Articles

Phorbol Ester-Mediated Downregulation of Tropoelastin Expression Is Controlled by a Posttranscriptional Mechanism†

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ABSTRACT: Expression of tropoelastin, the principal precursor of elastic fibers, is tissue-specific and is limited to a brief developmental period. Little is known, however, about the mechanisms that regulate the tissue- and temporal-specific expression of elastogenesis. The tropoelastin promoter contains putative phorbol ester responsive elements, or AP-1 binding sites, but the functional significance of these sequences is unknown. To test if tropoelastin expression is influenced by phorbol esters, we exposed elastogenic fetal bovine chondrocytes to 10^{-7} M 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Tropoelastin mRNA levels decreased greater than 10-fold in response to TPA, and this downregulation was paralleled by a decline in the secretion of tropoelastin protein into the culture medium. As determined by nuclear-runoff assay and transient transfection with a human gene promoter–CAT construct, tropoelastin transcription was unaffected after exposure to TPA. As indicated by actinomycin D experiments, the half-life of tropoelastin mRNA in control cells was about 20 h, but exposure to TPA resulted in an accelerated decay of the tropoelastin transcript ($t_{1/2}$ = 2.2 h). These data indicate that downregulation of tropoelastin expression was controlled by a posttranscriptional mechanism and that the AP-1 elements in the bovine tropoelastin promoter may not be involved in regulation of production.

Resiliency in tissues is provided by elastic fibers, and tissues such as aorta, lung, and skin, which are subjected to repeated deformation, have an extracellular matrix that is rich in elastin. The developmental production of elastin is unique among structural proteins in that expression of tropoelastin, the secreted precursor of elastin, and the formation of fibers are limited to a relatively brief period. The bulk of elastogenesis occurs during late fetal and early neonatal periods, and by maturity production is complete and synthesis of new tropoelastin ceases (Davidson & Giro, 1986). Because of the extreme durability and extended half-life of elastin, fiber function and tissue integrity are not compromised by this limited production (Rucker & Dubick, 1984; Shapiro et al., 1991).

The tissue- and temporal-specific production of elastin suggests that expression of tropoelastin is accurately controlled, but the mechanisms which govern elastogenesis are not known. In essentially all models studied, the production of tropoelastin correlates with the steady-state levels of its mRNA [for example, see Burnett et al. (1982), Davidson et al. (1984), Foster et al. (1990), Hinek et al. (1991), and Parks et al. (1988)]. Although these collective findings indicate that expression is regulated pretranslationally, the contributions of transcriptional and posttranscriptional mechanisms to the control of elastogenesis have not been delineated.

Analogous to other genes, the 5′ flanking region of the tropoelastin gene contains several cis elements associated with the initiation of gene transcription, such as SP-1, AP-1, and AP-2 consensus sequences and a CAAT box (Bashir et al., 1989; Yeh et al., 1989). The promoter region of the tropoelastin gene does not contain a canonical TATA box, and this, along with the presence of several G/C-rich domains and multiple transcription start sites, suggest that the tropoelastin promoter resembles regulatory sequences of genes which are constitutively expressed (Boyer et al., 1989, and references within). In addition, the sequences that direct basal transcriptional activity have recently been identified (Fazio et al., 1990; Kähäri et al., 1990), but no promoter sequences have yet been characterized which confer age or tissue-specific production. In fact, the promoter–CAT constructs used in these studies demonstrated similar transcriptional activity in both elastogenic and nonelastogenic cells. Following consideration of the precise temporal- and tissue-specific production of elastin, these features of the promoter are somewhat surprising and may indicate that important regulatory elements lie elsewhere in the gene or that diverse mechanisms regulate tropoelastin production.

The AP-1 recognition element binds c-jun/c-fos heterodimers (Curren & Franza, 1988), and transcriptional activation through this site is characterized as an immediate-early response upon exposure to compounds, such as phorbol ester and tumor necrosis factor-α, that activate protein kinase C (Brenner et al., 1989; Chiu et al., 1988; Lee et al., 1987; Mitchell et al., 1985). Treatment with phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA),[1] transiently upregulates expression of the c-*jun* and c-*fos* resulting in transcriptional regulation of various genes. Although the

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binding of AP-1 to its appropriate gene element usually induces or stimulates transcription, occupancy of this element by c-jun/c-fos heterodimers may also be involved in phenotypic suppression of gene transcription (Owen et al., 1990). The presence of an AP-1 recognition element in a promoter, however, does not necessarily mean that gene expression will be influenced, either positively or negatively, by factors that affect c-jun/c-fos expression.

