

# The Expression of Sarcomeric Muscle-specific Contractile Protein Genes in BC3H1 Cells: BC3H1 Cells Resemble Skeletal Myoblasts that Are Defective for Commitment to Terminal Differentiation

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**Abstract.** The BC3H1 cell line has been used widely as a model for studying regulation of muscle-related proteins, such as the acetylcholine receptor, myokinase, creatine kinase, and actin. These cells, derived from a nitrosourea-induced mouse brain neoplasm, have some of the morphological characteristics of smooth muscle and have been shown to express the vascular smooth muscle isoform of  $\alpha$ -actin. To provide further information about the contractile protein phenotype of BC3H1 and to gain additional insights into the possible tissue of origin of these cells, we have examined the expression of a battery of contrac-

tile protein genes. During rapid growth, subconfluent BC3H1 cells express the nonmuscle isoform of  $\alpha$ -tropomyosin ( $\alpha$ -Tm) and the nonsarcomeric isoforms of myosin heavy and light chains (MHCs and MLCs, respectively), but do not express troponin T (TnT). However, when BC3H1 cells differentiate in response to incubation in serum-deprived medium or upon approaching confluence, they express TnT as well as sarcomeric muscle isoforms of MHC, MLC 2 and 3,  $\alpha$ -Tm, and  $\alpha$ -actin. These results suggest that BC3H1 is a skeletal muscle cell line of ectodermal origin that is defective for commitment to terminal differentiation.

THE BC3H1 cell line (Schubert et al., 1974) has received considerable attention as a model for studying muscle cell differentiation and the regulation of muscle-related proteins. BC3H1 cells can be induced to differentiate by removal of growth factors from the culture medium or by allowing cultures to reach confluence. Under these conditions, BC3H1 cells accumulate a variety of muscle-specific gene products, such as muscle creatine phosphokinase (MCK)<sup>1</sup> (Schubert et al., 1974; Munson et al., 1982; Olson et al., 1983), myokinase (Schubert et al., 1974), and  $\alpha$ -actin (Strauch and Rubenstein, 1984; Strauch et al., 1986) as well as the nicotinic acetylcholine (Patrick et al., 1977; Olson et al., 1984) and insulin receptors (Standaert et al., 1984). Differentiation is reversible, either by adding mitogens to the culture medium (Lathrop et al., 1985; Spizz et al., 1986) or by replating cells in serum-rich medium (Munson et al., 1982; Olson et al., 1983).

The BC3H1 cell line was originally isolated from mouse brain neoplastic tissue and characterized by Schubert et al. (1974). Results of morphological, biochemical, and physio-

logical analyses led to the suggestion that BC3H1 cells were of smooth muscle origin. This concept was further strengthened by the demonstration that differentiation of BC3H1 resulted in the accumulation of smooth muscle-specific  $\alpha$ -actin (Strauch and Rubenstein, 1984; Strauch et al., 1986; Wang and Rubenstein, 1988). In contrast, the expression of MCK and the nicotinic acetylcholine receptor in BC3H1 suggested these cells behaved more like skeletal muscle. In addition, it has recently been shown that BC3H1 cells express a skeletal muscle-type voltage-gated  $\text{Ca}^{2+}$  channel (Caffrey et al., 1987) as well as the skeletal muscle isoform of  $\alpha$ -actin (Reeser, J. C., and A. R. Strauch, unpublished results). None of these markers, however, is specific for striated skeletal muscle. Given the widespread use of the BC3H1 culture system as an in vitro model for studying the differentiation and regulation of muscle-specific genes, it is important to establish the phenotype of muscle-specific gene expression in BC3H1 and to determine whether this phenotype supports a smooth or skeletal muscle origin for this cell line. Accordingly, we have performed RNA blot and S1 nuclease analyses using specific cDNA probes for a variety of contractile proteins representing both the thin and thick filaments. We now report that subconfluent, growing BC3H1 cells ex-

1. *Abbreviations used in this paper:* LSM, low serum medium; MCK, muscle creatine kinase; MHC, myosin heavy chain; MLC, myosin light chain; RLC, regulatory light chain;  $\alpha$ -Tm,  $\alpha$ -tropomyosin; TnT, troponin T.

press the nonmuscle isoform of  $\alpha$ -tropomyosin ( $\alpha$ -Tm) and the nonsarcomeric isoforms of myosin heavy and light chains (MHCs and MLCs, respectively), but do not express tropomyosin T (TnT). However, when BC3H1 cells differentiate in response to incubation in serum-deprived medium or upon approaching confluence, they express TnT as well as sarcomeric muscle isoforms of MHC, MLC 2 and 3,  $\alpha$ -Tm, and  $\alpha$ -actin. These results suggest that the BC3H1 cell line most closely resembles a skeletal muscle cell that is defective for commitment to terminal differentiation.

## Materials and Methods

### Cell Culture

The BC3H1 mouse cell line (Schubert et al., 1974) was a generous gift of Dr. Eric N. Olson (Department of Biochemistry and Molecular Biology, University of Texas, M. D. Anderson Hospital and Tumor Institute). Cultures were grown on 15-cm tissue culture dishes in DME containing 20% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin as described by Olson et al. (1983). The medium was changed every 2 d. To minimize differentiation of BC3H1 cells and induction of contractile protein genes, cells were passaged while no more than 75% confluent. To initiate differentiation, medium containing 20% serum was removed and replaced with medium, designated as LSM (low-serum medium; Lathrop et al., 1985), containing DME and F12 in a ratio of 3:1, 50  $\mu$ g/ml transferrin, 250  $\mu$ g/ml BSA, 0.25% FCS, and antibiotics.

