Journal of Cellular Biochemistry 61:194–215 (1996)

Posttranscriptional Aspects of the Biosynthesis of Type 1 Collagen Pro-Alpha Chains: The Effects of Posttranslational Modifications on Synthesis Pauses During Elongation of the Pro α1(I) Chain

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Abstract Early studies indicated that chain elongation pauses were prominent during the in vivo synthesis of type I procollagen chains, and it was postulated [Kirk et al., (1987): J Biol Chem 262:5540–5545.] that these might have a role in the coordination of procollagen I molecular assembly. To examine this postulate, polysomes isolated from [^{14}C]-Pro-labeled 3T6 cells were subjected to SDS-PAGE. The resulting gels were Western blotted and screened with a monoclonal antibody (SP1.D8) directed against the N-terminal region of the pro α1(I) chain. The blots were fluorographed, which also permitted analysis of the pro α2(I) chain. There was a prominent pro α1 synthesis pause near the completion of full-length chain elongation, not matched by a pro α2 pause. The amount of labeled polysome-associated near-full length pro α1 chains increased in parallel with labeling time. After 24 h in culture −[^{14}C-Pro], collagen synthesis ceased but unlabeled polysome-associated pro α1 chains were readily detected by SP1.D8. Change to fresh culture medium +[^{14}C-Pro] reinitiated synthesis and permitted tracing of the newly synthesized labeled pro α chains through the polysome and intracellular compartments. The secreted procollagen molecules had a 2:1 pro α1(I):pro α2(I) chain ratio but the polysome-bound peptides did not. Pulse-chase experiments showed that near-full length pro α1(I) chains remained bound to polysomes as long as 4 h after reinitiation of translation but there was no evidence for pro α2(I) chain accumulation. The hydroxylation inhibitor α,α′-dipyridyl, and triple-helix inhibitors *cis*-hydroxyproline and 3,4 dehydroproline had minimal effects on the buildup of polysome-associated pro α1 chains. The glycosylation inhibitor tunicamycin also failed to change the final pro α1 chain pausing, but it did cause the appearance of several discrete lower molecular weight pro α1-related polypeptides that could not be accounted for simply as the result of lack of N-linked glycosylation in the C-propeptide regions. Disulfide bond experiments showed that some of the paused nascent polysome-associated pro α1(I) chains were disulfide bonded. Thus, while synthesis of pro α1(I) and pro α2(I) chains proceeds in parallel within the same ER compartments, their elongation rates are not coordinated. Interactions leading to heterotrimer formation are a late event which may affect the rate of release of the completed pro α1(I) chain from the polysome. The release of completed nascent pro α1(I) chains from their polysomal complexes is regulated by a mechanism not operating in the synthesis of pro α2(I) chains. The pro α1(I) chain release process is not connected directly with hydroxylation, glycosylation or triple-helix formation.

INTRODUCTION

The synthesis and assembly of the heterotrimeric procollagen I molecule is a complex process. The principal problems are those of the temporal and spatial coordination of translation of the pro α1(I) and pro α2(I) chains at the endoplasmic reticulum (ER) surface [Brownell and Veis, 1976; Veis and Brownell, 1977; Veis et al., 1985; Hu et al., 1995a]. However, proline and lysine hydroxylation, and hydroxylysine glycosylation, take place as co-translational processes during chain elongation [Lazarides et al., 1971; Uitto and Prockop, 1974; Brownell and Veis, 1975]. Further, chaperone protein interactions with the nascent chains may modulate premature pro α chain association and prevent misfolding within the ER or mediate correct chain selection and folding [Hu et al., 1995b]. Earlier work also raised the possibility that chain elongation synthesis pauses might be an important component of nascent chain processing [Kirk et al., 1987; Veis and Kirk, 1989]. However, a direct comparison of the translation of the pro α1(I) and pro

Received September 1, 1995; accepted October 13, 1995.

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α2(I) mRNAs suggested that the individual pro α1(I) and pro α2(I) chains are elongated independently for the most part, so that chain interaction takes place late during synthesis [Hu et al., 1995a]. Inhibition of the hydroxylation reactions blocks secretion of the completed collagen [Ramaley and Rosenbloom, 1971] but the relationship between the cotranslational hydroxylation and glycosylation modifications and the synthesis pauses has not been examined.

These data have led us to consider that although the ribosomal assemblies for pro α1(I) and pro α2(I) chains must be directed to the same ER compartments so that the nascent chains can ultimately interact, the concept of "coordination" of synthesis must be kept open in the sense that the rates of chain elongation and chain modification, as influenced by synthesis pauses and modifications, may be different for the two chains. In such a scheme the interaction between nascent co-localized pro α1(I) and pro α2(I) chains synthesized within the same time frame would take place just prior to release of the chains from their ribosomal complexes. The present work was undertaken to examine the nature of the elongation pauses more directly, and to determine the effects of the process of co-translational modification on the pauses.

EXPERIMENTAL PROCEDURES

Cell Culture

Swiss Embryo Murine Fibroblasts (3T6) were purchased from the American Type Tissue Culture Collection (ATCC, Rockville, MD). Cells were cultured in sterile 150 cm^2 flasks (Corning Costar, Cambridge, MA) with 6% CO_2/94% air. Incubation media (≈25 ml/flask) consisted of Dulbecco's Modified Medium (DMEM); 10% Fetal Bovine Serum; 1:100 Penicillin-Streptomycin; 1:100 Amphotericin B; 10 mM glutamine (all cell culture reagents were from Gibco, Grand Island, NY). Ten μg/ml ascorbate was included for at least 24 h prior to collection of polysomes. The Penicillin and Amphotericin B were omitted from the incubation media 24 h prior to polysome collection and during radioactive isotope labeling periods. The 3T6 cells were obtained from ATCC at passage #85. They were not used beyond passage #105, since collagen production decreased after that point.

Polysome Collection

Four flasks of subconfluent cells, ≈3 × 10^9 cells/flask, were grown for 24 h in ascorbate. The media was removed and fresh media of the same composition, but including 2 μCi/ml ^{14}C-proline (Amersham, Arlington Heights, IL, sp. act. >250 mCi/mmol) or 20 μCi/ml ^{35}S-methionine (Amersham, sp. act. >500 mCi/ml), was added. The cells were incubated for the requisite period, then 100 μg/ml cycloheximide was added to block further synthesis and stabilize the polysome complexes. After a 10 min incubation at 37°C, the media was removed for ammonium sulfate precipitation of the secreted procollagen. Four ml of lysis buffer (10 mM HEPES; 10 mM KCl; 3 mM $MgCl_2$; 0.2% Triton X-100 [Pierce, Rockford, IL]; 0.05% Na deoxycholate; 200 μg/ml heparin; 2 mM DTT; 100 μg/ml cycloheximide; 0.05 μg/ml leupeptin [Boehringer Mannheim, Indianapolis, IN]; 0.7 μg/ml pepstatin [Boehringer Mannheim]; and 0.25 M sucrose) was added to each flask of cells. All stock solutions used in cell lysis and polysome collection were treated with diethylpyrocarbonate (DEPC) to a final volume of 0.1% for 24 h at room temperature, with the exception of the protease inhibitor solutions. After treatment, solutions were autoclaved in order to remove DEPC. Protease inhibitors were dissolved in deionized, DEPC-treated water. Cells were incubated for 10 min at 4°C, then dislodged from the flasks by agitation. The cells were lysed by pipetting up and down through a sterile Falcon 10 ml plastic pipette 3 times. The lysates were transferred to cooled, DEPC-treated polycarbonate tubes and centrifuged for 10 min at 12,000 rpm in a Beckman (Palo Alto, CA) Model L8-70 Ultracentrifuge and 13.1 rotor. The nuclei, membranes and unlysed cells were pelleted. The supernatant was then centrifuged at 100,000 × g for 1 h in a SW40.1 rotor. Polysomes were collected as the pellet.

Co- and/or Posttranslational Modification Effects

To determine the effects of protein modification on the translation process, cells were preincubated in the presence of 3,4 dehydroproline (90 min, 25 μg/ml), tunicamycin (3 h, 4 μg/ml), α,α′-dipyridyl (30 min, 0.3 mM) or cis-hydroxyproline (30 min, 200 μg/ml) at 37°C. These effector-containing media were replaced with fresh labeling media containing the effector plus ^{14}C-proline (2 μCi/ml) and incubated for an

additional 30 min. Synthesis was terminated and the polysomes were collected as above. In one experiment cells were incubated with cis-hydroxyproline but labeled with ^{14}C-lysine (2 μCi/ml) (Amersham, >250 mCi/mmol).

Sucrose Gradients and Polysome Fractionation

A DEPC-treated Hoeffer (San Francisco, CA) SG series gradient maker was used to prepare 12 ml linear 15–50% sucrose gradients. The stock sucrose solutions were prepared in 10 mM HEPES; 10 mM KCl; 3 mM $MgCl_2$; 0.2 mg/ml heparin; 2 mM DTT; 100 μg/ml cycloheximide; 10 mM phenylmethylsulfonyl fluoride (PMSF) solution. The post-nuclear cell lysate supernatant or polysome pellet in lysate buffer was overlayed onto the gradient and centrifuged at 41,000 rpm for 110 min in a Beckman SW40.1 rotor. Gradient fractions were collected with an Isco (Lincoln, NE) Model 185 Density Gradient Fractionator.

Antibody Purification

Monoclonal anti-pro α1(I)-N-propeptide antibody, SP1.D8, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine and the Department of Biology, University of Iowa, under contract N01-HD-6-2915 from the NICHD. A recombinant Protein G column (Pierce) was used to purify the SP1.D8 ascites fluid. The column was prewashed with 0.1 M glycine, pH 3.0, and equilibrated with PBS at pH 6.0. Ascites fluid diluted 1:1 in PBS was loaded (2 ml) and the column was eluted with 0.1 M glycine, pH 3.0. The eluate was immediately neutralized and dialyzed vs. PBS, pH 6.0. The final concentration of antibody was 1.4 mg/ml.

Gel Electrophoresis and Immunoblotting

Acrylamide gels were prepared according to the Laemmli (1970) procedure and transferred to nitrocellulose and immunoblotted according to the procedure of Towbin et al. [1979]. The gel sample buffer was 0.05 M Tris, pH 6.8, 1.0% SDS, 6% sucrose, 4% β-mercaptoethanol and 0.0006% w/v Bromophenol Blue. In disulfide bonding experiments the β-mercaptoethanol was omitted. Blots were blocked by incubation in 20 mM Tris, pH 7.4, 0.5 M NaCl, 2% bovine serum albumin overnight at 4°C or for 2 h at room temperature. Blots were incubated with 1:4000 SP1.D8 as primary antibody and developed with the Vector ABC avidin/biotin system (Vector Laboratories, Burlingame, CA) and 250 μg/ml diaminobenzimidine. Washes were made with 20 mM Tris, pH 7.4, 0.5 M NaCl, 0.1% Tween-20 solution.

Q-Sepharose Chromatography-Isolation of t-RNA Associated Peptides From the Polysome Pellet

The procedure of Bergman and Kuehl [1977] was used, essentially as modified by Kirk et al. [1987]. Q-Sepharose Fast Flow beads (Pharmacia, Piscataway, NJ) in 2 ml columns, were equilibrated in start buffer (STB) consisting of 6 M urea, 0.1% Brij-35, 0.1 M NaCl, 0.1 M NH_4 formate, pH 4.7. The samples, either polysome pellets, 100 μg E. coli tRNA (BRL, Grand Island, NY) or 80,000 cpm of standard procollagen were dissolved in 400 μl suspension buffer (SUB) 6 M urea, 1.0% Brij-35, 0.1 M NaCl, 0.9 M NH_4 formate, 0.5% SDS, 20 mM EDTA) and centrifuged in a Fisher (Pittsburgh, PA) Model 235 Micro-centrifuge for 10 min. The supernatant was diluted to 4 ml in STB and the RNA content determined by optical density at 260 nm. The total volume was then loaded onto the Q-Sepharose column, and the column was washed with STB. The eluate optical density was monitored at 260 nm and 1 ml fractions were collected. The total void volume (designated as "flowthrough" [FT]) was collected. After the OD was at baseline, the column was washed with STB containing 1.0 M NaCl until baseline absorbance was obtained again. The eluted fractions were designated as "retained" (RT). When ^{14}C-proline labeled polysomes or media procollagen were examined, 10 μl of each fraction was dissolved in 5 ml Beckman Ready Solv scintillation cocktail and counted in a Beckman Model 6800 scintillation counter.

When disulfide bonding in the polysome pellets was examined, a smaller 200 μl column was prepared in a blue Rainin (Woburn, MA) sterile pipette tip, plugged with sterile glass wool. Cells were lysed in lysis buffer prepared with 50 mM iodoacetamide to prevent disulfide bonding or disulfide bond interchange during cell lysis. In the disulfide bonding experiments, all of the Q-Sepharose buffers contained 50 mM iodoacetamide.