TPA can promote or otherwise alter cellular differentiation. For example, TPA mediates differentiation of U937 cells, a human premonocytic cell line, to a phenotype resembling that of circulating monocytes or tissue macrophages (Mitchell et al., 1985), and in chick chondrocytes, TPA exposure leads to apparent dedifferentiation as indicated by a switch from expression of type II to type I collagen (Finer et al., 1985). In addition, TPA mediates the regulation of cell-specific protein expression by transcriptional or posttranscriptional mechanisms (Sherman et al., 1990; Zhu et al., 1991). The presence of putative AP-1 sites in the tropoelastin gene promoter suggests that transduction mechanisms activated by phorbol esters may be active in the in vivo regulation of elastogenesis. Our findings indicate that tropoelastin expression is markedly downregulated by exposure to TPA and that this effect is mediated primarily by a posttranscriptional mechanism. These data indicate that phorbol-activated pathways influence elastin production and that the putative AP-1 site in the bovine gene is not active in this regulatory process.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment. Fetal bovine ears were obtained from a nearby slaughterhouse within 20 min after the death of the mother and were kept on ice in Earle's balanced salt solution containing antibiotics. Fetal age was estimated by measurement of the forehead to rump length. Skin and perichondrium were removed, and the cartilage was minced and incubated with gentle stirring in 10 mM calcium acetate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, containing 2 mg/mL bacterial collagenase for 4 h at 37 °C. Released chondrocytes were collected by centrifugation and suspended in Dulbecco's-modified Eagle's medium (DMEM) supplemented with glucose, bicarbonate, nonessential amino acids, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% calf serum. Primary chondrocytes were plated at 1.2×10^6 cells/P100 dish, grown to visual confluence, and passed 1:4. Cells were used at passage 1 or 2 for all experiments. Under these conditions, primary auricular cartilage cells retain the morphological characteristics of cultured chondrocytes and the ability to deposit an extensive, insoluble elastic matrix (Mecham, 1987).

Confluent chondrocytes were exposed to TPA at the indicated concentration for various times. Control cultures were treated with the same volume of dry dimethyl sulfoxide (0.5% final concentration). At the beginning of experimental treatments, cells were refed with serum-containing medium, and cross-linking inhibitors β-aminoproprionitrile (100 μg/mL) and penicillamine (50 μg/mL) were added. At the indicated times, medium and cell layer were collected and processed for the assays described below. To assay RNA turnover, actinomycin D was added to 5 μg/mL to inhibit transcription. At this concentration of actinomycin D, RNA synthesis was inhibited greater than 97% as determined by the incorporation of [^{3}H]UTP into trichloroacetic acid precipitable material.

Quantification of Tropoelastin Synthesis. Tropoelastin production in medium and cell extracts was quantified by an enzyme-linked protein binding immunoassay using rabbit antiserum to bovine tropoelastin. Culture medium from treated samples was assayed directly, and cell layers were extracted in 0.5 M acetic acid, neutralized with 1 N NaOH, diluted 1:1 in culture medium containing 20% calf serum, and assayed. Diluted samples (100 μL) were added to 96-well microtiter plates and assayed as described (Prosser et al., 1991). Tropoelastin levels were determined from a standard curve using 0–16 ng of purified tropoelastin diluted in culture medium and were normalized to micrograms of DNA per plate (Burton, 1956).

RNA Isolation and Hybridization. Cell cultures were washed twice with phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.4) and lysed in 4 M guanidine isothiocyanate buffer, and total RNA was isolated by extraction with acidic phenol/chloroform and ethanol precipitation (Chomczynski & Sacchi, 1987). Purified RNA was denatured in 1 M formaldehyde in the presence of 50% formamide and 50 ng/μL ethidium bromide, resolved by electrophoresis though 1% agarose, and transferred passively to nitrocellulose. Hybridization and wash conditions were as described (Parks et al., 1988) except tropoelastin mRNA was detected with ^{32}P-labeled 12-1, a 2.2-kb bovine tropoelastin cDNA (Parks et al., 1992). Relative tropoelastin mRNA levels were determined by densitometric scanning of autoradiographs and, in some experiments, were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Probes were prepared by nick translation or random priming using [α-^{32}P]dCTP.

