Elastin mRNA Levels and Insoluble Elastin Accumulation in Neonatal Rat Smooth Muscle Cell Cultures†

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ABSTRACT: Insoluble elastin accumulation, elastin mRNA translational efficiencies, and elastin mRNA levels were evaluated in cultures of neonatal rat aortic smooth muscle cells grown for several days in consecutive passages. When the products of in vitro translation were immunoprecipitated with an anti-α-elastin antibody, a single 79 000-Da protein was obtained. Northern blot analysis also indicated an elastin mRNA species corresponding to approximately 4.2 kilobases. Insoluble elastin accumulation increased in cells cultured for 7–21 days in first through fourth passages, while with one exception, relative levels and translational activity of elastin mRNA decreased with time in culture. The data indicated that a simple relationship between elastin accumulation and elastin mRNA levels was not evident.

The study of elastin biosynthesis has mainly been focused on the identity of the insoluble elastin protein(s) synthesized from several different species in vitro and in vivo. However, controversy has developed over the true identity of the primary translation product. Reports on the number of species and their molecular weights have varied. Several investigators have reported the in vitro translation of two elastin proteins with molecular weights of 70 000 and 73 000 (Foster et al., 1980, 1983; Davidson et al., 1982; Saunders & Grant, 1984). These proteins have been referred to as tropoelastin a and b. Still others have reported the existence of three tropoelastins that are products of unique mRNAs (Wrenn et al., 1987; Raju & Anwar, 1987). The possibility exists that these forms arise from differential splicing of a single elastin primary gene transcript.

In contrast, other investigators have reported the in vitro translation of a single elastin protein of 70 000 Da (Ryhanen et al., 1978; Burnett & Rosenbloom, 1979; Burnett et al., 1980; Rosenbloom, 1984). The appearance of a single translated protein is more in line with current evidence, which indicates the presence of only one elastin gene (Rosenbloom, 1984). Furthermore, the exact number of elastin genes and/or gene products may vary from species to species.

A neonatal rat aortic smooth muscle cell culture system capable of producing large quantities of insoluble elastin has been described (Oakes et al., 1982; Barone et al., 1985). Recently, we observed that these rat cell cultures accumulate large amounts of soluble elastin proteins when grown in the presence of β-aminopropionitrile fumarate (BAPN) (Chipman et al., 1985). Of these proteins, substantial quantities of two soluble elastin-like proteins were detected. Pulse–chase studies led to the hypothesis of a specific, extracellular processing step between a 77 000-Da soluble protein (designated protropoelastin) and a large proteolytic fragment having a molecular weight of 71 000. The data suggested that this 71 000-Da species in the rat system may be the moiety incorporated into the insoluble elastin.

The present study reports on the levels and translational efficiency of elastin mRNA from neonatal rat aortic cultures. The data indicate that a single 79 000-Da primary translation product is produced in this system. Also, the relative levels of elastin mRNA as measured by Northern blot hybridization paralleled the relative translational efficiency levels of the elastin mRNA as measured by in vitro translation, indicating a lack of translational control.

Experimental Procedures

Neonatal Rat Smooth Muscle Cell Cultures. Neonatal rat smooth muscle cells were isolated and grown from the aortas of 1–3-day-old rats as described previously by Oakes et al. (1982). Essentially, a collagenase (10 mg; Sigma type I)–elastase (2.5 mg; Sigma type III) mixture in 20 mL of Dulbecco's modified Eagle's (DME) medium containing 3.7 g/L sodium bicarbonate, penicillin (100 units/mL), and streptomycin (100 μg/mL) without bovine calf serum was added to 20 aortas that had been cut into small pieces. Digestion was carried out for 30–45 min at 37 °C with stirring. The resulting cell suspension was centrifuged at 400*g* for 5 min, and the cell pellet obtained was washed twice with DME containing 3.7 g/L sodium bicarbonate, penicillin (100 units/mL), streptomycin (100 μg/mL), and 20% fetal bovine serum. The cell pellet was then resuspended in 2–4 mL of fresh DME medium. Cells were seeded into primary culture at a density of 5×10^5 cells/25-cm^2 tissue culture flask and maintained for 7 days in 5 mL of DME medium. The cells were then subcultivated (first passage) by trypsinization (0.05% trypsin–0.02% EDTA; Gibco) for 5–8 min at 37 °C. They were subsequently seeded at 5×10^5 cells/25-cm^2 flask and maintained with 5 mL of medium or at 1.5×10^6 cells/75-cm^2 flask and maintained with 20 mL of medium. The medium was changed twice weekly. In some instances the trypsinization procedure was repeated, and cells were also examined in second, third, and fourth passages.

At specified times, cell layers were harvested in the following manner. The medium was removed, and each cell layer was washed twice with Puck's saline G. The cell layers were harvested in water with the aid of a rubber policeman and homogenized with a motorized glass–glass homogenizer. Samples of the homogenate were removed for analyses of DNA content, amino acid composition, and elastin content.

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All results presented below represent the average of three separate flasks.

DNA Determination. DNA content of the cell layers was determined by the diphenylamine method of Burton (1956).

Insoluble Elastin Preparation. For analyses of the elastin content, aliquots of the cell layer homogenate were lyophilized, resuspended in 2 mL of 0.1 N NaOH, and incubated at 98 °C for 45 min with occasional shaking according to the method of Lansing et al. (1952). Samples were then cooled and centrifuged, and the residues and supernatants were each hydrolyzed separately as described below.

Amino Acid Analysis. Supernatants from the Lansing procedure were made to 6 N HCl by the addition of an equal volume of concentrated HCl, and the residues (which consisted of the insoluble elastin) were suspended in 6 N HCl. All samples were hydrolyzed, in vacuo, at 110 °C for 20 h, then reconstituted in 0.01 N HCl, and analyzed on a Beckman Model 119 amino acid analyzer. Protein content was evaluated by adding the total micromolar amount of amino acids in both the Lansing supernatant and residue fractions.

