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Translation of Embryonic-Chick Tendon Procollagen Messenger Ribonucleic Acid in Two Cell-Free Protein-Synthesizing Systems

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Embryonic-chick tendon poly(A)-containing RNA was translated in the wheat-germ and mRNA-dependent rabbit reticulocyte-lysate systems. The ability of each system to synthesize polypeptides similar to pro-α chains of collagen was tested on the bases of electrophoretic mobility and susceptibility to highly purified bacterial collagenase. Very small amounts of polypeptides in the size range of pro-α chains were synthesized in the wheat-germ system, whereas efficient synthesis of two polypeptides similar to pro-α1 and pro-α2 chains was achieved in the reticulocyte lysate. The collagenous nature of the major high-molecular-weight products synthesized was demonstrated by their susceptibility to collagenase and ability to act as a substrate for purified collagen proline hydroxylase. Determinations of the relative amounts of these translation products suggest that the 2:1 ratio of pro-α1 and pro-α2 chains found in type I procollagen is reflected in proportional amounts of translatable mRNA for pro-α1 and pro-α2 chains. Comparisons of the electrophoretic mobilities of hydroxylated and unhydroxylated reticulocyte-lysate translation products were made with appropriate standards of hydroxylated and unhydroxylated procollagen polypeptides. The results suggest that, in common with a number of secreted proteins, procollagen is synthesized as pre-pro molecules consistent with the 'Signal Hypothesis'.

Over the past decade considerable advances have been made in our knowledge of the types, structure and post-translational processing of collagen, which is the most important protein component of virtually all vertebrate connective tissues. At least four genetically distinct types of collagen molecules (types I–IV) have been identified, and these collagen types are present in the different connective tissues in characteristic amounts. Types I and II collagens have been studied in greatest detail both chemically and biosynthetically. *In vivo* these molecules are synthesized on membrane-bound polyribosomes as three large precursor pro-α chains (approx. mol.wt. 150000), which are hydroxylated and glycosylated within the endoplasmic reticulum and subsequently secreted into the surrounding cell matrix as soluble triple-helical procollagen molecules. Before these collagenous molecules can be laid down as fibres within the extracellular environment the non-helical extension peptides at both *C*- and *N*-termini are removed enzymically (for reviews see Grant & Jackson, 1976; Prockop *et al.*, 1976; Fessler & Fessler, 1978).

Although there is considerable information on procollagen synthesis and assembly at the post-translational level, little is known of factors influencing transcription, post-transcriptional events and translation. One approach to investigating these aspects of procollagen biosynthesis requires the isolation and characterization of procollagen mRNA. An important means of detecting the presence of any mRNA species is its translation into authentic protein in cell-free protein-synthesizing systems, but until relatively recently little success has been achieved in synthesizing pro-α chains of collagen *in vitro*. Early workers were able only to synthesize pro-α chains by allowing isolated polyribosomes to complete their polypeptides *in vitro* (Kerwar *et al.*, 1972, 1973). Where RNA extracts were used to programme cell-free protein-synthesizing systems derived from Krebs II ascites cells (Benveniste *et al.*, 1973; Wang *et al.*, 1975) or wheat germ (Benveniste *et al.*, 1976; Zeichner & Rojkind, 1976), collagenous polypeptides synthesized were mainly of the size of collagen α chains (mol.wt. 100000) and smaller (for review see Harwood, 1979). The first report of successful synthesis of pro-α chains *in vitro* was by Harwood *et al.* (1975), and since then other workers have reported the complete translation of procollagen mRNA from chick calvaria (Boedtker *et al.*, 1976), fibroblasts in culture and tendon cells (Adams *et al.*, 1977; Rowe *et al.*, 1978; Howard *et al.*, 1978).

The inability to demonstrate the cell-free synthesis of complete pro-α chains in the early studies can probably be attributed to difficulties with the trans-

Abbreviation used: SDS, sodium dodecyl sulphate.

lation apparatus used *in vitro*. For example, cell-free systems from Krebs II ascites cells have the disadvantage of endogenous collagen-synthesizing abilities (Benveniste *et al.*, 1976), and unprocessed reticulocyte lysates have a high endogenous messenger activity which hampers the efficient translation of procollagen mRNA (Boedtker *et al.*, 1974). This latter problem is not encountered with the wheat-germ cell-free system (Roberts & Paterson, 1973), which has low endogenous mRNA content. However, the efficiency of translation by this system has been found to be very variable between batches of wheat germ (Marcu & Dudock, 1974; Carlier & Peumans, 1976), and particular difficulty has been encountered in translating a number of large mRNA molecules (Shih & Kaesberg, 1976; Davies *et al.*, 1977).

The present paper describes studies of the translation of chick tendon procollagen mRNA in the wheat-germ cell-free system and also in the messenger-dependent reticulocyte-lysate system described by Pelham & Jackson (1976), in which endogenous mRNA is removed by pretreatment with micrococcal nuclease. We have found this latter system to be much more efficient in the synthesis of pro-α chains. In addition, preliminary evidence is presented suggesting that procollagen mRNA is initially translated into a polypeptide of higher molecular weight than appropriate pro-α-chain standards, as would be expected of a secreted protein synthesized as proposed by the 'Signal Hypothesis' of Blobel & Dobberstein (1975).

Experimental

Materials

Trypsin (grade TRL) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Dulbecco's modification of Eagle's Minimum Essential Medium was purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Proteinase inhibitors and other chemicals used for the preparation of chick tendon cells and collagenous standards were obtained from sources described by Harwood *et al.* (1977). Heparin, dithiothreitol, ATP, GTP, phosphocreatine, spermine, spermidine, amino acids, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid], bovine serum albumin, *N*-lauroylsarcosine and $CaCl_2$ were supplied by Sigma Chemical Co., Poole, Dorset, U.K. Highly purified bacterial collagenase was obtained from Boehringer Corp., Lewes, Sussex. Oligo(dT)–cellulose (type 3) was purchased from Collaborative Research, Waltham, MA, U.S.A. The wheat-germ cell-free system was prepared from raw wheat germ (type 'Old Stone Mill') from Niblack Foods, New York, NY, U.S.A. L-[U-^{14}C]Proline (270mCi/mmol), L-[2,3,4,5-^{3}H]-proline (105Ci/mmol) and L-[^{35}S]methionine (915–970Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Purified acrylamide, *NN'*-methylenebisacrylamide and SDS were obtained from British Drug Houses, Poole, Dorset, U.K. 2,5-Diphenyloxazole (PPO, Scintillation Chemical Grade) was purchased from Intertechnique, Uxbridge, Middx., U.K. All other chemicals were of AnalaR grade.

Isolation of chick tendon cells

Cells were prepared from leg tendons of 8–10 dozen 17-day chick embryos by digestion with trypsin and partially purified bacterial collagenase as described by Dehm & Prockop (1971, 1972). An average of approx. 15×10^6 cells were obtained from a single embryo and cells were either stored in liquid N_2 before extraction of RNA or used for the preparation of ^{14}C-labelled collagenous polypeptides as outlined below.