In Vitro Transcription. Ongoing transcription was assessed in isolated nuclei by a modification of described techniques (Ley et al., 1989). Cells were harvested after 48 h of exposure to 10^{-7} M TPA, kept on ice, and lysed in 10 mM Tris-HCl, pH 7.4, containing 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40. Nuclei were isolated by centrifugation at 800g, washed once in the lysis buffer, suspended in transcription buffer, and counted with a hemacytometer. Synthesis of nascent transcripts was completed by incubating freshly isolated nuclei (2.5×10^7) for 15 min at 37 °C in transcription buffer consisting of 25 mM Tris-HCl, pH 7.0, 0.12 M KCl, 5 mM magnesium acetate, 10% (v/v) glycerol, 1 mM dithiothreitol, 1 mM ATP, 0.5 mM each CTP and UTP, and 200 μCi of [α-^{32}P]GTP (>3000 Ci/mmol). Radiolabeled RNA was isolated by guanidine isothiocyanate–phenol extraction as for cellular RNA, hybridized to excess gel-purified cDNA and plasmid DNA, and detected by autoradiography. Target DNAs were denatured by heating at 80 °C for 10 min in 4× standard saline–citrate buffer (SSC; 1× is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) containing 0.1 M NaOH, and the mixture was quenched on ice, neutralized with an equal volume of 2 M ammonium acetate, and adsorbed onto nitrocellulose filters using a slot-blot manifold. The blots were baked at 80 °C for 2 h and were hybridized for 48 h with the isolated radiolabeled RNA in 50% deionized formamide, 4× SSC, 2× Denhardt's solution, 20 μg/mL yeast RNA, 50 mM Na_2HPO_4, pH 7.5, and 0.1% sodium dodecyl sulfate (SDS). Blots were washed twice in 2× SSC, 0.1% SDS for 15 min and twice in 0.1× SSC, 0.1% SDS for 15 min

[1] Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMEM, Dulbecco's-modified Eagle's medium; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; SSC, standard saline–citrate buffer; CAT, chloramphenicol acetyltransferase; CMF, calcium magnesium-free medium; UTR, untranslated region.

at 55 °C. Tropoelastin transcription was assessed by hybridization to 12-1 tropoelastin cDNA and compared to hybridization to human β-actin cDNA and to an Alu I genomic clone derived from the human ε-globin gene. Background signal was assessed by hybridization to parental plasmid DNA.

Transient Transfection. An expression construct, pEP52CAT, containing 2.26 kb of the human tropoelastin promoter inserted upstream of sequences coding for chloramphenicol acetyltransferase (CAT) was generously provided by Dr. Jouni Uitto, Thomas Jefferson University. For controls, cells were transfected with an RSV-CAT expression vector or with plasmid DNA. DNAs were purified by repeated equilibrium centrifugation in CsCl–ethidium bromide density gradients. Chondrocytes were transfected by calcium phosphate precipitation (Gorman et al., 1982) with 20 μg of DNA/P100 dish about 24 h before reaching visual confluence. Preconfluent cells were fed with 10 mL of serum-containing DMEM 4 h before transfection. DNA was added to 25 mM Hepes, 140 mM NaCl, 0.75 mM Na_2HPO_4, pH 7, and the mixture was incubated at 37 °C for 30 min. While the solution was bubbled gently, 1 M $CaCl_2$ was added dropwise to 0.125 M. Precipitate was formed by incubating the mixture at ambient temperature for 15 min, and 1 mL was added dropwise per dish. After 4 h, the cultures were washed with calcium magnesium-free medium (CMF), and transfection was enhanced by shocking the cells with 15% glycerol in CMF for 3 min. Dishes were washed three times with CMF and fed with serum-containing DMEM with or without 10^{-7} M TPA. After 48 h, medium was sampled for determination of tropoelastin by ELISA, and cells were harvested by trypsinization, washed twice in PBS, suspended in 200 μL of 0.25 M Tris-HCl, pH 7.9, and lysed by freeze–thawing three times. Cell debris was separated by centrifugation at 8000g for 5 min. Protein concentration was determined using a Bio-Rad protein assay kit, and CAT activity was determined by incubating 100 μg of protein with 0.2 μCi of [^{14}C]chloramphenicol, 20 ng acetyl-coenzyme A, and 0.25 M Tris-HCl, pH 7.6, in a final volume of 130 μL at 37 °C for 18 h. Chloramphenicol was isolated by extraction with 750 μL of ethyl acetate, and the organic phase was isolated and dried. The extracted material was dissolved in 25 μL of ethyl acetate and spotted onto Bakerflex Silicagel 1B TLC plates (J. T. Baker, Thomasville, PA), and the acetylated forms were separated by chromatography in 95% chloroform, 5% methanol and detected by autoradiography.

RESULTS

TPA Represses Tropoelastin Production. Confluent fetal bovine chondrocytes were treated with various concentrations of TPA for 24 or 48 h. At 24 h, the levels of tropoelastin in cell cultures exposed to TPA were not markedly different from those in untreated cells, but at 48 h about 3-fold less tropoelastin had accumulated in cultures exposed to 10^{-7} M TPA and about 2-fold less was detected in cultures treated with 10^{-8} M TPA (Figure 1A). Chondrocytes exposed to 10^{-9} M TPA for 48 h had only a small drop in accumulated tropoelastin levels. Since the culture medium contained β-aminoproprionitrile and penicillamine, compounds which inhibit the cross-linking of tropoelastin into insoluble fibers, and because both cell-associated and soluble protein were assayed, these data represent total tropoelastin production. The proportion of tropoelastin in the medium to that in the cell layer was constant among control and treated chondrocytes indicating that exposure to TPA did not influence the secretion or extracellular compartmentalization of tropoelastin. Furthermore, the mass of DNA per dish was not affected by exposure to TPA up to 72 h (data not shown).

