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Phorbol Esters Selectively and Reversibly Inhibit a Subset of Myofibrillar Genes Responsible for the Ongoing Differentiation Program of Chick Skeletal Myotubes

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Phorbol esters selectively and reversibly disassemble the contractile apparatus of cultured skeletal muscle as well as inhibit the synthesis of many contractile proteins without inhibiting that of housekeeping proteins. We now demonstrate that phorbol esters reversibly decrease the mRNA levels of at least six myofibrillar genes: myosin heavy chain, myosin light chain 1/3, myosin light chain 2, cardiac and skeletal α-actin, and skeletal troponin T. The steady-state message levels decrease 50- to 100-fold after 48 h of exposure to phorbol esters. These decreases can be attributed at least in part to decreases in transcription rates. For at least two genes, cardiac and skeletal α-actin, some of the decreases are the result of increased mRNA turnover. In contrast, the cardiac troponin T steady-state message level does not change, and its transcription rate decreases only transiently upon exposure to phorbol esters. Phorbol esters do not decrease the expression of the housekeeping genes, α-tubulin, β-actin, and γ-actin. Phorbol esters do not decrease the steady-state message levels of MyoD1, a gene known to be important in the activation of many skeletal muscle-specific genes. Cycloheximide blocks the phorbol ester-induced decreases in transcription, message stability, and the resulting steady-state message level but does not block the tetradecanoyl phorbol acetate-induced rapid disassembly of the I-Z-I complexes. These results suggests a common mechanism for the regulation of many myofibrillar genes independent of MyoD1 mRNA levels, independent of housekeeping genes, but dependent on protein synthesis.

During normal skeletal myogenesis, a population of replicating myoblasts leave the cell cycle to become postmitotic myoblasts (34, 56). During this process, over 20 muscle-specific genes are coordinately activated and then maintained to produce the muscle phenotype (6). One subset of these genes, of which nothing is known, confers on the postmitotic myoblasts the distinctive capacity to fuse and form multinucleated myotubes (14, 21, 30, 31, 35). Cytoimmunofluorescence and electron microscopy studies have shown that independent of fusion, the temporal accumulation of a cohort of myofibrillar proteins is tightly linked both in normal postmitotic mononucleated myoblasts and in multinucleated myotubes (14, 21, 30, 31, 35). Kinetic studies on the accumulation of myofibrillar transcripts (9, 20), studies of agents that block fusion of immortalized myogenic cells (25, 49, 51, 57), and studies of nonmuscle cells converted to muscle cells by MyoD1 and related genes (7, 8, 14, 19, 42, 52, 59, 62, 72) demonstrate that the activation of one myofibrillar gene is generally linked to the activation of others. Unlike coordinate activation, there is little evidence supporting the existence of coordinate maintenance of muscle-specific expression after the initial activation. In fact, studies have emphasized the independent regulation of individual myofibrillar genes during normal maturation (28, 46, 58, 71) as well as in response to innervation (71) and hormone levels (36). It is unknown whether there is an underlying coordinate regulation of multiple myofibrillar genes, and it is unclear whether MyoD1 is involved in this regulation.

The phorbol ester 13-tetradecanoyl-*O*-phorbol acetate (TPA), a potent tumor promoter and activator of protein kinase C (PKC), reversibly inhibits the ongoing differentiation program of many cell types such as chondroblasts, melanocytes, and muscle (32). By studying the effects of TPA on the ongoing differentiation program of myotubes, we use TPA as a tool to study the regulation of continuous expression of myofibrillar genes. We reported that TPA promptly blocks spontaneous contractions in day 4 to 5 myotubes and over the next 72 h induces elongated myotubes to retract, forming multinucleated myosacs. Most myofibrillar structures are dismantled in 30-h myosacs (e.g., day 4 to 5 myotubes exposed to TPA for 30 h), and virtually all myofibrillar proteins are eliminated in 72-h myosacs. Nevertheless, the myofibril-depleted myosacs display a normal complement of desmin intermediate filaments, sarcoplasmic reticulum, T system, mitochondria, and so forth (18). [^{35}S]methionine labeling of these myosacs shows inhibited synthesis of many myofibrillar proteins but continued synthesis of all examined housekeeping proteins (2). These effects of TPA on skeletal myotubes are strikingly reversible. Seventy-two-hour myosacs transferred to normal medium begin to elongate within 15 to 20 h and by 48 h are indistinguishable from control myotubes.

In this study, we used Northern (RNA) blot and nuclear run-on (NRO) analyses to examine the effects of TPA on the expression of seven myofibrillar genes, three housekeeping genes, and a regulatory gene, MyoD1. TPA produced specific and reversible decreases in the transcription rates and steady-state message levels of many myofibrillar genes as well as decreases in message stabilities of at least the two α-actin mRNAs. In contrast, the expression of the house-

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keeping and MyoD1 genes did not decrease with TPA. Cardiac troponin T steady-state message levels did not change with exposure to TPA, but its transcriptional rate did transiently decrease. Studies using cycloheximide and TPA suggest that the TPA-induced decreases in the message stabilities, transcription rates, and resulting steady-state message levels are dependent on protein synthesis. In contrast, TPA-induced rapid disassembly of functional striated myofibrils is independent of protein synthesis.

MATERIALS AND METHODS

Culture. Chick skeletal muscle cultures were obtained as previously described (43). Cytosine arabinonucleoside (Ara-C; 10^{-5} M) was introduced 2 to 3 days after initial plating and was used throughout the experiments to remove replicating fibroblastic cells. Four days after initial plating, when greater than 95% of the nuclei are in multinucleated myotubes, various combinations of TPA (75 ng/ml), 0.1 mM cycloheximide, and actinomycin D (2 μg/ml) were added daily. Dermal fibroblasts were isolated from 11-day chick embryos and passaged four to five times to remove any contaminating muscle (12).

