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Supramolecular assemblies of mRNA direct the coordinated synthesis of type I procollagen chains

(chain association/triple helix formation/disulfide bonding)

ARTHUR VEIS, S. J. LEIBOVICH, JOHN EVANS, AND THERESA Z. KIRK

Department of Oral Biology, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611

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ABSTRACT **Registration of the three procollagen α chains and assembly of the triple-helical procollagen molecules takes place in the rough endoplasmic reticulum, but the exact location and timing of assembly is not known. As part of a study of the mechanism of molecular assembly, intact collagen-producing polyribosomes from embryonic chicken tendon fibroblasts have been examined by the techniques of rotary shadowing and electron microscopy. Intact mRNA strands corresponding in length to ≈4500 bases and complete procollagen α(I) chains have been observed. The mRNA strands are comprised of two mRNA chains. The ribosomes are present in pairs separated along the duplex strand by about 100 nm. The intact polysome is asymmetric; two duplex strands join, and large ribosome aggregates appear. These aggregates are dispersed by collagenase digestion, leaving separate duplex strands with ribosome pairs intact. Ribonuclease digestion yields mixtures of monosomes and ribosome aggregates. Sequential ribonuclease and collagenase digestions yield only monosomes. We propose that each ribosome reads one mRNA chain, so that each pair is thus translating two chains in synchrony. Thus, the complex morphology of the collagen-producing polyribosomes suggests that the organization of a single molecule begins by the organization of the mRNA chains themselves.**

The procollagen molecule is a complex structure comprised of three chains produced on individual monocistronic mRNAs. In its completed, fully assembled structure, the procollagen molecule is asymmetric. The three chains are extended and in register with their amino-terminal portions at one end of the long triple-helical rod-like central region, collagen, and with their carboxyl-terminal regions at the opposite end (1). In the most commonly occurring type of procollagen, type I, two of the chains are identical, pro-α1(I)-chains, while the third chain is distinctly different, pro-α2(I). Each pro-α-chain has a molecular weight of ≈140,000.

Pro-α-chain synthesis follows the usual pathway for secreted proteins. Each of the pro-α-chains is initiated in the cytosol with the translation of a signal peptide (2, 3) that directs continued synthesis into the secretory pathway by insertion through the endoplasmic reticulum (ER) into the ER cisternal space (4). Thus, elongating nascent chains are extruded through the ER as individual chains. The procollagen molecule then is assembled by a process involving selection of the three appropriate chains, followed by their precise registration and folding into the asymmetric triple-helical structure. Extensive co- and post-translational modifications that precede the final organization of the molecule (5, 6) include hydroxylation of proline and lysine residues and glycosylation of hydroxylysine. The logistics of this assembly process appear to be quite formidable.

Registration of the three chains is thought to begin, following release of the nascent chains into the cisternal space of the ER, by association and disulfide bonding of the newly completed procollagen COOH-terminal extension propeptide (COOH-peptide) regions (7–9). The triple-helix grows from the COOH-propeptide aggregation nucleation site to the NH_2 terminus of the molecule at a rate limited by the *cis–trans* isomerization of peptide bonds involving proline (10). Chain synthesis requires at least 6 min (1), and secretion from the cell requires an additional 15–20 min (11). Thus, the selection and folding processes must be highly efficient and take place over the span of only a few minutes.

Some years ago Brownell and Veis (12, 13) presented evidence that a portion of the nascent type I collagen chains, still on the polysome, exhibited a resistance to digestion with pepsin and showed typical collagen thermal denaturation behavior. The implication was that chain association might begin while the chains were still elongating, before completion and release. These data appeared to be incompatible with the later studies mentioned above on chain association and COOH-to-NH_2-terminal direction of helix formation (7–9) and were attributed to some cooling-induced artifact during polysome isolation. However, Bruckner and Eikenberry (14), have shown that the procollagen triple helix is more stable in the cell than *in vitro*, again by protease resistance. Their test for helicity was resistance to digestion with trypsin and chymotrypsin for 2 min at 20°C. These data suggested that, within both the cellular environment and the extracellular matrix, some components of the cells must be associated with the collagen to protect it from tryptic activity because isolated purified procollagen exhibited the appropriate collagen denaturation temperature. Since the polysome-associated nascent collagen already exhibited enzyme resistance, these new data (14) led us to reconsider the idea that chain synthesis and selection might be coordinated by organization within the biosynthetic system itself.