Pulse Experiments. At the time of pulsing, the spent medium was aspirated off and the cell layer was washed twice with calcium- and magnesium-free Puck's saline G solution. Prior to the pulse, the cells were incubated at 37 °C in a humidified atmosphere in 5% CO_2–95% air for 1 h in valine- and serum-free DME medium (starvation medium). The medium in each flask was then replaced with the same medium containing 20 μCi/mL L-[3,4-^{3}H]valine (sp act. 20 Ci/mmol). A labeling period of 6 h was used.

Soluble Elastin Preparation. (*A*) *Isolation from Cell Cultures.* Tropoelastin was prepared from the medium of first passage rat smooth muscle cells essentially by the method of Sandberg et al. (1971, 1975). The cells were maintained in culture as described above with the following changes. On days 11 and 14, the spent medium was removed and the cells were fed with medium supplemented with 25 μg/mL β-aminopropionitrile fumarate (BAPN). On day 14, the medium was replaced with serum-free medium for 6 h, after which time tropoelastin was isolated from the medium as described below. All of the following purification steps were performed at 4 °C in the presence of inhibitors. The proteolytic inhibitors used were *N*-ethylmaleimide (10 mM), ethylenediaminetetraacetic acid (EDTA) (5 mM), ε-amino-*n*-caproic acid (10 mM), phenylmethanesulfonyl fluoride (50 μM), and *p*-(hydroxymercuri)benzoate (300 μM).

The medium (with protease inhibitors) was dialyzed against water and lyophilized. The lyophilized material was dissolved in Puck's saline G at a concentration of 5–10 mg/mL and brought to 40% in ammonium sulfate by the slow addition of solid ammonium sulfate. The precipitate was collected by centrifugation, solubilized in 0.5 M ammonium formate, pH 5.5, and dialyzed overnight versus 0.5 M ammonium formate buffer, pH 5.5, with protease inhibitors. To 1 volume of the ammonium formate–protein solution was slowly added dropwise 1.5 volumes of 1-propanol, followed by the dropwise addition of 2.5 volumes of 1-butanol. After being stirred overnight, the material was filtered through Whatman no. 1 paper, and the filtrate was dried, washed with chloroform, and solubilized in 0.02 N formic acid. The formic acid solution was dialyzed against 0.02 N formic acid, pH 3.5, and lyophilized.

(*B*) *Purification by High-Performance Liquid Chromatography (HPLC).* The lyophilized tropoelastin material was dissolved in 0.02 N formic acid, pH 3.5, for further purification by HPLC. HPLC was performed on a Varian Model 5000 liquid chromatograph employing a 50 × 1.5 cm Toya Soda TSK G3000 SW molecular sieve column. A mobile phase of 0.02 N formic acid was utilized at 0.3 mL/min, and the eluant was monitored at 280 nm. Fractions of the column eluant was collected at 1-min intervals and samples hydrolyzed for amino acid analysis.

RNA Extraction and Purification. Neonatal rat aortic smooth muscle cells were cultured as described above. RNA was isolated by the guanidine extraction of Adams and Frank (1980) with some modifications. The cultures were extracted with 4 M guanidinium thiocyanate, 0.5% sodium lauryl sarcosine, 0.1 M 2-mercaptoethanol, and 25 mM sodium citrate, pH 7.0 (2.0 mL/75-cm^2 flask) and homogenized in a Polytron homogenizer at full speed for 30–60 s. After centrifugation (10 min, +10 °C, 8000*g*), 0.025 volume of acetic acid was added to the supernate, followed by 0.75 volume of cold 100% ethanol. The mixture was placed at −20 °C overnight. The following procedures were performed at 4 °C, unless otherwise indicated. After a brief centrifugation at −10 °C, the pellet was resuspended in 5 mL of 7.5 M guanidine hydrochloride, pH 7.0, and 5 mM dithiothreitol, followed by 0.025 volume of 1 M acetic acid and then 0.5 volume of 100% ethanol. The mixture was placed at −20 °C for at least 3 h. After centrifugation at −10 °C, this last guanidine–dithiothreitol solubilization step was repeated. The final pellet obtained was resuspended in 5 mL of cold ethanol and briefly centrifuged at −10 °C. The resultant pellet was solubilized in 2 mL of sterile water and centrifuged. The pellet was resuspended and centrifuged twice in an additional 1 mL of water, and all these supernates were combined. The combined RNA-containing supernates were reprecipitated by the addition of 0.1 volume of 2 M potassium acetate, pH 5.0, and 2 volumes of ethanol. The mixture was allowed to sit at −20 °C overnight. After brief centrifugation at −10 °C, the pellet was washed 3 times with ethanol and lyophilized (to remove the alcohol). Finally, the RNA was resuspended at 1 mg/mL in 0.1% sodium dodecyl sulfate (SDS) and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.6, and heated to 70 °C for 30 s. The RNA was quickly cooled on ice and adjusted to 0.5 M NaCl. Polyadenylated RNA was isolated by chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972).

In Vitro mRNA Translation. For translation, the mRNA was dissolved in sterile water to 2–4 μg/18 μL, and an appropriate sample was heated to 70 °C for 30 s and then quickly cooled in an ice bath. The reticulocyte lysate in vitro translation kit was obtained from Bethesda Research Laboratories and was used according to the manufacturer's specifications. This procedure is essentially the method of Rowe et al. (1978). L-[3,4(n)-^{3}H]Proline (Amersham; sp act. 500–600 Ci/mmol) or in some instances L-[^{35}S]methionine (Amersham; sp act. 1460 Ci/mmol) was utilized and the translation carried out at 26 °C for 90 min. The radiolabel incorporation was followed by the method of Pelham and Jackson (1976), which entailed trichloroacetic acid (TCA) precipitation of a small sample of the translation products for scintillation counting. The reaction was stopped by placing the sample at 4 °C. The translated proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), as described below. In some instances the sample was immunoprecipitated as described below prior to electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis. PAGE was performed according to the method of Laemmli (1970). A 3% stacking and 8% running gel (1.5 mm thick) was used. Gradient PAGE was also performed with a 5–10% gradient. Samples were reduced with 200 mM dithiothreitol

(DTT) and boiled for 1 min. Protein standards included ovalbumin (42 kDa), bovine serum albumin (68 kDa), and phosphorylase B (97 kDa). The protein bands were detected by Coomassie blue staining, followed by fluorographic techniques described below.

