Translation of Chick Calvarial Procollagen Messenger RNAs by a Messenger RNA Dependent Reticulocyte Lysate†

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ABSTRACT: A method was developed for the extraction of RNA from chick embryo calvaria which should be generally applicable to other connective tissues. Total RNA prepared by this method was translated by a mRNA-dependent reticulocyte lysate into discrete proα chains. Several criteria were used to identify these translation products, including (1) preferential labeling with [^{3}H]proline, (2) appropriate migration on sodium dodecyl sulfate-polyacrylamide gels, (3) selective sensitivity to collagenase digestion, and (4) specific precipitability by two different antisera against procollagen. Data from the immunoprecipitation experiments indicated that the majority of the proα chains contained the carboxy-terminal antigenic determinants. These results demonstrate that this translation system can be used as an assay for intact procollagen mRNAs and as a source of in vitro synthesized proα chains for future structural analysis.

Procollagen type I is a biosynthetic precursor of the triple-helical, structural protein, collagen type I. This precursor is comprised of three polypeptides, two identical proα 1 chains (~150 000 mol wt,[1] Monson et al., 1975) and a third, homologous proα 2 chain. Each of these polypeptides contains precursor-specific sequences located at both the amino and carboxy termini which are removed during the conversion of procollagen to collagen (Byers et al., 1975; Fessler et al., 1975; Davidson et al., 1975).

Although the biosynthesis, structure, and posttranslational modifications of collagen have been extensively studied (for reviews, see Bornstein, 1974; Martin et al., 1975), little is known about the regulation of procollagen gene expression. The recent identification of several genetically distinct types of collagen within a given organism suggests that the control mechanisms may be complex (for reviews, see Miller & Matukas, 1974; Miller, 1976). There is now evidence for tissue specificity in the expression of certain collagen types as well as variation in the relative proportions of individual collagen types during the development of certain tissues. Therefore, a study of the regulation of this class of structural proteins provides an opportunity to examine eukaryotic gene regulation throughout development.

Several experiments have been reported in which procollagen messenger RNA activity has been detected in RNA preparations (Benveniste et al., 1973, 1976; Boedtker et al., 1974, 1976; Harwood et al., 1975; Wang et al., 1975). The translation of procollagen mRNAs into complete proα chains has proven to be more difficult. Considerable progress in this area has been made by Benveniste et al. (1976) and Boedtker et al. (1976) and more recently by Adams et al, (1977) and Rowe et al. (1978).

Our investigation is confined to a study of the translation of the messengers coding for procollagen type I. Calvaria (frontal and parietal bones) from day 16 chick embryos were used as a source of RNA since only type I collagen has been detected in this tissue (Miller & Matukas, 1974) and approximately 60% of the protein synthesized in the frontal bone at this stage is collagen (Diegelmann & Peterkofsky, 1972).

We report here the development of a procedure for the extraction of RNA from chick embryo calvaria which should be generally applicable to other connective tissues. Total RNA prepared by this method was translated by a mRNA-dependent reticulocyte lysate into defined proα chains. Several criteria were used to identify these translation products including specific precipitation by two different antisera against procollagen. Data from the immunoprecipitation experiments indicated that the majority of the in vitro synthesized proα chains contained the carboxy-terminal antigenic determinants. This translational system can be used as a source of proα chains for future structural analysis in addition to serving as a reproducible assay for the intact proα chain mRNAs.

Experimental Procedures

Materials. L-[2,3-^{3}H]Proline (43.1 Ci/mmol) was purchased from New England Nuclear. L-[^{35}S]Methionine (406 Ci/mmol) was purchased from Amersham/Searle. Antiserum against native procollagen was a gift from Dr. Paul Bornstein; antiserum against the carboxy-terminal peptides of procollagen was a gift from Dr. Bjorn R. Olsen. Heat-killed, formalin-fixed *Staphylococcal aureus* was generously supplied by Dr. Richard B. Meagher. Micrococcal nuclease (17 000 U/mg) and purified *Clostridium histolyticum* collagenase were purchased from Worthington. Urea (Mann, UltraPure) solutions were deionized immediately before use with a mixed bed ion-exchange resin (Bio-Rex AG 501-X8).

Preparation of Total RNA from Chick Embryo Calvaria. Calvaria (frontal and parietal bones) were surgically removed from 20 dozens of day 16.5 Hubbard chick embryos and dropped directly into liquid nitrogen. The calvaria were either stored at −70 °C or powdered immediately in a mortar and pestle in the presence of liquid nitrogen (Benveniste et al., 1973). The powdered bones were homogenized in four batches each for 45 s at top speed with a Polytron (Tekman) in 50 mL of buffer A (20 mM Tris-HCl, pH 7.5, 2% $NaDodSO_4$, 25 mM EDTA, 0.2 mg/mL Pronase, 2 mg/mL heparin) which was

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminoethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MalNEt, *N*-ethylmaleimide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; mol wt, molecular weight.