### RNA Preparation and Blot Hybridization

Total RNA was extracted from BC3H1 cultures by the guanidinium isothiocyanate/CsCl procedure (Chirgwin et al., 1979). 15  $\mu$ g of total RNA was size fractionated by electrophoresis on 1% agarose gels in 200 mM 4-morpholinepropanesulfonic acid, pH 7.4, 1 mM EDTA, and 3% formaldehyde. Transfer to nitrocellulose and hybridization to  $^{32}$ P-labeled DNA probes was as described (Thomas, 1980). Filters were hybridized at 42°C for 14 h in a cocktail containing 50% formamide, 5 $\times$  SSC ( $1\times = 0.15$  M NaCl, 0.015 sodium citrate), 2 $\times$  Denhardt's solution, 0.1% SDS, 0.025 M sodium phosphate, and 50 mg/ml calf thymus DNA. At the end of hybridization, the filters were washed in  $0.1\times$  SSC at 55°C unless otherwise indicated. Filters were exposed to Kodak XAR-5 film for 2 d at  $-70^\circ\text{C}$  with one intensifying screen.

### Preparation of cDNA Probes

The following cDNA probes were labeled by nick translation (Rigby et al., 1977) using [ $^{32}$ P]dATP and [ $^{32}$ P]dCTP (400 Ci/mmol; Amersham Corp., Arlington Heights, IL) to a specific activity of  $\sim 10^8$  cpm/ $\mu$ g DNA: pMCK-4 (rat skeletal MCK; Benfield et al., 1984); pAC269 (chicken skeletal muscle  $\alpha$ -actin; Schwartz et al., 1980); pT1 ( $\alpha$ -tubulin; Valenzuela et al., 1981); rat fast skeletal muscle cDNA clones (Garfinkel et al., 1982); pTnT15 (TnT); pMLC-84 (MLC 1); pMLC2-18 (MLC 2); a cDNA clone encoding rat striated muscle  $\alpha$ -Tm (Wieczorek et al., 1988); a clone encoding a myosin regulatory light chain (RLC) isolated from a rat aortic smooth muscle cell cDNA library (Taubman et al., 1987); pMHC25 (rat embryonic MHC; Medford et al., 1980); and SMHC 29 (rabbit smooth muscle MHC; Nagai et al., 1988). Synthetic oligonucleotide probes comprised of the first 87 nucleotides of the 3' untranslated sequences of mouse skeletal  $\alpha$ -actin (Hu et al., 1986) and the first 55 nucleotides of the 5' untranslated sequences of mouse cardiac  $\alpha$ -actin (Minty et al., 1983) were end-labeled with T4 polynucleotide kinase and [ $^{32}$ P- $\gamma$ ]ATP.

### S1 Nuclease Mapping

RNA-DNA hybridization followed by S1 nuclease mapping analysis was performed as previously described (see Taubman et al., 1987; Wieczorek et al., 1988). The probes used were generated by digestion with restriction endonucleases and labeled at the 5' end with [ $^{32}$ P- $\gamma$ ]ATP and T4 polynucleotide kinase. Total cellular RNA (25  $\mu$ g) was hybridized to  $2 \times 10^4$  cpm of probe in 25  $\mu$ l of 80% deionized formamide, 400 mM NaCl, 10 mM Pipes, pH 6.4, 0.05% SDS, 1 mM EDTA. The hybridization mixture was incubated at 65°C for 1 h, the temperature was adjusted to 60°C, and the incubation was continued for 16 h. S1 nuclease (100 U) in 300  $\mu$ l of 200

mM NaCl, 20 mM sodium acetate, pH 4.5, 3 mM ZnSO<sub>4</sub> was added to each sample, and the samples were incubated at 25°C for 1 h. The reaction was terminated with 10 mM EDTA and precipitated with ethanol. Dried pellets were dissolved in 90% formamide and electrophoresed on 6% polyacrylamide-8 M urea sequencing gels. Gels were dried and exposed for autoradiography on Kodak X-Omat AR film.

## Results

BC3H1 cells were plated at low density ( $4 \times 10^4$  cells/15-cm dish) and subsequently grown in 20% FCS. At the indicated times, duplicate cultures were either harvested or switched to LSM, (see Materials and Methods) and incubated for an additional 48 h. To assay for the accumulation of muscle-specific mRNAs, blot hybridizations were performed using a variety of cDNA probes encoding muscle-related proteins.

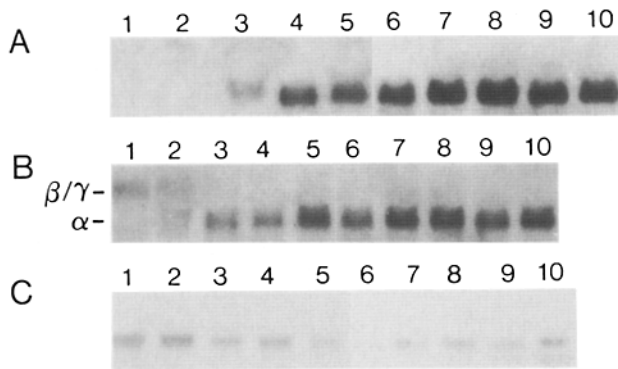
To confirm that the BC3H1 cells used in this study behaved as previously reported, the expression of creatine kinase and actin mRNA were examined. mRNA encoding MCK was absent in subconfluent, growing cultures, but its accumulation was induced when BC3H1 cultures approached confluence (Fig. 1 A, lanes 1-5) or when subconfluent cultures were switched to LSM (Fig. 1 A, lanes 6-10). mRNA compatible in size with either of the nonmuscle actin isoform(s),  $\beta$  and  $\gamma$ , was found in subconfluent, growing BC3H1 cultures (Fig. 1 B, lanes 1 and 2). However, when cultures approached confluence (Fig. 1 B, lane 3) or when subconfluent cultures were switched to LSM (Fig. 1 B, lanes 6-10), accumulation of an mRNA compatible in size with an  $\alpha$ -actin isoform was induced, while expression of the larger  $\beta$  or  $\gamma$  isoform(s) was no longer apparent. These results are similar to those demonstrated by other laboratories (Olson et al., 1983; Strauch et al., 1986). In contrast, levels of  $\alpha$ -tubulin mRNA were slightly diminished when BC3H1 cells reached confluence or were switched to LSM (Fig. 1 C), demonstrating that the increases in MCK and  $\alpha$ -actin mRNA levels did not simply represent a generalized increase in message levels.

