Isolation and Translation of Calvaria Procollagen Messenger Ribonucleic Acids†

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ABSTRACT: Procollagen messenger ribonucleic acid (mRNA) was isolated from the calvaria of 15-day-old chick embryos by chromatographing total RNA over oligo(dT)-cellulose two times, and then fractionating the twice-bound RNA on 85% Me_2SO/0–20% sucrose gradients. When analyzed on 99% formamide gels, the 27–30S fraction had three sharp fluorescent bands, one corresponding to 27S ribosomal RNA (rRNA), the others having mobilities lower than 27 S corresponding to molecular weights of 1 700 000 and 1 800 000. In wheat-germ, cell-free extracts, the 27–30S fraction directed the synthesis of two prominent collagenase sensitive polypeptides with mobilities corresponding to the calvaria pro-α chain markers. Twelve percent of this [^{3}H]proline-labeled, wheat-germ product could be hydroxylated with prolyl hydroxylase.

The isolation of messenger RNAs for specific proteins provides a tool to elucidate the regulation of protein synthesis in eucaryotes. The messenger RNA or the DNA complementary to it can serve as a probe to study the control of both transcription and translation. The isolation of messenger RNAs for the different collagen pro-α chains deserves special priority in view of its dominant role in a wide variety of structures. There are at least four distinct types of collagen expressed in different tissues of the same organism, or the same tissue at different developmental stages (Gross, 1973; Miller and Matukas, 1974). Clearly an understanding of the control of the expression of these collagen genes is central to the understanding of both normal and abnormal development.

Procollagen mRNA activity in various translation systems has been detected in a number of RNA preparations: chick embryo calvaria (Benveniste et al., 1973, 1974, 1976; Boedtker et al., 1974), chick embryo tendons and cartilage (Harwood et al., 1975), and from polysomes prepared from whole chick embryos (Wang et al., 1975; Neufang et al., 1975). But thus far the isolation of purified procollagen mRNAs has not yet been achieved. We report here the initial purification of microgram amounts of type I procollagen mRNAs having an estimated purity of 30% with 27S[1] rRNA being the only prominent contaminant. This was accomplished by two cycles of oligo(dT)-cellulose chromatography followed by size fractionation on fully denaturing 85% Me_2SO[2]–sucrose gradients.

Materials and Methods

Preparation of Total RNA from Embryonic Chick Calvaria. Calvaria from 15-day-old chick embryos were extracted as described previously (Boedtker et al., 1974) with the following modifications. All buffers were filtered and treated with diethyl pyrocarbonate (100 ppm), autoclaved, and stored frozen. The hot phenol-$CHCl_3$–isoamyl alcohol extraction was followed by a room temperature extraction of the aqueous phase with $CHCl_3$–isoamyl alcohol. Before pelleting through a 2.5-ml cushion of 6.1 M CsCl, the RNA–DNA mixture was dissolved in 1% recrystallized Sarkosyl (ICN, K & K Laboratories, Inc., Plainview, N.Y.)–0.01 M Na_2EDTA (pH 7.0) because of the insolubility of $CsDodSO_4$. Mineral oil instead of buffer was used to fill the tube.

Oligo(dT)-Cellulose Chromatography. Oligo(dT)-cellulose (T-2) was purchased from Collaborative Research Inc., Waltham, Mass. Chromatography was carried out as described by Aviv and Leder (1972), except that the RNA was first dissolved in sterile deionized water, heated to 65 °C for 1 min, chilled rapidly to room temperature, and then combined with an equal volume of two times binding buffer. After binding, the column was first washed with 0.1 M KCl– 0.01 M Tris-HCl, pH 7.6, and then the bound RNA was eluted with distilled deionized water. The bound RNA was applied to a smaller column and the RNA that rebound pooled.

Polyacrylamide Gel Electrophoresis under Denaturing Conditions. RNA samples were analyzed on 3% polyacrylamide–0.6% bisacrylamide gels in 99% formamide, a modification of the procedure described by Pinder et al. (1974). To ensure reproducible RNA mobilities, formamide was purified by stirring with 40 g/l. mixed bed resin (Bio-Rad AG 501-X8, 20–50 mesh) and 8 g/l. activated charcoal until the conductivity dropped to 70 $\mu\Omega^{-1}$. After filtering, the formamide was distilled at 200 mTorr and then stored at −20 °C in 50-ml aliquots. Electrophoresis was carried out for 4 h at 2.75 mA per tube in a buffer consisting of 0.01 M Na_2HPO_4 and 0.01 M NaH_2PO_4. After electrophoresis the gels were stained with ethidium bromide (1 μg/ml) in 0.1 M ammonium acetate (Bailey and Davidson, 1976) and the fluorescent bands photographed under an ultraviolet light.