Preparation of collagenous standards

[^{14}C]Proline-labelled tendon procollagen was prepared by incubating approx. 10^7cells/ml for 2h at 37°C in modified Krebs medium (Dehm & Prockop, 1971) containing 25μCi of L-[U-^{14}C]-proline. Procollagen secreted by these cells into the medium was isolated by $(NH_4)_2SO_4$ precipitation in the presence of proteinase inhibitors as described by Harwood *et al.* (1977).

For the preparation of [^{14}C]proline-labelled unhydroxylated procollagen (protocollagen), 10^7cells/ml were preincubated for 30min at 37°C in modified Krebs medium containing 0.3mM-$\alpha\alpha'$-bipyridine before the addition of 25μCi of [^{14}C]-proline. After incubation at 37°C for 2h, the cells were sedimented, cooled in ice and homogenized in 25ml of 0.2M-acetic acid containing 150μg of phenylmethanesulphonyl fluoride/ml, 10mM-*N*-ethylmaleimide and 15μg of $\alpha\alpha'$-bipyridine/ml. The homogenate was stirred for 16h at 4°C and centrifuged at 38000*g* for 30min at 4°C. After extensive dialysis of the supernatant at 4°C against 0.4M-NaCl/0.05M-Tris/HCl(pH7.4)/10mM-6-amino-n-hexanoic acid/10mM-*N*-ethylmaleimide/25mM-EDTA, protocollagen was precipitated by the addition of $(NH_4)_2SO_4$ to 30% saturation. Precipitated protocollagen was collected by centrifugation at 38000*g* for 1h at 4°C and shown to contain no detectable hydroxy[^{14}C]proline as judged by the method of Juva & Prockop (1966).

Labelled collagen α-chains were prepared by preincubating leg tendons from 36 embryos for 30min at 37°C in 10ml of Dulbecco's modification of Eagle's medium containing 64μg of β-aminopropionitrile fumarate/ml followed by a further 2h

incubation with 50μCi of [^{14}C]proline. The incorporation period was followed by a 5h chase in 25ml of Dulbecco's medium containing 64μg of β-aminopropionitrile fumarate/ml and 1% (w/v) proline. Collagen was then extracted by stirring the tendons in 0.2M-acetic acid for 16h at 4°C. The sample was centrifuged at 38000g for 30min at 4°C and acid-soluble collagen precipitated by the addition of solid NaCl to 5% (w/v). Precipitated collagen was collected by centrifuging at 38000g for 1h at 4°C, dissolved in 0.1M-acetic acid, dialysed against the same solution and freeze-dried.

The [^{14}C]proline-labelled samples of procollagen, protocollagen and collagen were dissolved in electrophoresis sample buffer (Laemmli, 1970), heated for 2min at 100°C and dialysed against the same buffer at 20°C before storage at −20°C.

Preparation of tendon poly(A)-rich mRNA

Poly(A)-rich RNA was prepared by a modification of the method of Brawerman (1974). Tendon cells (1.1×10^{10}) were homogenized in equal volumes of extraction buffer [0.1M-Tris/HCl, pH9.0, 0.5% (w/v) SDS, heparin (250μg/ml) and 0.3mM-spermidine] and phenol/chloroform/3-methylbutan-1-ol (50:50:1, by vol.). The emulsion was shaken for 20min at 20°C and the phenol and aqueous phases were separated by centrifugation at 3000g, 10°C for 30min. The aqueous phase was collected and the interphase material re-extracted with the above buffer / phenol / chloroform / 3 - methylbutan - 1 - ol mixture. The aqueous phases were pooled and the deproteinization procedure was repeated three times. Sodium acetate (2M, pH5.6) was added to the nucleic acid extract to a final concentration of 0.1M. Then 2vol. of ethanol was added and nucleic acids were precipitated overnight at −20°C.

The nucleic acid precipitate was washed five times with 80% (v/v) ethanol and dissolved in 15mM-NaCl/1mM-sodium citrate containing 4% (w/v) *N*-lauroylsarcosine. RNA was separated from DNA by centrifuging through a 5.7M-CsCl cushion (Glisin *et al.*, 1974). The pelleted RNA was dissolved in 0.2M-sodium acetate (pH5.6), and reprecipitated by the addition of 2vol. of ethanol. Precipitated RNA was washed five times with 80% ethanol, dissolved in cold sterile water and an equal volume of 0.02M-Tris/HCl buffer (pH7.5) containing 1.0M-NaCl and 0.2% (w/v) SDS was added. Poly(A)-rich RNA was bound to an oligo(dT)–cellulose column (10cm× 1cm), and rRNA was eluted with 0.01M-Tris/HCl (pH7.5)/0.5M-NaCl/0.2% SDS. The column was washed with 0.01M-Tris/HCl (pH7.5)/0.1M-NaCl/0.2% SDS to remove loosely bound RNA, and the poly(A)-rich bound RNA was eluted with 0.01M-Tris/HCl (pH7.5)/0.2% SDS. The poly(A)-rich RNA fraction was made 0.2M in sodium acetate (pH5.6) and the RNA precipitated with 2.5vol. of ethanol (RNA concentrations were determined by using the relationship $A_{260}^{1\,mg/ml}=20$).

Cell-free protein synthesis

Wheat-germ cell-free system. This cell-free protein-synthesizing system was prepared from wheat germ as described by Roberts & Paterson (1973), except that a 23000g supernatant (S-23) was prepared instead of a S-30 fraction. Protein-synthesis assays were in final volumes of 10μl as described by Gordon & Payne (1976). Each assay contained various amounts of K^+, Mg^{2+}, spermine or spermidine, tendon RNA and 10mM-Hepes, 2mM-dithiothreitol, 1mM-ATP, 20μM-GTP, 8mM-phosphocreatine, 50mM each of 19 unlabelled amino acids except methionine or proline, and [^{35}S]methionine or [2,3,4,5-^{3}H]proline. Samples were incubated for up to 3h at 23°C. At the end of the incubation period, 1μl samples were spotted on strips of 3MM Whatman paper, which were then left overnight at 4°C in 10% (w/v) trichloroacetic acid containing 1% (w/v) proline or methionine. The strips were washed three times in 5% (w/v) trichloroacetic acid, twice in ethanol and dried before determination of incorporated radioactivity by liquid-scintillation spectrometry in a toluene-based scintillant containing 0.7% (w/v) butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole].

Reticulocyte-lysate cell-free sytem. mRNA-dependent rabbit reticulocyte lysates were prepared as described by Pelham & Jackson (1976). Cell-free translation was carried out at 30°C in final volumes of 20μl containing 16μl of lysate, 1μl of tendon RNA (0.25–5μg), 1μl of [^{35}S]methionine (5μCi) or [^{3}H]-proline (10μCi) and 3μl of a master mixture consisting of 0.67M-KCl, 3.3mM-$MgCl_2$, 66mM-phosphocreatine and an amino acid mixture containing 0.3mM each of the 19 naturally occurring amino acids except methionine or proline. Incorporation of radioactivity into protein was measured by spotting 1 or 2μl portions on Whatman 3MM paper strips, followed immediately by 2×5μl of 1M-NaOH containing 0.5M-H_2O_2 (to decolorize the samples). After complete bleaching and drying at 37°C, incorporation was determined as for the wheat-germ assays.

