Elastin mRNA levels during foetal development of sheep nuchal ligament and lung

Hybridization to complementary and cloned DNA

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Elastin mRNA levels were quantified in sheep nuchal ligament and lung during the latter half of foetal development with elastin-specific cDNA (complementary DNA) probes using both hybridization in solution (saturation analysis) and hybridization on a fixed support (Northern analysis). For the solution-hybridization studies, cDNA prepared from nuchal-ligament mRNA was enriched to 65% for elastin sequences by hybridizing it to its template at a R_{0t} ($\text{mol} \cdot \text{s} \cdot \text{litre}^{-1}$) value that included only the abundant class of mRNA sequences. Hybridization of this probe to RNA extracted from nuchal ligament between 70 and 138 days after conception demonstrated elastin sequences increased about 10-fold (from 0.047 to 0.438% of total RNA). In contrast, lung elastin mRNA levels increased only 3-fold (from 0.009 to 0.022% of total RNA) during the same period. Over this developmental period these values correspond to increases in the average number of elastin mRNA molecules from 950 to 20000 molecules/ligament cell and from 130 to 330 molecules/lung cell. For Northern analysis, elastin mRNA was purified from near-term-sheep nuchal ligament on sucrose density gradients. Analysis of the translation products of this elastin mRNA showed that relative elastin precursor synthesis was at least 80% of total [^3H]valine incorporation. The M_r of this elastin mRNA, determined by methylmercury–agarose-gel electrophoresis, was approx. 1.25×10^6 . Northern hybridization of nuchal ligament and lung RNA to a [^{32}P]cDNA probe, transcribed from this sucrose-gradient-purified elastin mRNA, confirmed the developmental changes in elastin mRNA levels detected by solution-hybridization techniques. The specificity of this method was confirmed by using a cloned elastin gene fragment. These studies demonstrate that elastin mRNA levels in organs such as nuchal ligament and lung increase with foetal development, but that there are significant differences in the average cellular elastin mRNA content of these two organs.

Abbreviations used: cDNA, complementary DNA; R_{0t} value, normalized time, $\text{mol} \cdot \text{s} \cdot \text{litre}^{-1}$; mRNA_E , elastin mRNA; cDNA_E , elastin cDNA; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; $1 \times \text{SSC}$, 0.15M-NaCl/0.015M-sodium citrate, pH7.0; NaDodSO₄, sodium dodecyl sulphate; poly(A)⁺, polyadenylated; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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Elastin is a connective-tissue component whose rubber-like properties are essential to the normal function of organs such as skin, blood vessels, lung and elastic ligaments (Sandberg, 1976; Rucker & Tinker, 1977). Morphological and chemical approaches both indicate that there is a significant increase in the amount of elastin in developing nuchal ligament (Wirtschafter *et al.*, 1967; Cleary *et al.*, 1967) and lung (Loosli & Potter, 1959; Shibahara *et al.*, 1981).

Biosynthetic studies with explant cultures of sheep nuchal ligament and lung have demonstrated that there is a 20-fold increase in tropoelastin production in nuchal ligament (Davidson *et al.*, 1981, 1982) and a 3-fold increase in tropoelastin

production in lung (Shibahara *et al.*, 1981) during the latter half of foetal development. Although both of these developmental increases in elastin biosynthesis are closely paralleled by the amount of functional (translatable) elastin mRNA in the tissue, it has hitherto been uncertain whether these developmental changes represent an increase in total elastin mRNA as well as functional elastin mRNA (Davidson & Crystal, 1982). To clarify the regulatory mechanisms involved in this apparent increase in translational activity, the present study was designed to quantify the total number of elastin mRNA (mRNA_E) transcripts in these tissues during foetal development. To accomplish these, two types of hybridization with purified elastin cDNA were used: solution hybridization and Northern analysis. The specificity of these probes was confirmed by the use of a cloned elastin gene sequence.

Experimental procedures

Materials

Guanidinium chloride, distilled phenol, 'in vitro' reticulocyte lysate translation system, bacteriophage- ϕ X174 DNA, λ DNA and restriction endonucleases were from Bethesda Research Laboratories; *Escherichia coli* tRNA was from Boehringer-Mannheim; nuclease S-1 was from Sigma; formamide was from Matheson, Coleman and Bell, and was deionized as described by Tolstoshev *et al.* (1981a); methylmercuric(II) hydroxide was from Alfa Products, Danvers, MA, U.S.A.; poly(U)-Sephadex 4B was from Pharmacia. L-[2,3,4- ^3H]Valine (11.1 Ci/mmol) was from New England Nuclear Corp.; [^3H]dCTP (40 Ci/mmol) and [^{32}P]dCTP (410 Ci/mmol) were from Amersham International. Reverse transcriptase was obtained from Dr. J. W. Beard (Life Sciences, Gulfport, FL, U.S.A.) through the Office of Program Resources and Logistics, Viral Oncology Program, National Cancer Institute. All other chemicals were the highest grade commercially available. Foetal sheep were obtained from the Veterinary Resources Branch of the National Heart, Lung and Blood Institute with the generous co-operation of Dr. J. Pierce and Dr. D. Buckhold.

