Blockage of Tropoelastin Secretion by Monensin Represses Tropoelastin Synthesis at a Pretranslational Level in Rat Smooth Muscle Cells

STEVEN M. FRISCH,[1,2]* JEFFREY M. DAVIDSON,[3] AND ZENA WERB[1,4]

Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143[1]; Department of Biochemistry, University of California, Berkeley, California 94720[2]; Department of Pathology, Veterans Administration Medical Center, University of Utah, Salt Lake City, Utah 84148[3]; and Department of Anatomy, University of California, San Francisco, California 94143[4]

The blockage of protein secretion in the R22 cultured rat aortic smooth muscle cell strain with monensin repressed tropoelastin gene expression at the mRNA level by ca. 50-fold as measured by biosynthetic pulse-labeling, in vitro translation, and hybridization with a tropoelastin genomic DNA probe. These results suggest that tropoelastin gene expression is autoregulated, and they represent the first reported effect of monensin on gene expression.

Elastin, a major protein of the extracellular matrix of elastic tissues such as aorta and lung, is assembled by the covalent cross-linking of tropoelastin (TE), its 72-kilodalton (kDa) soluble precursor. Cells committed to elastogenesis devote as much as 25 to 40% of their total protein synthesis to TE in the later part of fetal life and in the newborn animal, yet they synthesize little or no detectable TE in adult connective tissues. These changes are reflected in altered levels of TE mRNA (1, 5, 24), although the nature of the regulation is unknown.

There is evidence that the synthesis of another secreted connective tissue macromolecule, procollagen, is subject to feedback repression by fragments of the protein itself (8). A similar general mechanism may be involved in the negative autoregulation of synthesis of tubulin (2) and simian virus 40 T antigen (22). We have used vascular smooth muscle cells as a model system in which to investigate the regulation of TE synthesis. To block protein secretion, we used monensin, a polycyclic ionophore that disrupts monovalent cation gradients across biological membranes and arrests the transit of secretory proteins from the early (proximal) to late (distal) Golgi subcompartments, causing an accumulation of secretory proteins within the cell (25). In this report, we demonstrate that monensin repressed TE synthesis at the mRNA level, suggesting that TE gene expression may be autoregulated.

The aortic smooth muscle cell strain, R22, which was isolated by Jones et al. (9) from embryonic rat heart, synthesizes and secretes several extracellular matrix components in large amounts (6, 29, 30), devoting 10 to 30% of its total protein synthesis to TE. We identified the TE gene product by its preferential labeling with [³H]valine, complete resistance to CNBr cleavage owing to its lack of methionine (23) (Fig. 1a), comigration in two-dimensional gel electrophoresis with purified chick TE (Fig. 1b), and partial chymotryptic peptide mapping in comparison with chick TE (data not shown).

First, we showed that TE is accumulated within cells treated with monensin. R22 cells were incubated with 20 μCi

of [³H]valine and 50 μg of β-aminopropionitrile per ml in minimal Eagle medium without nonradioactive valine in the presence of 0 to 20 μM monensin (Sigma Chemical Co.) for 4 or 10 h, and intracellular proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 2a). Because a significant amount of secreted TE spontaneously coacervated into an insoluble form in aqueous buffer despite the presence of β-aminopropionitrile, the following protocol was developed to exclude extracellular contamination. Cells were harvested by trypsinization with 0.5 mg of crystalline trypsin (Worthington Diagnostics) per ml for 6 min at the end of the labeling/treatment period, after which the trypsin was neutralized with a 10-fold excess of soybean trypsin inhibitor. Cells were transferred to microfuge tubes, washed twice with normal saline, and lysed in SDS sample buffer (12), in which TE is soluble. Monensin-treated cells showed a dramatic accumulation of TE, as well as a lesser accumulation of other classes of secreted proteins, including fibronectin (~220 kDa) and procollagens (~180 kDa). This result was consistent with data from other systems (25), demonstrating that monensin inhibits the secretion of a broad spectrum of proteins. In our smooth muscle cells, the larger accumulation of TE compared with that of other secretory proteins suggests either that multiple secretion pathways were being used (7) or that there was differential degradation of proteins intracellularly. We then determined the rates of TE synthesis by pulse-labeling monensin-treated cells with [³H]valine for 15 min (Fig. 2b), which was shorter than the average TE secretion time as measured in pulse-chase experiments (data not shown). A dose-dependent and time-dependent decrease in TE synthesis rates was seen, whereas overall protein synthesis rates were unaffected or only slightly stimulated by monensin. In addition to TE, the synthesis rates of several other major proteins were affected; most dramatically, the expression of several high-molecular-weight polypeptides (180 to 220 kDa) comigrating with fibronectin and collagens was repressed, and a 75-kDa protein was induced (Fig. 2b).