Size Fractionation on Denaturing Sucrose Gradients. Calvaria RNA was fractionated either on 70% formamide, 4–20% sucrose gradients following the procedure described by Suzuki et al. (1972), or on 85% Me_2SO/0–20% sucrose gradients (Scott, 1976). Me_2SO (85%) was prepared by adding 15 volumes of 0.05 M Tris-HCl, 5 mM Na_2EDTA, pH 7.6, to 85 volumes of Me_2SO (Matheson, Coleman and Bell, spec-

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[1] The large chick ribosomal RNA has been "renamed" 27 S rather than 28 S previously used because its molecular weight is 1 500 000 and corresponds to a sedimentation constant of 27 S; 28 S should be reserved for the large mammalian rRNA which has a molecular weight of 1 650 000.

[2] Abbreviations used: mRNA, messenger ribonucleic acid; Me_2SO, dimethyl sulfoxide; Na_2EDTA, disodium ethylenediaminetetraacetate; Sarkosyl, sodium *N*-lauroylsarcosinate; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, TLC, thin-layer chromatography; Tes, *N*-tris)hydroxymethyl)methyl-2-aminoethanesulfonic acid.

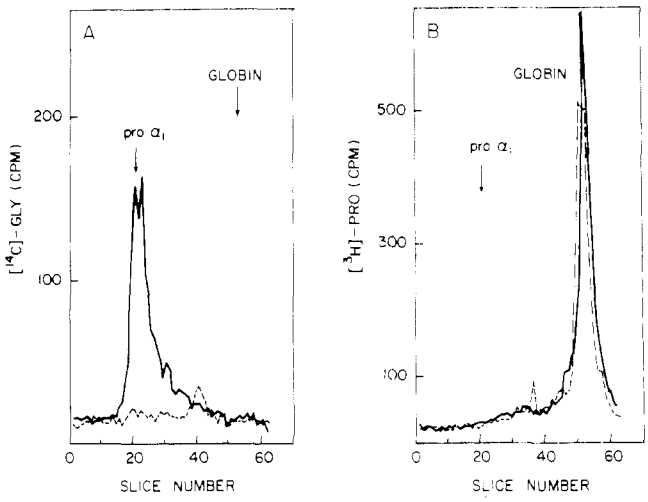

FIGURE 1: Comparison of the susceptibility of calvaria procollagen and rabbit globin to collagenase digestion. A mixture of [^{14}C]glycine in vivo labeled procollagen and [^{3}H]proline globin labeled in vitro in a reticulocyte lysate was digested with collagenase as described in Materials and Methods. Both the enzyme-treated and untreated mixtures were electrophoresed on 5% polyacrylamide gels in 1% $NaDodSO_4$ for 4 h and the gels sliced and counted as described previously (Boedtker et al., 1974). (A) Radioactivity profile of [^{14}C]glycine-labeled calvaria procollagen before (solid line) and after (dashed line) incubation with collagenase. (B) Radioactivity profile of [^{3}H]proline-labeled rabbit globin before (solid line) and after (dashed line) incubation with collagenase. The direction of migration is from left to right. The ^{14}C and ^{3}H radioactivity profiles are shown in separate panels for clarity.

troquality) titrated to pH 7.6. RNA was dissolved in 50 μl of 0.05 M Tris-HCl, 5 mM Na_2EDTA, pH 7.6, 200 μl of 85% Me_2SO added, and the solution heated at 65 °C for 1 min. After rapid cooling to 23 °C, the solution was layered on preformed, 0–20% sucrose gradients in polyallomer tubes. The latter were centrifuged for 26 h at 50 000 rpm in a Beckman SW56 Ti rotor at 23 °C. Fractions (0.1-ml) were collected and diluted with 1 ml of sterile distilled water, and A_{260} values were determined in a Gilford 2400S spectrophotometer.

