MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 1973–1977
0270-7306/87/051973-05$02.00/0

Vol. 7, No. 5

Autonomous Expression of c-*myc* in BC_3H1 Cells Partially Inhibits but Does Not Prevent Myogenic Differentiation

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Received 16 July 1986/Accepted 16 February 1987

Myogenic differentiation is obligatorily coupled to withdrawal of myoblasts from the cell cycle and is inhibited by specific polypeptide growth factors. To investigate the potential involvement of c-*myc* in the control of myogenesis, the BC_3H1 muscle cell line was stably transfected with a simian virus 40 promoter:c-*myc* chimeric gene. In quiescent cells in 0.5% serum, the exogenous c-*myc* gene was expressed at a level more than threefold greater than the level of endogenous c-*myc* in undifferentiated, proliferating cells of the parental line in 20% serum. The transfected *myc* gene partially inhibited the expression of both muscle creatine kinase and the nicotinic acetylcholine receptor, but was not sufficient to prevent the induction of these muscle differentiation products upon mitogen withdrawal.

Myogenic differentiation is accompanied by coordinate induction of a battery of tissue-specific gene products which include the muscle isoenzyme of creatine kinase (CK) and the nicotinic acetylcholine (ACh) receptor (5, 14, 21, 26, 28–33, 40). Activation of muscle-specific genes during myogenesis is coupled to cessation of cell division and is inhibited by serum components, fibroblast growth factor, and type β transforming growth factor (9, 22, 24, 29–32, 43). Several lines of evidence have suggested that the molecular control of myogenesis might involve the cellular oncogene c-*myc* (11, 38, 43), the putative intranuclear mediator of growth factor signals (2, 3, 16, 18). The onset of myogenesis has been reported to be preceded by a decline in c-*myc* expression (38). In certain variant muscle cell lines that cannot differentiate, c-*myc* fails to be down-regulated after removal of mitogens, despite withdrawal of myocytes from the cell cycle (38). These observations agree with reports that the viral *myc* oncogene can block differentiation of quail embryo myoblasts (11) and that deregulated expression of c-*myc* may block the differentiation of mouse erythroleukemia cells (7). However, it is conjectural whether the normal decline of this growth factor-inducible protein is a cause or, instead, a consequence of differentiation. For example, reinduction of c-*myc* was not sufficient to inactivate muscle-specific gene expression in L_6E_9 myotubes that were terminally differentiated (10). Furthermore, contrasting reports have questioned whether mitogen-inducible cellular oncogenes are either obligatory or sufficient to account for the changes in cell phenotype evoked by altered growth factor levels in a variety of other systems (4, 8, 23, 27).

In the present study, we have examined the potential role of c-*myc* in the control of myogenesis by stable transfection of BC_3H1 cells with a transcriptionally active c-*myc* gene. The BC_3H1 myogenic cell line was selected for these studies because differentiation in this system is accompanied by high-level induction of muscle CK and the ACh receptor, is relatively synchronous, and, most importantly, remains susceptible to modulation by growth factors (21, 22, 30–32, 37, 43). While the precise origin of this cell line is unknown, BC_3H1 cells have become a widely employed model for gaining insight into the mechanisms involved in regulation of myogenesis by mitogens. In this report, we present evidence that deregulated expression of c-*myc* partially inhibits, but does not prevent, myogenic differentiation of BC_3H1 cells after mitogen withdrawal.

The c-*myc* vector pSVc-*myc*-1 (20) comprised the murine c-*myc* coding sequences contained within exons 2 and 3, transcriptionally activated via the simian virus 40 (SV40) promoter. Transfection (13, 41) employed overnight incubation of 10^6 cells with 30 μg of carrier DNA, 0.1 μg of pSV2*neo* DNA to confer neomycin resistance (42), and 1 μg of the chimeric oncogene DNA. After an additional 24 h, the cells were fed with medium containing the neomycin analog G418 at 400 μg ml^{-1} and were refed weekly. Colonies resulting from transfection with the neomycin resistance gene alone were morphologically indistinguishable from parental BC_3H1 cells (Fig. 1A and B). c-*myc*-transfected myocytes typically were smaller, rounder, and more refractile (Fig. 1C and D). Seven independent clonal c-*myc*-transfected cell lines initially were screened for expression of muscle CK and ACh receptor as indicators of myogenic differentiation; a representative cell line was selected for more detailed characterization shown below. Southern hybridization (25) revealed the single 4.6-kilobase-pair *Hin*dIII c-*myc* restriction fragment in *neo* transfectants and confirmed stable incorporation of the exogenous DNA in myocytes transfected with the c-*myc* vector (Fig. 2).

