Dexamethasone-dependent Inhibition of Differentiation of C2 Myoblasts Bearing Steroid-inducible N-*ras* Oncogenes

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Abstract. *ras* proteins are localized to the plasma membrane where they are postulated to interact with growth factor receptors and other proximal elements in intracellular cascades triggered by growth factors. The molecular events associated with terminal differentiation of certain skeletal myoblasts are inhibited by specific polypeptide growth factors and by constitutive expression of transforming *ras* oncogenes. To determine whether the inhibitory effects of *ras* on myogenic differentiation were reversible and to investigate whether muscle-specific genes remained susceptible to *ras*-dependent repression in terminally differentiated myotubes, the murine myoblast cell line, C2, was transfected with a plasmid containing a mutationally activated human N-*ras* oncogene under transcriptional control of the steroid-sensitive promoter of the mouse mammary tumor virus long terminal repeat. Addition of dexamethasone to myoblasts bearing steroid-inducible *ras* oncogenes prevented myotube formation and induction of muscle creatine kinase and acetylcholine receptors. Inhibition of differentiation by dexamethasone occurred in a dose-dependent manner and was a titratable function of *ras* expression. In the presence of dexamethasone, myoblasts bearing steroid-inducible *ras* genes retained their dependence on exogenous growth factors to divide and exhibited contact inhibition of growth at confluent densities, indicating that the inhibitory effects of *ras* on differentiation were independent of cell proliferation. Removal of dexamethasone from N-*ras*-transfected myoblasts led to fusion and induction of muscle-specific gene products in a manner indistinguishable from control C2 cells. Examination of the effects of culture media conditioned by *ras*-transfected myoblasts on differentiation of normal C2 cells yielded no evidence for inhibition of differentiation via an autocrine mechanism. In contrast to the ability of N-*ras* to prevent up-regulation of muscle-specific gene products in myoblasts, induction of N-*ras* in terminally differentiated myotubes failed to extinguish muscle-specific gene expression. Together, these results suggest that oncogenic *ras* proteins reversibly activate an intracellular cascade that prevents establishment of the differentiated phenotype. The inability of *ras* to extinguish muscle-specific gene expression in terminally differentiated myotubes also suggests that *ras* may interfere with an early step in the pathway of myoblasts toward the differentiated state.

THE process of myogenic differentiation involves an ordered sequence of molecular events which include cessation of cell division, formation of multinucleated myotubes, and coordinate induction of a battery of muscle-specific genes encoding proteins involved in the specialized functions of the myofiber (for review, see reference 7). Among the gene products that are up-regulated during myogenesis are the components of the contractile apparatus, the muscle isoenzyme of creatine kinase (MCK),[1] and the nicotinic acetylcholine (ACh) receptor. The ability of certain skeletal myoblasts to differentiate is controlled in a negative manner by the extracellular concentration of serum mitogens, fibroblast growth factor (FGF) and type β transforming growth factor (TGFβ) (6, 14, 18, 28, 29, 32, 33, 38–40, 52, 53). In the presence of these growth factors, myoblasts fail to fuse or to express muscle-specific gene products. Conversely, reduction in the concentration of these growth factors below a critical level results in irreversible withdrawal from the cell cycle and terminal differentiation. After fusion, muscle-specific genes become refractory to the inhibitory effects of polypeptide growth factors. Little is known of the intracellular pathways whereby polypeptide growth factors influence the muscle differentiation program or of the mechanisms responsible for the loss of sensitivity of muscle-specific genes in myotubes to growth factors.

The products of proto-oncogenes have been postulated to play important roles in regulation of cellular proliferation and

1. *Abbreviations used in this paper*: ACh, acetylcholine; CK, creatine kinase; FGF, fibroblast growth factor; HS, horse serum; M, muscle isoenzyme; MMTV-LTR, mouse mammary tumor virus long terminal repeat; TGFβ, type β transforming growth factor.

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differentiation by transducing growth factor signals from the cell surface to the nucleus (3, 22, 50). As an initial step toward understanding the mechanisms involved in growth factor-mediated regulation of myogenesis, we have chosen to examine the consequences of autonomous expression of specific oncogenes on myogenic differentiation. Recently, Schneider et al. reported that deregulated expression of c-*myc*, the putative intranuclear mediator of growth factor signals, partially inhibited but did not prevent, induction of muscle-specific genes in the BC_3H1 muscle cell line (49). In contrast, mutationally activated *ras* oncogenes conferred a phenotype on myoblasts similar to that elicited by FGF and TGFβ and suppressed completely the ability of myoblasts to differentiate (41).

The mammalian *ras* family consists of the Harvey (H)-*ras*, Kirsten (K)-*ras*, and N-*ras* genes, each of which encodes a 21-kD GTP-binding proteins localized to the cytoplasmic surface of the plasma membrane (for review, see reference 30). *Ras* proteins appear to participate in transduction of mitogenic signals by interacting with specific cell surface growth factor receptors and other proximal elements in cascades triggered by growth factors (17, 27, 30, 37, 55, 59). Termination of transduced signals between receptors and enzyme effectors is postulated to be achieved through hydrolysis of GTP by endogenous GTPase activity of normal *ras* proteins. *ras* genes bearing point mutations, generally at codons 12 or 61, have been isolated from a wide range of tumors and have been shown to transform human and rodent cells (4, 30, 45, 57, 58). These mutations do not influence the subcellular localization of the proteins or their ability to bind guanine nucleotides, but in most cases result in diminished GTPase activity (12, 16, 34, 56). The loss of GTPase activity of mutant *ras* proteins has been associated with persistent activation of specific intracellular growth factor cascades and alleviation of growth factor requirements.

Previously, we reported that constitutive expression of the oncogenic forms of N- and H-*ras* prevented myoblast differentiation (41). Several important questions concerning the mechanism for *ras*-dependent inhibition of myogenesis, however, were not amenable to experimental analysis in differentiation-defective cell lines that expressed *ras* constitutively. For example, it remained unclear whether the inhibitory effects of *ras* on differentiation were reversible, and if so, whether the kinetics for induction of muscle-specific genes would be accelerated or delayed after release of myoblasts from *ras*-dependent repression. It was also unknown whether muscle-specific genes were susceptible to *ras* only during a specific period early in the differentiation program or whether these genes remained responsive to *ras* in terminally differentiated myotubes. The latter issue was of particular interest in light of observations that muscle-specific genes no longer respond to serum mitogens, FGF or TGFβ after myoblast fusion (6, 14, 32, 33, 40).

Here, we report that transfection of the C2 myoblast cell line with a mutationally activated N-*ras* gene linked to the steroid-inducible mouse mammary tumor virus long terminal repeat (MMTV-LTR), renders myoblasts sensitive to inhibition of differentiation by dexamethasone. Suppression of the differentiated state by dexamethasone occurred in a dose-dependent manner and required the continual presence of the steroid in the culture medium. Removal of dexamethasone led to fusion and induction of muscle-specific gene products with kinetics indistinguishable from control C2 myoblasts. The mechanism whereby *ras* inhibited differentiation did not require cell proliferation and was apparently not mediated by autocrine growth factors. In contrast to the ability of *ras* to prevent differentiation of myoblasts, induction of *ras* in terminally differentiated myotubes was without effect on myotube morphology or expression of muscle-specific genes. Together, these results indicate that *ras* inhibits myogenic differentiation through a highly transient and reversible mechanism and that this *ras*-dependent pathway interferes with an early step in the process of muscle-specific gene activation. In each of these respects, the mechanism for suppression of differentiation by *ras* resembles the mechanism used by TGFβ and FGF (6, 14, 32, 33, 40).

Materials and Methods

Cell Culture

The mouse skeletal muscle cell line, C2 (60) was grown in DME containing 20% FCS, as described (40). To initiate differentiation, cultures at ~80% confluence were transferred to DME containing 10% horse serum (HS). Dexamethasone (Sigma Chemical Co., St. Louis, MO) was prepared as a 0.1 mM stock in sterile deionized water and was added to culture media at the concentrations indicated. To obtain pure cultures of myotubes, confluent cultures that had been exposed to DME with 10% HS for at least 48 h were transferred to DME with 20% FCS and 0.1 mM cytosine arabinoside for 48 h. Aphidicolin (Sigma Chemical Co.) was stored as a 1 mg/ml stock in 100% ethanol and was added to a final concentration of 5 μg/ml to DME containing 10% HS as an alternative method of obtaining pure myotube cultures. Cell numbers were determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

In experiments that required coculturing of C2 cells with CO25 cells, barriers of silicon grease ~2-mm wide were applied to the centers of 35-mm culture dishes. Cells of each type were plated at equivalent densities on opposite sides of the barrier, which was of sufficient height to prevent exchange of media between the two sides. After cells had attached to the substratum, residual nonadherent cells were removed, the barrier was aspirated away such that only a thin line of silicon grease remained on the dish to separate the two cell types, and fresh medium with or without dexamethasone, as specified, was added to the cultures. Cultures were then placed on a rotating shaker at 60 rpm in a tissue culture incubator. After achieving 80% confluency, cultures were transferred to 10% HS and placed back on the shaker. At the indicated times, each cell type was harvested separately for CK assays and protein determination.