PAGE Fluorography. PAGE fluorography was performed essentially by the method of Bonner and Laskey (1975). The fluorograms produced by exposure to the polyacrylamide gels were scanned in a Shimadzu Chromato scanner, Model CS-930, at 600 nm.

Soluble Elastin Antibodies. The methods used were described by Chipman et al. (1975). New Zealand White rabbits (4–6 lb) were injected with α-elastin to raise the polyclonal antisera (Partridge & Davis, 1955). Initially, a 500-μg intradermal injection in complete Freund's adjuvant was performed and then followed by a 200-μg intradermal injection in incomplete adjuvant every 2 weeks. On the fifth week, and every 2 weeks following, approximately 50 mL of arterial blood was collected. Purification of these antisera was performed by utilizing an affinity technique with insoluble elastin as a specific immunoadsorbent as described below.

Purified calf ligamentum nuchae elastin (1 g) (Partridge et al., 1955) was homogenized and then washed twice in 150 mM sodium chloride. The elastin pellet was suspended in 5.0 mL of the antisera and shaken at 37 °C for 4 h, followed by centrifugation. A hemagglutination assay, performed on the supernatant, indicated that a negligible amount of antibody reactivity was present. To dissociate the antibody from the insoluble elastin, 10 mL of 3.5 M potassium iodide (KI) in phosphate-buffered saline, pH 7.4, was added, and the mixture was shaken gently for 18 h at 4 °C. Following centrifugation, the supernatant was dialyzed against 5 L of borate-buffered saline, pH 8.4, at 4 °C for 3 h. The anti-α-elastin antisera obtained before or after the affinity purification showed a titer of 1:10 000 when tested by an ELISA assay (Kessler, 1975) or the hemagglutination assay (Sykes & Chidlow, 1971). No cross-reactivity with collagen type I or III or fibronectin was detected.

Immunoprecipitations. Immunoprecipitations were performed essentially by the method of Davidson et al. (1982) with modifications. The α-elastin antibodies were prepared as described above. In a 1.5-mL microcentrifuge tube was aliquoted 1.0 mL of 150 mM NaCl and 50 mM Tris, pH 7.5 (Tris–NaCl buffer). The antigen (usually 25 μL of a translation) was added to the tube and mixed gently, and then 5 μL of antisera (1:10 000 titer) was added. This mixture was incubated for 1.5 h at 25 °C with gentle shaking. Fifty microliters of a protein A–bacterial adsorbent (Miles Catalog No. 79-700; washed and reconstituted as a 10% w/v suspension in Tris–NaCl buffer) was added to the mixture and allowed to incubate for 1 h at 25 °C with occasional stirring on a Vortex homogenizer. The mixture was centrifuged for 15 min. The supernatant was then removed. The pellet was resuspended in Tris–NaCl buffer and centrifuged as before, 3 times for 15 min, and the supernatants were combined. The pellet was boiled in 75 μL of Laemmli electrophoresis sample buffer (Laemmli, 1970) for 20 min and centrifuged at full speed for 10 min. The supernate and pellet were analyzed for radioactivity and protein profiles by SDS–PAGE (see above).

Western Blot. For Western blots (Burnette, 1981), soluble elastin and protein standards were subjected to PAGE as previously described on a 0.75 mm thick acrylamide slab gel. The gel was washed in Burnette electrode buffer (20 mM Tris base, 150 mM glycine, 20% methanol). The nitrocellulose paper (HA type, 0.45-μm pore) was allowed to soak in the Burnette electrode buffer prior to use. The washed gel was placed on a glass plate, covered with nitrocellulose paper, and overlayed with a piece of Whatman 3MM paper. This filter paper was wet with Burnette electrode buffer and covered with another glass plate. This assembly was clamped and immersed in a tray filled with Burnette electrode buffer, and electrophoretic transfer was accomplished at a constant current supply at 150–200 mA for 20 h.

The nitrocellulose paper was removed and rinsed twice for 10 min in 0.01 M sodium phosphate, 0.15 M NaCl, and 3% (v/v) Tween 20, pH 8.5, (Tween buffer) at room temperature and again 2 times at 37 °C. The blot was then placed in 100 mL of Tween buffer containing 0.5 mL of the rabbit anti-α-elastin antibody described previously, and it was incubated at 37 °C for 3 h. The blot was again rinsed 4 times for 20 min at 37 °C in Tween buffer, placed in 40 mL of Tween buffer containing 0.06 μCi/mL ^{125}I-labeled protein A (30.4 μCi/μg), and shaken slowly overnight. The blot was rinsed 7 times for 10 min at room temperature with water, air-dired, and exposed to Kodak XAR film for the indicated periods of time.

Nothern Blot. For Northern blot hybridization analyses, equal aliquots of total cellular RNA were denatured and separated by electrophoresis on 1.0% formaldehyde agarose gels (Lehrach et al., 1979). Following electrophoresis, RNA was stained with a 1 μg/mL ethidium bromide solution and then destained with water. Photographs were taken over a UV light box (Fotodyne; wavelights in the 300-nm range) to visualize the locations of the 28S and 18S rRNAs. To prepare for transfer, the gels were soaked for 30 min in 100 mM sodium chloride and 50 mM sodium hydroxide and neutralized for 15 min in 100 mM Tris-HCl, pH 7.6. Gels were equilibrated in 20× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 45 min, and the RNA was then transferred by blotting onto nitrocellulose paper for 16 h in the presence of 20× SSC.