MyoD1 retrovirus infection. Dermal fibroblast cultures were infected by exposure to an amphotropic retrovirus containing the MyoD1 coding region or a control virus (70) with Polybrene (8 μg/ml) for 8 h as previously described (14).

Immunofluorescence. Muscle cultures were fixed in 2% formaldehyde and double stained with rhodamine-labeled phalloidin and muscle specific α-actinin antibody and then with fluorescein-labeled secondary antibody as previously described (43).

Northern blot analysis. Total RNA was isolated by using guanidine-HCl, sarcosine extraction, and ethanol precipitation as described by Krawetz and Anwar (40). In accordance with the New England Nuclear protocol, 3 to 6 μg of glyoxated total RNA was fractionated, transferred to GeneScreen, fixed by baking at 80°C for 3 h, hybridized with a riboprobe at 250,000 cpm/ml at 60 to 65°C, and stripped for rehybridization.

In accordance with the Promega protocol, riboprobes were generated from constructs in which cDNAs were subcloned into pGem 1 or pGem 2, using standard methods described by Maniatis et al. (47). cDNAs were embryonic myosin heavy chain, nucleotide residues 1110 to 1211 (MHC) (C. Ordahl, University of California at San Francisco, unpublished probe); skeletal myosin light chain 2 fast, amino acid residues 68 to 111 (MLC2) (C. Ordahl, unpublished probe); skeletal α-actin, 3′ untranslated region (UTR) (S α-actin) (24); cardiac troponin T (C TnT) (16); skeletal myosin light chain 1/3 (MLC1/3) (5); cardiac α-actin, 3′ UTR (C α-actin) (23); β-actin, 3′ UTR (15); PA3 (15); α-tubulin (15); MyoD1 (19); and skeletal troponin T (S TnT) (27). The two unpublished probes have nucleic acid sequences virtually identical to those of published probes (61, 67). Cleveland et al. reported PA3 to be the 3′ UTR of chick γ-actin (15), whereas Chang et al. were unable to use PA3 to obtain a γ-actin genomic clone (11). Using PA3, we isolated a 1,000+-bp cDNA from a chick muscle library generously donated by B. Paterson (National Institutes of Health); nucleotide sequence analysis revealed actin coding sequences and homologies to the 3′ UTR of human and mouse γ-actin (data not shown; sequence submitted to EMBL). Northern blot analysis of 3 μg of skeletal muscle and fibroblast RNA revealed the expected cell type specificity and mRNA size for each riboprobe (data not shown).

NRO analysis. Nuclei were isolated from cells as described by Long and Ordahl (46). NRO assays, subsequent RNA isolation, and hybridization were done as described by Linial et al. (45) except that sodium dodecyl sulfate was increased from 0.2 to 1.0% in the hybridization buffer. Labeled transcripts were hybridized to Southern blots containing 5 μg of the following cDNAs: clone 251 (MHC) (67), 5′ *Eco*RI-*Bam*HI fragment of MLC2 (61), 3′ *Hae*III-*Pst*I fragment of MLC1/3, S α-actin, C TnT, β-actin, γ-actin, and α-tubulin. The latter five cDNAs are the same as those used for the Northern blot analyses. Longer cDNAs for MHC and MLC2 were required because their Northern blot counterparts gave little to no signal in an NRO assay. Full-length MLC2 and MLC1/3 cDNAs contained sequences that hybridized to fibroblast NRO transcripts (data not shown) and required the removal of 351 bp from the 3′ end of MLC2 and 99 bp from the 5′ end of MLC1/3 for muscle specificity. The blots were hybridized with 2 ml containing equal amounts of labeled transcripts (between 2×10^6 and 20×10^6 cpm) for 36 h at 65°C, washed, and exposed to preflashed film for 2 to 14 days at −70°C with intensifying screens.

The specificities of the NRO probes were verified by comparing their relative hybridizations to fibroblast and muscle NRO transcripts; to verify specificity to mRNAs, probes were hybridized to muscle nuclei transcripts in which the RNA polymerase II had been inhibited with α-amanitin (1 μg/ml) (data not shown). Muscle-specific probes hybridized only to muscle nuclear transcripts, while housekeeping probes hybridized to both muscle and fibroblast transcripts. As an internal control, there was no hybridization to pGem vectors. Hybridizations to all probes were decreased when transcripts were isolated from α-amanitin-treated muscle nuclei.

RESULTS

Effect of TPA on steady-state message levels. TPA selectively inhibits the synthesis of several muscle contractile proteins in a chick skeletal muscle culture. To characterize this phenomenon further, we examined the steady-state mRNA levels of seven myofibrillar genes and three housekeeping genes in Ara-C-selected skeletal myotubes during TPA treatment and during recovery from TPA treatment. The seven muscle-specific mRNAs are C α-actin, S α-actin, MHC, MLC2, MLC1/3, C TnT, and S TnT; the three housekeeping mRNAs are β-actin, γ-actin, and α-tubulin. The effects of TPA treatment and recovery in the absence of Ara-C were similar to those with Ara-C (data not shown).

Day 4 muscle cultures were treated with TPA for various time periods, and their RNAs were isolated; 3 μg of the total RNA was analyzed by Northern blot analysis. Blots were hybridized and stripped multiple times to generate composite figures. Figure 1a demonstrates that TPA treatment resulted in significant decreases in myofibrillar mRNAs for MHC, MLC1/3, MLC2, C α-actin, and S α-actin within 24 h. Although significantly decreased, the C α-actin mRNA level at 24 h increased reproducibly from that at 12 h. By 48 h, the mRNA levels decreased by 50- to 100-fold. In contrast, muscle-specific C TnT showed little response to TPA. There were modest threefold increases of β-actin and γ-actin mRNAs and no significant change in α-tubulin transcripts.