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preheated to 37 °C. The combined homogenates were incubated for an additional 5 min at 37 °C. The pH of the homogenate was lowered by the addition of 0.1 volume of 1 M CH_3COONa, pH 5, prior to extraction with an equal volume of phenol chloroform isoamyl alcohol (25:25:1) for 3 min at 37 °C. The aqueous phase and the interphase were removed and reextracted with another equal volume of the organic mixture. Subsequently, only the aqueous phase was reextracted two times. One-tenth volume of 2.5 M CH_3COONH_4, pH 6, was added to the final combined aqueous phases and the solution precipitated overnight with 2.5 volumes of 95% ethanol at −20 °C.

Contaminating heparin, other sulfated polysaccharides, and low molecular weight RNA and DNA were removed by washing the crude RNA with 3 M CH_3COONa using a modification of the procedure of Palmiter (1973). The ethanol precipitate was collected by centrifugation at 9000*g* for 20 min at −15 °C. The precipitate was dissolved in 10 mL of 0.1 M CH_3COONa, pH 5, and vortexed in the presence of a glass rod to shear the contaminating DNA. To this solution, 30 mL of 4 M CH_3COONa, pH 6, was added at 0 °C and the RNA precipitated overnight at 0 °C. The fluffy white precipitate was collected by centrifuging at 30 000*g* for 10 min at 0 °C. The pellet was smeared and vortexed with a glass rod in 20–30 mL of 3 M CH_3COONa, pH 6, and the suspension was allowed to stand on ice for 15 min before the RNA was again collected by centrifugation. This washing procedure was repeated three to four times. The final pellet was dissolved in 10 mL of buffer B (0.1 M CH_3COONa, pH 5, 0.5% $NaDodSO_4$, 0.005 M EDTA) and extracted with an equal volume of phenol–chloroform–isoamyl alcohol. The phenol layer was extracted with 10 mL and then 5 mL of buffer B and the combined aqueous phases were ethanol precipitated. A yield of 28 mg of total, washed RNA was routinely obtained from 20 dozens of chick embryo calvaria.

RNA was prepared for translation by washing the ethanol precipitate two times with 95% ethanol at −20 °C for 10 min. The RNA was collected by centrifuging at 16 000*g* for 15 min at −10 °C, dried and under nitrogen, dissolved in sterile H_2O.

Preparation of the Proα Chain Standards. Procollagen type I was radiochemically labeled by incubating day 17 chick embryo calvaria in a defined medium for 18 min with L-[2,3-^{3}H]proline as described (Monson et al., 1975). Incubations were terminated by freezing the calvaria in liquid nitrogen. After the calvaria were powdered in a mortar and pestle, they were homogenized for 20 s with a Polytron and extracted as described (Monson et al. (1975)) except Cl_3CCOOH precipitation was omitted.

Lysate Preparation. Rabbit reticulocyte lysates were prepared as described by Schimke et al. (1974) with the exception that blood was collected by cardiac puncture into chilled 50-mL polypropylene centrifuge tubes containing 1000 U of sodium heparin.

A mRNA-dependent translation system was generated by digesting the reticulocyte lysate with micrococcal nuclease according to a modification of the method Pelham & Jackson (1976). Freshly thawed lysate (300 μL) was digested with a final concentration of 10 μg/mL micrococcal nuclease in the presence of 1 mM $CaCl_2$ for 8 min at 16–18 °C. The digestion was terminated by the addition of EGTA to a final concentration of 2 mM. After thorough mixing, this lysate was aliquoted directly into translation reactions and translation begun immediately.

Translation Conditions. A standard translation mixture of 62.5 μL contained 25 μL of reaction mixture, 25 μL of freshly thawed, reticulocyte lysate or micrococcal nuclease digested lysate, and 12.5 μL of RNA or H_2O. The reaction mixture was prepared immediately before use from frozen stock solutions. The final concentrations of reagents contributed by the reaction mixture were 50 μM of each unlabeled amino acid, 20 mM Hepes, pH 7.6, 80 mM KCl, 2 mM Mg $(C_2H_3O_2)_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 10 μg/mL creatine phosphokinase (83 U/mg), 0.06 μM $PhCH_2SO_2F$, and either 248 μCi/mL L-[^{35}S]methionine or 208 μCi/mL L-[2,3-^{3}H]proline. All incubations were carried out in 1.5 mL of polypropylene centrifuge tubes (Eppendorf) for 90 or 120 min at 26 °C.

The kinetics of incorporation were monitored by removing 5-μL aliquots at specified times and processing them for counting as described (Pelham & Jackson, 1976). The dried filters were counted in Omnitol in a LS230 Beckman scintillation counter.

Collagenase Sensitivity of the Translation Products. Collagenase was purified according to the method of Peterkofsky & Diegelmann (1971). It rendered 90% of the counts in [^{3}H]proline-labeled collagen Cl_3CCOOH soluble, while <1% was solubilized when [^{3}H]tryptophan fibroblast proteins were used as a substrate. Since no tryptophan is found within the collagen molecule, these results suggest specificity. Furthermore, no collagenase digestion of the [^{35}S]methionine-labeled proteins synthesized by the endogenous mRNAs of the reticulocyte lysate could be observed.