Cetyltrimethylammonium Bromide (CTAB) Precipitation of tRNA-Bound Polysome-Associated Nascent Peptides

Polysome pellets isolated from 3T6 cells in the presence of 50 mM iodoacetamide were resuspended in 125 μl of a buffer containing 0.5 M Na acetate, pH 5.7, and 5 mM EDTA. This suspension was centrifuged for 10 min in a microfuge. Following the procedure of Gilmore and Blobel (1985) the supernatant was mixed with 125 μl 2% CTAB containing 100 μg yeast tRNA (BRL) as a carrier and allowed to incubate at 30°C for 10 min. Peptidyl-tRNA was pelleted by centrifuging for 10 min at room temperature in a Fisher micro-centrifuge. Pellets were washed twice in acetone:HCl (19:1) to remove the CTAB, then suspended in Laemmli sample buffer (no pH adjustment) either with or without β-mercaptoethanol. After incubation at 50°C for 30 min to hydrolyze the peptidyl tRNA bond, the released nascent chains were loaded onto 5–15% acrylamide gradient gels.

Isolation of Media Procollagen I as Standard

The protease inhibitors PMSF (1 mM), pepstatin (0.7 μg/ml), and leupeptin (0.5 μg/ml) were added to media collected from labeled 3T6 cells. Ammonium sulfate (176 mg/ml) was added slowly to the media at 4°C. The solution was stirred slowly until all of the salt dissolved. Stirring was stopped and the mixture was left overnight to precipitate the procollagen. The precipitate was collected by centrifugation at 10,000 rpm in a Beckman JA-14 rotor for 1 h. The pellets were resuspended in Collagenase buffer (0.1 M Tris-HCl, pH 7.5, 0.4 M NaCl, 30 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, 0.7 μg/ml pepstatin, and 0.5 μg/ml leupeptin) and stirred for 1 h at 4°C. The solutions were clarified by centrifugation for 10 min at 12,000 rpm.

Protease Digestions

Collagenase. Approximately 24–48 units (20 μl) Advanced Biofactures (Lynbrook, NY) Form III bacterial collagenase was added to 100 μl of sample dissolved in the Collagenase buffer described above. Digestion was carried out for 1 h at 37°C, then the mixture was placed on ice and diluted with Laemmli sample buffer.

Chymotrypsin. Samples dissolved in 100 μl collagenase buffer were digested with chymotrypsin at 100 μg/ml for 1 h at 4°C. Digestion was terminated by the addition of Laemmli sample buffer.

In Vitro Translations

The complete cDNAs for human pro α1(I) and pro α2(I) were transcribed to RNA from plasmids pHHα1 and pHHα2 and then translated in a rabbit reticulocyte lysate exactly as described by Hu et al. [1995a]. The translated proteins were labeled with [^{35}S]-Met and analyzed by gel electrophoresis and fluorography as described. Alternatively the polysomes were obtained from the translation mixture as pellets following centrifugation as described above. The proteins associated with the polysomes were then assayed by gel electrophoresis and fluorography.

RESULTS

Near Full Length Nascent pro α Chains Can be Detected on Intact 3T6 Cell Polysomes

Intact 3T6 cells were labeled with [^{14}C]-Pro for 30 min and then lysed. The lysate was either directly layered on a 15–50% sucrose gradient, or the polysomes were pelleted and then fractionated on a sucrose gradient. As shown in Figure 1 the cell lysate components, measured by absorbance at 260 nm, were distributed all along 15 to 50% sucrose gradients. The 40S and 60S ribosomal subunits were prominent in the total cell lysate supernatant (Fig. 1A) but were essentially removed during the prior polysome pelleting procedure (Fig. 1B). The fully assembled 80S and larger polysomal units were the principal constituents of the polysome pellets and were distributed through the higher density regions of the gradient. EDTA-treatment of a lysate, a process which extracts Mg^{2+} from fully assembled ribosome-mRNA complexes and releases the ribosomes from the mRNA, still yielded a pellet after centrifugation but this pellet produced no A_{260} peaks in the polysome region of a sucrose gradient (data not shown), indicating that the components distributed over the gradient in Figure 1B were indeed intact polysomes [Hu et al., 1995a]. The newly synthesized protein components collected in intact and EDTA-dissociated polysome pellets were compared. The pellets were prepared in otherwise identical fashion from 3T6 cells labeled with ^{14}C-proline for 30 min after a 24 h culture period in unlabeled DMEM. The EDTA-treated pellets contained fewer than 1.0% of the counts of the untreated pellets. The newly synthesized pro-

tein components of the intact and EDTA-dissociated pellets were examined by gel electrophoresis (Fig. 2). Figure 2A shows the direct fluorography of the intact (lane 2) and EDTA-dissociated pellets (lane 3). Western blots of the two preparations were stained with SP1.D8, an anti-pro α1(I) collagen antibody reactive with an epitope region located at the junction of the N-propeptide and N-telopeptide. The intact pellet, Figure 2B, lane 2′, showed several antibody-reactive bands, the most prominent of which corresponded to nearly-full length pro α1 chain. In contrast, the EDTA-treated pellet was essentially devoid of SP1.D8-reactive components (lane 3′). These data demonstrated that the majority of the radioactive and/or SP1.D8-reactive proteins associated with the polysome pellet were initially on intact polysomes and that these polysomes had a marked accumulation of near-full length pro α1(I)-chains. A control experiment was carried out to verify the specificity of the SP1.D8 antibody. The antibody recognized pro α1(I) and pNα1(I) chains obtained from the procollagen secreted into the culture media but did not react with pro α2(I) or pNα2(I) (Fig. 3, compare lanes 1 and 1′). Neither the α1(I) nor α2(I)-chains present after chymotrypsin digestion of the undenatured media procollagen reacted with the antibody (Fig. 3, compare lanes 3 and 3′). Figure 3A, lane 3 demonstrates that the secreted procollagen had the correct α1(I):α2(I) chain ratio.

In the polysome preparations, every nascent pro α1(I) chain which had elongated sufficiently for the chain to have entered the main collagenous domain should have contained the SP1.D8-reactive pro α1(I)-N-propeptide domain. The very heavy antibody reactivity of the band near pro α1(I) in the polysome pellet shown in Figure 2B, lane 2′, indicated that, under the conditions of preparation, the polysomes were not uniformly loaded with nascent chains of all lengths. Instead, there was a major polysome-associated concentration of nearly full length pro α1 chains. However, several lower weight bands reacted with the SP1.D8 indicating the presence of accumulations of intact (N-propeptide containing) incompletely elongated chains. These chain lengths represent points of elongation pauses, and are revealed more sensitively by the separate in vitro translations of the human pro α1(I) and pro α2(I) RNAs transcribed from the appropriate plasmids (Fig. 4). As previously noted by Hu et al. [1995a] there are striking differences in the apparent pause sites in the synthesis of the two chains in this membrane-free system in which no post translational modifications take place.

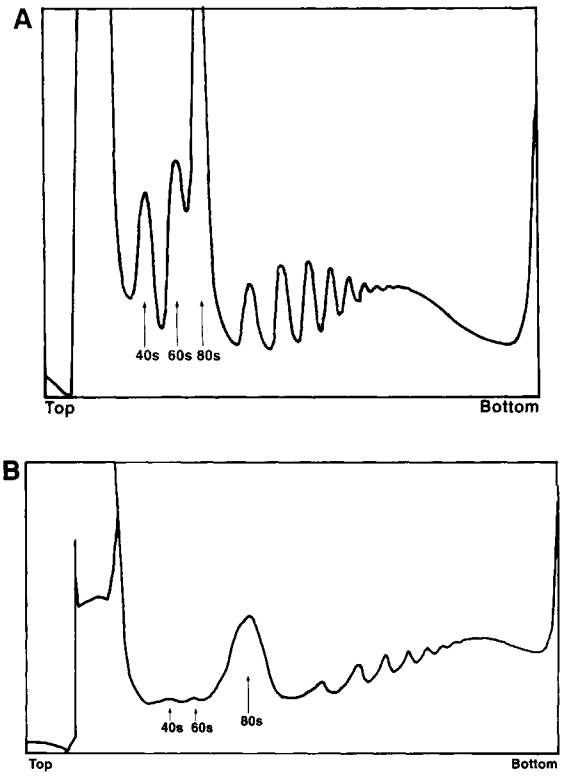

Fig. 1. Sucrose gradient distribution of the total cell lysate and polysome pellet. 3T6 cells were lysed as described in Experimental Procedures. After centrifugation to remove cell nuclei and debris, cell lysates were either loaded directly onto 15–50% sucrose gradients (**A**) or ultracentrifuged in sucrose buffer and the resulting pellet loaded onto the same kind of gradient (**B**). The sedimentation position of the small and large ribosomal subunits are indicated (40s and 60s, respectively) as well as that of assembled ribosome (80s) and polysomes (>80s). The presence of a large amount of material sedimenting in the <80s regions near the top of the gradients was apparent in total cell lysates. After pelleting, pre 80s-sedimenting structures were markedly reduced, while 80s and larger polysomes remained unchanged.

Most of the prominently labeled 3T6 cell polysome pellet radioactive bands in lanes 2 and 3 of Figure 2A were not reactive with the SP1.D8 antibody. Further, as shown in Figure 5B, which presents a fluorograph of the polysome-associated proteins at a variety of labeling time points, the majority of the radioactive nascent protein bands present in the polysome pellets were not removed or depleted by collagenase digestion. This can be seen most readily in the 2 and 4 h labeling time points where the near-full length pro α1 chain bands are most evident. These data

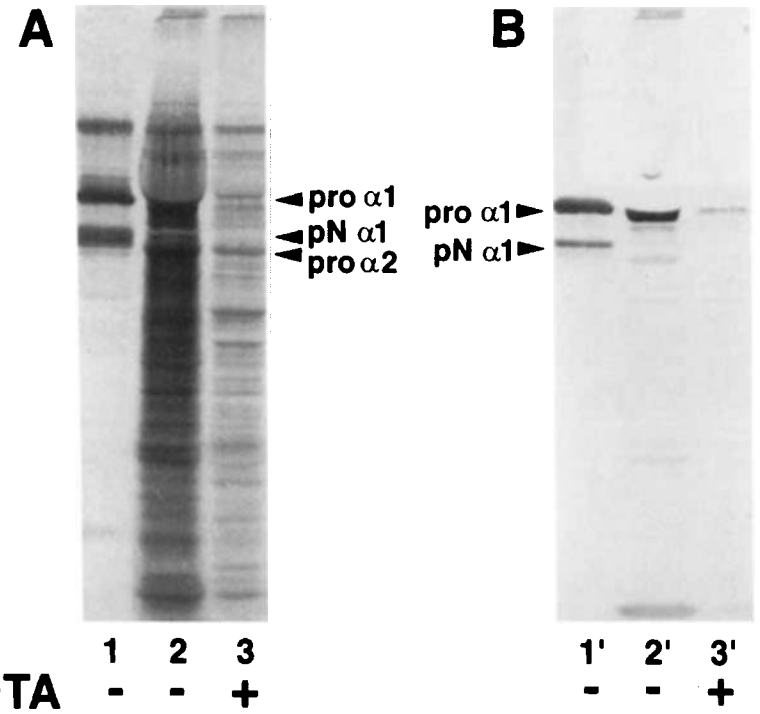

Fig. 2. EDTA release of polysome-associated procollagen. Eight 150 cm^2 flasks of 3T6 fibroblasts were labeled for 30 min with ^{14}C-proline after an overnight incubation in unlabeled DMEM. Polysomes were collected as described in Experimental Procedures. Prior to the final ultracentrifugation to pellet the polysomes 20 mM EDTA was added to half of the sample in order to dissociate ribosomal subunits. Both EDTA-treated and untreated polysomes were subjected to SDS-PAGE and Western blotting with anti-N-propeptide (SP1.D8). After staining, blots were exposed to X-ray film for 4 weeks. *Lanes 1–3* (**A**) represent the fluorogram and *lanes 1'–3'* (**B**) represent the Western blot. Lanes 1 and 1': labeled procollagen collected from the media of 3T6 cells (the positions of pro α1(I), pN α1(I), and pro α2(I) chains are indicated); lanes 2 and 2': proteins comprising the untreated polysome pellet; and lanes 3 and 3': proteins remaining in the "polysome" pellet after EDTA-treatment. The Western blot (lane 2') shows that nearly full length pro α1(I) chains are prominent in the untreated polysomes, while the fluorogram (lane 2) shows abundant proline labeling in many very large as well as low M_r proteins. EDTA-treatment (lanes 3 and 3') dissociated pro α1(I) chains and most other nascent polypeptides from the polysome pellet.

emphasize the important point that the polysomes pelleted by high speed centrifugation contained many strongly ^{14}C-proline labeled nascent proteins other than collagen. Thus, while collagen might represent a high proportion of the protein secreted from the 3T6 cells, collagen synthesis accounts for only a small fraction of the total (cell + secreted) protein synthesis at any particular time.