To determine whether the repression of TE synthesis was due to inhibition of TE translation or to decreased TE mRNA levels, we isolated total RNA from R22 cells treated with 20 μM monensin for 24 h by the method of Tolstoshev

___

* Corresponding author.

et al. (28) and assayed the TE mRNA content of each sample by both in vitro translation and Northern blot analysis. The total RNA was translated in standard micrococcal nuclease-treated rabbit reticulocyte lysates (20) that lacked valine and were supplemented with [^{3}H]valine (Fig. 3a, lanes 1 to 4). For the study of TE mRNA pSS1, a sheep genomic TE DNA

probe (4a) was shown first to hybridize specifically with rat TE mRNA by the criterion of hybrid-selected translation. Nitrocellulose filters containing 20 μg of the tropoelastin genomic DNA probe pSS1, which had been linearized with SalI, or 20 μg of salmon sperm DNA, were prepared according to Miller et al. (17). Hybridization with 50 μg of
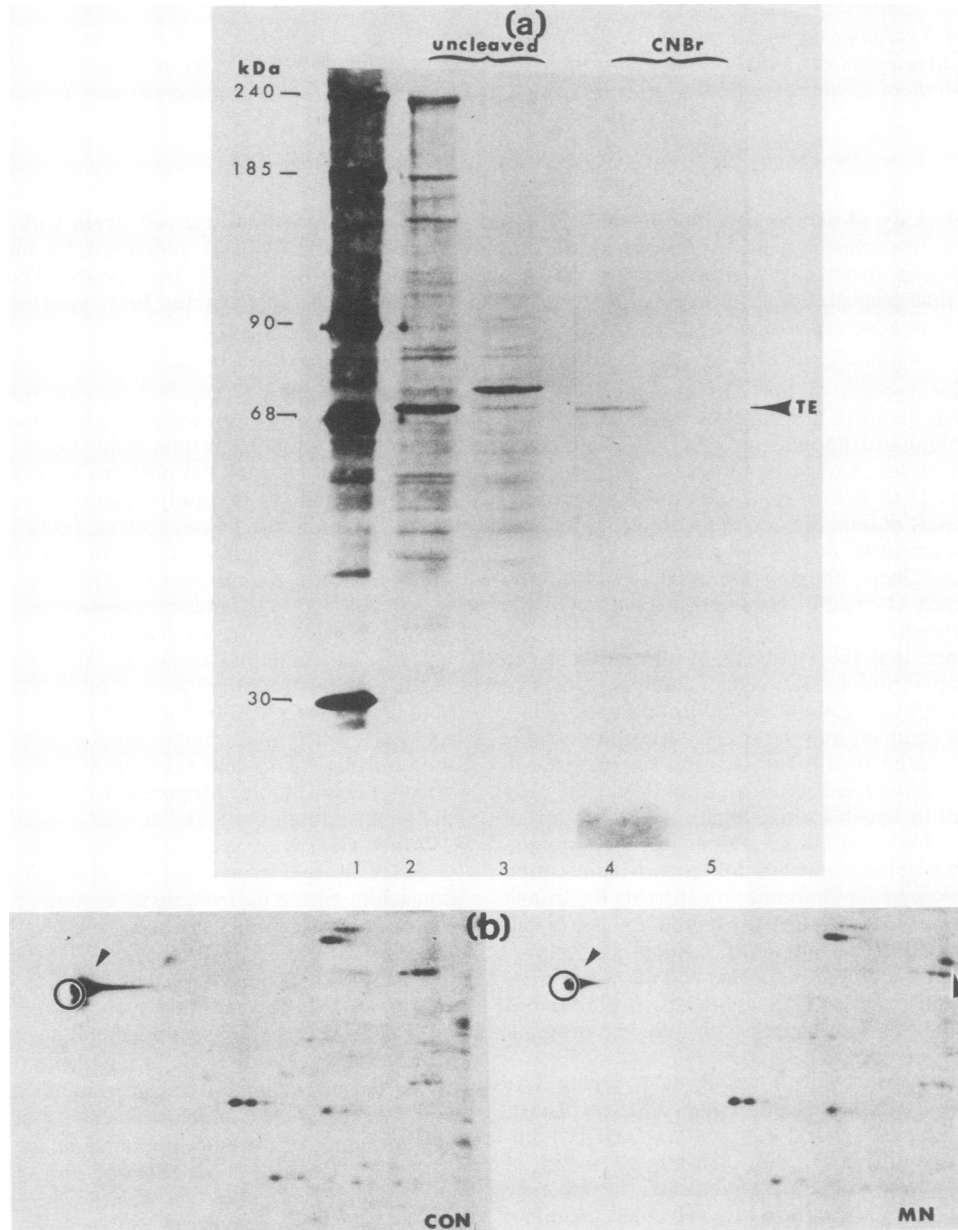