Two 15-μL aliquots of [^{35}S]methionine-labeled translation reactions were incubated for 30 min at 37 °C with an equal volume of 20 mM Hepes, pH 7.5, 5.0 mM MalNEt, 10 mM $CaCl_2$ either with or without 5 μg of collagenase. Digestions were terminated by the addition of 5 μL of 0.25 M EDTA, an equal volume of twice-concentrated, $NaDodSO_4$ sample buffer was added, and the samples were immediately heated for 5 min in boiling H_2O. Twenty-three microliters of each sample (corresponding to 5 μL of original lysate) was analyzed by slab $NaDodSO_4$-polyacrylamide gel electrophoresis. For quantitative analysis, 5 μL of the lysate was incubated in duplicate in 200 μL of 20 mM Hepes, pH 7.5, 5 mM $CaCl_2$, and 2.5 mM MalNET at 37 °C with or without 10 μg or collagenase. The samples were then processed for counting as described above.

Antibody Precipitation of Translation Products. Translation reactions were terminated by bringing the reactions of 10 mM EDTA, and 100 μg/mL pancreatic ribonuclease A and 50 U/mL T1 and digesting at 37 °C for 15 min. One-fifth volume of 5× buffer C (1× = 0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 2 mM proline, 2 mM methionine, and 1 mM NaN_3) was added and insoluble material was removed by centrifuging for 5 min at 12,000*g*. Aliquots of the supernatant (equivalent to 25 μL of the original translation reaction) were precipitated with 3 μL of antisera for 2–4 h at 25 °C or overnight at 0 °C. A protein A bearing strain of *Staphylococcus aureus* (SA) was substituted for a second antibody in indirect immune precipitation (Kessler, 1975). To each chilled reaction, 40 μL of a 30% (w/v) suspension of heat-killed, formalin-fixed SA was added and the suspension overlayed onto 300 μL of cold 1 M sucrose in buffer C. The antigens bound to the immunoadsorbant were collected by centrifuging for 1 min at 12 000*g*. The tubes were frozen and the pellets recovered by cutting off the bottom portion of the tube. Each pellet was transferred to a fresh tube and washed two times with 1 mL of buffer C. Immunoprecipitation was quantitated by counting the pellets in Aquasol. For $NaDodSO_4$-polyacrylamide gel electrophoresis, the antigens and antibodies were eluted with 75 μL of 5 M urea-$NaDodSO_4$

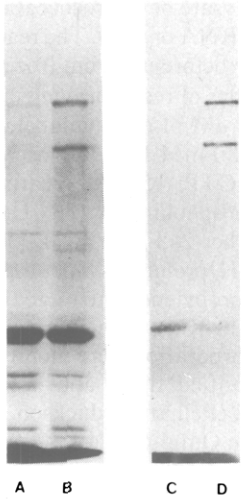

FIGURE 1: NaDodSO$_4$–polyacrylamide gel electrophoresis of undigested reticulocyte lysate translations. (A) [^{35}S]Methionine-labeled endogenous products. (B) [^{25}S]Methionine-labeled products from endogenous RNA and exogenous chick calvarial RNA (25 μg/125 μL of reaction). (C) [^{3}H]Proline-labeled endogenous products. (D) [^{3}H]Proline-labeled products from endogenous RNA and exogenous chick calvarial RNA (25 μL reaction).

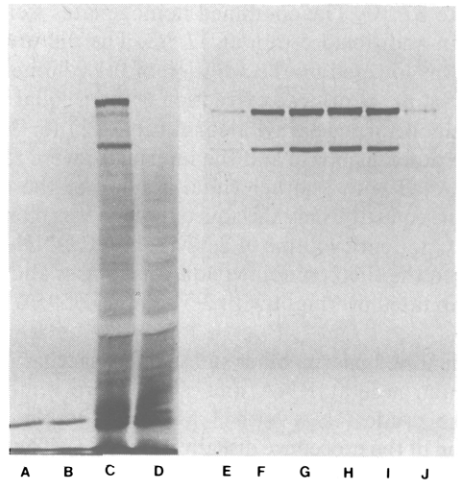

FIGURE 2: NaDodSO$_4$–polyacrylamide gel electrophoresis of mRNA-dependent reticulocyte lysate translation reactions labeled with [^{35}S]-methionine (slots A–D) or [^{3}H]proline (slots E–J) of the following samples: (A) control (no added RNA) incubated without collagenase; (B) control incubated with collagenase; (C) calvarial RNA translation products incubated without collagenase; (D) calvarial RNA translation products incubated with collagenase; (E–J) [^{3}H]proline-labeled calvarial RNA translation products obtained with 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM magnesium acetate, respectively.

sample buffer by incubating for 10 min at 25 °C. The immunoadsorbant was pelleted by centrifuging for 1 min at 12 000g, washed again with 25 μL of sample buffer. The combined eluants were made 5% in 2-mercaptoethanol, heated for 5 min in boiling H_2O for analysis by NaDodSO$_4$–polyacrylamide gel electrophoresis.