One possible explanation of the high content of the full length pro α1(I) chains on the polysomes (and relatively low content of shorter length, incomplete chains) could have been that during the cell lysis step of polysome isolation some of the secreted extracellular procollagen, or some of the completed intracellular collagen in the process of secretion, interacted and pelleted with the polysomes. To examine this possibility, 3T6 cells were cultured for 24 h in DMEM as described above. Instead of labeling with ^{14}C-proline at this point, the culture media was replaced with fresh DMEM containing exogenous ^{14}C-labeled type I procollagen. Polysomes were collected from these cells after 4 h incubation. The radioactivity of the polysomes and the recovered ^{14}C-procollagen in the media were determined. Virtually no activity above background was found in the polysome pellet, and there was essentially quantitative recovery of the ^{14}C-procollagen radioactivity in the media, Table 1, in agreement with similar experiments by Kirk et al. [1987]. Thus, the presence of the nearly full length pro α1(I) chains on the polysomes was not an artifact of polysome preparation.

The Dynamics of pro α Chain Production

The high concentration of polysome-associated near-full length nascent pro α1(I) chains had been noted earlier in chick embryo fibro-

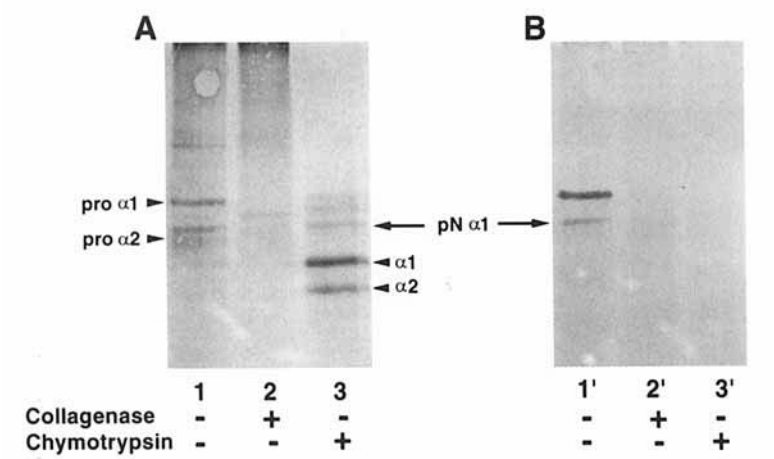

Fig. 3. Demonstration of the specificity of SP1.D8 for pro α1(I) and pNα1(I) chains. ^{14}C-proline labeled type I procollagen was ammonium sulfate-precipitated from the media of 3T6 fibroblasts. Precipitates were resuspended in collagenase buffer and digested with either buffer (control *lanes 1 and 1'*); collagenase (*lanes 2 and 2'*) or chymotrypsin (*lanes 3 and 3'*). Protein digests were subjected to SDS-PAGE and Western blotting with anti-N-propeptide (SP1.D8). After staining, blots were exposed to X-ray film for 2 weeks. Lanes 1–3 **(A)** represent the fluorogram and Lanes 1'–3' **(B)** represent the Western blot of the protein digests. The migration positions of α1(I)-, α2(I)- collagen chains, and pN α1(I) and pro α1(I) chains are indicated. SP1.D8 showed no reaction with pro α2(I)- or α2(I)-chains.

blasts [Kirk et al., 1987; Veis and Kirk, 1989], but not to such an extent. The content of these chains was found to be dependent on the preincubation conditions and the capacity of the cells to secrete the procollagen. As shown below, after 24 h incubation in the same media, synthesis of new collagen was totally inhibited in the 3T6 cells, probably the result of a buildup of N-propeptide in the 24 h-conditioned media [Horlein et al., 1981; Perlish et al., 1985]. Replacement with fresh, unconditioned media allowed renewed secretion and synthesis of collagen. In essence, the 3T6 cells could be synchronized with respect to their synthesis and secretion of type I procollagen. This phenomenon permitted studies of the build-up of the polysome-associated collagen chains.

Specifically, after 3T6 cells were cultured for 24 h in unlabeled DMEM, the media was replaced with fresh media containing 2 μCi/ml of ^{14}C-proline. Cells were labeled for the times indicated in the caption of Figure 5, then cycloheximide was added to block further chain elongation and lock the nascent chains on the polysomes. Next, the cells were lysed and the postnuclear supernatant was fractionated into polysome pellet and post-ribosomal supernatant pools. The secreted media was collected at each time point. Samples from each of the 3 pools at each time point were loaded onto 6% acrylamide gels and, after electrophoresis, electroblotted onto nitrocellulose and stained with the SP1.D8. Autoradiograms of the blots were then obtained and densitometric analysis was applied. Figure 5 shows the incorporation of the ^{14}C-proline into the polysome pellet as a function of time. As seen in panel A the incorporation of proline into nascent polysome pellet-associated proteins was not detectable for the first 15 min. At 30 min a number of labeled bands were evident and some were collagenase sensitive (Fig. 5B, 0.5 h ± lanes). Notably, the most heavily labeled, collagenase sensitive band was that corresponding to the near-full length band seen in Figure 2(A,B), indicating that this band was accumulating on the polysomes at a faster rate relative to any of the lower weight collagenase-sensitive bands. This concentration effect became more evident as labeling time increased. While lower weight collagenase-sensitive bands also increased in content to detectable levels over the 4 h period, none of the intermediate weight collagenase sensitive bands approached even 50% of the labeling intensity of the near-full length pro α1(I) band. On the other hand, several other polysome pellet-associated proteins that were not collagenase sensitive also accumulated steadily and were as intensely proline labeled as the full-length pro α1(I) band. Components at M_r 120,000, 97,000, and 47,000–46,000 were especially prominent (see arrowheads, Fig. 5B).

SP1.D8-stained immunoblots of the same gels were very revealing (Fig. 6). At the zero-time point, where no new radioactivity was detect-

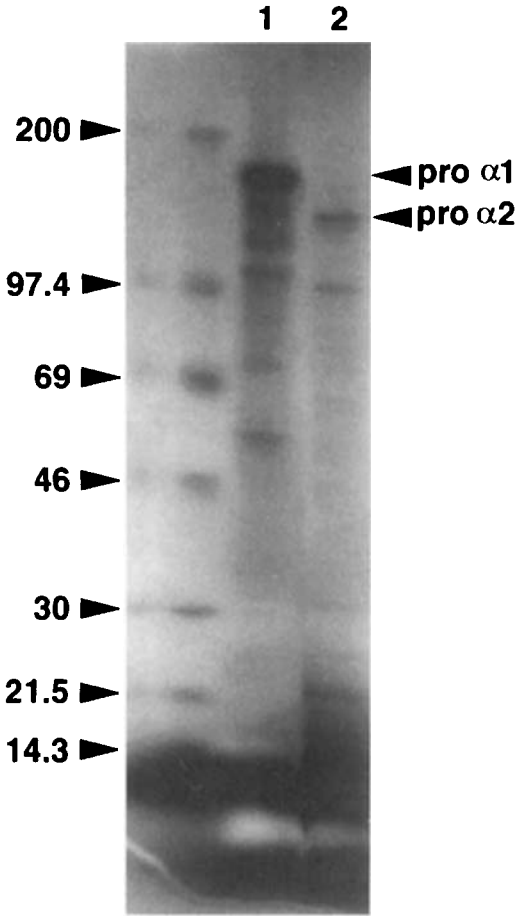

Fig. 4. In vitro translation of pro α1 and pro α2 in vitro transcribed RNAs. Fluorographs of 6% PAGE of the polysomes recovered from rabbit reticulocyte lysate in vitro translations of the in vitro transcribed RNAs from human pro α1(I) and pro α2(I) cDNAs. *Lane 1,* pro α1(I). *Lane 2,* pro α2(I). The migration positions of protein standards and media pro α1(I)- and pro α2(I)-chains are indicated. Identical amounts of RNA were used under identical translation conditions. Neither the pattern of peptides nor the intensities (indicative of translation efficiency) were comparable.

able, the SP1.D8 showed a single, heavily reactive band at the ≈pro α1(I) position. This non-radioactive antibody-reactive band gradually diminished in intensity until it was depleted below detectable limits between 30 and 60 min (Figure 6B). The concentration of this component subsequently increased so that it was again detectable at the 2 hour time period. There was a parallel rise in radioactivity of this band during the same period, indicating that it represented newly synthesized protein.

Upon completion and folding within the ER the collagen moves through the Golgi and into secretory vesicles for export to the extracellular space. In the pelleting procedure, the polysome fraction is separated from the "post-ribosomal" intracellular pool. This provided the opportunity to determine the relative rates of movement of the newly synthesized collagen from the ER, through the post-ribosomal intracellular compartments and into the extracellular media. The relative intensity of both the radioactivity and immunostaining of the collagenase-sensitive, near-full length pro α1(I) band at each time point was determined by densitometric scanning of the fluorograms and immunoblots for each pool as a function of labeling time. As shown in Figure 7B, at the point where the media was changed (zero time) both polysome and intracellular pools held substantial amounts of SP1.D8-reactive pro α1 chain (non-radioactive). Thus, the synthesis block effected by the conditioned medium led to an initial accumulation of collagen in both intracellular pools. The intracellular post-ribosomal pro α1-pool content decreased markedly during the first 15 min, as the intracellular collagen was secreted. Depletion of the polysome-associated pro α1 was slower but those chains transferred to the intracellular compartment, with the result that the intracellular pool was increased. By 60 min the transfer was complete so that the polysome-associated pro α1 pool had dropped to a near zero level while the post-ribosomal pool was increased. Within 2 h of the media change the post-ribosomal pool had decreased and by 4 h, the intracellular pool was virtually cleared from the cell. Thus, in the absence of feed-back regulation of secretion, the movement of completed collagen from the intracellular compartment is rapid and collagen is not retained within the cell. However, a portion of the newly synthesized pro α1(I)-chains are retained in the polysome-associated pool. Synthesis of new nascent polysome-associated collagen was not detected until 30 min after the media was changed and the unlabeled polysome-associated collagen had begun to transfer to the intracellular pool (Fig. 6).

Secreted (non-radioactive) collagen was detected in the extracellular media by SP1.D8 staining 15 min after the addition of fresh media. It's content increased slowly as the intracellular pool was cleared. After 120 min of labeling, the rate of increase in the extracellular pool was greater than the rates of collagen accumulation in either the polysome-associated or intracellular pools. At some later point which has not yet been determined, the rates of synthesis and

