EVIDENCE THAT THE MESSENGER RNA FOR COLLAGEN IS MONOCISTRONIC

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SUMMARY

Polysomes synthesizing Type I and II procollagens were isolated from rat calvaria and from a murine chondrosarcoma respectively. All collagenase sensitive polypeptides synthesized in vitro, as well as the collagen specific immunoprecipitated materials were smaller than β components. Polysomes contained nascent peptides consistently smaller than α chains. In low K^+ (10mM) nonspecific adsorption of collagen to polysomes and aggregation of polysomes were observed. Such effects were reversed in the presence of 250mM K^+. We find no evidence supporting the polycistronic mRNA hypothesis for collagen and suggest rather that large polysomal aggregates are artifacts generated at low K^+. We propose that collagen is synthesized on polysomes as a precursor polypeptide smaller than 185,000.

INTRODUCTION

Studies of collagen biosynthesis at the polysomal level have led to controversy regarding the size of procollagen synthesizing polysomes. Polysomes ranging in size from 300-400S (1-3) to as large as 2,000S (4-6) have been reported. Such apparently contradictory data underscore the difficulties in working with polysomes engaged in collagen synthesis. Significant differences in data may result from minor variations in experimental conditions. Recent reports of large collagen synthesizing polysomes and attempts to isolate them by specific immunoprecipitation suggest that the mRNA for procollagen is polycistronic (7). According to such an hypothesis, the mature triple helical molecule is derived from a precursor polypeptide at least three times the size of a single pro α chain, which is subsequently cleaved. However, very large polysomes have been interpreted as artificial aggregates (8). It has also been reported that antibody binding techniques may create artifacts due to nonspecific adsorption of completed and released chains to polysomes during the isolation procedure (9).

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In this communication, polysomes were isolated from rat calvaria and a murine chondrosarcoma under conditions of high and low K^+ ion concentrations. The products of synthesis were characterized by gel electrophoresis, collagenase sensitivity, and by specific immunoprecipitation. We present evidence that heavy aggregates of polysomes and large molecular weight products are artifacts generated at low K^+ concentrations.

MATERIALS AND METHODS

Tissue Sources. Nine day old chick embryos were obtained from Truslow Farms, MD, and maintained in a humidified incubator at 37°. Newborn Sprague-Dawley rats, less than 12 h old were used as a source of calvaria. Carcasses were prepared by evisceration and decapitation of 12 day old chick embryos. The transplantable chondrosarcoma (10-12) was injected into three week old Sprague-Dawley rats and sacrificed after 5-6 weeks when the tumors had reached 15-20 g. Tumors were dissected free of the capsule and digested with a mixture of collagenase (0.1%) and hyaluronidase (0.05%) for 2-3 h at 37° to obtain cell suspensions.

Isolation of Total Polysomes. Total polysomes were isolated from newborn rat calvaria and rat chondrosarcoma cell suspensions as described elsewhere (13). In brief, tissue or cells were homogenized in 2.5 vol of Buffer A (w/v) containing: 120mM sucrose; 50mM Tris-HCl, pH 7.5; 10mM KCl; 10mM $MgCl_2$; 3mM DTT; heparin, 200 µg/ml; and cycloheximide, 100 µg/ml. The homogenate was made 1% Triton X-100, and then the K^+ ion concentration was adjusted to 250mM. The homogenate was centrifuged at 8,000xg for 20 min and the supernatant fraction was layered over 2M and 1M sucrose cushions in Buffer A, but without cycloheximide. Polysomes were harvested by centrifugation at 105,000xg for 20 h at 0-2°. The polysomal pellets were rinsed with cold H_2O and stored at -70°.

Evaluation and Labeling of Polysomes In Vitro. Polysomal pellets were resuspended in Buffer B containing: 10mM Tris-HCl, pH 7.5; 10mM $MgCl_2$; 10mM KCL; 1mM DTT; and 1mM EDTA, and the absorbancy at 260 nm was determined. Each assay mixture contained in a total volume of 250 µl: 40mM Tris-HCl, pH 7.5; 80mM KCl; 4.5mM $MgCl_2$; 0.25mM ATP, pH 7.0; 0.1mM GTP; 8mM creatine phosphate; 40 µg creatine phosphokinase; 1mM DTT; 100 µg pH 5 and S_{200} fractions (14); 2 µM of each of 19 unlabeled amino acids but not proline; 3µCi of (^{3}H) proline, 1µCi/µmol; and 0.5-3.0 A_{260} units of the polysome preparation (15). Samples were incubated for varying periods at 37° and the reaction was terminated by the addition of cycloheximide, 20 µg/ml, at 0-4°. Pooled reaction mixtures were applied to a 30 x 2.5 cm column of BioGel P-2 and eluted with Buffer B. The excluded material was layered over discontinuous sucrose gradients as described above and centrifuged at 105,000xg for 20 h at 0-2°. Labeled polysomes were resuspended in Buffer B and radioactivity determined. When polysomal cell-free product was to be obtained, the reaction was terminated by the addition of RNase, 80 µg/ml, and the incubation continued for an additional 15 min at 37°. After BioGel P-2 column chromatography, the excluded material was digested with collagenase as previously described (16). Conditions for SDS gel electrophoresis of the labeled polysomal products were as described by Guenther et al. (17).