Slab NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. The buffer system employed was that of Laemmli (1970). Samples were prepared by dissolving pellets or diluting aliquots of translation reactions directly into 5 M urea–NaDodSO$_4$ sample buffer (3% NaDodSO$_4$, 0.0625 Tris-HCl, pH 6.8, 5 M urea, 10% glycerol, and 5% 2-mercaptoethanol) followed immediately by heating for 5 min in boiling H_2O. Electrophoresis was performed as described by O'Farrell (1975) using a 3% stacking and a 6% running gel. The slab gels were poured and run in place in a locally constructed gel apparatus similar to the Hoefer vertical slab apparatus. Gels were stained with 0.1% Coomassie Blue in 50% Cl_3CCOOH and destained with 7.5% acetic acid. The dried gels were autoradiographed using No-screen Kodak medical X-ray film for ^{35}S or for ^{3}H prepared for fluorography with preflashed RP-Royal X-omat X-ray film (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Results

Translation of Calvarial RNA in a Reticulocyte Lysate. Initially, the optimal conditions for the translation of chick muscle mRNA (2.0 mM Mg^{2+} and 80 mM KCl; Strohman et al., 1977) were used in the translation of total RNA from chick calvaria by the reticulocyte lysate. The products were analyzed by slab NaDodSO$_4$–polyacrylamide gel electrophoresis and the bands visualized by autoradiography in the case of [^{35}S]methionine labeling and fluorography in the case of labeling by [^{3}H]proline (Figure 1). Although the pattern of polypeptides resulting from the translation of endogenous reticulocyte mRNAs is particularly complex in the case of [^{35}S]methionine labeling (Figure 1A), two high molecular weight bands which are synthesized in response to calvarial RNA (Figure 1B) can be seen above this background. With [^{3}H]proline as a label, the pattern resulting from the endogenous translation is greatly simplified (Figure 1C) and two additional bands in the appropriate size range for proα chains are clearly visible when exogenous calvarial RNA is translated (Figure 1D). Since approximately 20% of the amino acids in the proα chains are proline, these results suggest that the two high molecular weight bands (Figure 1D) represent translated proα chains.

Translation of Calvarial RNA in a mRNA-Dependent Reticulocyte Lysate. Predigestion of the reticulocyte lysate with micrococcal nuclease reduces endogenous protein synthesis (Pelham & Jackson, 1976); only one major band can be seen when [^{35}S]methionine is used as a label (Figure 2A). As a result the spectrum of proteins synthesized in response to added total calvarial RNA is clearly visualized (Figure 2C). Two prominent and several minor high molecular weight bands are seen; a third prominent band migrates near the front. When [^{3}H]proline was used as a label, no visible bands were observed in the absence of added RNA, whereas two prominent and several minor high molecular weight bands were seen when total calvarial RNA was translated (Figure 2E–J).

Collagenase Sensitivity of the Translation Products. The susceptibility of the translation products to digestion by a highly specific collagenase was tested by incubating aliquots of the [^{35}S]methionone translation reaction with or without the enzyme and analyzing the digests by slab NaDodSO$_4$–polyacrylamide gel electrophoresis (Figures 2C,D). A comparison of slot C with D shows that collagenase digestion results in the disappearance of the high molecular weight bands, while the remainder of the pattern appears to be unaltered. All the [^{3}H]proline-labeled, high molecular weight bands (Figure 2E–J) also disappeared as a result of collagenase treatment (data not shown). These results indicate that all of these high molecular weight translation products are collagenous and, therefore, related to proα chains.

Optimization for the Translation of Proα Chains. The micrococcal nuclease treated lysate was specifically optimized for the synthesis of proα chains by monitoring the collagenase sensitivity of [^{3}H]proline incorporation and analyzing the translation products by slab NaDodSO$_4$–polyacrylamide gel electrophoresis. Translations were performed at 2.0 mM exogenous $Mg(C_2H_3O_2)_2$, while the exogenous KCl concentration was varied from 40 to 180 mM. Aliquots of each reaction

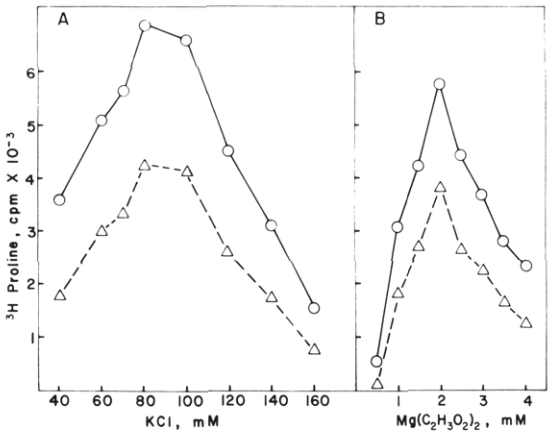

FIGURE 3: Potassium chloride (A) and magnesium acetate (B) optima for the translation of procollagen mRNAs by a mRNA-dependent reticulocyte lysate as monitored by [^{3}H]proline incorporation (O—O) and collagenase-sensitive, [^{3}H]proline incorporation (Δ- - -Δ).