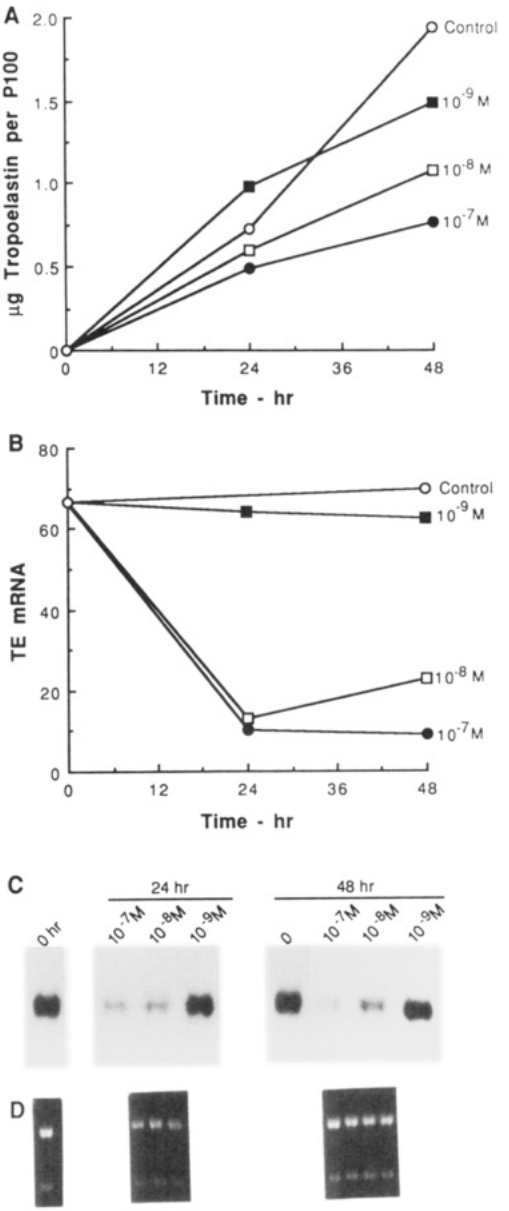

FIGURE 1: Effect of TPA dose on tropoelastin expression. Confluent cultures of bovine chondrocytes derived from a fetus of 220 d were exposed to the indicated concentrations of TPA in serum-containing medium. At 24 or 48 h post-TPA, the medium and cell layer were sampled for ELISA of secreted tropoelastin, and total RNA was isolated from the cell layer of parallel dishes. (A) The mass of tropoelastin in the culture medium and cell layer was assayed as described under Experimental Procedures, and the values per dish were totaled. The data represent the mean of triplicate determinations expressed as the total amount of tropoelastin per dish. Background signal was determined by ELISA of unconditioned medium and subtracted from the values determined from experimental samples. (B) Total RNA (5 μg per lane) was isolated from control chondrocytes and from cells exposed to TPA for 24 or 48 h. Tropoelastin (TE) mRNA was detected by Northern hybridization (shown in panel C) and quantified by densitometry. The relative levels of tropoelastin mRNA are expressed in arbitrary units. (C) The drop in tropoelastin steady-state mRNA levels after exposure to TPA for 24 or 48 h was assayed by Northern hybridization with a ^{32}P-labeled bovine tropoelastin cDNA. Autoradiography was for 6 h with an intensifying screen. Tropoelastin mRNA migrates at 3.5 kb. (D) The equivalency of loading among lanes was demonstrated by ethidium bromide staining of samples. Shown are photomicrographs of the gel lanes represented in panel C before transfer to nitrocellulose.

