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Correlation of Procollagen mRNA Levels in Normal and Transformed Chick Embryo Fibroblasts with Different Rates of Procollagen Synthesis†

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ABSTRACT: Procollagen mRNA obtained from chick embryonic tendon cells, cultured fibroblasts, and fibroblasts transformed by Rous sarcoma virus (RSV) was measured by translation in a modified reticulocyte lysate and by hybridization with a complementary DNA (cDNA). The lysate was treated with *Staphylococcal* nuclease to destroy endogenous mRNA and chromatographed on a Sephadex column to reduce the endogenous amino acid content. The optimal conditions for procollagen mRNA translation included 40 mM sodium, 6 mM phosphate, and 0.4 mM spermidine in addition to the usual reagents. With these methods, procollagen mRNA activity could be detected in cells synthesizing only 1–2% procollagen. The immunoprecipitable product was degraded by collagenase and migrated slightly slower than authentic secreted procollagen on polyacrylamide gel electrophoresis. Procollagen mRNA was isolated from embryonic chick tendons and purified by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation. The mRNA activity was located in the 27S region of the gradient; this RNA was used to synthesize procollagen cDNA. The purified cDNA was about 900 nucleotides long, was specific for procollagen mRNA, and hybridized to 75% with RNA from cells synthesizing procollagen. The concentration of procollagen mRNA correlates well with rates of procollagen synthesis in tendon fibroblasts in situ, fibroblasts in culture, and in RSV-transformed fibroblasts. When tendon fibroblasts were grown in culture, the procollagen mRNA concentration fell about threefold; the concentration was 1.5-fold lower at high cell density compared to low density. Transformation by RSV lowered procollagen mRNA levels another four- to tenfold at both high and low density. These results indicate that the rate of procollagen synthesis is determined primarily by the steady-state concentration of procollagen mRNA rather than by translational control.

Highly specialized cells such as reticulocytes, myoblasts, and lens cells are characterized by a limited proliferation potential and by predominant synthesis of a few specific proteins. As their progenitor cells differentiate, they accumulate the specific mRNAs for their cell-specific proteins (Chan et al., 1974; Ramirez et al., 1975; Clissold et al., 1977; John et al., 1977; Bloemendal, 1977). The synthesis of these cell-specific proteins typically correlates well with the cellular concentration of their respective mRNAs; however, in developing myoblasts there is evidence that actin and myosin mRNA accumulate in a nontranslated (masked) form prior to their expression (Bag and Sarbar, 1975; Bester et al., 1975).

In the process of dedifferentiation, a population of cells develops an enhanced proliferative potential and often reduces its synthesis of cell-specific proteins. Certain fibroblasts illustrate this process. In embryonic chick tendons, fibroblasts devote approximately 60% of their total protein synthesis to procollagen (Dehm and Prockop, 1971), while chick embryo fibroblasts maintained in cell culture synthesize 5–10% collagen (Peterkofsky, 1972). However, the level of procollagen synthesis in vitro is influenced by a number of factors including ascorbate and serum concentrations (Peterkofsky, 1972; Schwartz and Bissell, 1977). The proportion of procollagen drops to 1% if chick cells are transformed by Rous sarcoma virus (RSV)[1] (Levinson et al., 1975; Kamine and Rubin, 1977). A similar decline in procollagen synthesis is observed

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[1] Abbreviations used are: RSV, Rous sarcoma virus; cDNA, complementary DNA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SET buffer, 1% sodium dodecyl sulfate, 5 mM EDTA, 10 mM Tris-HCl (pH 7.5); DMEM, Dulbecco's modified Eagle's medium; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline (150 mM NaCl, 20 mM KH_2PO_4, pH 7.5); IgG, immunoglobulin G; DEAE, diethylaminoethyl.

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in transformed mammalian cells (Green et al., 1966; Peterkofsky and Prather, 1974). The molecular mechanisms underlying these changes in procollagen synthesis are just beginning to be explored (Adams et al., 1977).

The decline in procollagen synthesis might reflect either translational control of procollagen mRNA or a transition to a lower steady-state level of mRNA. In order to distinguish between these possibilities, we have developed a translation assay for procollagen mRNA employing a modified reticulocyte lysate and immunoprecipitation for product identification. A cDNA, enriched in sequences complementary to procollagen mRNA, was prepared to test for the possibility that procollagen mRNA is modified to a nontranslatable form in dedifferentiated fibroblasts. These studies reveal a close correlation between the relative rates of procollagen synthesis and mRNA levels, measured either by translation or by hybridization, in fibroblasts at different stages of dedifferentiation. The data suggest that the extent of procollagen synthesis in culture, and after viral transformation, is determined predominantly by the level of procollagen mRNA rather than by translational control.

Experimental Procedures

Preparation of the Reticulocyte Lysate and Antibodies to Procollagen. Rabbits weighing 2–4 kg were injected daily with freshly prepared phenylhydrazine (7 mg/kg) for 5 days. On day 6, reticulocyte counts were performed, and those animals with reticulocyte counts less than 75% were given 10 mg/kg. Animals with reticulocyte counts greater than 75% received a maintenance dose on day. 6. No injections were given on day 7, and on day 8 blood was collected by heart puncture and the lysate was prepared as described by Palmiter (1973).

Aliquots of the lysate were incubated with *Staphylococcal* nuclease to destroy the endogenous globin mRNA activity (Pelham and Jackson, 1976). After the nuclease was inactivated with 2 mM EGTA, 10 mL of the lysate was applied to a 1.5 × 20 cm Sephadex G-50 (fine) column equilibrated with 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 25 mM KCl, 1 mM dithiothreitol, 0.5 mM EGTA, and 1 mM $MgCl_2$ at 4 °C. The fractions from the column which contained 80% of the input absorbance at 415 nm were pooled and frozen at −70 °C. Preliminary studies demonstrated that this method separated these fractions from free amino acids.

Procollagen isolated from chicken calvaria and purified by DEAE-cellulose chromatography (Davidson et al., 1975) was used to immunize rabbits. The resulting antisera have been shown to recognize both NH_2- and COOH-terminal nonhelical determinants in procollagen (Nist et al., 1975). Anti-$p_N\alpha 1$ antisera were prepared as described by von der Mark et al. (1973). Purified rabbit IgG was used to produce goat anti-rabbit IgG antisera.