Isolation and analysis of mRNA_E

Total cellular RNA was prepared from foetal-sheep nuchal ligament and lung by using 8M-guanidinium chloride (Deeley *et al.*, 1977; Tolstoshev *et al.*, 1981b; Davidson *et al.*, 1982) and stored in liquid N_2 vapour before analysis. Poly(A)-containing RNA (mRNA) was isolated by affinity chromatography on poly(U)-Sephadex 4B (Jelinek *et al.*, 1973) from 140-day foetal-sheep nuchal-ligament RNA, a developmental period

when this organ is enriched in translatable mRNA_E (Davidson *et al.*, 1982). With this procedure, about 1.3% of the input RNA was recovered as poly(A) $^+$ mRNA.

The purity and the M_r of the isolated mRNA from poly(U)-Sephadex were determined by gel electrophoresis under fully denaturing conditions in 1% agarose containing 10mM-methylmercuric hydroxide (Bailey & Davidson, 1978). Gels (4mm thick) were prepared in a horizontal-slab-gel apparatus (model H-2; Bethesda Research Laboratories) and electrophoresis was performed with constant voltage (20V) for 16h. After electrophoresis, the RNA species in the gel were stained with ethidium bromide (10 $\mu\text{g}/\text{ml}$) in 0.5M-ammonium acetate and photographed under u.v. light with Polaroid type 55 film. The M_r values for the RNA size markers were estimated to be 1.75×10^6 for 28S rRNA, 1.03×10^6 for 23S rRNA, 0.68×10^6 for 18S rRNA and 0.55×10^6 for 16S rRNA (McMaster & Carmichael, 1977).

The translational activity of the isolated mRNA was determined in a rabbit-reticulocyte-lysate-system and the elastin precursor translated was identified with a specific anti-(α -elastin) serum (Davidson *et al.*, 1982). The total translation products labelled with [^3H]valine were analysed by NaDodSO $_4$ /polyacrylamide gel electrophoresis on an 8% polyacrylamide gel as described previously (Davidson *et al.*, 1982).

Synthesis of cDNA used for solution-hybridization studies

cDNA was transcribed from mRNA prepared from 140-day foetal-sheep nuchal ligament with avian-myeloblastosis-virus reverse transcriptase. The reaction mixture (100 μl) contained 100mM-Tris/HCl, pH 8.3, 10mM-MgCl $_2$, 60mM-KCl, 10mM-dithiothreitol, 2.5 μg of oligo(dT) $_{12-18}$, 5 μg of actinomycin D, 0.5mM-dATP, 0.5mM-dTTP, 0.2mM-[^3H]dCTP (20 Ci/mmol), 5 μg of mRNA, and 20 units of reverse transcriptase. Before the reaction was initiated, the solution of mRNA was heated to 70°C for 2 min. After incubation at 42°C for 1 h, the following were added (final concns.) to the reaction mixture: 2.5mM-EDTA, 0.5% SDS, 0.1mM-NaCl, 50 μg of *Escherichia coli* tRNA/ml, and 10mM-sodium acetate, pH 6.0 (final volume 500 μl), and extracted with phenol/chloroform/isopentanol (50:50:1, by vol.) (Boyd *et al.*, 1980). The aqueous phase was chromatographed over a Sephadex G-50 column in water. The excluded cDNA fraction was adjusted to 0.3M-NaOH and 15mM-EDTA and incubated at 37°C for 16 h to hydrolyse template mRNA. After neutralization of the reaction by the addition of HCl, with Phenol Red as an indicator, the labelled cDNA was precipitated with 2.5 vol. of 95% (v/v) ethanol in the

presence of 50 μg of *E. coli* tRNA. The precipitated cDNA was dissolved in water, chromatographed on Sephadex G-50 (as described above), and the excluded cDNA was used for RNA excess hybridization in solution (see below). The specific radioactivity of cDNA was 3.2×10^7 d.p.m./ μg and the transcriptional efficiency was about 20%. The size of cDNA was determined by both 5%-polyacrylamide/8.3M-urea gel electrophoresis (200 V, 1–2 h, 55°C) (Trapnell, 1981) and methylmercury-agarose-gel electrophoresis as described above.

