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Relationships Between Translation of Pro α1(I) and Pro α2(I) mRNAs During Synthesis of the Type I Procollagen Heterotrimer

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Abstract Final assembly of the procollagen I heterotrimeric molecule is initiated by interactions between the carboxyl propeptide domains of completed, or nearly completed nascent pro α chains. These interactions register the chains for triple helix folding. Prior to these events, however, the appropriate nascent chains must be brought within the same compartments of the endoplasmic reticulum (ER). We hypothesize that the co-localization of the synthesis of the nascent pro α1(I) and pro α2(I) chains results from an interaction between their translational complexes during chain synthesis. This has been investigated by studying the polyribosomal loading of the pro α-chain messages during in vitro translation in the presence and absence of microsomal membranes, and in cells which have the ability to synthesize the pro α1 homotrimer or the normal heterotrimer. Recombinant human pro α1(I) and pro α2(I) cDNAs were inserted into plasmids and then transcribed in vitro. The resulting RNAs were translated separately and in mixture in a cell-free rabbit reticulocyte lysate ± canine pancreatic microsomes. Cycloheximide (100 μg/ml) was added and the polysomes were collected and fractionated on a 15–50% sucrose gradient. The RNA was extracted from each fraction and the level of each chain message was determined by RT-PCR. Polysomes from K16 (heterotrimer-producing), W8 (pro α1(I) homotrimer), and A2′ (heterotrimer + homotrimer) cells were similarly analyzed. Translations of the pro α1(I) and pro α2(I) messages proceeded independently in the cell-free, membrane-free systems, but were coordinately altered in the presence of membrane. The cell-free + membrane translation systems mimicked the behavior of the comparable cell polysome mRNA loading distributions. These data all suggest that there is an interaction between the pro α chain translational complexes at the ER membrane surface which temporally and spatially localize the nascent chains for efficient heteromeric selection and folding.

Key words: polysomes, in vitro translation, chain initiation, synthesis pauses, pro α chains

Formation of the triple helical heterotrimeric procollagen I molecule requires the synthesis of the constituent nascent pro α1(I) and pro α2(I) chains, translated from two distinct mRNAs. Molecular assembly, including polypeptide chain registration and triple helix formation, takes place within the endoplasmic reticulum (ER) compartment. Transfer to the Golgi apparatus for movement into the secretory pathway requires completion of both post translational modifications and triple helix formation. The [pro α1(I)]$_2$ [pro α2(I)] heterotrimer is the structure produced in overwhelming amount. However, a stable [pro α1(I)]$_3$ homotrimer can be formed from pro α1(I)-chains and small amounts of [pro α1(I)]$_3$ homotrimer are often detected in most systems. On the other hand, a [pro α2(I)]$_3$ homotrimer has not been detected. Thus, chain selection for molecular assembly has considerable specificity. Efficient procollagen I heterotrimer assembly appears to require that appropriate sets of elongating nascent chains be inserted into the same compartments of the ER, and that a mechanism for chain selection must be operative within the ER.

Most investigators have addressed the coordination of collagen chain synthesis as a problem of coordinate transcription of the pro α1(I) and pro α2(I) genes [Karsenty and de Crombrugghe, 1990; Lyons and Schwarz, 1984; Rossi et al., 1988; Vuust et al., 1985], with the finding that

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in normal fibroblasts, there is coordinate expression of both genes in an approximate 2/1 ratio [Vuust et al., 1985; Kosher et al., 1986; Vuorio and de Crombrugghe, 1990]. However, as shown by Olsen and Prockop [1989] levels of pro α1(I) and pro α2(I) messages can deviate appreciably from the 2/1 ratio, although at the protein level the secreted collagen I maintains the heterotrimer 2/1 chain ratio. This, together with our previous work [Brownell and Veis, 1976; Veis and Brownell, 1977; Veis et al., 1985; Kirk et al., 1987; Veis and Kirk, 1989], indicated that there must be a mechanism regulating chain selection. In the present work we have focused on the next steps in biosynthesis, that is, on the processes surrounding the translation of the mRNAs.

We have demonstrated previously that interactions leading to registration of nascent pro α(I) chains could take place while the chains were still polysome-associated [Brownell and Veis, 1976] and that triple helix formation could also be initiated [Veis and Brownell, 1977]. Direct observation of polysomal aggregates using rotary shadowing electron microscopy showed that nascent collagen chains were being translated on complex polyribosomal aggregates associated with more than one strand of mRNA. The suggestion was made that the organization of triple helical type I procollagen molecules begins with the organization of the mRNA translocons themselves [Veis et al., 1985]. Further studies of the coordinate synthesis of the pro α1(I)- and pro α2(I)-chains [Kirk et al., 1987; Veis and Kirk, 1989] supported a molecular assembly mechanism in which chain selection and folding were related to the attachment of the ribosomes to the ER surface. Elongation pauses were also a prominent feature of chain synthesis [Veis and Kirk, 1989]. The present study was undertaken to examine procollagen I heterotrimer synthesis in terms of a molecular mechanism which could encompass both independent message readout and coordinated synthesis.

Logically, the translation of secreted, multimeric proteins can be divided into those processes which deal with the cytosolic side of the ER surface and relate to the initiation of chain synthesis and message readout, and those which deal with the interactions of the nascent chains within the ER lumen, ultimately leading to polypeptide chain selection and folding. The strategy adopted in this work has been to make a direct examination of the possibility of interaction between pro α1(I) and pro α2(I) mRNAs or their translational complexes while they are actively engaged in the translation of procollagen I. If the mRNAs or translational complexes interact during translation, then systems with different pro α1 and pro α2 message levels should have different patterns of pro α1 and pro α2 message translation rates, synthesis pauses during readout, and consequently differences in polysomal loading. The specific approach was to examine the extent of chain synthesis and ribosomal loading in cell-free rabbit reticulocyte lysate translation systems containing pure pro α1(I) or pro α2(I) mRNAs or mixtures, and then compare these data with that from the same systems plus microsomal membrane. These data were then to be compared with the ribosomal loading patterns within cells synthesizing either the normal heterotrimer or the pro α1(I) homotrimer. As shown below, the synthesis and ribosomal loading patterns were indeed different, indicating a membrane related interaction between translation of the two messages.

EXPERIMENTAL PROCEDURES

Cell-Free Translation

The full-length cDNAs of human pro α1(I) and pro α2(I) chains were excised by Hind III and Hpa I double digestion from recombinant eukaryotic expression plasmids pSTL2 [Lee et al., 1992], and pSTL29 [Lee et al., 1988, 1990] and cloned into subcloning vector pGEM-3Z (Promega, Madison, WI) between the Hind III and Sma I sites so as to place the sense strand of both cDNAs under the control of the SP6 promoter. The plasmids, designated pHHα1 and pHHα2, respectively, were linearized by digestion with Nde I at its recognition site immediately downstream of the 3′ end of the cDNA insert. We are indebted to Dr. Dan Greenspan of the University of Wisconsin for the gift of the pSTL2 and pSTL29 plasmids.

In vitro transcription of the linearized cDNA was then carried out with phage SP6 RNA polymerase. The RNA transcripts were recovered from the reaction mixture by DNase digestion, phenol/chloroform extraction, and ethanol precipitation. Purity of the RNAs was analyzed by UV absorbance at 260 nm and the integrity of the mRNAs was verified by denaturing agarose gel electrophoresis. Each preparation yielded a single component. The pro α1(I) transcript was approximately 5.5 kb and the pro α2(I) transcript was 4.5 kb.

Cell-free translation was carried out using the Promega rabbit reticulocyte lysate translation system. The initial uncapped RNAs were able to be translated satisfactorily after optimization of the K^+ ion concentration. The maximum amounts of full-length polypeptide products of both mRNAs were obtained at 100–120 mM K^+ and at RNA concentration to 2.0–5.0 μg/50 μl. Reactions were carried out in total volume of 50 or 100 μl, at 30°C. The translations of the two RNAs were carried out under identical conditions with [^{35}S]-Methionine (0.8–1.0 mCi/ml) in the lysate. The distribution of molecular size of the synthesized polypeptides was determined by SDS-PAGE on 6% gels. After electrophoresis the gels were fixed in 30% CH_3OH-10% CH_3COOH for 20 min, then washed in H_2O for 10 min, and Fluoro-Hance (Research Products International, Mt. Prospect, IL.) for 30 min. The gels were dried and then exposed to Kodak XAR5 film for 12–24 h at −80°C. Collagen components were identified by digestion of the translation mix with bacterial collagenase (Advanced Biofactures, Type III, Lynbrook, NY) and electrophoresis on 6% gels as described by Ouellette et al. [1981]. A control translation of luciferase mRNA was carried out in every case to assure that the translation system was active.

In the initial experiments the pro α1(I) and pro α2(I) RNA transcripts were translated separately. A second set of translations was carried out under identical conditions except that canine pancreatic microsomes (Promega) were added to make a final concentration of 1 u/μl in the reaction mixture [Walter and Blobel, 1993]. Finally, the pro α1(I) and pro α2(I) RNAs were mixed in 2:1 molar ratios at the final RNA content of 0.1 μg/1 μl (50 μl final volume). The translations were then carried out ± the pancreatic microsomes.

When the cell-free translations were used for polysome isolation and determination of RNA distributions, 20 nM complete amino acid mixture was used in place of the 20 nM amino acid mix minus Met. The translations were terminated by the addition of cycloheximide to a final concentration of 100 μg/ml. When microsomal membranes had been added to the translation mix, Triton X-100 and sodium deoxycholate were also added to final concentrations of 0.2 and 0.05%, respectively, to dissolve the membranes.

Cell Lines

K16 and W8 rat liver epithelial cell lines were a gift from Dr. Barbara Smith [Smith and Niles, 1980]. The K16 cells produce normal collagen type I heterotrimer [Marsilio et al., 1984]. The W8 cell line was derived from chemically transformed K16 cells. The W8 cells produce pro α1(I) mRNA and secrete triple helical pro α1(I) homotrimer but do not transcribe the pro α2(I) message [Smith and Niles, 1980]. The A2 cell line [Lee et al., 1988], a gift from Dr. Dan Greenspan, had been constructed by transfecting the W8 cell line with a full-length cDNA clone (pSTL29) for the human pro α2(I) gene, the same cDNA used in the in vitro transcription-cell-free translation study described above. The transfected A2 cells synthesize human pro α2(I) chains and secrete a mixture of [pro α1(I)]$_2$[pro α2(I)] heterotrimer and [pro α1(I)]$_3$ homotrimer. During the course of this work the A2 cells were lost and no further supply was available. We therefore transfected a new batch of the W8 cells with the pSTL29 essentially according to the procedure of Lee et al. [1988]. Transfection was accomplished with 5 μg pSV2neo (Clontech, Palo Alto, CA) and 25 μg pSTL29/10 cm culture dish (10^6 cells) using the calcium phosphate precipitation method [Wigler et al., 1979] and the Promega mammalian transfection system. The W8 cells were incubated in the transfection medium for 16 h then glycerol shocked for 2 min. The cells were further incubated in culture medium with 10% FBS for 24 h, then 0.4 mg/ml G418 (Gibco-brl, Gaithersburg, MD) was added to the medium. The G418-resistant clonal cells were picked with a cloning cylinder and maintained in culture medium with 10% FBS and G418 (0.2 mg/ml).