Transfection and Isolation of Dexamethasone-responsive Cell Lines

Cultures of C2 cells in 10-cm culture dishes containing ~5×10^6 cells were transfected by calcium phosphate precipitation (19) with pSV2neo, which confers resistance to G418 as a selectable marker (51), and with the oncogenic form of N-*ras* containing a substitution of lysine for glutamine at amino acid 61 (4). The N-*ras* gene was under transcriptional control of the MMTV-LTR, conferring glucocorticoid inducibility to the gene. The details of this plasmid were described previously (35). At 16-24 h after transfection, cells were transferred from 1 10-cm dish to two 15-cm dishes and at 48 h after transfection 400 μg/ml of G418 (Gibco, Grand Island, NY) was added to the cultures in growth media. Individual colonies were isolated after 14 d and passaged into stable cell lines. Clonal cell lines were exposed to a series of concentrations of dexamethasone from 10 nM to 2 μM and CK activity and the extent of fusion were determined. Cell lines that showed a dose-dependent inhibition or retardation of differentiation were selected and tested further.

Assays for CK Activity and ACh receptor

Creatine kinase (CK) was assayed as described previously (38). Acetylcholine receptors (AChR) were assayed by the specific binding of [^{125}I]α-bungarotoxin to cell monolayers (39).

Assay for Expression of p21 ras Proteins by Western Transfer

A mouse anti-*ras* p21 monoclonal antibody, 259, (Cetus Corp., Emeryville, CA) was used for quantitation of *ras* p21 protein (15). Cell lysis and protein extraction were carried out according to manufacturer's instructions and protein transfer was performed as described previously (38). Equivalent quantities of protein from cells under each condition were applied to the lanes. After incubation of nitrocellulose blots with the primary anti-*ras* p21 antibody, an ^{125}I-labeled rabbit anti-mouse secondary antibody was added. After subsequent washing, blots were exposed to Kodak X-AR5 film using intensifying screens. Relative levels of p21 *ras* were quantitated by densitometry of autoradiographs.

RNA Isolation and Northern Blot Hybridization

RNA was prepared from cell cytoplasm by magnesium precipitation of polyribosomes (44). The relative abundance of individual mRNAs was determined by Northern blot hybridization followed by densitometry, as described previously (52). Equivalent quantities of RNA from cells under each condition were applied to the lanes. N-*ras* mRNA was measured using a 665 base-pair Sal I/Eco RI fragment from a human N-*ras* cDNA containing exons II, III and IV (57, 58). DNA probes were labeled with ^{32}P by the method of Feinberg and Vogelstein (10).

Results

N-ras Reversibly Inhibits Myoblast Fusion

When maintained at subconfluent densities in DME with 20% FCS, C2 myoblasts proliferate and exhibit none of the biochemical or morphological manifestations of the myogenic phenotype (25, 40, 41, 60). The onset of differentiation, after transfer of cultures at 80% confluence to medium with 10% HS (fusion-promoting medium), is marked by the appearance of multinucleate myotubes and the accumulation of muscle-specific gene products such as MCK and the ACh receptor.

Constitutive expression of transforming *ras* genes completely inhibits fusion and induction of muscle-specific gene products in C2 cells (41). To extend our understanding of the mechanisms involved in *ras*-dependent inhibition of myogenesis, C2 cells were transfected with a human N-*ras* gene, activated by a missense mutation at position 61 (4). Transcription of N-*ras* was controlled by the MMTV-LTR, rendering the gene inducible by dexamethasone. *ras* genes were cotransfected with the selectable marker pSV2*neo*, which confers resistance to the neomycin analog, G418. Colonies arising from the transfection were screened initially by adding fusion-promoting medium containing 2 μM dexamethasone. Of a total of 26 independent colonies examined, 10 failed to form myotubes when exposed to dexamethasone under fusion-promoting conditions. These dexamethasone-responsive colonies were isolated and passaged into stable cell lines. Subsequent analysis revealed that seven of the cell lines fused normally in the absence of dexamethasone but remained as mononucleate cells in the presence of dexamethasone. The remaining three of the clones were either partially or completely differentiation-defective.

In our previous studies using constitutively expressed *ras* oncogenes, we found that the effects of *ras* were dominant and that virtually all clones bearing mutant *ras* genes were unable to differentiate (41). Therefore, in the present study, we limited our analysis to clones that showed dose-dependent inhibition of morphological differentiation in response to dexamethasone. Clones that differentiated in the presence of dexamethasone were considered to lack functional steroid-inducible *ras* genes, whereas clones that never differentiated were assumed to express the transfected *ras* oncogene constitutively or to represent clones that originated from differentiation-defective cells in the original population.

Fig. 1 shows the morphology of a representative clonal cell line, designated CO25, bearing the steroid-inducible N-*ras* oncogene, under a series of culture conditions. In growth medium, CO25 cells were morphologically indistinguishable from nontransfected C2 myoblasts (data not shown). Transfer of cultures at 80% confluence to fusion-promoting medium led to formation of myotubes with kinetics identical to normal C2 cells (Fig. 1 *A*). In contrast, addition of 100–2,000 nM dexamethasone to fusion-promoting medium resulted in a complete block in the ability of CO25 myoblasts to form myotubes (Fig. 1 *B*). The inhibitory effects of dexa-

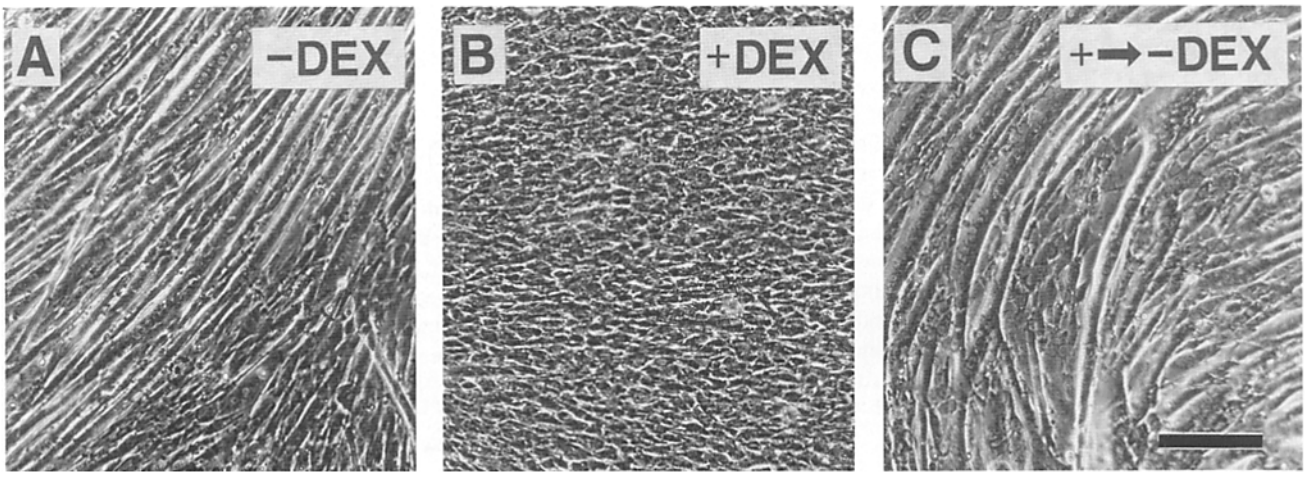


Figure 1. Effects of dexamethasone on morphology of myoblasts bearing steroid-inducible N-*ras* oncogenes. The CO25 cell line, bearing the steroid-inducible N-*ras* oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency, cultures were transferred to fusion-promoting medium for 5 d (*A*). A separate set of cultures was treated identically but was exposed to 500 nM dexamethasone 48 h before transfer to fusion-promoting medium containing dexamethasone (*B*). After 3 d in fusion-promoting medium containing dexamethasone, cultures shown in Panel B were transferred to fresh medium without dexamethasone for 3 d (*C*).