RNA excess hybridization in solution

Each reaction mixture (10 μl) contained 0.2 ng of [^3H]cDNA and from 3 ng to 3 μg of template mRNA. Hybridization was carried out at 43°C for 18 h in deionized 50% (v/v) formamide/0.5 M-NaCl/50 mM-Hepes (pH 6.8)/0.5 mM-EDTA/*E. coli* tRNA (50 $\mu\text{g}/\text{ml}$) (Young *et al.*, 1974).

Each hybridization mixture was sealed in silicone-treated 20 μl capillary tubes and heated in boiling water for 10 min before the incubation. The amount of hybridized cDNA was determined by digestion with S1 nuclease (Deeley *et al.*, 1977). The percentage hybridization was defined as the ratio of S1 nuclease-resistant, trichloroacetic acid-insoluble radioactivity to the trichloroacetic acid-insoluble radioactivity present in an undigested, parallel portion of each hybridization mixture. Corrections for self-annealing of cDNA were made by subtracting the percentage of hybridization obtained in reaction mixtures without mRNA.

Enrichment of elastin cDNA

RNA-excess-hybridization analysis (see the Results section) showed that the cDNA prepared as described above contained approx. 30% elastin-specific sequences. This elastin-specific component was further enriched by hybridizing 200 ng of cDNA with a 20-fold excess of its template mRNA, just below the first transition R_0t value in an RNA-excess-hybridization analysis [$R_0t = 1.60 \text{ mol} \cdot \text{s} \cdot \text{litre}^{-1}$ [$\log(R_0t) = 0.2$]]. Preparative hybridization was performed in a 25 μl reaction mixture as described above. After incubation at 43°C for 1 h, the mixture was diluted to decrease the concentration of formamide and digested with S1 nuclease as described above. cDNA-mRNA hybrids were ethanol-precipitated in the presence of 50 μg of *E. coli* tRNA. The cDNA enriched for elastin sequences (cDNA_E) was freed from its complementary mRNA by alkaline hydrolysis and then deproteinized with phenol/chloroform/isopentanol as described above. The aqueous phase was chromatographed over a Sephadex G-50 column with water and the elastin-enriched cDNA_E was recovered in the void volume.

Quantification of mRNA_E levels in nuchal ligament and lung by saturation hybridization

Total cellular RNA was prepared from foetal-sheep nuchal ligament and lung at different developmental ages. The elastin-specific sequences in each RNA preparation were measured by titration-curve analysis (Young *et al.*, 1974) with [^3H]cDNA. A constant amount (0.1 ng) of cDNA_E was hybridized with increasing amounts of total RNA (up to 30 μg) in 2.5 μl of hybridization mixture at 43°C for 88 h. The amount of RNA required to achieve 50% S1 nuclease protection of the cDNA_E probe was used to calculate the percentage of elastin-specific sequences in total RNA, with the assumption that the size of cDNA_E (600 nucleotides) was one-sixth that of mRNA_E (3500 nucleotides, see below).

To estimate the number of elastin mRNA molecules per cell, the RNA/DNA ratios for each tissue at each gestational stage were determined as described previously (Davidson *et al.*, 1982). The amount of mRNA_E per sheep cell was calculated by using the RNA/DNA ratios for each tissue, together with the known amount of DNA per sheep cell (6.2 pg/cell; Vendrely, 1955; Enesco & Leblond, 1962), the percentage of elastin sequences in total RNA determined as described above, Avogadro's number, and the size of mRNA_E ($M_r = 1.25 \times 10^6$; see below).

Purification of elastin mRNA used in Northern analysis

To purify mRNA_E, a solution of mRNA (50 μg) prepared from 140-day-foetal-sheep nuchal ligament was made to 1 mM-EDTA (final volume 250 μl), heated at 65°C for 10 min, chilled on ice, mixed with equal volume of 0.2 M-NaCl/2 mM-EDTA/20 mM-sodium acetate, pH 5.0, and layered over a 10–35% linear sucrose gradient in 0.1 M-NaCl/1 mM-EDTA and 10 mM-sodium acetate, pH 5.0 (Harris *et al.*, 1973). Centrifugation was performed in a Beckman SW 41 rotor (30000 rev./min 16 h, 4°C). The gradients were fractionated, and RNA was precipitated from each fraction (500 μl) (Tolstoshev *et al.*, 1979). The total mRNA activity of each fraction was determined by using the reticulocyte-lysate system described above, and the purity and the size of mRNA_E was evaluated by methylmercury-agarose-gel electrophoresis.