After 48 h in TPA, muscle cultures were fed with normal medium and allowed to recover (Fig. 1b). Within 24 h, there were increases in muscle-specific transcripts. By 48 h, the levels of muscle-specific transcripts were equivalent to those in the control. In contrast, α-tubulin levels remained con-

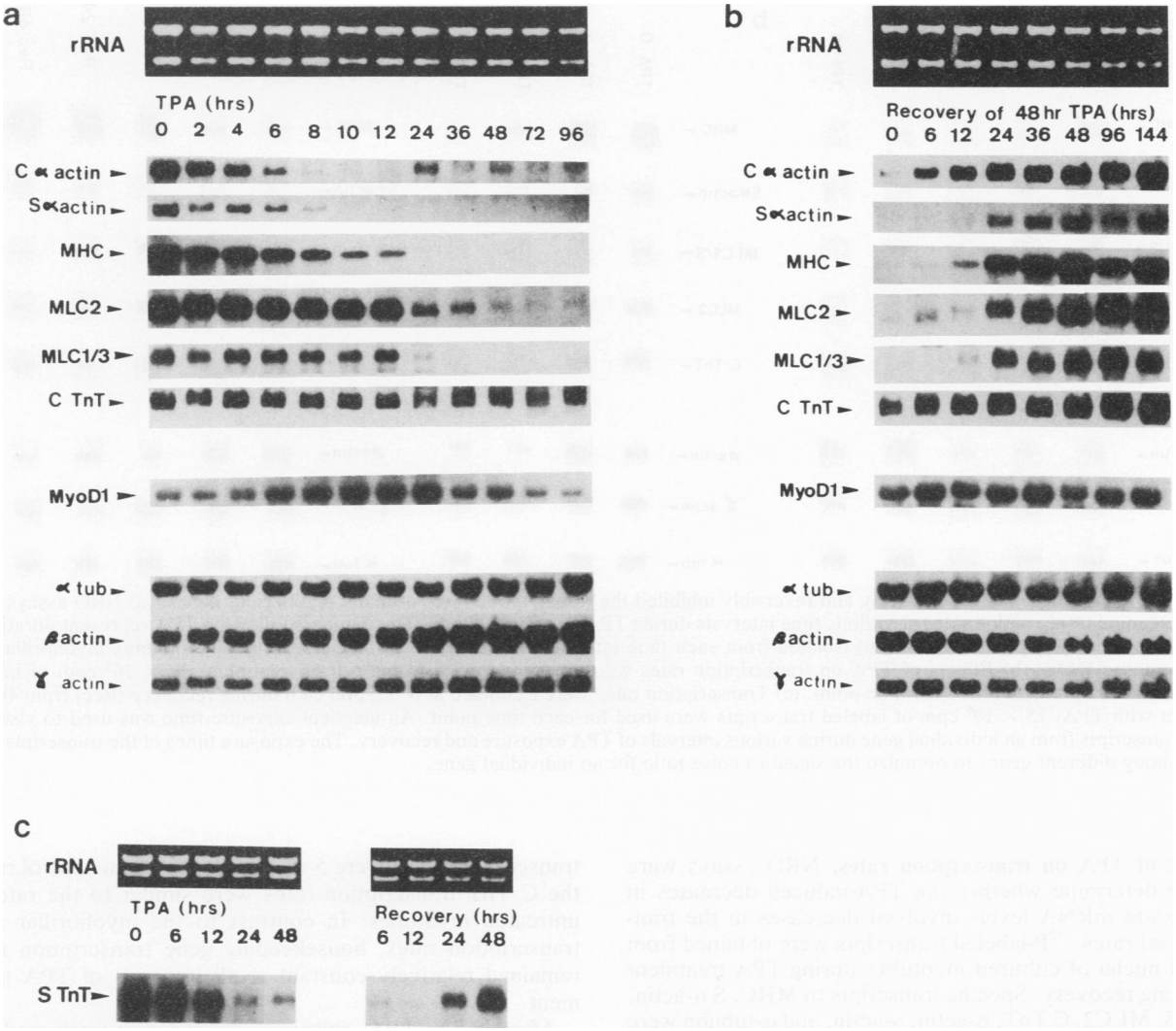

FIG. 1. Evidence that TPA selectively and reversibly decreased the mRNA levels of many myofibrillar genes. (a) TPA was added to 4-day-old chick muscle cultures (0 = time of TPA addition). After various time intervals, RNA was extracted and analyzed by Northern blot. A single Northern blot was hybridized and stripped multiple times to generated a composite figure. The upper seven rows of bands represent myofibrillar and muscle regulatory mRNAs, while the lower three rows represent housekeeping mRNAs. In all figures, α tub represents α-tubulin. Relative quantities and qualities of the RNAs are indicated by ethidium bromide staining of rRNA. (b) After 48 h in TPA, normal medium was added to the muscle cultures and mRNA levels were examined over time (0 = time of normal medium addition). (c) The effect of TPA on the skeletal isoform of TnT (S TnT) was examined.

stant, while β-actin and γ-actin levels actually decreased. C TnT mRNA levels, which did not decrease with TPA treatment, did increase upon recovery. However, the time, amplitude, and duration of induction of C TnT varied among experiments (data not shown).

To determine whether the lack of cardiac TnT response is common to all muscle isoforms of TnT, we studied the TPA response of a skeletal isoform of TnT (Fig. 1c). S TnT mRNA, like most myofibrillar transcripts and unlike C TnT mRNA, decreased reversibly upon treatment with TPA.

