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Density-Dependent Modulation of Vascular Smooth Muscle α-Actin Biosynthetic Processing in Differentiated BC3H1 Myogenic Cells

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Abstract The expression of vascular smooth muscle (VSM) α-actin mRNA during BC3H1 myogenic cell differentiation is specifically stimulated by conditions of high cell density. Non-proteolytic dissociation of cell-cell and cell-matrix contacts in post-confluent cultures of BC3H1 myocytes using EDTA promotes loss of the differentiated morphological phenotype. EDTA-dispersed myocytes exhibit an undifferentiated fibroblastoid appearance and contained reduced levels of both VSM and skeletal α-actin mRNA. Muscle α-actin mRNA levels in EDTA-dispersed myocytes were not restored to that observed in confluent myocyte preparations by experimental manipulation of cell density conditions. Pulse-labeling techniques using L-[^{35}S]cysteine to identify muscle actin biosynthetic intermediates revealed that EDTA-dispersed myocytes expressed nascent forms of both the VSM and skeletal muscle α-actin polypeptide chains. However EDTA-dispersed myocytes were less efficient in the post-translational processing of immature VSM α-actin compared to non-dispersed myocytes. Simple cell-to-cell contact may mediate VSM α-actin processing efficiency since high-density preparations of EDTA-dispersed myocytes processed more VSM α-actin intermediate than myocytes plated at low density. The actin isoform selectivity of the response to modulation of intercellular contacts suggests that actin biosynthesis in BC3H1 myogenic cells involves mechanisms capable of discriminating between different isoform classes of nascent actin polypeptide chains.

Key words: actin, muscle cells, differentiation, cell contacts, peptide mapping, posttranslational control, EDTA

Actin gene expression is modulated during vertebrate myogenesis largely by transcriptional control mechanisms. While the molecular biological aspects of actin gene transcription have been described in some detail [Carroll et al., 1988; Elder et al., 1988; Min et al., 1990; Sartorelli et al., 1990; Lee et al., 1991], comparatively little is understood about the extracellular signals which initiate changes in actin gene expression during muscle development. Microenvironmental conditions have been shown to influence the growth, differentiation, and protein biosynthetic properties of muscle cells particularly smooth muscle cells present in the blood vessel wall [Davies, 1986; Schwartz and Reidy, 1987; Carey, 1991]. A variety of growth stimulatory factors such as platelet derived growth factor [Blank and Owens, 1990], fibroblast growth factor [Klagsbrun and Edelman, 1989], insulin-like growth factor-I [Elgin et al., 1987], and transforming growth factor β [Chen et al., 1987; Goodman and Majack, 1989], as well as growth inhibitory basement membrane constituents including laminin [Hedin et al., 1988] and heparan sulfate glycosaminoglycans [Castellot et al., 1987; Clowes et al., 1988; Desmoullière et al., 1991], all have observable effects on gene expression in vascular smooth muscle cells. Developmentally balanced levels of growth-permissive and growth-restrictive substances in the vessel wall may permit the controlled expansion of phenotypically stable, contractile-stage vascular smooth muscle cells during embryogenesis or collateral formation associated with normal tissue repair and remodelling processes. Likewise, maladaptations which affect the blood vessel wall microenvironment may contribute to the

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onset and progression of proliferative vascular diseases such as arteriosclerosis, which has been shown to involve altered patterns of actin expression [Kocher and Gabianni, 1987].

Expression of the vascular-specific isoform of α-actin by cultured smooth muscle cells [Owens et al., 1986; Blank et al., 1988; Liau and Chan, 1989; Strauch et al., 1989] appears to be stimulated at high cell density by a mechanism that operates independently from density arrest of cell growth. Vascular α-actin levels in vivo also were significantly reduced when smooth muscle myocytes in the tunica media were stimulated to dissociate from neighboring cells during atherogenesis provoked by experimental endothelial cell denudation [Barja et al., 1986; Kocher and Gabbiani, 1987; Schwartz et al., 1986; Clowes et al., 1988]. Expression of VSM α-actin polypeptide in the mouse BC3H1 myogenic cell line also was shown to be induced by high cell density conditions [Strauch and Rubenstein, 1984a; Strauch et al., 1986; Strauch and Reeser, 1989]. Cytodifferentiation of postconfluent BC3H1 myoblasts appears to require the accumulation of differentiation-permissive macromolecules on the culture substrate [Strauch et al., 1991a]. The demonstrated ability of BC3H1 myogenic cells to respond dynamically to both positive and negative microenvironmental cues points to their utility as a unique model system for basic studies aimed at understanding how modulation of cell-cell and cell-substrate contact affects expression of the VSM α-actin gene [Min et al., 1990].

In this report we present evidence showing that high cell density selectively stimulates expression of VSM α-actin mRNA during BC3H1 myogenic cell differentiation. Non-proteolytic dispersion of cell-to-cell and cell-to-matrix contacts in post-confluent BC3H1 myocyte preparations reduces the level of muscle actin transcripts and specifically attenuates the rate of VSM α-actin post-translational biosynthetic processing. Alteration of cell to cell interactions during myoblast cytodifferentiation may represent a fine tuning mechanism for governing the production and/or stability of VSM α-actin polypeptides in BC3H1 cells. Since dispersed BC3H1 myocytes exhibit an undifferentiated fibroblastic appearance, the continuous expression of fully processed VSM α-actin polypeptides may be important for maintaining the cytoarchitectural features of differentiated myocytes. The BC3H1 myogenic cell line may be useful for investigating how modulation of cell-to-cell contacts influences muscle cell phenotypic switching in a manner that does not necessarily promote the irreversible loss of the ability to express VSM α-actin mRNA and protein.