Extraction of RNA. Embryonic chick calvaria or leg tendons (Remlington Farms, Redmond, Wash.) were frozen in liquid N_2 and stored at −70 °C. A 5% homogenate was prepared by disrupting 0.5 g of tissue in 10 mL of SET buffer containing 65 μg/mL of proteinase K (Merck) at 20 °C using a Polytron (Tekman) for 10–15 s at midspeed setting, followed by several strokes with a tight-fitting Dounce homogenizer (Kontes). Cultured fibroblasts were extracted with 3 mL of the same buffer per 100-mm Petri dish. Samples were incubated for 1 h at 40–50 °C and then shaken with 0.5 vol of phenol saturated with 0.5 M Tris-HCl (pH 7.5) followed by 0.5 vol of chloroform. After a brief centrifugation to separate the phases, the aqueous phase was removed and reextracted with phenol/chloroform. Total nucleic acids in the resultant aqueous phase were precipitated overnight with 0.1 M NaCl and 2 vol of ethanol at −20 °C. The precipitate was collected by centrifugation at 13 000*g* for 10 min, washed three times with 0.04 M NaCl in 66% ethanol to remove traces of phenol, and lyophilized. Total nucleic acids were dissolved in water at 1–2 mg/mL. Aliquots were taken for DNA determination by the diaminobenzoic acid method (Fujimoto et al., 1977). The RNA was precipitated by the addition of 2 volumes of 3 M sodium acetate, 5 mM EDTA, pH 7.0. The precipitate was washed twice with 3 M sodium acetate, 5 mM EDTA and once with 0.04 M NaCl, 66% ethanol and then lyophilized. The RNA was dissolved in water at an approximate concentration of 1 mg/mL and stored at −70 °C. The yield of salt-washed RNA from embryonic tendons or calvaria was approximately 2.5 mg/g wet weight (5–7 $\mu g/10^6$ cells), while fibroblasts in culture produced 8–11 $\mu g/10^6$ cells.

mRNA was prepared by passage through an oligo(dT)-cellulose column (Aviv and Leder, 1972). The RNA was loaded onto the column in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 4 mM EDTA, and the mRNA was eluted with H_2O. The water eluate was made 0.5 M with NaCl, heated to 60 °C for 5 min, cooled, and recycled through the column. Sucrose gradient analysis of total RNA or oligo(dT)-purified RNA was performed on linear 5–20% sucrose gradients in 10 mM Hepes, pH 7.5. The RNA sample was heated to 60 °C for 5 min, cooled, layered over the sucrose, and centrifuged for 10 h at 40 000 rpm at 2 °C (SW41 rotor, Beckman). The absorbance at 254 nm was measured with an Isco Model 160 sucrose gradient fractionator.

Translation of Procollagen mRNA. During the course of this study, the translation conditions for procollagen mRNA were modified from those described by Palmiter (1973). The optimal conditions now in use are given here. A 125-μL reaction mixture contained 25 μL of nuclease and Sephadex-treated lysate, up to 5 μg of total RNA (less of purified mRNA fractions), 60 mM KCl, 30 mM NaCl, 4 mM KH_2PO_4 (pH 7.5), 1.75 mM $MgCl_2$, 0.3 mM spermidine, 15 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, 50 μg/mL creatine kinase, and 40 μCi/mL [^{3}H]proline (25 Ci/mmol). Incubation was for 2 h at 26 °C. The reaction was terminated with a 50-μL solution of 5% sodium deoxycholate, 5% Triton X-100 and 1 mM proline, containing a 1:50 dilution of anti-procollagen serum. After a 1-h incubation at 20 °C, the mixture was centrifuged at 13 000*g* for 10 min to remove insoluble material. The procollagen–antibody complexes in the supernatant were then precipitated in a separate tube with an excess of goat anti-rabbit IgG serum (100 μL; see text). After another 1-h incubation at 20 °C, the immunoprecipitates were collected by centrifugation through a sucrose–detergent solution as described by Palmiter (1973).

Product Characterization. The products made during in vitro translation were immunoprecipitated with antibody to procollagen or to the $p_N\alpha 1$ chain and were dissolved in sample buffer which contained 50 mM dithiothreitol and 0.5 M urea. The dissolved samples were applied to a 5% slab acrylamide gel (Laemmli, 1970), and the bands were identified by fluorography (Bonner and Laskey, 1974). [^{3}H]Procollagen standards were prepared by DEAE-cellulose chromatography (Davidson et al., 1975). Collagenase digestion (Peterkofsky and Diegelmann, 1971) was performed on the immunoprecipitate using purified bacterial collagenase (Advanced Biofactures). The reaction was terminated by addition of the gel sample buffer and heating to 100 °C. The solution was applied directly to the acrylamide gel.

Preparation of Procollagen cDNA and Hybridization. Procollagen cDNA was synthesized as described by Lee et al. (1978). The reaction mixture (20 μL) contained 50 mM

Tris-HCl (pH 8.3); 6 mM $MgCl_2$; 50 μg/mL 27S mRNA fraction (see Results); 10 mM dithiothreitol; 125 μM [^{3}H]-dCTP (50 μCi, 20 Ci/mmol, Schwarz-Mann); 333 μM each of dGTP, dATP, and dTTP, 12.5 μg/mL oligo(dT)$_{16}$ (Pabst Laboratories); 1 mg/mL RNase inhibitor (Searle Products for Research); and 1.5 units of reverse transcriptase (supplied by Dr. J. W. Beard, Life Sciences Inc.). Incubation was for 30 min at 45 °C. The reaction was terminated by the addition of 180 μL of SET buffer, and the RNA was hydrolyzed with 0.25 M NaOH at 60 °C for 5 min. The sample was neutralized and passed through a Sephadex G-50 column in SET buffer. The cDNA was layered over linear 8–18% sucrose gradients containing 0.1 M NaOH, 0.9 M NaCl, and 5 mM EDTA and centrifuged for 16 h at 40 000 rpm at 4 °C (SW41 rotor, Beckman). Fractions containing cDNA longer than 200 nucleotides were pooled, neutralized, and precipitated with ethanol after the addition of carrier yeast tRNA. This preparation was purified further by incubation under hybridization conditions for 4 h, followed by passage through a hydroxylapatite column at 60 °C in 10 mM NaH_2PO_4, pH 7.2. The single-stranded cDNA was eluted with 0.14 M NaH_2PO_4, passed through a Sephadex column, and precipitated as above. The cDNA was then back-hybridized for 24 h with 10 μg/mL of the RNA used as template; hybrids were separated from single-stranded molecules by passage through hydroxylapatite at 60 °C in 0.12 M NaH_2PO_4 and elution of the hybrids with 0.4 M NaH_2PO_4. RNA in the hybrids was hydrolyzed with 0.3 M NaOH overnight at 37 °C, and the cDNA was passed through Sephadex and concentrated as above.

Hybridizations were performed at 68 °C for a constant time (typically 24 h) in 20 μL of 0.6 M NaCl, 4 mM EDTA, 0.15% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 7.5), containing 500 cpm of cDNA and varying amounts of RNA. Hybrid formation was assayed by S1 nuclease digestion (McKnight and Schimke, 1974). The relative concentration of procollagen mRNA in RNA samples was determined by comparison with a standard curve constructed with tendon RNA. The theoretical basis of this approach is discussed by Young et al. (1974). In practice, three different concentrations of RNA were chosen that would give less than 30% hybridization in order to enhance the sensitivity of the comparisons with the standard, since in this range the curves are nearly linear.