To that end, we began an investigation of the morphology of the polyribosomal assemblies synthesizing collagen chains. We present here direct rotary-shadowing electron microscopic evidence that collagen-synthesizing polyribosomes, containing nascent collagen chains, already exhibit a supramolecular organization that might permit the coordination of assembly of chains from different mRNAs.

MATERIALS AND METHODS

Preparation of Intact Type I Collagen Polysomes. Matrix-free cells were obtained from the leg tendons of 17-day-old White Leghorn chicken embryos as described by Dehm and Prockop (15). Cells (10^8 per ml) were incubated in 50 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10,000 units of penicillin G, 5,000 μg of streptomycin, and ascorbic acid (40 μg/ml) for 30 min at 37°C in humidified 95% air/5% CO_2. Protein synthesis was terminat-

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Abbreviations: pro-α-chain, procollagen α chain; ER, endoplasmic reticulum; COOH-peptide, COOH-terminal extension propeptide.

ed by adding cycloheximide (100 μg/ml) and chilling at 4°C for 10 min. All subsequent steps were conducted at 4°C. Cells were pelleted by centrifugation at 10,000 rpm for 10 min in a Sorvall RC5B centrifuge. The cells were suspended in 50 mM Tris·HCl, pH 7.4/240 mM KCl/7.5 mM $MgCl_2$ (TKM buffer) containing 120 mM ribonuclease-free sucrose, cycloheximide (100 μg/ml), heparin (200 μg/ml), 2 mM dithiothreitol, phenylmethanesulfonyl fluoride (160 μg/ml), and 1 mM benzamidine·HCl and were incubated for 10 min. The suspension was subjected to a freeze–thaw cycle twice (dry ice/acetone–37°C water bath); after addition of Tween 20 and sodium deoxycholate to final concentrations of 1% (vol/vol) and 0.5%, respectively, the suspension was incubated for 15 min. The mixture was centrifuged as before, and the supernatant was layered over 0.5–1.5 M linear sucrose gradients (in TKM alone) and centrifuged in a Beckman SW50.1 rotor at 37,000 rpm for 60 min at 4°C. Polysomes were obtained as the fastest sedimenting peak fraction on the gradient, as analyzed at 260 nm. This fraction was diluted 1:1 with TKM. The polysomes were then pelleted by centrifugation at 45,000 rpm for 12 hr and diluted to a concentration of 175 μg/ml, as determined by absorption at 260 nm in the assay buffer described below. Samples were stored at −70°C in 100-μl aliquots.

Enzyme Digestion. Polysome samples (100 μl; 175 μg/ml) were suspended in assay buffer (50 mM Tris·HCl, pH 7.2/100 mM NaCl/10 mM $MgCl_2$/10 mM Ca acetate/10 mM *N*-ethylmaleimide; in the case of the collagenase assay, the buffer also contained 1 mM benzamidine). Polysomes were digested separately with 2.2 μg of collagenase (form III, Advance Biofactures, Lynbrook, NY; ABC, chromatographically pure), equivalent to 17 collagenase ABC units per assay reaction, and ribonuclease A (bovine pancreas, Sigma; 1 μg per assay reaction) at 37°C for 1 hr. The final reaction volume was 300 μl. The controls consisted of polysome samples with an appropriate volume of assay buffer, incubated as described above. Reactions were terminated by quick chilling to 4°C, followed by dilution with glycerol to 70% (vol/vol) to produce a polysome suspension.

Electron Microscopy. Control and enzyme-digest glycerol suspensions (52 μg of polysomes per ml) were sprayed onto freshly cleaved mica from a distance of 30–40 cm at an angle of 30°. The mica sheets were immediately mounted on the rotary table of an Edward's vacuum coating unit (model E306-A) and evacuated to 10^{-5}–10^{-6} torr (1 torr = 133.3 Pa). After the specimen was allowed to dry under reduced pressure, the mica sheet was rotary-shadowed with platinum/carbon by using an electron-beam gun at an angle of 8° from a distance of 15 cm. The specimen table was rotated at 120 rpm during the shadowing procedure. Film thickness during the evaporation was monitored using an Edward's film-thickness monitor (model FTM4), and 1.5–2 nm of platinum was deposited in a controlled manner over a 15- to 20-sec period. After shadowing at a low angle, the film was stabilized by evaporation of carbon at right angles to the specimen from a distance of 10 cm. The carbon-coated replica was then stripped from the mica sheet by flotation onto distilled water, and the stabilized replicas were collected on