Total RNA from each of the clonal transfected cells was isolated using a RNA isolation kit (Tel-Test Inc., Friendswood, TX) and dot-blotted to Nylon membrane (Amersham, Arlington Heights, IL) at 2 μg total RNA/blot using a Dot-blot filtration manifold [Schleicher and Schuell, Keene, NH]. The filter was baked at 80°C for 2 h and prehybridized using Quick-Hybrid hybridization solution (Stratagene, La Jolla, CA). A nucleotide probe specific for the human pro α2(I) gene was radiolabeled with [α-^{32}P]dCTP (ICN Biomedicals, Costa Mesa, CA) by a random priming kit (Stratagene), following the manufacturer's directions.

Cell Culture Techniques

Cells were grown at 37°C in an atmosphere of 93% air and 7% CO_2 in DMEM medium [DMEM + 10% FCS with penicillin (1,000 U/ml), streptomycin (1 mg/ml), and fungizone

(0.25 μM/ml) added as antibiotic/antimycotic agents (Northwestern Univ. Cancer Center Media Preparation)] or in α-MEM with 10% FBS (GIBCO-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. When used, ascorbate was added at 50 or 150 μg/ml of medium.

When the cells were to be stored they were grown in the DMEM medium plus 150 μg/ml ascorbate, which was added fresh daily. The cells were detached from the plates with 0.05% trypsin + 0.5 mM NaEDTA and the cell suspension was pelleted by centrifugation at 600*g*. The cells were washed twice with DMEM medium. The final pellet was resuspended in DMEM in 10% glycerol. The cells were held at room temperature for 30 min, then aliquoted into microfuge vials. The vials were held at −20°C for 1 h, then transferred to −70°C for 4–16 h. Long-term storage was at liquid nitrogen temperature.

Collagen Analysis

For analyses of the collagens produced by the different cells, the cells were grown to ≈95% confluence in T150 flasks. At that point 0.2 μCi [^{14}C]-proline/ml and 64 μg/ml β-aminopropionitrile was added to the medium and the cells were incubated for 24 h. The procollagen was precipitated from the medium with 148 mg/ml ammonium sulfate. The precipitate was collected by centrifugation in a JA20 (Beckman) rotor at 15,000 rpm for 20 min. The pellet was dissolved in 100 μl 0.4 M NaCl and centrifuged at 12,000 rpm for 10 min to remove any undissolved material. The proteins remaining in the supernatant were analyzed on 6% SDS-polyacrylamide gels under reducing conditions. A portion of each preparation was digested with 48 U bacterial collagenase (Advanced Biofactures, Type III; or Calbiochem, La Jolla, CA) at 37°C for 20 min in 5 mM $CaCl_2$, 30 mM KCl, 100 mM Tris.HCl, pH 7.5. The reaction was stopped by addition of the SDS-PAGE sample buffer to the mixture. Chymotryptic digestion was carried out in the same digestion buffer, with 100 μg/ml chymotrypsin, for 60 min on ice. After electrophoresis the gels were prepared for fluorography by Fluoro-Hance as described above. Exposure to Kodak XAR5 film was from 5 to 7 days at −80°C depending on the level of radioactivity.

Isolation of Polysomes

For polysome isolation the cells were grown to confluence in complete amino acid mixtures without the addition of labeled amino acids. The cells were detached from the plates with 0.05% EDTA-free trypsin. Cells were combined fresh from four T150 flasks, or approximately 5×10^9 frozen cells were thawed, and lysed in 1 ml of RNase-free Lysis Buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.2% Triton X-100 [Pierce-HPLC Grade], 0.05% sodium deoxycholate, 0.2 mg/ml of heparin). The cell sludge was vortexed in and out of ice for a total time of 2 min. The suspension was then transferred into a 1.5 ml microfuge tube and centrifuged for 5 min at ≈14,000*g* at 4°C. The supernatant was taken up with a pipette in such a way as to avoid the pellet as well as the white flocculent material on the top. This supernatant (approximately 900 μl) was layered onto a 15–50% linear sucrose gradient.

Linear 15–50% sucrose gradients with a total volume of 12 ml were prepared using a Hoefer SG Series gradient maker. The stock sucrose solutions were treated with diethylpyrocarbonate (DEPC) and autoclaved. Subsequently the stock solutions were diluted with DEPC treated and autoclaved 10× Gradient Buffer, to yield a final Gradient Buffer composition of 10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, and 0.02 mg of heparin/ml. In some cases the final Gradient Buffer also contained 100 μg/ml cycloheximide. A P-3 peristaltic pump (Pharmacia, Piscataway, NJ) was used to transfer the gradient into an UltraClear ultracentrifuge tube (Beckman, Fullerton, CA). The gradient was poured from the bottom of the tube, with the 15% sucrose coming in first. The total volume of the sucrose gradient was 12 ml and allowed the use of 900 μl of sample.

The gradients were spun at 40,000 rpm in a Beckman SW41 rotor to $\omega^2 t$ of 1.15×10^{11} rad^2/sec, at 4°C. Following the centrifugation the gradients were collected using an ISCO gradient maker (Model 185, ISCO, Lincoln, NE), a flow cell, and a Gilson 205 fraction collector. The fractions were monitored in an ISCO Model 228 Absorbance Detector flow cell at 254 nm and the absorbance was plotted as a function of time. The fractions were collected either at 5 drops/tube for radioactivity count (protein labeling) or at 32 or 50 drops/tube for RNA isolation.

Polysomes obtained from the cell-free translation reaction mixtures were analyzed and fractionated on 15–50% sucrose gradients in the same way except that the total volume of the gradient was 3.6 ml. The total volume of translation mix was layered onto the gradients and spun at 300,000*g* in a Beckman SW 50.1 rotor

for 2 h at 4°C. Ten 360 μl fractions were collected, then 50 μl aliquots were taken from each fraction and diluted with 250 μl DEPC-treated water. These diluted fractions were used for RNA isolation.

RNA Extraction From the Polysomes

Total RNA from cells. Two methods were used. In the first experiments, phenol saturated with water was mixed with chloroform and isoamyl alcohol in ratio 25:24:1. Sucrose gradient fractions were suspended in water containing 0.1% SDS. Each tube was vortexed briefly and the phenol mix was added. The tube was vortexed again and then centrifuged for 5 min. The water layer was transferred to a new tube and the phenol extraction repeated. The extractions were continued until no precipitate was seen at the phenol/water interface. A final extraction was done and the aqueous phase transferred to a new tube. The RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.6, and 3 volumes of ethanol. The precipitated RNA was resuspended in 100 μl of DEPC-treated water, then reprecipitated twice in the same way. The concentration of RNA was determined by measuring the optical density at 260 nm of a 10 μl aliquot of the final solution. The remaining solution was kept at −70°C.

The simplified single step guanidinium thiocyanate-phenol-chloroform procedure of Chomczynski and Sacchi [1987] provided equivalent yields of total RNA and was the ultimate method of choice.

mRNA extraction. Messenger RNA was isolated from both the cells and isolated polysome fractions using the mRNA Isolation Kit from Becton Dickson (Lincoln Park, NJ) with 10 μg polyinosinic acid as carrier [Winslow and Henlkart, 1991].

mRNA Detection

Slot blots. Slot blots were done using a S & S Minifold II (Schleicher and Schuell Inc.) according to the recommendations of the manufacturer, published in the technical bulletin "Sequences," 371. Between 1 and 10 μg of total RNA was placed in a total volume of 100 μl of water and mixed with 300 μl of 6.15 M formaldehyde in 10 × SSC. The solution was incubated at 65°C for 15 min. The slots were washed with 500 μl of 10 × SSC and 0.5 μg of isolated RNA was loaded into each slot. The filters were baked for 2 h at 80°C under vacuum. The nitrocellulose was prehybridized for 2 h in hybridization buffer containing 50% formamide, then hybridized overnight in the same solution with the probes at 42°C, washed in 2 × SSC plus 0.1% SDS 3 times, and autoradiographed.

The autoradiograms were scanned using a Hoefer Scanner, Model 1650, and analyzed using the Hoefer GS365 software program.

The blots were probed with either pα1R1 or pα2R2 nucleotide probes specific for the rat pro α1(I) and pro α2(I) mRNAs, respectively [Genovese et al., 1984]. These probes are the cDNA sequences coding for the C-terminal part of the respective procollagen chains. Random priming was done using the Prime-It kit (Stratagene), following manufacturer's directions. The probe specific activity was determined by TCA precipitation on glass fiber filters (GF/C, Whatman, Clifton, NJ). Prior to hybridization the probes were denatured by adding 0.1 volume of 1 N NaOH and incubating at 37°C for 5 min.

Amplification of cDNA by RT-PCR. Messenger RNA from each cell polysome fraction (1 ng) was reverse-transcribed to cDNA with oligo-d(T) nucleotide primers using a Gibco-brl synthesis kit with Moloney murine leukemia virus reverse transcriptase (MMLV). The reaction was stopped with 10 mM EDTA. Taq buffer (20 mM Tris.HCl, pH 8.4, 50 mM KCl, 2.5 mM dithiothreitol, 0.4 mM dNTPs) and a set of nested sense and antisense primers, constructed as described below, were added. A set of rat β-actin primers (Clontech, Palo Alto, CA) was also used. The reaction mixture was incubated 5 min at 94°C and 5 U Taq polymerase (Perkin-Elmer, Norwalk, CT) was added. PCR was performed for 30 cycles consisting of 45 s at 94°C, 2 min at 58°C (for rat pro α1(I) and pro α2(I) primers) or 62°C (for human pro α2(I) and rat β-actin primers), and 3 min at 72°C. During the last cycle the extension time was lengthened to 15 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and detected with ethidium bromide fluorescence.

The polysome fractions from the cell-free translations were analyzed in a similar fashion. In this case human pro α1(I) and pro α2(I) primers were used. PCR proceeded for 28 cycles of 94°C for 1 min, 61°C for 1.5 min, and 72°C for 2 min. During the final cycle the extension time was 15 min. The PCR products were analyzed on 1.8% agarose gels.

Rat specific probes for pro α1(I) and pro α2(I) messages. The K16 and W8 cells were derived from rat. Primers for rat specific sequence probes were designed from the C-terminal sequences of the rat pα1R2 and pα2R2 clones [Genovese et al., 1984]. For the pα1R2 clone, the

forward and reverse primers were GATGGCTGCACGAGTCACAC and GTCTGGGGCACCAACGTCCA, respectively. For the pα2R2 primers, the corresponding sequences were CCTGCTGGTCCTTCTGGCCC and CCACTCTGGGTGGCTGAGTC. Total RNA from K16 cells was used as a template for reverse transcription (Superscript II, GIBCO-BRL). The cDNAs for rat pro α1(I) and pro α2(I) chains were also amplified with PCR. The rat pro α1 primers produced a 117 bp band and the pro α2 primers produced a 356 bp band as predicted from the initial cDNA sequences. The bands were purified from the gels and subcloned into pCR II vector using the TA cloning system (Invitrogen, San Diego, CA). The sequences of the inserts were confirmed using a Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

Human specific probes for pro α1(I) and pro α2(I) message. The cDNA probes were obtained by PCR amplification of specific N-propeptide-telopeptide regions of the pro α1(I) pSTL2 and pro α2(I) pSTL29 genes. The primers for the human pro α1(I) specific probe, yielding a 269 bp PCR product, were:

Forward

CAAGAGGAAGGCCAAGTCGAG

Reverse

GGTGGTTTCTTGGTCGTGGGT

The human pro α2(I) primers, yielding a 196 bp product, were:

Forward

AAACTGTAAGAAAGGGGCCCCA

Reverse

AGGGCCAAGTCCCAATCCTTTTC.