### Differentiated BC3H1 Cells Produce TnT mRNA

TnT is a critical component of the sarcomeric contractile apparatus. In contrast, TnT does not play a role in smooth muscle contraction (for review see Adelstein and Eisenberg, 1980). Neither TnT protein nor mRNA has been found in smooth muscle tissue or cells (Medford et al., 1984; Marston and Smith, 1985). TnT mRNA was not detected in 25-30% confluent proliferating BC3H1 cells (Fig. 2 A, lane 1) and was detected in only trace amounts at  $\sim 50\%$  confluence (Fig. 2 A, lane 2). However, high levels of TnT mRNA were found when BC3H1 cultures approached confluence (Fig. 2 A, lanes 3-7) or when subconfluent cultures were incubated in LSM (Fig. 2 B). Differentiated BC3H1 cells thus synthesize an mRNA found only in sarcomeric tissues or cells.

### Differentiation of BC3H1 Cells Results in a Switch from the Nonmuscle to Striated Muscle Isoform of $\alpha$ -Tm

Rat  $\alpha$ -Tm is encoded by a single gene whose transcripts are alternatively spliced to produce distinct nonmuscle and smooth and striated muscle isoforms (Wieczorek et al., 1988). The nonmuscle and smooth muscle mRNAs are the same size and are significantly larger than the striated muscle



**Figure 1.** mRNA expression in BC3H1 cells. RNA blot hybridizations were performed using 15  $\mu$ g of total cellular RNA and hybridized with the following cDNA probes (see Materials and Methods): (A) striated MCK; (B) chicken skeletal muscle  $\alpha$ -actin; and (C)  $\alpha$ -tubulin. BC3H1 cultures were plated at low density in DME containing 20% FCS. Cultures were grown in the same medium and harvested 2, 3, 4, 5, and 7 d after plating (lanes 1–5, respectively). Duplicate cultures were harvested after a 48-h incubation in LSM beginning 2, 3, 4, 5, and 7 d after plating (lanes 6–10, respectively). Cultures maintained in DME + 20% FCS appeared confluent 5 d after plating.

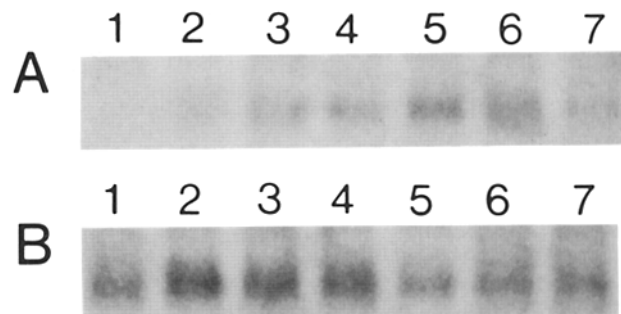
isoforms. This is due to the presence in smooth muscle and nonmuscle transcripts of an alternative 3' exon with a significantly larger 3' untranslated region than that of the striated muscle-specific 3' exons (Ruiz-Opazo et al., 1985; Wieczorek et al., 1988). As demonstrated (Fig. 3 A, lanes 1–3), subconfluent, growing BC3H1 cultures express an  $\alpha$ -Tm mRNA of a size compatible with either the nonmuscle or smooth muscle isoform. To further establish the identity of the hybridizing species, S1 nuclease mapping was performed using probes which differentiate between the nonmuscle and smooth muscle isoforms (Wieczorek et al., 1988). These two isoforms differ only at the 5' end, where the smooth muscle isoform contains exon 2 while the nonmuscle (and striated muscle) isoform uses exon 3. Fig. 4 A shows the results of S1 mapping using a smooth muscle cDNA restriction fragment. Full protection of this probe (355 nucleotides; e.g., uterus, Fig. 4 A, lane 4) is seen only with smooth muscle, while partial protection (116 nucleotides; e.g., L<sub>6</sub>E<sub>9</sub>, Fig. 4 A, lane 3) is seen with striated and nonmuscle tissues (Wieczorek et al., 1988). RNA from subconfluent, proliferating BC3H1 cells (Fig. 4 A, lane 5) produced mainly partial protection, consistent with the nonmuscle isoform. Fig. 4 B shows the results of S1 mapping using a striated muscle cDNA restriction fragment. Full protection of this probe (355 nucleotides; e.g., L<sub>6</sub>E<sub>9</sub>, Fig. 4 B, lane 3) is seen with striated and nonmuscle tissues, while partial protection (116 nucleotides; e.g., uterus; Fig. 4 B, lane 4) is seen with smooth muscle (Wieczorek et al., 1988). RNA from subconfluent, proliferating BC3H1 cells (Fig. 4 B, lane 5) produced full protection, again consistent with the nonmuscle isoform.