Fibroblast Culture Conditions. Primary cultures of chick embryo fibroblasts were obtained from 17-day-old leg tendons by the method of Dehm and Prockop (1971). The cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 25 mM Hepes, 100 μg/mL penicillin, and 100 units/mL streptomycin in a humidified incubator under a 5% CO_2/95% air atmosphere. Chick embryo fibroblasts were infected with Prague C strain of RSV and allowed to undergo two to three passages before use to ensure a high percentage of transformed cells.

At the start of an experiment, replicate cultures of normal or transformed cells (1×10^6, unless otherwise stated) were plated into 100-mm^2 Petri dishes. When the cells had reached either early confluence or high density, plates were removed for RNA extraction and pulse-labeling. For the latter, the cells were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min at 37 °C in DMEM without fetal calf serum and containing 50 μg/mL sodium ascorbate. The medium was changed and the cells were incubated at 37 °C for 30 min with 50 μCi of [^{3}H]proline in 5 mL of DMEM containing ascorbate. The medium was discarded and the cell layer washed three times with cold PBS. The cell layer was scraped into 5 mL of 1 M NaCl, 30 mM Tris-HCl (pH 7.5), 25 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM

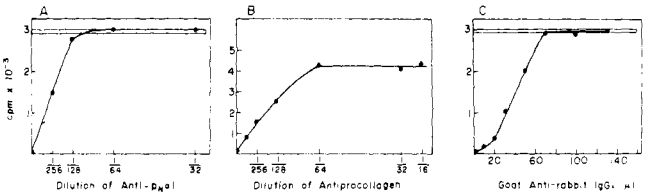

FIGURE 1: Titration of procollagen and goat anti-rabbit IgG antisera. (A) Precipitation of [^{3}H]procollagen with antisera to pNα1 chains. Fifty microliters of serially diluted antiserum to pNα1 chain was added to a 125-μL solution of PBS containing 0.7% Triton X-100, 0.7% deoxycholate, and 3000 cpm of ^{3}H-labeled procollagen isolated from chick calvaria by DEAE-cellulose chromatography. The total amount of rabbit serum was kept constant by the addition of nonimmune serum. After incubation for 1 h at room temperature, 100 μL of goat antirabbit γ-globulin serum was added. After another 1-h incubation period, the immunoprecipitate was isolated through a sucrose pad as described by Palmiter (1973). (B) Precipitation of procollagen made in vitro with antisera to intact procollagen. Replicate translations of total calvarial RNA were terminated with the addition of 50 μL of serially diluted procollagen antiserum added to the translation stop mix. Nonimmune serum was added to keep the β-globulin concentration constant in all the tubes prior to the addition of the goat anti-rabbit IgG serum. The immunoprecipitates were collected as in A. (C) Precipitation of the [^{3}H]procollagen–anti-procollagen complex with goat anti-rabbit IgG. To replicate tubes containing [^{3}H]procollagen synthesized in organ culture, 125 μL of 0.7% Triton X-100 and 0.7% deoxycholate in PBS were added along with 50 μL of a 1/32 dilution of rabbit anti-pNα1 serum. After a 1-h incubation, varying amounts of goat anti-rabbit IgG serum were added and immunoprecipitates were collected as described in A.

N-ethylmaleimide and disrupted with a Polytron. After the insoluble material was removed by centrifugation at 13 000*g* for 5 min, the procollagen content was determined by collagenase digestion. Procollagen synthesis was expressed as a percentage of total protein synthesis after correcting for the 5.4-fold enrichment of proline in the collagenous regions of procollagen, as compared to average proteins (Peterkofsky and Diegelmann, 1971), and the contribution of the nonhelical regions of procollagen to the collagenase-resistant cpm (von der Mark et al., 1973; Murphy et al., 1975), viz:

$$\% \text{ procollagen} = \frac{\text{collagenase sensitive cpm} \times 100}{[5.4\ (\text{collagenase resistant cpm}) - 0.51(\text{collagenase sensitive cpm})]}$$

Results

The translation of procollagen mRNA was performed in a modified reticulocyte lysate and the product was characterized and quantitated by specific immunoprecipitation. The novel aspects of this method are described below.

Immunoprecipitation of Procollagen. Procollagen synthesis was measured by a double-antibody procedure. Antibodies prepared against type I procollagen or procollagen chains (pNα1) containing NH_2-terminal, but lacking COOH-terminal, extensions were titered against a [^{3}H]procollagen standard under conditions used in the translation mixture. Figure 1A shows that the rabbit antibody to pNα1 was capable of precipitating all of the [^{3}H]procollagen added to a nonradioactive translation mixture. The same dilution of antibody to either procollagen or pNα1 gave optimal recovery of the [^{3}H]procollagen synthesized in the reticulocyte lysate (Figure 1B). These antibodies did not precipitate procollagen in the absence of goat anti-rabbit IgG antiserum; the optimum concentration for the second antibody was determined as shown in Figure 1C.

Nonspecific radioactivity trapped in the immunoprecipitates was measured in a precipitate of similar size formed with rabbit antiovalbumin and goat anti-rabbit IgG or in a separate

TABLE I: Effect of Lysate Modifications on [^{3}H]Proline Incorporation into Procollagen.[a]

Lysate	Incorporation (cpm × 10^{-3}/125 μL) Total	Procollagen	Background	Procollagen/background
Unmodified	197.8	0.65	0.22	3.0
Nuclease treated	10.0	1.15	0.13	8.9
Nuclease and Sephadex treated	43.1	2.0	0.63	3.2
Nuclease and Sephadex treated (optimized)	54.2	5.3	0.58	9.1

[a] Each 125-μL reaction mixture containing 0.8 μg of total tendon RNA was incubated at 26 °C for 2 h. Procollagen was recovered by immunoprecipitation. The same preparation of reticulocyte lysate was used in all four test conditions.

TABLE II: Incorporation of Various Amino Acids into Procollagen with Modified Nuclease-Treated Lysate.[a]

Amino acid (Ci/mmol)	Total isotope (cpm × 10^{-7})	Total incorp (cpm × 10^{-5}) −mRNA	+mRNA	Procollagen (− background) (cpm × 10^{-4})	Procollagen as a % of mRNA-dependent protein synthesis (%)
[^{3}H]Pro (52)	2.08	1.03	9.25	11.10	13.5
[^{3}H]Ala (53)	1.55	2.10	6.40	5.20	12.1
[^{3}H]Gly (11)	3.92	0.40	2.39	4.66	23.4
[^{3}H]Glu (21)	5.33	1.55	4.15	4.40	16.9
[^{35}S]Met (?)	2.85	3.84	6.10	3.35	14.8
[^{3}H]Leu (62)	1.85	2.45	7.62	2.68	5.2
[^{3}H]Phe (54)	1.91	2.65	5.35	2.08	7.7

[a] Reaction mixtures of 250 μL were incubated for 2 h at 26 °C with or without 2.9 μg of sixfold enriched procollagen mRNA; procollagen was isolated by immunoprecipitation.

