjection of antibodies against oncogenic *ras* proteins into transformed fibroblasts results in a loss of the transformed morphology and a normal growth rate (13, 14, 33, 52, 54). Moreover, microinjection of oncogenic *ras* proteins into quiescent fibroblasts induces membrane ruffling and pinocytosis, followed by proliferation (4). *ras*-transformed cells also exhibit elevated levels of diacylglycerol and the intermediates of the phosphatidylinositol turnover cycle, suggesting a potential regulatory role for *ras* in the phosphatidylinositol-4,5-bisphosphate breakdown pathway in the plasma membrane (16).

Little is known of the transmembrane signaling pathways by which FGF and TGFβ inhibit myogenesis; however, the similarities between the effects of these growth factors and of activated *ras* gene products on myocytes raise the intriguing possibility that each of these regulators of myogenic differentiation may operate by a common intracellular pathway. Because of the known ability of activated *ras* proteins to modulate phosphatidylinositol turnover (16), it is tempting to speculate that FGF and TGFβ might influence differentiation through a mechanism involving this pathway. Diacylglycerol, one of the products of phosphatidylinositol turnover, can act as a substrate for the synthesis of prostaglandins, which in turn activate adenylate cyclase. It may be relevant in this regard that prostaglandin E_1, cAMP analogs, and other compounds that elevate intracellular levels of cAMP, each inhibit myogenic differentiation through a mechanism independent of cell proliferation (J. S. Hu, E. A. Sternberg, and E. Olson, unpublished results).

Using the nonfusing muscle cell line, BC_3H1, Lathrop et al. showed that FGF allowed quiescent myocytes to progress from G_0 to a new restriction point several hours into G_1 that was nonpermissive for differentiation (27). Because *ras* proteins participate in the pathway leading to DNA synthesis, it will be interesting to determine whether transforming *ras* proteins advance quiescent myocytes to a similar region of the cell cycle. Fusion of normal C2 cells precludes direct comparison of the position in G_0-G_1 of myotubes relative to *ras*-transfected myocytes; therefore, this question will have to be addressed using BC_3H1 cells or using culture conditions that allow C2 cells to differentiate in the absence of fusion.

Although several retroviral transforming genes have been shown to prevent terminal differentiation of myoblasts (11), oncogene expression does not inhibit myogenesis in all situations. In BC_3H1 cells, for example, autonomous expression of c-*myc* (46) or of v-*erbB* (M. D. Schneider and E. N. Olson, unpublished results) fails to suppress the differentiated phenotype after withdrawal of cells from the cell cycle. Similarly, expression of c-*myc*, v-*erb*, or v-*abl* in C2 cells does not prevent fusion or induction of muscle-specific genes (E. Olson, R. Cruz, and G. Spizz, unpublished results). When expressed at high levels, proto-oncogenic *ras* genes have been reported to elicit phenotypes similar to those of their oncogenic homologs (31). Ongoing studies are aimed at determining whether over-expression of proto-oncogenic *ras* genes also can influence myogenesis.

In the future, it will be interesting to test directly the hypothesis that the receptors for FGF or TGFβ interact with cellular *ras* proteins to initiate an intracellular cascade that culminates in suppression of myogenic differentiation. If, for example, TGFβ or FGF function through a *ras*-dependent mechanism, myoblasts expressing elevated levels of proto-oncogenic *ras* proteins might be predicted to exhibit hypersensitivity to the inhibitory effects of these growth factors on the differentiated phenotype. Elucidation of the pathways whereby activated *ras* gene products inhibit muscle-specific gene expression will extend understanding of the mechanisms involved in coordinate regulation of gene expression during cellular differentiation and of the pathways for intracellular communication between cell surface and nucleus.

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