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Regulation of type I collagen mRNA levels in fibroblasts

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Type I procollagen mRNA levels, as well as total RNA and poly(A)-rich mRNA, remain constant when rapidly growing human fetal lung fibroblasts (HFL-1 cells) are compared with quiescent cells. Polysome profiles of cells in both growth states revealed that the distribution of type I collagen mRNA in the mRNP fraction and in polysomes also remained constant even though total RNA and poly(A)-rich mRNA were shifted from polysomes to the mRNP pool in resting cells. Similar results were obtained when RNA fractions in polysomes associated with the cytoskeletal framework were examined. It is known that procollagen production is unaffected by the growth state of cells [Breul, S. D., Bradley, K. H., Hance, A. J., Schafer, M. P., Berg, R. A. and Crystal, R. G. (1980) *J. Biol. Chem. 255*, 5250–5260] although total protein synthesis is markedly decreased in resting cells. It would therefore appear that the translational control responsible for reduced synthesis of non-collagenous proteins in resting cells does not extend to procollagen and that transcriptional control can account for levels of type I procollagen produced by cultured human fibroblasts.

Fibroblasts in culture have been used extensively to study the regulation of collagen synthesis and to monitor the effects of agents that are thought to influence collagen production (see [1] for a brief review). In such experiments an important variable is the growth state of the cells, since differences in collagen mRNA levels and consequent protein synthesis as a function of growth rate or degree of confluence of cells *in vitro* could markedly influence the results that are observed.

Breul et al. [2] performed an extensive study of collagen production by human diploid lung fibroblasts (HFL-1) during periods of rapid and relatively slow cell growth over 25 population doublings. These workers concluded that, when allowance was made for changes in specific activity of intracellular free proline, collagen production, as measured by production of labeled hydroxyproline, was constant even during periods of rapid cell growth. Subsequently Tolstoshev et al. [3] determined that confluent HFL-1 cells contained twice the levels of type I procollagen mRNA compared to logarithmically growing cells and degraded less than half the newly synthesized procollagen intracellularly, but they confirmed that net collagen production did not change with growth state. Since isolated RNA from log-phase and confluent cells was equally translatable in a cell-free translation system, these workers suggested that some of the procollagen mRNA in confluent cells may be compartmentalized. Such segregation may make this mRNA less efficiently translatable or, alternatively, some aspect of the translational apparatus of log-phase cells may enhance the translatability of log-phase procollagen mRNA [3].

Our laboratory has been interested in studying the regulation of collagen synthesis by fibroblasts in culture and in defining the mechanisms by which putative transcriptional and translational controls are exerted. We reasoned that, if confluent cells were subject to translational control of collagen synthesis, it might be possible to demonstrate differences in the distribution of procollagen mRNA between mRNP and polysome pools in log-phase and confluent (slowly growing) fibroblasts. Our studies show that total poly(A)-rich RNA as well as type I collagen mRNA levels do not change with growth state. However, while the proportion of total poly(A)-rich RNA in polysomes decreases as cells become confluent, the distribution of collagen mRNA does not change. Thus, under the culture conditions used in this study, collagen synthesis appears to be defined by mRNA levels, and the translational control exerted on the synthesis of many non-collagenous proteins does not appear to extend to type I collagen.

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Abbreviations. mRNP, mRNA-ribonucleoprotein particles, SDS, sodium dodecyl sulfate; ^{35}S-UTP, uridine 5′-(α-[^{35}S]thio)triphosphate.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium was obtained from Gibco and fetal calf serum was from HyClone Laboratories. Proteinase K was from Boehringer Mannheim, RNase A and RNase T1 were from Sigma. RNA polymerase SP6 and T7, [^{3}H]leucine (592 Ci/mmol) and ^{35}S-UTP (1200 Ci/mmol) were from New England Nuclear. [^{3}H]Polyuridine was from Amersham. The plasmids containing the SP6 and T7 promotors (Gemini Plasmids) were obtained from Promega Biotec. Nitrocellulose (BA 85) was from Schleicher & Schüll, GF/C glass-fiber filters were from Whatman.

Cell culture

Human fetal lung fibroblasts, HFL-1, from the American Type Culture Collection (CCL 153), were grown in Dulbecco's modified Eagle's medium at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin, 0.5 μg/ml 2-hydroxybenzoate, 50 μg/ml ascorbate, and 584 μg/ml glutamine.