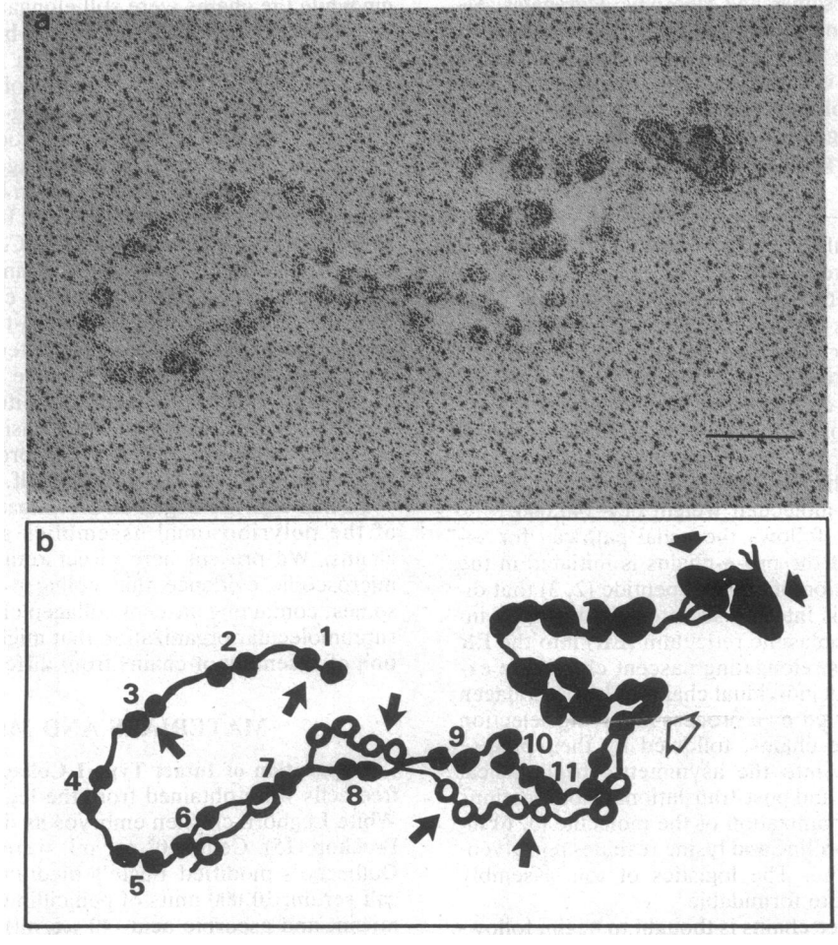

FIG. 1. (*a*) Rotary-shadowed replica of an intact collagen-synthesizing polyribosomal aggregate. [Bar = 100 nm in this and all subsequent micrographs; all are printed at the same magnification.] (*b*) A diagramatic representation of *a*. The pairs of ribosomes along one of the strands are numbered up to the point where larger ribosomal aggregates are seen. At least 12 ribosome pairs are seen to this point. →, pairs of ribosomes distributed along the mRNA strands; ◊, two mRNA strands merge, and larger ribosomal aggregates appear; ◆, large ribosomal aggregates.

copper grids. Specimens were examined in a JEOL-100-S electron microscope with an operating voltage of 60 KV and instrumental magnifications of ×50–×100,000.

RESULTS

Polysomes prepared from [^{3}H]proline-labeled chicken tendon cells exactly as described above were shown by gel electrophoretic analyses to contain nascent collagen chains. The polysomes were sufficiently intact to be capable of continued elongation of the nascent chains in an *in vitro* "readout" system that followed the procedure of Blobel and Dobberstein (4). Collagenase digestion of the polysomes removes all of the collagen from the heavy, fastest-sedimenting polysomes (12, 13). Ribonuclease digestion of [^{3}H]proline-labeled polysomes moves the nascent collagen chains from heavy-polysome to monosome regions on a sucrose gradient (12). Detailed studies of the nascent chains on the polysomes will be described elsewhere (16). The results of these studies are cited here to indicate that, as isolated, the polysomal assemblies are fully functional.