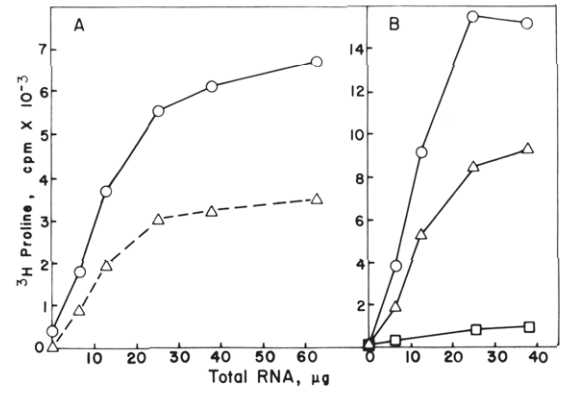

FIGURE 4: (A) [^{3}H]Proline incorporation (O—O) and collagenase-sensitive, [^{3}H]proline incorporation(Δ- - -Δ) as a function of total chick calvarial RNA translated per 125 μL of mRNA-dependent, reticulocyte lysate translation reaction. (B) Immunoprecipitable [^{3}H]proline counts as a function of total chick calvarial RNA per 125 μL of translation reaction. Aliquots (25 μL) were precipitated with antisera against both the amino- and carboxy-terminal extensions of procollagen (O—O), or with antisera specific for the carboxy-terminal extension (Δ—Δ) or control sera (□—□).

were incubated in duplicate either with or without collagenase as described in the Experimental Procedures. Aliquots which were precipitated directly with Cl_3CCOOH indicated that no measurable degradation occurred in the absence of collagenase. The collagenase sensitivity paralleled the [^{3}H]proline incorporation and the exogenous KCl optimum was broad, 80–100 mM (Figure 3A). Similarly, the $Mg(C_2H_3O_2)_2$ optimum, determined at 80 mM KCl, occurred at 2.0 mM for this preparation of lysate (Figure 3B). Under optimal conditions, 55–65% of the incorporated counts were rendered Cl_3CCOOH soluble by collagenase.

At optimal Mg^{2+} and KCl concentrations, the temperature optima of the translation of total calvarial RNA was determined. The kinetics of [^{3}H]proline incorporation were measured for reactions performed at 26, 30, and 37 °C. After 90 min the incorporation was 4170, 4526, and 1307 cpm/5 μL for each of these temperatures, respectively. Although the incorporation was slightly higher at 30 °C, 26 °C was chosen as the optimal temperature since the kinetics of incorporation were more linear at this temperature.

Incorporation of [^{3}H]proline and collagenase sensitivity were also determined as a function of total calvarial RNA added to the translation reaction (Figure 4A). There was a correspondence between the collagenase susceptibility and the [^{3}H]proline incorporation with saturation occurring at approximately 25 μg per 125 μL of reaction. Samples from each of these optimization experiments were also analyzed by slab $NaDodSO_4$–polyacrylamide gel electrophoresis. As a representative example, the samples obtained as a function of Mg^{2+} concentration are shown (Figure 2E–J). The results confirmed the data obtained by [^{3}H]proline incorporation and collagenase sensitivity. In every case where there were collagenase digestible counts, prominent proα bands could be seen. The relative migration of these bands remained constant but the intensities of the two bands changed with the various translation conditions.

Antibody Precipitability of the in Vitro Synthesized Proα Chains. Synthesis of the proα chains as a function of added calvarial RNA was also quantitated using specific precipitation with anti-procollagen antibodies. Three identical aliquots of each reaction (corresponding to 25 μL of the original lysate) were precipitated for 4 h at 25 °C as described in Experimental Procedures with an excess of either control sera, antisera directed against native chick procollagen type I (von der Mark et al., 1973), or antisera prepared against the disulfide-bonded, COOH-terminal peptide of procollagen (Olsen et al., 1977).