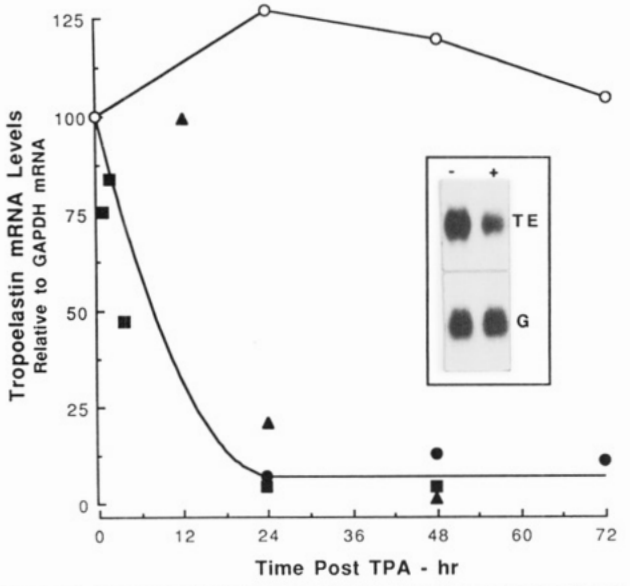

FIGURE 2: Downregulation of tropoelastin mRNA steady-state levels by exposure to TPA. Confluent cultures of bovine chondrocytes derived from 180- (▲), 220- (○,●) and 230-d (■) fetuses were treated with 10^{-7} M TPA (solid symbols) for the indicated times. The data presented are from three experiments, and in one experiment time-matched controls (○) were included to demonstrate the stability of tropoelastin mRNA steady-state levels in confluent cultures. Total RNA was isolated, and nitrocellulose blots containing 5 μg per lane were hybridized simultaneously for tropoelastin and GAPDH mRNAs. Autoradiographs were scanned with a densitometer. Tropoelastin mRNA values were normalized to GAPDH mRNA, and the data are presented as a percent of 0-h controls for each experiment. (Inset) Autoradiogram of 24 h control (–) or TPA-treated (+) samples probed for 3.5-kb tropoelastin (TE) and 1.3-kb GAPDH (G) mRNAs.

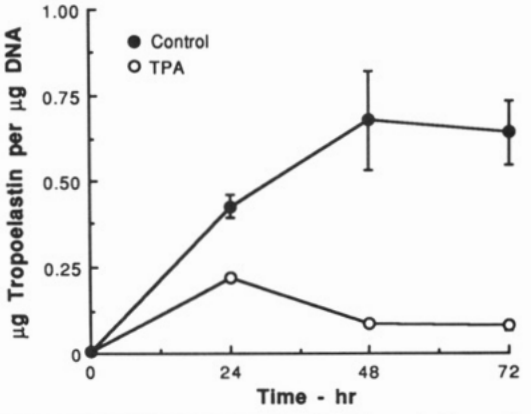

FIGURE 3: TPA mediates decreased levels of tropoelastin production. Chondrocytes from a 180-d fetus were treated with 10^{-7} M TPA for the indicated times in the presence of elastin cross-linking inhibitors. Tropoelastin in the medium and cell layer was quantified by ELISA, and the values were totaled and normalized to the mass of DNA per dish. The data are presented as the mean ± standard deviation of triplicate determinations of both medium and cell layer from three plates per point.

Northern analysis demonstrated that tropoelastin mRNA levels were reduced about 7-fold in cells exposed to 10^{-8} or 10^{-7} M TPA for 24 h, and this downregulation was sustained at 48 h post-TPA (Figure 1B,C). Consistent with the ELISA data, exposure to 10^{-9} M TPA for 24 or 48 h had no appreciable affect on tropoelastin mRNA levels. The slight increase in tropoelastin mRNA levels seen in cells treated with 10^{-8} M TPA for 48 h probably indicates variation in the assay rather than recovery from extended exposure. Indeed, repressed levels of tropoelastin protein and mRNA were maintained up to 72 h post-TPA (Figures 2 and 3). The difference in the magnitude of downregulation between tropoelastin protein levels (about 3-fold) and mRNA levels (about 7-fold) does not necessarily indicate translational regulation. The ELISA measures the total accumulation of tropoelastin over the 24- or 48-h treatment period. Between 24 and 48 h, tropoelastin levels increased in control cells about 3-fold, but in cells exposed to 10^{-7} M TPA, the levels of secreted tropoelastin increased less than 50%. Thus, after 24 h of exposure to TPA, the rate of tropoelastin production was diminished about 6-fold, paralleling the drop in steady-state mRNA levels.

A more detailed time course revealed that the decline in tropoelastin steady-state mRNA levels was detected within a few hours after TPA exposure (Figure 2). These data also demonstrate that TPA induced a persistent repression of tropoelastin expression as indicated by the sustained low levels at 72 h (Figures 2) which was also seen in the levels of secreted protein (Figure 3). TPA exposure did not influence GADPH mRNA levels indicating a specific effect on tropoelastin expression (Figure 2, inset).

Exposure to TPA Does Not Affect Tropoelastin Transcription. The presence of an AP-1 element in the bovine tropoelastin promoter sequences suggests that the gene transcription may be influenced by exposure to phorbol esters. Nuclear runoff assay, however, demonstrated that the rate of tropoelastin transcription in fetal bovine chondrocytes was not affected by a 48-h exposure to 10^{-7} M TPA (Figure 4). In this assay, tropoelastin transcription was compared to total transcription, as determined by hybridization of Alu I containing transcripts, and to transcription of β-actin pre-mRNA. The strong hybridization signal for Alu I indicates that in vitro transcription proceeded properly. High copy numbers of Alu I type repeat elements are present in the introns of many genes transcribed by RNA polymerase II, and, thus, hybridization to these elements provides a representation of total transcription. The decrease in tropoelastin transcription relative to that for β-actin (Figure 4B) reflects a nearly 2-fold increase in β-actin transcription relative to Alu I. This finding is consistent with the TPA-mediated increase in β-actin expression seen in other cells types (Gewirtz & Shen, 1990) and indicates that β-actin is not a constitutively expressed gene.