Analysis of cell-free translation products

Electrophoresis. At the end of the reaction period, 3-times-concentrated electrophoresis sample buffer (Laemmli, 1970) was added to each incubation, the mixture heated at 100°C for 2min and proteins were analysed by discontinuous SDS/polyacrylamide-slab-gel electrophoresis with a 3% stacking gel and 10% separating gel (Laemmli, 1970). Electrophoresis was for 5–6h at 25mA or 16h at 10mA, 20°C. Gels were fixed in 10% (v/v) acetic acid/25% (v/v) meth-

anol and then impregnated with PPO for fluorography, dried and exposed to pre-flashed Kodak Royal X-Omat X-Ray film XH-1 (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Collagenase treatments. The collagenous nature of the translated products was assessed by their susceptibility to digestion by highly purified bacterial collagenase. At the end of the incorporation period, 2μl of a solution containing 100mM-$CaCl_2$, 50mM-*N*-ethylmaleimide, 1mM-methionine or 1mM-proline and 2μl of bacterial collagenase (1mg/ml) was added per 18μl of cell-free reaction mixture. Collagenase digestions were for 1h at 30°C. The proportion of incorporated radioactivity susceptible to collagenase digestion was determined by removing 1μl portions for trichloroacetic acid precipitation before and after incubation with enzyme. As controls some samples were incubated under the same conditions except that 2μl of sterile water was substituted for collagenase. The reaction was stopped by the addition of 2μl of 100mM-EDTA (adjusted to pH7.0 with 1M-NaOH) and 3-times-concentrated electrophoresis sample buffer for subsequent analysis on SDS/polyacrylamide gels.

Hydroxylation of reticulocyte-lysate translation products. Purified proline hydroxylase was prepared from 13-day embryonic chicks by the method of Tuderman *et al.* (1975). Reticulocyte-lysate translation products were incubated with purified proline hydroxylase (0.03–0.1μg/μl) for 1h at 37°C in a final volume of 60μl containing 0.05M-Tris/HCl buffer, pH7.8, 2μg of bovine serum albumin/μl, 0.1mM-dithiothreitol, and the cofactors 0.05mM-$FeSO_4$, 0.1μg of catalase/μl, 2mM-ascorbate and 0.5mM-α-oxoglutarate (Halme *et al.*, 1970). Proline hydroxylase was omitted from control assays. In some samples at the end of the hydroxylation reaction, highly purified bacterial collagenase was added as described in the previous section. The enzyme reaction was terminated either by the addition of 3-times-concentrated electrophoresis sample buffer for subsequent polyacrylamide-gel analysis or by

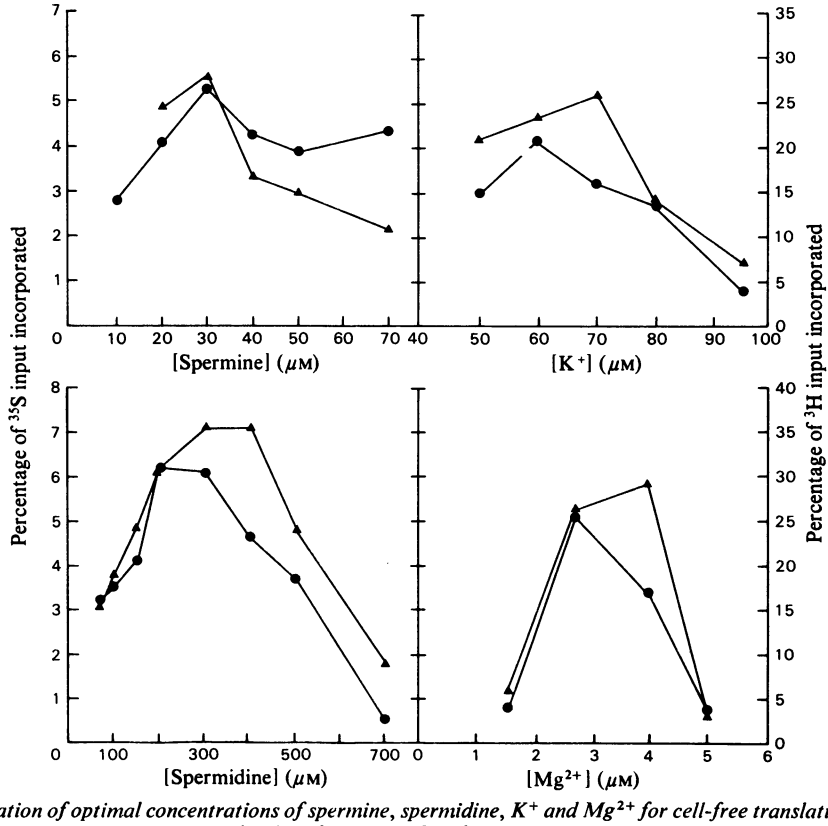

Fig. 1. *Determination of optimal concentrations of spermine, spermidine, K^+ and Mg^{2+} for cell-free translation of chick tendon poly(A)-rich RNA in the wheat-germ system*

Translation assays contained 0.2μg of RNA/μl, 5μCi of [^{35}S]methionine (●) or 10μCi of [^{3}H]proline (▲) and various amounts of spermine, spermidine, K^+ and Mg^{2+}. Samples were incubated for 3h at 23°C.

adding 50% (w/v) trichloroacetic acid (containing 0.1% proline) to 10% (w/v) final concentration for hydroxy[^{3}H]proline determinations. Trichloroacetic acid precipitates were washed three times with 80% (v/v) acetone, and the dried precipitates hydrolysed in 6M-HCl for 24h at 110°C. Hydroxy[^{3}H]proline in the hydrolysates was determined by the method of Juva & Prockop (1966).

Results

Is procollagen mRNA translated efficiently in the wheat-germ cell-free system?

An important prerequisite for the detection of procollagen mRNA in RNA extracts is a cell-free system capable of synthesizing pro-α chains of collagen. We initially chose the wheat-germ cell-free system (Roberts & Paterson, 1973) for its ease of preparation, low endogenous mRNA content and because it had been used successfully by others to translate procollagen mRNA (Harwood *et al.*, 1975; Boedtker *et al.*, 1976; Adams *et al.*, 1977; Frischauf *et al.*, 1978; Howard *et al.*, 1978). Because wheat germ from different commercial sources yields cell-free systems of differing efficiencies, the wheat germ used in this study was obtained from the source reported by Marcu & Dudock (1974) to give extracts with the best cell-free synthesis.