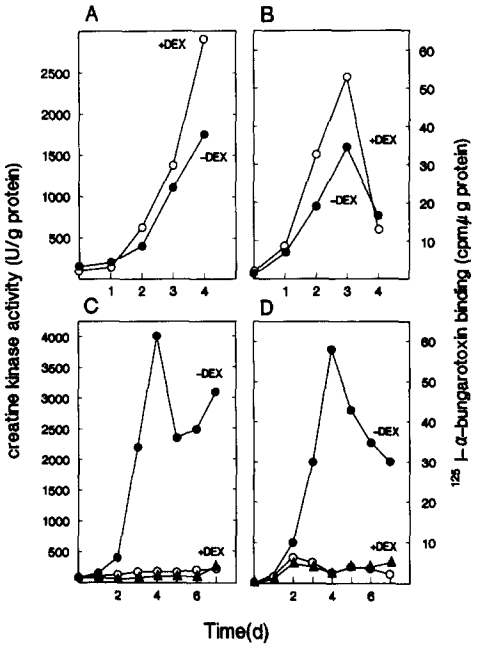


Figure 2. Expression of CK activity and ACh receptors in normal myoblasts and in myoblasts bearing steroid-inducible N-*ras* oncogenes. Normal C2 cells (*A* and *B*) or CO25 cells (*C* and *D*), bearing the steroid-inducible N-*ras* oncogene, were cultured in DME with 20% FCS. After achieving 80% confluency (day 0), cultures were transferred to fusion-promoting medium in the presence (○) or absence (●) of 500 nM dexamethasone. A separate set of CO25 cultures was exposed to 500 nM dexamethasone for 48 h before exposure to fusion-promoting medium containing dexamethasone (▲). CK activity and ACh receptors were assayed on consecutive days.

methasone on fusion were not observed in normal C2 myoblasts (data not shown). Maintenance of CO25 cells in fusion-promoting medium with dexamethasone for up to 7 d failed to result in detectable formation of multinucleate myotubes, indicating that dexamethasone inhibited rather than delayed the morphological alterations associated with terminal differentiation. In the presence of dexamethasone at concentrations lower than 100 nm, intermediate levels of myoblast fusion were observed (data not shown).

To determine whether the inhibitory effects of dexamethasone on morphological differentiation were reversible, fusion-blocked CO25 myoblasts, maintained in the presence of fusion-promoting medium with dexamethasone for 3 d, were transferred to fusion-promoting medium without dexamethasone. As shown in Fig. 1 *C*, these cultures formed large branching myotubes within 3 d after removal of dexamethasone. Thus, the inhibitory effects of dexamethasone on morphological differentiation of myoblasts bearing steroid-inducible *ras* oncogenes were fully reversible and required the continual presence of the steroid in the culture medium.

Transforming N-ras Oncogenes Inhibit Muscle-specific Gene Expression

To determine whether muscle-specific genes in CO25 cells were subject to regulation by dexamethasone, undifferentiated cultures in growth medium were exposed to 500 nM dexamethasone for 48 h before transfer to fusion-promoting medium, or were transferred directly to fusion-promoting medium containing the steroid. In the absence of dexamethasone, the muscle-specific gene products, CK and ACh receptor, were induced in a similar manner in normal C2 cells and in CO25 cells (Fig. 2). In the presence of dexamethasone, however, induction of CK and ACh receptors was inhibited dramatically in CO25 cells. Suppression of differentiation by dexamethasone occurred rapidly as demonstrated by the nearly complete inhibition of expression of muscle-specific gene products in cultures exposed to fusion-promoting medium with dexamethasone without pretreatment with the steroid (Fig. 2, *C* and *D*). Induction of CK activity and ACh receptors in C2 myoblasts was not inhibited by dexamethasone (Fig. 2, *A* and *B*). Together, these results demonstrate that the morphological and biochemical events associated with myogenesis become sensitive to inhibition by dexamethasone after transfection of myoblasts with a steroid-inducible *ras* oncogene.

Time Course for Induction of N-ras Protein and mRNA by Dexamethasone

The ability of dexamethasone to inhibit differentiation of CO25 myoblasts with no apparent lag (Fig. 2) suggested that the N-*ras* protein accumulated relatively rapidly in response to steroid induction. To determine the kinetics for *ras* induction, CO25 myoblasts were exposed to 500 nM dexamethasone for a series of times, cell extracts were prepared, and levels of p21 *ras* protein were quantitated by Western blot analysis using the anti-*ras* monoclonal antibody, 259 (15). In the absence of dexamethasone, a 21-kD polypeptide that comigrated with purified *ras* protein, was evident in CO25 cells (Fig. 3 *A*) and in C2 myoblasts (data not shown). Because the antibody used for these studies recognizes the endogenous proto-oncogenic forms of H-*ras* and N-*ras* from mouse, we conclude that this band represents one or both of these normal *ras* proteins. We do not know the identity of the polypeptide of higher molecular weight that is sometimes detected by the anti-*ras* antibody. After addition of dexamethasone to CO25 cells, the p21 polypeptide began accumulating ~3 to 5 h after exposure of cells to the steroid and continued to increase over a period of 48 to 60 h. The maximum steady state level achieved by exogenous p21 *ras* was four- to fivefold higher than the level of the endogenous *ras* proteins (Fig. 3, *A* and *B*).

The kinetics for induction of N-*ras* mRNA also were determined by Northern blot hybridization using a ^{32}P-labeled human N-*ras* cDNA. The hybridization probe used for this experiment hybridizes only to human N-*ras* transcripts and therefore permits accurate measurement of exogenous N-*ras* mRNA expression without complications from the endogenous proto-oncogenic mRNA species. As shown in Fig. 3 *C* this probe hybridized to an mRNA species of 2.2 kb, corresponding to exogenous N-*ras* mRNA (21, 35). The kinetics of induction of N-*ras* mRNA were similar to those of p21 N-*ras* protein. The pattern of expression of N-*ras* mRNA in response to dexamethasone was similar in myoblasts growing in 20% FCS (Fig. 3 *C*) and in differentiating cultures after 2 d in 10% HS (data not shown).

It is of interest to note that steroid induction of N-*ras* via the MMTV-LTR occurred with delayed kinetics in C2 cells compared with previous reports in 3T3 cells (26, 43). We also observed little or no feed-back inhibition of *ras* expression as was observed in 3T3 cells transfected with MMTV-