To further assess transcription, we determined the effect of TPA on the ability of tropoelastin promoter sequences to initiate transcription of a heterologous gene reporter. The expression construct, pEP52CAT, contains the region from –2260 to –14 of the human tropoelastin gene. This fragment includes two putative AP-1 elements and confers responsiveness to TPA and TNF-α in human dermal fibroblasts and rat aortic smooth muscle cells (J. Uitto, personal communication). In fetal bovine cells, however, no effect on CAT activity was noted after a 48-h exposure to 10^{-7} M TPA (Figure 5A) although endogenous tropoelastin expression, as determined by ELISA, was markedly repressed (Figure 5B).

Downregulation of Tropoelastin Is Associated with an Accelerated Decay of the mRNA. The lack of an effect on transcription indicates that TPA mediates a repression of tropoelastin expression at a posttranscriptional level. To assess if this mechanism involves degradation of tropoelastin mRNA, we exposed TPA-treated cells to actinomycin D to shut off new transcription and tracked the decay of tropoelastin mRNA by Northern hybridization of total RNA. In control cells, tropoelastin mRNA was relatively stable with a half-life of about 20 h (Figure 6).

After a 12- or 24-h exposure to TPA, tropoelastin transcripts decayed rapidly with a half-life of about 2.2 h, and this rate

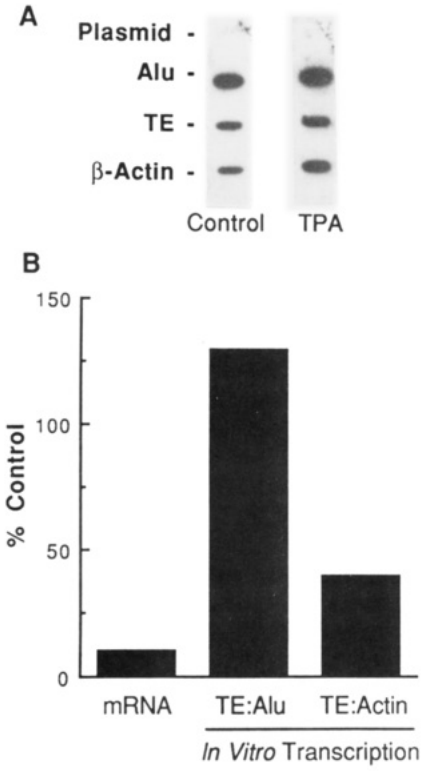

FIGURE 4: TPA does not affect tropoelastin transcription. Nuclei were isolated from 220-d fetal chondrocytes treated with 10^{-7} M TPA for 48 h and used in a runoff assay as described under Experimental Procedures. (A) Transcribed, ^{32}P-labeled RNA was hybridized to parental plasmid DNA, a genomic fragment containing Alu I repeat sequences (Alu) and cDNAs for a bovine tropoelastin cDNA (TE) and β-actin. Autoradiography was for 5 d with an intensifying screen. (B) The density of the autoradiographic signal for tropoelastin transcripts was normalized to that for Alu I or β-actin, and the values are presented as the percent change of the transcription rate in TPA-treated cells relative to control cells. Percent change of tropoelastin steady-state mRNA levels in 48-h TPA-treated chondrocytes (from Figure 2) is included for comparison.

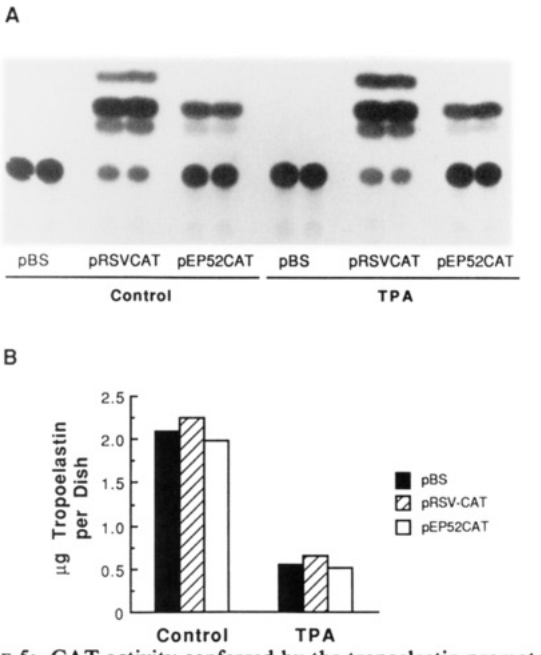

FIGURE 5: CAT activity conferred by the tropoelastin promoter is not influenced by TPA in transiently transfected bovine cells. (A) Chondrocytes derived from a 220-d fetus were transfected with 20 μg of reporter DNA as described under Experimental Procedures. After transfection, cells were exposed to 10^{-7} M TPA or were kept in control medium, and CAT activity was assayed 48 h later. Cells were transfected with plasmid DNA (pBS), pRSV-CAT, or a CAT construct containing 2.2 kb of the human tropoelastin promoter (pEP52CAT). CAT activity was assessed by the conversion of [^{14}C]-chloramphenicol to acetylated forms which migrate faster on thin-layer chromatography plates. (B) At the time cells were harvested for the CAT activity assay, culture medium was sampled for determination of tropoelastin protein levels by ELISA.