Assay for in Vitro Synthesis of Procollagen. Preparation of Wheat-Germ, Cell-Free Extract. Wheat germ was obtained from the Pillsbury Co. and the S-30 prepared according to the procedure of Roberts and Paterson (1973). The homogenate was centrifuged at 23 000*g* rather than 30 000*g*. The wheat-germ extract was recentrifuged prior to use.

Protein Synthesis Assays. Reaction mixtures (100 μl) contained 20 μl of wheat-germ S-30, 34 mM Hepes, pH 7.6, 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 20 μg/ml creatine phosphokinase (Calbiochem), 20 μM amino acids except proline, 0.3–8 μg of RNA, 5 μCi of [^{3}H]proline (26 Ci/mmol), 64 mM KCl, 2.2 mM magnesium acetate, and 40 μM spermine (Sigma). The last three components were optimized.

The reaction mixture was incubated at 24 °C for 3 h. At the end of the reaction, a 5-μl aliquot was spotted on Whatman 3MM paper, treated with 2 drops of concentrated ammonia, and was then immersed four times in 5% cold Cl_3CCOOH, and finally cold ethanol, each for 10 min. The filters were then dried and counted in ScintiLene (Fisher Scientific Co.).

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Five microliters of 20% $NaDodSO_4$ and 0.5 μl of mercaptoethanol were added to the reaction mixture, and the mixture was heated at 80 °C for 10 min. After cooling, 10 μl of 0.05% bromphenol blue in glycerol was added and the sample applied to a 7.5% polyacrylamide slab gel with a 5% stacking gel as described by Studier (1973) in the buffer system of Laemmli (1970). Gels were fluorographed according to the method of Bonner and Laskey (1974) using preflashed x-ray films (Laskey and Mills, 1975).

Collagenase Digestion of Wheat-Germ Product. The specificity of the clostridial collagenase used was first tested by its inability to solubilize *Escherichia coli* proteins labeled with [^{3}H]proline to a specific activity of 1.1×10^6 cpm/mg (Schwarz et al., 1976) and by its inability to affect [^{3}H]proline-labeled rabbit globin under conditions that eliminate [^{14}C]glycine-labeled calvaria procollagen. The radioactivity profile of a $NaDodSO_4$–polyacrylamide gel of both proteins before and after collagenase treatment is shown in Figure 1. Calvaria procollagen is completely degraded by collagenase while the mobility of globin remains unaltered.

To determine the collagenase sensitivity of the wheat germ product, the incubation mixture was divided in half, and 5 μl of 0.05 M Tes (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), pH 7.5, and 5 mM $CaCl_2$ were added to both. One microliter of collagenase (17 μg/ml) was added to one and then both were incubated at 37 °C for 30 min.

Hydroxylation of Wheat-Germ Product. Prolyl hydroxylase was prepared by the method of Peterkofsky and DiBlasio (1975) and the hydroxylation carried out using a modification of their assay. A final volume of 1 ml contained 100 μl of wheat-germ reaction mixture, 0.2 ml of prolyl hydroxylase, and 50 mM Tris-HCl, 70 μM $Fe(NH_4)_2(SO_4)_2$ 0.6 mM α-ketoglutarate, 2 mM ascorbic acid, 0.3 mM dithiothreitol, 0.1 mg/ml beef liver catalase (Sigma) and the solution was incubated at 37 °C for 30 min. The mixture was then alkylated according to Monson et al. (1975), by addition of iodoacetic acid to a final concentration of 0.11 M. After standing in the dark at 23 °C for 40 min, a sevenfold excess of mercaptoethanol was added. The solution was made 0.5% in $NaDodSO_4$, heated at 90 °C for 2 min, and then passed over a Bio-Gel P-6 column equilibrated with 0.02 M NH_4HCO_3 to remove salts and excess radioactive proline. The sample was then lyophilized, hydrolyzed in 6 M HCl for 5 h in the autoclave, dried in a rotary evaporator, and dissolved in 50 μl of water, and 20 000–50 000 cpm were applied to precoated cellulose TLC sheets (EM Reagents, E. Merck, Darmstadt, Germany) and chromatographed in butanol–acetic acid–water (63:27:10) for 4 h (Stepka, 1958). Strips (0.5 cm) of the TLC sheets were scraped off and counted in 8 ml of ScintiLene (Fisher). Full hydrolysis and the maximal separation on TLC sheets were first determined with in vivo labeled calvaria procollagen. There was no radioactivity at the origin, or anywhere except at the position of the proline and hydroxyproline standards.