The above results suggest that the predominant  $\alpha$ -Tm species produced by growing BC3H1 cells is the nonmuscle isoform. Upon reaching confluence, BC3H1 cultures stopped producing this mRNA and began to accumulate an  $\alpha$ -Tm mRNA of a size compatible with a striated muscle isoform (Fig. 3 A, lanes 4–7). S1 nuclease mapping (Fig. 4, lanes 6

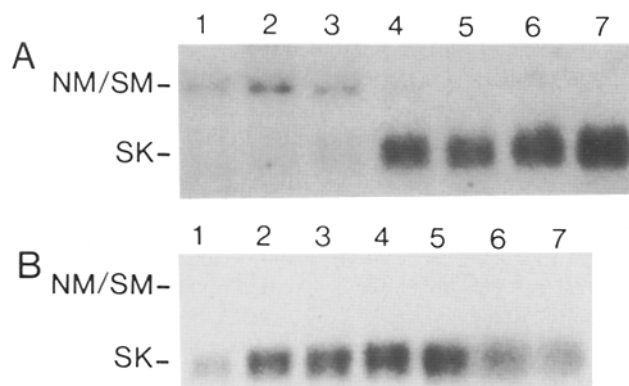
and 7) showed no change in the splicing pattern at the 5' end, confirming that this was a striated muscle isoform. This isoform switch was also noted when subconfluent cultures were switched to LSM (Fig. 3 B). Similar changes in  $\alpha$ -Tm splicing patterns (i.e., from nonmuscle to striated muscle isoforms) are seen upon differentiation of striated muscle cells (Wieczorek et al., 1988; our unpublished observations).

### Differentiated BC3H1 Cells Produce Skeletal Muscle MLC 3 mRNA

Fast skeletal muscle contains three types of MLC: two "alkali" light chains, MLC 1 and MLC 3, and one RLC, MLC 2. In contrast, smooth muscle contains one alkali and one regulatory light chain. The rat skeletal alkali light chains are generated by alternative splicing of primary transcripts from a single gene containing two promoters (Periasamy et al., 1984; Strehler et al., 1985). This gene is expressed in a developmental and tissue-specific manner (Whalen et al., 1981; Periasamy et al., 1984) and, in particular, is not expressed in smooth muscle. As shown (Fig. 5 A, lanes 1–3), subconfluent growing BC3H1 cultures do not contain detectable amounts of skeletal muscle alkali light chain mRNAs. However, upon reaching confluence, high levels of MLC 3 mRNA were detected (Fig. 4 A, lanes 4–7). No mRNA corresponding in size to MLC 1 was detected at any time in culture. MLC 3 mRNA was also induced by switching subconfluent cells to LSM (Fig. 5 B). The pattern of expression is similar to that seen in the embryonic skeletal muscle line L<sub>6</sub>E<sub>9</sub> (Nadal-Ginard, 1978), where only MLC 3 mRNA is expressed (our unpublished observations). In other skeletal muscle cells both MLC 1 and MLC 3 mRNAs are expressed with the ratio of MLC 3 to MLC 1 highest in cells derived from adult skeletal muscle (Periasamy et al., 1984). The presence or absence of smooth muscle alkali light chain mRNA in BC3H1 cultures could not be assayed because of the lack of cross-hybridization of smooth muscle mRNA with the skeletal muscle probes and because of the lack of availability of specific cDNA probes for mouse smooth muscle alkali light chains.



**Figure 2.** Expression of TnT mRNA. RNA blot hybridizations were performed using the rat TnT cDNA probe. (A) BC3H1 cultures were plated at low density and grown in DME + 20% FCS for 2, 3, 4, 5, 7, 9, and 11 d (lanes 1–7, respectively). Cultures appeared confluent 5 d after plating. (B) Duplicate cultures were grown in DME + 20% FCS and subsequently switched to LSM for 48 h, 2, 3, 4, 5, 7, 9, and 11 d after plating (lanes 1–7, respectively).



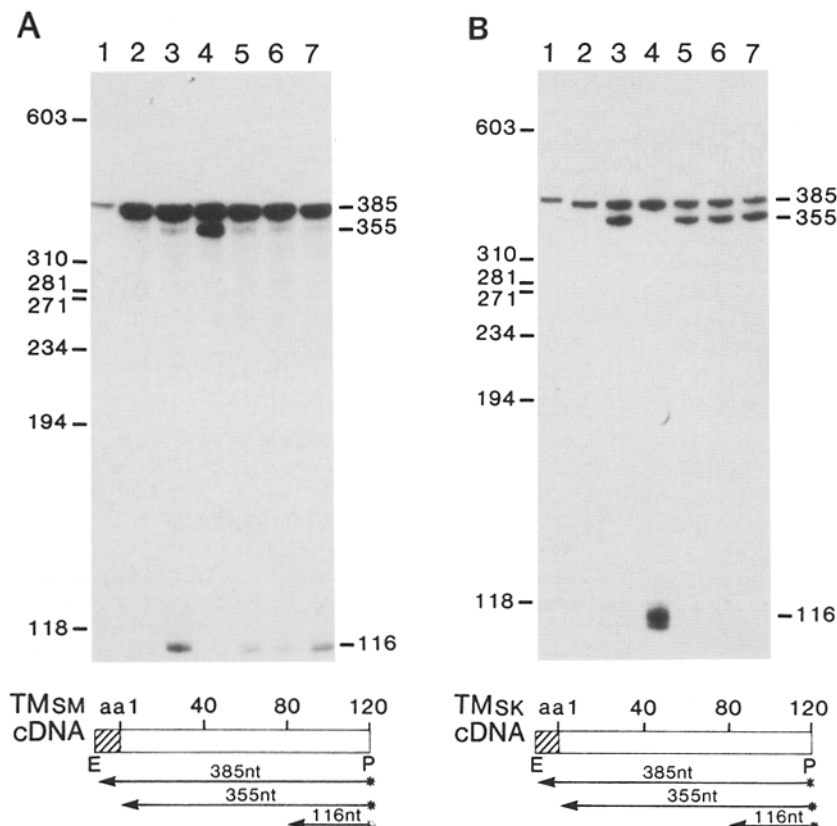
**Figure 3.** Expression of  $\alpha$ -Tm mRNA. RNA blot hybridizations were performed using the rat  $\alpha$ -Tm cDNA probe. (A) BC3H1 cultures were plated at low density and grown in DME + 20% FCS for 2, 3, 4, 5, 7, 9, and 11 d (lanes 1-7, respectively). Cultures appeared confluent 5 d after plating. (B) Duplicate cultures were grown in DME + 20% FCS and subsequently switched to LSM for 48 h 2, 3, 4, 5, 7, 9, and 11 d after plating (lanes 1-7, respectively). NM/SM, nonmuscle and smooth muscle isoforms of  $\alpha$ -Tm. SK, skeletal muscle isoform.