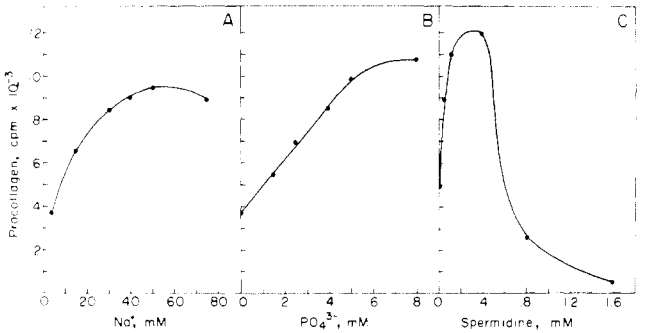

FIGURE 2: Optimization of procollagen mRNA translation in the modified reticulocyte lysate. Calvarial RNA (3 μg) was translated in 125-μL reaction mixtures containing 60 mM KCl, 1.75 mM magnesium acetate, and varying amounts of (A) NaCl, (B) KH_2PO_4 (pH 7.5), or (C) spermidine hydrochloride.

translation mixture to which oviduct RNA had been added and the product precipitated with antiprocollagen antibodies. The background was routinely determined by the latter method. Several different concentrations of oviduct RNA were translated in order to construct a curve relating background to total incorporation. The background fell from about 1.2% of total incorporation at low oviduct RNA concentrations to about 0.3% at the highest concentrations. Up to 30% of the total isotope incorporation was immunoprecipitable with tendon or calvarial RNA.

Optimization of the Lysate for Procollagen mRNA Translation. Table I summarizes the effect of various lysate conditions on the incorporation of [^{3}H]proline into procollagen. In the unmodified lysate, the level of incorporation was low due to competition of added procollagen mRNA with endogenous mRNAs and due to a high endogenous concentration of proline (about 50 μM). The endogenous mRNA was destroyed by *Staphylococcal* nuclease treatment as described by Pelham and Jackson (1976); this treatment not only increased total incorporation into procollagen but, more importantly, increased the sensitivity of the assay, since the background decreased. After the nuclease-treated lysate was passed through a Sephadex G-50 column, the proline concentration was reduced to 4–6 μM. This resulted in improved incorporation of proline into procollagen, although not to the extent that would be expected by the increase in isotopic specific activity. However, when the lysate was optimized (see below), the labeling of procollagen increased about fivefold compared to the nuclease-treated lysate, and the ratio of incorporation into procollagen to background was improved (Table I).

With the nuclease- and Sephadex-treated lysate, maximal incorporation of proline into procollagen occurred at 1.75 mM magnesium and 60 mM potassium (data not shown). Spermidine in a concentration range of 0.1–0.4 mM greatly improved procollagen synthesis (Figure 2C). The value of adding polyamines to the wheat germ system has been described (Hunter et al., 1977).

The activity of the lysate was potentiated by the presence of sodium and phosphate (Figure 2A,B). The maximal effect of sodium occurred at approximately 40 mM while that of phosphate was at 6 mM. In subsequent experiments, we found that diluting the lysate with an equal volume of PBS had the same effect on procollagen synthesis as did the same concentration of sodium and phosphate in the full-strength lysate. Because of the above experiments, all subsequent procollagen translations were performed with the nuclease- and Sephadex-treated lysates that had been diluted with an equal volume of PBS. Table II shows the extent of incorporation of several different amino acids into procollagen using this modified reticulocyte lysate system. [^{3}H]Proline gave the best overall incorporation with the lowest background, but the specific activity per residue was higher with [^{35}S]methionine or [^{3}H]phenylalanine [see Monson et al. (1975) for amino acid composition of procollagen].

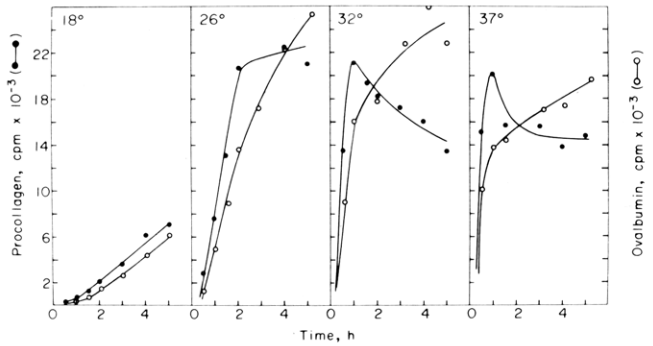

FIGURE 3: Effect of the incubation time and temperature on the translation of procollagen and ovalbumin mRNA. Replicate translation mixtures containing 3.0 μg of calvarial RNA and 1.0 μg of oviduct RNA were incubated under optimal conditions for several times at temperatures shown. All the samples were assayed simultaneously for the procollagen (●-●) and ovalbumin (O-O) content by immunoprecipitation.

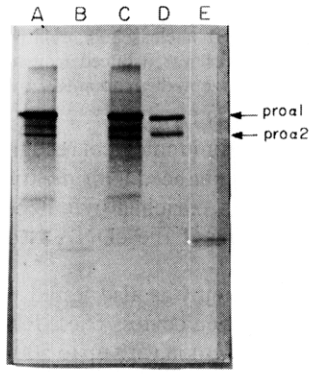

FIGURE 4: Identification of the procollagen made in the reticulocyte lysate. Immunoprecipitates from translation of calvarial RNA or calvarial [^{3}H]procollagen labeled in organ culture were analyzed by acrylamide gel electrophoresis. The radioactivity was identified by fluorography. Antibody to intact procollagen was used for all samples. Track A: Immunoprecipitated cell-free product translation (20 000 cpm). Track B: Immunoprecipitated cell-free translation that was digested with purified bacterial collagenase. Track C: A sample treated identically as in B except that bacterial collagenase was omitted from the incubation medium. Track D: Immunoprecipitated ^{3}H-labeled procollagen synthesized in organ culture (30 000 cpm). Track E: Immunoprecipitated ^{3}H-labeled procollagen synthesized in organ culture and treated with bacterial collagenase.