[^{14}C]*Glycine in Vivo Labeled Calvaria Collagen.* Calvaria were removed from four dozen 16-day-old chick embryos and incubated for 2 h with 250 μCi of [^{14}C]glycine, as described by Bornstein et al. (1972), except that 1.4 mM 2,2′-bipyridine was added.

The labeled calvaria were homogenized in 0.5 M acetic acid–0.1% Triton X-100 and then stirred for 16 h at 4 °C. The suspension was centrifuged at 15 000 rpm for 30 min and the supernatant dialyzed against 2 l. of 0.5 M acetic acid. The solution was made 5% NaCl (w/v) and stirred for another 16 h. The precipitate was then collected by centrifuging at 7000 rpm for 10 min, redissolved in 0.5 M acetic acid and dialyzed against the same for another 6 h after which it was lyophilized. The yield was 12 mg of procollagen with a specific activity of about 100,000 cpm/mg. Analysis on $NaDodSO_4$–slab gels showed this procollagen preparation contained two bands with mobilities corresponding to molecular weights of 150 000 and

TABLE I: Wheat Germ Analysis of Formamide–Sucrose Gradient Fractions of Calvaria RNA.

Fraction	μg of RNA/ Fraction	pmol of Proline/ 2.5 μg of RNA	% Solubilized by Collagenase	Total pmol of Proline in Procollagen/Fraction[a]
>30 S	6.7	4.8	27	3.47
27–30 S	22.2	9.1	50	40.5
<27 S, >18 S	11.2	10.4	2	0.94
18 S	13.0	15.7	−2	0
<18 S	18.2	23.9	10	17.4
−RNA		0.93		

[a] Total pmol of proline in procollagen per fraction equals μg of RNA/fraction × pmol/μg RNA × fraction solubilized by collagenase.

120 000, using calf skin α_1, β_{11}, and γ_{111} as molecular weight standards. The more rapidly migrating band contained p-α_1 as well as pro-α_2 chains as a result of enzymatic scission during the preparation (Martin et al., 1975). Recently pulse-labeled calvaria procollagen was prepared using a 20-min pulse and neutral salt extraction in the presence of protease inhibitors (Monson et al., 1975). The mobility of the slowest migrating species in this preparation was identical with that in the earlier preparations used as size markers here.

Results

Formamide–Sucrose Gradient Fractionation of Oligo(dT)-Cellulose Bound Calvaria RNA. When fractionated on aqueous sucrose gradients, procollagen mRNA activity was found in near equal amounts in all fractions sedimenting faster than 18 S (Boedtker et al., 1974; Crkvenjakov, 1974). To obtain a better fractionation, 70% formamide/4–20% sucrose gradients were used to fractionate oligo(dT)-cellulose bound calvaria RNA, as described in Materials and Methods. Five fractions, corresponding to >30 S, 27–30 S, <27 S >18 S, 18 S, and <18 S, were pooled and 1.5-μg aliquots of each were analyzed by polyacrylamide gel electrophoresis in 99% formamide. After staining with ethidium bromide, the gels were photographed and the results are shown in Figure 2. Fraction I contains some 27S rRNA but most of the RNA is located in a band of considerably lower mobility generated by the molecular weight independent movement of very large RNA molecules in this gel system (see Discussion). These molecules are presumed to be of nuclear origin. This band is also clearly visible in fraction II whose major component is 27S rRNA. However, this fraction also contains a faint but distinct band with a slightly lower mobility than 27S rRNA. Fractions III, IV, and V all contain 18S rRNA as well as a continuous distribution of RNA molecules ranging from larger than 18 S in fraction III to smaller than 18 S in fraction V, as expected. Fractions III and IV, however, both contain RNA molecules considerably larger than one would expect to find sedimenting between 27 and 18 S, or at 18 S. The nonribosomal RNAs sediment more slowly in these gradients than one would expect from their size.