### Differentiated BC3H1 Cells Contain both Sarcomeric and Nonsarcomeric Myosin RLCs

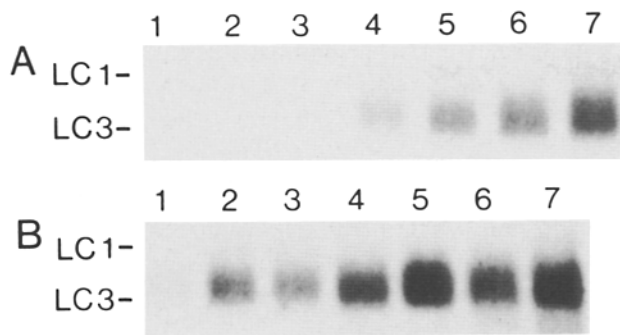
The rat skeletal muscle RLC, MLC 2, gene is expressed in a developmental and tissue-specific manner (Devlin and Emerson, 1978; Shani et al., 1981). Under the conditions of hybridization used in this experiment, the rat MLC 2 probe

does not cross-hybridize with RNA derived from smooth muscle tissues or cells, but does cross-hybridize with other sarcomeric MLC 2 mRNAs, such as those isolated from cardiac muscle (our unpublished observations). Subconfluent growing BC3H1 cultures contained very small or undetectable amounts of MLC 2 mRNA (Fig. 6 A, lanes 1 and 2). However as cultures approached confluence, increasing amounts of mRNA accumulated which hybridized with the rat skeletal muscle MLC 2 probe (Fig. 6 A, lanes 3-7). High levels of MLC 2 mRNA were induced by switching subconfluent cells to LSM (Fig. 6 B). These results demonstrate that, in addition to accumulating sarcomeric MLC 3 mRNA, differentiated BC3H1 cells also contain a sarcomeric isoform of the myosin RLC, MLC 2.

We have recently isolated a cDNA clone from rat aortic smooth muscle cells which corresponds to a nonsarcomeric myosin RLC (Taubman et al., 1987). The corresponding mRNA is larger than that encoding the skeletal muscle MLC 2 and is readily distinguished from MLC 2 mRNA on RNA blots. This mRNA is found in a wide variety of muscle and nonmuscle tissues and thus does not exhibit the tissue specificity found for other contractile protein genes (Taubman et al., 1987). Whether this cDNA encodes a smooth muscle- or nonmuscle-specific RLC has yet to be conclusively determined. Growing, but subconfluent, BC3H1 cells expressed high levels of RLC mRNA (Fig. 6 C). As these cells continued to grow in culture, smaller amounts of RLC mRNA were detected. By switching subconfluent cells to LSM, a rapid decrease in the levels of this mRNA was noted (Fig. 6 D) corresponding to levels seen in postconfluent density arrested cells (Fig. 6 C). This pattern of expression is opposite



**Figure 4.** S1 nuclease analysis of  $\alpha$ -Tm mRNA expression. (Lane 1) probe; (lane 2) tRNA; (lane 3) L<sub>6</sub>E<sub>9</sub> rat skeletal muscle myoblasts; (lane 4) rat uterus; (lanes 5-7) BC3H1 cells at 30% confluence, 90% confluence, and 2 d after 100% confluence, respectively. (A) The smooth muscle (SM) cDNA Eco RI-Pst I restriction fragment with a polylinker tail (hatched) depicted at the bottom was 5' end labeled at a nucleotide corresponding to codon 120. Significant full protection of this probe (355 nt) is seen only with smooth muscle samples, while partial protection (116 nt) is seen in striated and nonmuscle samples (Wieczorek et al., 1988). (B) The striated muscle cDNA restriction fragment with a polylinker tail (hatched) (SK) depicted at the bottom was 5' end labeled at a nucleotide corresponding to codon 120. Full protection (355 nt) is seen with striated and nonmuscle samples while partial protection (116 nt) is seen with smooth muscle (Wieczorek et al., 1988).



**Figure 5.** Expression of skeletal muscle MLC 1 and MLC 3. RNA blot hybridizations using the rat skeletal muscle MLC 1 cDNA probe. MLC 1 and 3 are transcribed from the same gene and both mRNAs can be identified using the MLC 1 transcript. Only MLC 3 is seen in BC3H1 cultures under the conditions used. (A) BC3H1 cultures were plated at low density and grown in DME + 20% FCS for 2, 3, 4, 5, 7, 9, and 11 d (lanes 1-7, respectively). Cultures appeared confluent 5 d after plating. (B) Duplicate cultures were grown in DME + 20% FCS and subsequently switched to LSM for 48 h, 2, 3, 4, 5, 7, 9, and 11 d after plating (lanes 1-7, respectively).

that seen in rat aortic smooth muscle cells, where increasing amounts of RLC mRNA are detected at confluence (Taubman et al., 1987). Thus, BC3H1 cells express both sarcomeric and nonsarcomeric forms of myosin RLC, with the former present predominantly in the growth-arrested, differentiated state and the latter predominantly during rapid cell growth.