Effect of TPA on MyoD1 mRNA levels. Since MyoD1 has been shown to be important in the activation of many muscle-specific genes (19) and has been reported to bind to the *cis* elements of many muscle genes (41), we examined the effect of TPA on the MyoD1 steady-state message level. Figure 1a demonstrates that the MyoD1 mRNA level did not decrease with TPA treatment; instead, it reproducibly increased after 24 h in TPA before returning to the control level after 48 h in TPA. To verify this finding independently, we examined the effects of TPA on primary dermal fibroblasts converted to muscle cells by a retrovirus containing MyoD1 driven by a viral promoter. These MyoD1-converted fibroblasts form normal striated myofibrils, spontaneously contract, and are indistinguishable from normal muscle in all details examined (14). Despite constant to increased expression of exogenous MyoD1 mRNA in these converted cells, TPA treatment resulted in reversible disassembly of myofibrils and reversible and selective decreases in muscle-specific mRNA levels (data not shown). These results indicate that the mechanism by which TPA affects muscle expression does not involve continuous suppression of MyoD1 mRNA levels.

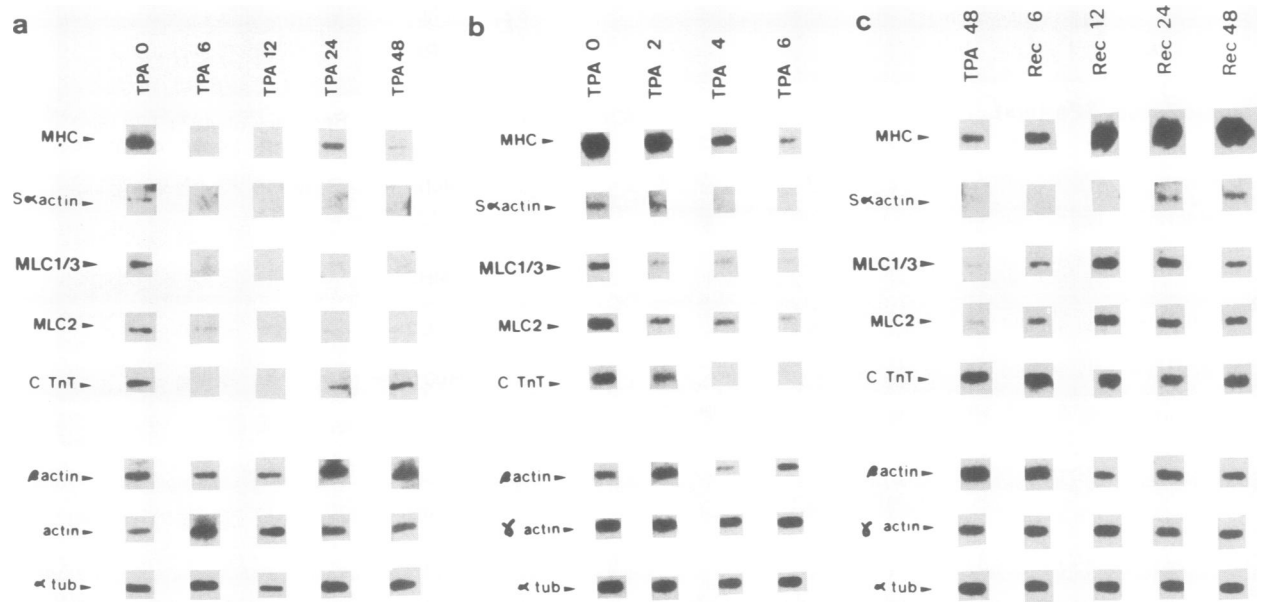

FIG. 2. Evidence that TPA selectively and reversibly inhibited the transcription rates of many myofibrillar genes. (a) NRO assays were used to examine transcription rates at various time intervals during TPA treatment (TPA). The numbers following TPA represent duration in hours. Labeled transcripts (4×10^6 cpm) isolated from each time interval were hybridized to Southern blots containing myofibrillar and housekeeping cDNAs. (b) Effects of TPA on transcription rates were examined at 2, 4, and 6 h of treatment; 10×10^6 cpm of labeled transcripts were hybridized for each time point. (c) Transcription rates were examined at 6, 12, and 24 h during recovery (Rec) from 48 h of treatment with TPA; 15×10^6 cpm of labeled transcripts were used for each time point. An identical exposure time was used to visualize labeled transcripts from an individual gene during various intervals of TPA exposure and recovery. The exposure times of the transcripts were varied among different genes to optimize the signal-to-noise ratio for an individual gene.

Effect of TPA on transcription rates. NRO assays were done to determine whether the TPA-induced decreases in steady-state mRNA levels involved decreases in the transcriptional rates. ^{32}P-labeled transcripts were obtained from isolated nuclei of cultured myotubes during TPA treatment and during recovery. Specific transcripts to MHC, S α-actin, MLC1/3, MLC2, C TnT, β-actin, γ-actin, and α-tubulin were detected by hybridizing nuclear transcripts to a Southern blot containing their respective cDNAs. Figure 2 shows the results of three experiments in which the effects of TPA and recovery on transcription rates were examined.