METHODS

Cell Culture Methods

BC3H1 myogenic cells were obtained from Dr. David Schubert, the Salk Institute, LaJolla, CA and maintained in logarithmic stage growth as previously described [Schubert et al., 1974; Strauch and Rubenstein, 1984a] using Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco/BRL, Grand Island, NY). Undifferentiated myoblasts were continuously maintained below 40% confluency by passaging cultures using a 1:9 split ratio to minimize the formation of extensive cell-cell contacts and prevent premature cytodifferentiation. Culture medium was replaced every third day. To initiate the cytodifferentiation process, post-confluent BC3H1 myoblasts were treated for the times indicated in the text with hormone-supplemented, serum-free N2 medium [Bottenstein and Sato, 1982] containing RPMI 1640, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), pH 7.2 (Sigma Chemical Company, St. Louis, MO), 5 μM bovine serum albumin (Pentex, fatty acid free, ICN Biochemicals, Costa Mesa, CA), 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite (all from Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin/100, and μg/ml streptomycin (Gibco/BRL, Grand Island, NY). EDTA-dispersed myocytes (EMC) were prepared from fully differentiated myocyte cultures following a 5 day exposure to N2 medium. Myocyte dispersion was accomplished by adding 4 ml of 0.1% EDTA in Dulbecco's calcium- and magnesium-free phosphate buffered saline (PBS) to each 100 mm tissue culture dish. Following a 15 min incubation at 37°C, the detached myocyte aggregates were collected, transferred to a 50 ml polypropylene centrifuge tube containing 25 ml of 0.1% EDTA, and further processed by repeated trituration using a standard bore size, 10 ml serological pipette and moderate speed vortexing (1 s bursts) until a uniform suspension of individual myocytes was obtained as determined by phase-contrast microscopy. Clumps of myocytes that could not be completely dispersed were removed after settling to the bottom of the tube during a 10 min incubation at 1g. EMC

were collected by centrifugation (10 min at 250g), diluted to the required density with N2 medium and seeded into multiple 100 mm tissue culture dishes. The dishes were inspected after 24 h and only those containing EMC exhibiting minimal cell to cell contact were selected for further analysis.

Actin Isoform N-Terminal Peptide Analysis

For isotope pulse labeling studies, BC3H1 cells were exposed to cysteine-free N2 medium containing 120 μCi/ml L-[^{35}S]cysteine (600 Ci/mmol, Amersham Corp., Arlington Heights, IL) for 20–50 min at 37°C [Strauch and Rubenstein, 1984b; Strauch and Reeser, 1989]. For some experiments, radiolabeled cells were rinsed three times following the pulse and then chased for 18 h in complete N2 medium to allow for post-translational processing of the newly synthesized actins. Radiolabeled cells were collected into 4 ml of ice-cold actin depolymerization buffer containing 0.75 M guanidine hydrochloride, 0.5 M sodium acetate, 10 mM Tris-HCl, pH 8.0, 0.5 mM calcium chloride, 0.5 mM ATP, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride and homogenized on ice by sonication for 45 s [Strauch and Rubenstein, 1984a]. Homogenates were repeatedly passed (a total of four times) over individual 1 ml bed volume DNAse-I agarose affinity columns and bound actin monomers were released from the washed column bed using elution buffer containing 3 M guanidine hydrochloride, 0.5 M sodium acetate, and 2 mM calcium chloride. Actin samples were dialyzed against distilled water, lyophilized to dryness, performic acid oxidized, digested with TPCK-trypsin (Worthington, Freehold, NJ), and analyzed by pH 6.3 cellulose thin layer electrophoresis (TLE) as previously described [Vandekerckhove and Weber, 1981; Strauch and Rubenstein, 1984a]. Selected actin N-terminal peptides were eluted from the cellulose plates with water, lyophilized, and analyzed by pH 3.3 TLE to distinguish peptides derived from vascular smooth and skeletal muscle α-actins from those corresponding to non-muscle β- and γ-actins [Strauch and Reeser, 1989]. The relative amount of actin N-terminal peptide in each preparation was estimated by computer-assisted image analysis of thin layer plate autoradiographs using a Tracor Northern TN8502 imaging system and IPa85 software (Tracor Northern, Middleton, WI).

Preparation of a Mouse VSM α-Actin cDNA Probe

A lambda gt10 cDNA library was prepared from poly(A)+ RNA that was isolated using the procedure of Chirgwin et al., [1979] from post-confluent BC3H1 cells following a 48 h exposure to N2 serum-free differentiation medium. Replica nitrocellulose lifts prepared from each 150 mm agar plate of phage were processed as described by Benton and Davis [1977], prehybridized for at least 2 h at 42°C in BLOTTO solution (6× SSC, 20 mM sodium phosphate, pH 6.5, 0.25% instant non-fat dry milk, 50% de-ionized formamide), and hybridized for 12 h at 42°C in BLOTTO solution containing a [^{32}P]dATP (3,000 Ci/mmol, Amersham)-labeled [Feinberg and Vogelstein, 1983] 1,250 bp *Sac*I-*Sac*II coding region subfragment of a full-length human skeletal α-actin cDNA (pHMαA1, provided by Dr. L. Kedes, University of Southern California). Positive actin clones were amplified by the plate lysate method described by Maniatis et al., [1982]. Purified phage (10^8 phage/clone) and 10 ng samples of actin cDNAs (provided by Dr. L. Kedes) encoding the 3′-UT regions of human fibroblast β-actin (pHFβA-3′UT), human fibroblast γ-actin (pHFγA-3′UT), human skeletal α-actin (pHMαA1-3′UT), and human cardiac α-actin (pHMcA-3′UT) were loaded into adjacent slots of a slot-blot apparatus (Schleichter and Schuell, Keene, NH) fitted with a 0.45 μm nitrocellulose filter. Replicate air-dried blots were denatured, neutralized, baked, pre-hybridized with BLOTTO solution as described above, and each was then probed with a different 3′-UT region actin cDNA from the above list. A VSM α-actin cDNA clone, by virtue of its unique 3′-UT region sequence [Carroll et al., 1986; Min et al., 1988], should not hybridize to any of these diagnostic 3′-UT actin probes. The largest actin cDNA insert from a lambda gt10 clone, which appeared to be specific for VSM α-actin, was excised by *Eco*R1 digestion and subcloned into pUC118 and pUC119 (derivatives of pUC18 and pUC19 respectively that contain the intergenic region of bacteriophage M13) to prepare single strand template [Schreier and Cortese, 1979] for DNA sequence analysis using the dideoxy chain termination method [Sanger et al., 1977]. One 1,100 bp cDNA insert that was determined by sequence analysis to encode VSM α-actin was subcloned into the *Eco*R1 site of the pGEM3Zf(−) Riboprobe Gemini™ vector

(Promega, Madison, WI) and designated pMαVSM-1.