To determine the distribution of procollagen mRNA in these five fractions, equal aliquots were translated in a wheat-germ S-30, and the size distribution as well as collagenase sensitivity of each product was measured. The total incorporation into Cl_3CCOOH-precipitable material and the collagenase sensitivity of the product are listed in Table I for each fraction. Although the overall stimulation of the wheat-germ system is highest for the lowest molecular weight RNA (fraction V), over 75% of the synthesis of collagenase-sensitive polypeptides is stimulated by fraction II. This is more dramatically demonstrated in the fluorogram obtained after polyacrylamide gel electrophoresis of the product obtained with each fraction; this is shown in Figure 3. Each pair of strips represents the product before and after treatment with collagenase. Only fractions I and II produce heavily labeled bands comigrating with the calvaria pro-α chain standards as well as some smaller polypeptides, all of which are totally degraded by collagenase. Although a faint pro-α band is visible in the product programmed by fraction III, the dominant products as well as those produced by fractions IV and V are considerably smaller than procollagen and are quite resistant to collagenase.

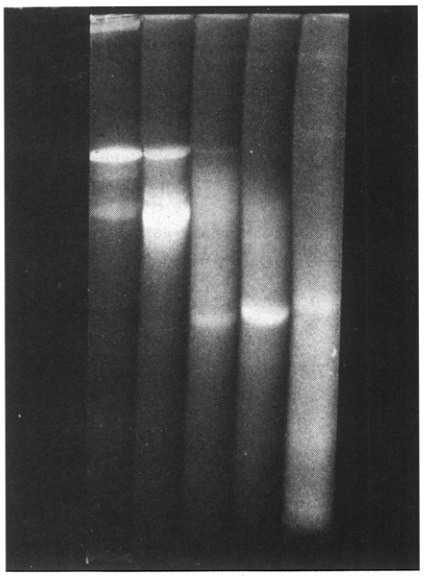

FIGURE 2: Polyacrylamide gel electrophoresis of five formamide–sucrose gradient fractions of oligo(dT)-cellulose bound calvaria RNA. RNA (50 μg) was sedimented through a 4–20% sucrose gradient in 70% formamide at 36 000 rpm in a Beckman SW40 Ti rotor for 23 h at 20 °C; 1.5 μg of each of five fractions was electrophoresed on 3% polyacrylamide gels in 99% formamide for 4 h and then stained with ethidium bromide and photographed as described in Materials and Methods. From left to right, RNA sedimenting at: (I) >30 S; (II) 27–30 S; (III) <27 S to >18 S; (IV) 18 S; and (V) <18 S. Direction of migration is downward.

The finding that most of the procollagen synthesized in wheat-germ extracts consisted of complete or nearly completed chains confirms and extends the reports of successful translation of procollagen mRNAs in wheat-germ extracts (Beneveniste et al., 1974, 1976; Harwood et al., 1975). The absolute requirement for high KCl concentrations reported by both of the former groups can apparently be replaced by using spermine which favors the synthesis of longer polypeptides (Atkins et al., 1975). We consistently failed to find an increase in pro-α chain synthesis at higher KCl concentration when spermine was used.

Although the specificity of the collagenase used was well

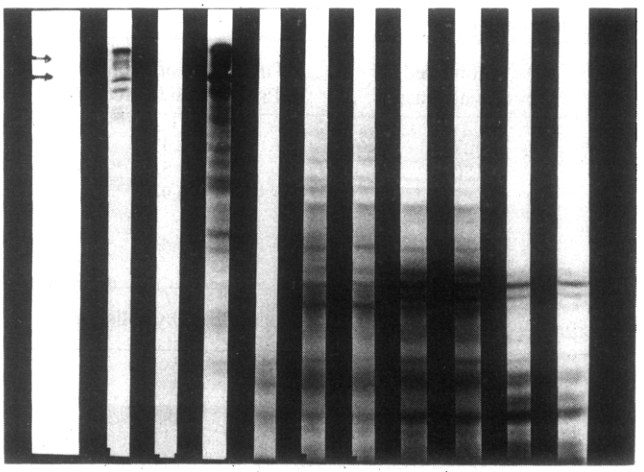

FIGURE 3: Fluorogram of wheat-germ product obtained with five formamide–sucrose gradient fractions. From left to right: calvaria procollagen standard: (I) >30 S, minus collagenase, >30 S, plus collagenase; (II) 27–30 S, minus collagenase, 27–30 S, plus collagenase; (III) <27 S to >18 S, minus collagenase, <27 S to >18 S, plus collagenase; (IV) 18 S, minus collagenase, 18 S, plus collagenase; (V) <18 S, minus collagenase, <18 S, plus collagenase. Translation in wheat-germ S-30, collagenase digestion, and analysis of product on slab gels were carried out as detailed in Materials and Methods.