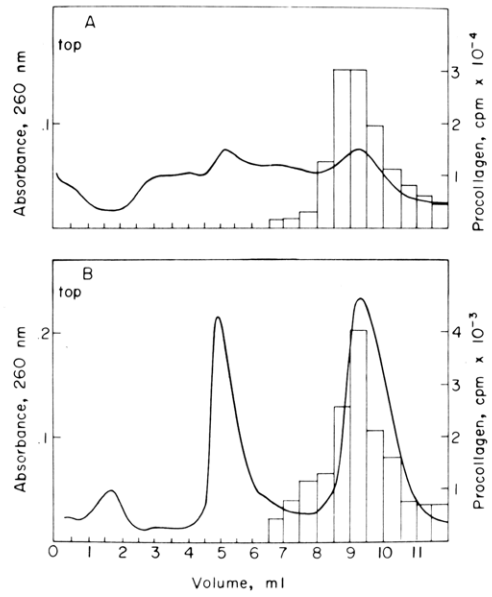

FIGURE 5: Identification of procollagen mRNA activity in a sucrose gradient profile. RNA samples were centrifuged as described under Experimental Procedures. Aliquots (25 μL) from the gradient fractions were assayed directly for procollagen mRNA activity. The solid line represents the A_{254} profile of the gradient, while the histogram expresses the radioactivity of the procollagen immunoprecipitate from each fraction. (A) Tendon mRNA purified by two passages through oligo(dT)-cellulose (1 A_{260} unit). (B) Total RNA from chick embryo fibroblasts (5 A_{260} units).

The optimum time and temperature for the synthesis of procollagen in vitro was 2 h at 26 °C (Figure 3). Under these conditions, the synthesis of procollagen was linear and loss of procollagen due to degradation was not observed. At 15 °C, degradation was not observed but total incorporation was much less than at 26 °C. At 32 and 37 °C, the formation of procollagen was more rapid than at 26 °C, but the total incorporation did not exceed that obtained at 26 °C for 2 h. Furthermore, prolonged incubation at the elevated temperatures resulted in significant loss of procollagen, presumably due to degradation. In contrast to procollagen, loss of radioactive ovalbumin was not observed at the higher temperatures and net accumulation continued up to 5 h of incubation, although not in a linear manner (Figure 3).

The Assay for Procollagen mRNA. The incorporation of proline into procollagen was linear with the amount of total RNA added up to 20 μg/mL with tendon RNA, up to 15 μg/mL with calvarial RNA, and up to 8 μg/mL with chick embryo fibroblast RNA. To quantitate procollagen mRNA, five aliquots of total RNA ranging from 0.5 to 5 μg were incubated for 2 h at 26 °C in the translation mixture; the points which fell within the linear range of the assay were used to calculate the amount of procollagen synthesized per microgram of RNA added to the reaction mixture.

Characterization of the Translation Products. Procollagen made by translation in vitro or by intact calvaria was immunoprecipitated, dissolved in a sodium dodecyl sulfate buffer, and subjected to electrophoresis on 5% acrylamide gels (Figure 4). The major band precipitated by the antiprocollagen antibody (track A) migrated slightly slower than proα1 chains made by intact calvaria (track D). Bands of smaller size, but still larger than collagen α chains, were present in the immunoprecipitate; we have not determined whether these bands are incomplete proα1 or proα2 chains. However, many of the smaller bands were not present when antisera specific for the $p_N\alpha$1 chain were used (data not shown), suggesting that some of these bands are related to proα2. All of the bands from the in vitro synthesized products were degraded by purified bacterial collagenase (track B), but the bands were unchanged by incubation in the absence of collagenase (track C). Amino acid analysis of procollagen made in the lysate failed to show the presence of hydroxyproline, indicating the absence of prolyl hydroxylase activity in the reticulocyte lysate.

Partial Purification of Procollagen mRNA. mRNA was purified from total tendon RNA by two passages through oligo(dT)-cellulose. It was then heated to 60 °C for 5 min and fractionated on a 5–20% sucrose gradient. The procollagen mRNA activity was determined by direct translation of aliquots from the gradient fractions. Procollagen mRNA activity appeared as a single peak of activity in the 27S region of the gradient (Figure 5A). The procollagen mRNA in total RNA derived from chick embryo fibroblasts eluted in a similar position (Figure 5B). Table III summarizes the purification of procollagen mRNA activity after oligo(dT)-cellulose and sucrose gradient fractionation.

Preparation and Characterization of Procollagen cDNA.

TABLE III: Partial Purification of Procollagen mRNA from Chick Tendon.[a]

Purification step	RNA assayed (μg)	Procollagen (cpm)	Sp act. (cpm/μg)
Total RNA	1.54	13 700	8 900
Oligo(dT)	0.20	16 700	83 500
Oligo(dT) and sucrose gradient	0.23	30 500	132 600

[a] mRNA was translated as indicated under Experimental Procedures.

The peak fractions of procollagen mRNA activity from a sucrose gradient such as that shown in Figure 5A were pooled and used as a template for reverse transcriptase. The resulting cDNA preparation was fractionated further to remove double-stranded DNA and DNA complementary to mRNA species present in low abundance (see Experimental Procedures). These procedures yielded a cDNA preparation that hybridized to about 75% in the presence of tendon RNA (Figure 6) and gave negligible background in the absence of RNA.

To estimate the abundance of the mRNAs that hybridize with this cDNA, its hybridization with tendon RNA was compared with the hybridization of pure ovalbumin cDNA with hen oviduct RNA.[2] This comparison suggests that the mRNAs to which presumptive procollagen cDNA hybridizes are present at about 15 000 molecules per tendon cell. Considering that this mRNA is 27S and has on the order of 5000 bases, 15 000 molecules represents about 40 ng/1 000 000 cells with a total RNA content of about 6000 ng; thus, it represents about 0.67% of total RNA. (The entire mRNA population of a cell typically represents 1–2% of total cellular RNA.) The only mRNA of this size and abundance in tendon cells would be procollagen mRNA.

To establish further that this cDNA measures procollagen mRNA, procollagen-synthesizing polysomes were enriched by immunoprecipitation, and the enrichment of sequences complementary to the cDNA was determined. Polysomes were isolated from chick fibroblasts that were synthesizing procollagen at about 20% of total protein synthesis. They were then incubated with anti-$p_N\alpha 1$ IgG followed by an excess of goat anti-rabbit IgG essentially as described by Shapiro et al. (1974). RNA was extracted from an aliquot of the sample just before centrifugation of the immunoprecipitate and from the pellet after centrifugation. The pellet was 2.5-fold enriched in sequences that hybridized with this presumptive collagen cDNA when compared to unfractionated material. Since this

[2] The presumptive procollagen cDNA gave half-maximal hybridization with 25 ng of total tendon RNA under standard conditions, compared to 6 ng of hen oviduct RNA required for the same extent hybridization with pure ovalbumin cDNA under the same conditions. Both cDNAs were of similar size (~900 bases) and were labeled with dCTP of the same specific activity. Thus, the difference in the amount of RNA required to achieve the same extent hybridization is directly proportional to the molecular abundance of the respective mRNAs. The concentration of ovalbumin mRNA in laying hens has been measured by several groups to be in the range of 75 000–150 000 molecules/cell. The RNA content of hen oviduct cells is about 4 μg/μg of DNA compared to about 2.5 μg of RNA/μg of DNA in tendon. Thus, the concentration of mRNAs in tendon that hybridize with procollagen cDNA is approximately 15 000 molecules/tendon cell based on an average ovalbumin mRNA content of 100 000 molecules/cell [(6/25) × (2.5/4) × 100 000 = 15 000]. This number is approximate, in view of the assumptions made and the possible errors in measurements, but it is likely to be within a factor of 2 of the real value.