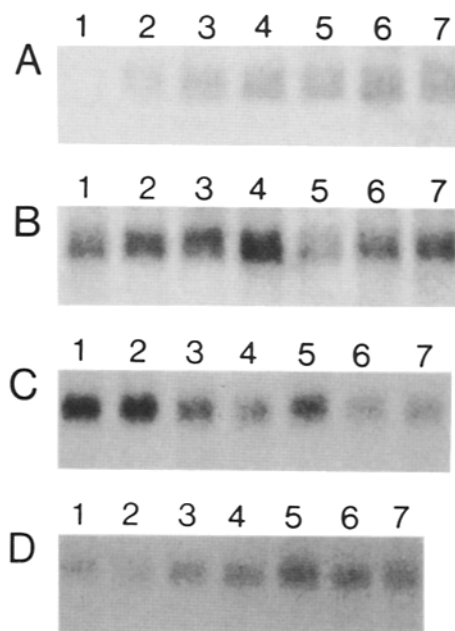
#### **Differentiation of BC3H1 Cells Is Accompanied by the Induction of a Sarcomeric Isoform and the Deinduction of a Nonsarcomeric Isoform of MHC**

Rat MHCs are members of a multigene family and are expressed in a developmental and tissue-specific fashion (for review see Mahdavi et al., 1986). RNA from BC3H1 cultures was hybridized with a cDNA probe encoding a rabbit aortic MHC isoform (gift of M. Periasamy, Department of Physiology and Biophysics, University of Vermont College of Medicine). At high stringency, this cDNA hybridizes strongly only to RNA derived from smooth muscle cells or tissues, but does hybridize weakly to RNA from nonmuscle or striated muscle (Nagai et al., 1988; our unpublished observations). Subconfluent growing BC3H1 cultures contained mRNA which hybridized with this smooth muscle MHC probe. As these cells approached confluence, the amount of hybridizing species was diminished (Fig. 7 A, lane 1-4). Switching subconfluent cultures to LSM also resulted in a diminution of this mRNA (Fig. 7 A, lanes 5-8). Duplicate blots were hybridized with a cDNA probe encoding an embryonic skeletal muscle MHC. This probe hybridizes to all sarcomeric muscle isoforms, but not to smooth muscle or nonmuscle isoforms (Wydro et al., 1983). Subconfluent growing BC3H1 cultures contained very small amounts of mRNA hybridizing to this cDNA probe (Fig. 7 B, lane 1). However, markedly increased amounts of mRNA were seen when cells approached confluence (Fig. 7 B, lanes 2-4) or when subconfluent cells were switched to LSM (Fig. 7 B, lanes 5-8).

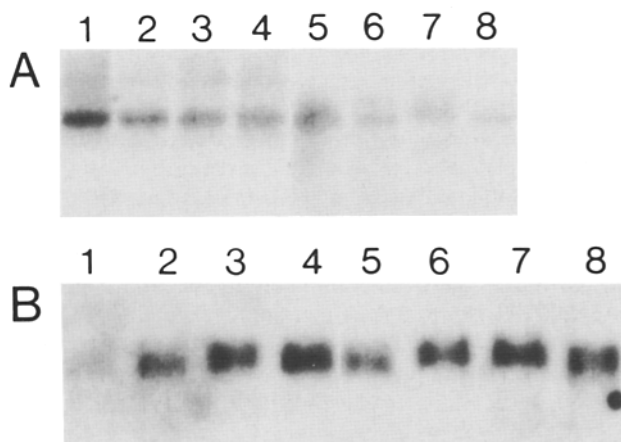
The results of hybridization with the embryonic skeletal muscle MHC probe are opposite those seen with the smooth muscle MHC cDNA. In particular, the strongest signal detected with the smooth muscle probe (Fig. 7 A, lane 1) occurs at a time when only a trace signal is noted with the embryonic skeletal MHC probe (Fig. 7 B, lane 1). This argues strongly that the predominant MHC mRNA detected by the smooth muscle MHC cDNA in growing subconfluent BC3H1 cultures is a nonsarcomeric isoform(s). In contrast, growth-arrested BC3H1 cells predominantly express a sarcomeric muscle MHC isoform(s). Differentiation of BC3H1 cells thus appears to be accompanied by the induction of a sarcomeric and deinduction of a nonsarcomeric MHC gene.

#### **BC3H1 Cells Express Sarcomeric Isoforms of $\alpha$ -Actin**

It has recently been reported that induction of BC3H1 cells results not only in the synthesis of vascular smooth muscle  $\alpha$ -actin, but of skeletal  $\alpha$ -actin as well (Reeser, J. C., and A. R. Strauch, unpublished results). RNA from BC3H1 cells was hybridized to probes specific for the 5' untranslated end of mouse cardiac  $\alpha$ -actin (Minty et al., 1983) and 3' untranslated end of mouse skeletal  $\alpha$ -actin (Hu et al., 1986)



**Figure 6.** Expression of skeletal muscle MLC and the smooth/non-muscle myosin RLC. RNA blot hybridizations using the rat skeletal MLC 2 (A and B) and aortic RLC (C and D) cDNA probes. The skeletal muscle MLC 2 probe is specific for sarcomeric MLC 2 mRNAs. The aortic RLC probe hybridizes with sarcomeric MLC 2 as well as smooth muscle and nonmuscle RLC mRNAs. However, the sarcomeric MLC 2 mRNAs can be distinguished on 1% agarose gels from the larger smooth/nonmuscle RLC mRNAs. Only the bands corresponding to the smooth/nonmuscle mRNAs are shown in C and D. (A and C) BC3H1 cultures were plated at low density and grown in DME + 20% FCS for 2, 3, 4, 5, 7, 9, and 11 d (lanes 1-7, respectively). Cultures appeared confluent 5 d after plating. (B and D) Duplicate cultures were grown in DME + 20% FCS and subsequently switched to LSM for 48 h, 2, 3, 4, 5, 7, 9, and 11 d after plating (lanes 1-7, respectively).



**Figure 7.** Expression of sarcomeric and nonsarcomeric MHCs. RNA blot hybridizations using rabbit smooth (A) muscle and rat skeletal muscle (B) MHC probes. The smooth muscle probe hybridizes strongly only with RNA derived from smooth muscle cells or tissues, but does give weak signals with RNA derived from sarcomeric and nonmuscle tissues. The skeletal muscle probe is specific for sarcomeric RNAs. Cultures were grown in DME + 20% FCS for 2, 4, 6, and 8 d (lanes 1–4, respectively). Cultures appeared confluent on day 5. Cultures were switched to LSM for 48 h, 2, 4, 6, and 8 d after plating (lanes 5–8, respectively).