Total RNA was extracted from embryonic-chick tendon cells and poly(A)-rich RNA was then isolated by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972) as described in the Experimental section. This poly(A)-rich RNA was used to prime the wheat-germ system in the presence of [^{35}S]methionine or [^{3}H]proline. Concentrations of K^+, Mg^{2+}, spermine or spermidine, and RNA were varied to obtain optimal conditions for maximum synthesis of procollagen polypeptides on the basis of both incorporation of radioactivity and size of translation products as judged by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). As shown in Figs. 1 and 2, incorporations of [^{35}S]methionine and [^{3}H]-proline were optimal when cofactors were present at the following concentrations: 60–70mM-K^+, 2.7–4.0mM-Mg^{2+}, 300–400μM-spermidine and 0.2μg of poly(A)-rich RNA/μl. In experiments where spermidine was replaced by spermine (10–70μM), the incorporation of radioactivity and the molecular size of the translation products were significantly lower. Hence in all subsequent experiments 300μM-spermidine was used for cell-free translation together with 60mM-K^+, 2.7mM-Mg^{2+} and 0.2μg of poly(A)-rich RNA/μl. All cell-free translations with the wheat-germ system were conducted at 23°C, since incubation at 30°C resulted in less synthesis of high-molecular-weight polypeptides (K. S. E. Cheah, unpublished work). Under these optimal conditions,

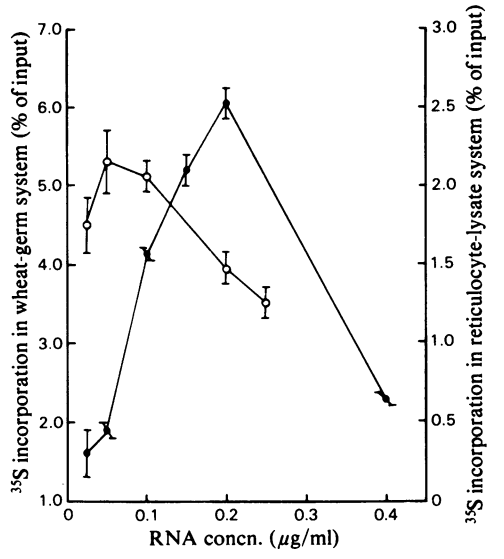

Fig. 2. *Determination of optimal concentrations of chick tendon poly(A)-rich RNA for cell-free translation in the wheat-germ and reticulocyte-lysate systems*

Various concentrations of chick tendon poly(A)-RNA were translated in the wheat-germ (●) and reticulocyte-lysate (○) systems. Wheat-germ assays contained 60mM-K^+, 2.7mM-Mg^{2+}, 300μM-spermidine and 5μCi of [^{35}S]methionine and were incubated at 23°C for 3h. The composition of reticulocyte-lysate assays was as described in the Experimental section and incubations were for 2h at 30°C. Values given are ± range of duplicate assays.

incorporation of [^{35}S]methionine reached a maximum after an incubation of 3h (Fig. 3).

The collagenous nature of the cell-free translation products was assessed by determining their susceptibility to digestion by highly purified bacterial collagenase. This enzyme specifically cleaves amino acid sequences that are characteristically present in collagen (for review see Weiss, 1976). Using this approach we found that the amounts of incorporated radioactivity made trichloroacetic acid-soluble by collagenase treatment were approx. 16% for both [^{35}S]methionine-labelled and [^{3}H]proline-labelled translation products (Table 1).

To determine whether complete synthesis of pro-α chains had been achieved, the translation products were analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 4 (slots 4 and 6) shows the spectrum of [^{35}S]methionine-labelled proteins synthesized under optimal conditions, and similar patterns were obtained with [^{3}H]proline. Most of the labelled polypeptides synthesized had molecular weights less than 100000. Polypeptides with mobility corresponding to pro-α chains were present in very small amounts

and were seen as very faint bands only after over-exposure of the fluorograms. Collagenase treatment of the translation products resulted in the removal of the faint bands migrating in the region of α- and pro-α-chains (Fig. 4). In addition, the radioactivity forming a background between bands was decreased

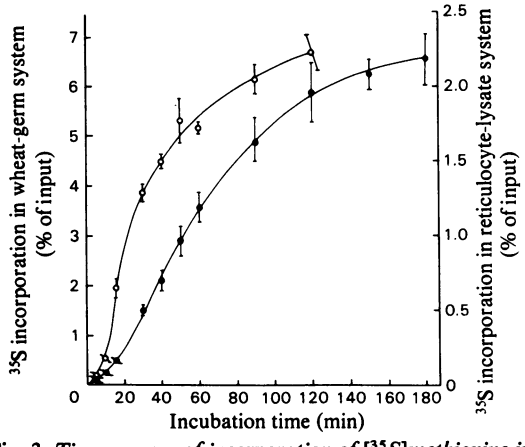

Fig. 3. *Time-courses of incorporation of [^{35}S]methionine in the wheat-germ (●) and reticulocyte-lysate (○) systems in response to chick tendon poly(A)-rich RNA*
Incubations were conducted under optimal conditions as described in the text.

Table 1. *Translation of chick tendon poly(A)-rich RNA in wheat-germ and reticulocyte-lysate cell-free systems: collagenase-sensitivity of cell-free translation products*
Chick tendon poly(A)-rich RNA was translated in the wheat-germ and reticulocyte-lysate cell-free systems in the presence of either [^{3}H]proline or [^{35}S]methionine under optimal conditions (see the Results section). Cell-free products were then incubated with highly purified bacterial collagenase for 1h at 30°C. Percentage collagenase-sensitivity was determined by subtracting the percentage of radioactivity (c.p.m.) made trichloroacetic acid-soluble in control samples (incubated without enzyme) from that solubilized in the presence of collagenase. Endogenous proteinase activity was 4% for wheat germ and 20% for reticulocyte lysate. Values given are means of triplicate determinations ± range of values.

Cell-free system	Amino acid incorporated	Percentage of incorporated radioactivity that is collagenase-sensitive
Wheat germ	[^{3}H]Proline	16.9 ± 3.0
	[^{35}S]Methionine	15.6 ± 6.0
Reticulocyte lysate	[^{3}H]Proline	33.4 ± 2.0
	[^{35}S]Methionine	17.1 ± 3.6

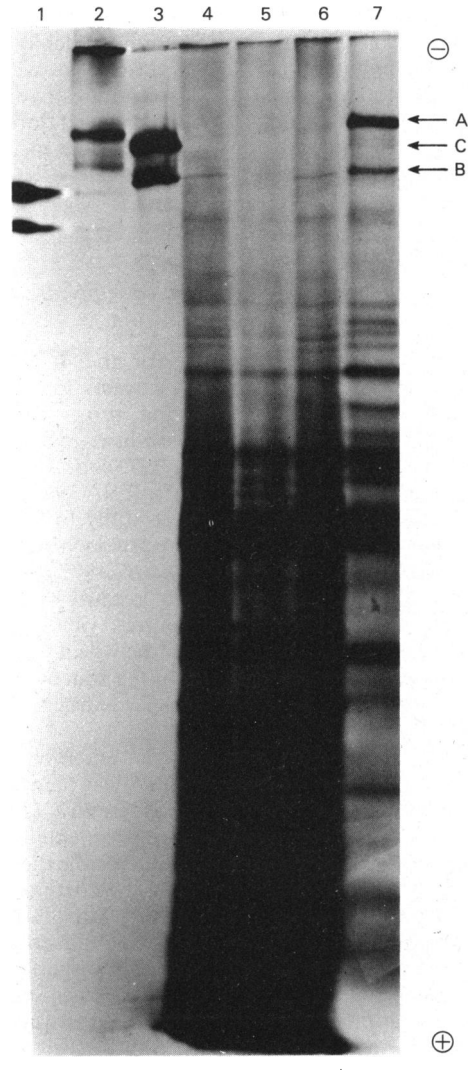

Fig. 4. *Fluorogram of [^{35}S]methionine-labelled polypeptides synthesized in the wheat-germ and reticulocyte-lysate systems in response to chick tendon poly(A)-rich RNA*
Products of translation were compared with [^{14}C]-proline-labelled standards by electrophoresis on a 10% SDS/polyacrylamide gel for 6.5h at 25mA, 20°C. The collagenous nature of the wheat-germ products synthesized under optimal conditions (see Figs. 1 and 2) was assessed by collagenase treatment for 1h at 30°C. The samples in slots 1–7 are as follows: 1, α1 and α2 chains; 2, pro-α1 and pro-α2 chains; 3, unhydroxylated pro-α1 and pro-α2 chains; 4, wheat-germ products; 5, wheat-germ products and collagenase; 6, wheat-germ products, control incubation with water; 7, reticulocyte-lysate products. The major high-molecular-weight collagenous polypeptides have been designated A and B for reference purposes (see the text).

after collagenase treatment, presumably owing to the removal of the spectrum of incomplete collagenous polypeptides.