All muscle-specific gene transcription rates, including that for C TnT, decreased significantly after 6 to 12 h of treatment with TPA (Fig. 2a and b). Note that the steady-state message levels of C TnT remained constant in TPA-treated myotubes. During the first 6 h, the transcription rates of myofibrillar genes decreased as a group, as opposed to a sequential pattern in which one myofibrillar transcription rate decreased prior to others (Fig. 2b). This finding argues against the hypothesis that TPA decreases the rate of one myofibrillar gene, which then feeds back to decrease the rates of others. As a control, the transcription rates, measured after 6 h in fresh normal medium and Ara-C, remained constant (data not shown). After 24 h of TPA treatments, transcription rates for MHC and C TnT were higher than those after 6 to 12 h of TPA treatment (Fig. 2a). After 24 h of TPA treatment, the increased transcription rate of MHC remained four- to fivefold less than the control rates, as determined by densitometry; the increased rate of C TnT was 50% of control rates. Repeat NRO experiments showed that after 24 h of TPA treatment, the C TnT rates ranged between 50 to 100% of control rates (data not shown). By 48 h, with the exception of C TnT, the myofibrillar gene transcription rates were 5- to 10-fold less than control rates; the C TnT transcription rates were similar to the rates in untreated myotubes. In contrast to the myofibrillar gene transcription rates, housekeeping gene transcription rates remained relatively constant at all intervals of TPA treatment.

After 48 h in TPA, cultures were fed with fresh medium. For MHC, MLC1/3, and MLC2, recovery was detected within 6 h; for S α-actin, the recovery was first detected at 24 h (Fig. 2c). After 24 h in fresh medium, the transcription rates were comparable to the control rates. Rates for housekeeping genes and C TnT remained constant throughout the recovery period. The constant transcription rate of C TnT during recovery suggests that the induction of C TnT steady-state message levels seen in recovering myosacs is due to increase in message stability.

Effect of TPA on message stability. To determine whether TPA-induced decreases in myofibrillar steady-state message levels involved decreases in message stabilities, we compared the decreases with the normal message turnover rates. Normal turnover rates were examined by blocking all transcription with actinomycin D and monitoring the drop in steady-state message levels over time. TPA-induced decreases for the two α-actin mRNA levels were much greater than those with actinomycin D (Fig. 3a and b). This finding suggests that even if TPA completely blocked transcription of the two α-actin mRNAs, it also must decrease their message stabilities in order to produce the rapid decreases in the steady-state message levels. While a more complicated interpretation cannot be ruled out, the premise that actinomycin D completely blocks transcription but does not affect normal mRNA turnover is consistent with the rapid decrease

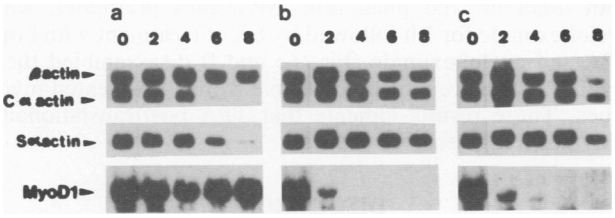

FIG. 3. Evidence that TPA decreased the message stabilities of skeletal and cardiac α-actins. Northern blot analysis was done on 6 μg of RNA isolated from cultures treated with TPA (a), actinomycin D (b), or TPA plus actinomycin D (c). The effectiveness of actinomycin D in blocking transcription is demonstrated by the rapid decrease in the level of a short-half-life mRNA, MyoD1.

in the level of MyoD1 mRNA, which has a half-life less than 30 min (66).

To determine which of two possibilities, synthesis or posttranslational modification, was involved in the mechanism by which TPA decreased myofibrillar message stability, Northern blot analyses were done on RNA isolated from cultures treated with TPA plus actinomycin D (Fig. 3c). In myotubes treated with both drugs, the decreases in mRNAs levels are virtually identical to those seen with actinomycin D alone and different from those seen with TPA. These results suggest that actinomycin D blocks TPA-induced synthesis of a protein(s) responsible for increased degradation of specific mRNAs.

Cycloheximide blocks the effects of TPA on steady-state mRNA levels. To determine the mechanism by which TPA might selectively inhibit the expression of several myofibrillar genes, the effects of TPA were analyzed in the presence of a protein synthesis inhibitor. Northern blot analyses were done on cultures treated with TPA, cycloheximide, or TPA plus cycloheximide (Fig. 4). If TPA works via posttranslational activation, inactivation, or degradation of a factor(s), then cycloheximide treatment should not prevent

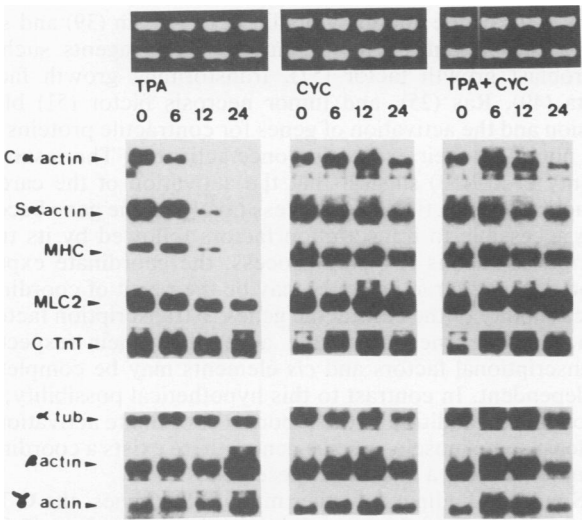

FIG. 4. Evidence that cycloheximide blocked the effects of TPA on mRNA levels. Northern blot analysis was done on 3 μg of total RNA isolated from muscle cultures after various hours of treatment with TPA, cycloheximide (CYC), or TPA plus cycloheximide (TPA+CYC).