For cloning purposes and construction of a probe for the detection of the human pro α2(I) in the A2′ cell line, the primers, yielding a 190 bp product, were:

Forward

CGGCGCGAATTCCAATCTTTACAAGAGG

Reverse

TAACATGTCGACAGGGCCAAGTCCAACT.

The underlined sequences denote EcoRI and SalI restriction sites, respectively. Using pSTL29 as the template, PCR amplification with these primers produced the predicted 190 bp agarose gel band. The PCR product was purified from the band, digested with SalI and EcoRI (Promega) and ligated into a EcoRI/SalI digested pGER-3Z vector (Promega). The recombinant plasmid was transformed into competent JM 109 cells (Promega) and the sequence of the insert was confirmed using the Sequenase 2.0 kit.

Northern blot analysis

After electrophoretic separation in an 2.2 M formaldehyde 1% agarose gel, mRNA and total RNA were transferred to Nylon membranes using a Schleicher and Schuell blotting system. The membrane was baked for 2 h at 80°C and/or UV cross-linked (1.2×10^5 joules), prehybridized in hybridization solution (Stratagene), and hybridized with the [^{32}P]-labeled DNA probes for 1 h. After hybridization the filter was washed in 2 × SSC and 0.2% SDS for 15 min at room temperature and in 0.1 × SSC and 0.2% SDS for 15 min at 65°C. Autoradiography was carried out at −70°C using Kodak XAR film and an intensifying screen.

RNase protection assays

The plasmids with the rat pro α1(I) or pro α2(I) cDNA inserts were digested with Bam HI (Promega) and the plasmid with human pro α2(I) cDNA insert was digested with EcoRI (Promega). The linearized plasmids were purified from a 2% agarose gel. The purified plasmids containing the rat cDNAs were transcribed with T7 RNA polymerase and the plasmid with the human cDNA was transcribed with SP6 RNA polymerase in the presence of 1X transcription buffer, 10 mM DTT; 1 U RNasin ribonuclease inhibitor; ATP, GTP, and CTP at 0.5 mM each; 12.5 μM UTP; and 50 μCi [α-^{32}P]UTP (ICN Biomedicals). After the "run-off" transcription the template DNA was digested with RNase-free DNase (Ambion, Austin, TX). In each case radiolabeled antisense RNA probes were purified with a phenol/chloroform extraction and ethanol precipitation. Total RNA from the cells was then hybridized overnight with the labeled antisense RNA probes at 45°C using a Ribonuclease protection assay kit (Ambion). After hybridization the RNAs which were not hybridized with the antisense RNA were degraded with ribonuclease. The protected duplex fragments were separated electrophoretically on 6% sequencing gels and analyzed by autoradiography.

RESULTS

Intrinsic Translational Efficiency of Pro α1(I) and Pro α2(I) Messages

The purpose of this set of experiments was to determine the relative translational efficiencies of the mRNAs. The complete cDNAs for the human pro α1(I) and pro α2(I) chains were ligated into pGEM-3Z. The corresponding RNAs were obtained by in vitro transcription. As shown in Figure 1A both in vitro transcribed RNAs migrated as single bands on denaturing gels, with the expected full-length size. These RNAs were then used, individually, at equal molar concentrations and under identical optimized conditions in the cell-free, membrane-free rabbit reticulocyte lysate translation system. The ^{35}S-methionine labeled translation products were examined by SDS-PAGE (Figure 1B, lanes 2 and 4). The collagen bands, which represented virtually all of the protein synthesized were identified by their susceptibility to digestion with bacterial collagenase (Figure 1B, lanes 3 and 5).

The expectation was that under these identical cell-free translation conditions in the absence of membranes both pro α chains would be produced in equal amount. This proved not to be the case. The pro α2(I) chains contain 63% of the methionine content of pro α1(I) chains and hence the labeling density of the band corresponding to full-length pro α2 chain, assuming complete translation, would always be less than the density of the pro α1 band. However, as seen in the gels of Figure 1B, the pro α1 mRNA yielded a substantially higher concentration of full-length pro α1 chains than did the pro α2 mRNA, relative to their methionine content. The distinctive patterns of collagenase sensitive bands obtained were highly reproducible. In both cases shorter chains, either prematurely terminated and released incomplete chains or accumulations of ribosome-associated, paused nascent chains, were evident in addition to the full-length pro α chains.

Quantitative densitometry of the pro α1 and pro α2 lanes (Fig. 1C) not only indicated, as noted above, that the pro α2 mRNA was less efficiently translated in terms of total incorporation of ^{35}S-Met but also showed that the relative content of full-length pro α2 chains produced (34% of the total collagenase sensitive bands) was less than for the full-length pro α1 translation (64% of collagenase sensitive bands). There was indeed a difference in intrinsic translational efficiency for the two mRNAs. There was no difference in relative translational efficiency when the mRNAs were mixed and translation was carried out together in the membrane-free translation mix. The bands seen at less than full length positions could be attributed to the components of the individual translations, the net result being the sum of the independent translation gels (Fig. 2, lanes 2, 4, and 6).

The addition of the canine pancreas microsomes to the translation mixture improved the translation efficiency of the separate mRNAs, as shown by the increase in proportion of full-length chains in each case (Fig. 2, lanes 2, 3 and 4, 5). However, when the two mRNAs were cotranslated the situation changed. While there was a further improvement in the translation of pro α2, with more full-length pro α2 chain being produced, the relative amount of full-length pro α1 chain was decreased. The sum of the band patterns in Figure 2, lanes 2 and 4, was equivalent to that of lane 6, but the sum of lanes 3 and 5 was not equivalent to the pattern of lane 7. Clearly, the addition of the microsomal membranes to the translation mixture introduced an interaction which tended to make the translations of the two mRNAs coordinated rather than entirely independent as in the translations without membrane present.

mRNA Distributions Along the Polysomes During Translation

In the optimized in vitro system the majority of the synthesized procollagen chains are fully elongated and released, the ribosome-associated nascent growing chains represent only a small fraction of the total collagen polypeptides. It is possible to detect these nascent chains at the protein level [Veis and Kirk, 1989]. However, it is difficult to study the collagen polysome distributions since the volumes of translation mixtures are so small. This is even more difficult in the cells since even in the best of circumstances, the collagen represents only a small portion of the total proteins being synthesized at any given time. On the other hand, with appropriate probes and techniques it seemed more feasible to study the ribosomal distribution of the mRNAs for the pro α1 and pro α2 chains. As postulated above, changes in synthesis rate and synthesis pauses engendered by synthesis coordination should be reflected by changes in polyribosomal loading of the mRNAs. The techniques for polysome fractionation were developed for the cell lysates and

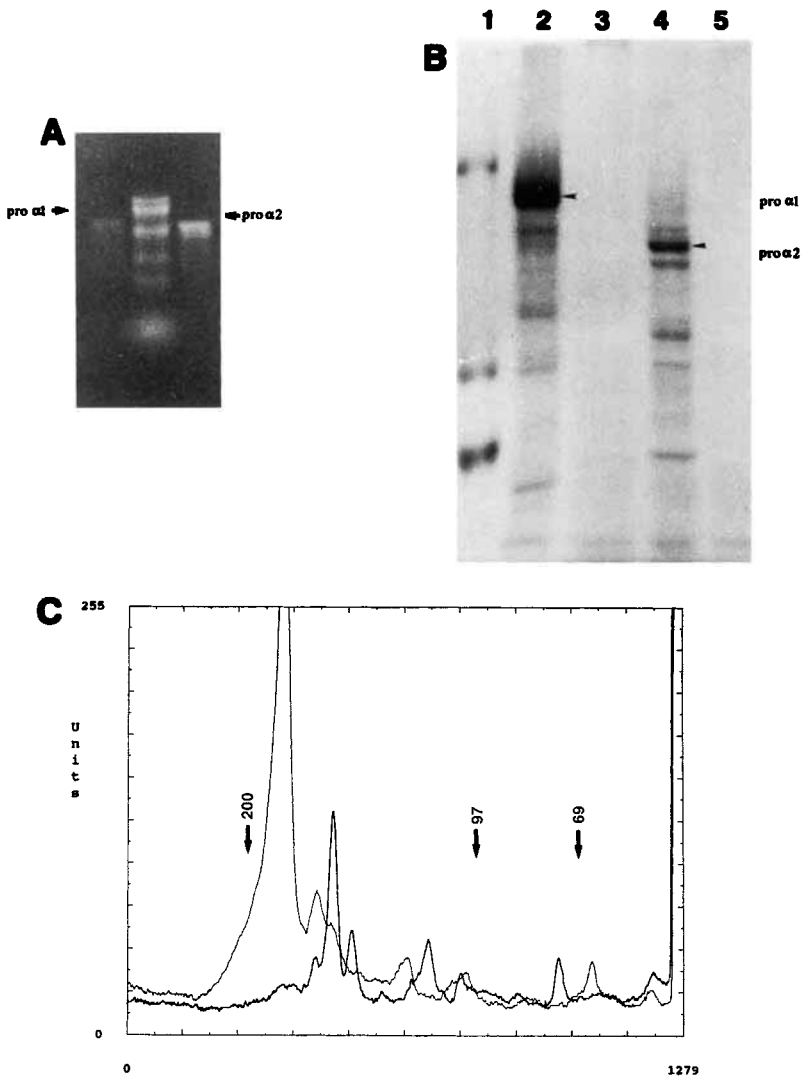

Fig. 1. In vitro translation of human pro α1(I) and pro α2(I) mRNAs under identical conditions in a rabbit reticulocyte lysate, labeling with ^{35}S-Methionine. **A:** Denaturing agarose gel electrophoresis of the mRNAs obtained following transcription of the pro α1(I) and pro α2(I) cDNAs. *Left lane:* Pro α1(I) mRNA at 5.5 kb. Right lane: Pro α2(I) mRNA at 4.5 kb. *Center lane:* Standards. Each mRNA was essentially a single band. **B:** SDS-PAGE on 6% gels of the translation products from the transcribed mRNAs. Lane 1: Molecular weight standards, M_r 200, 97, and 69 K, respectively. *Lane 2:* Pro α1(I) translation products. *Lane 4:* Pro α2(I) translation products. *Lanes 3* and *5:* Collagenase digests of lanes 2 and 4, respectively. **C:** Densitometric analysis of lanes 2 and 4 in B. All parameters were identical for these analyses, including volumes and compositions of translation mix, molar amounts of the mRNAs, specific activity and amounts of ^{35}S-Met, and densitometer settings. The major peaks are the full-length, non-hydroxylated pro α chains. *Thin line,* pro α1(I); *bold line,* pro α2(I).

were suitable for the smaller volume but less complex translation mixtures.