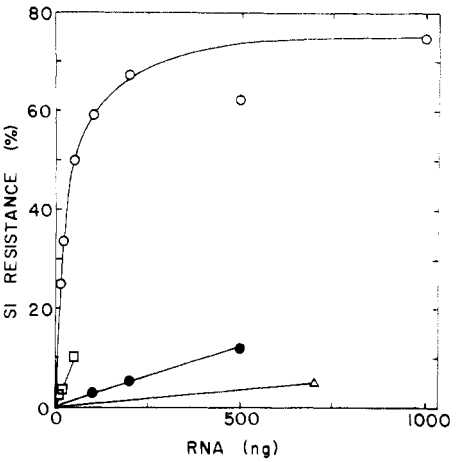

FIGURE 6: Titration of procollagen mRNA sequences with cDNA. RNA from embryonic chick tendons (○), fibroblasts grown in culture to low density (□), transformed fibroblasts grown to low density (●), and reticulocytes (△) were hybridized for 24 h with procollagen cDNA as described under Experimental Procedures. The extent of hybridization was determined by resistance to S1 nuclease. The relative concentration of procollagen mRNA sequences was deduced from the horizontal displacement of the curves relative to the tendon RNA standard.

method relies solely on immunological recognition of nascent procollagen chains, the enhanced hybridization of the cDNA prepared against mRNA enriched by independent methods implies that at least half of the cDNA is directed against procollagen mRNA.

This cDNA preparation was also tested with RNA from chick liver, brain, and reticulocytes. These RNA preparations gave less than 3% hybridization with up to 500 ng of RNA (the maximum range used to measure procollagen mRNA in all fibroblastic RNA preparations), with much higher concentrations of RNA, significant hybridization was achieved; e.g., 3.5 μg of reticulocyte RNA protected 7% of the cDNA, 11 μg of brain RNA protected 17% of the cDNA, and 9 μg of liver RNA protected 26% of the cDNA against S1 nuclease. The values obtained with brain and liver may reflect a low level of procollagen mRNA in these organs, contributed by resident fibroblasts, as well as hybridization of rare cDNA sequences. Since reticulocytes are not thought to synthesize procollagen, the 7% hybridization in this case is presumably all due to cDNA sequences other than procollagen cDNA.

All lines of evidence indicate that this cDNA preparation is predominantly composed of procollagen cDNA, although a low level of sequences complementary to mRNAs present in low abundance is probably inevitable, considering the method of its preparation. Nevertheless, the results below indicate that hybridization with this cDNA preparation parallels procollagen mRNA activity when RNAs from tissues with over a 50-fold range of procollagen-synthesizing activity are compared.

Correlation of Collagen Synthesis with Procollagen mRNA Levels. Previous investigators have found that freshly isolated tendon fibroblasts direct 60% of their synthetic effort into collagen production (Dehm and Prockop, 1971), whereas fibroblasts in culture make only 5–10% collagen (Peterkofsky, 1972). This percentage may depend on the number of divisions the cells have undergone in culture and their degree of confluence. To determine the stability of the rate of procollagen synthesis and of procollagen mRNA levels during culture, freshly isolated cells were plated at varying densities and grown to early confluence. Replicate cultures were then pulse-labeled with [^{3}H]proline to measure the rate of procollagen synthesis. Figure 7 shows that the relative rate of procollagen synthesis

TABLE IV: Characterization of the RNA Content and the Synthesis of Procollagen and Total Protein in Normal and RSV-Transformed Chick Fibroblasts.[a]

	Tendon fibroblasts		Transformed fibroblasts	
	Low density	High density	Low density	High density
Total DNA (μg/plate)[b]	28	45	23	148
RNA/DNA	4.2	3.0	4.5	2.4
Total proline incorporated (cpm × 10^{-3}/μg of DNA)	1.96	1.55	2.78	2.60
Collagenase-sensitive proline incorporated (cpm × 10^{-3}/μg of DNA)	1.35	0.87	0.15	0.10

[a] Replicate fibroblast cultures were either extracted to measure total nucleic acids or labeled with [^{3}H]proline. DNA, RNA, collagen, and total protein determination were performed as described under Experimental Procedures. [b] 100 μg of DNA/plate is equivalent to 5 × 10^5 cells/cm^2.

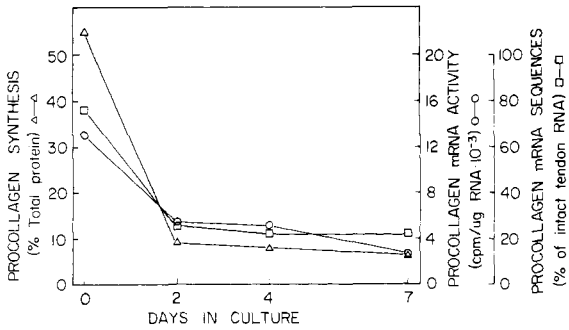

FIGURE 7: Comparison of the rate of procollagen synthesis with the amount of procollagen mRNA after growth of chick tendon fibroblasts in tissue culture for varying periods of time. The rate of procollagen synthesis (Δ–Δ) was determined as described under Experimental Procedures. The amount of mRNA was determined by cell-free translation (O–O) and by hybridization (□–□) as described under Experimental Procedures.