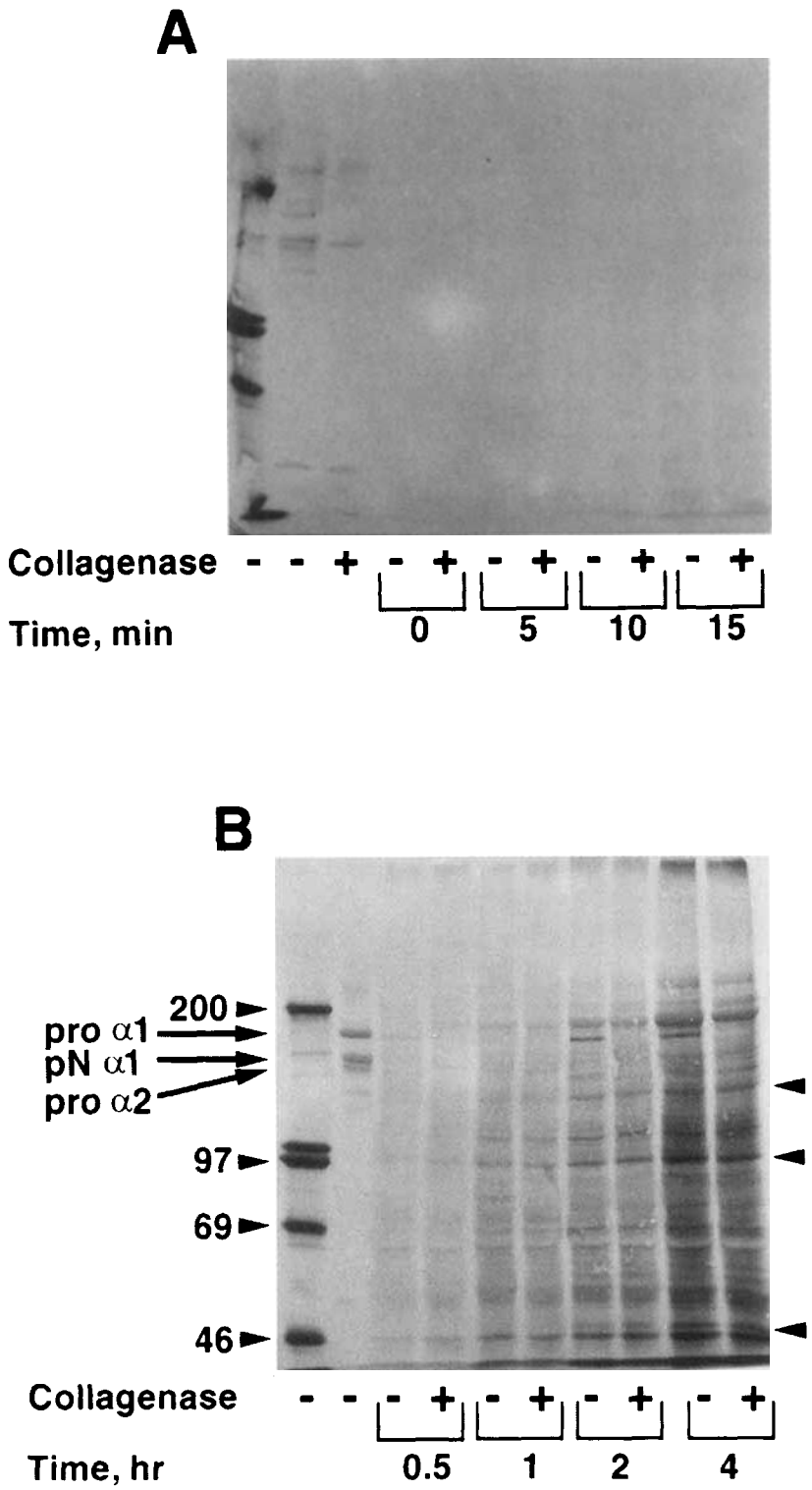

Fig. 5. Radioactive labeling of the nascent procollagen-synchronization of translation initiation after release of inhibition. 3T6 cells were cultured for 24 h in unlabeled media. The conditioned media was removed and fresh ^{14}C-proline labeling media was added for the various times indicated. Polysomes were then isolated and subjected to SDS-PAGE. The resolved proteins were transferred to nitrocellulose and fluorographed. Before loading into the gels, each polysome preparation was divided in two. One half was digested with bacterial collagenase (+) the other served as buffer control (−). In **A**, the first three lanes represent molecular weight standards, standard procollagen isolated from the media of 3T6 fibroblasts, and standard procollagen digested with collagenase, respectively. In **B**, the first two lanes represent the molecular weight standards and the procollagen standard, respectively. The migration positions of pro α1(I), pro α2(I) and pN α1(I) collagen are indicated. No radioactive labeling was detected for the first 15 min but there was a prominent collagenase digestible nascent chain near the pro α1(I) position by 1 h. *Arrowheads* mark prominent collagenase resistant, labeled chains which also increased in density with time. (See also Fig. 9.)

TABLE I. Control for Contamination of Polysomes With Secreted Procollagen*

Incubation time Minutes	CPM Polysome associated	CPM Media
0	—	26,000
5	—	24,600
10	—	28,500
15	—	23,600
30	19	16,600
60	11	21,400
120	17	17,800
240	22	25,400

*Cell were incubated in unlabeled DME media. After 24 h, the media was removed and replaced with fresh media containing ^{14}C-labeled procollagen prepared by ammonium sulfate precipitation from the media of a separate culture. The cells were incubated for the periods indicated, then lysed in the usual manner. The polysomes were harvested and counted for radioactivity. The media was also collected and subjected to ammonium sulfate precipitation. The radioactivity of the recovered procollagen was determined. The CPM in column 2 were obtained after subtraction of the background. Essentially no radioactivity was recovered in the polysome fraction.

secretion must begin to slow in response to the conditioning of the media. The radioactivity data, Figure 7A, which describe only the freshly synthesized pro α1(I) chain content, provide similar insights. The presence of newly synthesized full-length chains on the polysomes is not seen until 15 min after release of the synthesis block, but they are rapidly released to the intracellular compartment. Between 1 and 2 h, however, the radioactivity in the polysome-associated near-full length pro α1(I) chains exceeds that of the post-ribosomal chains in the secretory pathway.

Pulse-Chase Analysis of Polysome Associated Collagen

The synchronization of collagen synthesis after 24 h of culture, followed by release of the secretion-synthesis block, was repeated with one modification. After the 24 h culture in cold media, fresh, unconditioned media containing ^{14}C-proline was added for 30 min, then chased with fresh media containing unlabeled proline. The polysome, intracellular and media pools were collected as in the previous protocol. Gels were run, Western blotted and stained with SP1.D8. These pulse-chase polysome pellet pool data (not shown) duplicated that in Figure 7B, with a clearance of polysome-associated pro α1(I) chains at 60 min.

The pulse-chase polysome radioactivity data are shown in Figure 8. Polysome-associated labeled proline in pro α1(I)-chains was maximal at the start of the chase period but decreased until, at about 60 min total elapsed time (30 min chase), a minimum labeling level was reached. However, this level did not decline to zero. A low level of radioactive pro α1(I)-chains remained associated with the polysomes for the entire 4 h chase period, many times longer than required for synthesis and secretion of a procollagen molecule. These data agree with those of Kirk et al. [1987] who showed that a 15 min chase period was not sufficient to clear radiolabeled pro α chains from chick tendon polysomes.

Nature of the Polysome-Retained Collagen

A surprising aspect of the radiographic data of Figure 5 was that the pro α1(I) and pro α2(I)-chains did not appear to accumulate on the polysomes in the expected 2:1 ratio after synchronized renewal of collagen synthesis. This is especially apparent in Figure 5B in the 2h and 4h lanes. Figure 9 shows an enlargement of the pro α regions for these gels. The arrows mark the putative positions of full-length, fully processed pro α1(I) and pro α2(I) chains. As shown both visually and by densitometric analysis, there was no dense band corresponding to polysome-associated pro α2(I) to match the accumulated intensity of the very prominent pro α1(I) band at either time point, in spite of the fact that an appropriate 2:1 pro α1:pro α2 ratio was maintained in the labeled secreted media procollagen. This is a further indication that while the normal heterotrimer was produced, the individual pro α1(I) and pro α2(I) messages and/or nascent chains were not translated or processed in the same manner.

A second important observation was that the polysome-associated near-full length pro α1(I) band was not homogeneous in composition. The SP1.D8-stained bands were broad and variable in width. The bands obtained at different times after release of the synthesis block by media change were analyzed by densitometry. The broad polysome-associated pro α1(I) band seen at zero time in Figure 6A had the complex structure shown in the densitometer scan in Figure 10 (solid line). In contrast, 2 h after the initiation of new synthesis, the SP1.D8-stained a more narrow single band which migrated in the same position as the low M_r component of the 0 h peak (dashed line, Fig. 10). Similar

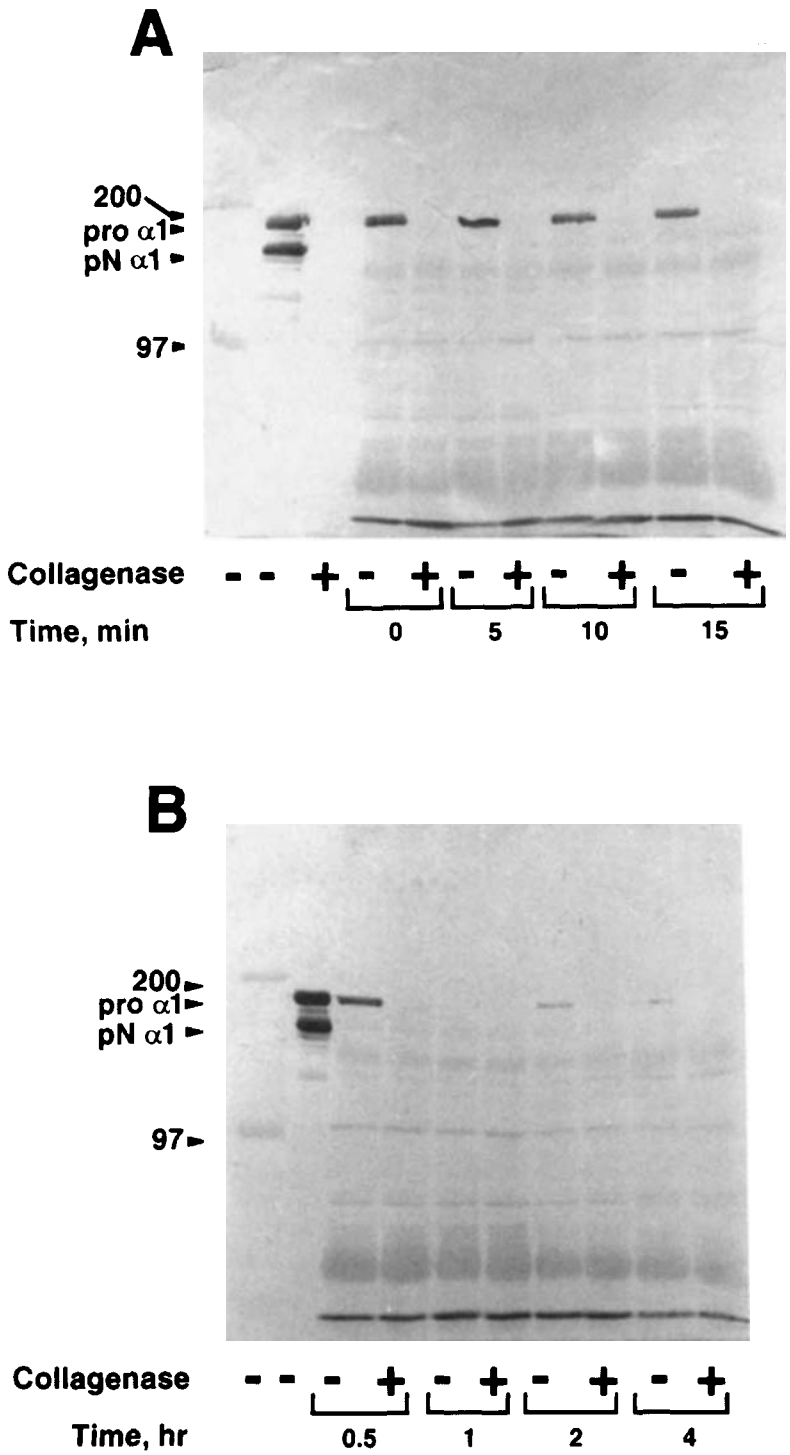

Fig. 6. Western blots of pro alpha 1(I) nascent chains showing their accumulation and release over the course of labeling. The nitrocellulose blots described in Figure 5 were immunoblotted with SP1.D8 antibody. The migration positions of pro α1(I), pro α2(I), and pN α1(I) are indicated. Near-full length pro α1(I) chains had accumulated on polysomes during the prelabeling incubation, but were released and secreted between 30 and 60 min post-labeling. After 60 min, newly synthesized pro α1(I) chains begin to reaccumulate.

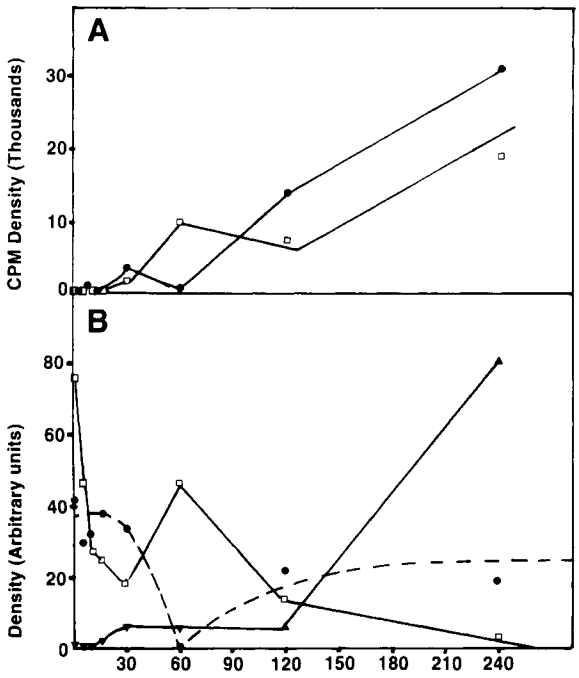

Fig. 7. Movement of pro α1(I) chains between polysome-associated, intracellular and extracellular pools. The fluorograms depicted in Figure 5 and Western blots shown in Figure 6 were scanned with a densitometer and the area corresponding to the near-full length pro α1(I) chains were quantitated for each time point. The intracellular, non-polysome-associated material from each time point (supernatants obtained after ultracentrifugation of cell lysates) were also collected and electrophoresed, Western blotted, fluorographed, and scanned. **A:** The density of radioactive near-full length pro α1(I) chains as a function of time in the polysome-associated (*closed circles*) and intracellular (*open squares*) pools. **B:** The density of SP1.D8 immunolabeling of the pro α1(I) chains as a function of time. *Closed circles and dashed* line—nascent chains bound to polysomes. *Open squares*—intracellular pool. *Closed triangles*—extracellular pool. A small quantity of collagen was released to the extracellular pool within 30 min of release of the synthesis block but the level remained constant for almost 2 h. However, within the cell, transfer from the polysome associated pool to the intracellular, postribosomal pool was evident, causing a transient increase in the intracellular pool.

analysis of the autoradiograms of the pulse-chased polysomes at 10 min and 4 h showed that the first detectable radioactive band corresponded to the low molecular mass region (zone I, Fig. 10) of the zero-time pro α1(I)-band, whereas at 4 h the peak of radioactivity had moved to the higher mass zones (II,III). These data show that the components in zone I are more newly synthesized than the components of region III. Thus, the components of the wide immunostained polysome-associated pro α1(I)-related band had accumulated at discrete points of chain elongation and/or post-translational modification. Final chain completion and release of the polysome-associated pro α1(I) chains take place at a slower rate than the initial elongation rate at which the nascent chains are synthesized.