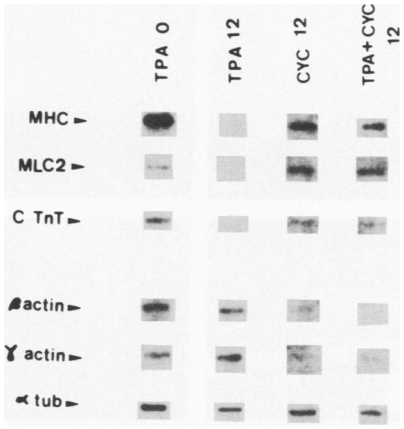

FIG. 5. Evidence that cycloheximide blocked the effects of TPA on transcription rates. NRO analysis was done on muscle cultures treated for 12 h with TPA (TPA 12), cycloheximide (CYC 12), or TPA plus cycloheximide (TPA+CYC 12). TPA 0 designates results from control cultures. A total of 2×10^6 cpm of labeled transcripts was hybridized for each treatment.

the effect(s) of TPA. However, if the effects of TPA required the synthesis of a factor(s), then cycloheximide should prevent the down regulation of muscle gene expression. As demonstrated previously, TPA treatment decreased the myofibrillar mRNA levels except that of C TnT. Treatment with cycloheximide alone produced a complicated pattern in which some mRNA levels transiently increased (C α-actin, β-actin, and γ-actin), others decreased (S α-actin and α-tubulin), and others remained relatively constant (MHC, MLC2, and C TnT). This difference between the effects of cycloheximide and TPA emphasizes the specificity of TPA for myofibrillar genes. Not all perturbations of the ongoing myogenic differentiation program selectively affect myofibrillar gene expression. Treatment with TPA plus cycloheximide resulted in a pattern that was similar to that seen with cycloheximide and different from that seen with TPA.

Cycloheximide blocks the effects of TPA on transcription rates. To determine which of two possibilities, synthesis or posttranslational modification of a muscle-inhibiting factor, was involved in the mechanism by which TPA decreased myofibrillar transcription rates, we examined the effects of TPA in the presence of cycloheximide. NRO assays were done on myotubes treated for 12 h with TPA, cycloheximide, or TPA plus cycloheximide (Fig. 5). Low signal-to-noise ratios prevented the analysis of MLC1/3 and S α-actin transcription rates. As demonstrated previously, TPA treatment decreased the transcription rates of all examined myofibrillar genes. Treatment with cycloheximide alone decreased the transcription rates of all genes, both myofibrillar and housekeeping, except for the MLC2 and α-tubulin genes, which retained their original transcription rates. Again, this finding emphasizes that not all perturbations of the ongoing myogenic differentiation program selectively affect myofibrillar gene expression. Treatment with TPA plus cycloheximide resulted in a pattern of transcription rates that was virtually identical to that seen with cycloheximide and different from that seen with TPA. These results indicate that protein synthesis is necessary for selective TPA-induced decreases in myofibrillar transcription rates.

Cycloheximide does not block the disassembly of striated myofibrils in myotubes treated with TPA. To determine

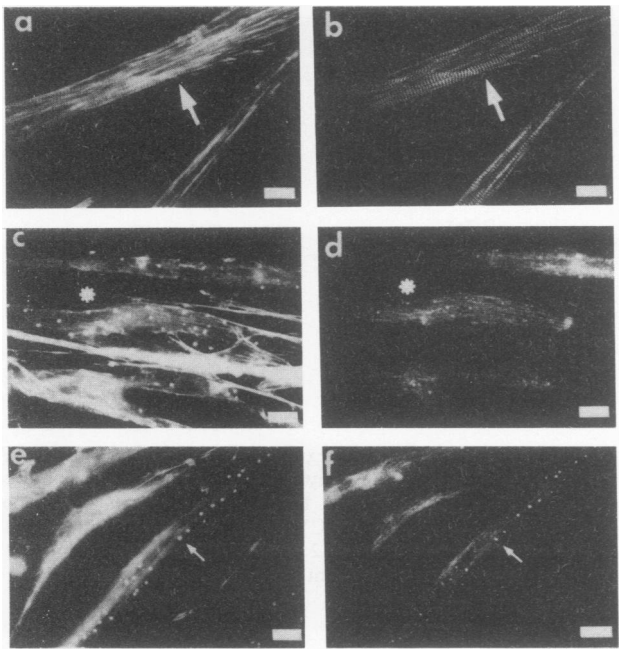

FIG. 6. Evidence that cycloheximide did not block the TPA-induced disassembly of the contractile apparatus. Four-day striated myotubes were treated with cycloheximide, TPA, or TPA plus cycloheximide. After 6 to 7 h of treatment, the myotubes were fixed and double stained with fluorescein-labeled antibodies to α-actinin (a, c, and e) and with rhodamine-labeled phalloidin (b, d, and f). (a and b) Myotubes treated with cycloheximide for 7 h; (c and d) myotubes treated with TPA for 6 h; (e and f) myotubes pretreated for 1 h with cycloheximide and then treated with TPA and cycloheximide for an additional 6 h. Note that cycloheximide did not block the disassembly of the I-Z-I complex and did not prevent the appearance of the 3.0-μm bodies, findings seen in TPA-treated myotubes. Arrows and asterisks are for orientation. Bar = 200 μm.

whether the TPA-induced disassembly of the contractile apparatus results from a block in synthesis of myofibrillar proteins and the normal turnover of the apparatus or whether it involves the activation of a disassembly process, we examined the contractile apparatus after the myotubes have been treated for 6 to 7 h with cycloheximide, TPA, or TPA plus cycloheximide. Treated myotubes were stained with fluorescein-labeled antibodies to α-actinin and with rhodamine-labeled phalloidin, which binds to all filamentous actin. Lin et al. (43) reported that the earliest TPA-induced disassembly of the contractile apparatus is observed with these two strains. Treatment with TPA plus cycloheximide consisted of pretreatment with cycloheximide for 1 h prior to the addition of TPA. Identical results were obtained when cycloheximide and TPA were added simultaneously (data not shown).