was consistent between two experiments (Figure 6). The decline in transcript stability (about 9-fold) closely approximated the drop in steady-state mRNA levels (about 9-fold). In control and TPA-treated cells, relative tropoelastin mRNA levels increased slightly yet reproducibly after a 3-h exposure to actinomycin D (Figure 6). This small increase in relative steady-state mRNA levels may reflect variation in the Northern assay, or it may indicate that actinomycin D mediated a repression of a labile mechanism involved in but not necessary for TPA-induced destabilization of tropoelastin mRNA.

DISCUSSION

The experiments reported here demonstrate that the expression of tropoelastin in bovine cells is markedly repressed by exposure to phorbol ester and that this effect is controlled by a posttranscriptional mechanism characterized by an accelerated decay of tropoelastin mRNA. Exposure to TPA did not influence GAPDH mRNA levels or alter the proliferation of cells, as determined by DNA content, indicating that the repressive effect was specific for tropoelastin and was not a secondary effect due to general cellular catabolism mediated by extended exposure to phorbol esters. As demonstrated by nuclear runoff assay, the rate of tropoelastin transcription was not affected by exposure to TPA. Similarly, transcription of a heterologous gene, under control of 2.26 kb of the human tropoelastin promoter, was not influenced by TPA. In our experiments, tropoelastin steady-state mRNA levels dropped about 10-fold after a 24–48-h exposure to TPA, and the magnitude of this decrease can be accounted by the change in the decay kinetics of the mRNA. In control cells, tropoelastin mRNA turned over with a half-life of about 20 h, but in TPA-treated chondrocytes, the transcript half-life was reduced about 9-fold to 2.2 h. Although other mechanisms may contribute to the decline in tropoelastin production, we have found that the translatability of tropoelastin mRNA is not influenced during developmental expression (Parks et al., 1988) or by repression with 1,25-dihydroxyvitamin D_3 (Pierce et al., 1992). Thus, transcript destabilization may be an important and primary mechanism in the regulation of tropoelastin expression.

TPA influences the transcription of many genes, and this effect is typically an early response mediated by the production and binding of c-jun/c-fos, or AP-1 heterodimers, to precise cis-regulatory sequences, called TPA-responsive elements or TREs, within the promoter regions of affected genes. The bovine gene has one putative AP-1 site, at bases –585 to –578 (Yeh et al., 1989), but the sequence of this element (TGTC-TCT) suggests that it may not be responsive to TPA or capable of interacting with AP-1 complexes. The consensus sequence for the AP-1 recognition element is 5′ TGA(G/C)TCA 3′, and, as shown by mutational analysis, enhancer activity is dependent on the first T and A bases (Angel et al., 1987; Lee et al., 1987; Schüle et al., 1990). Since the putative AP-1 element in the bovine tropoelastin gene has a substitution of an A for a T in the third position, this site may not be involved in TPA-mediated downregulation of tropoelastin of expression.

Although the sequence of the putative bovine TRE does not indicate regulation at the level of the gene, AP-1 may influence