Labeling of Tissue Polysomes in Organ Culture. Forty 12 day old chick embryo carcasses were placed in Dulbecco-Vogt modified Eagle's medium

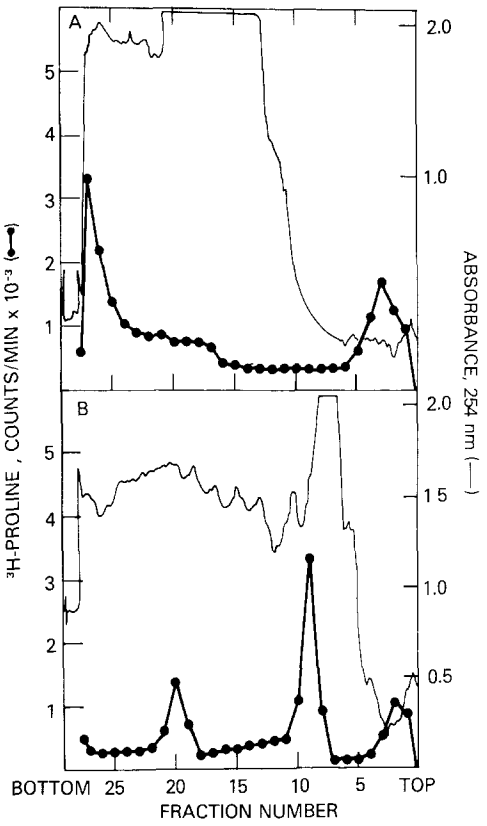

Figure 1. Sucrose density gradient profile of polysomes isolated from 12 day chick embryo carcasses. Labeling in organ culture for 20 min with (^{3}H)proline was performed as described in Materials and Methods. Polysomes were layered over a 10-50% linear sucrose gradient. Centrifugation was at 105,000xg for 90 min at 4° in a Beckman SW41 rotor. Polysome isolation and centrifugation were performed at (A) 10mM and (B) 250mM K^+ concentrations.

supplemented with 0.1 mg/ml ascorbic acid plus penicillin and streptomycin. The tissue was preincubated for 20 min at 37° in a shaking water bath. The medium was discarded, the tissue rinsed twice with fresh medium, and 100 ml of fresh medium containing 250 µCi of (^{3}H)proline were added. Incubation was continued for an additional 20 min at 37° and terminated by the addition of cycloheximide, 20 µg/ml, and chilling. Total polysomes were isolated as described.

RESULTS

The effect of low and high levels of K^+ during isolation and centrifugation on the profile of chick embryo polysomes is illustrated in Fig. 1. At 10mM K^+, a peak of radioactivity cosedimented with the heaviest material at the bottom of the gradient. In contrast, rapidly sedimenting

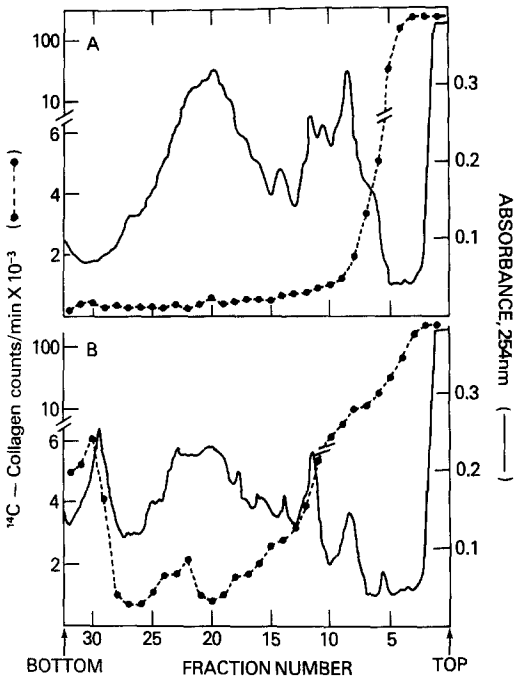

Figure 2. Sucrose density gradient profile of rat liver polysomes isolated in the continuous presence of (^{3}H)-labeled collagen chains in a 2:1 ratio of α1: α2. Polysomes were centrifuged on a 10-50% linear sucrose gradient as described in Figure 1. Polysomes were isolated and centrifuged in (A) 250mM and (B) 10mM K^+ ion concentrations.

material was not observed in polysomes isolated and centrifuged in the presence of 250mM K^+. Instead, two distinct peaks of radioactivity of intermediate size were present in the gradient (Fig. 1b).