(Fig. 8, A and B, respectively). Growing, but subconfluent, BC3H1 cells expressed neither isoform of  $\alpha$ -actin (Fig. 8, lanes 1 and 2). However, when cultures reached confluence (Fig. 8, lanes 3 and 4) or when subconfluent cultures were switched to LSM (Fig. 8, lanes 5 and 6), both cardiac and skeletal muscle  $\alpha$ -actin isoforms were expressed.

#### **The Contractile Protein mRNA Phenotype of Differentiated BC3H1 Cells Is Reversible**

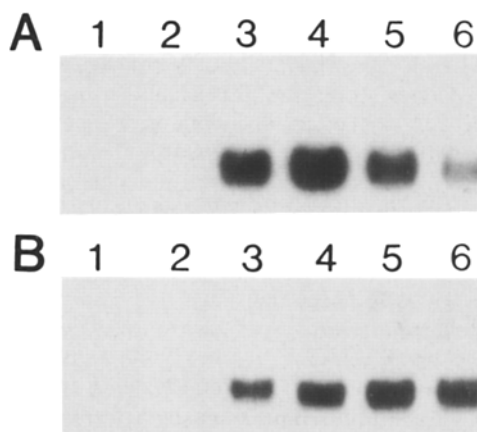
It has been demonstrated that the expression of MCK and  $\alpha$ -actin mRNAs in BC3H1 cells is reversible: they can be deinduced by either adding growth medium or growth factors to subconfluent, induced BC3H1 cultures or by replating induced cultures at low density in growth medium (Munson et al., 1982; Olson et al., 1983; Lathrop et al., 1985; Spizz et al., 1986). Similarly, the expression of the contractile protein mRNAs described above was reversible. When confluent, induced cultures were replated at low density in growth medium, TnT (Fig. 9 A) and embryonic skeletal muscle MHC (Fig. 9 F) mRNA levels were significantly decreased within 24 h of replating and were absent by 72 h. There was a rapid return of the nonmuscle  $\alpha$ -Tm isoform and a slower loss of the striated muscle isoform (Fig. 9 B). The long time courses of disappearance probably reflect the high levels and long half-lives ( $\sim 50$  h; Garfinkel, 1982; Medford et al., 1983) of these mRNAs. The deinduction of MLC 3 mRNA is likely rendered particularly slow by such a cause. When induced, confluent cultures were replated at low density in growth medium, there was a very slow loss of MLC 3 mRNA, with significant amounts still detectable at 72 h (Fig. 9 C, lanes 1–4). However, when induced, subconfluent cultures were replated at low density in growth medium, there was a more rapid loss of MLC 3 mRNA, with little detectable at 72 h (Fig. 9 C, lanes 5 and 6). As expected, replating

resulted in increased levels of the smooth/nonmuscle RLC (Fig. 9 D) and nonsarcomeric MHC (Fig. 9 E) mRNAs.

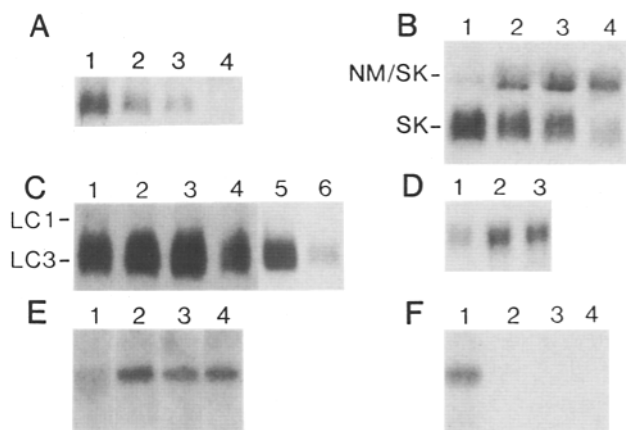
#### **Discussion**

The results presented above demonstrate that BC3H1 cells exhibit two distinct phenotypes of contractile protein mRNA expression. Subconfluent, growing cultures possess predominantly nonmuscle or smooth muscle mRNA isoforms of  $\alpha$ -Tm, MHC, and myosin RLC. Under these conditions, they have minimal or undetectable levels of mRNA for TnT, or sarcomeric muscle isoforms of MHC, MLCs, or  $\alpha$ -Tm. In contrast, BC3H1 cultures that are growth arrested either by confluency or by switching to a nongrowth (LSM) medium express predominantly sarcomeric muscle mRNAs. These include all the sarcomeric mRNAs tested: TnT and sarcomeric muscle isoforms of  $\alpha$ -Tm, MHC, MLC 2, and MLC 3. In the case of  $\alpha$ -Tm, MHC, and myosin RLC, the induction of the sarcomeric muscle isoform is associated with decreased expression of the smooth or nonmuscle isoform. The induced phenotype is reversible: replating BC3H1 cells in growth medium results in a loss of sarcomeric muscle and the reappearance of nonsarcomeric muscle contractile protein mRNAs.

The pattern of induction of contractile protein mRNAs in BC3H1 closely resembles that previously described for sarcomeric myoblasts. Myoblasts grown in the presence of sufficient growth factors remain in the proliferative state and do not express sarcomeric muscle-specific genes. When the concentration of growth factors is reduced, the cells cease DNA synthesis and begin to express sarcomeric muscle-specific genes, including MHC, MLCs,  $\alpha$ -actin,  $\alpha$ - and  $\beta$ -Tm, and TnT (Devlin and Emerson, 1978; Shani et al., 1981; Caravatti et al., 1982; Garfinkel et al., 1982; Nadal-Ginard et al., 1984). This pattern of contractile protein gene expression can occur even in the absence of fusion of myoblasts to



**Figure 8.** Expression of sarcomeric  $\alpha$ -actin isoforms. RNA blot hybridizations using cDNA probes from the 3' untranslated region of mouse skeletal (A) and 5' untranslated region of mouse cardiac (B)  $\alpha$ -actin (see Materials and Methods). Cultures were grown in DME + 20% FCS for 1, 3, 5, and 8 d (lanes 1–4, respectively). Cultures, grown for 1 d in DME + 20% FCS, were switched to LSM for 2 and 4 d (lanes 5 and 6, respectively).