A representative growth curve of HFL-1 cells is shown in Fig. 1. One day after plating almost 40% of the cells are

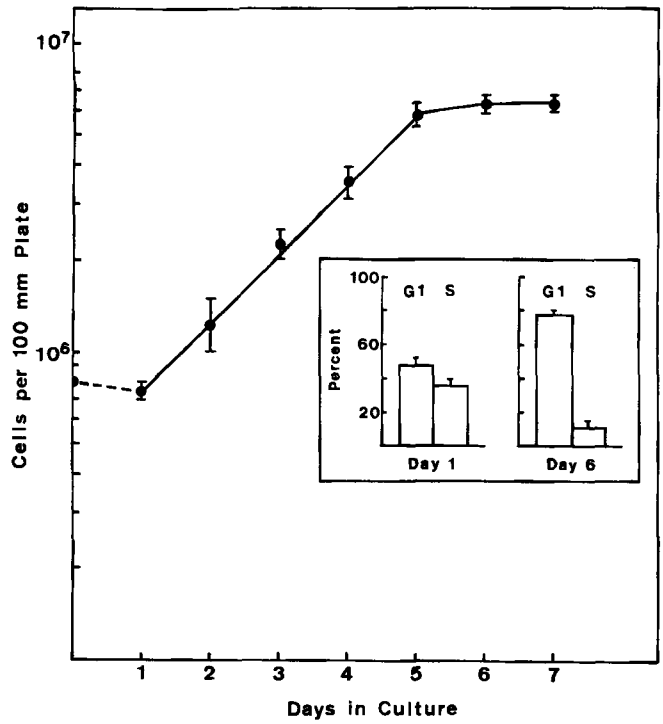

Fig. 1. *A growth curve of HFL-1 cells in culture.* 8×10^5 cells were plated in a 100-mm dish on day zero. The bars indicate the standard error of three experiments. Inset: cell-cycle analysis of cells on day 1 and day 6. The percentage of cells in the G1 and S phases of the cell cycle are plotted. The bars indicate the standard error of three experiments

synthesizing DNA and the culture retains logarithmic growth characteristics until day 5. At day 6 10% or less of the cells appear to be synthesizing DNA. Cells were counted in a Coulter counter and cell-cycle analysis was performed by cytofluorimetry. Cells were harvested 2 days after plating when the culture was in a logarithmic growth phase and 6 days after plating when the majority of the cells were quiescent.

Polysome preparation

Trypsinized cells were washed three times with phosphate-buffered saline containing 2 μg/ml cycloheximide. The cells were suspended in 0.5 ml or 1.0 ml buffer A (25 mM Tris/HCl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 2 μg/ml cycloheximide). After 5 min on ice the same volume of buffer A, containing 0.3 M sucrose, 2% Triton X-100, 0.5% sodium deoxycholate and 1 mg/ml heparin, was added. The cells were homogenized in a Dounce homogenizer with 10 strokes using the tight pestle. Cell fragments were removed by centrifugation at $20000 \times g$ in a Sorvall centrifuge for 5 min. The supernatant was layered over a linear 15–50% sucrose gradient and a 70% sucrose pad in buffer A. The samples were centrifuged in a SW40 rotor (Beckman) at 36000 rpm for 110 min at 4°C. The gradients were fractionated and their absorbance monitored at 254 nm with an ISCO density gradient fractionator (model 640).

Preparation of cytoskeletal fractions

The trypsinized and washed cells were suspended in 1 ml buffer A, containing 0.3 M sucrose, 0.1 M KCl, 1 mg/ml heparin and 0.5% Triton X-100. The cells suspension was kept on ice for 20 min and then centrifuged at $20000 \times g$ in a Sorvall centrifuge for 5 min to separate the cytoskeletal fraction from the cytosol. The pellet was then extracted in a buffer containing 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 1 mg/ml heparin, 1% Tween 20, 0.5% sodium deoxycholate by passing the resuspended pellet 10 times through a 21-gauge needle. Nuclei and cell fragments were precipitated by centrifugation as described above.

Preparation of total nucleic acids and RNA

For the preparation of total nucleic acids, trypsinized cells were suspended in 1 × SET buffer (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1% SDS), containing 0.1 mg/ml proteinase K, and incubated at 40°C for 1 h. The cytoskeletal fraction was treated in the same way. SDS and proteinase K were added to the sucrose gradient fractions and to the cytosol fraction to a final concentration of 1% and 0.1 mg/ml, respectively. The conditions for digestion were as described above. Subsequently the samples were extracted with equal volumes of redistilled, water-saturated phenol and chloroform. After adjusting the NaCl concentration to 0.1 M, the nucleic acids were precipitated with 2 vol. 100% ethanol at −70°C for 1 h. After centrifugation, the pellets were washed with 70% ethanol and dissolved in H_2O or in 0.2 × SET buffer.