Previous studies on the translation of procollagen mRNA in the wheat-germ system have indicated that, when spermidine was not included in the incubation mixture, maximal synthesis of collagenous polypeptides did not necessarily coincide with maximal incorporation of radioactivity (Benveniste *et al.*, 1976; Harwood *et al.*, 1975). In particular it was found that concentrations of K^+ between 150 and 180mM increased the yield of high-molecular-weight collagenase-susceptible polypeptides. Accordingly, we analysed the cell-free products after translation in the presence of 180mM-potassium acetate, but no improvement in the yield of high-molecular-weight products was observed. This finding is in agreement with the work of Boedtker *et al.* (1976), where increasing K^+ concentrations in the presence of spermine did not improve yields of pro-α-chain-sized polypeptides. In other experiments the amino acid composition of the cell-free incubation mixture was altered, for it was considered that the limited ability to synthesize complete pro-α chains might arise from a restricted availability of the amino acids glycine and proline, which occur in very high proportions in collagenous molecules. Amino acid mixtures made up in the proportions characteristic of procollagen (Monson *et al.*, 1975) were used, but no increased synthesis of high-molecular-weight polypeptides was observed.

In the wheat-germ system, therefore, although good incorporation of radioactivity was achieved, only small amounts of completed pro-α chains were synthesized. There are a number of possible explanations which singly or in combination could have given this result. For example, the poly(A)-rich RNA from chick tendon cells could have been degraded during the isolation procedures, although stringent precautions against nuclease activity were used throughout. Alternatively the added mRNA could have been degraded during the cell-free translation period, for endogenous nuclease activity has been detected in the wheat-germ system (Hunter *et al.*, 1977; Tse & Taylor, 1977). A further real possibility is the consideration that the wheat-germ preparation was unable to complete the synthesis of pro-α chains through lack of appropriate factors. To distinguish between the first possibility and the other two, the same poly(A)-rich RNA preparation was used to programme an alternative cell-free protein-synthesizing system.

Evidence for the cell-free synthesis of pro-α chains in the reticulocyte-lysate system

The poly(A)-rich RNA isolated from chick tendon cells was translated in a reticulocyte-lysate cell-free system pre-treated to remove endogenous mRNA (Pelham & Jackson, 1976). Maximum incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable polypeptides was achieved with RNA concentrations between 0.05 and 0.1 μg/μl after incubation for 2h at 30°C (Fig. 2). However, radioactivity incorporated by the reticulocyte lysate was lower than in the wheat-germ system. This observation can probably be attributed to the larger endogenous pool of amino acids in reticulocyte lysates than in wheat germ (Palmiter *et al.*, 1977; Rowe *et al.*, 1978).

The susceptibility of the reticulocyte-lysate translation products to digestion by bacterial collagenase was again used to assess the collagenous nature of the synthesized polypeptides. To compensate for the presence of EGTA in the reticulocyte lysate (Pelham & Jackson, 1976), excess $CaCl_2$ was added to satisfy the Ca^{2+} requirement of collagenase. It is noteworthy that the specificity of collagenase under these conditions was confirmed by its ability to digest completely [^{14}C]proline-labelled procollagen (type I), whereas [^{35}S]methionine-labelled globin remained unaffected. As shown in Table 1, approx. 17% of the [^{35}S]methionine-labelled translation products became soluble in cold trichloroacetic acid after collagenase digestion. There was therefore no significant difference in the amounts of collagenase-sensitive [^{35}S]methionine-labelled polypeptides in both the reticulocyte-lysate and wheat-germ systems. In contrast, where the reticulocyte-lysate translation products were labelled with [^{3}H]proline, over 30% of the synthesized polypeptides were made trichloroacetic acid-soluble by collagenase digestion. This value represents twice that obtained with the wheat-germ products.

Analyses of the reticulocyte-lysate translation products by SDS/polyacrylamide-gel electrophoresis revealed the synthesis of two heavily labelled high-molecular-weight polypeptides (bands A and B, Fig. 5) which migrated in the region of standards of chick tendon pro-α1 and pro-α2 chains. Also of interest was band C, which migrated in a position intermediate between bands A and B and had an intensity which varied between different translation assays (Figs. 4 and 5). The collagenous nature of bands A and B was demonstrated by their susceptibility to collagenase digestion (Fig. 5). Several other high-molecular-weight bands were removed by collagenase, including band C, and also bands D and E, which have molecular weights of approx. 130000 and 90000. As with the wheat-germ system, collagenase treatment decreased the background radioactivity between bands throughout the gel (Fig. 5).

Bands A and B had mobilities similar to, but not identical with, those of pro-α1 and pro-α2 chains respectively. Although little difference was observed between the mobilities of band A and the pro-α1 chain, band B migrated markedly ahead of the pro-α2