Northern Blot and Transcription Run-On Analyses of Actin mRNA

Total cellular RNA was purified from BC3H1 cell preparations using the acidic guanidinium thiocyanate method [Chomczynski and Sacchi, 1987] and analyzed by electrophoresis on 1.4% agarose gels containing 6.5% de-ionized formaldehyde as described previously [Corces et al., 1981; Strauch et al., 1986]. Nitrocellulose blots were prehybrized for 2 h at 44°C in a diethylpyrocarbonate-treated (1%) BLOTTO solution containing 0.25% non-fat dry milk, 6× SSC, and 50% freshly de-ionized formamide [Siegel and Bresnick, 1986]. The filters were hybridized for 16 h in the same solution containing one of the following random oligonucleotide-labeled [Feinberg and Vogelstein, 1983] [^{32}P]dATP probes as indicated in the Results: 1) a gel-purified (NuSieve agarose, FMC Corp., Rockland, ME), 160 bp subfragment from pMαVSM-1 containing only the 3′-UT region sequence from the mouse VSM α-actin cDNA. 2) A gel-purified 688 bp subfragment of pEMSV688SK (J.C. Reeser, Ph.D. thesis) encompassing the 5′-UT region (exon 1) with the adjacent 5′-flanking region and intron 1 sequences from the mouse skeletal α-actin gene [Sharp et al., 1989; Hu et al., 1986]. This 688 bp fragment was subcloned from the parent M13 clone 18-5 (generously provided by Dr. Sandra Sharp, California State University at Los Angeles) and placed into pEMSV to facilitate plasmid propagation. A 175 bp 3′-UT region probe from a mouse skeletal muscle α-actin cDNA (pJ3′, provided by Dr. S. Sharp) also was used for some northern blot analyses (quantitative comparison of skeletal and VSM α-actin mRNA levels shown in Fig. 1). Hybridizations were performed at 44°C using BLOTTO solution containing approximately 5 × 10^6 CPM of radiolabeled probe. The blots were washed four times at 22°C with 2× SSC, twice at 63°C in 0.2× SSC, 0.1% SDS, air dried, and exposed for varying periods to Kodak XAR-5 film at −70°C using Cronex Lightening Plus intensifying screens (DuPont, Wilmington, DE). The amount of non-muscle and muscle actin mRNAs on blot autoradiographs was estimated by computer-assisted image analysis as described above. Exposure periods were selected which fell within the linear response range of the film.

Procedures outlined in Ausubel et al., [1987] were used for nuclear run-on studies of actin mRNA transcription. BC3H1 cells at various developmental stages were scraped into cold PBS, collected into a pellet by low-speed centrifugation, and extracted in 4 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO). Lysis buffer was added while vortexing which continued for 10 s after addition. An additional 4 ml of lysis buffer was added following a 5 min incubation on ice and the suspension was centrifuged at 500g for 5 min at 4°C to sediment cell nuclei. The resulting pellet was gently resuspended in 200 μl of glycerol storage buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), transferred to sterile microcentrifuge tubes, frozen in liquid nitrogen, and stored at −70°C until needed. Run-on transcription assays were performed as described by Ausubel et al. [1987] and terminated by a 5 min treatment at 30°C with a high salt quenching buffer (0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, and 300 units/ml of RNase-free DNase-I) followed by 20 mg/ml proteinase K in 5% SDS, 0.5 M Tris-HCl, pH 7.4, and 0.125 M EDTA for 30 min at 42°C. After phenol/chloroform/isoamyl alcohol extraction, radiolabeled RNA in the aqueous phase was precipitated with 10% trichloroacetic acid/60 mM sodium pyrophosphate and collected onto Whatman GF/A (Whatman, Maidstone, England) glass fiber filters. Filters were transferred to glass scintillation vials and incubated with 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$, containing 25 μg/ml DNase-I for 30 min at 37°C. The digestion was terminated by addition of EDTA to 15 mM and SDS to a final concentration of 1% and the filters were heated to 65°C to release the RNA which was collected, digested with proteinase K, extracted with phenol/chloroform, denatured with 0.2 M NaOH, neutralized with 1 M HEPES, and ethanol precipitated. Aliquots of RNA containing equivalent amounts of radioactivity was diluted and then hybridized for 36 h at 65°C to nitrocellulose slot blots previously prepared by applying plasmid DNA (5 μg per slot) containing exon 1/intron 1 specific sequences (5′-end probes) for either the VSM [pSB12b-8-hGH, Min et al., 1990] or skeletal (pEMSV688SK, as described above) α-actin genes. Following hybridization, the filters were washed twice with 0.2× SSC at 65°C and then treated for 30 min at 37°C with RNase A (0.01 mg/ml in 2× SSC) to remove non-specifically bound