Quantification of DNA and RNA

DNA was quantified using a fluorescence assay as described [4]. RNA was determined by absorbance at 260 nm. In the samples containing total nucleic acid, the amount of RNA was obtained by subtracting the amount of DNA obtained by the fluorescence assay from the amount of total nucleic acid, determined by absorbance at 260 nm. Cell number was calculated using a value of 9.2 pg DNA per HFL-1 cell [2].

Poly(A)-rich mRNA assay

1–2 μg RNA or total nucleic acid was hybridized with 50 nCi [^{3}H]polyuridine in 2 × SSC buffer (20 × SSC = 3 M NaCl, 0.3 M sodium citrate, 30 mM EDTA). The samples were kept at 65°C and then at 37°C for 3 h each. Unbound [^{3}H]polyuridine was digested with RNase A (10 μg/ml containing 0.5 mg/ml herring sperm DNA in 2 × SSC) at 37°C for 10 min. The remaining material was precipitated with trichloroacetic acid at a final concentration of 10%, filtered through GF/C filters and counted in 0.4% Omnifluor in toluene.

Quantification of procollagen type I mRNA levels

Type I procollagen mRNA levels were quantified by a solution hybridization assay using RNA probes complementary to the α1 (I) procollagen cDNA clone HF 677 [5] and the α2(I) cDNA clone HF 1131 [6]. Briefly the cDNA was cloned into the polylinker site of the plasmid pUC12 containing the SP6 promotor [7] or the Gemini plasmid containing the T7 and SP6 promotors on either side of the polylinker region. The plasmids were linearized at the end of the cDNA insert, downstream of the promotor, using an appropriate restriction enzyme. Transcription into antisense RNA was carried out with either the SP6 or T7 polymerase using ^{35}S-UTP as a radioactive marker according to the manufacturer's

protocol. An RNA standard (sense RNA) was obtained in the same way by using a plasmid with the opposite orientation of the cDNA insert or in the case of the Gemini plasmid by using the other polymerase. For hybridization, aliquots of RNA were brought up to a final volume of 10 µl with 0.2 × SET buffer. Then, 20 µl buffer containing 0.6 M NaCl, 20 mM Tris/HCl, pH 7.5, 4 mM EDTA, 0.2% SDS and 10000–30000 cpm labeled RNA per sample was added. To prevent evaporation, the samples were covered with a drop of mineral oil. The samples were denatured at 95 °C for 4 min and hybridization was then carried out at 68 °C overnight. RNase digestion of single-stranded RNA was carried out at 37 °C for 1 h by adding 300 µl buffer containing 0.3 M NaCl, 10 mM Tris/HCl, pH 7.5, 4 mM EDTA, 0.1 mg/ml herring sperm DNA, 40 µg/ml RNase A and 2 µg/ml RNase T1. The remaining double-stranded RNA was precipitated on ice at a final concentration of 10% trichloroacetic acid. The precipitates were collected on GF/C filters, washed first with 3% trichloroacetic acid/1% $NaPP_i$, then with ethanol and counted in 0.4% Omnifluor in toluene.

Northern blots were performed as described by Melton et al. [7].

Total protein synthesis

To measure total protein synthesis, cell cultures were labeled for 24 h with 10 µCi/ml [^{3}H]leucine in serum-free medium containing 1 mg/ml bovine serum albumin. The cell layer was solubilized in 0.5 M NH_4OH. Aliquots of the cell layer and the culture medium were precipitated with trichloroacetic acid at a final concentration of 10%, filtered on GF/C filters, and the incorporated radioactivity counted as described above. Values for the cell layer and medium were combined.

RESULTS

In order to quantify reliably the level of type I procollagen mRNA in cultures of rapidly growing (log phase) and confluent human fetal lung fibroblasts (HFL-1 cells), a solution hybridization assay was established. To obtain antisense RNA, cDNAs coding for α1(I) and α2(I) mRNA were cloned into plasmids containing bacterial SP6 or T7 RNA polymerase promotors. These plasmids, which were linearized downstream from the cDNA, were then transcribed into radiolabeled RNA. Unlabeled sense RNA, which was used as a standard, was obtained in the same way by transcribing plasmids containing the cDNA in the appropriate orientation.

Fig. 2 shows a typical example of a standard curve plotted as radioactivity (cpm) hybridized as a function of sense RNA input. In this case the labeled RNA represents synthetic antisense α1(I) RNA and the standard RNA represents synthetic sense α1(I) RNA. Background values after RNase digestion were usually less than 3% of the input radioactivity and maximal hybridization reached 70–80% of input activity. Given the known molecular size of the transcribed sense RNA, and the extent of hybridization derived from the standard curve, the number of mRNA molecules per cell can be calculated. However, reliable data can only be obtained in the linear range of the standard curve.