Subcloning of an elastin genomic clone

A restriction-endonuclease-*Bam*HI/*Eco*RI digest of the DNA from sheep genomic clone SE1 [see the accompanying paper (Davidson *et al.*, 1984)] was preparatively fractionated by agarose-gel electrophoresis. A 1.3 kb fragment, which represented the 3'-terminus of the genomic insert,

was isolated by electroelution (Trapnell, 1981) and subcloned into bacterial plasmid pUC9 (Vieira & Messing, 1982) which had been similarly digested. After ligation and transfection into host *E. coli* strain JM103 by standard procedures, a large number of ampicillin-resistant, β -galactosidase-negative colonies were produced. One recombinant, designated 'pSS1', was selected for further amplification, and plasmid DNA was purified by standard procedures.

Evaluation of elastin mRNA levels by Northern analysis

RNAs isolated from nuchal ligament and lung were electrophoresed in 10 mM-methylmercury/1% agarose gel and then transferred to diazophenylthioether paper, which was then pretreated with hybridization buffer (Alwine *et al.*, 1977) for 3 h at 42°C and then hybridized at 42°C for 48 h with heat-denatured [32 P]cDNA in the hybridization buffer minus glycine and salmon spermatozoa DNA. The [32 P]cDNA used for the studies was transcribed from sucrose-gradient-purified mRNA_E by using [32 P]dCTP (140 Ci/mmol) as described above. In one experiment, hybridization was performed with a cloned sheep elastin gene fragment, pSS1, prepared as described above. Plasmid DNA (0.25 μ g) was nick-translated in the presence of [32 P]dATP to a specific radioactivity of 2×10^8 c.p.m./ μ g and denatured at 70°C for 5 min in hybridization buffer. After hybridization with cDNA or pSS1 DNA, the paper was washed three times with $2 \times$ SSC/0.1% SDS at room temperature, three times with $0.1 \times$ SSC/0.1% SDS at 55°C, dried, and exposed to X-ray film (Kodak X-Omat R) at -70°C. Exposure time was adjusted to obtain a linear dose-response relationship between the amount of 140-day-foetal-sheep nuchal-ligament RNA and the absorbance of the 24-25S region of the gel, as determined by an integrating densitometer (Ortec). Cellular abundance of unknown concentrations of mRNA_E was extrapolated from the standard curve and the relative response of a parallel sample of gradient-purified mRNA_E of known size (3500 nucleotides) and purity (~80%).

Results

Preparation of an elastin-specific cDNA probe

When mRNA isolated from 140-day-foetal-sheep nuchal ligament was electrophoresed in a denaturing methylmercury-agarose gel, a single band was clearly visible at a position corresponding to M_r 1.25×10^6 (Fig. 1a, lane 1); as described below, this corresponded to the molecular size of mRNA_E. Other minor RNA species were present, as well as a minor contamination

with rRNA. Translation of this mRNA preparation in a rabbit-reticulocyte-lysate system yielded one prominent protein with M_r approx. 70000-72000 (Fig. 1b), the known M_r of the primary translation product of mRNA_E (Burnett & Rosenbloom, 1979; Foster *et al.*, 1980; Davidson *et al.*, 1982). No larger cell-free translation products were visible on this gel. Relative elastin synthesis, determined by specific immunoprecipitation of the cell-free product, was 40% of total [3 H]valine incorporation. Densitometric scanning of the gels also confirmed that 36% of the total radioactivity was in the 70000-72000- M_r band (not shown). Thus mRNA_E was the major component of this mRNA preparation.

The cDNA synthesized from total nuchal-ligament mRNA using reverse transcriptase had an average length of 600 nucleotides as determined by gel electrophoresis in urea/polyacrylamide (Fig. 1c) and methylmercury-agarose (results not shown). When hybridized back to its template under conditions of RNA excess, about 30% of this cDNA annealed to a low-complexity high-abundance class of RNA (Fig. 2). This abundant-class cDNA was further enriched in elastin-specific sequences such that it represented 65% of the total sequences in the cDNA probe (Fig. 2).

Quantification of mRNA_E levels in developing foetal-sheep nuchal ligament and lung using saturation-hybridization

RNA species from foetal-sheep nuchal ligament and lung at different developmental stages were hybridized with a cDNA_E probe under saturation-hybridization conditions (Fig. 3). The extent of hybridization of each of the nuchal ligament and lung RNA species was approx. 65%, suggesting that only mRNA_E in each RNA preparation hybridized with the cDNA_E probe at the R_{0t} values used.