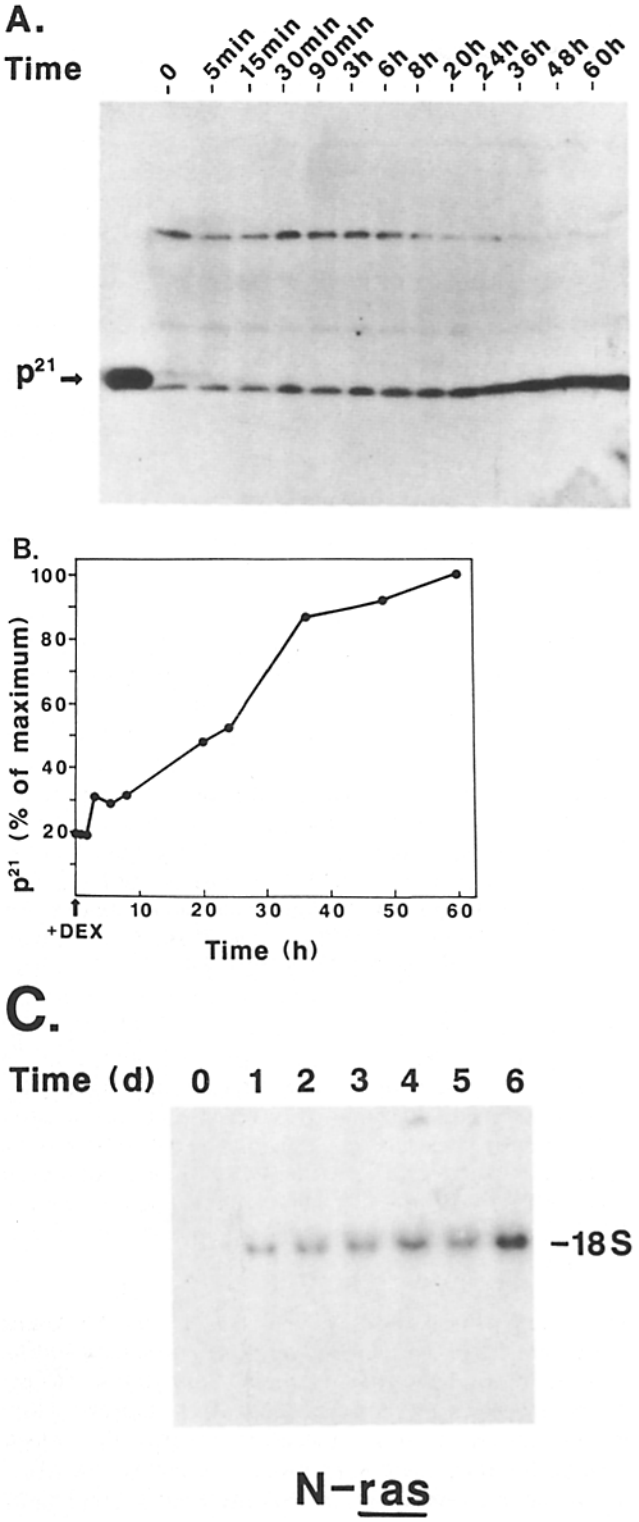


Figure 3. Induction of N-*ras* protein and mRNA by dexamethasone in myoblasts bearing steroid-inducible N-*ras* oncogenes. The CO25 cell line, bearing the steroid-inducible N-*ras* oncogene, was cultured in DME with 20% FCS. 500 nM dexamethasone was added to the cultures at time 0 and cells were harvested at the indicated times after exposure to the steroid. (*A*) levels of $p21^{N\text{-}ras}$ were determined by Western blot analysis using anti-p21 antibody, 259, as described in Materials and Methods. Purified p21 *ras* protein was used as a standard in the left lane. (*B*) levels of N-*ras* protein from *A* were quantitated by densitometry of the autoradiographs. (*C*) Levels of exogenous N-*ras* mRNA were determined by Northern blot analysis at the indicated times. The position of 18 S ribosomal RNA is indicated.

LTR-driven H-*ras* genes. The molecular basis for the contrasting pattern of expression of N-*ras* in C2 myoblasts is unclear, but may reflect a lower level of glucocorticoid receptors in these cells, although this has not yet been tested.

Dose-dependent Induction of N-ras and Inhibition of Differentiation by Dexamethasone

It was of interest to correlate directly the levels of N-*ras* expression with the extent of differentiation and thereby determine the minimum level of *ras* required to completely extinguish the differentiated phenotype. The dose dependence for inhibition of differentiation of CO25 cells by dexamethasone is shown in Fig. 4 *A*. Partial inhibition of CK expression was observed at concentrations of dexamethasone as low as 5 nM. The ID50 for dexamethasone was ~7 nM, with maximum inhibition occurring at 100 nM. These results indicate that *ras* does not inhibit myogenesis through an absolute all-or-none event. Instead, it appears that the effects of *ras* occur in a graded manner through a concentration-dependent mechanism.

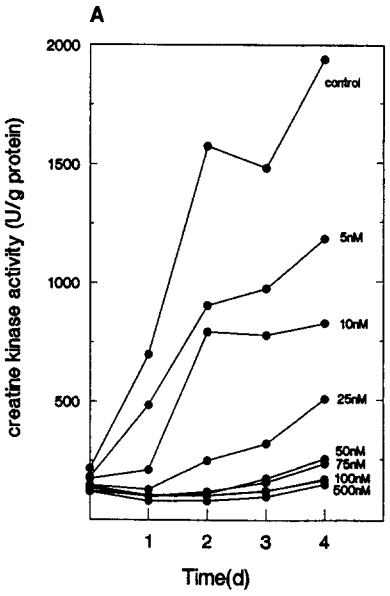
The dose-dependence on dexamethasone of N-*ras* mRNA induction was also analyzed in CO25 cultures exposed to varying concentrations of the steroid for 72 h. As shown in Fig. 4 *B*, N-*ras* mRNA was detectable in the presence of dexamethasone at concentrations as low as 5 nM. Maximal levels of N-*ras* mRNA expression occurred at 25 to 100 nM dexamethasone. Comparison of the dependence on dexamethasone of N-*ras* mRNA accumulation with the extent of inhibition of differentiation, showed an inverse relationship (Fig. 4 *C*); the ED_{50} for both processes was 7 nM with maximal responses occurring between 25 and 100 nM.

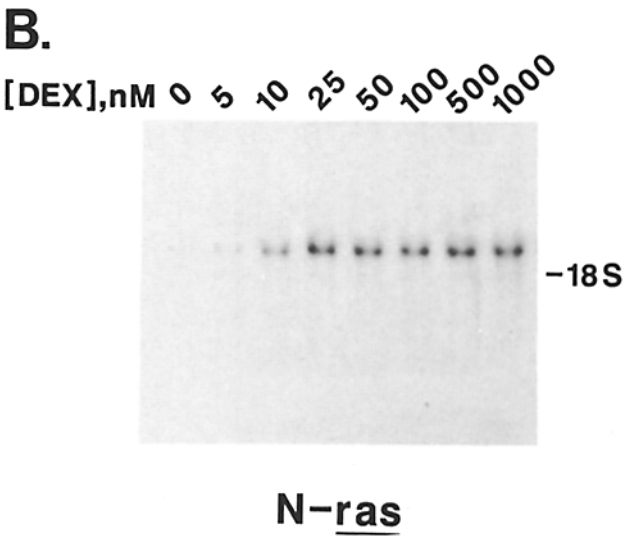
Reversibility of Ras-dependent Inhibition of Differentiation

To determine whether the inhibitory effects of *ras* on muscle-specific gene expression were reversible, CO25 cells were maintained in fusion-promoting medium containing 500 nM dexamethasone for 3 d and were subsequently transferred to fresh fusion-promoting medium lacking the steroid. As shown in Fig. 5, removal of dexamethasone from differentiation-inhibited CO25 cells led to appearance of CK and ACh receptors with kinetics indistinguishable from those of the initial induction of these muscle gene products in C2 cells or in non-dexamethasone-treated CO25 cells. These results indicate that the inhibitory effects of *ras* on muscle-specific gene expression are readily reversible and that prolonged expression of *ras* in myoblasts neither accelerates nor delays subsequent induction of muscle-specific gene products after release of myoblasts from *ras*-dependent inhibition.

Terminal Differentiation After Release from ras-dependent Repression Does Not Require a Round of DNA Synthesis

It was of interest to determine whether CO25 myoblasts, released from *ras*-dependent inhibition of differentiation by removal of dexamethasone, required a round of cell division to be reprogrammed before induction of the differentiated phenotype. To address this question, we tested whether inhibition of DNA synthesis interfered with differentiation of CO25 cells after transfer from fusion-promoting medium containing dexamethasone to fresh medium lacking the ste-





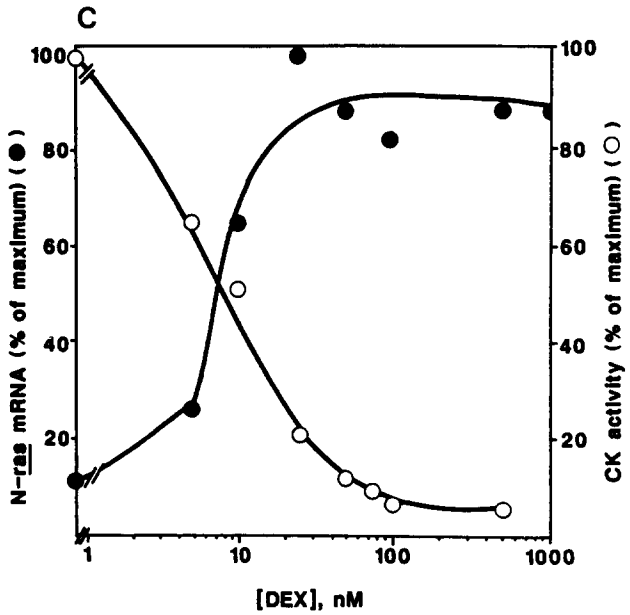


Figure 4. Dose-dependent inhibition of differentiation and induction of N-*ras* mRNA by dexamethasone. The CO25 cell line, bearing the steroid-inducible N-*ras* oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency (day 0), cultures were transferred to fusion-promoting medium containing a range of concentrations of dexamethasone. (*A*) CK activity was assayed on consecutive days after exposure to the steroid. (*B*) Levels of exogenous N-*ras* mRNA were determined by Northern blot analysis after exposure to fusion-promoting media containing dexamethasone for 72 h. (*C*) Levels of N-*ras* mRNA expression in response to dexamethasone were determined by densitometry of the Northern blot shown in *B*. CK activity after exposure of CO25 cells to dexamethasone for 72 h was obtained from *A*. Levels of N-*ras* mRNA are expressed relative to the level in the presence of 25 nM dexamethasone, which was assigned a value of 100%. Levels of CK activity are expressed relative to the level in control cultures without dexamethasone, which was assigned a value of 100%.

roid. In the presence of 5 μg/ml aphidicolin, which inhibits DNA synthesis, fusion (data not shown) and induction of CK activity occurred normally after removal of dexamethasone from differentiation-inhibited cultures (Fig. 6). Similar results were obtained in the presence of 0.1 mM cytosine arabinoside, which eliminates replicating cells (data not shown). It can be concluded, therefore, that a round of DNA synthesis is not required for myoblasts to overcome the inhibitory effects of *ras* on differentiation.