Selection of model cell lines. Smith and Niles [1980] characterized a rat liver epithelial cell line, designated as K16, which produced normal procollagen I heterotrimer in reasonable amounts. By chemical transformation they also developed a line, W8, which had the singular inability to transcribe message for the pro α2(I) chain, while transcription of the pro α1(I) mRNA was not affected. The W8 cells produce and secrete pro α1(I) homotrimer [Marsilio et al., 1984]. Further, stable transfection of the W8 cells with the cDNA encoding the human pro α2(I) mRNA chain produced a new cell line, A2, with the ability to produce a mixture of a rat-human chimeric procollagen I heterotrimer and [pro α1(I)]$_3$ homotrimer [Lee et al., 1988]. This set of related cells was selected for study. The phenotypes of these cell lines under our conditions (data not shown) were confirmed by culturing the cells in the presence of [^{14}C]-Proline. The

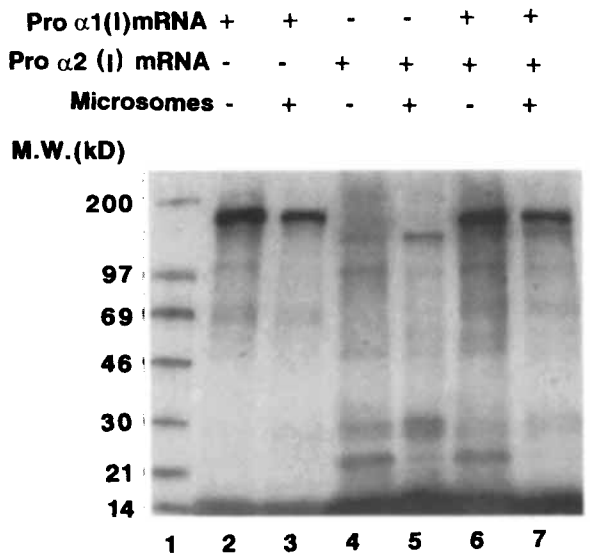

Fig. 2. In vitro translation of human pro α1(I) and pro α2(I) mRNAs separately and in mixture, under identical conditions in a rabbit reticulocyte lysate, ± canine pancreatic microsomes. The conditions were as in Fig. 1 except for the addition of the microsomes. *Lane 1:* Standards. *Lane 2:* Pro α1(I) mRNA alone − microsomes. *Lane 3:* Pro α1(I) mRNA alone + microsomes. *Lanes 4* and *5:* Pro α2(I) mRNA alone ± microsomes. *Lanes 6* and *7:* Co-translation of pro α1(I) mRNA and pro α2(I) mRNA ± microsomes.

secreted media proteins were electrophoresed in SDS-polyacrylamide gels under reducing conditions. They were also digested with either bacterial collagenase or chymotrypsin prior to electrophoresis. In confirmation of the data shown originally by Smith and Niles [1980] and again by Lee et al. [1988] the secreted media protein from K16 cells contained the expected procollagen pro α1(I) and pro α2(I) bands, along with many other ^{14}[C] Pro-labeled proteins. Collagenase digestion of the K16 proteins removed the bands corresponding to pro α1(I) and pro α2(I) chains. Digestion with chymotrypsin converted the pro α bands to α1(I) and α2(I) chain size. The secreted media proteins from W8 cells contained only pro α1(I) chains. The A2 cells produced both pro α1(I) and pro α2(I) chains. However, in A2 cultures the media pro α1/pro α2 ratio was >2, reflecting a lower than normal level of pro α2(I) production and the presence of the pro α1(I) homotrimer [Lee et al., 1992]. The A2′ cells duplicated the behavior of the A2 line.

Polysome distribution and fractionation. A typical polysome optical density vs. gradient fraction profile is illustrated for the parent K16 line in panel A of Figure 3. Similar analyses of the W8, A2, and A2′ cell polysomes yielded virtually identical distribution patterns. The 40S and 60S ribosomal subunits and the complete assembled 80S ribosome peaks are readily apparent, along with a series of well-resolved peaks corresponding to polyribosomes with increasing numbers of ribosomes. RNase treatment yielded a flat baseline in the polyribosome area (Fig. 4A) and the addition of EDTA dissociated the ribosomes from the polysomes, leaving only the intact 40 and 60S subunits (Fig. 4B). Polysomes obtained from cells which were starved (0% FCS) for 24 h prior to freezing yielded a profile (Fig. 4C) demonstrating that the quiescent state of the cells was indeed reflected in the very low overall translation of proteins [Stanners, 1966; Wettstein et al., 1964; Colombo et al., 1966]. These data confirmed that the profile seen in Figure 3A reflected the distribution of the K16 (and W8, A2, and A2′) cell polyribosomes in their intact state. It was not unexpected that each of the cell types yielded essentially the same distribution of polysomes as a function of density gradient position as measured by optical density since type I collagen is not the major product of these epithelial cells.

Distribution of pro α1(I) and pro α2(I) mRNAs on the cell polysome gradients. Equal volume fractions were collected throughout each gradient. At low ribosomal loading the fractions represented polysomes containing essentially a single number of translocons. However, as evident in Figure 3A, as ribosomal loading increased, the higher density fractions contained broader mixtures of mRNAs with increasing numbers of mRNA-associated ribosomes per mRNA strand per fraction. Similarly, each fraction had a different optical density and hence contained a different total amount of RNA. To answer the question of where the collagen messages might be preferentially accumulated, 0.5 μg of the total RNA isolated from each gradient fraction were loaded into the wells of the slot blot apparatus. The blots were then probed by hybridization with either pα1R1 or pα2R2, nucleotide probes specific to pro α1(I) and pro α2(I) mRNAs, respectively [Genovese et al., 1984]. The resulting autoradiograms were developed and scanned using a densitometer.

Collagen message distribution in K-16 cells. Panel B of Figure 3 shows the labeling density distribution of a set of slot blots of the mRNA derived from K16 cell polysomes after probing with pα1R1. The same blot was stripped and then reprobed with pα2R2 (Fig. 3C). Since there was less pro α2(I) message than pro α1(I)

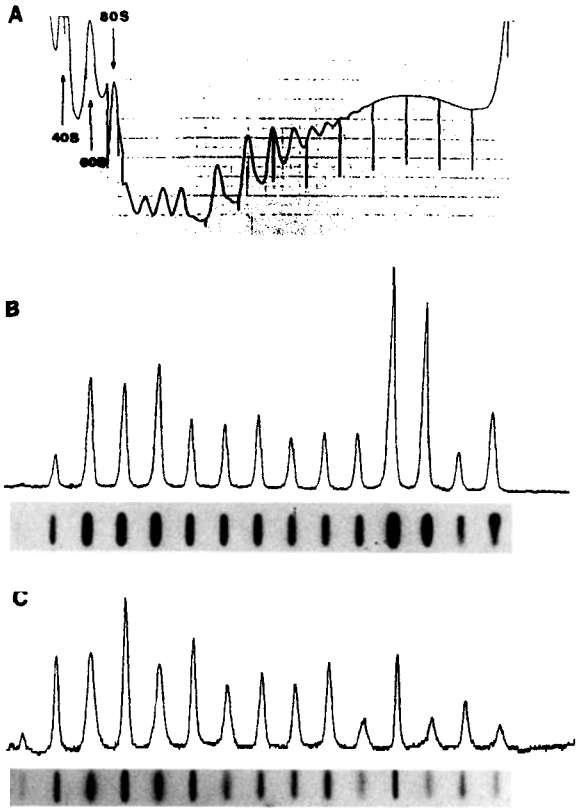

Fig. 3. Size distribution of K16 cell polysomes as determined by density gradient centrifugation, and the corresponding pro α1(I) and pro α2(I) mRNA distributions as determined by probes pα1R1 and pα2R2. **A:** Optical density of the K16 cell polysomes at 254 nm as a function of gradient density fraction number. The positions of the 40, 60, and 80S ribosomal complexes are noted. **B:** The pro α1(I) mRNA distribution as determined by densitometric measurements of slot blots of 0.5 μg total RNA per slot, probed with pα1R1. **C:** The same K16 cell RNA slot blot as in B stripped and reprobed with pα 2R2 for the pro α2(I) mRNA distribution.

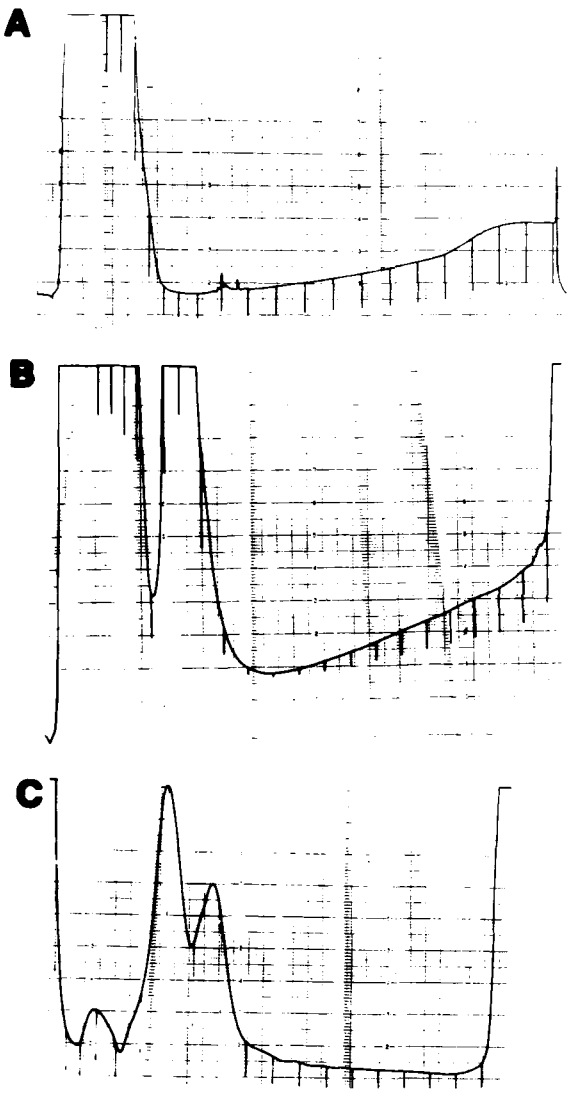

Fig. 4. Size distribution of K16 cell polysomes after various treatments of the cells or lysate as determined by optical density at 260 nm. These data are to be compared with the profile seen in Figure 3A. **A:** The polysome profile following RNase digestion of the lysate. The monosome and polysome complexes were eliminated. **B:** The polysome profile of the cell lysate in the presence of 50 mM EDTA. The mRNA associated ribosomes were disrupted but the 40S and 60S components remained intact. **C:** The polysome profile from lysates of K16 cells which had been cultured for 24 h in 0% FCS. The 80S ribosomal complex assembled but translation of most proteins had ceased or drastically diminished. These data indicate that the 80S and higher density components seen in the untreated K16 polysome profile do indeed represent polyribosomal assemblies.

message the pro α2 blots had to be developed longer and the densitometer gain adjusted differently during analysis, although the two probes were labeled to approximately the same extent. Thus, the most important feature of these data is the relative intensity of probe label as a function of polysome size. Replicate analyses of the polysome distributions from different preparations of K16 cells reproduced the probe labeling density patterns ± one fraction.

To assure that the levels of probe binding were not artifactual and to examine probe cross reactivity, the slot blot in Figure 3C was stripped again and reprobed with pα1R1, a process which yielded the original pro α1(I) mRNA distribution (not shown). This demonstrated that the probes did see different mRNAs and did not cross-react. Stripping did not significantly reduce the amount of collagen mRNA in the blots.