Abundance of mRNA_E was based on half-saturation R_{0t} values. The percentage of elastin-specific sequences in nuchal-ligament total RNA increased about 10-fold during foetal development (0.047% at 70 days, 0.172% at 108 days, and 0.438% at 138 days after conception) (Fig. 4a). By contrast, the elastin-specific sequences in lung total RNA increased about 3-fold during the same period (0.008% at 68 days, 0.013% at 107 days, and 0.022% at 147 days after conception) (Fig. 4a). To calculate the average number of elastin mRNA molecules per cell in these tissues, the RNA/DNA ratio was determined in each tissue at each developmental stage. In the lung, the RNA/DNA ratio remained constant during development; by contrast, the ratio in nuchal ligament increased (results not shown). By using these RNA/DNA ratios, the number of elastin mRNA molecules was found to

Fig. 1. Analysis of mRNA prepared from near-term-foetal-sheep nuchal ligament and its transcribed cDNA
 (a) Poly(A)⁺ RNA was electrophoresed in a 1%-agarose gel containing 10mM-methylmercuric hydroxide. The gel was stained with ethidium bromide and photographed under u.v. light. Lane 1, mRNA (5 μ g) isolated from 140-day-foetal-sheep nuchal ligament using poly(U)-Sephacrose 4B chromatography; lane 2, ribosomal RNA markers with relative sizes indicated. (b) Fluorogram of an NaDodSO₄/8%-polyacrylamide gel of [³H]valine-labelled translation products directed by the mRNA shown in (a). M_r standards included dansylated phosphorylase *a* (93 000), bovine serum albumin (68 000) and ovalbumin (43 000). The dense band migrating at approx. M_r 70 000 (pre-tropoelastin) represented 40% of the cell-free product. (c) Size estimation of ³H-labelled cDNA transcribed from the mRNA shown in (a). The size of this cDNA was determined by electrophoresis in a 5%-polyacrylamide/8.3M-urea gel and subsequent fluorography. Lane 1, ³H-labelled cDNA; lane 2, DNA size markers (bacteriophage- ϕ X174 DNA digested with restriction endonuclease *Hae*III) run in a parallel slot and stained with ethidium bromide are indicated as the number of bases.

increase from 950 to 20 000 molecules per nuchal-ligament cell and from 130 to 330 molecules per lung cell during same period (Fig. 4b).

Isolation and analysis of partially purified mRNA_E used in Northern analysis

To characterize further the nature of mRNA_E in these tissues, mRNA prepared from 140-day-foetal-sheep nuchal ligament was purified on an aqueous sucrose density gradient, yielding one major RNA peak in the 25S region of the gradient (Fig. 5a). When cell-free translation was performed on these peak fractions, the highest elastin mRNA activity was found to be coincident with this 25S mRNA peak (Fig. 5b). About 80% of total [³H]valine incorporation of this translation product was elastin, indicating that the sucrose gradient yielded at least a 2-fold purification of mRNA activity. Electrophoresis of the 25S mRNA under denaturing conditions in a methylmercury-agarose gel showed one predominant band (Fig. 5c; cf. Fig. 1a) with an M_r 1.25×10^6 (approx. 3500 bases). Saturation-hybridization analysis of a [³H]cDNA_E species with 25S mRNA_E showed that the extent of hybridization to mRNA_E was also about 65%

(Fig. 5d) and that elastin sequences were enriched 2-fold in this preparation.

Evaluation of relative elastin mRNA levels in developing foetal-sheep nuchal ligament and lung using Northern analysis

Various amounts of total RNA prepared from 138-day-foetal-sheep nuchal ligament were electrophoresed in a methylmercury-agarose gel, transferred to diazophenylthioether-paper, and hybridized with [³²P]cDNA (sp. radioactivity 2.2×10^8 d.p.m./ μ g) that had been transcribed from the sucrose-gradient-purified elastin mRNA (Fig. 6a, lanes 1–5). In all lanes, one major band was observed, on autoradiography, at the region corresponding to M_r 1.25×10^6 , the same as that of mRNA_E (Fig. 6a, lane 6). The amount of [³²P]cDNA hybridized to elastin mRNA (calculated by scanning each lane with a densitometer) was proportional to the amount of total RNA applied to gel (results not shown; see Fig. 6a, lanes 1–5). The relative amount of elastin mRNA was determined in RNA preparations from nuchal ligament and lung by this procedure (Fig. 6). Essentially identical results were obtained in a subsequent experiment

Fig. 2. RNA-excess hybridization of total and abundant-class $[^3H]$ cDNA

The $[^3H]$ cDNA (0.2 ng) shown in Fig. 1(c) was hybridized with its template mRNA to the R_0t values indicated (●). Abundant-class cDNA was selected by hybridizing 200 ng of cDNA with a 20-fold excess of its template mRNA to $R_0t = 1.60 \text{ mol} \cdot \text{s}^{-1} \cdot \text{l}^{-1}$ [$\log(R_0t) = 0.2$] as described in the Experimental procedures section. This purified, abundant cDNA (0.1 ng) was then hybridized back to its template mRNA (see Fig. 1a) under RNA-excess conditions (○).