Post-Translational Modification of the Polysome-Associated Collagen

Effects of hydroxylation and triple-helix inhibitors. The relationship between nascent chain cotranslational modification and polysomal nascent chain retention was investigated by examining the effects of the hydroxylation inhibitor α,α′-dipyridyl; the triple-helix formation inhibitors *cis*-4-hydroxy-L-proline and 3,4-dehydroproline; and the N-glycosylation inhibitor tunicamycin. Each of these inhibitors was incubated with 3T6 cells in unlabeled DMEM for at least 4 h. The cultures were then labeled for 30 min with ^{14}C-proline. In the case of *cis*-hydroxyproline, labeling was also carried out with ^{14}C-lysine.

cis-Hydroxyproline is incorporated to the extent of only about 14 residues per nascent collagen α-chain and does not interfere with the hydroxylation of the remaining prolines [Rosenbloom and Prockop, 1971]. However, subsequent intracellular triple-helix formation and secretion are affected. Figure 11A shows the fluorograph of ^{14}C-lysine-labeled polysomes. Control ^{14}C-lysine-labeled procollagen from culture media clearly demonstrated the positions of pro α1(I), pNα1(I) and pro α2(I) chains (lane 3). The pro α1(I) and pNα1(I) of the cell media were sharply delineated by the SP1.D8 staining (lane 3′). The SP1.D8 staining of the control and *cis*-hydroxyproline treated polysome-associated bands (lanes 1′ and 2′) showed similar pro α1(I) bands. As anticipated [Rosenbloom and Prockop, 1971], *cis*-hydroxyproline appeared to have little effect on the incorporation of labeled proline into the nascent collagen, data virtually identical to controls were obtained with ^{14}C-proline labeling (Fig. 11B, lanes 5, 5′). The distributions of protein within the *cis*-proline and non-treated polysome-associated pro α1(I) peaks were similar, except that the apparent molecular weight of the *cis*-proline treated cell polysomes was shifted to a slightly lower M_r range (Fig. 12A).

α,α′-Dipyridyl inhibition of hydroxylation broadened the SP1.D8-reactive polysome-associated pro α1(I) band (Fig. 11B, lane 4′) somewhat but shifted the entire band to a lower M_r range (Fig. 12B). Incorporation of 3,4 dehydroproline

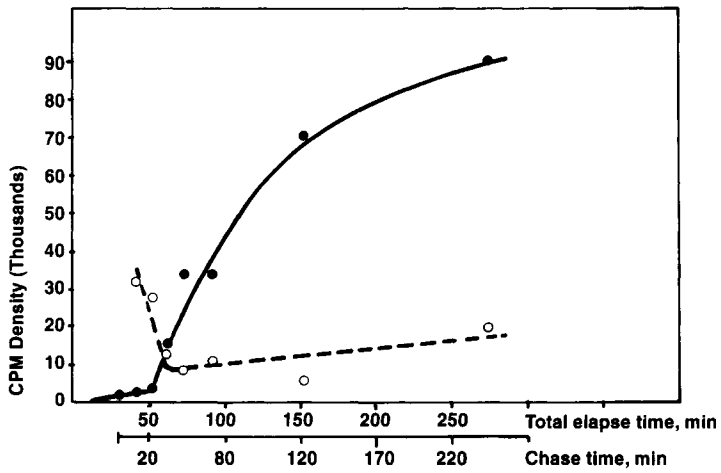

Fig. 8. Pulse-chase experiment demonstrating the retention of newly-synthesized pro α1(I) chains on the polysomes as long as 4 h postlabeling. Polysomes from the synchronized 3T6 cells were isolated after ^{14}C-proline labeling for 30 min, followed by a chase with DMEM containing unlabeled proline for the various time periods indicated. Each of the polysome-associated and extracellular pro α1(I) chains were blotted on to nitrocellulose and exposed to X-ray film for fluorography. Autoradiograms were analyzed by densitometry, then arbitrary densitometer units were plotted versus both chase time and total elapsed time (chase time + 30 min). ^{14}C-labeled polysome-associated pro α1(I) chains (*open circles, dashed line*) decreased with chase time, but their content plateaued after 60 min. Even after a 4 h chase time, they were not yet released from polysomes. Radiolabeled extracellular procollagen first appeared after 20 min chase, plateaued, then increased sharply after 60 min chase time (*closed circles, solid line*).

similarly reduced the apparent molecular weights of all components of the peak (Figures 11B and 12C), but the band width was markedly narrowed. Uitto and Prockop [1974] reported that in addition to blocking triple helix formation, 3,4-dehydroproline reduced hydroxylation by as much as 87% of control values. The differences in M_r are consistent with the known dependence of α-chain electrophoretic mobility on the extent of collagen hydroxylation. The presence of the retained polysome-associated pro α1(I)-related components at nearly the same levels in control and treated polysomes suggest that neither hydroxylation reactions nor triple-helix formation are the primary basis for the retention of this fraction on the polysomes.

Figure 13 compares the contents of the intracellular, post-ribosomal pools from control cells, αα′-dipyridyl, *cis*-hydroxyproline, 3,4 dehydroproline, and EDTA treated cells. As indicated earlier, treatment of the cells with EDTA disrupts the polysomes and releases the bound collagen from the polysome pellet into the post ribosomal supernatant. This was evidently the case as comparison of lanes 2 and 3 of the fluorogram in Figure 13A demonstrates. The putative pro α1 band was stronger in the EDTA post ribosomal supernate than in the untreated cell supernate. This was confirmed by the heavier

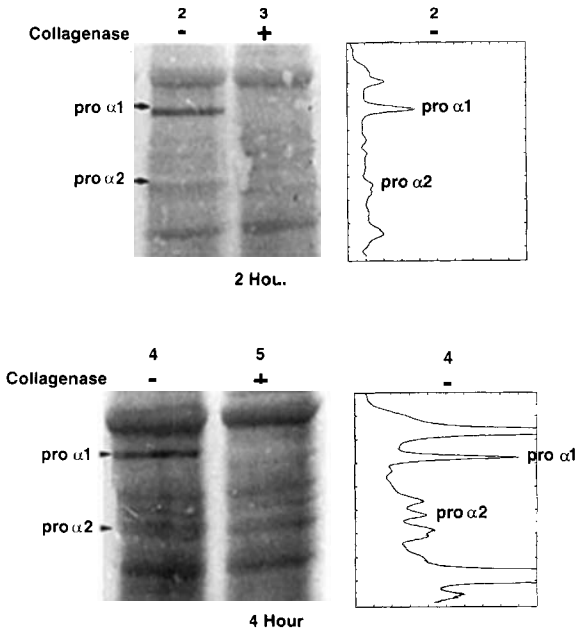

Fig. 9. Simultaneously synthesized near-full length nascent pro α1(I) and pro α2(I) nascent chains are not present in a polysome-associated state in a 2:1 ratio. After 2 or 4 h of coordinated synthesis, as shown if Figure 5B, a prominent polysome-associated pro α1(I) band is quite evident, but no equivalent polysome-retained pro α2(I) band is present. The appropriate regions of lanes 2, 3, 4 and 5 of Figure 5B were subjected to densitometry, as shown on the right. The collagenase-sensitive full-length pro α1 and pro α2 bands were identified. It is evident that the pro α1:pro α2 $\gg$ 2:1.

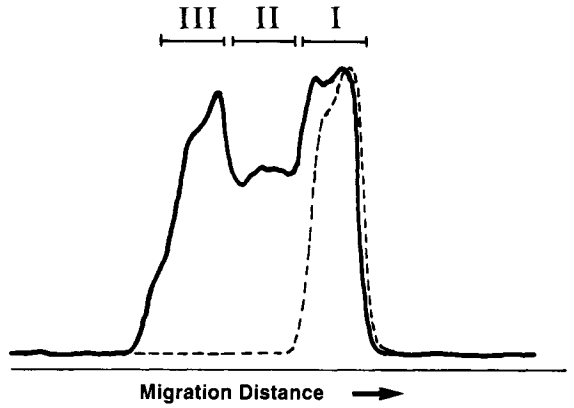

Fig. 10. Near-full length polysome-associated pro α1(I) nascent chains are not homogeneous. The near-full length pro α1(I) nascent chain appearing at zero labeling time (*solid line*) and at 4 h post-labeling (*dashed line*) (see Fig. 6) were scanned with a densitometer after immunostaining with the SP1.D8. The multinodal nature of the peak occurring at zero time indicates that pro α1(I) chains in several elongation or processing states were present on the translation inhibited polysomes. After 4 h post-labeling, the retained unlabeled pro α1(I) had been cleared and only newly accumulated pro α1 was retained. The radioactivity data (Fig. 5) also showed that translation had resumed but only the fastest migrating, lowest M_r newly synthesized species was present. Three zones of M_r are evident within this complex band retained or accumulated on the polysomes.

staining of the EDTA treated supernate pro α1 band with SP1.D8 (lanes 2′ and 3′). The αα′-dipyridyl treatment also increased the intracellular, post ribosomal content of pro α1-chains. However, much more of the intracellular collagen was held within the ER in every treatment, except for the EDTA disruption of the polysomes.

Effects of Glycosylation Inhibition

Tunicamycin is an inhibitor of N-linked glycosylation. Each pro α(I) chain has a single N-glycosylation site in it's carboxyl-propeptide region [Clark, 1979]. To determine the effect of C-propeptide-region glycosylation, 3T6 cells were treated with tunicamycin prior to labeling with ^{14}C-proline. In contrast to the minor effects of the helix region hydroxylation and triple-helix inhibitors, tunicamycin had a marked effect on the polysome-associated chain size distribution. As seen in Figure 14A (lanes 1′ and 3′), three new SP1.D8 labeled bands with apparent M_r less than that of the completed pro α1(I)-chain appeared after tunicamycin treatment in addition to the typical pro α1(I)-band. The proteins in these bands were collagenase digestible (lane 4′) and heavily ^{14}C-proline-labeled (Fig. 14A, compare lanes 1 and 2 with lanes 3 and 4) and hence represented an accumulation of newly synthesized chains. Thus, inhibition of the C-propeptide N-glycosylation reveals at least 3 processing stages for the polysome-associated pro α1(I)-chains that are reflected in changes in apparent M_r. The difficulties in determining the molecular mass of the pro α1(I)-chain from gel electrophoresis arise from the double complexities of mixed globular and helix regions and different extents of chain processing [Sandell and Veis, 1980]. The apparent M_r values for the glycosylation inhibited intermediates range from 150K to 164K based on globular protein standards. On this same basis the highest mass of the final retained chains would be 175K.

Densitometric tracings of the SP1.D8 labeled bands, Figure 14A, lanes 1′ and 3′, showed (Fig. 14B) that the typical pro α1(I) polysome band had the same intensity and structure in both inhibited and control polysome pellets. The concentrations of the lower M_r collagenase-sensitive bands were well above the SP1.D8 detection limits in the tunicamycin-inhibited polysomes. The presence of the low M_r bands in addition to the "full-length" paused chains suggest that processing through the final pause (complex high M_r band) and the ultimate release of the nascent chain from the polysome is not related to N-linked carboxyl-propeptide glycosylation. No radioactive collagen was detected in the ^{14}C-Pro labeled tunicamycin-treated cell media. Thus, the heavily radiolabeled polysome-associated collagenase-digestible components produced by tunicamycin treatment of the 3T6 cells (lane 3′ of Fig. 14A), were not secreted but were retained at the polysome-ER level during the 4h labeling period (data not shown).