Cells transfected with c-*myc* exhibited doubling times equivalent to *neo* transfectants in medium with 20% fetal bovine serum (FBS) and exhibited contact inhibition of cell proliferation at confluent densities. Moreover, for both *neo* and *myc* transfectants, transfer to medium with 0.5% FBS was accompanied by withdrawal from the cell cycle within ~48 h even at subconfluent densities (Fig. 3A). Thus, the c-*myc* expression vector was unable by itself to release BC_3H1 cells from their dependence on exogenous growth factors for proliferation. These results do not exclude more subtle alterations in growth factor requirements, as have been reported for c-*myc*-transfected fibroblasts (2, 17).

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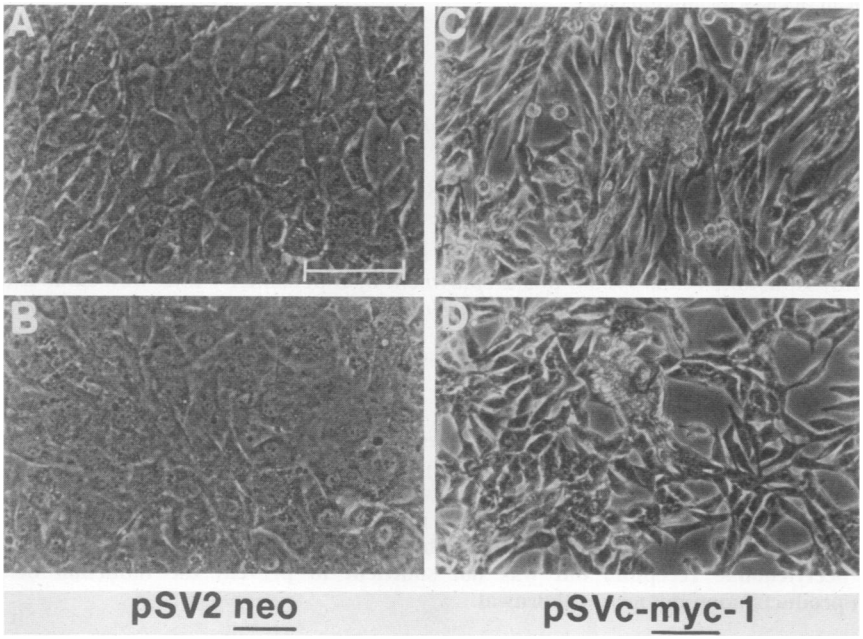

FIG. 1. Morphology of transfected BC_3H1 myocytes. Phase-contrast microscopy is shown for pairs of independent replicate colonies, photographed after 18 days in selective medium: (A and B) pSV2*neo* alone; (C and D) pSVc-*myc*-1 + pSV2*neo*. Subsequent experiments were performed on gelatin-treated polystyrene to ensure stable monolayers. Bar, 100 μm.

As reported previously (30–32, 43), exposure of BC_3H1 cells to medium with FBS reduced to 0.5% was accompanied by induction of CK activity and cell surface ACh receptors (Fig. 3B and C). These muscle-specific gene products were also induced after mitogen withdrawal in cells transfected with c-*myc*. However, expression of these muscle differentiation products was inhibited by 50 to 75%. Because CK and ACh receptors were expressed with similar kinetics and reached maximum levels within 4 to 5 days after exposure of *neo* and *myc* transfectants to medium with 0.5% FBS, the reduced expression of these muscle-specific gene products in the presence of an exogenous c-*myc* gene may be due to interference in the differentiation program, rather than to a simple delay in the onset of differentiation.