Fig. 3. Solution-hybridization of purified elastin cDNA against various RNA species under saturation conditions. Hybridization was carried out at 43°C for 88 h. (a) Control titration curve with the template mRNA used to prepare the cDNA probe. (b) Titration curves with total RNA prepared from 70-day(●), 108-day(■), and 138-day(▲)-foetal-sheep nuchal ligament. (c) Titration curves with total RNA prepared from 68-day(○), 107-day(□) and 147-day(△)-foetal-sheep lung.

Fig. 4. $mRNA_E$ levels in sheep nuchal ligament and lung during foetal development as determined by solution-hybridization with a purified elastin cDNA probe

These results are based on half-saturation values derived from the titration analysis described in Fig. 3. (a) The percentage of elastin sequences in nuchal ligament total RNA (●) and lung total RNA (○) as a function of foetal age. (b) The average number of elastin mRNA molecules per nuchal-ligament cell (●) and lung cell (○) as a function of foetal age. The data in (b) were determined from (a) by using RNA/DNA values at each age.

Fig. 5. Purification and analysis of elastin mRNA used for Northern hybridizations

mRNA (50 μ g) was fractionated in a sucrose density gradient as described in the Experimental procedures section. (a) A_{260} profile. Arrows indicate the positions of ribosomal RNA markers in a parallel gradient. (b) Distribution of total mRNA and elastin mRNA activity. mRNA, recovered from 20 μ l of each 500 μ l fraction, was translated in a rabbit-reticulocyte-lysate system in the presence of [3 H]valine. Total mRNA activity in the presence of [3 H]valine (open bars) was evaluated by precipitation with trichloroacetic acid, and elastin mRNA activity (hatched bars) was quantified with a specific anti-(α -elastin) serum. (c) Size determination of purified elastin mRNA. Gradient-purified mRNA (2 μ g of the 25S peak of the gradient shown in (a)) was electrophoresed in a 1%-agarose gel containing 10 mM-methylmercuric hydroxide and stained. rRNA markers are indicated. (d) Titration-curve analysis of the purification of mRNA_E using abundant-class [3 H]cDNA_E. Elastin-specific cDNA_E (0.1 ng) was hybridized with increasing amounts of purified elastin mRNA (○) (0.3–20 ng) as well as the original template mRNA (●). See Fig. 3(a) for comparison.

which used a cloned elastin gene fragment (pSS1) as the hybridization probe (Fig. 7).

Since mRNA_E levels in lung RNA were much lower than in nuchal-ligament RNA, a 3-fold amount of lung RNA (30 μ g) was electrophoresed in the same gel used in Fig. 6(a) (results not shown). Radioactivity bound in the 1.25×10^6 - M_r -mRNA region was quantified (Fig. 8a) to establish the developmental increase in mRNA_E levels in lung RNA. In addition, the qualitative presence of mRNA_E in total lung RNA was detected in a second experiment using a [32 P]cDNA species of higher specific radioactivity (5.6×10^8 d.p.m./ μ g; Fig. 6b). By this method, the number of mRNA_E molecules per cell increased more than 10-fold in the nuchal ligament and about 3-fold in the lung during the same developmental period (Fig. 8). These results closely paralleled those obtained by solution-hybridization.

Discussion

The considerable abundance of functional mRNA_E in near-term sheep nuchal ligament (Davidson *et al.*, 1981, 1982) permitted the synthesis and purification of elastin cDNA probes with which to analyse further the mechanisms that regulate the expression of elastin genes. Two methods, namely (1) back-hybridization to elastin-specific sequences, and (2) transcription from a purified mRNA_E, were used to prepare elastin-specific cDNA probes that specifically and quantitatively interacted with mRNA_E. Subsequently, it was established that a cloned DNA probe gave the same quantitative results. The genomic and cDNA_E probes were used to demonstrate increases in mRNA_E in nuchal ligament and lung during foetal-sheep development. In both tissues, mRNA_E levels increased substantially during the latter half

Fig. 6. Northern hybridization of sucrose-gradient-purified elastin mRNA and total RNA species prepared from sheep nuchal ligament and lung during stages of foetal development