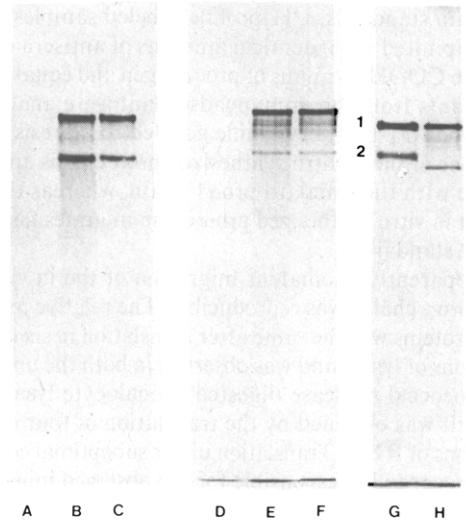

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of [^{3}H]proline-labeled (A–C) and [^{35}S]methionine-labeled (D–F) calvarial RNA translation products precipitated with an excess of either control sera (A, D), antisera directed against both the NH_2- and COOH-terminal peptides of procollagen (B, E), or antisera directed against the disulfide-bonded, COOH-terminal peptides of procollagen (C, F). The migration of standard proα1 and proα2 chains (slot G, 1 and 2) is compared with the migration of the in vitro synthesized proα chains (slot H).

The results are depicted in Figure 4B. Approximately 50% of the [^{3}H]proline counts were precipitated by native procollagen antisera as compared with 30% for the COOH-terminal antisera and 3% for the control sera. Similar percentages were obtained by precipitating overnight at 0 °C.

Analysis by slab $NaDodSO_4$–polyacrylamide gel electrophoresis of the immunoprecipitates served to validate identification of the material corresponding to the high molecular weight bands as in vitro synthesized proα chains. Antisera directed against the COOH terminus of procollagen precipitated only the material corresponding to the two prominent high molecular weight bands and the bands migrating between them (Figure 5C). In contrast, antisera containing antibodies against both the NH_2- and COOH-terminal regions of procollagen precipitated this same material together with a distribution of smaller, presumably incomplete chains (Figure

5B). No bands were visible in the sample precipitated by control sera (Figure 5A).

The specificity of these antibody precipitations was demonstrated with [^{35}S]methionine-labeled translation reactions since a much broader spectrum of proteins are labeled with this amino acid than with [^{3}H]proline. Results identical with those with [^{3}H]proline were obtained with the antisera against both the NH_2- and COOH-termini of procollagen (Figure 5E) or antisera against the COOH terminus (Figure 5F) and the control sera (Figure 5D). Furthermore, all the stained bands of the lysate disappeared and the only stained bands remaining corresponded to the antibodies themselves.

Undigested reticulocyte lysate translations of calvarial RNA were also precipitated with antisera directed against procollagen. The slab $NaDodSo_4$-polyacrylamide gel electrophoresis profiles of these immunoprecipitates were similar to those shown for the mRNA-dependent lysate. This result confirms the identification of the material corresponding to the high molecular weight bands synthesized in response to added RNA (Figure 1B,D) as proα chains.

Comparison of the Migration of the in Vitro Synthesized Proα Chains with Standards. In order to compare the migration of the in vitro synthesized proα chains with proα1 and proα2 chain standards, [^{3}H]proline-labeled samples of each were precipitated with identical amounts of antisera directed against the COOH terminus of procollagen and equal aliquots of the eluants from the immunoadsorbant were analyzed by slab $NaDodSO_4$-polyacrylamide gel electrophoresis (Figure 5G,H). One of the in vitro synthesized proα chains appears to comigrate with the standard proα1 chain, whereas the other prominent in vitro synthesized proα chain migrates faster than the proα2 standard.

This apparently anomalous migration of the in vitro synthesized proα chains was reproducible. The relative migration of these proteins was the same after translation in six different preparations of lysate and was observed in both the undigested and micrococcal nuclease digested reticulocyte lysates. The same result was obtained by the translation of four different preparations of RNA. Translation under suboptimal conditions did not appear to be responsible for the observed migration of the in vitro synthesized proα chains since $NaDodSO_4$-polyacrylamide gel electrophoresis analysis of the samples from the optimizations with respect to Mg^{2+}, KCl, temperature, and added RNA all resulted in the same gel pattern.

Proteolysis of the translation products did not appear to account for the observed migration. The presence of $PhCH_2SO_2F$, an inhibitor of serine proteases, during translation did not change the migration. Samples which were not precipitated by antibodies migrated similarly, indicating that degradation was not occurring during immunoprecipitation. There were no apparent differences between samples treated with EDTA and ribonuclease prior to $NaDodSO_4$-polyacrylamide gel electrophoresis and those which were pipetted directly into hot $NaDodSO_4$ sample buffer. Furthermore, aliquots removed at different times during the course of translation all revealed the same pattern when analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis.

Discussion

An essential requirement for examining transcriptional regulation of collagen synthesis during embryonic development of connective tissues such as calvaria is an RNA extraction procedure which is not only efficient for early stages of development but also equally effective at later stages when the extracellular matrix is both extensive and, in the case of calvaria, also partially calcified. The method presented here facilitates direct and rapid extraction of total RNA from such tissues. Interference of the extracellular matrix is minimized by homogenization of the powdered tissue in the presence of $NaDodSO_4$ with simultaneous digestion by Pronase, while the RNA is protected from degradation with an RNase inhibitor, heparin. Sulfated polysaccharides, such as chondroitin sulfate, which are commonly present in connective tissues are presumably removed along with DNA and heparin by washing the RNA with 3 M sodium acetate. This is thought to be a critical step since other sulfated polysaccharides, like heparin, might fractionate with RNA and inhibit translation unless removed. Consequently, this procedure should be generally applicable to other connective tissues such as cartilage which contains substantial quantities of condroitin sulfate. It should, thereby, facilitate the quantitation of procollagen mRNAs as a function of tissue development and also provide a convenient method for obtaining large quantities of intact mRNAs not only for type I but also for other types of procollagen.