The N-ras Oncogene Does Not Abrogate the Requirement for Growth Factors to Divide

Differentiation of skeletal myoblasts depends absolutely upon cessation of cell division. Because transforming *ras* genes have been reported to release some cell types from their dependence on exogenous growth factors to divide, it was important to determine whether the failure of CO25 cells to differentiate in the presence of dexamethasone might be attributable to continued proliferation of these cells in fusion-promoting medium. As shown in Fig. 7, neither the rate of cell division in 20% FCS nor the saturation density achieved by CO25 cells were affected appreciably by dexamethasone. Moreover, dexamethasone-treated CO25 cells withdrew from the cell cycle at subconfluent densities in medium with 0.5% FCS in a manner identical to C2 myoblasts. These experiments were performed in medium containing 0.5% FCS rather than 10% HS because HS contains a low level of mitogenic activity that supports a slow rate of growth of C2 myoblasts at low densities. To determine whether CO25 cells were able to stop dividing completely in the presence of dexamethasone it was necessary to analyze their growth rate under conditions in which there was no proliferation in control cultures. The inhibitory effects of

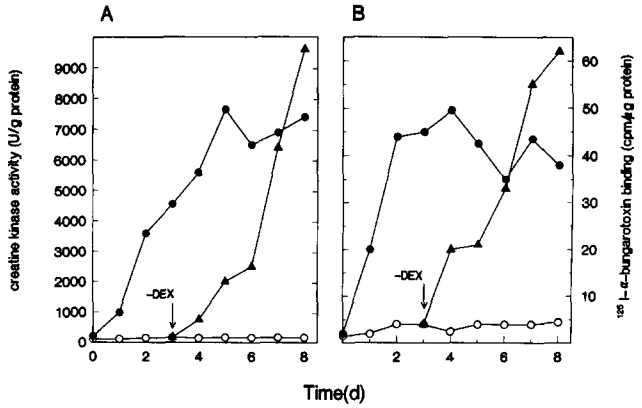


Figure 5. Reversibility of the differentiation-defective phenotype of myoblasts bearing steroid-inducible N-*ras* oncogenes. The CO25 cell line, bearing the steroid-inducible N-*ras* oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency (day 0), cultures were transferred to fusion-promoting medium in the presence (○) or absence (●) of 500 nM dexamethasone (*DEX*). Half of the cultures maintained in dexamethasone were transferred to fresh fusion-promoting medium without dexamethasone on day 3 (▲). CK activity and ACh receptors were assayed on consecutive days.

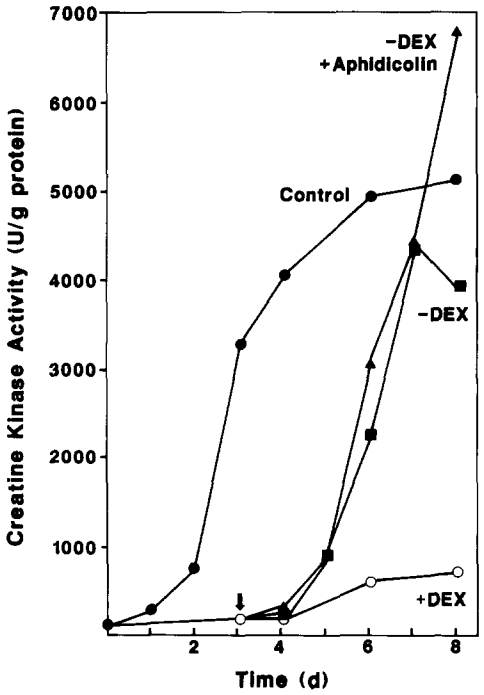


Figure 6. Effects of inhibitors of DNA synthesis on induction of CK activity following removal of dexamethasone from myoblasts bearing steroid-inducible N-*ras* oncogene. The CO25 cell line, bearing the steroid-inducible N-*ras* oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency, cultures were transferred to fusion-promoting medium in the presence (○) or absence (●) of 500 nM dexamethasone (*DEX*). After 3 d, dexamethasone-treated cultures were transferred to fresh fusion-promoting medium lacking the steroid, but containing 5 mM aphidicolin (▲) or no addition (■). CK activity was determined on consecutive days.

dexamethasone on differentiation of CO25 cells were also observed in the presence of 0.5% FCS (data not shown). We conclude that dexamethasone-dependent inhibition of differentiation of CO25 cells occurs through a mechanism independent of continuous cell proliferation. These results do not rule out the possibility, however, that *ras* may confer subtle alterations in sensitivity to specific growth factors that would not be detectable by the assays employed here.

ras Does Not Inhibit Myogenic Differentiation through An Autocrine Mechanism

The ability of *ras* oncogene proteins to inhibit myogenic differentiation could be due to direct activation by *ras* of an intracellular pathway that interferes with muscle-specific gene expression. Alternatively, *ras* could prevent myogenesis indirectly through an autocrine mechanism involving release of an inhibitory growth factor. The latter possibility is particularly relevant, in light of reports that some *ras*-transformed cell types secrete transforming growth factors (1, 54), which are known to inhibit myogenesis (14, 33, 40). To distinguish between these possibilities, normal C2 cells and CO25 cells were cultured on opposite sides of a barrier that allowed free exchange of medium between the two cell types. Cultures were maintained in growth medium containing 2 μM dexamethasone for 48 h and were subsequently transferred to fusion-promoting medium with the steroid for 3 d. Cells were then harvested separately from each side of the permeable barrier and CK activity was assayed. As shown in Fig. 8, differentiation of C2 cells was not affected significantly by the presence of medium conditioned by dexamethasone-treated CO25 cells. These results argue against the involvement of autocrine factors in *ras*-dependent inhibition of differentiation and suggest that *ras* activates an intracellular cascade that interferes directly with the differentiation program.

Induction of N-ras in Terminally Differentiated Myotubes Does Not Extinguish Muscle-specific Gene Expression

The ability to modulate N-*ras* expression in CO25 cells with dexamethasone made it possible to investigate whether in-

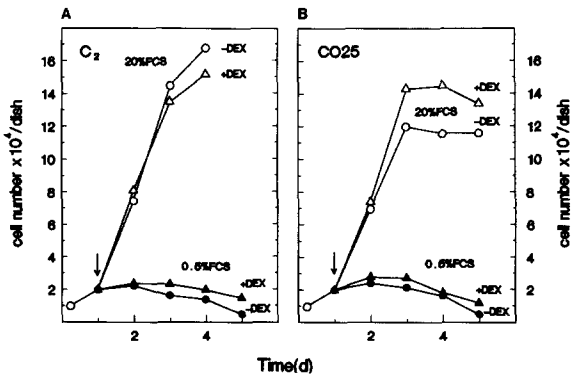


Figure 7. Effects of dexamethasone on proliferation of myoblasts bearing steroid-inducible N-*ras* oncogenes. Normal C2 cells (*A*) or CO25 cells (*B*), bearing the steroid-inducible N-*ras* oncogene, were cultured in DME with 20% FCS. On day 1, cultures were exposed to DME with 20% or 0.5% FCS in the presence (△) or absence (○) of 500 nM dexamethasone (*DEX*), as specified. Cell numbers were determined on consecutive days using a Coulter Counter. (*Open symbols*), 20% FCS; (*closed symbols*), 0.5% FCS.