When sprayed on mica the intact polysomes appeared to be highly tangled filamentous masses covered with globules of various sizes. In favorable cases where the filaments were spread, structures such as that shown in Fig. 1*a* (and depicted schematically in Fig. 1*b*) were typically observed. Very clearly, ribosomes of about 25-nm diameter decorate long strands of mRNA. However, there are two notable features in the micrographs. First, the ribosomes are not distributed uniformly but are paired regularly along each strand (longer arrows in Fig. 1*b*). Second, where two ribosome-bearing strands come together, (open arrowhead in Fig. 1*b*), the ribosomes aggregate into groups larger than 25 nm in diameter. The largest ribosome aggregates have major dimensions of >50 nm (filled arrowheads in Fig. 1*b*). Close inspection of many micrographs suggested that each mRNA strand in Fig. 1*a* is, in fact, a duplex containing two individual chains of mRNA.

After collagenase digestion, the ribosome aggregates of >25-nm diameter were disrupted, and one observed only the long strands of mRNA decorated with uniformly spaced pairs of ribosomes (Fig. 2). In these micrographs, the duplex chain nature of the mRNA strands is clearly seen (arrowheads). The two mRNA chains cross on opposite sides of the ribosomes and appear to be loosely wound around each other in the intervening spaces. The ribosome pairs are separated from each other at a distance of ≈100 nm. The duplex, two-chain nature of the mRNA strand is also clearly seen in Fig. 2 *a* and *c*, where at one end of the strand the two mRNA chains within the duplex are separated. A fragment of an isolated single strand of mRNA, devoid of ribosomes is shown in Fig. 2*d*. The extra thickness of the duplex mRNA–chain strands in Fig. 1*a* and Fig. 2 *a* and *b* is evident. The thickness of the isolated chain agreed with that of the single filament ends seen in Fig. 2*c*.

Digestion with RNase destroyed the mRNA strands and left behind single ribosomes and ribosome aggregates (Fig. 3*a*). Sequential digestion with collagenase and ribonuclease left only single 25-nm ribosomes (Fig. 3*b*).