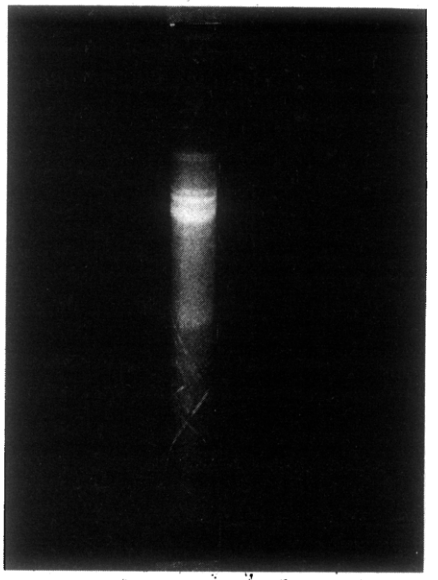

FIGURE 4: Polyacrylamide gel electrophoresis in 99% formamide of calvaria RNA bound to oligo(dT)-cellulose two times. RNA (1.5 μg) was electrophoresed and the gel stained and photographed as described in Materials and Methods. The direction of migration is downward.

established (see Materials and Methods), additional evidence for the synthesis of collagen polypeptides in wheat germ in response to 27–30S RNA was obtained by showing that the [^{3}H]proline-labeled product could be hydroxylated by prolyl hydroxylase. The wheat-germ product, obtained when fraction II, fraction IV, and total RNA were used as mRNA, was hydroxylated and hydrolyzed. Proline and hydroxyproline were separated by thin-layer chromatography, and the amount of radioactivity comigrating with proline and hydroxyproline standards was determined as described in Materials and Methods. Twelve percent, or 1800 cpm, of the total [^{3}H]proline in the wheat-germ product was converted to hydroxyproline when fraction II was used as mRNA while there was no hydroxylation with fraction IV and less than 1% with total RNA.

Clearly procollagen mRNAs are concentrated in fraction II which consists of 27S rRNA, some very large RNA as well as RNA near 27 S in size, and an additional small but distinct RNA band found only in this fraction.

Dimethyl Sulfoxide–Sucrose Gradient Fractionation of Oligo(dT)-Cellulose Rebound Calvaria RNA. The location of most of the procollagen mRNA activity at 27–30 S on formamide–sucrose gradients is obscured because of the large amount of 27S rRNA still present after a single binding to and release from oligo(dT)-cellulose. Therefore this step was repeated. RNA having been through two such cycles was reduced in amount by nearly 50% over that in one cycle. This was analyzed by electrophoresis in 99% formamide. The gel electropherogram shown in Figure 4 displays three close-lying bands in addition to a slow moving band corresponding to very large RNA. The band with the highest mobility is 27S rRNA: the two other bands of slightly lower mobility are clearly visible: one about as intense as 27S rRNA while the other is less intense.

To size fractionate this doubly bound RNA which represents 0.6% of total RNA, Me_2SO–sucrose gradient fractionation was used rather than formamide–sucrose gradients since the former provides both more stringent denaturing as well as disaggregating conditions. The resultant gradient is shown in

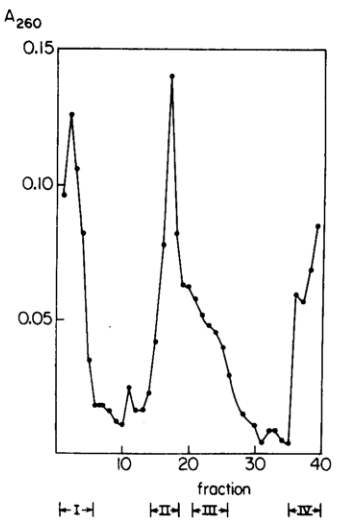

FIGURE 5: Absorbance profile of twice oligo(dT)-cellulose bound calvaria RNA fractionated on a Me_2SO–sucrose gradient. Total calvaria RNA was bound to oligo(dT)-cellulose two times as described in Materials and Methods. RNA (35 μg) was sedimented through a 0–20% sucrose gradient in 85% Me_2SO at 50 000 rpm in a Beckman SW 56 Ti rotor for 25 h at 23 °C. Fractions were collected, and the absorbance was read as described in Materials and Methods.