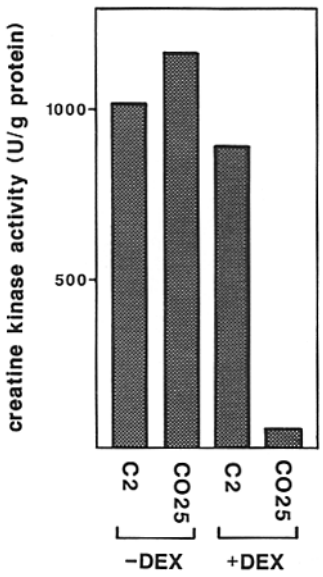


Figure 8. Effects of medium conditioned by myoblasts bearing steroid inducible N-*ras* oncogenes on differentiation of normal C2 cells. Normal C2 cells and CO25 cells, bearing the steroid inducible N-*ras* oncogene, were plated at equivalent densities in 35-mm culture dishes on opposite sides of a permeable barrier. Cultures were maintained in DME with 20% FCS and 2 μM dexamethasone (*DEX*). After achieving 80% confluency, cultures were transferred to fusion-promoting medium and dexamethasone. 3 d later cells were harvested separately from each half of the dish and CK activity was determined. 2 μM dexamethasone was used in these experiments to ensure maximum persistent expression of N-*ras* in CO25 cells throughout the course of the experiment. This high level of dexamethasone does not have adverse effects on cell viability.

duction of N-*ras* in terminally differentiated myotubes was sufficient to extinguish expression of muscle-specific genes. Preliminary experiments indicated that myotube cultures that contained a significant percentage of nuclei in unfused myocytes showed a decline in expression of CK activity and mRNA in response to dexamethasone. We believe this inhibitory response may reflect the down-regulation of muscle-specific gene products in partially differentiated mononucleate cells, since fusion is not a prerequisite for muscle-specific gene expression in C2 cells (Hu, J. S., and E. N. Olson, unpublished results). Our primary purpose was to examine whether muscle-specific genes in terminally differentiated myotubes were susceptible to *ras*-dependent repression. Therefore, to obtain cultures of pure myotubes, CO25 cells were cultured in fusion-promoting medium for 2 d, at which time large, branching myotubes were clearly visible. Nonfused myocytes were then eliminated from the cultures by stimulation with growth medium containing 20% FCS and 0.1 mM cytosine arabinoside for 2 d. Pure cultures of terminally differentiated myotubes were exposed subsequently to 1 μM dexamethasone and CK activity was assayed at 24-h intervals after addition of the steroid. Under these conditions, CK activity (Fig. 9) and MCK mRNA (data not shown) continued to increase after addition of dexamethasone to cultures of CO25 myotubes. The morphology of CO25 myotubes also remained unchanged in the presence of dexamethasone.

The failure of *ras* to suppress muscle-specific gene expression in myotubes cannot be attributed to a loss of responsiveness of the transfected *ras* gene to steroids because N-*ras* mRNA was induced in myotubes in response to dexamethasone (Fig. 10). We did observe, however, that 1,000 nM dexamethasone was required to induce *ras* expression in myotubes to levels comparable to that induced in myoblasts by 100 nM dexamethasone (data not shown). Together these results demonstrate muscle-specific genes are susceptible to *ras*-dependent repression during the early stages of the differentiation process. However, in pure cultures of terminally differentiated myotubes muscle-specific genes appear to become refractory to the inhibitory signals generated by transforming *ras* proteins.

Discussion

The transition of certain types of myoblasts to terminally differentiated myotubes has been shown previously to be inhibited by TGFβ and FGF through a mechanism independent of cell proliferation (6, 14, 28, 29, 33, 40, 52). Inhibition of fusion and muscle-specific gene expression requires the continual presence of these growth factors in the extracellular milieu and is rapidly reversible after their removal. These results are consistent with the notion that inhibition of myogenesis by TGFβ and FGF requires continuous occupancy of their respective cell surface receptors and involves an intracellular signal (or signals) that is extremely short-lived.

In an effort to identify the intracellular signalling pathway(s) used by TGFβ and FGF to regulate myogenesis, we have introduced a series of mutationally activated *ras* oncogenes into myoblasts and have investigated whether the proteins encoded by these oncogenes might confer a phenotype on myoblasts similar to that elicited by TGFβ and FGF. *ras* oncogenes were selected for these studies because *ras* proteins are localized to the plasma membrane where they are believed to function at an early step in intracellular growth factor cascades by transducing signals between specific cell surface growth factor receptors and intracellular enzyme effectors (2, 11, 27, 30, 35, 37, 55, 59). Recently, we reported that transfection of C2 cells with mutationally activated N- or H-*ras* oncogenes, under constitutive transcriptional control, completely inhibited the molecular events associated with myogenic differentiation (41). Similar inhibition of differentiation by *ras* was observed in the BC3H1 muscle cell line (5, 46, Klein, D. J., and A. Connally, personal communication).

In the present study, we have extended these previous observations by constructing myoblast cell lines in which the entire developmental program is subject to negative control

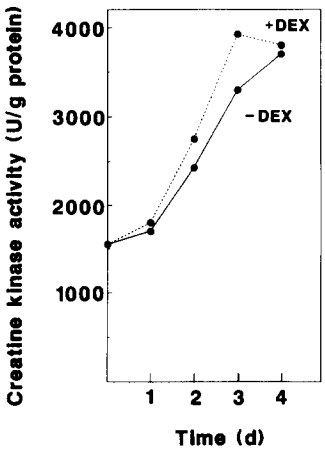


Figure 9. Effects of dexamethasone on CK activity in terminally differentiated myotubes. CO25 cells were allowed to achieve 80% confluency and were transferred to medium with 10% HS for 48 h. Cultures were then fed with medium containing 20% FCS and 0.1 mM cytosine arabinoside to eliminate unfused cells. After 48 h in cytosine arabinoside, half of the cultures were exposed to 1 μM dexamethasone and CK activity was determined on consecutive days. Time 0 on the graph represents the time in which dexamethasone was added to cultures of pure myotubes which had initiated differentiation 4 d earlier.

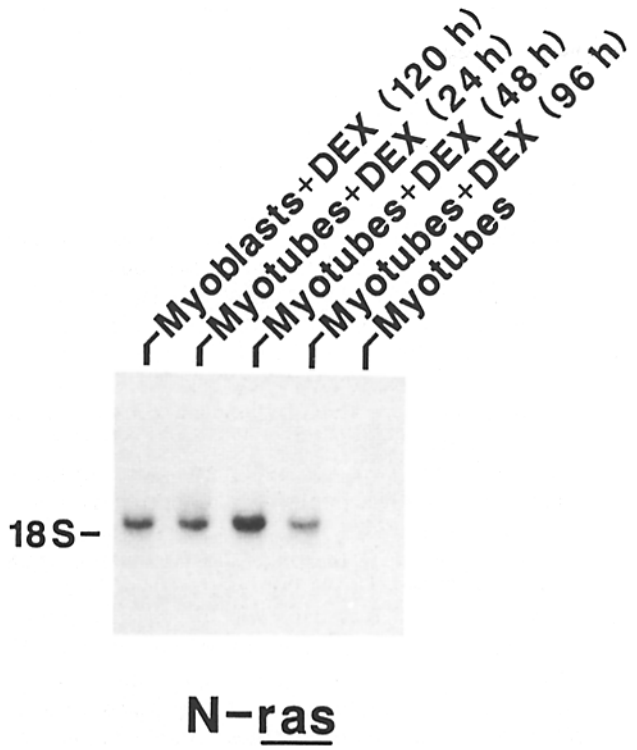


Figure 10. Effects of dexamethasone on expression of N-*ras* mRNA in terminally differentiated myotubes. CO25 cells were allowed to achieve 80% confluency and were transferred to medium with 10% HS for 48 h. Cultures were then fed with medium containing 20% FCS and 0.1 mM cytosine arabinoside to eliminate unfused cells. Dexamethasone (1 μM) was also added to the cultures at the time of addition of cytosine arabinoside. At the indicated times, RNA was isolated and steady state levels of N-*ras* mRNA were determined by Northern blot analysis. Levels of N-*ras* mRNA in myoblasts exposed to 1 μM dexamethasone and in myotubes in the absence of the steroid are also shown.

by dexamethasone. These myoblast cell lines, bearing steroid-inducible N-*ras* oncogenes, permit cell cycle withdrawal to be reversibly dissociated from muscle-specific gene induction and allow fine-tuning of the differentiated phenotype in a manner not previously possible. The precise control over the differentiation program afforded by these cell lines should facilitate detailed analysis of the mechanisms whereby growth factor signalling cascades impinge on muscle-specific genes.