Disulfide Bonding in the Polysome-Associated Collagen

Two methods were used to assess the presence of disulfide bonding among the near-full length polysome-associated pro α1(I)-chains. Following the procedure of Kirk et al. [1987], the polysome pellet components collected after a 30 min labeling period were passed over Q-Sepharose columns under denaturing conditions. Iodoacetamide was added to the solutions to prevent artifactual disulfide bond interchange. The column flow-through fraction (FT) contained polysome-associated but tRNA-free chains. The retained fraction (RT) contained the nascent, elongating tRNA-bound chains. Aliquots of each

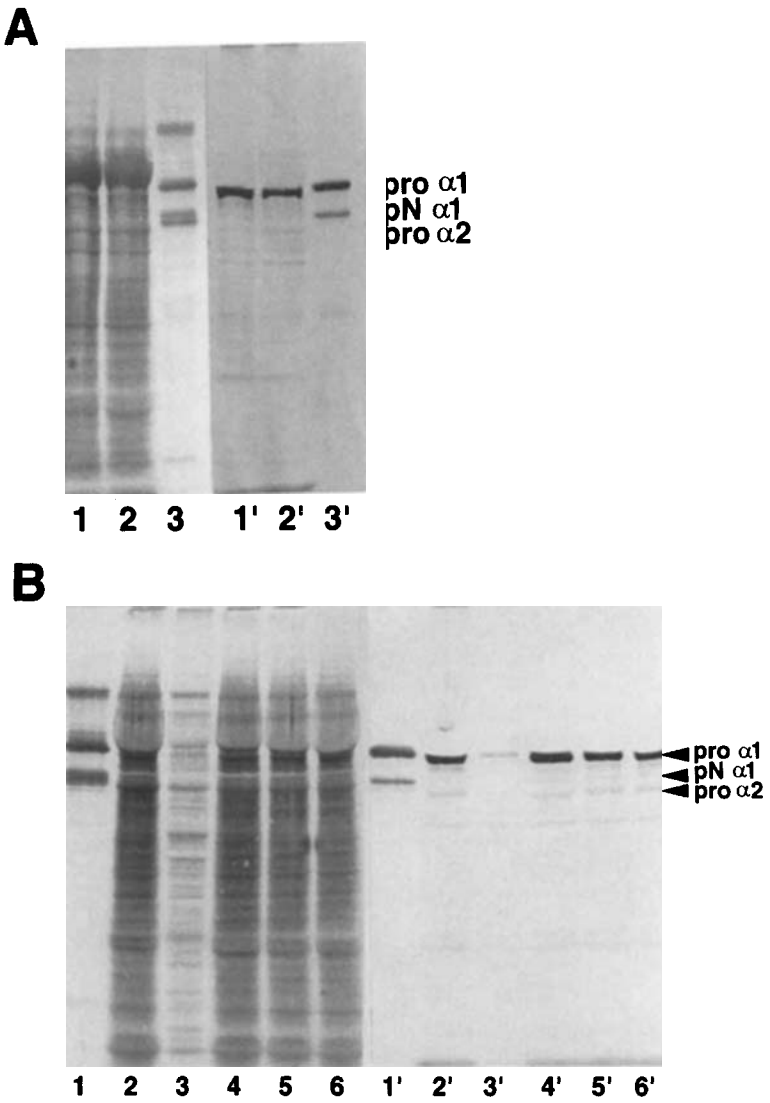

Fig. 11. Effects of hydroxylation and triple helix inhibitors on polysome-associated pro α1(I) chains. **A:** Polysomes were collected after preincubation of 3T6 fibroblasts with *cis*-hydroxyproline (both an hydroxylation and triple helix formation inhibitor) and labeling with ^{14}C-lysine. Polysomes were Western blotted and stained with SP1.D8 then exposed to X-ray film for 4 weeks. *Lanes 1–3* represent fluorograms of labeled polysomes, while *lanes 1'–3'* represent immunoblots. Lanes 1 and 1', untreated polysomes; lanes 2 and 2', polysomes from *cis*-hydroxyproline-treated cells; lanes 3 and 3', standard procollagen isolated from the media of 3T6 fibroblasts. The migration positions of pro α1(I), pro α2(I), and pNα1(I)-chains are indicated. **B:** Polysomes were collected after preincubation of 3T6 fibroblasts with either *cis*-hydroxyproline, the hydroxylation inhibitor α,α dipyridyl, or the triple helix inhibitor 3,4 dehydroproline and labeled with ^{14}C-proline. Polysomes were Western blotted and stained with SP1.D8 then exposed to X-ray film for 4 weeks. *Lanes 1–6* represent fluorograms of labeled polysomes, while lanes 1'–6' represent immunoblots. Lanes 1 and 1', standard procollagen; lanes 2 and 2', untreated polysomes; lanes 3 and 3', polysomes treated with EDTA before pelleting; Polysomes collected from either, α,α dipyridyl (lanes 4 and 4'), *cis*-hydroxyproline (lanes 5 and 5') or 3,4 dehydroproline (lanes 5 and 5') treated cells.

chromatographic fraction were either counted directly, or Western blotted under reducing and non-reducing conditions.

In an alternate procedure with the same objective, the components of the polysome pellets were precipitated with CTAB. The anionic covalent complexes of tRNA and nascent chains were precipitated (equivalent to the Q-Sepharose RT fraction), while the other polysome-associated components remained in the supernatant (equivalent to FT). Table II shows that the two methods did not yield identical results. About 50% of the counts were precipitated by CTAB whereas only 36% of the counts were bound to

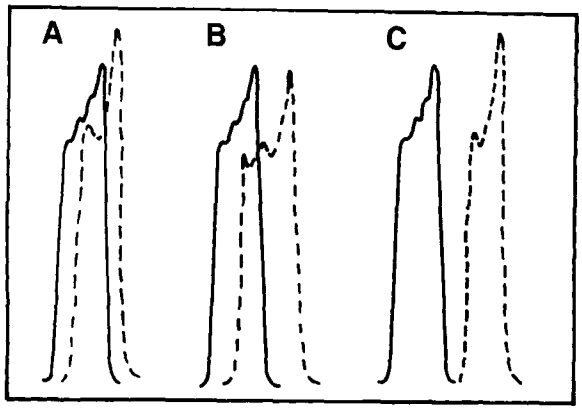

Fig. 12. Densitometer scans of polysome-associated pro α1(I) near-full length chains collected from inhibitor-treated cells. Western blots in Figure 10B were scanned and inhibitor-treated polysomes (lanes 4'–6') compared to controls (lane 2'). *Dashed lines* represent **A:** *cis*-hydroxyproline, **B:** α,α dipyridyl, and **C:** 3,4 dehydroproline treated cells. *Solid lines* represent controls. Hydroxylation inhibition caused faster migration of nascent pro α1(I) chains, while triple helix inhibition caused little change in migration or amount of pro α1(I) nascent chains.

the Q-Sepharose (RT). Kirk et al. [1987] had found ≈ 75% of counts from labeled chick embryo fibroblasts in a comparable QAE-Sephadex RT fraction, but a shorter 15 min labeling period had been used.

Each of the fractions from both procedures was subjected to gel electrophoresis, run under reducing and non-reducing conditions, and Western-blotted. The SP1.D8 reactive bands were analyzed semi-quantitatively by densitometry.

Full-length pro α-chains were present in both the Q-Sepharose FT and CTAB-supernatant fractions. Both FT and CTAB-supernatant fractions yielded a component corresponding to pro α-chain trimer under non-reducing conditions, but this component was not present under reducing conditions. The SDS-PAGE gels of RT and CTAB precipitate fractions yielded essentially identical patterns under reducing and non-reducing conditions, with no evidence of pro α-chain trimer.

The SP1.D8-reactive pro α and pro α-chain trimer contents in each FT and CTAB-supernate gel lane were analyzed semi-quantitatively. As shown in Table III, approximately 30% of the total SP1.D8-reactive protein in these non-tRNA containing fractions appeared to be disulfide-linked, compared to 40% bonding in total polysome preparations prior to CTAB precipitation or RT-FT fractionation. It should be noted that use of the above procedure with standard, fully completed and secreted procollagen isolated from the cell culture media consistently yielded a value of 50% disulfide bonding. Thus, since the secreted procollagen is presumed to be fully disulfide bonded, it is likely that this approach may provide an underestimate of the level of disulfide bonding in the polysome-associated fraction.

DISCUSSION

The main features of the synthesis of a polypeptide chain are well known. They involve, sequentially, the assembly of the first 80S ribosomal unit at the start codon of the mRNA, initiation of synthesis, movement of the initiated ribosomal assembly along the mRNA via repetitive translocations of the peptidyl-tRNA from the ribosomal A to P sites with corresponding chain elongation [Moazed and Noller, 1989], possible cotranslational modification of the nascent polypeptide chain, and finally, at the mRNA termination codon, release of the ribosomal assembly and completed polypeptide chain. The released start codon is a site for reinitiation of a new peptide chain. Each initiation, reinitiation, and translocation step may be regulated by interactions with other cytosolic protein factors or by the secondary structure of the mRNA. The large mRNA required for the synthesis of a high molecular weight protein may be read simultaneously by a number of ribosomal assemblies, thus, the rate of production of a polypeptide chain can be further regulated by the density of reading of the message. At the same time, the folding of the nascent chain may be modulated by interaction with various accessory, chaperone, proteins.

Synthesis of a protein destined for secretion extracellularly adds additional layers of complexity, the first of which requires the insertion of the nascent polypeptide chain into the ER compartment. This is accomplished by interaction of the nascent signal peptide (SP) and its ribosomal translocon (R) with the complex called the signal recognition particle (SRP) [Walter and Blobel, 1981; Walter and Johnson, 1994]. This interaction arrests further elongation until the SRP-R-SP complex is bound to the ER surface by ER membrane-associated SRP receptors [Wolin and Walter, 1988; 1989]. Upon insertion of the nascent chain into the lumen of the ER cotranslational processing of the nascent chain and folding take place within the sequestered ER compartment, divorced from the cytosolic-

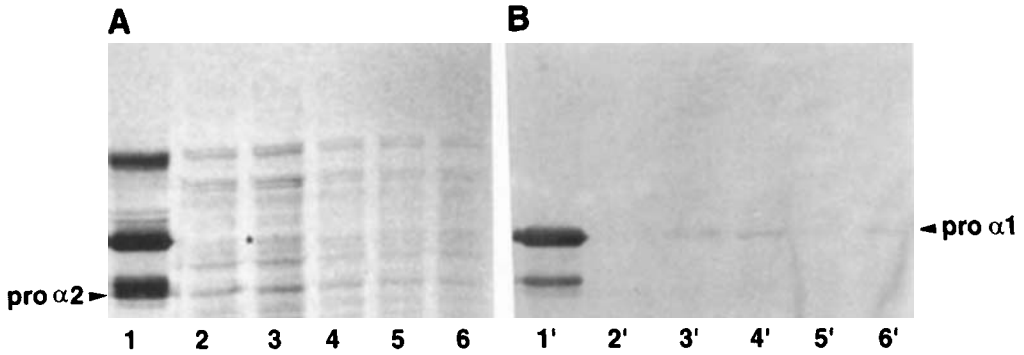

Fig. 13. Effect of inhibition of hydroxylation and triple helix formation on intracellular α1(I) procollagen. Post-ribosomal supernatants from the polysomes pelleted in Figure 10B were Western blotted, stained with SP1.D8, and exposed to X-ray film. *Lanes 1–6* represent autoradiograms of labeled intracellular proteins, while lanes 1′–6′ represent immunoblots of α1(I) chains. Lanes 1 and 1′: standard procollagen. Lanes 2 and 2′: intracellular material from untreated cells. Lanes 3 and 3′: supernatants of cell lysates treated with EDTA before pelleting (equivalent to nascent + intracellular chains); Lanes 4 and 4′, 5 and 5′, 6 and 6′: intracellular material from α,α dipyridyl, *cis*-hydroxyproline or 3,4 dehydroproline treated cells, respectively. There are very low levels of intracellular pro α1 (*) in the post-ribosomal supernatant from untreated cells but a greater amount in the EDTA treated cells.

face synthesis reinitiation, chain elongation and translocation events.

If, as in the case of procollagen I, the secreted molecule is a heteromeric construct requiring the products of translation of two genetically distinct mRNAs, then a mechanism must also exist to target the separate gene products to the same ER compartments. Further, since the chains must fold at the same time to form the compound helix, the interaction of the nascent chains must be well-regulated.