Figure 5. Approximately half of the RNA applied sediments at 27–30 S as a sharp peak followed by a slower sedimenting shoulder. A substantial part of the absorbance seen at both the top and the bottom of this gradient is not due to RNA. Only 20% of the bottom fraction and 10% of the top fraction were recovered as RNA after alcohol precipitation, while over 90% of the 27–30S fraction was recovered as RNA. This fraction corresponds to 0.3% of total RNA used as starting material.

When equal aliquots of all four fractions were analyzed on gels, only the 27–30S fraction and the slower sedimenting shoulder were visible: these are shown in Figure 6. The 27–30S fraction is now seen to contain only three bands corresponding to 27S rRNA and two slightly slower migrating species. The band corresponding to very large RNA species seen both before Me_2SO size fractionation (Figure 4) and after formamide size

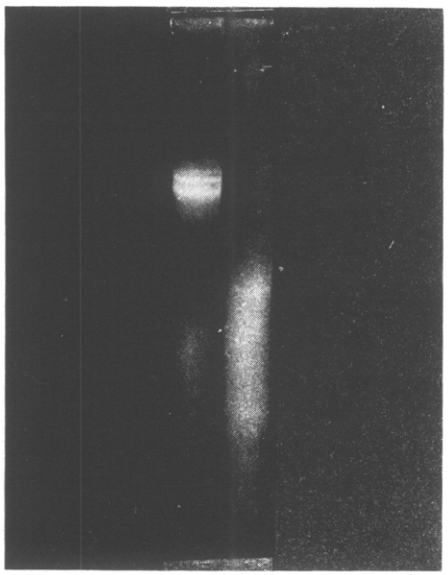

FIGURE 6: Polyacrylamide gel electrophoresis in 99% formamide of Me_2SO–sucrose gradient fractions of calvaria RNA bound to oligo(dT)-cellulose twice. Fractions II and III (1.5 μg each; Figure 5) were electrophoresed, stained, and photographed as described in Materials and Methods. (Left) The 27–30 S Me_2SO–sucrose gradient fraction (fraction II in Figure 5); (right) <27–18 S Me_2SO–sucrose gradient fraction (fraction III in Figure 5).

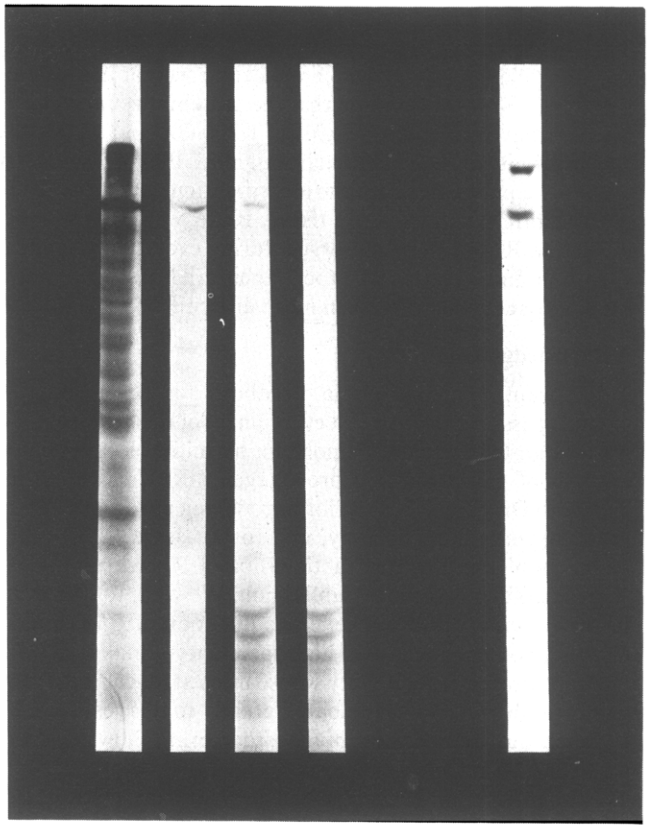

FIGURE 7: Fluorogram of wheat-germ product obtained with Me_2SO–sucrose gradient fractions. From left to right: 27–30S fraction (fraction II in Figure 5), minus collagenase, 27–30S fraction, plus collagenase; <27–18S fraction (fraction III in Figure 5), minus collagenase, <27–18S fraction, plus collagenase; calvaria procollagen standard. Translation in wheat-germ S-30, collagenase digestion, and analysis of product on slab gels were carried out as described in Materials and Methods.

fractionation (Figure 2) has been quantitatively removed.