From these data it was evident that both the pα1R1 and pα2R2 collagen probe-reactive mRNAs were distributed throughout the su-

crose gradient, showing that collagen mRNAs with all different degrees of ribosomal loading were present. Moreover, a substantial portion of both mRNAs was accumulated near the initiation and low ribosomal loading regions of the gradient, fractions 3 to 6. Finally, the two mRNA distribution patterns were not identical. These data were quite different from our initial expectations.

Since there were significantly different levels of pro α1(I) and pro α2(I) mRNA in the K16 cells, and the possibility of different affinities and specific activities of the probes existed, the densitometric scans of the blots were normalized to the total content of each mRNA. After carefully adjusting the baseline, each peak on the same probed blot was integrated using the Hoeffer Scan 365 software and manual integration mode. The sum of all integrated peaks was then used to determine the percent probe-reactive mRNA in each fraction. The gradient distributions for the K16 pro α1(I) and pro α2(I) mRNAs are shown in Figure 5. These plots accentuate the fact that a significant portion of each mRNA was lightly loaded with ribosomes. Most importantly, however, the normalized profiles demonstrate that in the K16 cells, which make and secrete the normal procollagen I heterotrimer, the pro α1(I) and pro α2(I) messages were not loaded with ribosomes in an equivalent fashion. There was a major accumulation of pro α1(I) message in polysomes containing about 20 ribosomes (Fractions 12–13, Fig. 5), but this was not duplicated in the pro α2(I) polysomes.

Pro α1(I) message distribution in W8 cells. The W8 cell polysomes were isolated, fractionated, and placed on slot blots exactly as those from the K-16 cells. The results of probing with pα1R1 are shown in Figure 6. The blots were stripped and reprobed with the pα2R2. These blots were entirely blank, confirming that there was no transcribed pro α2(I) message, and verifying that the probes were neither cross-reacting nor detecting messages for extraneous proteins. As seen in Figure 6A, and compared in Figure 6B with the normalized form of the K16 cell data, the pro α1(I) message in the W8 cells was accumulated either near the translation initiation complex region of the gradient, or at the very bottom of the gradient on fully loaded polysomes. These data showed that in the absence of the pro α21(I) message the translation of the pro α1(I) message proceeded in a different fashion, one in which there was near maximal loading of the mRNA with ribosomes. The pro α1(I) mRNA polysome distribution data were distinctly different for the W8 and K16 cells.

mRNA distributions in the A2 cells. The human pro α2 message was detected weakly by the rat pα2R2 probe. Nevertheless, as shown in Figure 7A, the majority of the polysome-associated pro α2(I) message appeared to be located near the initiation-low ribosomal loading region. This accumulation was more pronounced than seen in the distribution of the K16 cell pro α2 message (Fig. 7B).

The change in distribution of the pro α1(I) message in the A2 cells, as compared to that in

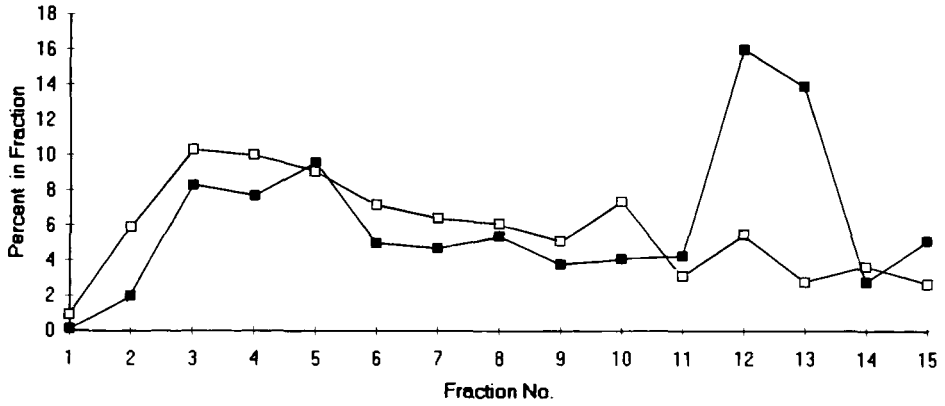

Fig. 5. The normalized distributions of pro α1(I) and pro α2(I) messages on the isolated K16 cell polysomes. The slot-blot autoradiograms for each of the individual runs were quantitated by densitometry. The fraction of the total radioactivity (film density) in each density gradient fraction was calculated by integration after baseline correction. This method of data presentation avoids the problems of film exposure time and development, possible loading of different total amounts of RNA on the gradient in replicate blots, differences in probe specific activity and binding affinity, and focuses entirely on the distribution of a particular probe-reactive mRNA in the gradient. ■, K16 polysome distribution probed with pα1R1; □, K16 polysome distribution reprobed with pα2R2.

W8, was profound. The relatively high content of heavily loaded polysomes in W8 was sharply reduced in favor of a major accumulation of the pro α1(I) mRNA near the initiation-low ribosomal loading region in A2 cells (Fig. 8). The reintroduction of the transcribable pro α2(I) message into W8 cells clearly altered the ribosomal translational pattern of the normal pro α1(I) message.

RT-PCR determination of pro α1(I) and pro α2(I) mRNA distributions The use of the relatively large pα1R1 and pα2R2 probes left open the questions of specificity and sensitivity of the probes. We therefore also explored polysome gradients with the specific primer sets described above. The rat pro pα1 primer set yielded a PCR product of 117 bp, whereas the rat pro α2 primers yielded a 356 bp PCR product. The human pro α2 primers yielded a 190 bp PCR product. There was no cross-reactivity between human and rat pro α2 PCR primers or probes, and no cross-reactivity between pro α2 and pro α1 chain primer sets. The RNase protection assay applied to the total RNA from the A2′ cells showed that only human pro α2 mRNA was protected by the human pro α2 antisense RNA probe (data not shown). Although the total RNA could be used, the mRNA gave more satisfactory results and it was used to provide the data presented below.

Sucrose gradients of the polysome fraction from the cell lysates from K16, W8, and A2′ cells were divided into 10 fractions. The mRNA was recovered from each fraction and the message levels determined after amplification as described. Note that in these experiments, the A2′ cells rather than the original A2 cells were used. The A2′ cells had a somewhat higher content of pro α2 mRNA than the A2 cells but otherwise behaved in a similar manner, producing hetero and homotrimer. Figure 9 shows the PCR analyses of the 7 polysome-containing gradient fractions, 4 through 10. Fractions 1–3 contained the low density pre-polysome components. These data confirmed and refined the slot blot data on the total RNA. First and foremost, the rat K16

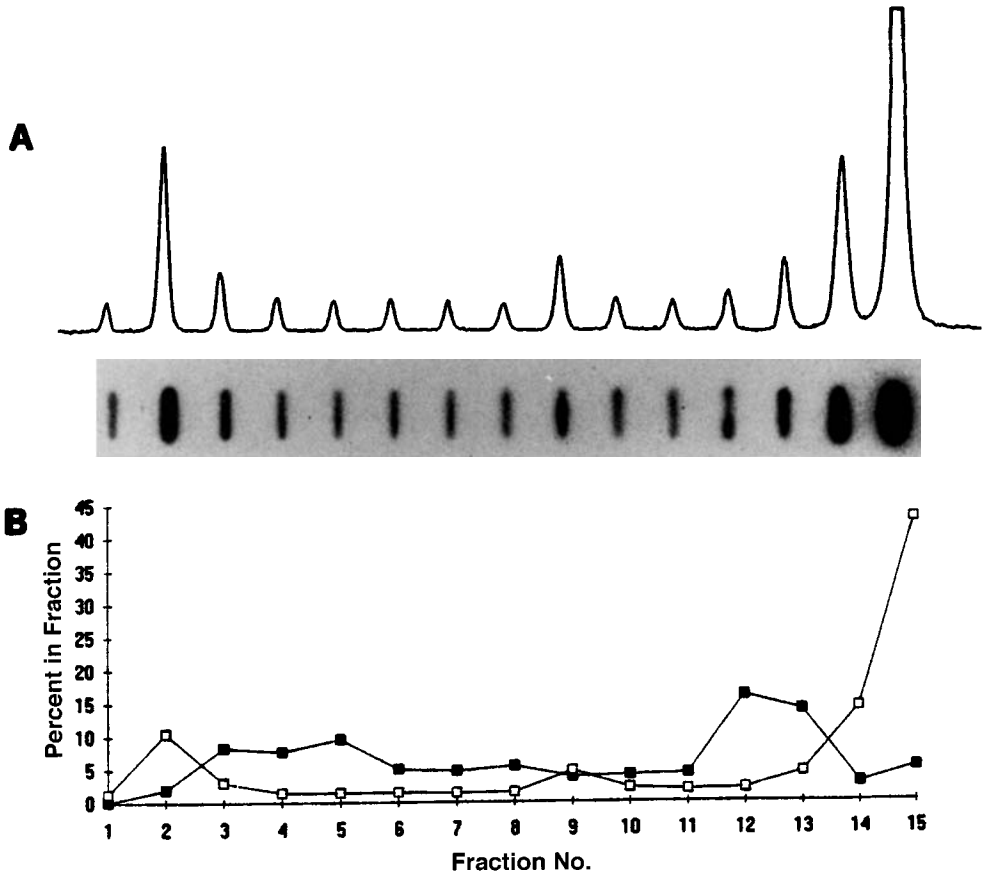

Fig. 6. The distribution of pro α1(I) mRNA along the density gradient fractionated polysomes of W8 cells. **A:** The densitometric tracing from a slot blot probed with pα1R1. **B:** The normalized plot of percent pro α1(I) mRNA in each fraction as a function of fraction number. Comparison of the W8 and K16 distributions. □, W8 mRNA; ■, K16 mRNA.

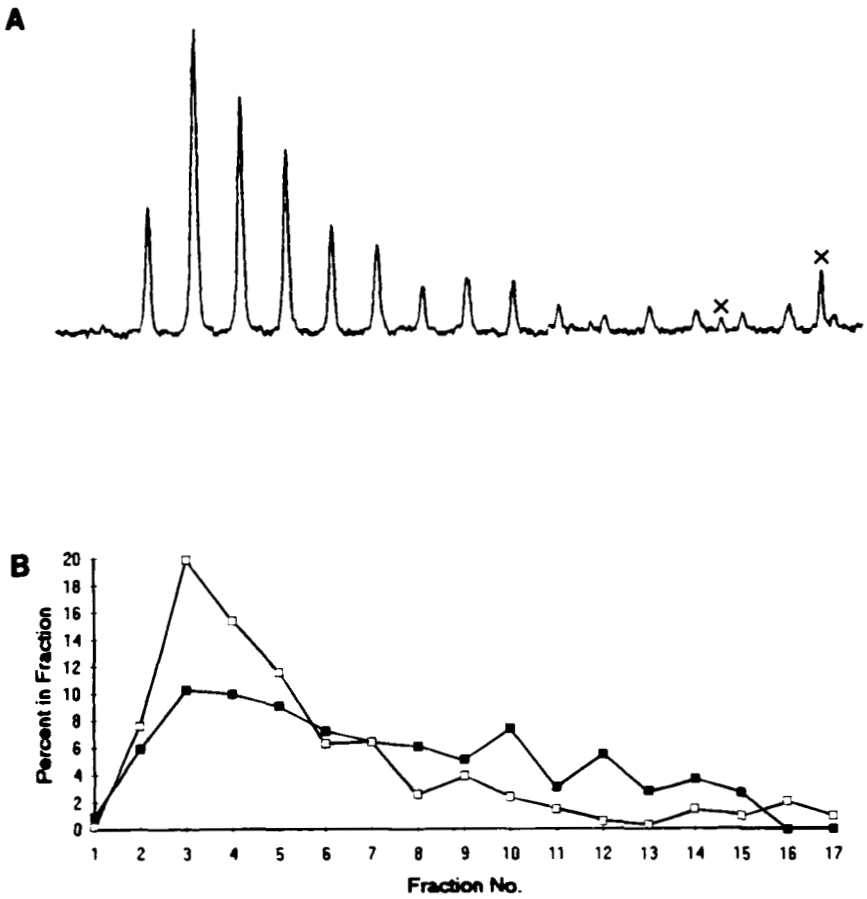

Fig. 7. The distribution of pro α2(I) mRNA along the density gradient fractionated polysomes of A2 cells. **A:** The densitometric tracing from a slot blot probed with pα2R2. The two minor peaks marked "x" were spurious background not corresponding to a slot and were not included in the integrations. **B:** The normalized plot of percent pro α2(I) mRNA in each fraction as a function of fraction number. Comparison of A2 and K16 distributions. □, A2 pro α2(I) mRNA. ■, K16 pro α2(I) mRNA.