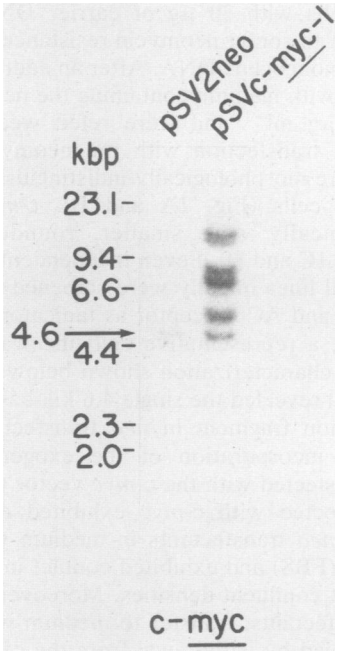

FIG. 2. Southern hybridization analysis of transfected BC_3H1 myocytes. DNA was extracted from clonal BC_3H1 myocytes transfected with pSV2*neo* or pSVc-*myc*-1. DNA was digested with *Hin*dIII, size fractionated (10 μg per lane), transferred to nitrocellulose, and hybridized to the ^{32}P-labeled *Xba*I-*Hin*dIII fragment of pSVc-*myc*-1 (12, 25). The endogenous 4.6-kilobase-pair *Hin*dIII fragment is indicated (arrow).

c-*myc* mRNA is generally expressed at basal levels in cells under conditions of serum deprivation and rapidly accumulates after mitogenic stimulation (2–4, 6, 8, 10, 18, 45). To compare relative levels of endogenous and exogenous *myc* transcripts in the different cell lines and to determine whether exogenous *myc* transcripts were subject to regulation similar to the endogenous mRNA, we analyzed levels of c-*myc* mRNA under conditions of serum deprivation and after exposure of quiescent cells to medium with 20% FBS for 2 and 4 h (Fig. 4A). The pSVc-*myc*-1 transcript, which lacks exon 1, is 200 to 300 nucleotides shorter than the 2.1-kilobase endogenous transcript and can be readily distinguished. In 0.5% FBS, exogenous c-*myc* mRNA levels were 20 to 40-fold higher in c-*myc* transfectants than were the endogenous c-*myc* mRNA levels in controls (compare lanes 1 and 4, Fig. 4A). Serum stimulation resulted in an approximate 10-fold increase in c-*myc* mRNA levels in each cell type. Because SV40-driven c-*myc* was induced in parallel with the endogenous c-*myc* transcript found in control cultures, it will be interesting to determine whether this reflects transcriptional induction of the SV40 promoter (15), an alteration in c-*myc* mRNA stability, or both. Though the SV40 promoter-linked gene was regulated by serum components and was significantly down-regulated upon serum withdrawal, the minimal level of exogenous c-*myc* expression observed in 0.5% serum (nominally "differentiating" conditions) was at least threefold higher than the level of