The Me_2SO–sucrose gradient fractions were translated in the wheat-germ S-30 and the products obtained analyzed on gels. The results obtained are shown in Figure 7. The 27–30S fraction stimulated the synthesis of two polypeptides that comigrate with calvaria procollagen standards. In addition a number of polypeptides of intermediate size are also present. While the collagenase digestion appears to have been incomplete, these intermediate bands were digested at least as much as those comigrating with procollagen. The <27–18S fraction stimulated the synthesis of a very small amount of pro-α chain as well as much smaller polypeptides which were resistant to collagenase. From this we conclude that almost all procollagen mRNA activity is in the 27–30S fraction.

Molecular Weights of Procollagen mRNAs. Having established that calvaria procollagen is programmed by RNA fractions sedimenting at 27–30 S and containing two RNA bands with mobilities less than 27S rRNA when analyzed on denaturing gels, the correspondence between these bands and procollagen mRNAs is highly probable. When analyzed on gels with TMV RNA and *E.* coli rRNA as size markers, the molecular weight of these two RNA species is 1 700 000 and 1 800 000.

Discussion

By translating calvaria mRNA fractions in wheat-germ extracts, we have been able to demonstrate that most of the procollagen synthesis activity is located in the 27–30S fraction on both 70% formamide–sucrose and 85% Me_2SO–sucrose gradients. The identification of the product as pro-α chain is based on its comigration with calvaria pro-α chain standards on $NaDodSO_4$ gels, the collagenase sensitivity of the comigrating bands, and on the hydroxylation of the wheat-germ product by prolyl hydroxylase. Furthermore we have been able to show that the majority of the [^{3}H]proline-labeled product seen on gels is collagenase sensitive when the 27–30S RNA fraction is used, while the product obtained with smaller size fractions is collagenase insensitive. Therefore the 27–30S RNA fraction is not contaminated significantly with other mRNA molecules but a considerable quantity of incomplete procollagen chains appears to be produced.

After binding calvaria RNA to oligo(dT)-cellulose and releasing it twice and then sizing the product on Me_2SO gradients, only three bands appear on polyacrylamide gels in 99% formamide. One comigrates with 27S rRNA. The other two bands have molecular weights of 1 700 000 and 1 800 000, the former always appearing in higher concentration than the latter. Both of these molecular weights represent mRNA sufficiently large to code for type I calvaria pro-α chains with molecular weights of 150 000 requiring a minimum of 1 600 000 daltons for their encodement (Monson et al., 1975). The identification of these bands as pro-α_1- and proα_2-collagen mRNAs certainly seems the most likely interpretation.

The molecular weights of procollagen mRNAs reported here are in good agreement with that reported previously (Boedtker et al., 1974) in spite of the fact that a part of the interpretation presented at that time must be revised. The appearance of a single major pulse-labeled species, other than rRNA, has now been shown to be an artefact of the 3.4% acrylamide–99% formamide gel electrophoresis analysis. In these gels RNA with molecular weights greater than about 1 600 000 move into the gel with a "limiting mobility" independent of their molecular weight (Lehrach et al., 1976). Therefore, pulse-labeled nuclear RNAs of this molecular weight or higher enter the gel and form a very sharp band with a mobility indicating an artificially low molecular weight. Both large RNA species (silk fibroin mRNA) and large DNA species (λ DNA) migrate with the

same mobility as pulse-labeled calvaria RNA on these gels. Thus in the earlier report we were only justified in claiming that procollagen mRNA had a molecular weight in excess of 1 600 000. Now we can state that the presumptive procollagen mRNAs have molecular weights of 1 700 000 and 1 800 000. The proximity of these molecular weights to that of 27S rRNA certainly complicates both the identification and purification of these mRNAs from avian tissue. If the molecular weights of these mRNAs were conserved during evolution, the identification and isolation of procollagen mRNAs from mammalian tissues should be even more difficult.

Acknowledgments

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