Figures 6a and b demonstrate the stable nature of the contractile apparatus in the absence of protein synthesis for 7 h. Myotubes treated for 7 h in cycloheximide retained their contractile apparatus, as demonstrated by the normal distribution of phalloidin and anti-α-actinin to the I bands and the Z bands, respectively. The staining patterns were virtually identical to those observed in myotubes of a typical day 4 muscle culture (data not shown). Figures 6c and d demonstrate two of the earliest findings in TPA-induced disassembly of the myofibrils: the disassembly of Z bands and the appearance of 3-μm circular bodies that strain positive for both α-actinin and phalloidin. Myotubes pretreated with cycloheximide for 1 h followed by 6 h of treatment with both TPA and cycloheximide (Fig. 6e and f) disassembled their myofibrils and are indistinguishable from TPA-treated myotubes. These results indicate that TPA posttranslationally activates a disassembly process.

DISCUSSION

Coordinate maintenance. TPA was used as a tool to study the regulation of the continuous expression of multiple myofibrillar genes. In the postmitotic myotube, we demonstrate that even after the contractile genes are activated, there is still a coordinate regulation of a subset of contractile genes that is independent of housekeeping genes but is dependent on protein synthesis.

The term coordinate regulation was introduced by Holtzer and colleagues to describe the simultaneous appearance of a cohort of proteins specific for the postmitotic myoblasts which was previously absent in replicating presumptive myoblasts (14, 21, 30, 31, 35). This phenomenom implied the existence of a master switch such as the recently discovered myogenic helix-loop-helix family of proteins. Coordinate regulation was redefined by Emerson and colleagues to describe the similar temporal accumulation of myofibrillar proteins, mRNAs, and transcripts and implied a common mechanism in the activation of multiple genes (9, 20). Gunning et al. (26) defined coordinate regulation in terms of identical accumulation of mRNAs. Finding differences in the accumulation of muscle-specific mRNAs at short time intervals, they concluded that the genes for these mRNAs were not coordinately regulated (26). We do not favor the last definition because it disregards common mechanisms of regulation that may coexist with mechanisms of individual modulation. We define coordinate regulation as a similar response in the expression of selective genes to a given internal or external signal suggestive of a regulatory mechanism common and distinctive but not necessarily comprehensive to these multiple genes.

An underlying assumption in our study is that the mechanism(s) involved in the activation of a gene is not identical to that involved in its maintenance. This difference has been indicated for the immunoglobulin heavy-chain (39) and simian virus 40 genes (68). In muscle, many agents such as fibroblast growth factor (57), transforming growth factor beta (49), Ras (25), and tumor necrosis factor (51) block fusion and the activation of genes for contractile proteins but do not affect their expression once activated. The results of Minty et al. (53) suggest that the activation of the cardiac α-actin gene is a two-step process involving the gene becoming accessible to transcription factors followed by its transcription. In this two-step process, the coordinate expression of the contractile genes may be the result of coordinate accessibility of the contractile genes to transcription factors. Once these genes are made accessible, their respective transcriptional factors and *cis* elements may be completely independent. In contrast to this hypothetical possibility, we show that in addition to the reported coordinate activation of at least some muscle-specific genes, there exists a coordinate maintenance of a subset of these genes.

Similar to findings for other myofibrillar genes, the C TnT transcription rate decreases significantly upon 6 to 12 h of TPA exposure. In contrast to other contractile genes, the C TnT transcription rate returned to near the control rate after 24 to 48 h in TPA. In addition, TPA had little effect on the C TnT steady-state levels. Presumably, the transient decrease

in the transcription rates could not be detected on a Northern blot because of a long half-life of the C TnT mRNA or a compensating increase in message stability. Depletion of TPA is ruled out since increasing the frequency of TPA dosage from 75 ng/ml/day to 75 ng/ml/12 h did not change the results. Possible explanations for the exceptional recovery of C TnT transcription rates include a generalized inactivation of the TPA response with the C TnT gene recovering first or a different TPA-responding/compensatory transcriptional mechanism unique to an individual gene. We do not favor the first explanation because other myofibrillar mRNA levels remain reversibly and selectively decreased for 144 h (data not shown). The second explanation is consistent with our definition of comaintenance as a common regulatory mechanism superimposed with individual modulation and may also explain the slight but reproducible recovery of the MHC transcription rate and of the C α-actin steady-state message level at 24 h in TPA.

Qualitatively, the changes in transcription rates as measured by NRO analysis are consistent with the changes in steady-state message levels as measured by Northern blot analysis. The changes in rates temporally precede those in steady-state levels. However, the amplitudes of the changes, 50- to 100-fold in Northern blots versus 10- to 20-fold in NRO, differ. In addition to changes in transcriptional rates, there may be changes in message stabilities. Our findings with use of actinomycin D are consistent with this hypothesis, at least for the α-actins. Although we cannot rule out a more complicated interpretation involving actinomycin D inhibition of generalized mRNA turnover, [^{3}H]uridine pulse-chase experiments by other investigators also indicate increased myofibrillar mRNA turnover. Crow found decreased stability of MLC1/3 mRNA in cultured postmitotic muscle cells exposed to TPA (18a). If the reported half-lives of MHC and α-actin of 50 and 18 h, respectively, in L6 cells (50) are comparable to those in primary chick myotubes, then TPA decreases MHC and α-actin mRNA stabilities. A less likely explanation for the discrepancy between transcription rates and mRNA levels is based on a report by Bentley and Groudine (4), who observed transcription of both strands of the c-*myc* gene in an NRO assay but detected only the sense mRNA in the cytoplasm. In our study, the actual decreases in correct transcriptional rates may be obscured by transcriptions of the opposite strands of the DNA which do not respond to TPA. Also, NRO assays have weaker signal and higher background than do Northern blots.