Following transfer of the RNA to nitrocellulose paper, the filters were rinsed with 2× SSC, air-dried for 1 h, and baked in vacuo at 80 °C for 2 h. Filters were then prehybridized for 4 h to overnight at 42 °C in a solution of 5× SSC, 0.1, SDS, 1× Denhardt's solution, 50% deionized formamide, 0.1 M sodium phosphate, pH 7.0, and 100 μg/mL salmon sperm DNA as described (Dean et al., 1983).

Probes were radiolabeled by using a BRL nick translation kit. The cDNA probes used were a sheep elastin clone (pcSEL1) (Yoon et al., 1984) and a chicken glyceraldehyde-3-phosphate dehydrogenase clone (Stone et al., 1985). The label used was [α-^{32}P]dCTP with specific activity greater than 3000 Ci/mmol (New England Nuclear). Each nick translation reaction contained 0.5 μg of the indicated cDNA and 50 μCi of [α-^{32}P]dCTP/25-μL reaction; the specific activity of the resulting cDNAs ranged from 10^7 to 10^8 cpm/μg of plasmid DNA. Hybridization was performed at 42 °C for 48 h with prehybridization buffer plus 1.0×10^6 cpm of the appropriate nick-translated cDNA probe/mL of prehybridization buffer. Unhybridized radioactivity was removed by washing the blots for 30 min at 68 °C with two changes of 2× SSC followed by two changes of 1× SSC and 0.1% SDS. Autoradiography was performed with Kodak XAR-5 film, and the resulting autoradiograms were quantitated by scanning densitometry.

Results

The neonatal rat aortic smooth muscle cell cultures have been shown to be extremely active in synthesizing and depositing large amounts of insoluble elastin in their extracellular matrix, making this an ideal system for studying elastin bio-

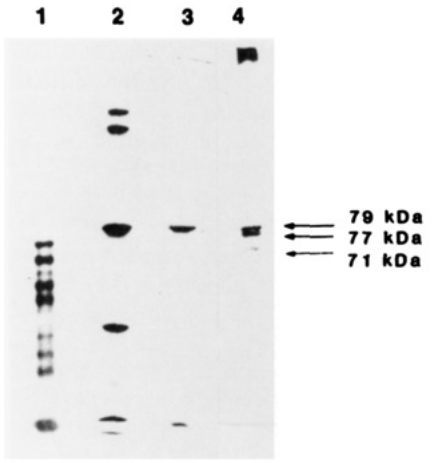

FIGURE 1: SDS–PAGE of pulse medium and products of in vitro translated mRNA from neonatal rat aortic smooth muscle cell cultures. First passage, 14-day cultures were either pulsed for 6 h with [^{3}H]valine or extracted for mRNA and in vitro translated with [^{3}H]proline. Lane 1, Sandberg-purified soluble elastin from pulse medium; lane 2, translation of poly(A+) mRNA; lane 3, immunoprecipitate of mRNA translation products with anti-α-elastin antibody; lane 4, combination of Sandberg-purified soluble elastin preparation and RNA translation products immunoprecipitated with an anti-α-elastin antibody.

synthesis. Recently, we observed that these rat cell cultures accumulate large amounts of soluble protein when grown in the presence of BAPN (Chipman et al., 1985). Of these proteins, substantial quantities of two soluble elastin-like proteins were detected having apparent molecular weights of 77 000 and 71 000.

Neonatal rat aortic smooth muscle cells (75-cm^2 flasks) were grown in first passage for 14 days in culture. The medium was supplemented with BAPN on days 11 and 14, and on day 14, 2 h after the BAPN supplement, the cells were either pulsed for 6 h with [^{3}H]valine or mRNA extracted. The PAGE profile of the resulting radioactive soluble elastin proteins can be seen in Figure 1. The Sandberg-purified soluble elastin preparation obtained from the medium of the culture (lane 1) revealed several radioactive bands, with the two largest protein bands having apparent molecular weights of 77 000 and 71 000. Also present were a series of major bands that appeared to be discrete soluble elastin fragments. Such fragmentation has been reported by many laboratories including our own (Narayanan & Page, 1974; Sandberg et al., 1975; Mecham et al., 1976; Chipman et al., 1985).

The cell cultures were also extracted for total RNA. The mRNA fraction was isolated by affinity chromatography on oligo(dT)–cellulose and translated in the presence of [^{3}H]-proline for 90 min as described. The profile of the in vitro translation (lane 2) showed a series of proteins; however, the most abundant was a protein band that migrated to a position slightly above the 77 000-Da solution elastin protein. On the basis of ^{14}C protein standards, this protein has a molecular weight of 79 000. Approximately 20% of the total translated proteins can be attributed to the 79 000-Da band. Immunoprecipitation of the translation products with a specific elastin antibody (lane 3) produced a single protein band having an apparent molecular weight of 79 000, strongly suggesting that there is one primary elastin protein synthesized by these vascular smooth muscle cells. To determine whether the immunoprecipitated elastin translate did indeed migrate to a position above the 77 000-Da protein, the Sandberg-purified soluble elastin preparation was combined with the products of the cell-free translation reaction and then immunoprecipitated with an anti-α-elastin antibody. The mixture was then

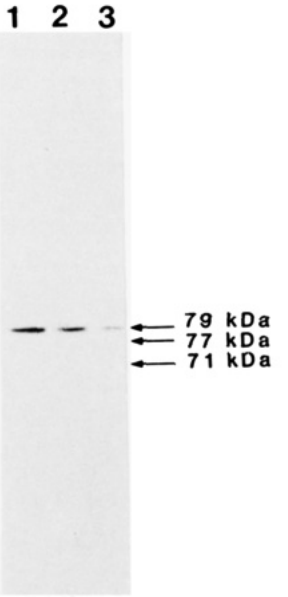

FIGURE 2: SDS–gradient PAGE of the products of the in vitro translation reaction. Total RNA was isolated from first passage neonatal rat smooth muscle cells maintained for 14, 21, and 28 days in culture, translated in the presence of [^{3}H]proline, and immunoprecipitated with an anti-α-elastin antibody. Immunoprecipitated translation products were separated by electrophoresis on 5–10% linear gradient polyacrylamide–SDS gels. Lane 1, day 14; lane 2, day 21; lane 3, day 28.