RNA species were electrophoresed in a 1%-agarose gel containing 10 mM-methylmercuric hydroxide, transferred to diazophenylthioether-paper and hybridized with [32 P]cDNA_E transcribed from purified mRNA_E. Bound radioactivity was detected by autoradiography at -70°C . Ribosomal RNA size markers are indicated. (a) Identification, dose-response calibration, and quantitative analysis of mRNA_E in nuchal-ligament RNA. Lanes 1–5 contained (respectively) 1, 2, 4, 8 and 12 μg of total RNA prepared from 138-day-foetal-sheep nuchal ligament; lane 6, 100 ng of gradient-purified mRNA_E; lanes 7–9, 10 μg of 70-, 108-, and 138-day-foetal-sheep nuchal-ligament total RNA. (b) mRNA_E content in lung total RNA species. Lanes 1–3 contained (respectively) 30 μg of 68-, 108-, and 147-day-foetal-sheep lung RNA. For the data in (b), a 2-fold-greater-specific-radioactivity [32 P]cDNA_E was employed to increase the sensitivity of the method.

of foetal development, but both the absolute content and the developmental increase of mRNA_E in the nuchal ligament was much more dramatic. In addition, for the near-term nuchal ligament, elastin mRNA was shown to exist as a major component of the total mRNA population. This mRNA could be clearly resolved by sucrose-density-gradient centrifugation and was shown to have an M_r of 1.25×10^6 (3500 bases) by analysis on denaturing agarose gels.

Quantification of mRNA_E using saturation-hybridization

The cDNA probe used to quantify mRNA_E levels by saturation-hybridization was transcribed from total nuchal-ligament mRNA and subsequently purified by partial back-hybridization to its original template. Several lines of evidence suggested that this cDNA could be used to measure accurately mRNA_E levels.

First, total template mRNA was isolated from a tissue that devotes 20–40% of its protein-synthesizing machinery to elastin synthesis (Mecham *et al.*, 1981; Davidson *et al.*, 1982). The mRNA template used for cDNA synthesis directs the incorporation

of [^3H]valine in a cell-free system into protein products of which 40% are elastin precursor (as determined by NaDodSO₄/polyacrylamide gels or immunoprecipitation with specific anti-elastin antiserum). Near-term-foetal-sheep nuchal-ligament mRNA does not direct the synthesis of any other major protein. Thus, although a variety of cDNA species are likely transcribed from total nuchal-ligament mRNA, elastin-specific cDNA will be the major cDNA species in the absence of selective reverse-transcriptase activity.

Second, the cDNA was further purified by partial back-hybridization to its template. Since mRNA_E is the major mRNA species in the template, the region up to first major transition of the mRNA-excess R_{ot} analysis likely represents specific hybridization of the cDNA to the elastin sequences in the mRNA template. Of the cDNA probe selected from this class of RNA-DNA hybrids, 65% was hybridizable, even to an appreciable excess of nuchal ligament or lung mRNA.

Third, titration analysis of this isolated cDNA_E with gradient-purified mRNA_E showed that the maximal extent of hybridization was also about 65% and that the purified mRNA_E was 2-fold

Fig. 7. Northern hybridization of sheep nuchal-ligament RNA to a cloned elastin gene fragment

Electrophoretic and transfer conditions were as described in Fig. 6. Hybridization was to ^{32}P -labelled pSS1 DNA. Lanes A, B and C contain (respectively) 1.25, 2.5 and 5.0 μg of total RNA from 138-day-foetal-sheep nuchal ligament. The hybridizing mRNA had an electrophoretic mobility identical with that in Fig. 6.

enriched in elastin sequences compared with the original template, consistent with the 2-fold increase in mRNA_E translational activity.

With this elastin-specific cDNA probe, solution-hybridization studies demonstrated that both nuchal-ligament and lung mRNA_E levels (% elastin sequences) increased significantly during the latter half of foetal development. The average number of mRNA_E molecules per lung and nuchal-ligament cell also increased during the same period, and this increase was much more pronounced in the nuchal-ligament cell. This development increase in absolute mRNA_E levels closely paralleled that of functional mRNA levels in nuchal ligament (Davidson *et al.*, 1981) and lung (Shibahara *et al.*, 1981).

Fig. 8. Estimate of mRNA_E content of developing foetal-sheep lung and nuchal ligament by Northern hybridization

A dose-response relationship was established by linear-regression analysis of quantitative densitometric data derived from scanning and digitally integrating the region containing $1.25 \times 10^6 \cdot M_r$ RNA in the methylmercuric hydroxide/agarose gels of Fig. 6(a). Exposure time was adjusted to obtain linear response of the film to [^{32}P]cDNA_E. The calculation of cellular abundance of mRNA_E molecules was based on the known size (~ 3500 nucleotides) and purity ($\sim 80\%$) of sucrose-gradient-purified mRNA_E. (a) Abundance of mRNA_E molecules in foetal-sheep nuchal ligament as a function of foetal age. (b) Abundance of mRNA_E molecules in foetal-sheep lung as a function of foetal age.