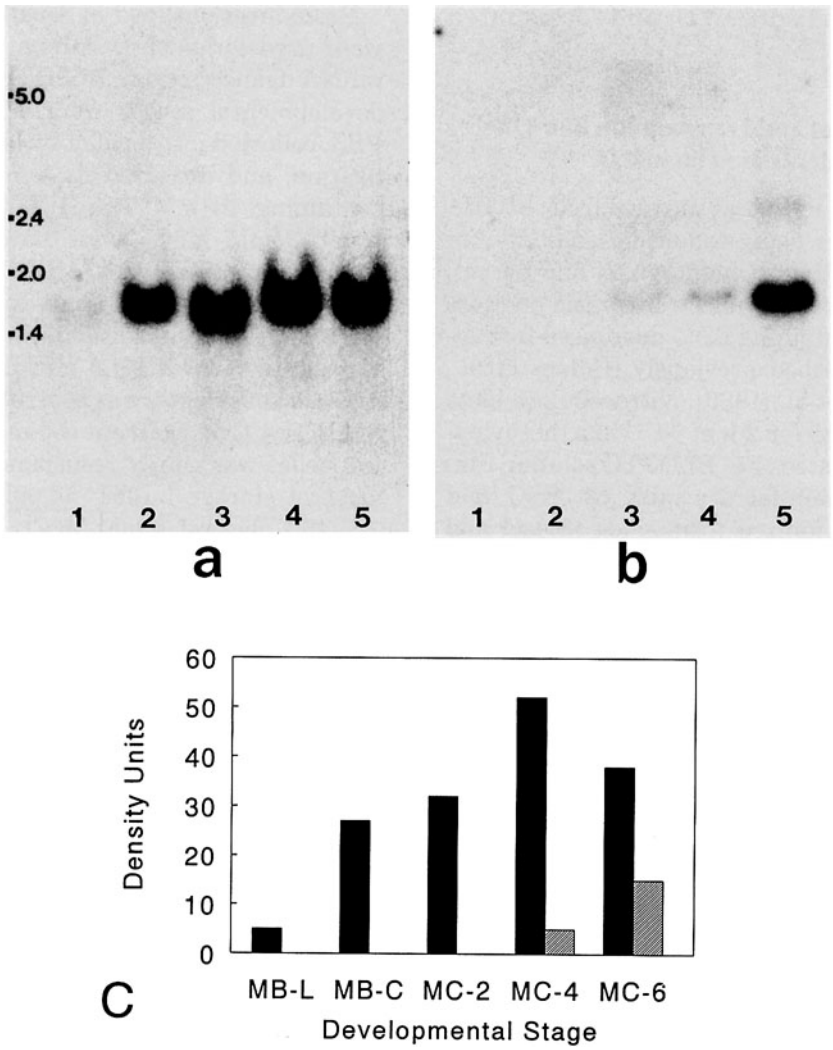

Fig. 1. Northern blot analysis of VSM and skeletal α-actin mRNA levels during BC3H1 cell differentiation. **a:** Total RNA (20 μg/lane) from subconfluent myoblasts (**lane 1**), confluent myoblasts (**lane 2**), and post-confluent myocytes induced by 2, 4 or 6 day treatments with N2 serum-free medium (**lanes 3–5,** respectively) probed with the 3′-UT subfragment of pMαVSM-1. **b:** Thirty microgram samples of total RNA from subconfluent myoblasts (**lane 1**), confluent myoblasts (**lane 2**), and post-confluent myocytes induced by 4 or 6 day treatments with N2 medium (**lanes 3 and 4,** respectively) probed with a mouse skeletal α-actin 3′-UT cDNA probe. Total RNA from mouse skeletal muscle tissue (10 μg) was included in (b), **lane 5** as a positive control. The relative amount of α-actin on each blot was estimated by densitometry and is presented in (c). Y-axis, level of expression in arbitrary units; MB-L, log phase myoblasts; MB-C, confluent myoblasts; MC-2,4,6, myocytes induced by 2, 4, or 6 days of treatment with N2 medium. Closed bars denote the level of VSM α-actin mRNA and cross-hatched bars indicate skeletal α-actin mRNA levels.

single-stranded RNA. Blots were exposed to X-ray film for periods within the linear response range of the film and hybridization was evaluated using computer assisted image analysis as described above.

RESULTS

Northern blot analysis employing a 3′-UT subfragment probe from a mouse VSM α-actin cDNA clone (pMαVSM-1) was used to examine relationships between VSM α-actin mRNA levels and cell density during BC3H1 myoblast cytodifferentiation (Fig. 1). Total RNA was isolated from subconfluent BC3H1 myoblasts during logarithmic phase of growth, confluent myoblasts, and post-confluent myoblasts induced to differentiate into myocytes by cultivation in N2 serum-free differentiation medium for 2, 4, or 6 days. The VSM α-actin probe hybridized exclusively to a developmentally regulated 1500 nucleotide

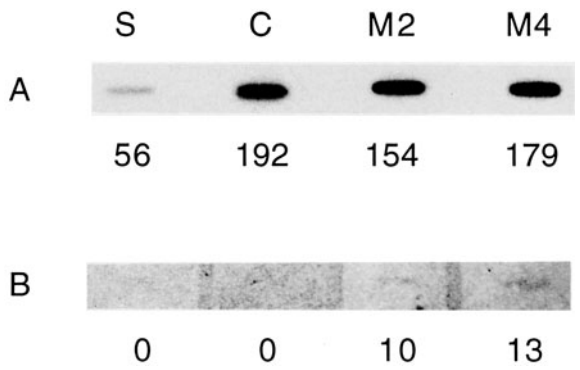

Fig. 2. Run-on transcription assay of VSM and skeletal muscle α-actin mRNA during BC3H1 cell differentiation. Run-on assays were performed on nuclei isolated from subconfluent myoblasts (S), confluent myoblasts (C), and post-confluent myocytes induced by 2 day (M2) or 4 day (M4) treatment with N2 medium. Slots contain 5 μg of 5′-end subfragments from either the mouse VSM α-actin gene (row **A**) or mouse skeletal α-actin gene (row **B**). Numbers shown below each slot blot series indicate densitometric values in arbitrary units. The hybridization signal corresponding to skeletal α-actin gene transcripts, although barely detectable on high contrast photographic images prepared from slot blot autoradiographs (row B), was clearly discernable on original autoradiographs using computer-assisted image analysis.

RNA previously shown by in vitro translation techniques to encode an α-actin polypeptide [Strauch et al., 1986]. Confluent BC3H1 myoblasts in serum-supplemented medium accumulated approximately eight times more VSM α-actin mRNA than sub-confluent myoblasts (Fig. 1a,c). Although serum withdrawal did not appear to be an absolute requirement for induction of VSM α-actin mRNA expression in BC3H1 myoblasts, exposure of confluent cells to N2 differentiation medium did elicit a slight additional increase in the level of this mRNA (Fig. 1a,c). Sustained exposure to serum-free medium caused a slight reduction in VSM α-actin mRNA expression. In contrast, accumulation of skeletal α-actin mRNA in confluent BC3H1 cells required prolonged exposure of the cells to serum-free medium (Fig. 1b,c). In numerous analyses of actin mRNA expression, skeletal muscle α-actin mRNA has not been observed to be induced in BC3H1 myoblasts by high cell density alone. Nuclear run-on assays were performed using similar sized, 5′-untranslated region probes to examine whether α-actin mRNA transcriptional activity was enhanced in confluent myoblasts. As shown in Figure 2, VSM α-actin was significantly enhanced in confluent myoblasts and remained elevated during myoblast maturation into myocytes. In contrast, transcriptional activity from the skeletal α-actin gene was not evident in confluent cells and could barely be detected in nuclei isolated from confluent myoblasts that were treated with N2 differentiation medium for either 2 or 4 days.