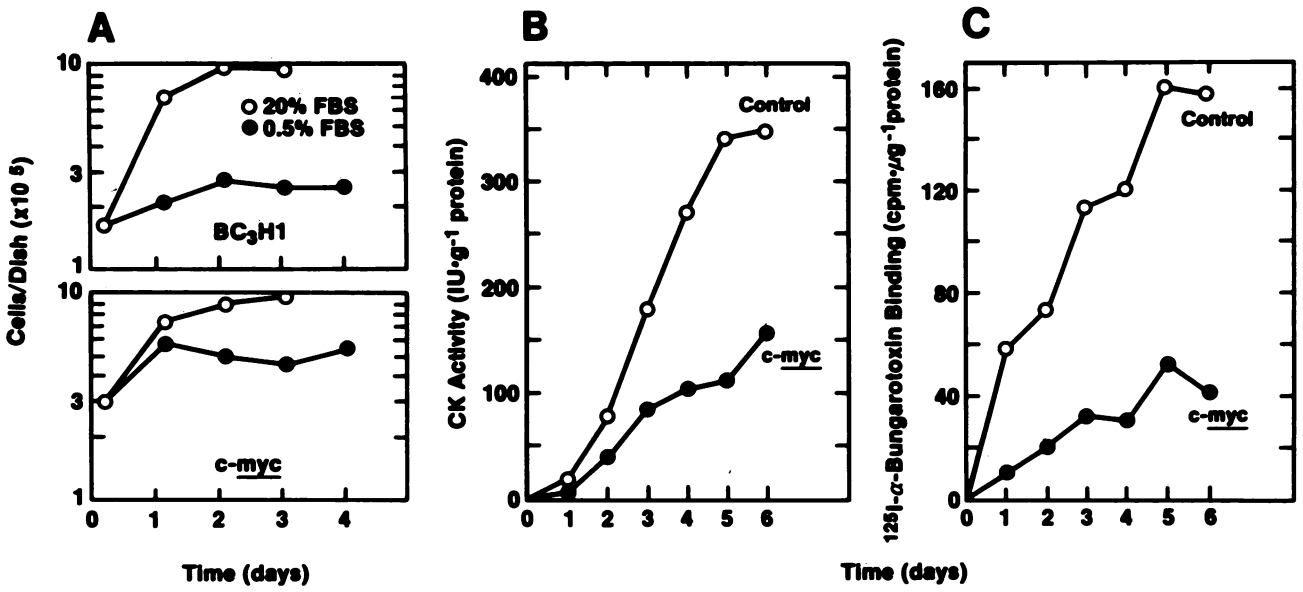

FIG. 3. (A) Proliferation of transfected BC_3H1 myocytes. BC_3H1 myocytes transfected with pSV2*neo* (control) or pSVc-*myc*-1 (c-*myc*) were cultured to ~25% confluency in Dulbecco modified Eagle medium with 20% FBS. On day 0, half of the cultures were exposed to medium with 0.5% FBS, and cells were counted on subsequent days using a Coulter counter. Each cell line became quiescent with respect to growth within 2 days. Results are shown on a semilogarithmic scale. (B and C) Expression of CK and ACh receptors in transfected BC_3H1 myocytes. BC_3H1 myocytes transfected with pSV2*neo* (control) or pSVc-*myc*-1 (c-*myc*) were cultured in Dulbecco modified Eagle medium with 20% FBS. On day 0, cultures were exposed to medium with 0.5% FBS. On consecutive days, (B) CK activity was determined enzymatically (36), and (C) ACh receptors were measured by specific binding of α-^{125}I-bungarotoxin (31).

endogenous c-*myc* expression found in the parental line during logarithmic growth in 20% serum.

As anticipated from the induction of CK activity in the SV40:c-*myc* transfectants, *mck* transcripts measured by Northern blot hybridization also were induced in these cells at 4 days of mitogen withdrawal and were diminished in abundance by ~75% (Fig. 4B). Transfection with c-*myc* did not influence expression of all cellular mRNA, as demonstrated by the constitutive expression of glyceraldehyde-3-phosphate dehydrogenase mRNA in all cell lines examined (unpublished data).

We have examined the phenotypic consequences of transfecting BC_3H1 cells with an exogenous c-*myc* gene, a "distal" element in the cascade postulated for transduction of growth factor signals. There are at least four biochemical mechanisms through which deregulated expression of the