FIG. 1. (a) CNBr cleavage of TE and a 75-kDa monensin-induced protein. Untreated cells (lanes 2 and 4) or cells treated with 1 μM monensin for 24 h (lanes 3 and 5) were incubated for 15 min in valine-free minimal Eagle medium containing 20 μCi of [^{3}H]valine per ml (38 Ci/ml, Amersham Corp.) and lysed in SDS sample buffer (12). Lysates were undigested (lanes 2 and 3) or digested with 20 mg of CNBr per ml (lanes 4 and 5) in 70% formic acid for 16 h at room temperature, lyophilized, redissolved in SDS sample buffer, and analyzed on a 5 to 12% SDS-polyacrylamide gel (12) by fluorography. Molecular weight standards are shown in lane 1. (b) Two-dimensional gel analysis of radiolabeled proteins from R22 cells that were untreated (CON) or treated with 1 μM monensin for 24 h (MN). Cells were labeled as in (a) and lysed in urea sample buffer (10), and proteins were separated on 13-cm nonequilibrium pH gradient electrophoretic gels (10) for 1,600 V-h. Second-dimension separation was performed on 5 to 12% SDS-polyacrylamide gels that were Coomassie stained and then fluorographed. The position of chick TE (a gift of R. Rucker, University of California, Davis) is indicated by an open circle, and radiolabeled R22 TE is shown by a downward arrowhead. The 75-kDa protein induced by monensin is indicated by an upward arrowhead. First-dimension migration was from right to left (acidic to basic). Repression of TE synthesis was minimal in this experiment because of the low monensin dose used; see Fig. 2b.
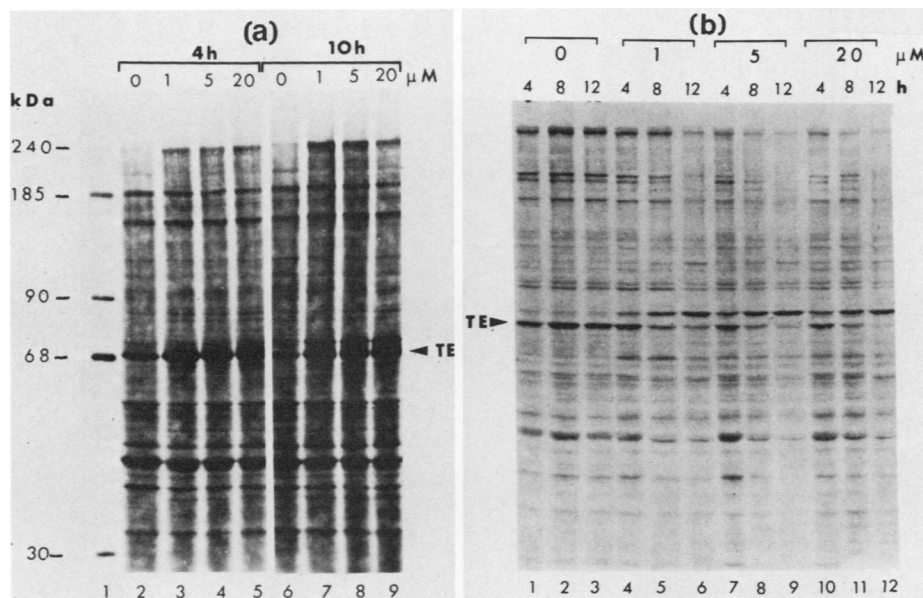
FIG. 2. (a) Intracellular accumulation of TE in monensin-treated R22 cells (continuous label experiment). Proteins from R22 cells continuously labeled with [³H]valine in the presence of monensin as described in the text were precipitated by the addition of 30 μl of Nonidet P-40 and 75 μl of 50% trichloroacetic acid, washed twice in cold acetone, evaporated, and dissolved in SDS sample buffer. Equal amounts of radioactivity were applied to each lane of the 5 to 12% SDS-polyacrylamide gel, followed by fluorography. The position of the TE band is indicated by an arrow, and molecular weight standards are indicated at the left. (b) TE synthesis rates in monensin-treated R22 cells. Cells were treated with 0 to 20 μM monensin for 4 to 12 h in minimal Eagle medium plus 10% fetal calf serum before incubation for 15 min in valine-free minimal Eagle medium containing 20 μCi of [³H]valine per ml. After two washes with phosphate-buffered saline, the cells were lysed in 0.5 ml of SDS sample buffer (12) and boiled, and the proteins were precipitated by the method described in (a). Equal amounts of radioactivity were applied to each lane of the 5 to 12% SDS-polyacrylamide gel, which was fluorographed.