Data presented in Figures 1 and 2 implied that de novo induction of VSM α-actin gene expression in BC3H1 myoblasts was an early event in the cytodifferentiation process perhaps stimulated by developing cell-to-cell or cell-matrix contacts. To determine if maintenance of a high cell density state was essential to sustain expression of VSM α-actin mRNA in BC3H1 cells, we examined how non-proteolytic cell dispersion effected actin mRNA levels in differentiated, post-confluent BC3H1 myocytes. Treatment with calcium- and magnesium-free, phosphate-buffered saline containing 0.1% EDTA released the myocytes from culture dishes as a continuous sheet of cells within 5 min at 37°C. Trituration was employed to fragment the detached myocyte sheet and disperse variably sized clumps of cells which insured that cell-to-cell linkages were disrupted prior to re-seeding the cells at low density in N2 differentiation medium. Dispersion of post-confluent cells caused a uniformly rapid and extensive change in myocyte morphology (Fig. 3). EDTA-dispersed myocytes (EMC) re-attached in N2 medium within 30 min after treatment and exhibited a fibroblastoid appearance that closely resembled that of immature, proliferating myoblasts cultivated in serum-supplemented medium (compare Fig. 3b,c). The results of northern blot analysis shown in Figure 4 indicated that after 48 h these cells were still expressing both VSM and skeletal α-actin mRNA, although at significantly reduced levels compared to high-density myocytes. EMC retained about 70% of the amount of VSM α-actin observed in non-dissociated myocytes, whereas 50% of the skeletal α-actin mRNA present in non-dissociated myocytes was retained by EMC. Dissociated myocytes seeded at low density in N2 medium remained quiescent during the 48 h observation period prior to RNA isolation in accordance with our previous findings on the behavior of non-proteolytically dispersed BC3H1 cells in N2 medium [Strauch et al., 1991a].

Dissociation of confluent myocyte preparations with EDTA results in loss of both cell-cell and cell-matrix contacts. Previously we showed that substrate-associated macromolecules (SAM) present in BC3H1 myocyte cultures were capa-

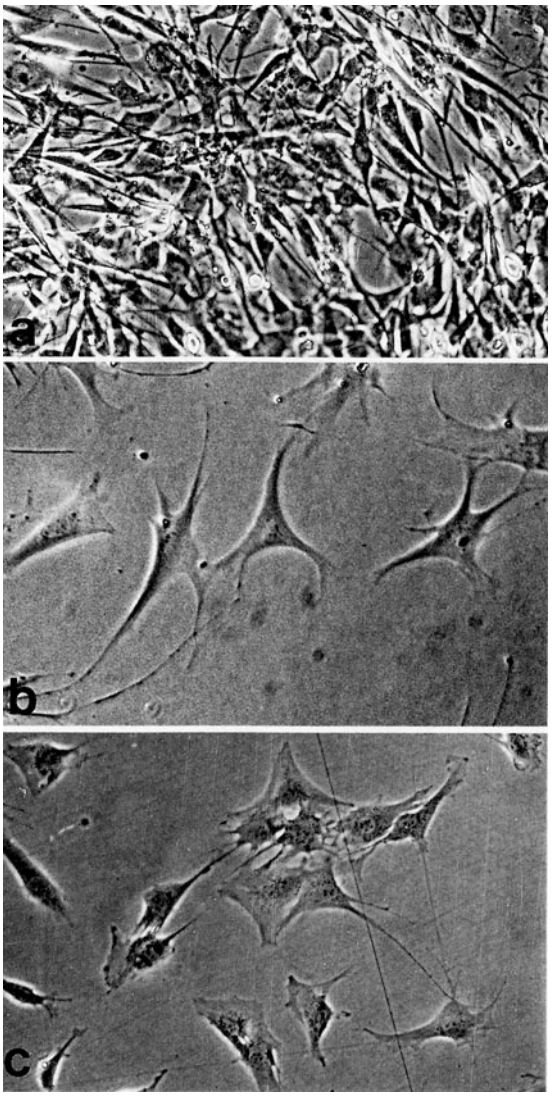

Fig. 3. Light micrographs depicting morphological features of BC3H1 myogenic cell preparations. **a:** Post-confluent myocytes induced by a 6 day treatment with N2 serum-free medium. **b:** Quiescent myocytes prepared as in (a) but 24 h after dispersion with EDTA. **c:** Proliferating undifferentiated myoblasts in medium containing 10% serum. Scale: 1 cm = 34 μm.

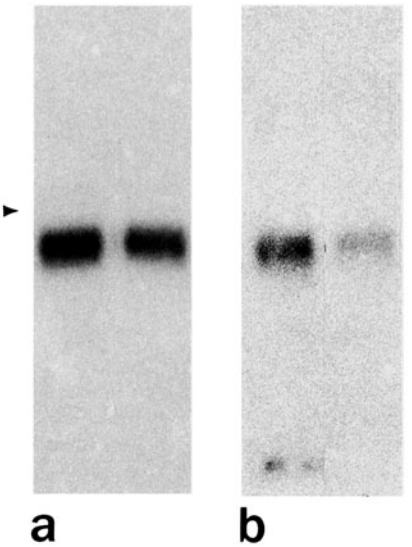

Fig. 4. Northern blots depicting α-actin mRNA levels in normal and EDTA-dispersed BC3H1 myocytes. Total RNA (5 μg/lane) isolated from normal myocytes or from myocytes 2 days after dispersion with EDTA was probed for α-actin mRNA using either VSM (**a**) or skeletal (**b**) α-actin gene specific probes. The normal myocyte RNA samples appear on the left side and RNA samples from EMC are shown on the right side of each panel. The arrow denotes the position of the 2 kb RNA size marker.