subjected to PAGE (lane 4). The three most intense bands migrated at 79 000, 77 000, and 71 000 Da, respectively. No other bands were detected between 77 000 and 71 000 Da. To make certain that the cell-free elastin translation product from the neonatal rat aortic smooth muscle cell cultures was indeed a single band, a PAGE with a 5–10% gradient was performed. Total RNA was isolated from first passage neonatal rat aortic smooth muscle cells grown for 14, 21, and 28 days in culture, then translated in the presence of [^{3}H]proline, and immunoprecipitated with an anti-α-elastin antibody. As can be seen in Figure 2, the translated elastin protein appears as a single band having a molecular weight of 79 000.

Elastin mRNA was analyzed by Northern blot hybridization. Total RNA was extracted from first passage cells grown for 14 days in culture and subjected to electrophoresis on 1.0% agarose gels. Northern blots of total RNA (15 μg) were than hybridized with a ^{32}P-labeled elastin cDNA probe (Yoon et al., 1984). A single visible band was located at approximately 26 S as estimated by the 18S (2.0 kilobases) and 28S (5.1 kilobases) ribosomal RNAs visualized by ethidium bromide staining. The size of this 26S elastin mRNA corresponded to approximately 4.2 kilobases in length. These results are consistent with other studies (Frisch et al., 1985).

Elastin gene expression was further investigated as a function of cell growth over time in different passage levels. The neonatal rat aortic smooth muscle cell cultures accumulate large amounts of insoluble cross-linked elastin. Changes in insoluble elastin protein and elastin mRNA production were therefore evaluated from neonatal rat aortic smooth muscle cells grown for different times in one culture passage and from cells grown in consecutive passage levels. Smooth muscle cells in first, second, third, and fourth passages (75-cm^2 flasks) were grown in culture for 28 days. The cell layers were analyzed for DNA, insoluble elastin, and total protein contents (Figure 3). The DNA contents remain essentially constant or increase slowly after 14 days in culture from cells obtained from first to fourth passages. The insoluble elastin associated with the cell layer as well as total protein content continued to increase

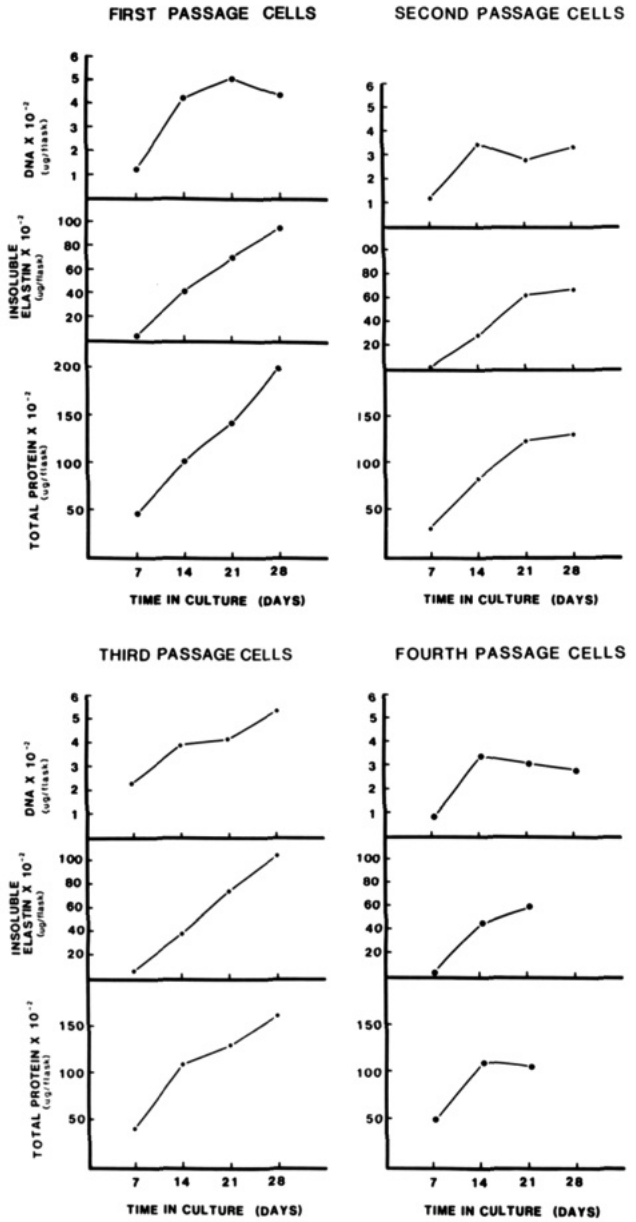

FIGURE 3: Cell growth and protein synthetic activity as a function of time in first, second, third, and fourth passages. Neonatal rat aortic smooth muscle cells were grown in first, second, third, and fourth passages for 28 days in culture (75-cm^2 flasks) and analyzed for DNA, insoluble elastin, and total protein contents. See the text for details.