**Figure 9.** Reversibility of contractile protein mRNA expression. RNA blot hybridization using the following probes: (A) TrT; (B)  $\alpha$ -Tm; (C) skeletal muscle MLC 1 and 3; (D) smooth/nonmuscle RLC; (E) embryonic skeletal muscle MHC; and (F) smooth muscle MHC. Cultures were grown in DME + 20% FCS for 5 d (lane 1). Duplicates of these confluent, induced cultures were then replated at low density in DME + 20% FCS for 1, 2, or 3 d (lanes 2-4, respectively). For MLC 1 and 3 (C), some cultures were also grown in DME + 20% FCS for 2 d and then induced by switching to LSM for 2 d (lane 5). Duplicate cultures were replated at low density and grown in DME + 20% FCS for 72 h (lane 6).

form myotubes (Nguyen et al., 1983; Endo and Nadal-Ginard, 1987). Moreover, the differentiated phenotype can be reversible: under the appropriate conditions, myoblasts can reenter the cell cycle and concomitantly deinduce the expression of the muscle-specific genes (Nguyen et al., 1983; Devlin and Konigsberg, 1983; Nadal-Ginard et al., 1984). In particular, the phenotype of BC3H1 cells closely resembles that of  $L_6E_9-3b_2$  cells (Nguyen et al., 1983). These cells are temperature sensitive for the commitment to terminal differentiation. At nonpermissive temperatures,  $L_6E_9-3b_2$  cells induce the sarcomeric contractile protein genes in the absence of cell fusion. Upon serum stimulation, they rapidly deinduce the contractile protein genes and reenter the cell cycle. BC3H1 cells thus behave like skeletal myoblasts that are defective for commitment to terminal differentiation. Like the  $L_6E_9-3b_2$  cells, they can reversibly express sarcomeric muscle-specific contractile protein genes.

The finding that differentiated BC3H1 cells produced the vascular smooth muscle isoform of  $\alpha$ -actin (Strauch and Rubenstein, 1984; Strauch et al., 1986) lent support to the speculation that BC3H1 cells were derived from vascular smooth muscle. We have found that BC3H1 cells express both cardiac and skeletal muscle  $\alpha$ -actin mRNA upon induction either by serum deprivation or reaching confluence. The pattern of expression of actin mRNA in BC3H1 cells is therefore similar to that seen in sarcomeric muscle (Garrels and Gibson, 1976; Whalen et al., 1976; Devlin and Emerson, 1978; Minty et al., 1982; Mohun et al., 1984). While growing, cells express  $\beta$ - and  $\gamma$ -actins; upon differentiation, they switch to sarcomeric  $\alpha$ -actin isoforms. The coexpression of the skeletal and cardiac isoforms of  $\alpha$ -actin is a common feature of both adult and fetal skeletal and cardiac muscle (Minty et al., 1982; Gunning et al., 1983; Mayer et al., 1984; Mohun et al., 1984; Paterson and Eldridge, 1984). While

smooth muscle  $\alpha$ -actin has not been found in skeletal muscle tissue, it has been found in dividing mouse L6 (Pinset and Whalen, 1984) and T984 (Buckingham et al., 1982) myoblasts. Upon differentiation, these cells ultimately express the sarcomeric forms of  $\alpha$ -actin. Thus, the presence of smooth muscle  $\alpha$ -actin in BC3H1 cells is not inconsistent with these cells possessing a predominantly sarcomeric contractile protein phenotype upon differentiation.

The work reported above suggests that the BC3H1 line behaves most like a nonfusing sarcomeric muscle cell, at least in regard to the expression of contractile protein genes. This is in agreement with previous findings that these cells express other proteins more typical of skeletal muscle, such as MCK, the nicotinic acetylcholine receptor and a skeletal muscle voltage-gated  $Ca^{2+}$  channel. BC3H1 cells were derived from a nitrosoethylurea-induced brain tumor (Schubert et al., 1974). Transplantation experiments have demonstrated that populations of neural crest cells can give rise to a wide variety of differentiated adult cell types (Noden, 1983; Le Douarin, 1986). Most significantly, neural crest cells are a source of craniofacial skeletal and connective tissues in the regions formed by frontonasal and branchial arch processes (for review see Noden, 1983) and contribute to the aorticopulmonary septum (Kirby et al., 1983). It has also been observed that when the rat neurotumor cell line RT4 is treated with 5-azacytidine, a variety of cell types can be isolated, one of which (RT4-B8) can form myotube-like structures and synthesize muscle-type myosin (Tomozawa, Y., unpublished observations). It is thus apparent that neural tissue can give rise to striated muscle and that the BC3H1 cell line may represent a nonfusing striated muscle cell. One cannot, however, disregard the possibility that BC3H1 represents a nonsarcomeric muscle cell whose transformation has resulted in its expression of sarcomeric contractile protein genes.

The BC3H1 culture system has proven of considerable value in the study of MCK and actin gene regulation (Olson et al., 1983; Strauch et al., 1986; Wang and Rubenstein, 1988). We have now demonstrated that a large battery of muscle-specific genes are regulated in BC3H1 cells. These include MHCs, MLCs,  $\alpha$ -Tm, TrT, and the skeletal and cardiac isoforms of  $\alpha$ -actin. The BC3H1 culture system should therefore provide an excellent model for studying the regulation of a variety of contractile protein genes in a milieu that allows for the reversible expression of both sarcomeric and nonsarcomeric forms.

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