The inset in Fig. 2 shows a Northern blot hybridization analysis of total RNA from HFL-1 cells. Polymorphic transcripts of 5.8×10^3 and 4.8×10^3 bases were identified as previously described by Chu et al. [8]. The finding of only two bands, identified as α1(I) mRNA in total fibroblast RNA,

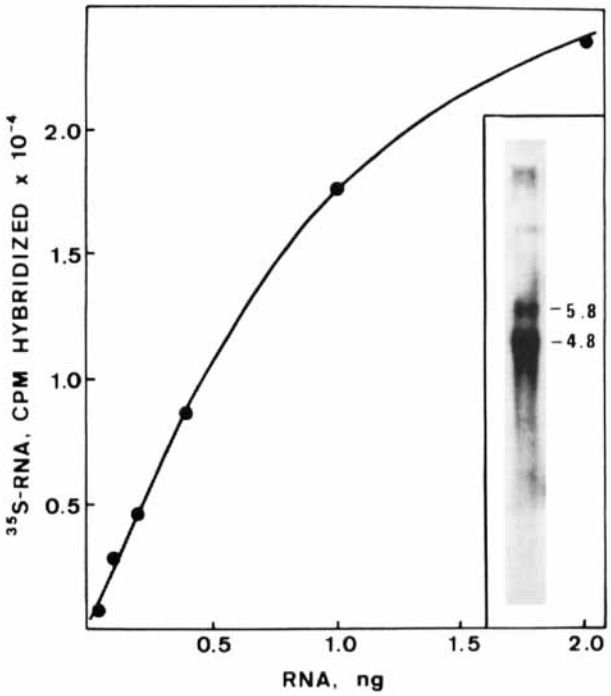

Fig. 2. *Typical standard curve for a solution hybridization experiment.* Increasing amounts of synthetic α1(I) RNA were hybridized to ^{35}S-labeled synthetic antisense α1(I) RNA. Synthesis was achieved using the SP6 RNA polymerase as described in Materials and Methods. Inset: Northern blot hybridization analysis of total HFL-1 RNA utilizing ^{25}S-labeled synthetic antisense α1(I) RNA

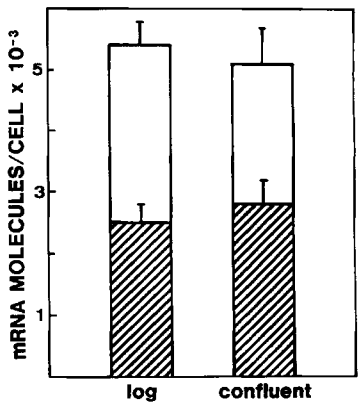

Fig. 3. *Type I procollagen mRNA content in rapidly growing (log phase) and confluent HFL-1 fibroblasts.* (□) α1(I); (▨) α2(I) mRNA. The bars indicate the standard error of the mean (SE) of 10 experiments

provides strong supporting evidence for the specificity and utility of synthetic antisense RNA as a probe for collagen α1(I) mRNA.

Fig. 3 shows the results of assays of α1(I) and α2(I) mRNA levels in rapidly growing and confluent cultures of HFL-1 cells. No significant differences were observed in either α1[I] or α2(I) mRNA content when rapidly growing and confluent cultures were compared. The ratio of α1(I) to α2(I) mRNA in log-phase cells was 2.2, and in quiescent cells, 1.8. This difference is not thought to be significant.

Fig. 4 shows the results of quantification of total RNA and poly(A)-rich mRNA in HFL-1 fibroblasts. There was no significant difference in either quantity when log-phase and

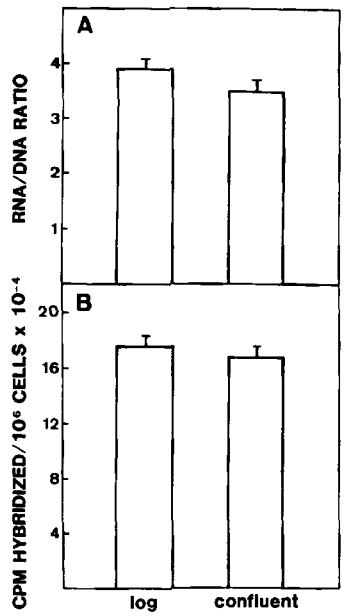

Fig. 4. *Levels of total RNA (A) and poly(A)-rich mRNA in log-phase and confluent cells.* The bars indicate the SE of 15 (A) and 7 (B) experiments. Poly(A)-rich mRNA was assayed by hybridization with [^{3}H]polyuridine as described in Materials and Methods.