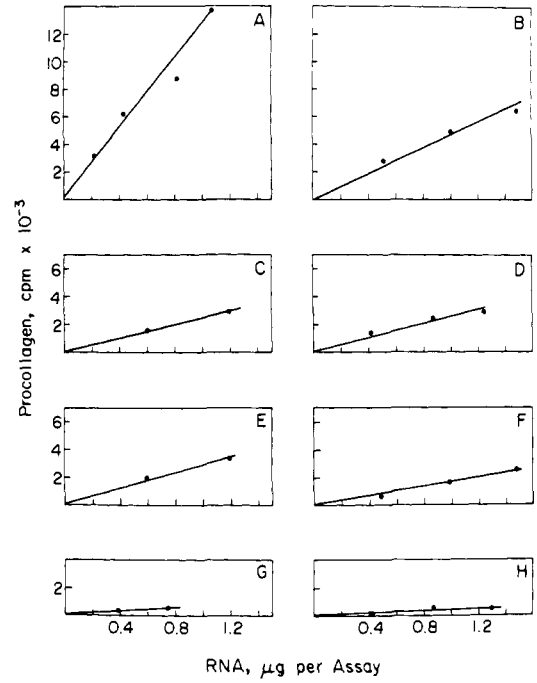

FIGURE 8: Procollagen mRNA activity determined by translation of RNA samples described in Figure 7 and Table V. The slope of the curve was used to determine the procollagen mRNA activity. Panels A–D: RNA from tendon fibroblasts cultured for 0, 2, 4 and 7 days, respectively. Panels E–H: the procollagen mRNA activity in low- and high-density normal (E, F) and RSV-transformed (G, H) fibroblasts.

fell fivefold within 2 days and established a new steady-state level that was maintained during the next 5 days (3–5 population doublings). Cells that were grown for 3 weeks and three passages (12–15 doublings) had a similar low level of procollagen synthesis.

The decline in procollagen synthesis in cultured tendon fibroblasts could be due to either a decreased translational efficiency of procollagen mRNA, an inactivation of procollagen mRNA, or a loss of mRNA sequences. To distinguish among these possibilities, RNA was isolated from half of the cultures at each time point and then translated in the reticulocyte lysate and hybridized with procollagen cDNA. Figure 8A–D illustrates the assay of procollagen mRNA activity with RNA from tendon fibroblasts after 0, 2, 4, and 7 days in culture. Those points in the linear range of the assay were used to calculate the relative procollagen mRNA activity. Figure 7 shows these data along with that obtained by hybridization. Both assays revealed a decline in procollagen mRNA levels that paralleled the decline in procollagen synthesis, suggesting that procollagen synthesis is a function of mRNA levels rather than translational control.

Several authors have reported that the relative rate of procollagen synthesis falls even further when chick fibroblasts are transformed with RSV (Levinson et al., 1975; Kamine and Rubin, 1977). To investigate the mechanism of this further decline in cell specialization, fibroblasts that were transformed by RSV (Prague C strain) and control tendon fibroblasts were plated at equal density and grown to either low or high density, at which time the relative rate of procollagen synthesis was measured and RNA was isolated for both mRNA translation and hybridization with procollagen cDNA.

Table IV shows that the amount of RNA in control and transformed cells (RNA/DNA) was similar and that high density is associated with a reduction in the cellular RNA content as well as with a small reduction in total protein synthesis. The level of total protein synthesis per cell is somewhat higher in the transformed cells. Procollagen synthesis was greatly reduced in the transformed cells and was also reduced in the high-density nontransformed cells, in agreement with previous results (see introduction section).

Table V shows the correlation of procollagen synthesis after these treatments with the level of procollagen mRNA measured by translation or hybridization. The values for functional (translatable) procollagen mRNA are derived from the assays shown in Figure 8E–H, while representative titrations of procollagen mRNA sequences are shown in Figure 6. The values for procollagen synthesis or mRNA levels are related to those of intact tendon for convenience. In all cases, the fall in procollagen synthesis, whether due to increased cell density or transformation, was associated with a reduction in procollagen mRNA levels measured by either assay. In these ex-

TABLE V: Relative Rates of Procollagen Synthesis and Content of Procollagen mRNA Activity and Procollagen mRNA Sequences in Normal and Transformed Chick Fibroblasts.[a]

Cell source	Procollagen synthesis		Procollagen mRNA		
			Translation		Hybridization with cDNA
	% total	Relative to intact tendon	Total cpm/μg of RNA	Relative to intact tendon	relative to intact tendon
Intact tendon	50	100	10 300	100	100
Tendon fibroblasts, low density	34	68	3 040	30	45
Tendon fibroblasts, high density	21	42	1 940	19	29
Transformed fibroblasts, low density	1	2	770	7.5	6.5
Transformed fibroblasts, high density	1	2	180	1.8	3.0

[a] Replicate cultures were either labeled with [^{3}H]proline to determine the percentage of procollagen synthesis or used to isolate RNA as described under Experimental Procedures. Procollagen mRNA activity was measured as shown in Figure 8. Hybridization with cDNA was performed as described under Experimental Procedures and in the text.

periments, fibroblast transformation produced a 20- to 30-fold reduction in procollagen synthesis, and this was associated with a four- to tenfold decrease in procollagen mRNA levels (Table V). The effect of transformation on functional procollagen mRNA is in agreement with recent observations by Adams et al. (1977). The present study shows that, in addition, the mRNA sequences are significantly decreased, indicating that the loss of functional mRNA is not due to covalent modification of the mRNA, rendering it untranslatable, or to selective degradation of procollagen mRNA during the isolation procedure.

The observation that the relative rate of procollagen synthesis decreased more than procollagen mRNA levels may be an indication of a lower translational efficiency of procollagen mRNA in transformed fibroblasts compared to controls, in addition to a lower steady-state level of mRNA. Alternatively, the difference may be a function of the way the data are expressed. For example, the mRNA levels are measured relative to total RNA, whereas synthetic rates are a function of only polysomal RNA. The ratio of total mRNA (including procollagen mRNA) to rRNA may increase after transformation and would be reflected as an increased ratio of polysomes to monoribosomes. Such a change would be consistent with the increased ratio of protein synthesis to cellular RNA shown in Table IV. At a more technical level, each of these assays is subject to different experimental errors which undoubtedly become exaggerated when the level of procollagen synthesis (mRNA) falls to very low levels as observed after viral transformation. In the hybridization assay, for example, we have not subtracted the low level of hybridization to reticulocyte RNA, although this is probably appropriate. To do so would lower the estimates of procollagen mRNA sequences in transformed cells into a range comparable to the rate of procollagen synthesis in those cells.

Discussion

The development of a reliable cell-free translation system is invaluable for studying the control of specific mRNA levels. There are two major problems in developing such a system. One is the isolation of undegraded RNA, and the other is optimizing mRNA translation. Both are facilitated by a fast and easy method of specific product identification. Because of its large size, the isolation of intact procollagen mRNA and full-length translation products from it has been relatively difficult. Recent studies by Benveniste et al. (1976) and Boedtker et al. (1976) demonstrate considerable mastery of these problems.

We have employed a different combination of techniques than previously used for procollagen mRNA which has all the characteristics required for a quantitative assay of mRNA levels. Although we have isolated some active procollagen mRNA by a variety of standard RNA isolation schemes, the most reliable method has involved homogenization of tissue in warm sodium dodecyl sulfate plus proteinase K, essentially as described by Hilz et al. (1975). This method is easily adapted to cells in culture and to soft or even partially calcified tissues such as embryonic calvaria. When a low-concentration homogenate (30–50 mg/mL) is prepared, the interface during subsequent phenol extraction is negligible, thus giving rise to an excellent recovery of total nucleic acid. Of even more importance, the nucleic acids are undegraded. The most convincing demonstration of the control of RNase by these procedures is the results of experiments in which ovalbumin mRNA was added to the homogenizing medium used to prepare tendon or calvarial RNA. In seven different preparations, the recovery of functional (translatable) ovalbumin mRNA was ~90% (range 70–100%).