total RNA was performed at 47°C for 4 h, the filters were washed, and specifically bound mRNA was eluted (17). The eluted mRNA was precipitated with ethanol and translated in rabbit reticulocyte lysates (Fig. 3a, lanes 5 to 7). The Northern blot hybridizations were performed with 5 to 10 μg of the same preparations of total RNA used in the in vitro translations. RNAs were fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde (13), transferred to nitrocellulose (26), baked, and prehybridized according to Maniatis et al. (15). The prehybridization mixture was then replaced with a hybridization mixture (50% deionized formamide, 50 mM sodium phosphate [pH 6.5], 4% dextran sulfate, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2× Denhardt solution, 200 μg of salmon sperm DNA per ml) containing 5×10^7 cpm (~0.5 μg) of pSS1, pA1 (3), or pRM223 (27) DNA nick-translated (21) with DNA polymerase I and DNase I (Bethesda Research Laboratories). As a control, the RNAs were hybridized with the chick actin plasmid pA1 (3) or the rabbit muscle aldolase plasmid pRM223 (27). After 16 h of hybridization at 42°C, the filters were washed three times in 2× SSC–0.1% SDS at room temperature and three times in 0.4× SSC–0.1% SDS at 50°C, dried, and autoradiographed with the use of an intensifying screen (Fig. 3b). Although monensin caused a 3- to 4-fold decrease in the actin mRNA level, aldolase mRNA actually increased 3- to 5-fold, and as much as a 50-fold repression of TE mRNA synthesis was observed in cells treated with the highest dose of monensin, as determined densitometrically from the autoradiograms of Northern blots.

A more detailed kinetic and dose-response analysis of TE mRNA levels by hybridization is shown in Fig. 4a, and the in vitro translations of the same RNA samples, densitometric-

ally scanned for ease of interpretation, are shown in Fig. 4b. (The magnitude of TE mRNA synthesis repression assayed by in vitro translation is probably underestimated because of saturation of the translation system with TE mRNA due to the unusual amino acid composition of TE [23], requiring large amounts of charged glycyl-, alanyl-, valyl-, and prolyl-tRNAs.) The translation and hybridization experiments (Fig. 4a and b) showed similar kinetics of inhibition of TE synthesis that paralleled those in the biosynthetic experiments. Thus, the repression of TE synthesis in R22 cells by monensin is probably attributable to a specific decrease in TE mRNA levels; however, we have not yet ascertained whether transcription, RNA processing, or RNA degradation steps were affected.