Partial purification and characterization of mRNA_E

The near-term-foetal-sheep nuchal ligament served as an excellent source of material for further characterization of mRNA_E. A well-resolved peak of both absorbance and mRNA_E activity in the 25S region of a sucrose density gradient indicated an unusually large template for elastin translation. This size estimate (about 3500 nucleotides) was confirmed under fully denaturing conditions, suggesting that a significant fraction (30%) of mRNA_E is untranslated (800–1000 nucleotides). Although a partial explanation for this observation is that a 100–200-base poly(A) tract is present at the 3'-terminus of this mRNA, these results suggest an extensive region of untranslated mRNA sequences. Consistent with this hypothesis, a cloned cDNA from chick mRNA_E (Burnett *et al.*, 1982), has been reported to contain at least 250 untranslated nucleotides at the 3'-terminus. In addition, partial DNA sequence analysis of clone pSS1 has confirmed this expectation (J. Rosenbloom, P. A. LuValle & J. M. Davidson, unpublished work).

Northern-blot analysis of mRNA_E

Hybridization of cDNA_E probe to Northern blots of total nuchal-ligament and lung RNA, as well as to purified mRNA_E, provided visual data on the relative amounts of mRNA_E in these tissues.

The specificity of the cDNA probe for its template was confirmed by the precise correspondence of maximal cDNA_E binding and the position of migration of the major, 25S, mRNA species of nuchal ligament, which was elastin mRNA (see above). Precisely the same pattern was detected by a cloned elastin gene fragment. Radioactivity bound in other regions of the blots may have been the result of hybridization to partially degraded mRNA_E or other minor mRNA species present in the original template for cDNA_E synthesis. These regions of the blots were therefore not included in the quantitative analysis of mRNA_E levels.

Preliminary evidence would suggest that the elastin gene and its initial transcript are much larger than 3.5kb (J. M. Davidson, S. Shibahara, P. A. LuValle, P. Tolstoshev & R. G. Crystal, unpublished work; Davidson & Crystal, 1982). Blotting of both cellular RNA and nuclear-RNA species from these tissues will be a useful approach to describe the processing of elastin gene transcripts.

Mechanisms controlling mRNA_E levels

These studies show that the developmental increases in elastin mRNA levels parallel the levels of elastin production and mRNA_E activity in nuchal ligament (Davidson *et al.*, 1981, 1982) and lung (Shibahara *et al.*, 1981). In this context, it is likely that the relative amounts of mRNA_E are the major determinants of the extent of elastin synthesis during the differentiation of these tissues. The production of procollagen, another connective-tissue component of embryonic organs, has also been shown to be paralleled by the levels of procollagen mRNA during development of foetal-sheep lung and tendon (Tolstoshev *et al.*, 1981b) and embryonic-chick calvarium (Moen *et al.*, 1979).

However, elastin production is likely not a simple function of mRNA_E concentration, since previous data indicate that the rate of protein production, and probably the rate of mRNA utilization, is substantially higher in lung than in nuchal ligament under conditions of short-term culture (Davidson *et al.*, 1981).

There are significant differences in the average cellular mRNA_E contents of nuchal ligament and lung. For example, the average near-term-foetal-sheep nuchal-ligament cell contains 60 times more mRNA_E molecules than the average near-term-foetal-sheep lung cells. However, this does not necessarily indicate that each elastin-producing nuchal-ligament cell possesses more mRNA_E than each lung elastin-producing cell, i.e. these data may simply be a reflection of the difference in the cell populations of each organ. In this regard, nuchal ligament appears to be composed of a popu-

lation of morphologically homogenous fibroblasts which synthesize elastin (Kewley *et al.*, 1978; Jones *et al.*, 1980; Mecham *et al.*, 1981), whereas lung is a very complex tissue composed of many types of epithelial, endothelial, mesothelial and mesenchymal cells (Kuhn, 1976). Thus the moderate increase in average mRNA_E levels in the developing foetal lung may be the consequence of a relatively large increase in mRNA_E levels in a minor population of specialized lung cells.

Although several factors may affect the expression of elastin genes (Mecham *et al.*, 1981; Eichner & Rosenbloom, 1979; Davidson & Crystal, 1982), it is still unknown how mRNA_E levels are regulated in elastin-producing cells. There are several possible sites of control which may affect mRNA_E levels: the number and transcription rate of elastin genes, the processing and transport of mRNA_E precursors, and the rate of degradation of mRNA_E. To evaluate such potential mechanisms, further studies will need to focus on the structure and function of the elastin gene.

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