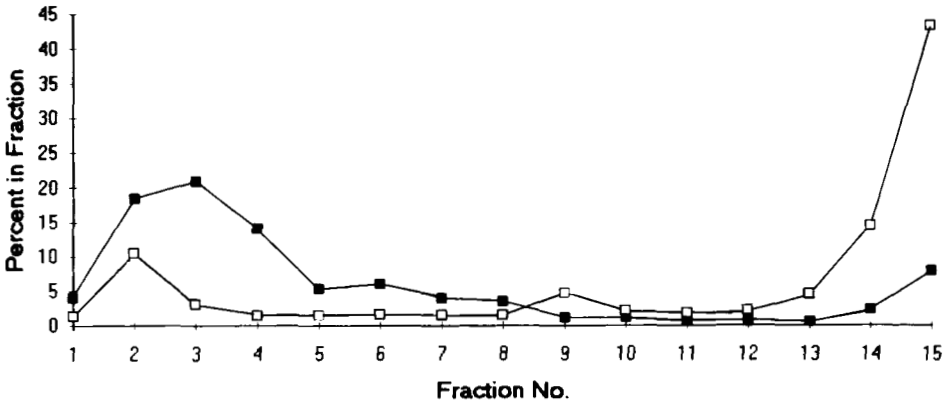

Fig. 8. A comparison of the distribution of pro α1(I) mRNA along the density gradient fractionated polysomes of W8 and A2 cells. Blots were probed with pα1R1. ■, A2 pro α1(I) mRNA; □, W8 pro α1(I) mRNA.

pro α1 and pro α2 mRNAs were not distributed equally along the gradients. Very strikingly, the pro α1 mRNA was distributed all along the gradient, with the maximum in fraction 8. In contrast there were two distinct pro α2 mRNA peaks, indicating that there must be a significant pausing or stacking at low pro α2 mRNA loading. Deletion of the pro α2 mRNA in W8

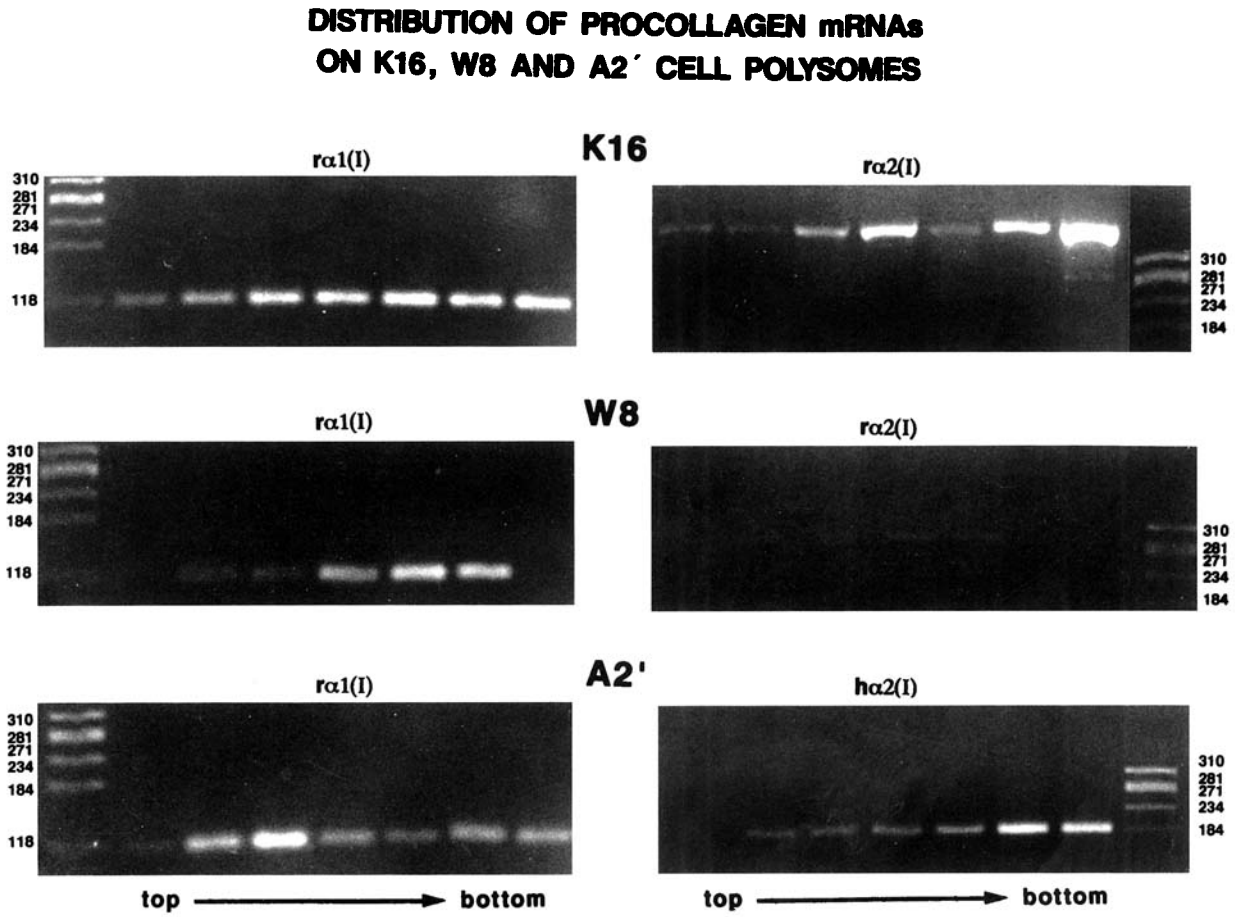

Fig. 9. The distribution of procollagen mRNAs along K16, W8, and A2′ cell polysome gradients as determined with RT-PCR. The pro α1 PCR products at 117 bp are shown on the left, the pro α2 PCR products are shown on the right, at 356 bp for the rat and 190 bp for human.

moved the pro α1 mRNA distribution to higher loading as also seen in the completely independently obtained data of Figure 6. The PCR data for the W8 pro α2-probed mRNA confirmed both the absence of rat pro α2 mRNA and the specificity of the rat pro α2 primer set. As seen in Figure 8 with the original A2 cells, the insertion of human pro α2 mRNA into A2′ down-regulated the pro α1 mRNA loading density. At the same time the human pro α2 mRNA loading, probed with the human pro α2 primer set, was shifted to more fully loaded polysomes. The quantitation of the slot-blot total RNA and PCR mRNA amplification data are not identical but the patterns of distribution are qualitatively the same.

Distribution of pro α1 and pro α2 mRNA along the polysome gradients of the in vitro translation system. The protein data indicating no differences in the cell-free, membrane-free translations of either pro α1 or pro α2 mRNAs in the presence or absence of the other mRNA (Fig. 2) were duplicated by the analysis of the polysome mRNA distribution data (Figs. 10 and 11, upper gels). The pro α1 mRNA was distributed identically in the membrane-free translations ± cotranslation with pro α2 mRNA (Fig. 10, upper gels). Similarly, the pattern of pro α2 mRNA distribution was similar in the membrane-free pro α2 mRNA translation ± cotranslation with pro α1 mRNA (Fig. 11, upper gels). Thus, the two messages can be considered to have been initiated and translated independently and without interaction, although they obviously compete for initiation factors, t-RNA acyl amino acids, elongation factors, and so on.

The addition of microsomal membrane to the translation mixtures had two distinct consequences. The addition of the microsomes in the independent pro α1 and pro α2 message translation systems led to similar changes. That is, the polysome distributions were shifted to heavier mRNA ribosome loading densities (Figs. 10 and 11, lower left) and apparent release of early pausing or stacking during chain elongation. On

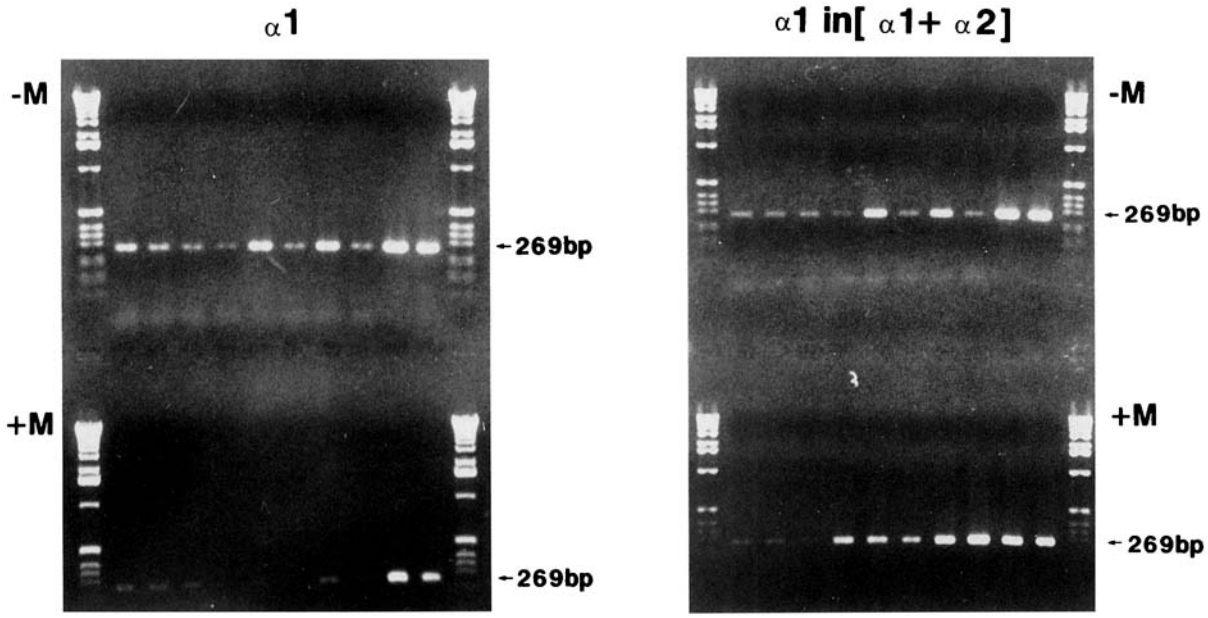

Fig. 10. Polyribosomal distribution of the pro α1(I) mRNA during in vitro translations in the presence or absence of pro α2 mRNA and presence or absence of microsomal membrane, as determined by PCR of polysome gradient fractions. **Left panel:** Pro α1(I) mRNA alone. **Right panel:** Pro α1(I) mRNA in mixture with pro α2(I) mRNA. Upper gels, no microsomal membrane. Lower gels, plus microsomal membrane.