Other interesting points emerged from the RNA data. Because the kinetics of induction of the 75-kDa protein were very similar to the kinetics of repression of TE synthesis (Fig. 2b), we deemed it possible that the two proteins were related. However, the in vitro translation experiment indicated that the 75-kDa protein induced by monensin was translated from an RNA distinct from TE mRNA; therefore, the two proteins are probably not related to each other by a protein processing mechanism that is perturbed by monensin. Other evidence makes it unlikely that the 75-kDa protein is a TE-like protein translated from an abnormally processed mRNA or an mRNA encoded by a different TE gene. First, the dramatic difference between this protein and TE in first-dimension migration on nonequilibrium pH gradient electrophoretic gels (Fig. 1b) suggests a large difference in amino acid content. Second, the 75-kDa induced protein was labeled with [³⁵S]methionine (data not shown) and was degraded by CNBr (Fig. 1a), which would indicate the
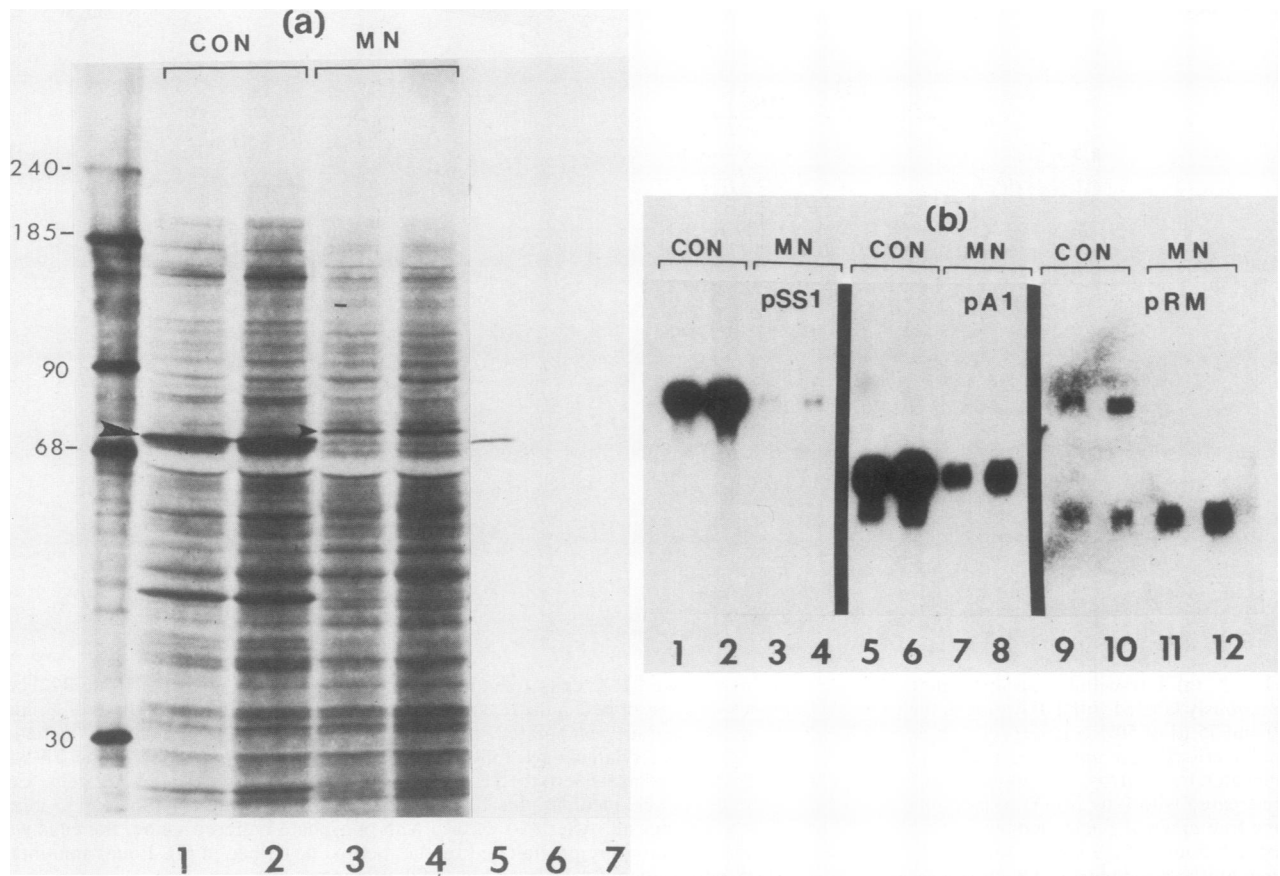