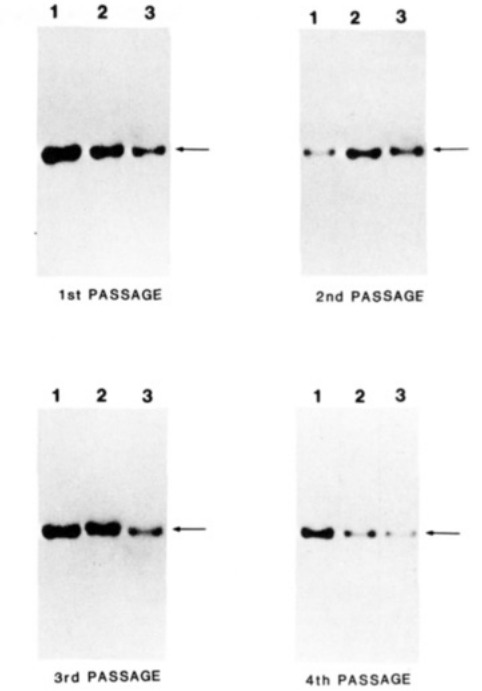

FIGURE 4: Northern blot hybridization analyses of elastin mRNA as a function of time in first, second, third, and fourth passages. Preparations of total RNA (15 μg/lane) extracted from neonatal rat aortic smooth muscle cells in first, second, third, and fourth passage levels were analyzed by Northern blots with an elastin-specific cDNA clone. Lane 1, day 14; lane 2, day 21; lane 3, day 28. The arrow indicates 26S elastin mRNA.

during the time course measured (7, 14, 21, and 28 days in each passage). This trend does not change if the data are normalized per microgram of DNA.

To measure relative elastin mRNA levels and translational efficiencies over the time course, RNA was isolated. Northern blots containing equal amounts of total RNA (15 μg) extracted from cells grown for 14, 21, and 28 days in first, second, third, and fourth passage levels were hybridized with the ^{32}P-labeled elastin cDNA clone. The resulting autoradiograms are shown in Figure 4. The relative amounts of elastin mRNA isolated independently from first, third, and fourth passages decreased with time. However, elastin mRNA levels from second passage cultures indicated an increase at day 21 and thereafter decreased. Densitometric determinations can be seen in Figure 5.

To control for RNA integrity and yield over the course of the experiments, the same RNA samples were tested for hybridization to a glyceraldehyde-3-phosphate dehydrogenase cDNA clone by Northern blot analysis (data not shown). No appreciable change in the level of glyceraldehyde-3-phosphate dehydrogenase mRNA was observed during the course of the study. The levels of glyceraldehyde-3-phosphate dehydrogenase mRNA did not vary significantly from passage to passage. To estimate the relative translational efficiencies of the isolated mRNAs, cell-free translations were performed. The same RNA samples used for Northern blot studies were employed. Total RNA (3 μg/30-μL reaction) was translated in a rabbit reticulocyte cell-free system in the presence of [^{3}H]proline for 90 min and subjected to SDS–PAGE. The proteins synthesized were visualized by fluorography. The resulting autoradiograms and subsequent densitometric determinations are shown in Figures 6 and 5, respectively. The mobility assignment made for the translated elastin RNA was based on previous work employing specific immunoprecipitation with an anti-α-elastin antibody. The patterns of functional elastin mRNA synthesis paralleled that seen in the Northern blot analyses. A good correlation was found between the relative levels of elastin mRNA as measured by Northern blot hybridization and the relative translational capacity of the elastin mRNA as measured by in vitro translation.

The same RNA samples were used to direct synthesis of proteins in a cell-free system using [^{35}S]methionine as a precursor (data not shown). This was performed to ensure that all proteins were being produced in the translations and also to ensure that all mRNAs were intact. These results indicated that no other protein was being selectively translated by the smooth muscle cells.

No significant differences can be seen in the extractable total RNA from cells over time in different passages (Table I). Therefore, the decrease in elastin mRNA levels cannot be attributed to a decrease in the steady-state cellular RNA levels. Since the relative elastin mRNA levels and translational efficiencies are similar, there appears to be a lack of translational control with respect to time in culture from each passage level.

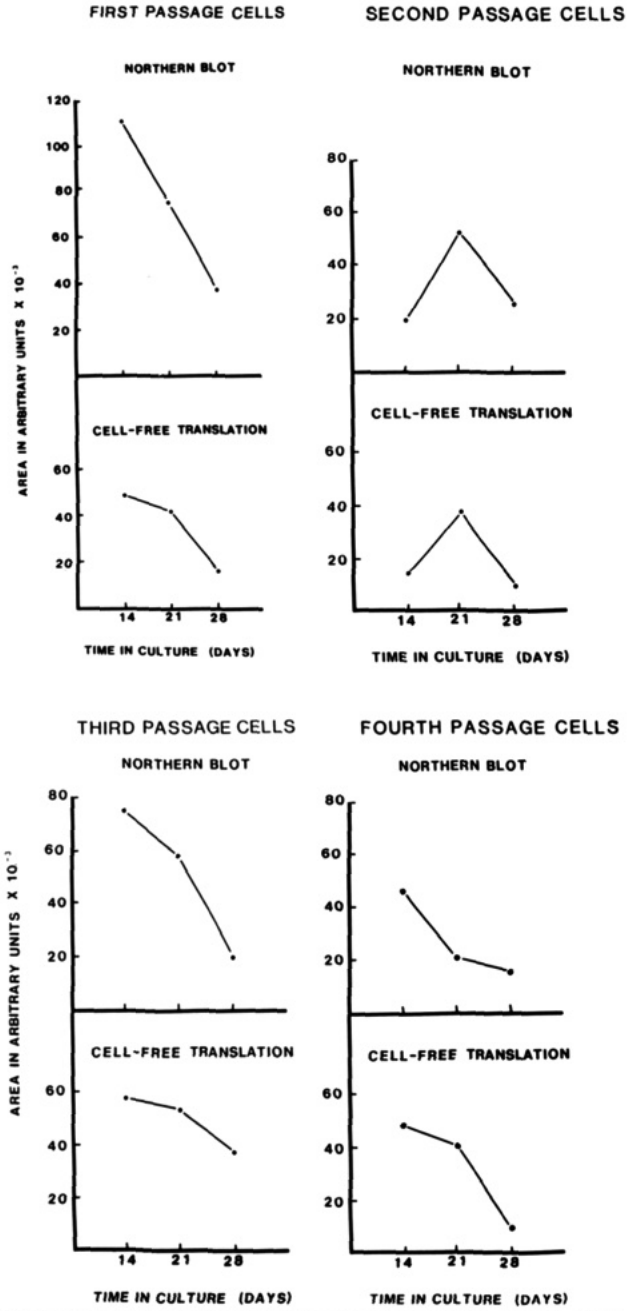

FIGURE 5: Quantification of elastin mRNA from first, second, third, and fourth passages by Northern blot analyses and cell-free translations. The autoradiograms were analyzed by densitometric scanning and the areas for the mRNA and elastin protein plotted as a function of time in culture in first, second, third, and fourth passages.