Chain-contour lengths were measured on a Zeiss Video-

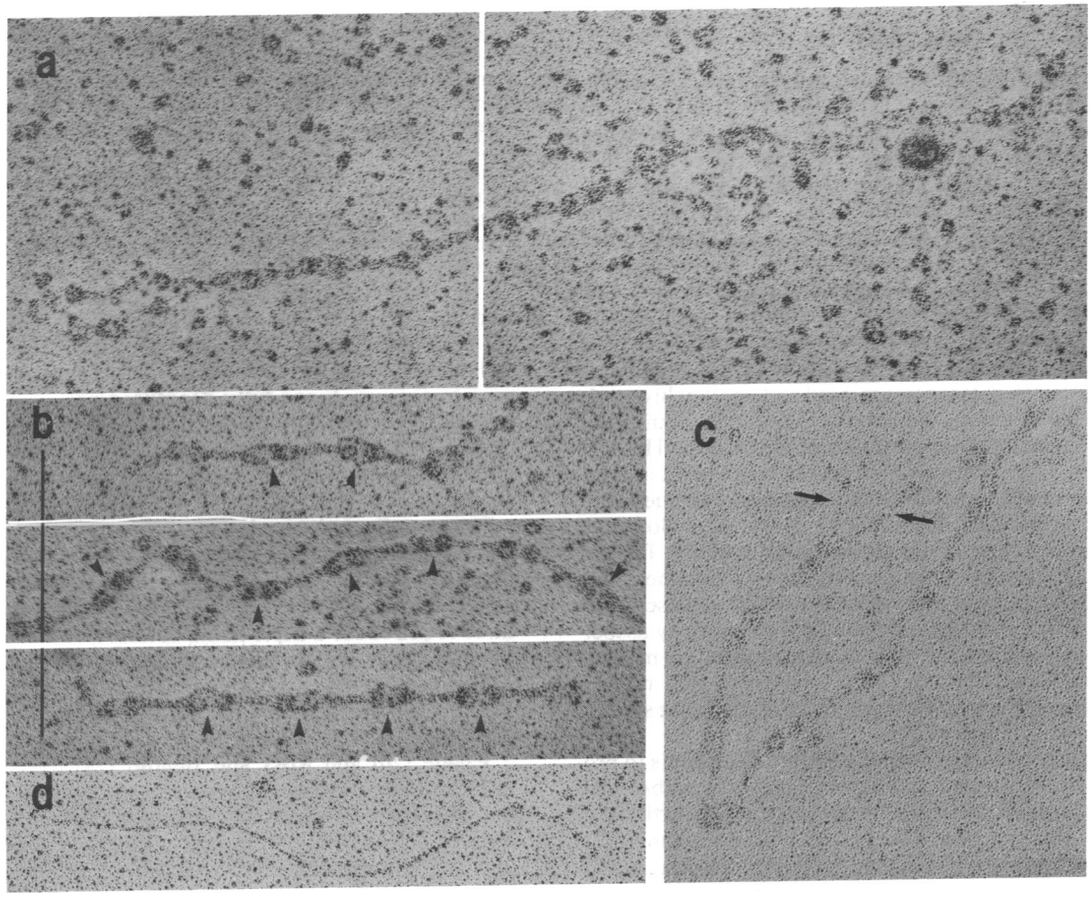

FIG. 2. Electron micrographs of rotary-shadowed collagenase-digested polysomes. (*a*) One of the longest segments found after digestion. (*b*) Numerous shorter sections showing duplex-chains (arrowheads). (*c*) Micrograph clearly showing the two-chain, duplex nature of the strand of mRNA. The mRNA chains come together at the first ribosome pair (arrows). (*d*) An isolated mRNA chain without attached ribosomes. Note the thickness relative to the strands between ribosome pairs in *a*, *b*, and *c*.

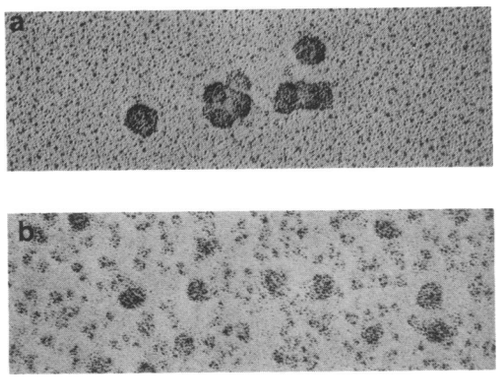

FIG. 3. (*a*) Electron micrograph of rotary-shadowed polysomes after digestion with RNase. Aggregates of ribosomes are present. (*b*) Appearance after digestion of polysomes with collagenase and RNase. Only single ribosomes are seen.

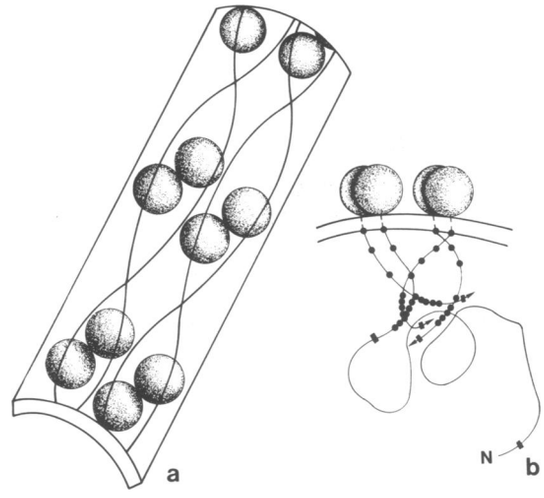

FIG. 4. Schematic surface and cross-section views of the disposition of ribosomes in polysomes along the ER surface. (*a*) A surface view showing aligned duplex mRNA strands of polysomes. (*b*) A cross section through two ribosome pairs and the cisternal space. The ribosomes, membrane, and nascent chains are drawn to scale to emphasize the spatial relationships: ribosomes, 25 nm; membrane, 7 nm. The COOH-peptide region of the α chain of procollagen has 246 residues with about 3 Å extension per residue. Near chain completion and before release, $\approx$700 Å of the contour length of the procollagen COOH-peptide will have been extruded into the ER lumen. The close spacing of the ribosomes, all with nascent chains elongated to near the same length permits many contacts between chains. The positions of cysteine residues along the procollagen COOH-peptide are noted by heavy dots in their appropriate sequence positions. Note the density of these residues near the amino-terminal end of the procollagen COOH-peptide. One of the chains is depicted complete to the molecular amino terminus. The other chains are not shown for the sake of clarity. The black box (■) along each chain in *b* represents the junction between the "noncollagenous" procollagen COOH-peptide and the "collagenous" sequence region. The ease of registering chains in the procollagen COOH-peptide region in this restricted volume just prior to chain release is obvious. It is also evident that each ribosome grouping is separated by about 100 nm from its neighbors along the RNA strand, not allowing such interactions between the nascent chains in the preceding or following pair.