FIG. 3. (a) In vitro translation of mRNA from untreated (CON) or monensin-treated (MN) R22 cells. Cells were treated with 20 μM monensin for 24 h, and total RNA was prepared. Two micrograms (lanes 1 and 3) or 4 μg (lanes 2 and 4) of each RNA was translated in 15 μl of a rabbit reticulocyte lysate system (20), 5 μl of which was analyzed on a 5 to 12% SDS-polyacrylamide gel (12) followed by fluorography. The larger arrow indicates the tropoelastin band; the smaller arrow indicates the 75-kDa monensin-induced protein. Lanes 5 to 7: hybrid-selected translation of TE mRNA. Lane 5: pSS1 DNA × control RNA; lane 6: pSS1 DNA × monensin RNA (20 μM, 24-h monensin treatment); lane 7: salmon sperm DNA × control RNA. (b) Northern blots of 5 μg (lanes 1, 3, 5, 7, 9, 11) or 10 μg (lanes 2, 4, 6, 8, 10, 12) of RNAs from control (CON) and monensin-treated (MN) R22 cells (described in a) hybridized with TE (pSS1), actin (pA1), or aldolase (pRM223) probes. (The filter that was hybridized with pRM223 was reused without probe elution after hybridization with pSS1, so the TE mRNA bands are visible.)
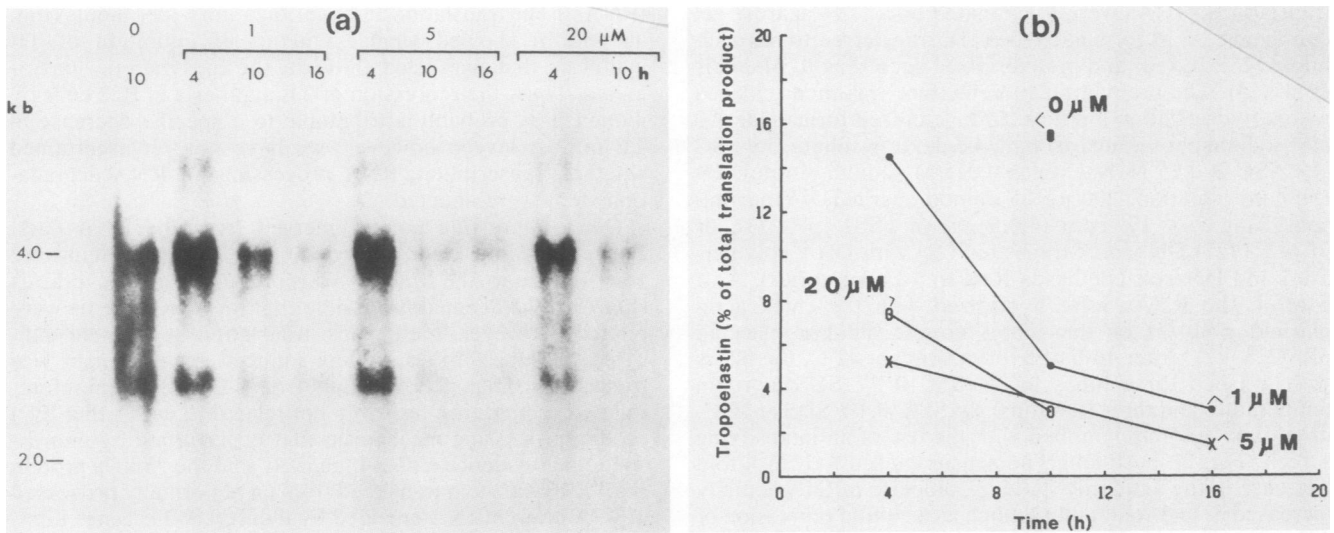