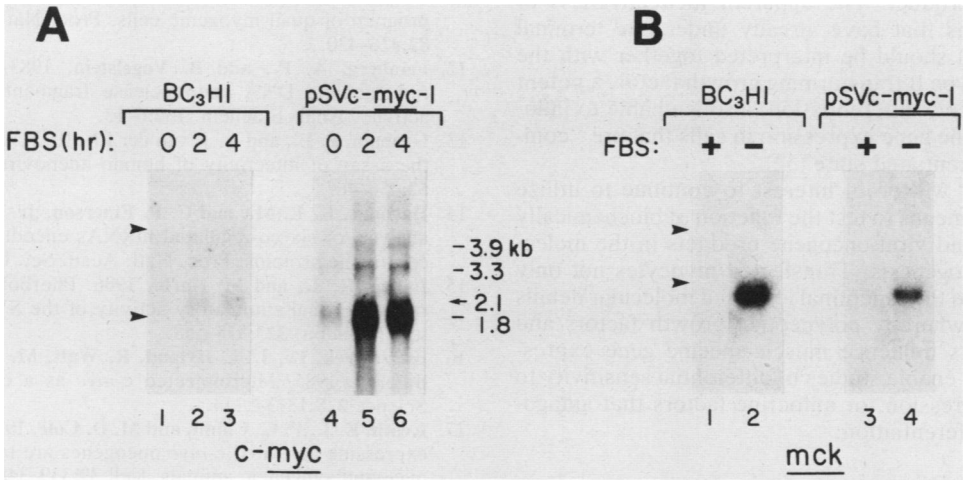

FIG. 4. Expression of c-*myc* mRNA and *mck* mRNA in transfected BC_3H1 myocytes. (A) BC_3H1 myocytes and SV40:c-*myc*-transfected cells were cultured in Dulbecco modified Eagle medium with 20% FBS and were exposed to medium with 0.5% FBS for 4 days. Total cellular RNA was isolated from (0) quiescent cells and after treatment with 20% FBS for 2 or 4 hours as indicated. Northern hybridization was performed using the *Xba*I-*Hin*d III fragment of pSVc-*myc*-1 (11, 23). Lanes: 1, 2, and 3, control BC_3H1 myocytes; 4, 5, and 6, c-*myc* transfectants. (B) Total cellular RNA was isolated from BC_3H1 cells and SV40:c-*myc* transfectants, (+) during proliferative growth in 20% FBS or (−) after mitogen withdrawal for 4 days. The *mck* probe comprised the 0.84-kilobase-pair *Sma*I-*Eco*RI fragment of R21 (35); comparable results were obtained with the 0.85-kilobase-pair *Pst*I fragment of pHMCK-Ia (34). Lanes: 1 and 2, control BC_3H1 myocytes; 3 and 4, c-*myc* transfectants.

c-*myc* gene product might impinge on the control of differentiation and cause partial inhibition of muscle-specific gene expression. The c-*myc* protein is found within the nucleus (1), and it has been suggested that c-*myc* protein might itself regulate cellular promoters of transcription, as do the nuclear products of *fos* (39) and adenovirus E1A genes (19). Alternatively, autonomous expression of c-*myc*, a member of the set of genes induced by "competence" growth factors (6, 8, 18, 45), can serve as a surrogate for those factors and enable cells to replicate DNA and divide in the presence of "progression" factors (16). Such observations are especially important in the context of evidence that certain competence factors can block or reverse myogenic differentiation (22, 43). Other possible functions suggested for c-*myc* include influences on activity of transcription complexes or on RNA processing within the nucleus (6). Finally, deregulated *myc* expression potentiates at least the proliferative responses of cells, elicited by various growth factors, and might, therefore, sensitize myoblasts to the negative effects on differentiation of certain growth factors (44).

Because *mck* and ACh receptor expression in *myc* transfectants were induced at least 50-fold after mitogen withdrawal, our observations argue strongly against the hypothesis that induction of muscle-specific genes is obligatorily coupled to down-regulation of c-*myc*. The conclusions we report here are also supported by our recent findings that functional sodium and calcium channels, characteristic of skeletal muscle cells, are formed in BC_3H1 myocytes only after mitogen withdrawal; their induction is delayed but not prevented by expression of the SV40:c-*myc* gene (J. M. Caffrey, A. M. Brown, and M. D. Schneider, Science, in press). These results complement a recent report that reinduction of c-*myc* in differentiated L_6E_9 myotubes was insufficient to inactivate muscle-specific gene expression (10) and that deregulated expression of c-*myc* in transgenic mice did not disrupt normal development (23).

Contrasting results obtained with the viral *myc* oncogene in primary quail myoblasts (11) may reflect cell type-specific responses to the *myc* gene product or differences in the procedures utilized, or might signify functional differences in the v-*myc* gene product. The apparent ineffectiveness of c-*myc* in myocytes that have already undergone terminal differentiation (10) should be interpreted together with the observation that type β transforming growth factor, a potent inhibitor of myogenic differentiation, also is unable to influence muscle-specific gene expression in cells that are "committed" to a differentiated state (32).

In the future, it will be of interest to continue to utilize gene transfer as a means to test the function of biochemically discrete cellular and viral oncogene products in the molecular events of myogenesis. Transfected myocytes not only provide insight into the functional level and molecular details of the pathways whereby polypeptide growth factors and oncogene products influence muscle-specific gene expression, but also may enable studies of differential sensitivity to competence, progression, or autocrine factors that antagonize myogenic differentiation.

We thank J. Michael Bishop, Arnold Strauss, and Robert Weinberg for the plasmids indicated and Sharon Mulvagh for helpful criticisms. We thank Mary Anne Bresser and Rebecca Montgomery for technical assistance and Ellen Madson for preparing the manuscript.

This investigation was supported by grants from the National Institutes of Health (M.D.S., E.N.O.) and the American Heart Association, Texas Affiliate (M.D.S., E.N.O.) and by a Basil O'Connor Starter Research Grant from the March of Dimes (E.N.O.). P.A.P. was a fellow of the Stanley J. Sarnoff Society of Fellows for Research in Cardiovascular Science. The Baylor Molecular Cardiology Unit is supported by the American Heart Association–Bugher Foundation Center for Molecular Biology of the Cardiovascular System.

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