Calvarial RNA prepared in this fashion was translated by a mRNA-dependent reticulocyte lysate to yield proα chains by a number of criteria. Analysis of the labeled cell-free products by slab $NaDodSO_4$-polyacrylamide gel electrophoresis with subsequent visualization by fluorography or autoradiography revealed two major bands which migrated in the region expected for proα chains when compared with standards (Figures 2 and 5). These prominent bands as well as several minor bands migrating between or slightly ahead of them were selectively absent from samples which had been pretreated with bacterial collagenase (Figure 2D). Antisera directed against both the amino- and carboxy-terminal extensions of procollagen and antisera directed specifically against the carboxy-terminal extension were used to confirm the identification of the prominent, high molecular weight cell-free products as proα chains (Figure 5). Although these results demonstrate the synthesis of proα chains, they do not identify individual chains. The relative migration of the prominent upper and lower bands (Figure 5G) suggests that they are related to proα1 and proα2, respectively. The identification of the minor proα chains which migrate between the major two proα chains remains to be established.

Translation of calvarial RNA into proα chains by a reticulocyte lysate indicated that no tissue specific factors are required for translation of the proα chain in RNAs in this heterologous, cell-free system. The proα chain mRNAs compete effectively with endogenous reticulocyte mRNAs in the untreated lysate (Figure 1B,D) and code for the majority of the cell-free products in the mRNA-dependent lysate (Figure 2C). The reticulocyte lysate is particularly well suited to the translation of proα chains since it produces very high molecular weight products from endogenous mRNAs (Figure 1A). The translation of proα chain mRNAs is reproducible over a wide range of conditions as judged by $NaDodSO_4$-polyacrylamide gel electrophoresis analysis of samples from the optimization experiments. By contrast, the translation of proα chains by wheat germ extracts has been reported to require either high KCl concentrations (Benveniste et al., 1973, 1976; Harwood et al., 1975) or spermine (Boedtker et al., 1976).

During the course of this investigation no evidence was obtained which would support the suggestion that the procollagen mRNA is polycistronic or that the initial translation product is larger than 300 000 (Church et al., 1971; Bankowski & Mitchell, 1973; Park et al., 1975). Rowe et al. (1978) have reported two immunoprecipitable, collagenase-sensitive translation products which migrate much slower than proα chain standards by $NaDodSO_4$-polyacrylamide gel electrophoresis. Unlike their findings, the immunoprecipitates (Figure

5) obtained with two distinctly different antisera against procollagen do not contain similar products with either [^{3}H]proline or [^{35}S]methionine labeling. Products having similar migration to those presented by Rowe et al. (1978) are visible when the lysate is analyzed directly by NaDodSO$_4$–polyacrylamide gel electrophoresis without immunoprecipitation. However, these products are not susceptible to cleavage by collagenase. The reason for this discrepancy is unclear.

The differences in migration observed (Figures 5G,H) between the in vitro synthesized proα chains and the proα1 and proα2 chain standards require cautious interpretation. Procollagen normally undergoes several posttranslational modifications such as hydroxylation of prolyl and lysyl residues and the addition of a disaccharide to a specific hydroxylysine residue (for review, see Bornstein, 1974). Recently additional carbohydrate has been demonstrated in procollagen (Murphy et al., 1975; Clark & Kefalides, 1976) of which a considerable amount has been localized to the carboxy-terminal extension (Olsen et al., 1977). Amino acid analysis of immunoprecipitated proα chains demonstrated that hydroxylation of prolyl residues was not occurring during translation. Presumably, the other modifications are lacking as well. On this basis alone, one would expect the in vitro synthesized proα chains to migrate faster than the corresponding, fully modified standards. However, since proα chains appear to be synthesized on the rough endoplasmic reticulum prior to transport into the cisternae (Diegelmann et al., 1973; Harwood et al., 1974; Weinstock & LeBlond, 1974; Weinstock et al., 1975), one would predict from the signal hypothesis (Blobel & Dobberstein, 1975) that they would contain additional sequences at their NH_2 terminal. The presence of such signal peptides or any other sequences not found in the standards would have the opposing effect of slowing the migration of the in vitro synthesized proα chains relative to the standards. As a consequence of these factors, the near comigration of one of the in vitro synthesized proα chains with standard proα1 might be taken as an indication that additional polypeptide sequences are present in the in vitro synthesized product. Rowe et al. (1978) have reported a similar migration of in vitro synthesized proα chains relative to proα chain standards. A detailed structural comparison between the in vitro synthesized proα chains and those derived from procollagen will be required to confirm the identification of the major translation products as proα1 and proα2 chains and to establish both the presence and nature of the postulated additional sequences.