To determine if completed released chains might have an effect on the polysomal profiles, nonspecific adsorption of collagen to polysomes was examined. Total polysomes were isolated from rat liver, a tissue not actively engaged in collagen synthesis (18) in the presence of high and low K^+ concentrations. Tritium labeled α1 and α2 chains were added immediately following homogenization. In high K^+, all the radiolabeled collagen appeared at the top of the gradient (Fig. 2a). In low K^+, a major peak of labeled collagen appeared at the bottom of the gradient cosedimenting with the heaviest polysome fraction. An additional peak

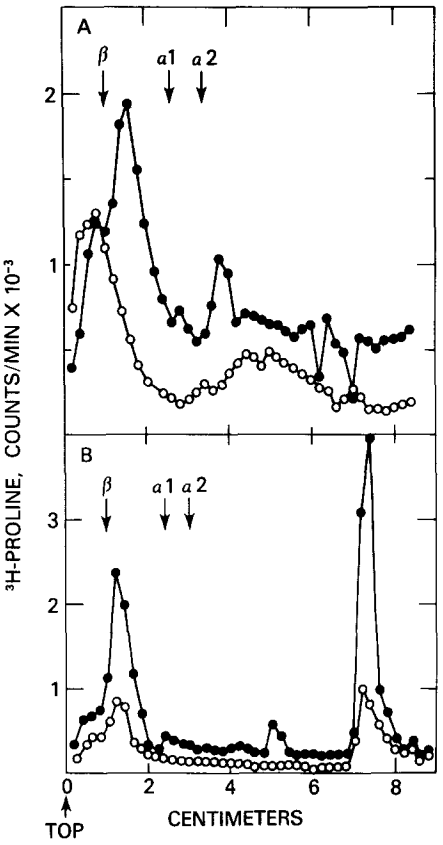

Figure 3. SDS polyacrylamide gel electrophoresis of (^{3}H)proline labeled total cell-free polysomal products, including released and nascent chains as described in Materials and Methods. (A) Cell-free products from rat calvaria polysomes before (●---●) and after (o---o) collagenase digestion. (B) Cell-free products from rat chondrosarcoma polysomes before (●---●) and after (o---o) collagenase digestion. The molecular weight markers were (^{14}C)-labeled α1 and α2 chains and β dimers obtained from rat skin collagen.

was present in the middle of the gradient, while the radioactive peak at the top of the gradient appeared broadened (Fig. 2b).

To characterize the products synthesized by these polysomes, the isolated radioactive material was subjected to polyacrylamide gel electrophoresis with and without collagenase pretreatment. Both the calvaria (Fig. 3a) and rat chondrosarcoma (Fig. 3b) polysomes exhibited a prominent peak of radioactive material on the gels, which was collagenase susceptible and which migrated between the collagen α1 and β markers. The peak of low

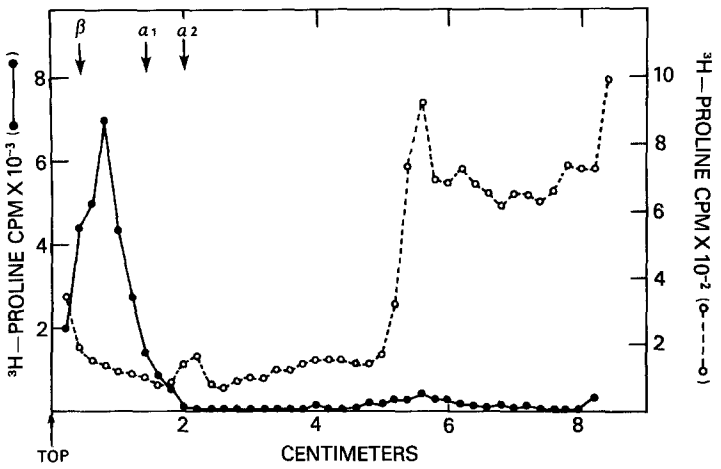

Figure 4. SDS polyacrylamide gel electrophoresis of (^{3}H)proline labeled cell-free products from rat chondrosarcoma polysomes. Both nascent (o---o) and completed released chains (●——●) were isolated from 10-50% linear sucrose gradients as described under Figure 1.

molecular weight material in Fig. 3b which was also susceptible to collagenase digestion may represent small incompleted collagenous peptides. However, much of the products of synthesis migrated in a range compatible with the size of complete pro α chains which are about 145,000 daltons (19-21). Approximately 65% of both the calvaria and the chondrosarcoma cell-free products were susceptible to digestion by purified bacterial collagenase. Under these conditions 85-90% of labeled rat skin Type I collagen was digested.