The very elegant study of the rate of synthesis of collagen chains carried out by Vuust and Piez [1972] gave compelling evidence that synthesis of the helical regions of the type I α1 and α2-chains were carried out in parallel and gave rise to the hypothesis that their synthesis was somehow coordinated. More recently, coordinate chain synthesis has been viewed as dependent on the coordinate expression of the pro α1(I) and pro α2(I) genes, based on data which indicated that pro α1(I) and pro α2(I) steady-state mRNA levels were maintained at close to a 2:1 ratio [de Wet et al., 1983; Vuust et al., 1985]. Olsen and Prockop [1989] demonstrated, however, that the pro α1(I):pro α2(I) transcription ratio could vary and reach levels as high as 4:1 in cultured human fibroblasts, depending on culture conditions. It was noteworthy that changes in transcription levels were restricted to the pro α1(I) mRNA. Nevertheless, the apparent steady-state mRNA levels were maintained at an approximate 2:1 ratio under all conditions. Olsen and Prockop postulated that some posttranscriptional regulation mechanism was necessary to explain the discrepancy, however, the assumption of a direct relationship between message levels and translation rates for both chains continues to exist.

Hu et al. [1995a] demonstrated that while translations of the pro α1(I) and pro α2(I) messages proceeded independently in cell-free, membrane-free translation systems, their translation became coordinately altered in the presence of nascent chain insertion-competent microsomes. This led to the suggestion that there were interactions which bring the two pro α chain translational complexes to the same ER compartment and enhance the efficiency of heteromeric chain selection and folding. It was also shown that, both in cell-free systems and in cells, ribosomal loading on the pro α1(I) and pro α2(I) messages was not identical, and apparent synthesis pause sites were not identical. Thus, while the translational systems were co-localized they were not coordinated with respect to individual chain synthesis.

Nascent pro α chain interaction, within the restricted environment of the ER lumen, also shows specific regulation. Hu et al. [1995b] showed that the ER-resident collagen specific chaperone, hsp47, interacts specifically with the pro α1(I) amino propeptide while it had no specific binding to the pro α2(I) chain. They proposed that the hsp47 had its main function in preventing pro α1(I) homotrimer formation, in essence allowing completed pro α2(I) chains the

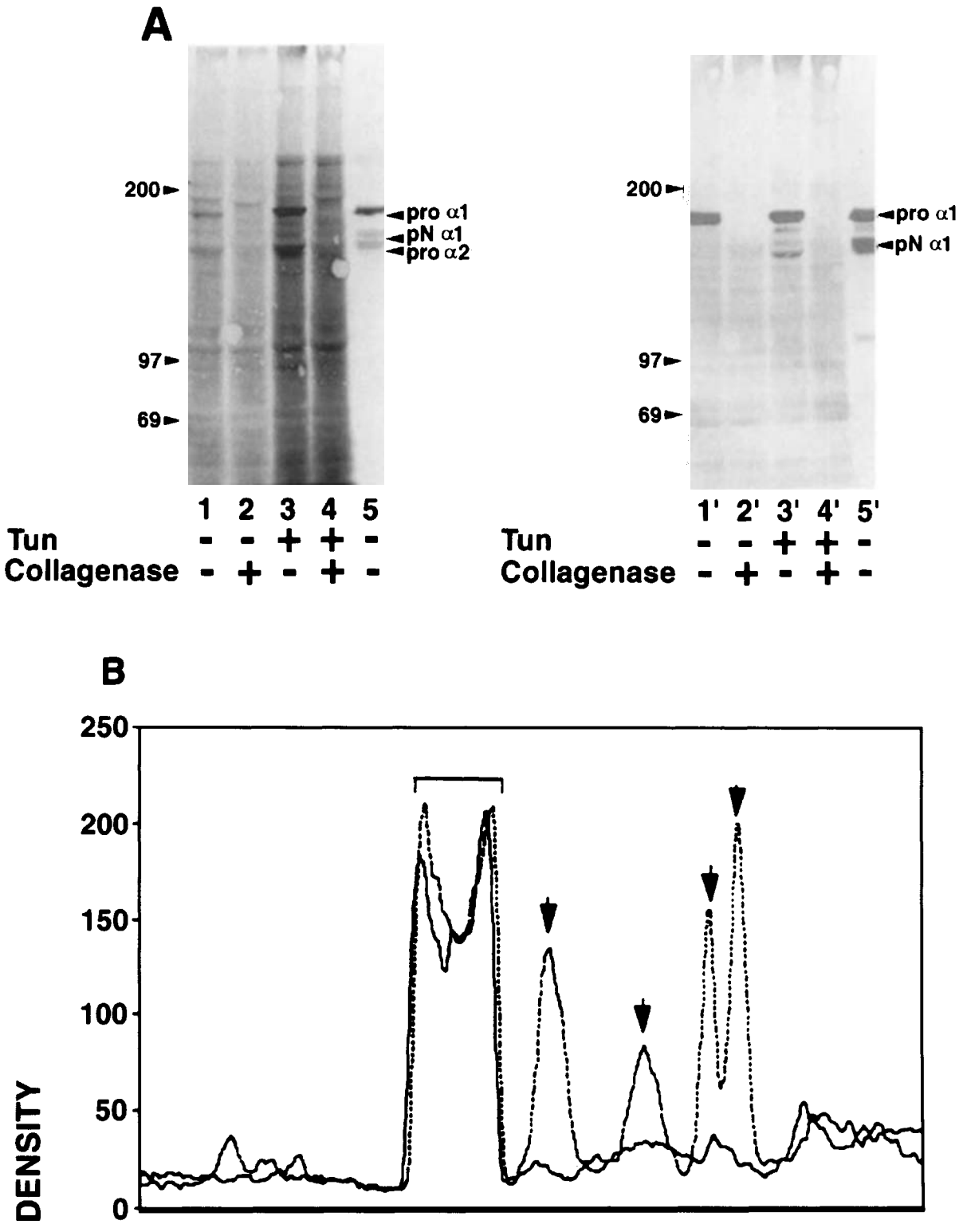

Fig. 14. Tunicamycin inhibition of N-glycosylation of pro α1(I) nascent chains. Polysomes were collected from 3T6 fibroblasts labeled for 30 min with ^{14}C-proline either in the absence (Tun−) or presence (Tun+) of tunicamycin (an inhibitor of N-linked glycosylation). The resulting peptides were Western blotted, stained with SP1.D8, and the blots fluorographed. **A:** *Lanes 1–5* represent the fluorogram, while lanes 1′–5′ represent the resulting immunoblot. Lanes 1 and 1′, polysomes collected from untreated cells; lanes 2 and 2′, collagenase digest of polysome-associated peptides; lanes 3 and 3′, polysomes isolated in the presence of tunicamycin; lanes 4 and 4′, collagenase digests of tunicamycin treated polysomes; lanes 5 and 5′, standard type I procollagen. The migration positions of α1(I)-, α2(I)-, and pNα1(I)-chains are indicated. **B:** Lanes 1′ and 3′ were scanned with a densitometer and compared. Tunicamycin-treated polysomes (*dashed line*) showed several collagenase-sensitive, faster migrating peptides (*arrows*) which accumulated in addition to the standard near-full length α1(I) chains seen in untreated control polysomes (*solid line*).

TABLE II. Total ^{14}C-Proline Incorporated Into Covalent Polysome Peptidyl-tRNA-Complexes

	Total polysome ^{14}C-Pro CPM	CTAB Precipitate or Q-Sepharose retained CPM	% of counts in tRNA-containing complexes
CTAB I	128,200	67,750	48.7
CTAB II	90,800	47,500	47.7
CTAB III	103,750	54,250	52.3
Q-Sepharose	126,960	81,254	36.0

***TABLE III.* Disulfide Bonding in tRNA-Bound and tRNA-Free Polysome Pellet Fractions**

	% of total CPM	% S-S bonded
CTAB-supernatant	39.6	28.2
Q-Sepharose FT	70.0	30.2
Total polysomes		39.7
Media standard procollagen		50.4

opportunity of interacting with completed pro α1(I) chains.

The studies presented here were undertaken with the objective of understanding the chain elongation phase of collagen synthesis more clearly in terms of the differences in pro α chain synthesis rates, and examining the effects of nascent chain cotranslational processing.

A salient feature in the data presented above was the marked differences in the elongation and processing of the nascent pro α1(I) and pro α2(I) chains in 3T6 cells. These cells do produce and secrete type I procollagen heterotrimer with a 2:1 pro α(I):pro α2(I) chain ratio, as demonstrated explicitly in Figure 3A, lane 3. Yet, when one examines equivalent fluorograms of the intact polysomes which should contain all of the nascent chains of both types, there is, instead, an overabundance of high molecular weight pro α1(I)-chains as demonstrated in Figures 5 and 9.

The sensitivity of the Western blotting procedure, using SP1.D8, was not sufficient to detect the lower molecular weight nascent pro α1(I)-chains which must have been present in the polysome pellets (Fig. 6B). Only the single, very prominent band near the migration position of full-length pro α1(I)-chain was evident. We do not have an antibody which will similarly exclusively detect the pro α2(I)-chain. However, one can postulate that if the pro α1(I) and pro α2(I)-chains had been synthesized in coordinated fashion, then a 0.5 equivalent amount of near-full length pro α2(I) should have been present. Consequently if the pro α1(I) band was strongly present in a fluorogram, then a near-full length pro α2(I) band should also have been observed. As seen in Figure 9, at 2 and 4 h after initiation of new collagen synthesis in the synchronized 3T6 cells, a polysome-associated dense, collagenase-sensitive radioactive band was present at the pro α1(I) position, while only a minor pro α2(I)-chain band was evident. The extracellular newly secreted collagen obtained under the same conditions nevertheless did show a 2:1 pro α1(I): pro α2(I) ratio of radiolabel density, as did the post-ribosomal intracellular pool. Only those molecules with the correct heterotrimer composition appear to leave the ER-compartment. Thus, the nascent full length pro α2(I)-chains could not have accumulated on the polysomes in the same fashion as the pro α1(I)-chains.

The polysome-associated proteins obtained in the pelleting procedure contain the entire set of nascent proteins being assembled and processed by the cell at the time of cell lysis. While it is common to think of 3T6 cells and other fibroblasts as having collagen as their major product, and this is true relative to the components destined for the extracellular matrix, it is obvious from Figures 2 and 5 that nascent procollagen I chains represent only a small portion of the total nascent polypeptides at any one time. Labeling with proline may suffice for identification of the secreted procollagen, but other intracellular proline-containing proteins are made in such abundance that the nascent collagen chains of all sizes are difficult to discern. Nevertheless, as shown in Figure 5, it is possible to pick out a few specific bands of radioactivity which are collagenase-digestible. The appearance of multiple distinct collagenase-digestible bands with M_r < pro α-chain size is evidence that synthesis pauses are a feature of nascent collagen-chain elongation in cells. The differences in pause sites and polysomal accumulation of the nascent chains of the two mRNAs was evident in independent cell free translations (Fig. 4), as reported by Hu et al. [1995a].

The pro α1(I)-chain is not the only polysome-associated protein which increased in content with time after labeling. Polypeptides with M_r's of 120,000, 97,000, and 47,000 steadily in-

creased in content over the 4 h labeling period examined (Fig. 5B). It is likely that the 47 K M_r component was the pro α1(I)-specific chaperone, hsp47 [Hu et al., 1995b]. A comparable 47 K protein was not detected among the ammonium sulfate precipitated extracellular media proteins.

If collagen hydroxylation is inhibited, collagen accumulates in the ER and secretion is blocked. However, Veis and Kirk [1989] reported that underhydroxylation during synthesis did not have any apparent effect on the distribution of putative pause sites on polysome-associated collagen chains. This was substantiated in the present work. Although the apparent molecular weights of the polysome-associated underhydroxylated chains were reduced, the hydroxylation and triple-helix formation inhibitors did not increase the amount of the polysome-associated full-length pro α1(I)-chains. Detailed analysis of the chain distribution within the major polysome-associated pro α1(I) band did indicate (Fig. 12) hydroxylation-related processing which broadened the range of apparent M_r. Some significantly lower M_r pro α1(I)-related components were present. There was no radiographic evidence to suggest that the rate of elongation of the pro α2(I)-chain was significantly altered by underhydroxylation.

Blocking triple helix formation with proline analogs did not alter the polysomal nascent chain distributions or cause the chains to accumulate in the postribosomal compartment, thus regulation of chain elongation is not related to the process of triple helix formation.

Tunicamycin was the only agent which did cause a significant alteration in the apparent pro α1(I) pause sites. Major accumulations of nascent polysome-associated paused peptides, at levels sufficient for antibody detection, were noted after 4 h incubation of the cells with tunicamycin. There is only one N-glycosylation site with an oligosaccharide M_r of 2,000–2,200 on each of the pro α-chain C-propeptides [Clark, 1979]. Thus, it was surprising to see that at least three additional pro α1(I)-chain pause sites were so prominent (Fig. 14). It is not clear at this time whether this was the result of a direct effect of tyunicamycin on the nascent procollagen chain glycation, or an indirect effect resulting from a defect in glycosylation of some other component important in the nascent chain processing, such as a chain elongation factor [Duksin and Bornstein, 1977] or a chaperone or other accessory protein.