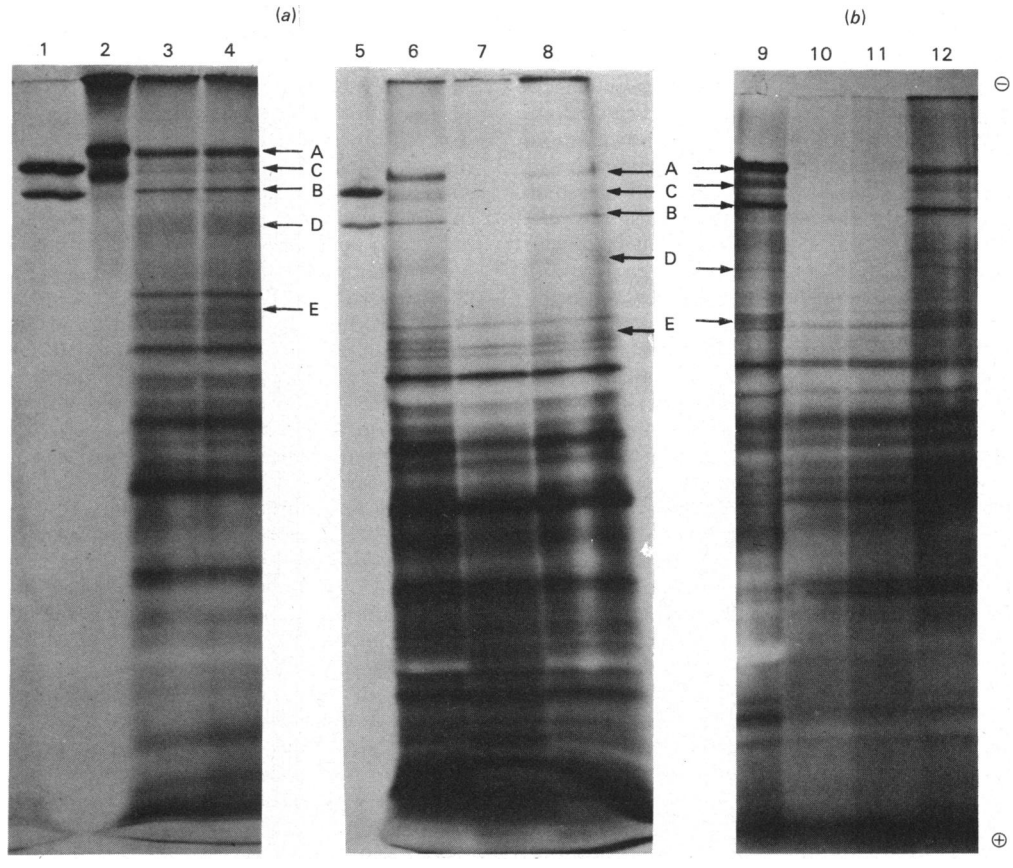

Fig. 5. *Fluorograms of [^{35}S]methionine- and [^{3}H]proline-labelled translation products synthesized in response to tendon poly(A)-rich RNA in the reticulocyte-lysate system and incubated with or without collagenase*

Cell-free products were analysed directly on 10% SDS/polyacrylamide gels or incubated with or without collagenase for 1 h at 30°C. (*a*) [^{35}S]Methionine-labelled products electrophoresed for 4.5 h at 25 mA, 20°C; (*b*) [^{3}H]proline-labelled products electrophoresed for 6 h at 25 mA, 20°C. The samples in slots 1–12 are as follows: 1 and 5, unhydroxylated pro-α1 and pro-α2 chains; 2, pro-α1 and pro-α2 chains; 3 and 4, 6 and 9, translation products of three different RNA preparations; 7, 10 and 11, collagenase-treated products; 8 and 12, control incubations with water. The major collagenous polypeptides have been designated A–E for reference purposes (see the text).

chain. However, since the translation products do not undergo hydroxylation in the reticulocyte-lysate system (Table 2), the cell-free products might be expected to migrate in positions corresponding to unhydroxylated pro-α1 and unhydroxylated pro-α2 chains, which have been shown to migrate faster than the corresponding hydroxylated polypeptide in composite agarose/polyacrylamide gels (Harwood *et al.*, 1977). This difference in mobility between hydroxylated and unhydroxylated pro-α chains is also demonstrable in SDS/polyacrylamide gels (Fig. 5). When bands A and B are compared with the unhydroxylated procollagen polypeptides, band A migrated markedly slower than unhydroxylated pro-α1 chains and band B migrated with a slightly lower mobility than unhydroxylated pro-α2 chains (Fig. 5).

Hydroxylation of the cell-free products with proline hydroxylase

To confirm that the products of translation included collagenous molecules, further experiments were undertaken to assess their ability to act as substrate for proline hydroxylase. [^{3}H]Proline-labelled polypeptides synthesized in the reticulocyte-lysate system were incubated with purified enzyme, and the amounts of hydroxy[^{3}H]proline in trichloro-

Table 2. *Hydroxylation of proline residues in reticulocyte-lysate translation products by collagen proline hydroxylase*
Translation products were incubated with or without proline hydroxylase (0.09 μg/ml) for 1 h at 30°C as described in the Experimental section. The enzyme reaction was terminated by the addition of cold trichloroacetic acid to 10%, and hydroxyproline content of the precipitate was determined by the assay of Juva & Prockop (1966) after hydrolysis of the samples in 6M-HCl for 24 h at 110°C. The proportion of hydroxyproline present in radioactive standards of chick tendon procollagen, protocollagen and collagen were also determined. No detectable hydroxyproline was found if samples were treated with collagenase after incubation with proline hydroxylase.

Expt. no.	Substrate	Incubation with or without proline hydroxylase	$\frac{100 \times \text{Hydroxyproline (d.p.m.)}}{\text{Total radioactivity (d.p.m.)}}$
1.	Translation products	−	0
		+	6.3
		+	4.9
	Unhydroxylated procollagen	−	0
		+	9.7
2.	Translation products	−	0
		+	6.8
		+	6.2
	Unhydroxylated procollagen	−	0
		+	15.7
3.	^{14}C-labelled procollagen standard	−	40.6
	^{14}C-labelled collagen standard	−	50.9

acetic acid-precipitable polypeptides were determined by the method of Juva & Prockop (1966). Approx. 6% of the ^{3}H in the translation products was present as hydroxy[^{3}H]proline after the enzyme incubation (Table 2), whereas up to 15% of the [^{14}C]proline residues in ^{14}C-labelled protocollagen were hydroxylated by the same enzyme preparation under similar conditions. No hydroxy[^{3}H]proline was detected in control assays from which proline hydroxylase was omitted, indicating that the reticulocyte lysate possessed no detectable proline hydroxylase activity. In further studies with the hydroxylated products of cell-free translation it was demonstrated that all the newly synthesized hydroxy[^{3}H]proline became soluble in cold trichloroacetic acid after digestion with bacterial collagenase.

Of considerable interest in this series of experiments was the observation that several of the products of cell-free translation had altered mobilities on SDS/polyacrylamide gels after hydroxylation with proline hydroxylase. The data in Fig. 6 demonstrate that hydroxylation resulted in a decrease in mobility of bands A, B and C as compared with the unhydroxylated cell-free translation products, and the extent of this change in mobility was influenced by the amount of enzyme used in the assay (Fig. 6). Maximal change was observed after hydroxylation with 0.06 μg of enzyme/μl (for details see the Experimental section), which resulted in band A migrating noticeably slower than normal pro-α1 chains. Under these conditions hydroxylated band B migrated more slowly than its unhydroxylated counterpart, but continued to migrate ahead of the fully hydroxylated and glycosylated pro-α2 chains of the procollagen standard (Fig. 6).

Determination of the relative proportions of the collagenous precursor polypeptides synthesized

Chick tendon procollagen is composed of two pro-α1 and one pro-α2 chains. This 2:1 ratio is also reflected in the proportions of pro-α1 and pro-α2 chains synthesized by chick tendon cells (Harwood *et al.*, 1977). To determine whether this ratio is maintained during the cell-free translation of procollagen mRNA, a comparison was made of the relative amounts of the bands corresponding most closely to pro-α1 and pro-α2 chains, i.e. bands A and B. The intensities of these bands were determined by densitometric measurements of the SDS/polyacrylamide-gel fluorograms, which were prepared under the conditions described by Laskey & Mills (1975). Under these conditions the X-ray plate is preflashed to ensure linearity between intensity of bands and amounts of radioactivity incorporated. These studies indicated that the ratio of radioactivity in band A to that in band B was 2:1 (Table 3), suggesting that the translatable mRNA species for pro-α1 and pro-α2 chains are also present in a 2:1 ratio.