ble of initiating myoblast cytodifferentiation in the absence of detectable cell-to-cell contact [Strauch et al., 1991a]. To study the contribution of cell-to-cell contact in governing VSM α-actin expression in BC3H1 cells independently from effects that are mediated by SAM, we replated EMC immediately after dispersion at two different densities into fresh tissue culture dishes and measured actin mRNA levels following a 48 h period to allow for reconstitution of cell-cell contacts. Under these particular experimental conditions, SAM would not be present since this material accumulates during the pre-/post-confluent myoblast period of cytodifferentiation [Strauch et al., 1991b] and thus would not be carried over into fresh culture dishes by the myocytes following EDTA treatment. EDTA effectively releases myocytes from SAM which remains adhered to the original plastic culture substrate in a biologically active form [Strauch et al., 1991a]. As shown in Figure 5, reconstitution of cell to cell contacts during the 48 h post-dissociation recovery period did not have a significant restorative effect on VSM α-actin mRNA levels in EMC. The level of VSM α-actin mRNA was not significantly different between high density (undiluted cells, HD-EMC) and low density (1:4 dilution of cells, LD-EMC). LD-EMC did not exhibit extensive cell-to-cell contacts. Interestingly, HD-EMC exhibited a more differentiated, highly elongated appearance compared to sparsely seeded LD-EMC (data not shown) implying that cytoarchitectural organization may be responsive to intercellular contacts which are restored in the high-density preparations during the post-dispersion recovery period. To investigate whether α-actin polypeptide biosynthetic mechanisms were responsive to cell density conditions, HD- and LD-EMC preparations were pulsed-labeled with

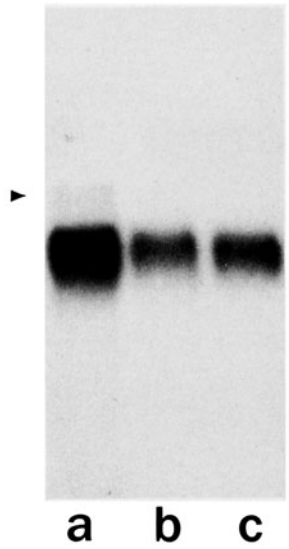

Fig. 5. Northern blot showing VSM α-actin mRNA levels in high and low density EDTA-dispersed myocytes. Myocytes were dispersed with EDTA, collected, and re-plated at their original high density into fresh culture dishes using 10 ml of fresh N2 medium (**lane b**) or diluted 1:4 with N2 medium prior to replating at low density (**lane c**). After 2 days, VSM α-actin mRNA levels were examined in each EMC preparation and compared to the level of expression observed in normal, non-dispersed myocytes (**lane a**). The arrowhead denotes the position of the 2 kb RNA size marker.

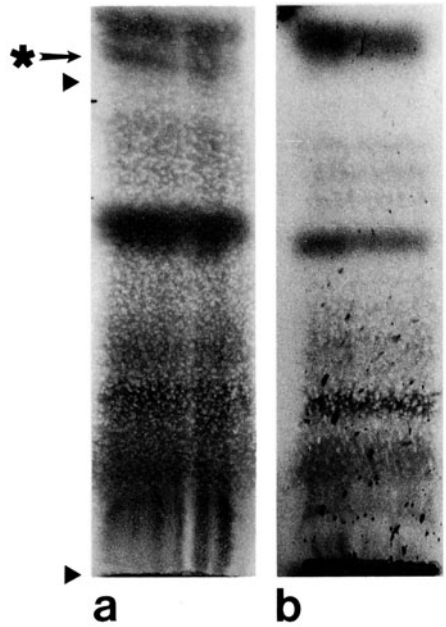

Fig. 6. Thin layer electrophoretograms showing actin N-terminal peptides derived from L-[^{35}S]cysteine pulse-labeled BC3H1 myocytes. **a:** Non-dispersed, normal myocytes. **b:** EDTA-dispersed myocytes (1:4 dilution). The asterisk denotes the position of the N-terminal peptide derived from the mature form of VSM α-actin which is detected in non-dispersed myocytes (a) but not EMC (b). The upper and lower arrowheads indicate the positions of the orange G marker dye front and origin of electrophoresis, respectively.

L-[^{35}S]cysteine and processed for actin N-terminal peptide analysis [Vandekerckhove and Weber, 1981; Strauch and Rubenstein, 1984a] to identify all newly synthesized actin isoforms. We previously showed that unprocessed muscle α-actin biosynthetic intermediates contain acetylcysteine at the amino terminus and represent the most abundant forms of α-actin expressed by pulse-labeled post-confluent BC3H1 myocytes [Strauch and Rubenstein, 1984b, Strauch and Reeser, 1989]. As shown in Figure 6, precursor-product conversion occurs fairly rapidly in post-confluent myocytes and approximately 20% of the total VSM α-actin precursor was found to be converted to the mature form during a 30 min pulse-labeling period. In contrast, LD-EMC did not express fully processed VSM α-actin polypeptide even though the amount of unprocessed VSM α-actin in these cells was comparable to the level observed in HD-EMC (Fig. 6). The altered rate of VSM α-actin processing between the two preparations of EMC appears to be related to cell density conditions alone and does not represent a non-specific effect of EDTA treatment, since EMC replated at high density were capable of converting a portion of the newly synthesized VSM α-actin intermediate into the mature form during the pulse labeling period (Fig. 7). Approximately 45% of the total VSM α-actin in HD-EMC was converted to the mature form during a 50 min pulse period whereas the VSM α-actin pool in LD-EMC remained largely unprocessed (Fig. 7). Differences in the efficiency of actin processing between HD-EMC and LD-EMC were more clearly evident when all the actin N-terminal peptides resolved at pH 6.3 were eluted for secondary analysis at pH 3.3, which permits better resolution of VSM and skeletal α-actin peptide species (Fig. 8). The results suggested that cell density-dependent actin processing was isoform selective. Preventing the restoration of cell-to-cell contacts following dispersion of myocyte preparations with EDTA appears to specifically inhibit the rate of VSM α-actin processing since the mature form of skeletal α-actin, as well as mature forms of non-muscle β- and γ-actins, appeared in both HD- and LD-EMC preparations (Fig. 8). Following the pulse labeling period, 32% and 36% of the total newly synthesized skeletal α-actin was estimated to be present in the mature form in HD- and LD-EMC, respectively.