The results of the present study extend our understanding of the mechanism for *ras*-dependent regulation of myogenesis in at least four important respects. First, suppression of differentiation of myoblasts bearing steroid-inducible *ras* oncogenes was demonstrated to be rapidly reversible after withdrawal of dexamethasone. This observation indicates that the *ras*-dependent phenotype requires the continual presence of *ras* to be maintained and implies that interference with the differentiation program by *ras* proteins requires the persistent transduction of highly transient intracellular signals. Second, the degree of differentiation of myoblasts bearing steroid-inducible *ras* genes was a titratable function of N-*ras* expression with partial inhibition of fusion and induction of muscle-specific genes occurring at low levels of *ras*. These results indicate the *ras* does not exert an all-or-none effect on the differentiation program, but rather, elicits a graded response proportional to the level of *ras* expression. Third, after release of myoblasts from *ras*-dependent inhibition, differentiation proceeded with normal kinetics, suggesting that *ras* arrests myoblasts at an early, rather than late step in the pathway toward differentiation. Induction of differentiation after release from *ras*-dependent inhibition also was shown to occur in the absence of an additional round of DNA synthesis, indicating that myoblasts do not need to be reprogrammed during a quantal cell cycle to overcome the differentiation-defective phenotype conferred by *ras*. Finally, induction of N-*ras* failed to down-regulate muscle-specific genes in terminally differentiated myotubes. In each of these respects, the effects of oncogenic *ras* proteins on myogenesis resemble the effects of TGFβ and FGF.

Several transformed cell types have been reported to secrete transforming growth factors (1, 54). Therefore, to test the possibility that *ras*-dependent inhibition of myogenesis might be mediated by an autocrine growth factor similar, or identical, to TGFβ or FGF, the ability of *ras*-transfected myoblasts to transfer their phenotype to normal myoblasts through culture medium was examined. No evidence for autocrine interactions between *ras*-transformed and normal myoblasts was detectable. Thus, we conclude that oncogenic *ras* proteins directly transduce an intracellular signal that operates in an antagonistic manner to the normal developmental signals that initiate myogenesis. It should be emphasized that *ras* proteins do not function directly as muscle-specific gene repressors. Instead, these proteins most likely activate an intracellular pathway that culminates in repression of the differentiation program.

What type of mechanisms might be responsible for *ras*-dependent inhibition of myogenic differentiation and how many intermediate steps may be involved? *ras* proteins are localized to the plasma membrane where they are postulated to function at an early step in the pathway for transduction of intracellular growth factor signals. Mutationally activated *ras* proteins have been reported to substitute for exogenous growth factors and to persistently activate intracellular signalling pathways that may normally be modulated by proto-oncogenic *ras* proteins (30). In light of the similarities between the effects of TGFβ, FGF and *ras* on myogenesis, it is tempting to speculate that signalling pathways similar or identical to those activated by TGFβ and FGF might be amplified in myoblasts bearing oncogenic *ras* genes. In this regard, several of the early events associated with growth factor receptor occupancy have been shown to be activated by oncogenic *ras* proteins. For example, microinjection of fibroblasts with oncogenic *ras* proteins or transfection with *ras* genes leads to increased activity of phospholipases A_2 and C, which modulate arachidonate release and phosphatidylinositol turnover, respectively (2, 13, 47, 59). *ras* oncogene proteins also have been reported to lead to an increase in intracellular pH, due to stimulation of the amiloride-sensitive Na^+/H^+ exchange (20). Finally, *ras* transformed cells have been demonstrated to exhibit increased rates of glycolysis and hexose transport which may contribute to a generalized alteration in cellular metabolism (48). The potential involvement of one or more of these biochemical events in *ras*-dependent repression of myogenesis is currently under investigation.

Terminal differentiation of mouse MM14 myoblasts has been shown by Hauschka and coworkers to be preceded by down-regulation of cell surface EGF and FGF receptors (31, 42). A similar pattern of regulation has been observed for TGFβ receptors during fusion of C2 and L6A1 myoblasts (8). This loss of cell surface growth factor receptors has been postulated as a mechanism to account for the inability of growth factors to suppress muscle-specific gene expression in myotubes. Because *ras* proteins are believed to function at a post-receptor step in growth factor cascades, it was possible to use CO25 cells to investigate whether muscle-specific genes in myotubes remained susceptible to repression by intracellular growth signals. The results reported here, which show that pure myotubes are insensitive to signals generated by *ras* proteins, suggest that irreversible activation of muscle-specific genes in myotubes may not result exclusively from down-regulation of growth factor receptors. The nature of the molecular events that place muscle-specific gene expression in myotubes beyond the control of *ras* remain to be established.

The consequences of expression of oncogenes encoding proteins presumed to function at early steps in intracellular growth factor cascades have been examined previously in cultures of primary myoblasts (9, 23, 24, 36). These studies demonstrated that v-*src* and v-*erb*B, which encode tyrosine protein kinases, prevent the molecular events associated with myogenesis. Like the effects of *ras* reported here, these oncogene products prevented differentiation through a mechanism independent of cell proliferation. It remains to be determined whether tyrosine kinases and mutationally activated *ras* proteins function through the same, or through parallel pathways to elicit their effects on the differentiation program.

Myoblast cell lines, modified by incorporation of exogenous oncogenes, offer the opportunity to dissect the mechanisms whereby intracellular signals generated at the cell surface culminate in alterations of gene expression. In the future, it will be particularly interesting to determine whether *ras* functions in the same intracellular pathway activated by TGFβ and FGF or whether these regulators of myogenesis utilize separate pathways that converge at a downstream step that governs the differentiation program.

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References

1. Anzano, M. A., A. B. Roberts, J. E. DeLarco, L. M. Wakefield, R. K. Assoian, N. S. Roche, J. N. Smith, J. E. Lazarus, and M. B. Sporn. 1985. Increased secretion of type β transforming growth factor accompanies viral transformation of cells. *Mol. Cell Biol.* 5:242–247.
2. Bar-Sagi, D., and J. Feramisco. 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. *Science (Wash. DC).* 233:1061–1068.
3. Bishop, J. M. 1985. Viral Oncogenes. *Cell.* 42:23–38.
4. Brown, R., C. J. Marshall, S. G. Pennie and A. Hall. 1984. Mechanism of activation of an N-*ras* gene in the human fibrosarcoma cell line HT1080. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1321–1326.
5. Caffrey, J. M., A. M. Brown, and M. D. Schneider. 1987. Mitogens and oncogenes can block the formation of specific voltage-gated ion channels. *Science (Wash. DC).* 236:570–574.
6. Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka. 1987. Growth factor control of skeletal muscle differentiation occurs in the G_1-phase and is repressed by fibroblast growth factor. *J. Cell. Biol.* 105:949–956.
7. Emerson, C., D. Fischman, B. Nadal-Ginard, and M. A. Q. Siddiqui, eds. 1986. Molecular Biology of Muscle Development. Alan R. Liss, New York. 957 pp.
8. Ewton, D. Z., G. Spizz, E. N. Olson, and J. R. Florini. 1988. Decrease in transforming growth factor-beta binding and action during differentiation in muscle cells. *J. Biol. Chem.* 263:4029–4032.
9. Falcone, G., F. Tato, and S. Alema. 1985. Distinctive effects of the viral oncogenes myc, erb, fps, and src on the differentiation program of quail myogenic cells. *Proc. Natl. Acad. Sci. USA.* 82:426–430.
10. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA endonuclease fragments to a high specific activity. *Anal. Biochem.* 132:6–13.
11. Feramisco, J. R., M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet. 1984. Microinjection of the oncogene form of the human H-*ras* (T-24) protein results in rapid proliferation of quiescent cells. *Cell.* 38:109–117.
12. Finkel, T. R., C. J. Der, and G. M. Cooper. 1984. Activation of *ras* genes in human tumors does not affect subcellular localization, post-translational modification, or guanine nucleotide binding properties of p21. *Cell.* 37:151–158.
13. Fleischman, L. F., S. B. Chahwala, and L. Cantley. 1986. Ras-transformed cells: Altered levels of phosphatidylinositol-4:5-biphosphate and catabolites. *Science (Wash. DC).* 231:407–410.
14. Florini, J. R., A. B. Roberts, D. Z. Ewton, S. L. Falen, K. C. Flanders, and M. B. Sporn. 1986. Transforming growth factor-β. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. *J. Biol. Chem.* 261:16509–16513.
15. Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick. 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and the cellular *ras* gene family. *J. Virol.* 43: 294–304.
16. Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick. 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. *Proc. Natl. Acad. Sci. USA.* 81:5704–5708.
17. Gilman, A. G. 1984. G-proteins and dual control of adenylate cyclase. *Cell.* 36:577–579.
18. Gospodarowicz, D. J., J. Weseman, J. S. Moran, and J. Lindstrom. 1976. Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. *J. Cell Biol.* 70:395–405.
19. Graham, F. L., and A. J. Vander Eb. 1973. A new technique for the assay of infectivity of human adenovirus DNA. *Virology.* 52:456–467.
20. Hagag, N., J. C. Lacal, M. Graber, S. Aaronson, and M. V. Viola. 1987. Microinjection of *ras* p21 induces a rapid rise in intracellular pH. *Mol. Cell. Biol.* 7:1984–1988.
21. Hall, A., and R. Brown. 1985. Human N-ras:cDNA cloning and gene structure. *Nucl. Acids Res.* 13:5255–5268.
22. Heldin, C.-H., and B. Westermark. 1984. Growth factors: mechanism of action and relation to oncogenes. *Cell.* 37:9–20.
23. Holtzer, H., J. Biehl, G. Yoeh, R. Meganathan, and A. Kaji. 1975. Effect of oncogenic virus on muscle differentiation. *Proc. Natl. Acad. Sci. USA.* 72:4051–4055.
24. Hynes, R. O., G. S. Martin, M. Shearer, D. R. Critchley, and C. J. Epstein. 1976. Viral transformation of rat myoblasts, effects on fusion and surface properties. *Dev. Biol.* 48:35–46.
25. Inestrosa, N. C., J. B. Miller, L. Silberstein, L. Ziskind-Conhaim, and Z. Hall. 1983. Developmental regulation of 16 S acetylcholinesterase and acetylcholine receptors in a mouse muscle cell line. *Exp. Cell Res.* 147: 393–405.
26. Jaggi, R., B. Salmons, D. Muellener, and B. Groner. 1986. The v-*mos* and -*ras* oncogene expression represses glucocorticoid hormone-dependent transcription from the mouse mammary tumor virus LTR. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2609–2616.
27. Kamata, T., and J. R. Feramisco. 1984. Epidermal growth factor stimulates the guanine nucleotide binding activity and phosphorylation of *ras* oncogene proteins. *Nature (Lond.).* 310:147–150.
28. Lathrop, B. K., E. N. Olson, and L. Glaser. 1985. Control by fibroblast growth factor of differentiation in the BC_3H1 muscle cell line. *J. Cell Biol.* 100:1540–1547.
29. Lathrop, B. K., K. Thomas, and L. Glaser. 1985. Control of myogenic differentiation by fibroblast growth factor is mediated by position in the G1 phase of the cell cycle. *J. Cell Biol.* 101:2194–2198.
30. Levinson, A. D. 1986. Normal and activated *ras* oncogenes and their encoded products. *Trends in Genet.* 2:81–85.