FIG. 4. (a) Time course and dose response of monensin effects on TE mRNA levels assayed by hybridization. RNAs from cells treated with monensin (0 to 20 μM) for 4 to 16 h were isolated as described in the legend to Fig. 3a, and Northern blot analysis was performed as described in the legend to Fig. 3b, using nick-translated pSS1 probe. (b) Time course and dose response of monensin effects on TE mRNA levels assayed by in vitro translation. RNA (2 μg) from cells treated as indicated was translated in 15-μl reactions, and 5 μl was analyzed on an SDS gel by fluorography; the autoradiogram was scanned with a Hoefer GS300 gel scanner. The ordinate represents the percentage of total peak area due to TE.

256

presence of several internal methionyl residues. Furthermore, our TE DNA probe failed to select any translatable mRNA from monensin-treated R22 cells.

The inhibition of TE gene expression by monensin may be explained by several models. Because monensin inhibits protein secretion, our data suggest feedback regulation of TE gene expression. Negative autoregulation of gene expression has been demonstrated in other systems by various approaches. Tubulin synthesis can be repressed either by increasing the intracellular concentration of monomer with colchicine (2) or by microinjecting purified tubulin into cells (4). Procollagen synthesis is repressed by inhibition of secretion (19) or by a peptide derived from the amino terminus of collagen (8). In *Escherichia coli*, mutations in *secA* or *secC* genes, encoding components of the secretory machinery, specifically repress the synthesis of secretory proteins, and the repression can be alleviated by signal sequence mutations (11). Simian virus 40 T antigen represses the transcription of its own gene in an in vitro transcription system (22). If the repression of TE synthesis is due to autoregulation of TE gene expression, then one would expect that an increased intracellular concentration of TE would cause a decreased rate of TE synthesis, perhaps by some mechanism such as transcriptional repression by a TE-TE receptor complex.

However, several alternative models cannot be ruled out. Monensin increases cellular pH, to which the expression of certain genes may be sensitive (18); in preliminary experiments with cellular alkalinizing agents such as ammonium chloride (15 mM) and chloroquine (100 μM), we have observed repression of TE synthesis, although these agents may inhibit protein secretion to some extent. (Interestingly, mitogens that transiently alkalinize cells, such as platelet-derived growth factor, stimulate glycolysis [18], which may be relevant to the aldolase mRNA induction we observed.) Monensin also prevents receptor recycling (25), and there is evidence for a receptor mediating chemotaxis to elastin peptides (16) that may be involved in modulating TE gene expression. Selective intracellular receptors may be involved in the transit of secreted proteins through the Golgi complex (14), and occupation of such a receptor because of monensin may also feed back to regulate mRNA levels. As another possibility, it has been proposed (Z. Werb, J. Cell Biol. 91:155a, 1981) that TE gene expression may be sensitive to changes in cytoskeletal structure, although the agents used in those experiments could have also inhibited TE secretion. It may be possible to test the autoregulation hypothesis by measuring TE synthesis rates in cells that have been microinjected with purified TE.

LITERATURE CITED

1. **Burnett, W., R. Eichner, and J. Rosenbloom.** 1980. Correlation of functional elastin messenger ribonucleic acid levels and rate of elastin synthesis in the developing chick aorta. Biochemistry **19:**1106–1111.
2. **Cleveland, D., and M. Kirschner.** 1981. Autoregulatory control of the expression of α- and β-tubulins: implications for micro-