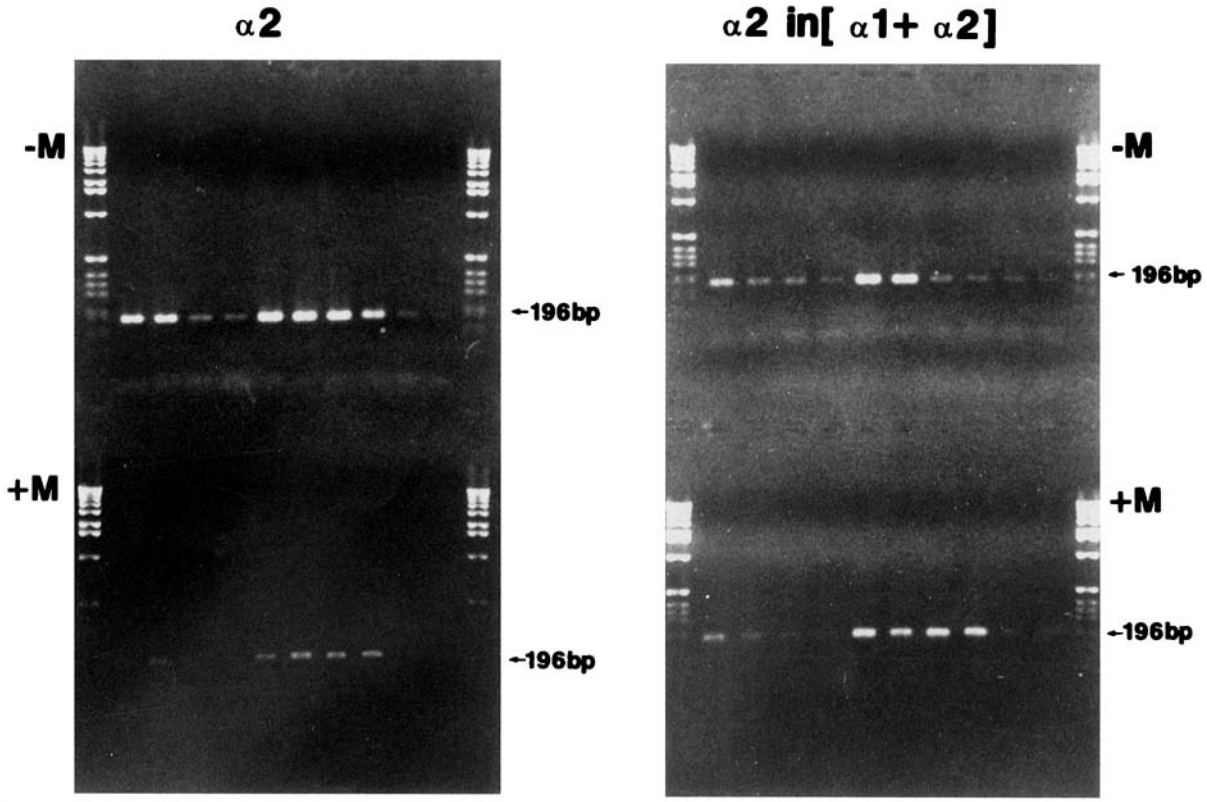

Fig. 11. Polyribosomal distribution of the pro α2(I) mRNA during in vitro translations in the presence or absence of pro α1 mRNA and presence or absence of microsomal membrane, as determined by PCR of polysome gradient fractions. **Left panel:** Pro α2(I) mRNA alone. **Right panel:** Pro α2(I) mRNA in mixture with pro α1(I) mRNA. Upper gels, no microsomal membrane. Lower gels, plus microsomal membrane.

the other hand, the cotranslation of the two mRNAs led to alterations which spread the mRNA ribosomal loading across the gradient in the case of pro α1 mRNA translation (down regulation of the membrane effect on pro α1 mRNA translation) but appeared to enhance the ribosomal loading and readout of the pro α2 message (Figs. 10 and 11, lower right) (upregulation of the membrane effect on pro α2 mRNA translation). Figure 12 presents a comparison of the cell and cell-free translation ribosomal distributions. The cell-free translations in the presence of membrane most closely resemble the situation during translation within the wild type K16 cells. These data suggest that membrane-related factors do interact to couple the translation of the messages involved in construction of the type I procollagen heterotrimer.

DISCUSSION

Production of the nascent chains of type I procollagen can be considered in terms of three distinct stages: initiation of translation; translocation of ribosomal complexes along the message during chain elongation; and final release of the completed chains from the polysome. Each of these processes may be regulated by a different set of parameters. Earlier data [Vuust and Piez, 1972] indicated that the procollagen I molecule was assembled from pro α1(I) and pro α2(I) chains synthesized within the same time frame; hence, it was suggested that synthesis might be coordinated. Subsequent work demonstrating that collagen chains were translated on complex polysomal aggregates [Veis et al., 1985] also indicated that chain selection and triple-helix formation might be facilitated by spatially coordinated translation [Kirk et al., 1987; Veis and Kirk, 1989]. Kirk et al. [1987] showed that type I procollagen heterotrimer synthesis in chick embryo tendon fibroblasts involved synthesis pauses, one of which was especially prominent and detected as occurring among nearly fully elongated chains. As shown in Figure 2,

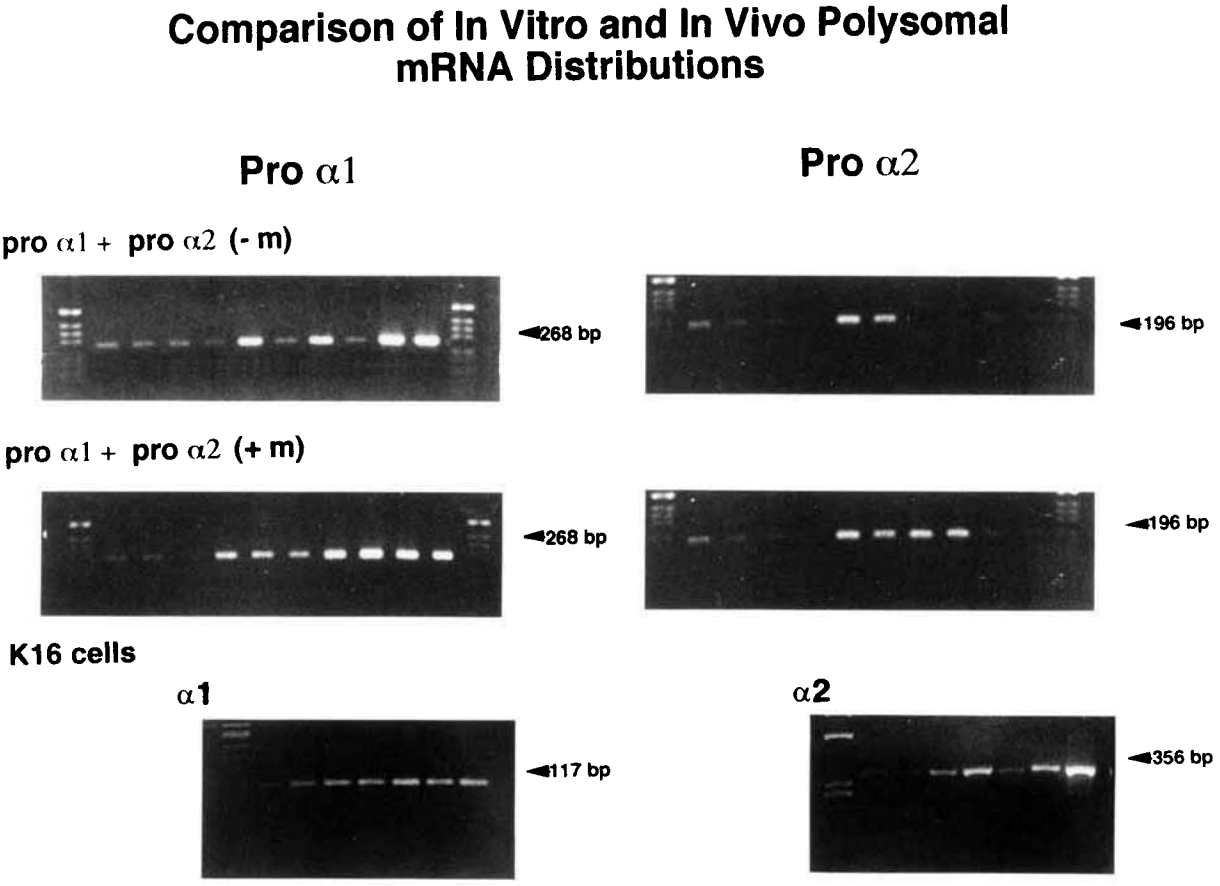

Fig. 12. A comparison of the polysomal procollagen I mRNA loading distributions in the wild type K16 cell and the rabbit reticulocyte in vitro translation systems. **Left panels:** Pro α1 mRNA. **Right panels:** Pro α2 mRNA. From top to bottom: In vitro translation—microsomes; In vitro translation + microsomes; K16 cell translation.

however, the apparent pauses do not occur with the same distribution or frequency during the independent readout of the pro α1(I) and pro α2(I) messages in the presence of microsomal membrane. Thus, the "coordination" of translation does not necessarily mean that chain elongation rates are same or that the messages are read out or translated in parallel, rather, "coordination" may mean that the chain synthesis is constrained to take place within the same ER compartments within the same time frame.

Comparison of the polypeptide chain patterns produced during in vitro cell-free translations of the separate messages, as shown in Figure 1, indicates that there are intrinsic differences in efficiency of translation of the pro α1(I) and pro α2(I) messages. The shorter-than-full-length nascent collagenase-sensitive chains seen in lanes 2 and 4 in Figure 1 were probably polysome associated since only completed, full-length chains were detected in the polysome-free low density protein fractions in sucrose gradients run on the translation mixtures (data not shown). One can thus hypothesize that the specific accumulations of nascent chains at specific sizes correspond to the presence of major pause sites, different for the pro α1 and pro α2 chains. In addition to the observation that translation does not appear to proceed at a uniform rate along the message for either mRNA, the cell-free translation of the same amounts of pro α1(I) and pro α2(I) mRNAs under identical conditions does not lead to the production of equal quantities of pro α1(I) and pro α2(I) chains.

Co-translation of the recombinant human pro α1(I) and pro α2(I) RNAs was selected as a means to examine possible interactions between the messages during translation. The data of Figure 2, lanes 2, 4, and 6, strongly indicate that in the membrane-free system there is no translational interaction. The products obtained were essentially the sum of the independent translations under the same conditions. When microsomal membranes were added to the independent translation mixtures, the efficiency of translation was markedly improved in both cases, with a greater portion of fully elongated chain present and a reduction in the content of intermediate nascent chains. However, when the two mRNAs were co-translated in the presence of the microsomal membranes, the resultant mixture was no longer the sum of the independent translations with membrane, and the lower M_r components attributable to nascent pro α2 chains were significantly reduced while the quantity of fully elongated chain was increased. At the same time, the amount of co-translated full length pro α1 chain was somewhat reduced. These data suggested that there is probably a membrane-mediated interaction between the translation complexes during co-translation. It is difficult to explore this issue further at the protein level because in the mixed system it is not feasible to quantitatively distinguish between the low levels of incomplete nascent pro α1(I) and pro α2(I) chains. We therefore turned to the analysis of the polysomes on the basis of the mRNA polysome loading.