31. Lim, R. W., and S. D. Hauschka. 1984. A rapid decrease in epidermal growth factor binding capacity accompanies the terminal differentiation of mouse myoblasts *In vitro J. Cell Biol.* 98:739–747.
32. Linkhart, T. A., C. H. Clegg, and S. D. Hauschka. 1980. Control of muscle myoblast commitment to terminal differentiation by mitogens. *J. Supramol. Struct.* 14:483–498.
33. Massagué, J., T. S. Cheifetz, T. Endo, and B. Nadal-Ginard. 1986. Type β transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. USA.* 83:8206–8210.
34. McGrath, J. P., D. J. Capon, D. V. Goeddel, and A. D. Levinson. 1984. Comparative biochemical properties of normal and activated human *ras* p21 protein. *Nature (Lond.).* 313:241–243.
35. McKay, I. A., C. J. Marshall, C. Cales, and A. Hall. 1986. Transformation and stimulation of DNA synthesis in NIH-3T3 cells are a titratable function of normal $p21^{N\text{-}ras}$ expression. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2617–2621.
36. Moss, P. S., N. Honeycutt, T. Pawson, and G. S. Martin. 1979. Viral transformation of chick myogenic cells. *Exp. Cell Res.* 123:95–105.
37. Mulcahy, L. S., M. R. Smith, and D. W. Stacey. 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature (Lond.).* 313:241–243.
38. Olson, E. N., K. C. Caldwell, J. I. Gordon, and L. Glaser. 1983. Regulation of creatine phosphokinase expression during differentiation of BC_3H1 cells. *J. Biol. Chem.* 258:2644–2652.
39. Olson, E. N., L. Glaser, J. P. Merlie, R. Sebbane, and J. Lindstrom. 1984. Regulation of surface expression of acetylcholine receptors in response to serum and cell growth in the BC_3H1 muscle cell line. *J. Biol. Chem.* 258:13936–13942.
40. Olson, E. N., E. Sternberg, J. S. Hu, G. Spizz, and C. Wilcox. 1986. Regulation of myogenic differentiation by type β transforming growth factor. *J. Cell Biol.* 103:1799–1805.
41. Olson, E. N., G. Spizz, and M. A. Tainsky. 1987. The oncogenic forms of N-*ras* or H-*ras* prevent skeletal myoblast differentiation. *Mol. Cell. Biol.* 7:2104–2111.
42. Olwin, B. B., and S. D. Hauschka. 1986. Identification of the fibroblast growth factor receptor and regulation during myogenic differentiation. *J. Cell Biol.* 103:1101*a*.
43. Owen, R. D., and M. C. Ostrowski. 1987. Rapid and selective alterations in the expression of cellular genes accompany conditional transcription of Ha-*ras* in NIH 3T3 cells. *Mol. Cell. Biol.* 7:2512–2520.
44. Palmiter, R. D. 1974. Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry.* 13:3606–3615.
45. Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg. 1982. Human EJ bladder carcinoma oncogene is a homologue of Harvey sarcoma virus *ras* gene. *Nature (Lond.).* 297:474–478.
46. Payne, P. A., E. N. Olson, P. Hsiau, R. Roberts, M. B. Perryman, and M. D. Schneider. 1987. An activated c-Ha-*ras* allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. *Proc. Natl. Acad. Sci. USA.* 84:8956–8960.
47. Preiss, J., C. R. Loomis, W. R. Bishop, R. Stein, J. E. Niedel, and R. M. Bell. 1986. Quantitative measurement of sn-1, 2,-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. *J. Biol. Chem.* 261:8597–8600.
48. Racker, E., R. J. Resnick, and R. Feldman. 1985. Glycolysis and methylaminoisobutyrate uptake in rat-1 cells transfected with *ras* or *myc* oncogenes. *Proc. Natl. Acad. Sci. USA.* 82:3535–3538.
49. Schneider, M. D., M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson. 1987. Autonomous expression of c-*myc* in BC3H1 cells partially inhibits but does not prevent myogenic differentiation. *Mol. Cell. Biol.* 7:1973–1977.
50. Schneider, M. D., and E. N. Olson. 1988. Control of myogenic differentiation by cellular oncogenes. *Mol. Neurobiol.* 2:1–39.
51. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV-40 early region promoter. *J. Mol. Appl. Genet.* 1:327–341.
52. Spizz, G., D. Roman, A. Strauss, and E. N. Olson. 1986. Serum and fibroblast growth factor inhibit myogenic differentiation through a mechanism dependent on protein synthesis and independent of cell proliferation. *J. Biol. Chem.* 261:9483–9488.
53. Spizz, G., J. S. Hu, and E. N. Olson, 1987. Inhibition of myogenic differentiation by fibroblast growth factor and type β transforming growth factor does not require persistent c-*myc* expression. *Dev. Biol.* 123: 500–507.
54. Stern, D. F., A. B. Roberts, N. S. Roche, M. B. Sporn, and R. A. Weinberg. 1986. Differential responsiveness of *myc*- and *ras*-transfected cells to growth factors: Selective stimulation of *myc*-transfected cells by epidermal growth factor. *Mol. Cell Biol.* 6:870–877.
55. Sullivan, N., W. Welch, R. Watt, D. Bar-Sagi, B. Ferguson, I. Chao, T. Kamata, R. Sweet, M. Gross, M. Rosenberg, and J. Feramisco. 1985. Microinjection of oncogene proteins causes DNA synthesis and proliferation in non-established cells. *Cancer Cells.* 3:243–259.
56. Sweet, R. W., S. Yokoyama, T. Kamata, J. R. Feramisco, M. Rosenberg, and M. Gross. 1984. The product of *ras* is a GTPase and the T24 oncogene mutant is deficient in this activity. *Nature (Lond.).* 311:273–275.
57. Tainsky, M. A., C. S. Cooper, B. C. Giovanella, and G. F. Vande Woude. 1984. An activated *ras*-N gene: detected in late but not early passage human PA1 teratocarcinoma cells. *Science (Wash. DC).* 225:643–645.
58. Taparowski, E., K. Shimizu, M. Goldfarb, and M. Wigler. 1983. Structure and activation of the human N-*ras* gene. *Cell.* 34:581–586.
59. Wakelam, M. J. O., S. A. Davies, M. D. Houslay, I. McKay, C. J. Marshall, and A. Hall. 1986. Normal $p21^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. *Nature (Lond.).* 323:173–176.
60. Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270: 725–727.