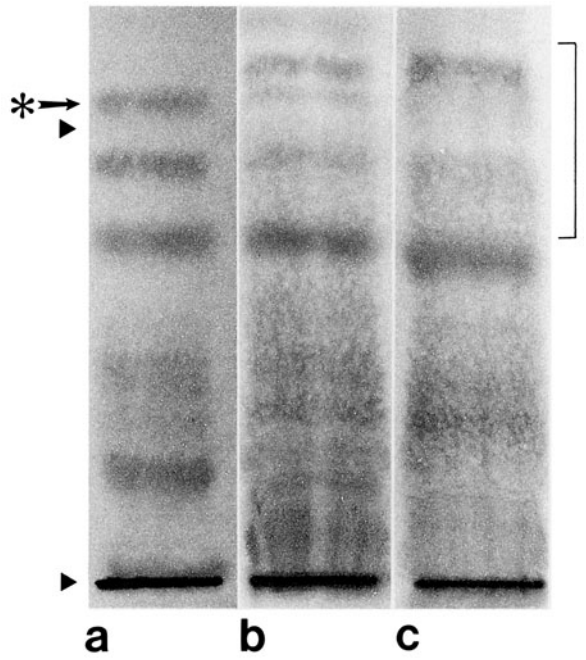

Fig. 7. Thin layer electrophoretograms depicting actin N-terminal tryptic peptides isolated from L-[^{35}S]cysteine-labeled EDTA-dispersed myocytes after culture at two different densities. Actin peptides also were prepared from pulse-labeled and chased (24 h), post-confluent myocytes (**a**) which revealed the fully processed form of VSM α-actin (asterisk). Pulse-labeled (no chase) high-density EMC contained both unprocessed and mature forms of the VSM α-actin N-terminal peptide (**b**), whereas similarly radiolabeled EMC plated immediately following a 1:4 dilution expressed predominantly the unprocessed form of VSM α-actin (**c**). The brackets denote the area of the pH 6.3 cellulose thin layer plate that was recovered and processed for secondary electrophoretic analysis at pH 3.3 (shown in Fig. 8). Upper and lower arrowheads indicate the positions of the orange G marker dye front and origin of electrophoresis, respectively.

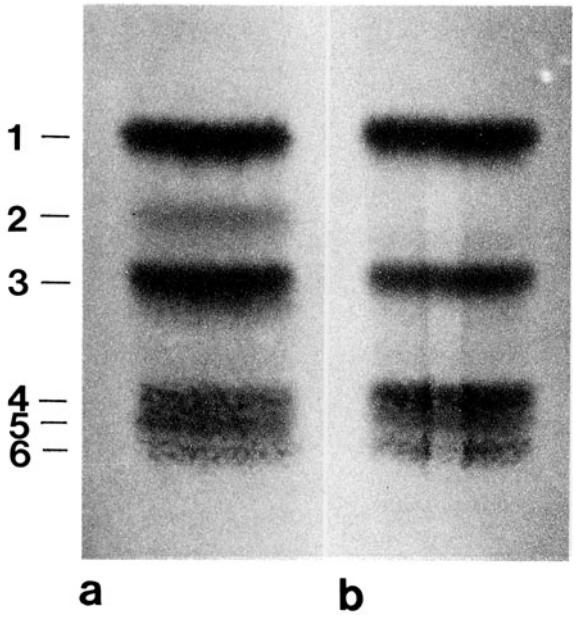

Fig. 8. Thin layer electrophoretogram depicting actin N-terminal peptides resolved from material eluted from pH 6.3 electrophoretograms (see bracketed area in Fig. 7). N-terminal peptides detected in high-density and low-density EMC are shown in (**a**) and (**b**), respectively. Peptide 1, unprocessed VSM α-actin; peptide 2, mature VSM α-actin; peptide 3, unprocessed skeletal α-actin; peptide 4, mature non-muscle β-actin; peptide 5, mature skeletal α-actin; peptide 6, mature non-muscle γ-actin.

DISCUSSION

High cell density appears to be required for the selective induction of VSM α-actin mRNA expression during BC3H1 myoblast cytodifferentiation. Although the cells used in this analysis are capable of expressing both VSM and skeletal muscle α-actin mRNA species [Strauch and Reeser, 1989], only the vascular-specific mRNA was inducible by cell to cell contact. Dispersion of intercellular contacts with EDTA also caused a decrease in both VSM and skeletal α-actin mRNA levels in BC3H1 myocytes suggesting that muscle actin mRNA expression may be sustained in these cells by regulatory mechanisms that depend on the maintenance of high cell density culture conditions. In this regard we recently determined that confluent BC3H1 cells secreted substrate-associated macromolecules (SAM) which are necessary and sufficient for myoblast cytodifferentiation and expression of muscle α-actin mRNA [Strauch et al., 1991a]. Dissociation of myocyte-SAM contacts may be the primary cause of attenuated muscle α-actin mRNA expression in EMC since restoring myocyte-myocyte contacts by itself had no significant inductive effect on muscle α-actin mRNA levels in dissociated cells. Biosynthesis of a cytodifferentiation-permissive matrix by confluent BC3H1 myoblasts may represent the important rate-limiting step in the induction of VSM α-actin gene expression during the cytodifferentiation process.

The establishment of intercellular contacts in post-confluent cells, however, does appears to be linked to efficient post-translational processing of newly synthesized VSM α-actin polypeptide chains. The finding that mature forms of non-muscle β- and γ-actin and skeletal muscle α-actin all accumulated to approximately the same extent in both low-density and high-density EMC indicated that the observed inhibitory effect of dispersion on VSM α-actin biosynthesis was neither a non-specific effect due to differences in