A Model for Pro α Chain Synthesis Coordination and Interaction During Procollagen I Heterotrimer Formation

The considerations presented above lead to the conclusions that the pro α1(I) and pro α2(I) chains of procollagen I are coordinated in synthesis by the process of parallel targeting of the respective mRNAs to the same ER compartments, but that the rates of chain elongation are not coordinated. The pro α1(I) nascent chain interacts with hsp47 within the lumen of the ER in a different fashion than does the pro α2(I) chain. The translation of the pro α1(I) mRNA is more efficient than that of the pro α2(I) chain, but the nearly completed pro α1(I) chains are held up and accumulate on the polysome. Pro α2(I) chains within the same compartments are not held up, but at, or near release may interact with the pro α1(I) chains and form the heterotrimer which then moves on to the Golgi in the secretory pathway. Chain processing per se within the ER lumen does not affect either the rate of chain elongation or the pattern of synthesis pauses. The polysome-retained excess pro α1(I) chains can either form a low level of [pro $\alpha1(I)]_3$ homotrimers which proceed to secretion through the Golgi route or are degraded intracellularly. Bienkowski et al. [1978] have shown that 10 to 15% of newly synthesized collagen never becomes secreted but is degraded intracellularly, although the pathway of that degradation process has not been determined. The polysome retained pro α1(I) chain which never becomes triple helical may be the source for this background of intracellularly degraded collagen. These considerations are summarized in the scheme shown in Figure 15. This scheme points out the three major problem areas which need further investigation: the basis for the coordinated pro α1(I)-pro α2(I) chain targeting to the ER; the basis for the strong synthesis pause-polysome retention of the near-full-length pro α1(I) chains; and, the mechanism whereby the pro α2(I) chain successfully competes with pro α1(I) chain in the homotrimer-heterotrimer competition.

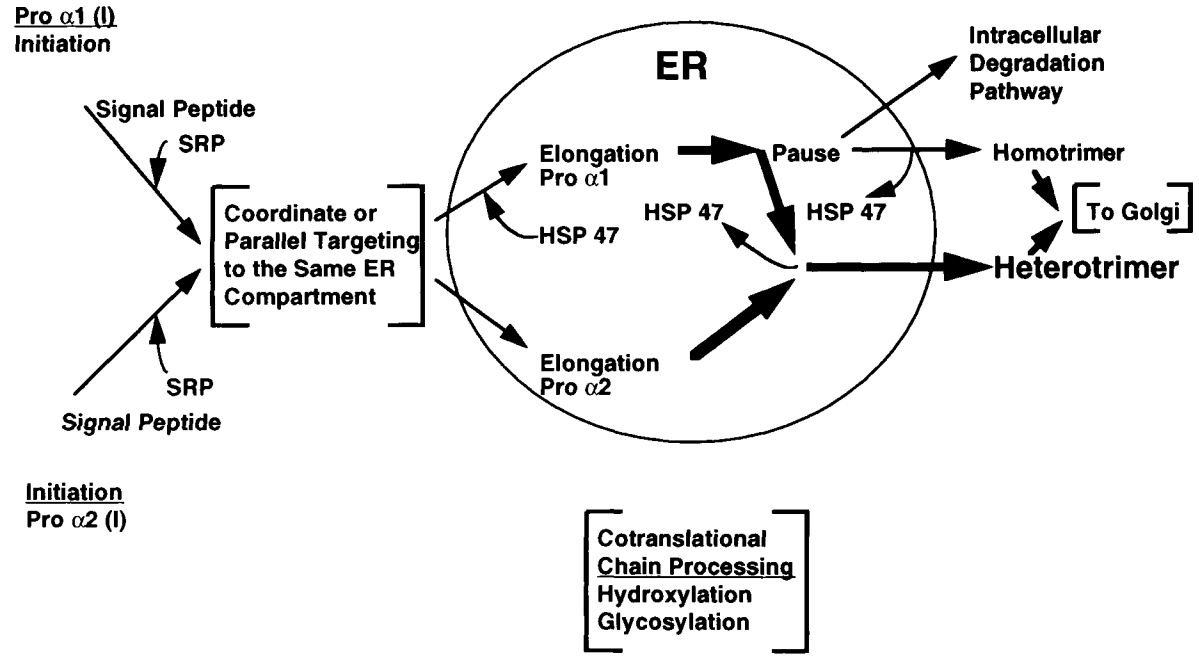

Fig. 15. A schematic representation of the points of intersection between pro α1(I) and pro α2(I) chains and their ribosomal complexes during their synthesis. In this scheme chain synthesis begins in the cytosol as usual for secretory proteins with the independent assembly of the first ribosome on each mRNA and initiation of chain elongation. We propose that after interaction of the nascent signal peptide with a SRP, and the consequent transient elongation arrest, the first unique event takes place with a clear cut [Hu et al., 1995a] but mechanistically unknown interaction or set of interactions which targets each initial translocon to the surface of the same ER compartment. Each polypeptide chain is inserted through the ER membrane and elongation continues independently, and at a somewhat different efficiency for each chain. The amino-terminal propeptide of the pro α1(I) chain interacts specifically with the ER resident chaperone hsp47 [Hu et al., 1995b], preventing pro α1(I) chain interactions. The usual steps of cotranslational processing (hydroxylation, O-linked glycosylation) follow. At a point near complete chain elongation, but before release of the chains from their polysomal complexes [Veis et al., 1985] the nascent pro α1(I) and pro α2(I) chains interact to form large polysome complexes. In this model, the elongation rates and synthesis pausing are governed by events taking place on the cytosolic face of the ER while the interactions between the colocalized complexes take place at the membrane or within the ER lumen. Near the completion of the more efficient synthesis of the pro α1(I) chains a small fraction of the chains are shunted off to a paused state, held on the polysomes. The less efficiently completed pro α2(I) chains, however, interact directly with other pro α1(I) chains to form the heterotrimers. The hsp47 is released for recycling within the ER and triple helix is formed. The released triple helical heterotrimers then move to the Golgi for further secretory processing. A portion of the paused pro α1(I)-chains may form triple helix and move as homotrimers in a parallel fashion along the secretory pathway. Cells which have been engineered to block pro α2(I)-mRNA readily form [pro α1(I)]$_3$ homotrimers [Marsilio et al., 1984; Lee et al., 1988]. Hsp47 is again released during this process. Finally, the pro α1(I) chains retained for too long a period (consuming pro α1(I)-mRNA and reducing pro α1(I)-chain potential net synthesis) are degraded intracellularly [Beinkowski et al., 1978]. The thickness of the arrows at the completion of elongation is meant to represent the relative amounts going to each pathway. This model emphasizes the need to examine the basis for the coordinated pro α1(I)-pro α2(I) chain targeting to the ER and the basis for the strong synthesis pause-polysome retention of the near-full length pro α1(I) chains, which are likely to have cytosolic regulatory factors, and the mechanism whereby the pro α2(I) chain successfully competes with pro α1(I) chain in the homotrimer-heterotrimer competition within the ER lumen.

ACKNOWLEDGMENTS

We are indebted to Drs. Farida Siddiqui and Boris Sabsay for their advice concerning this manuscript, and the study as it progressed. We thank Tom Dahl for his help in preparing the figures.

This work has been supported by NIH Grants AR13921 and DE01374 to A.V., and is derived, in part, from a doctoral dissertation submitted by T.G. to the Graduate School, Northwestern University, Evanston, IL, June 1992.

REFERENCES

Bergman LW, Kuehl WM (1977): Addition of glucosamine and mannose to nascent immunoglobulin heavy chains. Biochemistry 16:4490–4497.

Bienkowski RB, Cowan MJ, Mcdonal JA, Crystal RG (1978): Degradation of newly synthesized collagen. J Biol Chem 253:4356–4363.

Brownell AG, Veis A (1975): The intracellular location of glycosylation of hydroxylysine of collagen. Biochem Biophys Res Commun 63:371–377.

Brownell AG, Veis A (1976): Intracellular location of triple helix formation of collagen. Enzyme probe studies. J Biol Chem 251:7137–7143.

Clark CC (1979): The distribution and initial characterization of oligosaccharide units on the COOH-terminal propeptide extensions of the pro-α1 and pro α2 chains of type I procollagen. J Biol Chem 254:10798–10802.

de Wet WJ, Chu M-L, Prockop DJ (1983): The mRNAs for the pro-α1(I) and pro-α2(I) chains of type I procollagen are translated at the same rate in normal human fibroblasts and in fibroblasts from two variants of *Osteogenesis Imperfecta* with altered steady state ratios of the two mRNAs. J Biol Chem 258:14385–14389.

Duksin D, Bornstein P (1977): Impaired conversion of procollagen to collagen by fibroblasts and bone treated with tunicamycin, an inhibitor of protein glycosylation. J Biol Chem 252:955–962.

Gilmore R, Blobel G (1985): Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. Cell 42: 497–505.

Horlein D, McPherson J, Goh SH, Bornstein P (1981): Regulation of protein synthesis: Translational control by procollagen-derived fragments. Proc Natl Acad Sci USA 78:6163–6167.

Hu G, Tylzanowski P, Inoue H, Veis A: Relationships between translation of pro α1(I) and pro α2(I) mRNAs during synthesis of the Type I procollagen heterotrimer. J Cell Biochem 59:214–234.

Hu G, Gura T, Sabsay B, Sauk J, Dixit SN, Veis A: The Endoplasmic Reticulum Protein Hsp47 Binds Specifically to the N-Terminal Globular Domain of the Amino-Propeptide of the Procollagen I α1(I)-Chain. J Cell Biochem 59:350–367.

Kirk TZ, Evans JS, Veis A (1987): Biosynthesis of Type I Collagen. Characterization of the distribution of chain sizes and extent of hydroxylation of polysome associated pro α chains. J Biol Chem 262:5540–5545.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee S-T, Smith BD, Greenspan D (1988): Construction of a full length cDNA encoding human pro α2(I) collagen and its expression in pro α2(I)-deficient W8 rat cells. J Biol Chem 263:13414–13418.

Lazarides EL, Lukens LN, Infante AA (1971): Collagen polysomes: Site of hydroxylation of proline residues. J Mol Biol 58:831–846.

Marsilio R, Sobel ME, Smith BD (1984): Absence of procollagen α2(I) mRNA in chemically transformed rat liver epithelial cells. J Biol Chem 259:1401–1404.

Moazed D, Noller HF (1989): Intermediate states in the movement of transfer RNA in the ribosome. Nature 342: 142–148.

Olsen AS, Prockop DJ (1989): Transcription of human type I collagen genes. Matrix 9:73–81.

Perlish JS, Timpl R, Fleischmajer R (1985): Collagen synthesis regulation by the aminopropeptide of procollagen I in normal and scleroderma fibroblasts. Arthritis Rheum 38: 647–651.

Ramaley PB, Rosenbloom L (1971): Inhibition of proline and lysine hydroxylation prevents normal extrusion of collagen by 3T6 fibroblasts in culture. FEBS Lett 15:59–64.

Rosenbloom J, Prockop DJ (1971): Incorporation of cis-hydroxyproline into protocollagen and collagen. Collagen containing cis-hydroxyproline in place of proline and trans-hydroxyproline is not extruded at a normal rate. J Biol Chem 246:1549–1555.

Sandell L, Veis A (1980): The molecular weight of the cell-free translation product of α1(I) procollagen mRNA. Biochem Biophys Res Commun 92:554–562.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Uitto J, Prockop DJ (1974): Hydroxylation of peptide-bound proline and lysine before and after chain completion of the polypeptide chains of procollagen. Arch Biochem Biophys 164:210–217.

Veis A, Brownell AG (1977): Triple-helix formation on ribosome-bound nascent chains of procollagen: Deuterium-hydrogen exchange studies. Proc Natl Acad Sci USA 74: 902–905.

Veis A, Kirk TZ (1989): The coordinate synthesis and cotranslational assembly of Type I procollagen. J Biol Chem 264:3884–3889.

Veis A, Leibovich SJ, Evans J, Kirk TZ (1985): Supramolecular assemblies of mRNA direct the coordinated synthesis of type I collagen chains. Proc Natl Acad Sci USA 82:3693–3697.

Vuust J, and Piez KA (1972): A Kinetic Study of Collagen Biosynthesis. J Biol Chem 247:856–862.

Vuust J, Sobel ME, Martin GR (1985): Regulation of type I collagen synthesis. Total pro α1(I) and pro α2(I) mRNAs are maintained in a 2:1 ratio under varying rates of collagen synthesis. Eur J Biochem 151:449–453.

Wolin SL, Walter P (1988): Ribosome pausing and stacking during translation of eukaryotic mRNA. EMBO J 7:3559–3569.

Wolin SL, Walter P (1989): Signal recognition particle mediates a transient arrest of preprolactin in reticulocyte lysate. J Cell Biol 109:2617–2622.