With synthesis pauses, the number of ribosomes on a polysome of given message length must be less than the maximal loading value even if there is ribosomal stacking [Wolin and Walter, 1988]. Thus, it was reasonable to ask if the presence of the pro α2(I) message modified the translation of the pro α1(I) message (or vice versa), in terms of ribosomal loading.

The first step in translation of the pro α1(I) and pro α2(I) messages involves the ribosomal recognition of a particular AUG codon [Kozak, 1983] and assembly of the first complete ribosomal complex. The second step involves an SRP-mediated complexation with the signal peptide, accompanied by a translation arrest which is not released until the complex binds to the docking protein at the ER membrane [Lipp et al., 1987; Walter and Lingappa, 1986]. Releasing the translation arrest permits nascent polypeptide chain insertion into the cisternal space of the ER, while movement of the first assembled ribosome complex along the mRNA exposes the message for reinitiation and assembly of the next 80S ribosomal complex.

The collagen coding region of a pro α1(I) or pro α2(I) mRNA is ≈4,200 bases in length (1,400 amino acid residues). With a length of about 0.6 Mm/base in the mRNA [Saenger, 1984], and a ribosome coverage of about ≈30 nm along the mRNA, this corresponds to a maximal ribosomal loading of >80 ribosomes. Wolin and Walter [1988] have reported that ribosomes may be close packed with a coverage of as few as 27 nucleotides per ribosome during preprolactin synthesis. Although there may be differences between cell-free, membrane-free in vitro and

membrane-associated mRNA loading in cells, full loading of the type I procollagen messages should have been on the order of > 40 ribosomes per pro α-chain message. Such complexes would have sedimented at the very bottom of a 15–50% sucrose gradient. As demonstrated in Figures 3 and 9, both pro α1(I) and pro α2(I) mRNAs were non-uniformly distributed throughout the gradients of the polysomes isolated from the heterotrimer-producing K16 cells. Very little of either of the procollagen I mRNAs was involved with maximally-loaded polysomes. These data correlate well with the electron microscopic observations of Veis et al. [1985], which showed that in chick embryo fibroblasts the ribosomes were not densely packed along the type I collagen-producing messages.

There were clear differences between the pro α1 and pro α2 mRNA distributions along the polysome profiles. The accumulation of ribosomes on the K16 cell pro α1(I) mRNA in fractions 12 and 13 (Figs. 3 and 5) showed a maximal loading at a density corresponding to ≈ 20 ribosomes per mRNA strand, but this maxima was absent or much less pronounced with the pro α2(I) message. Surprisingly, a substantial fraction of both K16 procollagen I mRNAs were found around the region of the 80S, fractions 3–4, and in the lightly loaded polysomes of fractions 5 and 6. The more specific and sensitive PCR identification data (Fig. 9) confirmed that the pro α1 mRNA was distributed at varying ribosomal loading densities throughout the gradient, and that while the pro α2 mRNA was likewise distributed across the gradient the loading pattern was distinctly different from that of the pro α1 mRNA. The ribosomal distribution patterns varied somewhat from one cell culture experiment to the next but the characteristic differences between pro α1 and pro α2 mRNA distributions within the same set of cultured cells were consistently observed. Distinctive patterns of ribosomal loading during message readout are also seen in the cell-free, membrane-free translations of the individual pro α1 and pro α2 messages (Figs. 10 and 11, top left). These data unequivocally indicate that the rates of chain elongation and points of pause-stacking are intrinsically different for the two mRNAs. There was no interaction between the pro α1 and pro α2 translational complexes during co-translation in the cell-free, membrane-free system (Figs. 10 and 11, top right). However, the cell and cell-free, membrane-free translation polyribosomal distribution data were different.

Translation of pro α1 mRNA alone in the cell-free translation mixture plus microsomal membrane dramatically facilitated message ribosomal loading, and almost all the pro α1 message was associated with heavily loaded (> 40) polysomes (Fig. 10, lower left). That is, the translation coupled with the insertion and translocation of the nascent chains into the cisternal space of the microsomes and any co-translational modifications such as hydroxylation and glycosylation that might have occurred, had a marked effect on the manner in which the message readout was carried out. Translocation of nascent polypeptides through the ER is vectorial, and can involve both membrane proteins and lumenal proteins [Larriba, 1993; Vogel et al., 1990]. The addition of pro α2 mRNA to the cell-free + microsomal membrane translation mix (Fig. 10) lower right, brought about a further change in pro α1 message readout pattern, suggesting that there was an interaction between the translation complexes mediated by one or more of the microsomal membrane components. There were corresponding changes in pro α2 message loading patterns (Fig. 11) in translations ± microsomal membranes which showed that the interaction also modified the pro α2 mRNA readout.

These data were completely supported by the cell translation polysome analyses. Deletion of the pro α2(I) mRNA, in the W8 cells, led to pro α1 mRNA readout data (Figs. 6, 9, center panels) similar to that of the cell-free + microsomal membrane pro α1 mRNA translation. Restoration of the capacity to produce heterotrimer by introduction of the human pro α2(I) mRNA into W8 to yield the A2 and A2′ cell lines, altered the loading pattern of pro α1 mRNA, spreading loading to lower ribosomal densities (Figs. 8, 9, lower left). At the same time, the apparent loading of the pro α2 message was enhanced in both the cell-free + membrane (Fig. 11, lower right) and A2′ (Fig. 9, lower right) translations. Differences in the A2 and A2′ data may be the result of differences in pro α2(I) mRNA stoichiometry. It is probable that more human pro α2 message was introduced into A2′ than had been introduced into the A2 cells, but there was the further complication that the A2 products were

probed with the rat pα2R2 which cross-reacts weakly with the human pro α2 mRNA. We consider the A2′ cell data to be more reliable with respect to the pro α2 mRNA distributions.

The principal interpretation to be drawn from these data is that membrane-associated components, endoplasmic reticulum cytosolic-face membrane in the cells, and microsomal membranes in the cell-free translation system act to bring about an interaction between the translation complexes for the synthesis of pro α1 and pro α2 chains. This interaction may have two functions: to bring the translation complexes to the same regions of the ER-membrane to insure the colocalization of the nascent chains for interaction; and, to regulate or coordinate the rates of synthesis of the two polypeptide chains. In many systems producing type I collagen the pro α1/pro α2 message ratio is somewhat greater than 2/1 [Kosher et al., 1986; Vuorio and de Crombrugghe, 1990; de Wet et al., 1983]. In such situations it is likely that all of the pro α2 mRNA is engaged in heterotrimer synthesis, while the excess pro α1 may either produce homotrimer as in the A2 cell line [Lee et al., 1988] or be subject to intracellular degradation [Bienkowski et al., 1978].

Translation of secretory proteins begins with formation of the signal peptide in the cytosol. A translation block then arrests further elongation while interactions with the signal recognition particle ultimately direct the entire complex to the ER surface and the docking protein. The translation block is cleared and vectorial elongation of the nascent chain into the cisternal space of the ER follows. In the case of type I procollagen heterotrimer formation, an absolute requirement is that the nascent pro α1(I) and pro α2(I) chains be inserted into the same ER compartment so that appropriate chain interaction and helix formation can begin as soon as the carboxyl propeptide regions are reached. The conclusion noted in the previous paragraph that an interaction between messages takes place at the ER surface can be further refined by this requirement, with the proposal that the primary heterotrimer selection process is that of a membrane facilitated interaction between the SRP-Signal Peptide-mRNA complexes at the time of docking. It remains to be determined if this interaction is mediated through ER-membrane or cytosolic accessory (chaperone) proteins [Larriba, 1993].

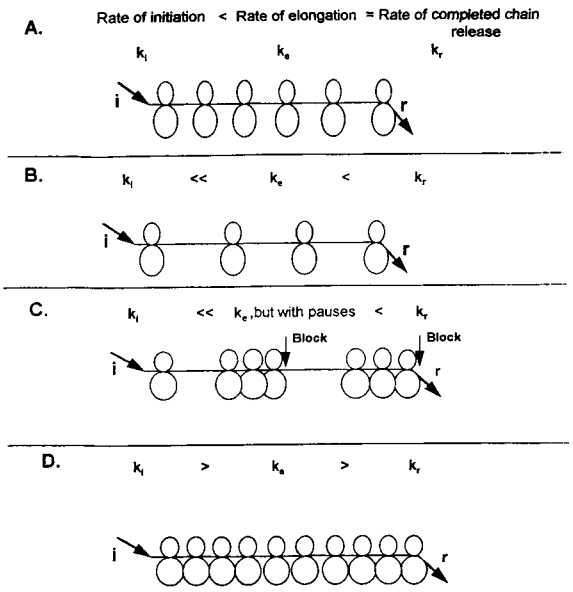

Fig. 13. Alternative models for the control of mRNA loading during polypeptide chain elongation. **A:** Model where rate of reinitiation (k_i) is marginally rate limiting, relative to the rates of elongation (k_e) and completed chain release (k_r). **B:** Model where k_i is much smaller than k_e. The consequence is a decrease of mRNA ribosomal loading. **C:** The same situation as in B, but with the imposition of a few elongation pause sites. In this case ribosomes stack and net loading is increased relative to Model B. **D:** Model where release of the completed chains is rate limiting. The mRNA is maximally loaded with ribosomes.

The present study does not specifically examine the kinetics of the initiation, elongation, or chain release processes but it is possible to speculate on relative rates from the steady state data considered above. The argument is illustrated schematically in Figure 13. If the rate of translation reinitiation after the first ribosomal complex is affixed to the endoplasmic reticulum surface (k_i) is somewhat slower than the rate of translocation during chain elongation (k_e) and the rate of release of the completed chains (k_r), then one would expect to have found the majority of the mRNA associated with the moderate ribosomal loading (Fig. 13A). If $k_i << k_e \leq k_r$ (Fig. 13B) the result would be a further reduction in average ribosomal loading of the message. The same situation, but with a few synthesis pauses, could lead to ribosomal stacking and an increase in the number of ribosomes/message [Wolin and Watter, 1988], the increase depending on the number and distribution of pause sites (Fig. 13C). If release of the fully elongated protein chains was the rate limiting step (Fig. 13D, effectively a major pause near

completion of chain elongation, $k_i > k_e > k_r$), then one would have expected to find the majority of the mRNA associated with full ribosomal loading, as was the case for the W8 cell and cell-free + membrane translations of the pro α1(I) message. The presence of pro α2(I) mRNA in the cell-free + membrane and A2′ and K16 cells obviously changed the ribosomal loading of the pro α1(I) message to a situation akin to those of Figures 8B or C, that is from $k_i > k_e > k_r$ to $k_i \leq k_e \leq k_r$. The same phenomenon is seen in the comparison of the loading of the pro α1(I) message in W8 and wild type K16 cells (Fig. 8). Thus, the most likely effect of the interaction of the translational complexes is to modify the relative rate of reinitiation of translation for each mRNA.

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