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Deficiency of Globin Messenger RNA in Reticulocytes of the Belgrade Rat†

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ABSTRACT: The anemia of the Belgrade laboratory rat, an autosomal recessive trait (*b/b*), is associated with diminished incorporation of iron into heme and amino acids into globin by reticulocytes. We have studied the basis of decreased globin synthesis in *b/b* animals. Stimulation of protein synthesis per total RNA by *b/b* RNA was 39–46% of that by normal RNA in the wheat germ cell-free system. Hybridization of total cytoplasmic RNA to radioactive DNA complementary to rat globin mRNA showed that the number of globin mRNA molecules per total RNA in *b/b* reticulocytes was about 45% of that in normal reticulocytes. RNA fractions were also characterized by an analysis of globin products on carboxymethylcellulose chromatography after translation of RNA in the wheat germ cell-free system. The globin chain patterns synthesized by total cytoplasmic RNA and polysomal RNA isolated from *b/b* reticulocytes were indistinguishable from those of normal reticulocytes. The globin patterns synthesized by poly(A)-containing RNA derived from either total cellular or polysomal RNA were also similar when *b/b* and normal animals were compared. Our results indicate that diminished globin synthesis in *b/b* animals reflects lowered globin mRNA content in *b/b* reticulocytes, although the functional composition of globin mRNA from normal and *b/b* animals is essentially the same. The Belgrade anemia probably results from defective uptake of iron in erythroid cells, leading to heme deficiency; it is attractive to speculate that a chronic heme deficit, in turn, leads to a shortage of globin mRNA.

Belgrade laboratory rats have a hypochromic, microcytic anemia inherited as an autosomal recessive trait (*b/b*) (Sladic-Simic et al., 1963). The primary defect leading to the anemia is not yet clear. Studies on iron metabolism indicate that there is malutilization of iron in the *b/b* rats (Sladic-Simic et al., 1966). The serum iron concentration is high and the iron-binding capacity of the serum is nearly saturated (Sladic-Simic et al., 1969). Nevertheless, there is virtually no stainable iron present in the body tissue and bone marrow (Sladic-Simic et al., 1969). Although *b/b* rats respond partially to parenteral iron treatment, erythrocytes remain hypochromic and microcytic (Sladic-Simic et al., 1966). Iron-uptake studies indicate that the transport of iron from plasma into reticulocytes is markedly decreased (Edwards et al., 1978). Transferrin binding and internalization are apparently normal; however, release of iron within the cell is defective for Belgrade rats (Edwards et al., 1977).

Hemoglobin synthesis in *b/b* rats is also diminished, as measured by the incorporation of radioactive amino acids into globin by intact reticulocytes (Edwards et al., 1978). Despite the strong resemblance in red cell morphology between the *b/b* anemia and human thalassemia, starch-gel electrophoresis of hemoglobins reveals no difference between *b/b* and normal rats (Sladic-Simic et al., 1966), and incorporation into six of the seven globin chains follows the same pattern comparing *b/b* to normal rats (Edwards et al., 1978). (At the time of the cited study, the identity of a seventh protein fraction as a globin chain had not been established.) Translation of poly(U) by a cell-free system derived from *b/b* reticulocytes suggests a defect in the translational machinery, possibly at the initiation step (Cusic and Becarevic, 1976). Messenger RNA isolated from *b/b* reticulocytes is active in directing globin synthesis in cell-free systems; however, the distribution of mRNA between polysomes and the postribosomal supernatant was significantly different in *b/b* reticulocytes as compared to normal reticulocytes (Crkvenjakov et al., 1976).

We have investigated the basis for the occurrence of decreased globin synthesis in Belgrade rats despite the absence of an alteration in the pattern of incorporation into globin chains. To accomplish this goal, we studied the amount and functional composition of globin messenger RNA in *b/b* reticulocytes.

Experimental Procedure

Materials. L-[4,5-^{3}H]Leucine (62 Ci/mmol), L-[^{14}C]leucine (3.9 mCi/mmol), and [5-^{3}H]dCTP (20 Ci/mmol) were obtained from Schwarz/Mann, Orangeburg, N.Y. Oligo(dT)-cellulose (type T2) and oligo(dT)$_{12-18}$ were purchased from Collaborative Research, Inc., Waltham, Mass. Preswollen carboxymethylcellulose (Whatman CM-52) was obtained from Reeve Angel Co., Clifton, N.J. S_1 nuclease was purchased from Seikagaku Kogyo Co., Tokyo, Japan. RNA-dependent DNA polymerase of avian myeloblastosis virus, Sephadex SP-50, and Chelex 100 were generously made available by Dr. Nick Hastie of Roswell Park Memorial Institute. All other chemicals are of reagent grade.

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