Table I: Total Extractable RNA Levels from Cell Cultures Grown over Time in Consecutive Passage Levels

	total RNA (μg/flask)		total RNA (μg/flask)
first passage		third passage	
day 14	166	day 14	162
day 21	164	day 21	194
day 28	207	day 28	275
second passage		fourth passage	
day 14	108	day 14	125
day 21	199	day 21	217
day 28	190	day 28	151

Discussion

Smooth muscle cells derived from neonatal rats that are enzymatically isolated from the heart (Jones et al., 1979) and

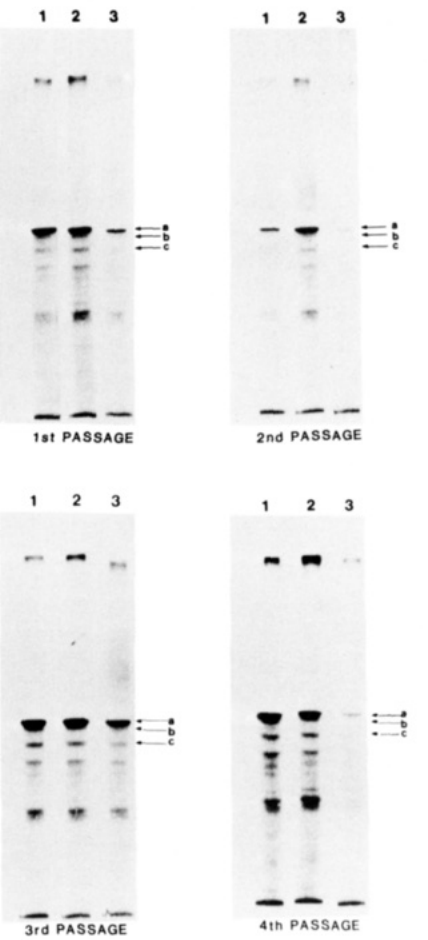

FIGURE 6: Total RNA translation activity as a function of time in first, second, third, and fourth passages. Equal amounts of total RNA (3 μg/30-μL reaction) extracted from neonatal rat smooth muscle cell cultures in first, second, third, and fourth passages were used to direct synthesis of proteins in a rabbit reticulocyte cell-free system in the presence of [^{3}H]proline. Lane 1, day 14; lane 2, day 21; lane 3, day 28. (a) indicates 79 000 Da; (b) indicates 77 000 Da; (c) indicates 71 000 Da.

medial layer of the aorta (Oakes et al., 1982; Barone et al., 1985) have been shown to be extremely active in synthesizing and depositing large amounts of cross-linked, insoluble elastin. This is not surprising since 3-day-old neonatal rats are at a stage in their development when both soluble and insoluble elastin production is extremely high. Two soluble elastin precursors (molecular weights of 77 000 and 71 000) have been identified in this culture system. However, considerable controversy has developed over the true identity of the primary translation product. Reports on the number of species and their molecular weights have varied.

For this report, mRNAs were isolated from cultures of neonatal rat aortic smooth muscle cells, translated in a rabbit reticulocyte lysate system, and immunoprecipitated with an anti-α-elastin antibody. The data obtained indicate that the primary translation product of elastin mRNA produced in these rat cultures is a single species with a molecular weight of 79 000. These findings are consistent with the "signal hypothesis" mechanism of extracellular processing and secretion (Blobel & Dobberstein, 1975; Davidson et al., 1982). The data would suggest that this 79 000-Da protein is secreted to the extracellular space with concomitant removal of a signal peptide by a specific peptidase on the lumenal side of the endoplasmic reticulum.

Elastin mRNA isolated from the neonatal rat aortic smooth muscle cell cultures was further analyzed by Northern blot

hybridization utilizing a cDNA probe specific for elastin mRNA (pcSEL1) (Yoon et al., 1984). A single 26S elastin mRNA was detected, corresponding to approximately 4.2 kilobases in length. The primary translation product of elastin mRNA from the neonatal rat smooth muscle cells has a molecular weight of 79 000 (corresponding to approximately 870 amino acid residues), yet the elastin mRNA is about 4200 nucleotides long. This is consistent with other reports, which also suggest that the elastin mRNA contains many untranslated nucleotides (Yoon et al., 1984).

Initial evaluation of the general biosynthetic pattern of the neonatal rat smooth muscle cells indicated a continuous increase in elastin accumulation over time in one culture passage. In an attempt to understand elastogenesis in the neonatal rat cells, cultures were evaluated for insoluble elastin protein production, elastin mRNA translational efficiency, and elastin mRNA levels in cells as a function of time in consecutive passages. The results indicated that the insoluble elastin accumulation continued to increase in cells grown in culture for 7–28 days in first through fourth passages. When the cells were analyzed for the relative levels and translational activity of elastin mRNA, a decrease was observed with time from cells grown in first, third, and fourth passages. Elastin mRNA levels and translational activity obtained from cells grown in second passage indicated an increase at day 21 and thereafter a decrease. However, reasons for the apparent differences in relative elastin mRNA levels and translation activity observed in second passage cells when compared with other passages still remain unclear.