To exclude the possibility that completed chains might be associated with (^{3}H)proline labeled polysomes, we examined the size of labeled peptides bound to polysomes and also completed released chains isolated from linear sucrose gradients. Materials from both the chondrosarcoma and rat calvaria were subjected to gel electrophoresis. Using high K^+, polysomes consistently contained labeled material of heterogeneous molecular weight which was collagenase digestible, but always smaller than α chains (Fig. 4). No completed chains were found associated with

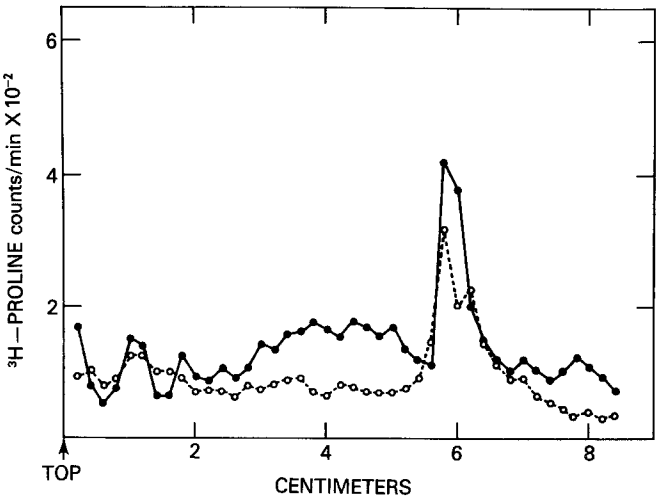

Figure 5. SDS polyacrylamide gel electrophoresis of (^{3}H)proline labeled cell-free products from rat calvaria polysomes. Cell-free product before (●——●) and after (o---o) immunoprecipitation with specific Type I procollagen antisera. The cell-free product before immunoprecipitation was coelectrophoresed with an equivalent amount of IgG.

polysomes. All the material with molecular weight similar to the precursor pro α chain was consistently found at the top of the gradients.

The cell-free products from rat calvaria polysomes were incubated with antiprocollagen Type I specific antibodies to precipitate collagenous peptides (Fig. 5). The gel electrophoresis was run for a longer period to allow better resolution between the presumptive pro α chains and the collagenase resistant high molecular weight material observed in Fig. 3a. This material was not immunoprecipitated by procollagen Type I antibodies as can be observed in the electrophoretic profile. The bimodal distribution of the major peak of radioactive material in the immunoprecipitate comigrating with the presumptive procollagen α chains may represent partial resolution between pro α1 and pro α2 chains.

DISCUSSION

In initial experiments, low K^+ concentrations were used routinely during isolation and centrifugation procedures. The major proportion of proline labeled collagenase digestible material was found consistently

at the bottom of the sucrose density gradients. Because collagen may bind to polysomes at K^+ concentrations below 120 mM (1), we decided to increase the K^+ levels. This prevented polysome aggregation (Fig. 1), presumably by abolishing the interaction of completed collagen chains with polysomes, and by decreasing the interaction of nascent chains. Procollagen is a protein which adheres nonspecifically to many biological complexes (20-22). A similar phenomenon has also been reported for immunoglobulins (9). In fact, nonspecific adsorption of many intracellular proteins to ribosomes during isolation procedures appears to be a general problem (23,24). Thus, very large polysome aggregates bearing presumptive polycistronic mRNA for collagen may instead be produced by the interaction of nascent chains or adsorbed collagen. Using low K^+ levels, we were able to produce such heavy polysomal-protein complexes by the addition of exogenous collagen to liver polysomes. These aggregates were readily dissociated by the inclusion of high K^+ (Fig. 2a).

Products larger than pro α chains were not observed in the nascent chains pool or in the products released from the polysomes in vitro. Larger peptides were neither collagenase sensitive nor precipitated by antiprocollagen antibodies.

Both Type I and II collagen synthesizing systems have been used in these studies supporting the hypothesis of a monocistronic mRNA for all collagens. Despite its unique hybrid structure, Type I collagen did not appear to differ from Type II collagen.

Previous reports of a polypeptide larger than 300,000 daltons associated with collagen synthesizing polysomes may be explained as artifacts of isolation (7). We suggest that in normal cells, the functional mRNA for procollagen is monocistronic and that the initial polysomal product of translation is a peptide comparable in size to the procollagen chain monomer described previously (19-21).

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