tubule assembly. Cold Spring Harbor Symp. Quant. Biol. **46:**171–183.
3. **Cleveland, D. W., M. A. Lopata, R. J. McDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell **20:**95–105.
4. **Cleveland, D. W., M. F. Pittenger, and J. R. Feramisco.** 1983. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. Nature (London) **305:**738–740.
4a.**Davidson, J. M., S. Shibahara, M. P. Schafer, P. Tolstoshev, and R. G. Crystal.** 1984. Sheep elastin genes: isolation and preliminary characterization of a 9.9 kilobase genomic clone. Biochem. J. **220:**643–652.
5. **Davidson, J. M., K. Smith, S. Shibahara, P. Tolstoshev, and R. G. Crystal.** 1982. Regulation of elastin synthesis in developing sheep nuchal ligament by elastin mRNA levels. J. Biol. Chem. **257:**747–754.
6. **De Clerck, Y. A., and P. A. Jones.** 1980. The effect of ascorbic acid on the nature and production of collagen and elastin by rat smooth-muscle cells. Biochem. J. **186:**217–225.
7. **Farquhar, M. G., and G. E. Palade.** 1981. The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. J. Cell Biol. **91(Suppl.):**77–103.
8. **Horlein, D., J. McPherson, S. Han Goh, and P. Bornstein.** 1981. Regulation of protein synthesis: translational control by procollagen-derived fragments. Proc. Natl. Acad. Sci. U.S.A. **78:**6163–6167.
9. **Jones, P. A., T. Scott-Burden, and W. Gevers.** 1979. Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells. Proc. Natl. Acad. Sci. U.S.A. **76:**353–357.
10. **Jones, P. P.** 1980. Analysis of radiolabeled lymphocyte proteins by one- and two-dimensional polyacrylamide gel electrophoresis, p. 398–440. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman & Co., San Francisco.
11. **Kumamoto, C. A., D. B. Oliver, and J. Beckwith.** 1984. Signal sequence mutations disrupt feedback between secretion of an exported protein and its synthesis in *E. coli*. Nature (London) **308:**863–864.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:**4743–4751.
14. **Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous.** 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. Nature (London) **304:**80–83.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Mecham, R. P., G. L. Griffin, J. G. Madras, and R. M. Senior.** 1984. Appearance of chemotactic responsiveness to elastin peptides by developing fetal bovine ligament fibroblasts parallels onset of elastin production. J. Cell Biol. **98:**1813–1816.
17. **Miller, J. S., B. M. Paterson, R. P. Ricciardi, L. Cohen, and B. E. Roberts.** 1983. Methods utilizing cell-free protein-synthesizing systems for the identification of recombinant DNA molecules. Methods Enzymol. **101:**650–674.
18. **Moolenaar, W. H., R. Y. Tsien, P. T. van der Saag, and S. W. de Laat.** 1983. Na$^+$/H$^+$ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature (London) **304:**645–648.
19. **Neblock, D. S., and R. A. Berg.** 1982. Lysosomotropic agents ammonium chloride and chloroquine inhibit both the synthesis and secretion of procollagen by freshly isolated embryonic chick tendon cells. Biochem. Biophys. Res. Commun. **105:**902–908.
20. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67:**247–256.

21. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.

22. Rio, D., A. Robbins, R. Myers, and R. Tjian. 1980. Regulation of simian virus 40 early transcription *in vitro* by a purified tumor antigen. Proc. Natl. Acad. Sci. U.S.A. 77:5706–5710.

23. Rucker, R., and D. Tinker. 1977. Structure and metabolism of arterial elastin. Int. Rev. Exp. Pathol. 17:1–47.

24. Shibahara, S., J. M. Davidson, K. Smith, and R. G. Crystal. 1981. Modulation of tropoelastin production and elastin messenger ribonucleic acid activity in developing sheep lung. Biochemistry 20:6577–6584.

25. Tartakoff, A. M. 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32:1026–1028.

26. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77:5201–5205.

27. Tolan, D. R., A. B. Amsden, S. D. Putney, M. S. Urdea, and E. E. Penhoet. 1984. The complete nucleotide sequence for rabbit muscle aldolese A messenger RNA. J. Biol. Chem. 259:1127–1131.

28. Tolstoshev, P., R. A. Berg, S. I. Rennard, K. H. Bradley, B. C. Trapnell, and R. G. Crystal. 1981. Procollagen production and procollagen messenger RNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth. J. Biol. Chem. 256:3135–3140.

29. Werb, Z., D. F. Bainton, and P. A. Jones. 1980. Degradation of connective tissue matrices by macrophages. III. Morphological and biochemical studies on extracellular, pericellular, and intracellular events in matrix proteolysis by macrophages in culture. J. Exp. Med. 152:1537–1553.

30. Werb, Z., M. J. Banda, and P. A. Jones. 1980. Degradation of connective tissue matrices by macrophages. I. Proteolysis of elastin, glycoproteins, and collagen by proteinases isolated from macrophages. J. Exp. Med. 152:1340–1357.