plan from tracings of the micrographs. An intact chain within a mRNA duplex, such as the one depicted in Fig. 1*b* by the filled-in ribosomes, has a contour length of 1703 nm. This length corresponds to 4865 bases, assuming 0.35 nm per base. This length is sufficient to code for a prepro-α1(I)-chain, which has at least 1441 amino acid residues in the pro-α1(I) region. The most intact collagenase-treated chains were all somewhat shorter, averaging 1240 nm (1180 amino acid residues) for the longest chains. The extra manipulations of the chains during digestion obviously lead to shearing and the appearance of many smaller chain segments.

DISCUSSION

Electron micrographs of ribosome-bearing mRNA chains have been presented before (17, 18). However, as in the micrographs of cell-free extracts of *Escherichia coli*, there has been evidence of neither ordering of the ribosomes along the mRNA chains nor association between mRNA chains (17).

Figs. 1 and 2 show two levels of organization in the isolated collagen polysomes. The collagenase-digested polysomes shown in Fig. 2 are constructed from two aligned and in-register mRNA chains forming a single duplex strand. The ribosomes are closely paired along the strand, with 100-nm intervals between pairs. It is probable that, within the pair, the ribosomes each read out a different chain. Thus, at least two monocistronic messages are read out in synchrony so that the nascent chains should be of nearly equivalent lengths within each pair at all stages of elongation. The second potential level of organization is shown in Figs. 1 and 3*a*. The presence of the large ribosomal aggregates implies that, in the intact polysomes, the collagen chains have elongated sufficiently (the 3′ end of the messages) to interact. The observation that the ribosomal oligomers hold together after RNase digestion but not after RNase and collagenase digestion indicates that the oligomers are held together by associated collagen chains from *two* duplex strands of mRNA, each duplex strand being comprised from two mRNA chains. Thus, up to four procollagen chains are being synthesized in virtual synchrony.

While the association of two duplex mRNA strands to produce the large ribosomal aggregates containing substantial amounts of nascent collagen chains may be an artifact of preparation, the specificity suggested by the asymmetry of the aggregates is more likely the result of collagen-chain interaction before the isolation process begins with the dissolution of the ER membrane. Certainly in the cell, the ER membrane is interposed between the mRNA–ribosome complex and the elongating nascent chains (Fig. 4). Kirk (16) has shown that completely elongated but not yet fully hydroxylated pro-α1(I)- and pro-α2(I)-chains remain associated with the polysome. These chains show resistance to pepsin (13) and chymotrypsin (16), yielding α-size chains, but no resistance to trypsin. Since fully denatured pro-α-chains are reduced to small peptides by chymotrypsin or pepsin, a correct chain registration must have been present to retain α-size degradation products in these incompletely hydroxylated chains.

The coordinated translation of two two-chain strands of mRNA might explain the puzzling intracellular degradation of collagen that always accompanies *in vivo* collagen synthesis (19). Four nascent α-chains may be produced for each three required for a triple helix, setting a baseline of 25% for potential degradation. Intracellular degradation as high as 33% of total collagen synthesis has been reported. Our morphological data do not provide any information as to the relative α1/α2 mRNA content of the duplex strands.

Palade (20) and Ross and Benditt (21) have shown that cells producing collagen have uniquely ordered arrays of ribosomes on the endoplasmic reticulum surface. As Ross and Benditt (21) stated, "Ribosomes appear in a characteristic and possibly specific configuration . . . grouped in rows of pairs which often curve . . . ." We now go a step further to propose that these ordered arrays reflect a specific ordering of the mRNAs being read out in the form of supramolecular organizations. These arrangements may be intrinsic to the mRNAs but are more likely to be the result of their interac-

tion with cytosolic or membrane proteins. Monson (22) has shown that collagen mRNA translated *in vitro* produces unhydroxylated pro-α-chains, which nevertheless register and aggregate to produce pepsin- and chymotrypsin-resistant structures. She argued that perhaps the collagen chains have the intrinsic property of rapid self-assembly. Our data suggest that this rapid registration could be the result of coordinated readout of the mRNAs due to the presence of specific supramolecular assemblies of mRNA and ribosomes.

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