Possible mechanisms. The comaintenance of many myofibrillar genes appears to involve regulations of both transcription and message stability. It is unlikely that a single factor would be directly involved as both a transcription and a message stability factor. Our studies using cycloheximide in combination with TPA are more consistent with an indirect effect of TPA; phorbol esters may regulate the transcriptions of genes that code for proteins that regulate muscle gene expression. Little is known about proteins that modulate the stabilities of specific mRNAs. More is known about the transcriptional regulators of myofibrillar genes, including the recently cloned myogenic helix-loop-helix proteins and the postulated muscle-specific *trans*-acting factors (for a review, see reference 63). None of these factors have been shown to be directly involved in continued expression of all of the myofibrillar genes examined in this study.

We hypothesized MyoD1 as a potential target by which TPA decreases myofibrillar gene expression. However, we find that TPA does not decrease MyoD1 mRNA levels. This lack of effect is verified by the effectiveness of TPA in inhibiting myofibrillar expression in MyoD1-converted fibroblasts which express exogenous MyoD1 containing no introns and a viral promoter. If inactivation of MyoD1 is involved in the TPA response, differential splicing and differential transcriptional initiation of endogenous MyoD1 are ruled out. We cannot rule out inactivation of MyoD1 via a translational block, a posttranslational modification, or a sequestering by the Id protein (3). The significance of the MyoD1 binding site in regard to muscle-specific gene expression is not clear given that nonmuscle genes contain these sites, a MyoD1 binding site contributes little activity to the promoter for the C TnT gene (48), and there is a lack of binding to the muscle creatine kinase enhancer by MyoD1 in mature myotubes (10). The involvement of other genes related to MyoD1, such as myogenin (72), Myf-5 (8), and MRF4/herculin/Myf-6 (7, 52, 62), as well as muscle-specific *trans*-acting factors cannot be ruled out in the TPA response.

Phorbol esters bind and activate PKC, a serine and threonine kinase (for a review, see reference 55). However, with long-term exposure to phorbol ester, myoblasts down regulate PKC (54). At this point, we have no evidence that the effects of TPA involve PKC. Even if PKC were involved, it is unclear whether the activation or down regulation of PKC is responsible for the inhibition of myofibrillar expression.

At the transcriptional level, Angel et al. (1) demonstrated that a short DNA consensus sequence, TGAGTCAG, is important in TPA-induced changes in transcription. This induced change in transcription has been reported to occur in the absence of protein synthesis. While AP-1 binding sites have generally been associated with increases in transcription, precedent for transcriptional inhibition is found for adipocyte p2 gene (60). We speculate that the *cis* elements of the genes for the putative transcription and message stability factors contain AP-1 binding sites. Alternatively, the response to TPA may be mediated by different *trans*-acting factors that bind to different sequences, as reported for the simian virus 40 enhancer (13).

Unlike TPA-induced decreases in transcription and stability, the TPA-induced rapid disassembly of the contractile apparatus is mediated by a posttranslational event. Under more physiological conditions, rapid and selective disassembly of I-Z-I complexes in functional myofibrils has been reported in cardiac myocytes during mitosis (38), resorbing tails in metamorphosing tadpoles (69), and pharmocologically damaged muscle (22). By studying TPA-induced disassembly, one might obtain insight into the mechanisms by which myofibrils are disassembled during normal development and during pathological states. Preliminary experiments indicate that the PKC inhibitors staurosporin and calphostin do not prevent TPA-induced disassembly.

Caution required in generalization to other systems. Caution should be used in generalizing the effects of TPA on cultured chick myotubes to other systems. The importance of the intracellular environment in dictating the cellular response to an exogenous signal cannot be overemphasized; different cell types can respond to TPA differently. TPA disassembles stress fibers in BSC-1 cells (65) but not in primary fibroblasts in skeletal muscle cultures (44). TPA blocks expression of the ongoing differentiation program of chondroblasts and melanoblasts (33) but activates differentiation in HL60 cells (64). Different responses in different cells to the same stimuli are not unique to phorbol esters and their receptors. Julius et al. (37) reported that the serotonin receptors can function as neurotransmitter receptors, as

growth factor receptors, or as cellular transforming/tumorigenic agents, depending on the intracellular environment.

Cossu et al. report that the effect of TPA is a function of the developmental age of mouse myoblasts (17). Myoblasts isolated from 3- to 18-day-old fetuses do not fuse in TPA, whereas myoblasts from older embryos do. While studies on myoblasts are difficult to compare with those on multinucleated myotubes, resistance to TPA is consistent with our observations that showed no morphological effect of TPA on primary 19-day fetal rat and newborn mouse muscle cultures.

Although generalization to other muscle culture systems may be limited, examination of the differences should yield information regarding the mechanism(s) for coordinate gene regulation of ongoing differentiation programs. Further understanding may be also obtained by comparisons between TPA and other agents known to produce myosacs, such as Rous sarcoma virus (29) and ethyl methanesulfonate (2). Just as bromodeoxyuridine was used as a tool for the study of myogenesis and led eventually to the isolation of myogenin (72), TPA may serve as a tool for the study of coordinate maintenance of gene expression and may be useful in isolating comaintenance factors. By understanding the mechanism(s) by which TPA reversibly blocks the ongoing differentiation program, we will understand better the normal mechanisms responsible for the maintenance of cell-specific phenotypes of muscle and of other specialized cells as well as the pathological states in which these mechanisms appear to be perturbed.

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