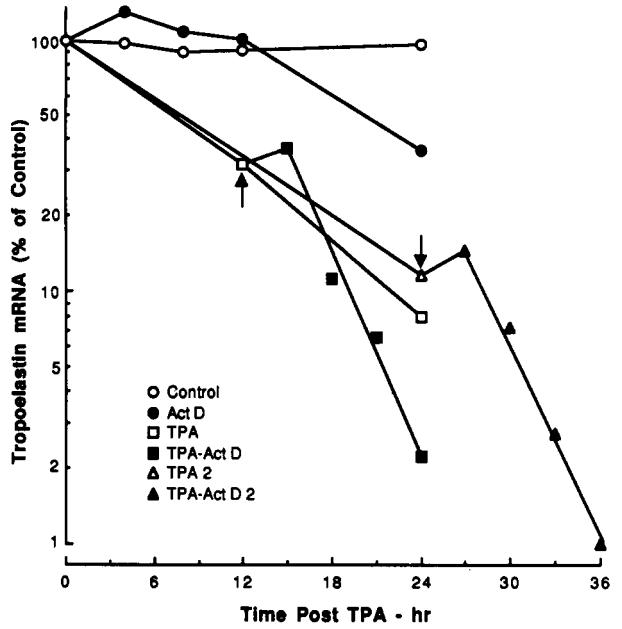

FIGURE 6: TPA mediates an accelerated decay of tropoelastin mRNA. Confluent chondrocytes derived from a 220-d fetal calf were exposed to 10^{-7} M TPA. At 12 or 24 h post-TPA (arrows), actinomycin D was added to a final concentration of 5 μg/mL, and cells were harvested at 3-h intervals over the next 12 h for isolation of total RNA. At 0 h, control cells were refed with fresh medium, and some dishes were given actinomycin D. Total RNA was isolated at 4, 8, 12 and 24 h post-actinomycin D. RNA (5 μg/mL) was analyzed by Northern hybridization for tropoelastin mRNA. Autoradiograms were scanned, and the values are expressed on a log scale relative to the 0-h control value.

the transcription of a trans-acting factor that directly regulates the stability of tropoelastin mRNA. Indeed, exposure of TPA to promonocytic U937 cells regulates a ribonuclease activity that specifically degrades transforming growth factor β1 mRNA (Wager & Assoian, 1990), and phorbol ester-mediated stabilization of c-*fms* mRNA is dependent on the synthesis of a labile protein (Weber et al., 1989).

TPA-responsive elements may be present elsewhere in the tropoelastin gene, but the methods we used to determine the regulatory mechanism indicate strongly that tropoelastin transcription was not affected by exposure to TPA. The nuclear runoff assay measures ongoing transcription from the endogenous gene and is an accurate indicator of the influence of transcription rate. Unlike the runoff assay, CAT assays of transfected promoter constructs indicate the effects of defined cis-acting sequences on the control of gene expression. The effects of chromatin structure, methylation, and other cis-acting sequences on gene expression do not influence promoter construct activity. The internal consistency of our runoff and transfection assays indicates that TPA does not influence tropoelastin transcription in bovine cells.

Posttranscriptional regulation of the tropoelastin gene expression may be a common regulatory mechanism. Stimulation of tropoelastin expression by human dermal fibroblasts in response to transforming growth factor-β1 is controlled by transcript stabilization (Kähäri et al., 1992), and marked downregulation mediated by exposure of fetal bovine chondrocytes and neonatal rat lung fibroblasts to 1,25-dihydroxyvitamin D_3 is regulated posttranscriptionally (Pierce et al., 1992). Although we do not yet know if posttranscriptional mechanisms regulate the cessation of elastin production in vivo, our findings indicate that accelerated decay of tropoelastin mRNA can completely turn off production in vitro. Posttranscriptional mechanisms are associated with the control of various products such as cytokines and cell cycle proteins and differentiation-specific proteins (Hargrove & Schmidt, 1989; Sachs, 1991; Shapiro et al., 1987; Shaw & Kamen, 1986; Shaw et al., 1986). Thus, it is possible that elastogenesis is regulated by controlling the decay kinetics of tropoelastin mRNA. Being similar to a constitutively expressed gene, the tropoelastin promoter may allow for permissive transcription thereby necessitating temporal and tissue-specific regulation at a posttranscriptional level. Although this proposed mechanism seems relatively inefficient, transcription is not necessarily an efficient pathway for control of gene expression. Regulated eukaryotic transcription requires the coordinate expression of multiple and specific trans-acting factors, enzyme, and ribonucleotide–protein complexes, and most of the primary transcript sequences are spliced before the mature mRNA is transported to the cytosol. In addition, repression of gene expression requires the continued production of silencer factors. Thus, it is possible that posttranscriptional mechanisms are involved in the temporally precise and tissue-specific production of elastic fibers.

Stabilization or specific degradation of mRNA contributes to the control of expression of many genes, and often sequences located in the 3′ untranslated region (UTR) are important determinants in transcript half-life (Brawerman, 1987; Shapiro et al., 1987). For example, the mRNAs for several lymphokines contain AU-rich sequence motifs in the 3′ UTR (Caput et al., 1986) which target the mRNAs for rapid degradation (Shaw & Kamen, 1986), and the 3′ UTR of ferritin mRNA contains sequence elements important in the regulation of steady-state mRNA levels (Casey et al., 1988). The 3′ UTR of tropoelastin mRNA does not contain sequences that have been associated with regulated degradation of other transcripts, and, thus, TPA-mediated decay of tropoelastin mRNA may be controlled by unique cis-acting sequences. The 3′ UTR of tropoelastin mRNA is similar in size and sequence among mammals, and portions of the 3′ UTR of tropoelastin mRNA have extensive identity among human, bovine, and rat sequences (Indik et al., 1990; Parks & Deak, 1990). Thus, cis elements in the 3′ UTR of tropoelastin mRNA may be involved in the regulation of tropoelastin gene expression.

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