Synthesis of procollagen in the modified reticulocyte lysate system was linear for 2 h at 26 °C. Higher temperatures increased the initial rate of translation, but the recovery of procollagen fell with prolonged incubation at 30–37 °C. This loss is probably due to preferential degradation, since a similar loss of ovalbumin synthesized in the same reaction mixture was not observed (Figure 3). Perhaps this degradation is related to the lack of normal triple helix formation of the unhydroxylated procollagen molecules. A protease in the reticulocyte lysate that degrades abnormal proteins has recently been described (Etlinger and Goldberg, 1977).

The reliability of the immunoprecipitation assay was checked in several ways. First, two different antisera were used—one was predominantly directed against the NH_2 terminus of proα1 (I) chains while the other was against procollagen. The specificity of these antisera has been documented elsewhere (von der Mark et al., 1973; Nist et al., 1975). Second, the major immunoprecipitated product of translation migrated close to the position of proα1 chains isolated from cultured cells when subjected to polyacrylamide gel electrophoresis. Since procollagen chains that are not glycosylated and hydroxylated migrate slightly faster than those with these modifications (unpublished observations), it is likely that the

product isolated from the reticulocyte lysate is slightly larger than the in vivo product. The product isolated from the lysate can be labeled with initiator fMet-tRNA and gives a unique sequence that is rich in hydrophobic amino acids (unpublished observations), indicating that it represents the primary translation product and probably has a NH_2-terminal extension that may function as a "signal peptide" in procollagen transport into the endoplasmic reticulum (Blobel and Dobberstein, 1975; Palmiter et al., 1977). Third, the polypeptides that migrate with procollagen are completely digested by prior treatment with bacterial collagenase. There are some smaller peptides observed on electrophoresis of the translation product, and the percentage of total protein synthesis that is procollagen in the translation assays is lower than that in vivo; however, these problems most likely relate to inefficient translation of large mRNAs and premature ribosome termination in vitro.

We prepared a cDNA probe, enriched in sequences complementary to procollagen mRNA, as an added control for differential procollagen mRNA degradation during RNA isolation and translation and to test for procollagen mRNA inactivation as a mechanism of modulating procollagen synthesis. This cDNA was prepared against oligo(dT)-cellulose-purified tendon RNA that sedimented at 27S and had maximal procollagen synthetic activity. There are undoubtedly other mRNAs in tendon cells that sediment at 27S; however, since procollagen mRNA represents the majority of total mRNA (~60% by weight, assuming equal translation of all mRNAs in vivo), it is unlikely that they represent a substantial fraction. We further selected against the very rare cDNA species by back-hybridization. Hybridization of RNA from total and immunoprecipitated polysomes demonstrates that at least 50% of the cDNA was against procollagen mRNA (since the mRNA was enriched 2.5-fold, whereas the maximum expected was about fivefold). From the titration curves of this cDNA with tendon RNA, we estimate that the predominant cDNA species reacts with a mRNA that is present at a concentration of approximately 15 000 molecules per cell.[2]

cDNA to procollagen mRNA will hybridize to a limited extent with RNA from tissues not thought to be synthesizing procollagen, but much higher concentrations of RNA are required to achieve significant hybridization. In the range of RNA concentrations used for fibroblast RNA, less than 3% of the cDNA was hybridized with liver, brain, or reticulocyte RNA. The RNAs that give rise to this hybridization are present at less than 30 molecules per cell. Since we wanted to use a cDNA probe to measure the relative concentration of procollagen mRNA, estimated to be in the range of 15 000 molecules/cell in tendon cells, and perhaps falling one or two orders of magnitude upon viral transformation, the characteristics of the cDNA seem adequate for this purpose.

We used the procollagen mRNA translation and hybridization assays to explore two aspects of the regulation of procollagen synthesis. First, we analyzed the drop in procollagen synthesis when highly differentiated tendon cells are dispersed and grown in culture. Then we studied the mechanisms responsible for the decline in procollagen synthesis upon transformation by RSV. Several possible mechanisms for these events were considered. First, the reduction in procollagen synthesis could reflect a decrease in translational efficiency of procollagen mRNA due to a covalent modification of the mRNA, e.g., removal of a 7-methylguanosine cap structure. If this were true, then the mRNA sequences would still be present and they would be detected even if the mRNA were untranslatable. Second, procollagen synthesis might be inhibited in a noncovalent manner by mRNA-specific translation factors (Heywood et al., 1974). Such inhibitors would probably be removed during mRNA isolation. In this case, we would expect both the translation and hybridization assays to show little change in procollagen mRNA levels during the course of tissue culture or cell transformation. Third, the drop in procollagen synthesis may reflect a decline in procollagen mRNA levels, in which case the relative rate of procollagen synthesis would correlate with procollagen mRNA levels measured by either assay. The data are most consistent with the latter hypothesis and are in agreement with the recent observations of Adams et al. (1977). However, we cannot rule out smaller contributing effects by either of the other mechanisms.

How procollagen mRNA levels are regulated is an open question. It seems most likely that the cellular environment and the *src* gene product from transforming viruses impinge upon the regulation of mRNA synthesis, degradation, or both to bring about a new steady-state level of mRNA. However, it is important to keep in mind that these cells are rapidly growing in culture, and procollagen mRNA levels reflect the average of the population. Within the tendon, a population of cells with high rates of procollagen synthesis, but with limited potential for proliferation, may exist; after dispersal of these cells in culture, cells with lower levels of procollagen synthesis, but higher rates of proliferation, may come to predominate. In a similar way, the level of procollagen synthesis in transformed cells may also reflect the emergence of a cell population with a low level of procollagen synthesis and a high proliferative potential. It is difficult, at present, to distinguish between these alternatives.

In any event, the results described in this paper indicate that translational control is unlikely to represent the major mechanism by which procollagen synthesis is regulated. Modulation of collagen production by cells in culture is known to occur as a function of ascorbate levels (Peterkofsky, 1972; Schwartz and Bissell, 1977) and serum and electrolyte concentration (Schwartz et al., 1976; Koch et al., 1977) in addition to the effects of cell density and viral transformation discussed in this paper. Genetic disorders of collagen metabolism in which the synthesis of specific collagen types is disturbed, perhaps in analogy with the hemoglobin thalassemias, are also known (Bornstein and Byers, 1978). The applications of the techniques described here in the study of the regulation of procollagen synthesis in such systems are therefore likely to lead to useful new information.

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