Discussion

Collagen accounts for a major proportion of protein synthesized by embryonic-chick tendon cells (Dehm & Prockop, 1971; Rowe *et al.*, 1978), and as a preliminary to the detailed characterization of procollagen mRNA the experiments described here were undertaken to establish an efficient system *in vitro* capable of synthesizing pro-α chains of collagen. Analysis by SDS/polyacrylamide-gel electrophoresis of the translation products synthesized in the wheat-

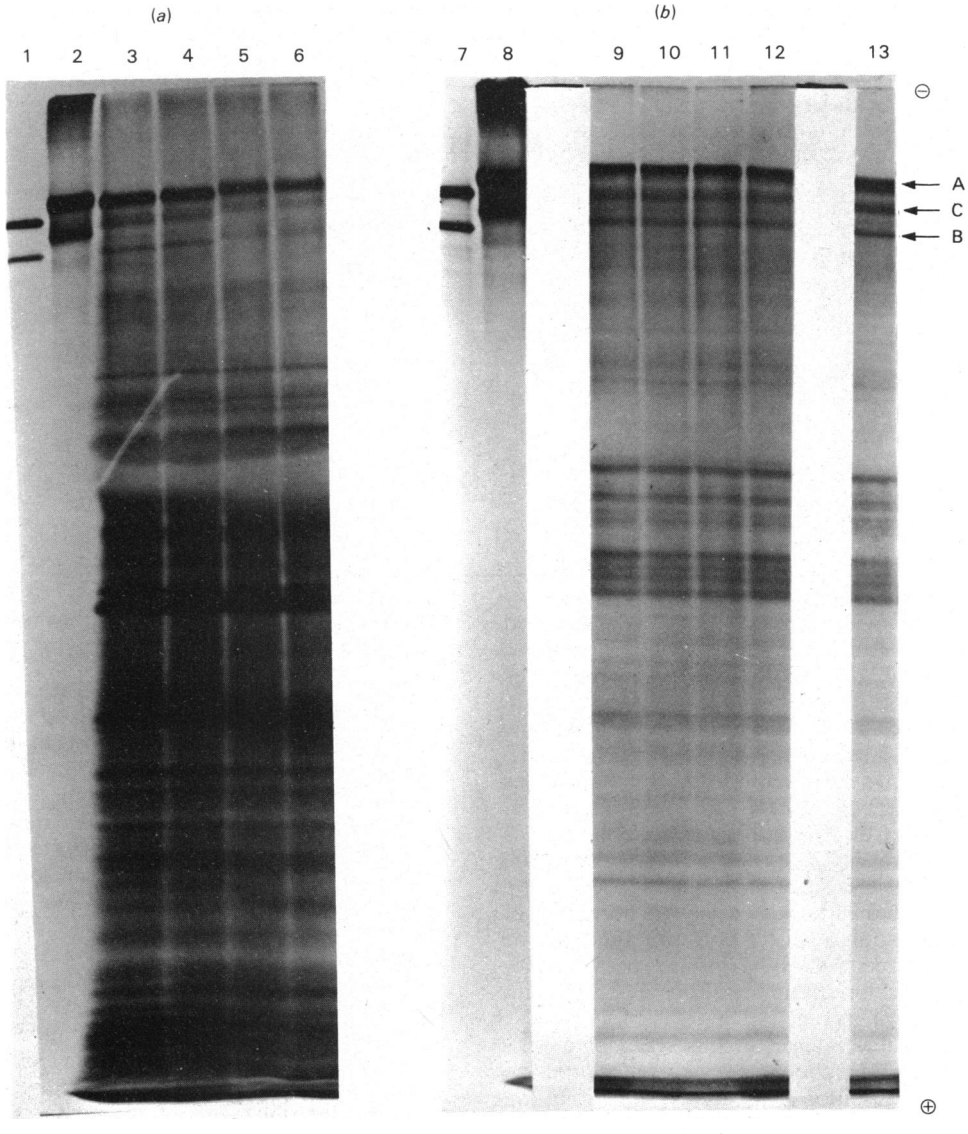

Fig. 6. *Fluorograms of [^{35}S]methionine- and [^{3}H]proline-labelled reticulocyte-lysate translation products incubated with and without proline hydroxylase*

Translation products were incubated for 1 h at 30°C with either proline hydroxylase or water (see the Experimental section) and analysed on 10% SDS/polyacrylamide gels. (*a*) [^{35}S]Methionine-labelled products; (*b*) [^{3}H]proline-labelled products. The samples in slots 1–13 are as follows: 1 and 7, unhydroxylated pro-α1 and pro-α2 chains; 2 and 8, pro-α1 and pro-α2 chains; 3, 4 and 13, control incubations with water; 5, 6, 10 and 11, incubated with 0.09 μg of enzyme/μl; 9, with 0.06 μg of enzyme/μl; 12, with 0.12 μg of enzyme/μl. The major collagenous polypeptides have been designated A, B and C for reference purposes (see the text).

germ system in response to poly(A)-rich RNA from tendon cells revealed the synthesis of only very small amounts of collagenase-sensitive polypeptides similar to pro-α chains (Fig. 4). A bias towards the synthesis of polypeptides smaller than pro-α chains has also been observed in other reports, where mRNA extracts from fibroblasts have been translated in wheat-germ systems (Benveniste *et al.*, 1976; Adams *et al.*, 1977). In contrast, when the same poly(A)-rich RNA sample from chick tendon cells was translated in mRNA-

Table 3. *Translation in the reticulocyte-lysate system: relative amounts of radioactivity incorporated into bands A and B*

Preflashed fluorograms (Laskey & Mills, 1975) of SDS/polyacrylamide gels of translation products and procollagen standards were scanned in a Joyce–Loebl densitometer and the areas under peaks corresponding to pro-α1 and pro-α2 chains (Bands A and B) computed. Values given are means ± S.D. obtained from scans of five different experiments, in each of which at least two slots were scanned.

Protein scanned	Amino acid incorporated	Radioactivity in band A relative to band B
Procollagen type I	[^{14}C]Proline	1.91 ± 0.30
Protocollagen type I	[^{14}C]Proline	2.30 ± 0.50
Translation products	[^{3}H]Proline	2.04 ± 0.66
	[^{35}S]Methionine	1.99 ± 0.63

dependent reticulocyte lysates we observed a significant synthesis of collagenase-susceptible polypeptides similar in mobilitiy to pro-α1 and pro-α2 chains (bands A and B, Figs. 4 and 5). Although the banding patterns obtained indicate that a number of the low-molecular-weight species synthesized were identical in both systems, there was no marked bias towards smaller polypeptides, as observed in the wheat-germ system (Fig. 4). Clearly, therefore, the extremely low yield of pro-α chains obtained with the wheat-germ system was not a consequence of degradation of the RNA sample during isolation, but impaired translation caused by endogenous nuclease activity in the wheat-germ preparation (Pelham & Jackson, 1976; Hunter *et al.*, 1977; Tse & Taylor, 1977) cannot be discounted. It is unlikely, however, that the discrete small polypeptides synthesized in the wheat-germ system were generated by specific endoribonuclease action on procollagen mRNA, since these low-molecular-weight products were collagenase-resistant (Fig. 4).