Furthermore, since no significant differences were observed in the extractable total RNA from cells over time in the different passage levels, the decrease in elastin levels could not be attributed to a decrease in the steady-state cellular levels. Results further suggest that the pattern of functional elastin mRNA synthesis paralleled the levels of elastin mRNA production. A good correlation was observed between the relative translational efficiency of elastin mRNA as measured by in vitro translation and of the relative levels of elastin mRNA as measured by Northern blot hybridization.

The regulation of synthesis of extracellular matrix proteins is poorly understood. In many other systems, this control is ultimately exercised through the adjustment of the steady-state cellular level of mRNA (Chan et al., 1973; Alton & Lodish, 1977; Soh & Sarkar, 1978). It appears that control in the elastin biosynthesis is expressed at the mRNA concentrations; however, it is not clear whether it is at the level of transcription. Since this study did not include measurements of soluble elastin levels, the translatability of elastin mRNA reticulocyte lysates suggests that there have been no structural changes in the mRNA altering its translation efficiency. However, it may be possible that there have been changes in translation control factors that alter the rate of initiation and/or propagation of polypeptide changes in vivo. Other possibilities that might explain the change in elastin mRNA levels could be due to the hnRNA processing and/or mRNA stability that occur in the neonatal rat smooth muscle cells.

Intracellular elastin biosynthesis may be influenced or controlled at several levels. These may include rate of mRNA translation, intracellular protein degradation, and/or turnover or stability of the elastin mRNA. However, since insoluble elastin protein production increases with time in culture and its elastin mRNA levels decrease, another feedback mechanism of control can be proposed. Namely, as a cell's extracellular matrix becomes more established with time of culture, the rate of insoluble matrix accumulation begins to slow down. A mechanism involving feedback control from component(s) of the insoluble extracellular matrix to the production of elastin mRNA within the cell is clearly feasible. It is even conceivable that one of the responsible components is the elastic fiber itself.

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Mechanism of Monofunctional and Bifunctional Alkylation of DNA by Mitomycin C†

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ABSTRACT: The relative amounts of monofunctional and bifunctional alkylation products of DNA with mitomycin C (MC) depend on whether one or both masked alkylating functions of MC are activated reductively; adduct **8** is the result of one function and adducts **7** and **9**, formed as a pair, are the result of both functions being activated [Tomasz, M., Lipman, R., Chowdary, C., Pawlak, J., Verdine, G. L., & Nakanishi, K. (1987) *Science (Washington, D.C.) 235*, 1204–1208]. To determine the mechanism governing this differential reactivity of MC with DNA, MC–*Micrococcus luteus* DNA complexes formed under varying conditions in vitro were digested to nucleosides and adducts. Adduct distribution, analyzed by high-performance liquid chromatography, served as the measure of monofunctional and bifunctional activation. H_2/PtO_2 and xanthine oxidase/reduced nicotinamide adenine dinucleotide (NADH) activated MC mostly monofunctionally, and $Na_2S_2O_4$ activated the drug bifunctionally under comparable conditions. Excess MC selectively suppressed, but excess PtO_2 selectively promoted, bifunctional activation by H_2/PtO_2; excess xanthine oxidase and/or NADH also had promoting effects. O_2 tested in the $Na_2S_2O_4$ system was inhibitory. 10-Decarbamoyl-MC acted strictly monofunctionally under all conditions. Monoadducts bound to DNA were converted to bis adducts upon rereduction. A mechanism with the following features was derived: (i) Activation of MC at C-1 and C-10 is sequential (C-1 first). (ii) A one-time reduction is sufficient for both. (iii) Activation of the second function may be selectively inhibited by kinetic factors or O_2. (iv) **7** and **9** are coproducts of bifunctional activation; their ratio depends on the DNA base sequence. (v) Activation of the second function involves an iminium intermediate. Direct applications to the action of MC in vivo are discussed.

Mitomycin C (MC,[1] **1**) is an antibiotic and antitumor agent used in clinical cancer chemotherapy. It is also a weak mutagen and carcinogen and inhibits bacterial cell division. One of its most interesting properties is the ability to bind covalently to DNA, both monofunctionally and bifunctionally, resulting in the latter case in the formation of covalent cross-links between the complementary strands (Szybalski & Iyer, 1967). These effects make MC unique among the known antibiotics. They also represent the ultimate molecular basis for its biological activity, as indicated by the parallels with a number of known DNA-targeted agents: selective inhibition of DNA replication, strong induction of the SOS response and sister chromatid exchange, and cross-resistance or cross-hypersensitivity of bacterial and mammalian cells to UV light and MC [see Tomasz et al. (1986) for specific references].

Under physiological conditions the covalent reactivity of MC with DNA requires enzymatic or chemical reduction (Iyer & Szybalski, 1964). This phenomenon led to designation of MC as a "bioreductive alkylating agent" (Lin et al., 1976). From their original observation, Iyer and Szybalski (1964) postulated a mechanism for the reductive activation of MC: the C-1 aziridine and C-10 carbamate groups are two masked alkylating functions, which become "allylic" (therefore activated) upon reduction of the quinone system and consequent spontaneous elimination of methanol from the 9/9a position (see **2**); they then may be displaced by two nucleophiles of DNA, resulting in a MC–DNA cross-link. Moore (1977) amended this hypothesis by speculating that both displacements are of S_N1 types, facilitated by resonance with the indolohydroquinone system of reduced MC, taking place sequentially as shown in Scheme I (**2** → **6**). Experimental investigations of the activation mechanism in model systems employing a large variety of enzymatic and chemical reducing systems as well as model low molecular weight nucleophiles verified most aspects of this scheme (Tomasz & Lipman, 1981; Kohn & Zein, 1983; Hornemann et al., 1983; Bean & Kohn, 1983; Pan et al., 1984; Peterson & Fisher, 1986; Danishefsky & Egbertson, 1986; Egbertson & Danishefsky, 1987). Evidence was

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[1] Abbreviations: MC, mitomycin C; UV, ultraviolet; br, binding ratio; NADH, reduced nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; CHO, Chinese hamster ovary.

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