L-[^{35}S]cysteine uptake between normal and EDTA-treated myocytes nor simply a consequence of EDTA treatment alone. Preliminary studies revealed that normal myocytes and EDTA-dispersed myocytes exhibited similar rates of L-[^{35}S]cysteine incorporation into acid-insoluble protein during short pulse labeling periods. In a previous study of contractile protein expression in fusion-competent rat skeletal muscle cells induced to differentiate in the presence of EGTA, discrepancies were noted between the synthesis and translational efficiency of mRNAs encoding a variety of muscle-specific contractile protein isoforms including skeletal α-actin [Endo and Nadal-Ginard, 1987]. The response of non-fusing BC3H1 myocytes to EDTA dispersion we have observed, however, appears to involve both translational and post-translational control steps. The translational efficiency of VSM α-actin mRNA expressed in BC3H1 myocytes appears to be enhanced by dispersion since peptide map analysis revealed that the nascent form of the VSM α-actin polypeptide is the dominant species in pulse-labeled cells both prior to and after EDTA treatment despite a reduction in the level of VSM α-actin mRNA following dispersion. The novel relationship between cell-to-cell contact and the processing of immature VSM α-actin polypeptide we have observed in BC3H1 myocytes as well as the apparent lack of correlation between α-actin protein and mRNA levels in arterial smooth muscle tissue [Barja et al., 1986; Kocher and Gabbiani, 1987] and isolated VSM cells [Corjay et al., 1989] and in BC3H1 myocytes following growth factor-induced de-differentiation [Wice et al., 1987; Wang and Rubenstein, 1988] all support the idea that both translational and post-translational controls may be available in muscle cells to regulate α-actin expression. Wice et al. [1987] reported that both FGF and serum blocked α-actin protein expression without invoking a significant reduction in the level of the corresponding mRNA. Since creatine phosphokinase expression was inhibited at both the protein and mRNA levels in treated cells, the authors speculate that the decrease in α-actin synthesis in modulated myocytes most likely was mediated by the selective inhibition of actin mRNA translation. FGF did not stimulate mitotic activity in the myocytes indicating that the observed decrease in α-actin expression was not due to dilution of protein and/or mRNA pools by renewed cycles of cell division. Likewise, Wang and Rubenstein [1988] found discrepancies between α-actin protein and mRNA levels in BC3H1 myocytes treated with non-mitogenic EGF that also could be explained by translational control mechanisms. In view of our findings in studies on EDTA-dispersed myocytes, it is interesting to speculate that an additional effect of differentiation-inhibitory growth factors on BC3H1 myocytes might be to reduce the efficiency of VSM α-actin post-translational processing. Indeed there are striking morphological similarities between EDTA-dispersed and FGF-treated BC3H1 myocytes [Strauch et al., 1989]. Since isoelectric focusing gel techniques used in previous studies of growth factor effects on actin expression in BC3H1 myocytes may lack the resolution required to unambiguously identify and quantitate levels of actin intermediates, it would be informative to perform actin N-terminal peptide map analysis on myocytes at various times after exposure to growth factors to determine if actin processing abnormalities occur as a consequence of these treatments. It also will be of interest in future studies to determine if altered VSM α-actin processing as we have observed in dispersed BC3H1 myogenic cells also occurs in modulated VSM cells that accumulate during proliferative vascular disease, in other types of isolated muscle cell preparations that are inducible for VSM α-actin expression, and in pleuripotent cell types such as chick embryo fibroblasts which appear to express VSM α-actin in a cell-density dependent manner [Carroll et al., 1988].

The phenomenology of actin isoform switching has been described for many types of muscle cell culture systems and in developing muscle tissue but the physiological significance of actin conversion during myogenesis remains obscure. The isoform selectivity of the response to dispersion of close cell contact we have observed suggests that post-translational actin processing in BC3H1 myogenic cells involves mechanisms capable of discriminating between different actin isoform polypeptide chains. We hypothesize that compartmentalization of nascent actin polypeptide chains within myocytes may be utilized as a means to govern isoform-selective post-translational processing. Actin isoform compartmentalization also may promote more efficient management of monomer pool size in vivo and facilitate rapid changes in the isoform content of pre-existing actin supramolecular assemblies that may be necessary for tissue remodelling during

development or pathogenesis. While very little actually is known about its functional significance, actin N-terminal processing might be linked to the assembly of certain types of contractile or cytoskeletal structures in the cell since several acidic amino acids localized in the highly exposed, 18 residue N-terminal region of the actin molecule have been identified as contact sites for basic amino acids in the functionally distinct actin-binding proteins depactin, α-actin, and fragmin [Sutoh, 1982, 1983; Sutoh and Mabuchi, 1984; Sutoh and Hatano, 1986; Mimura and Asano, 1987]. The N-terminal group of acidic amino acid residues also has been shown to be essential for ATP-dependent actin-myosin interaction [Sutoh et al., 1991]. There is some evidence that multiple actin isoforms are selectively utilized by certain types of cells. For example, VSM α-actin appears to be restricted from detergent-extractable actin networks found in the lamellipodia and other motile regions of vascular pericytes [DeNofrio et al., 1989]. In addition, a preliminary report by Peng and Fischman [1988] describing a novel in vitro assay for actin filament assembly indicates that α-actin subunits translated in reticulocyte lysates may have a greater affinity for the ends of preformed myofibrils compared to the non-muscle actins that were also present in these lysates. Recent studies by Hennessey et al. [1991] also suggest that unprocessed class II actin from *Drosophila,* which like VSM α-actin contains acetylcysteine at the N-terminus, is less well polymerized than fully processed actin. If this phenomena also pertains to vertebrate actins, then accumulation of unprocessed VSM α-actin may facilitate re-organization of the cytoskeleton following dispersion of myocyte cultures with EDTA. In this context, post-translational control mechanisms also may provide an economical means to regulate localized and/or transient changes in actin structural organization in the cell without invoking changes in developmentally fixed patterns of actin gene transcription. Since the fibroblastoid appearance of EDTA-dispersed BC3H1 myocytes contrasts significantly with the bipolar phenotype of differentiated myocytes, we speculate that insufficient VSM α-actin processing in EMC promotes cell shape destabilization. In vascular smooth muscle tissue, cytoarchitectural remodelling that occurs as a consequence of altered cell-cell or cell-matrix interactions during vasculogenesis may permit a limited degree of phenotypic modulation necessary for smooth muscle cell migration or proliferation without complete loss of the capacity to express VSM α-actin mRNA and protein. Reversion to the fully differentiated, contractile phenotype may not require de novo induction of the repressed gene but rather renewed high efficiency processing of intermediate forms of VSM α-actin as modulated cells reach a critical threshold density or encounter other microenvironmental signals which influence actin post-translational processing. We expect that the BC3H1 myogenic cell line will continue to be useful for investigating these dynamic relationships between actin isoform biosynthesis, utilization, and cytoarchitectural organization.

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