In the reticulocyte lysate, bands A and B were synthesized in a 2:1 ratio. This result correlates well with the synthesis of pro-α1 and pro-α2 chains in a 2:1 ratio observed in calvaria (Fessler *et al.*, 1975), tendon cells (Harwood *et al.*, 1977) and in studies with collagen-synthesizing polyribosomes (Kerwar *et al.*, 1972; Vuust, 1975). However, in the wheat-germ system the very faint bands corresponding to bands A and B were not in a 2:1 ratio. Instead, band B was more heavily labelled than band A, which was often so faint as to be undetectable except with marked over-exposure of the fluorogram. In this context, it is noteworthy that a close examination of fluorograms in some previous reports in which the wheat-germ system was used (Adams *et al.*, 1977; Frischauf *et al.*, 1978; Howard *et al.*, 1978) also show a similar heavier labelling of the band corresponding to pro-α2 chains as compared with pro-α1 chains. Since the pro-α1 chain is believed to be slightly longer than the pro-α2 chain (for review see Fessler & Fessler, 1978), any restricted ability of the cell-free system to complete the translation of long mRNA molecules might be likely to result in the synthesis of more of the shorter pro-α2 chains despite the potential for synthesis of pro-α1 and pro-α2 chains in a 2:1 ratio.

Comparison of the relative proportions of collagenase-sensitive [^{35}S]methionine- and [^{3}H]-proline-labelled polypeptides in both systems also suggests that, despite high incorporation values, maximum synthesis of collagenous polypeptides was not achieved in the wheat-germ system. In reticulocyte lysates approximately twice the proportion of incorporated [^{3}H]proline is collagenase-sensitive compared with incorporated [^{35}S]methionine (Table 1). This difference in collagenase susceptibility between proteins labelled with two different amino acids was not observed in the wheat-germ system. [^{35}S]Methionine may be assumed to be a general marker for proteins, whereas [^{3}H]proline preferentially labels collagenous proteins. Therefore, either a lack of completion of the relatively longer procollagen polypeptides or specific discrimination against collagenous molecules would explain the proportionately lower incorporation of collagenase-sensitive [^{3}H]proline. In the light of the SDS/polyacrylamide-gel analyses of wheat-germ translation products discussed above, the former explanation is more likely.

Variation in the viability and vigour of seeds and isolated cereal embryos is known to be affected by the conditions of growth, harvest and subsequent storage (for review see E. H. Roberts, 1972), and relatively small decreases in viability (e.g. from 99% to 86%) are accompanied by marked decreases in protein-synthesizing capabilities (Roberts & Osborne, 1973; Roberts *et al.*, 1973; Sen & Osborne, 1977). Decreases in rye-embryo viability are also associated with the development of lesions in rRNA and increases in endogenous nuclease activity (B. E. Roberts, 1972; Osborne *et al.*, 1974; Cheah & Osborne, 1978). All these factors could similarly contribute to the variability in translation efficiencies of cell-free systems derived from different wheat-germ stocks (Marcu & Dudock, 1974) and explain, at least in part, the complete translation of procollagen mRNA by some (Harwood *et al.*, 1975; Boedtker *et al.*, 1976; Adams *et al.*, 1977; Frischauf *et al.*, 1978; Howard *et al.*, 1978) and the failure or incomplete success by others (Neufang *et al.*, 1975; Benveniste *et al.*, 1976; Zeichner & Rojkind, 1976). It is of note, however, that, even where successful translation of procollagen mRNA has been reported, in most of these studies the synthesis of pro-α1 and pro-α2 chains in a 2:1 ratio was not achieved.

In contrast, the mRNA-dependent reticulocyte-lysate cell-free system appears to be a more reliable and successful system for the translation of procollagen mRNA. Several high-molecular-weight translation products (bands A–E, Fig. 5) were susceptible to digestion by highly purified bacterial collagenase. Because the products of cell-free translation are not hydroxylated (Table 2), direct comparison of the migration of these polypeptides in SDS/polyacrylamide gels with secreted procollagen polypeptides is not valid, for the post-translational modifications of hydroxylation and glycosylation markedly influence the electrophoretic behaviour of collagenous molecules (Fig. 5). However, since bands A and B are synthesized in a 2:1 ratio and their respective mobilities are lower than those of unhydroxylated pro-α1 and pro-α2 chains (Fig. 5), it appears that these translation products might represent slightly higher-molecular-weight precursor polypeptides of procollagen. The nature and origins of bands C, D and E is uncertain, but they may be incomplete translation products. Alternatively, bands C, D and E may arise from completed chains that have undergone cleavage by endogenous proteinases (Table 1) or proteinases synthesized during translation of tendon poly(A)-rich RNA, although such proteinases must have no requirement for Ca^{2+}, which is absent from the cell-free system.

Further evidence supporting the suggestion that bands A and B represent precursors of pro-α chains, i.e. pre-pro-α1 and pre-pro-α2 chains, was obtained from studies on the hydroxylation of the translation products. Of the total proline residues incorporated 6–7% were hydroxylated by proline hydroxylase (Table 2). Since one-third of the proline residues are present in collagenase-susceptible protein (Table 1), it can be calculated that the percentage hydroxylation of the collagenous polypeptides is approx. 20%, as compared with a value of 40% for secreted chick tendon procollagen (Table 2). Hence, only half of the potential sites become hydroxylated under the conditions used. These results are similar to those reported in other studies, where the proline residues in translation products of chick procollagen mRNA have been subjected to hydroxylation with proline hydroxylase (Boedtker *et al.*, 1976; Wilczek *et al.*, 1978). Although completion of hydroxylation was not achieved, the cell-free products were found to have altered mobilities after hydroxylation (Fig. 6). In particular, the partially hydroxylated band A migrated markedly more slowly than pro-α1 chains, suggesting that this collagenous product synthesized in the reticulocyte-lysate system must be significantly larger than the pro-α1 chain of secreted procollagen.

Studies on the assembly of proteins destined for secretion have revealed that cell-free translation of the appropriate mRNA species results in the synthesis of polypeptides some 15–30 amino acids longer than the precursor molecules previously identified *in vivo* (Blobel, 1977). The 'Signal Hypothesis' proposes a role for these *N*-terminal peptides (signal peptides), which are predominantly hydrophobic in nature, in determining that the polypeptides will enter the lumen of the endoplasmic reticulum and be secreted (Blobel & Dobberstein, 1975). The above studies on the cell-free translation of chick tendon procollagen mRNA are consistent with procollagen mRNA coding for pre-pro-α chains, although on the basis of the electrophoretic studies band A appears to be much larger than would be expected for a pro-α chain with a signal peptide of 15–30 amino acids. Further studies will be required to establish the exact nature of the precursor polypeptides and the role of the pre-sequence coded in the mRNA for procollagen.

Note Added in Proof (Received 6 April 1979)

Since the completion of this work, the successful translation of procollagen mRNA from chick calvaria in a mRNA-dependent reticulocyte-lysate system has been reported by Monson & Goodman (1978).

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