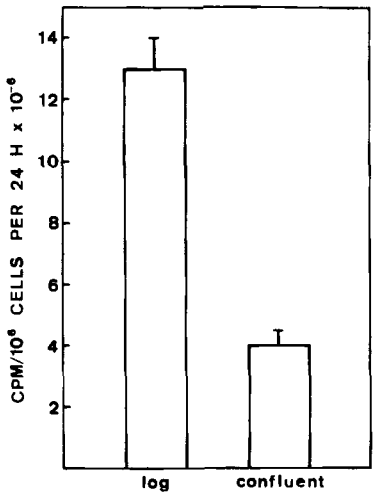

Fig. 5. *Total protein synthesis in log-phase and confluent cells as determined by incorporation of [^{3}H]leucine.* The values for cell layer and culture medium were combined. The bars indicate the SE of six experiments

confluent cells were compared. The production of total protein, measured by incorporation of [^{3}H]leucine during a 24-h labeling period, was more than three times higher in rapidly growing than in quiescent cells (Fig. 5). The production of type I procollagen, however, has been shown by other investigators, using well-established techniques, to be the same for both growth states [2, 3].

Since the amount of total poly(A)-rich mRNA and type I collagen mRNA does not change when cells become quiescent, these data, combined with those on total protein synthesis and collagen production, suggest that mRNA for non-collagenous proteins is translated less efficiently than type I collagen mRNA as cells become confluent. To examine this question cytoplasmic RNA was isolated under conditions which preserve intact polysomes. These extracts were centrifuged through sucrose gradients in order to obtain polysome patterns for rapidly growing and quiescent cells (Fig. 6). The absorbance profiles at 254 nm show substantial differences. Under conditions of logarithmic growth, a large proportion of the RNA is found in the polysome fraction (Fig. 6A). In quiescent cells, however, the non-polysomal mRNP particles represent the major RNA fraction (Fig. 6B). It has been reported that the distribution of poly(A)-rich mRNA follows the ultraviolet absorbance pattern [9]. In log-phase cells most of the poly(A)-rich mRNA is found in the polysomes, while in quiescent cells the majority of this RNA fraction sediments in the non-polysomal mRNP pool [9]. Type I collagen mRNA, however, did not follow this behavior. The distribution of this mRNA between the mRNP pool and polysomes, shown for α1(I) mRNA, seemed to be similar in both growth states (Fig. 6).

In order to quantify these findings, mRNP particles (fraction I) and polysomes (fraction II) were pooled as indicated in Fig. 6. These fractions were analyzed for their content of α1(I) mRNA, total RNA and poly(A)-rich mRNA. As shown in Fig. 7 the mRNP pool as well as the polysome pool contained the same number of α1(I) mRNA molecules in both growth states, suggesting that the same amounts of type I procollagen were being synthesized. These results were compared with the distribution of total RNA and poly(A)-rich mRNA in the polysome fraction. As shown in Fig. 8, while the proportion of total RNA and poly(A)-rich RNA in polysomes was reduced significantly in confluent cells, the proportion of α1(I) mRNA remained constant.

Comparison of the total number of α1(I) mRNA molecules per cell (Fig. 3) with the amount isolated in the combined RNP and polysome pools (Fig. 7) indicates that only a fraction of α1(I) mRNA molecules was extractable under conditions which do not degrade polysomes. Therefore, it was necessary to show that this polysome fraction is representative of the total polysome pool. It has been reported that active translation of mRNA occurs on polysomes that are bound to the cytoskeleton [10, 11]. Preparative conditions were therefore chosen which preserve the attachment of polysomes to the cytoskeleton. The results, shown in Fig. 9, indicate that the number of α1(I) mRNA molecules per cell in the cytoskeletal fraction and in the extractable cytosol fraction does not differ in logarithmically growing and confluent cells. We therefore conclude that our data on mRNP and polysome pools, as shown in Fig. 7, are representative of the total levels of type I collagen mRNA molecules in cells at the two different growth states.

DISCUSSION

The regulation of collagen synthesis is thought to play an important role in normal development and in the repair of injury in animals. Regulation could occur at a number of steps in the biosynthetic pathway for collagen, including transcription of the collagen genes, processing and translation of mRNAs, cotranslational and post-translational modification of collagen chains, secretion and extracellular processing, and assembly of (pro)collagen molecules. In addition, both intracellular degradation of newly synthesized collagen chains and extracellular proteolysis by collagenases and related collagenolytic proteases represent potential sites for control of net collagen production.

Although fibroblasts in culture are not well suited to the study of extracellular factors that control collagen production

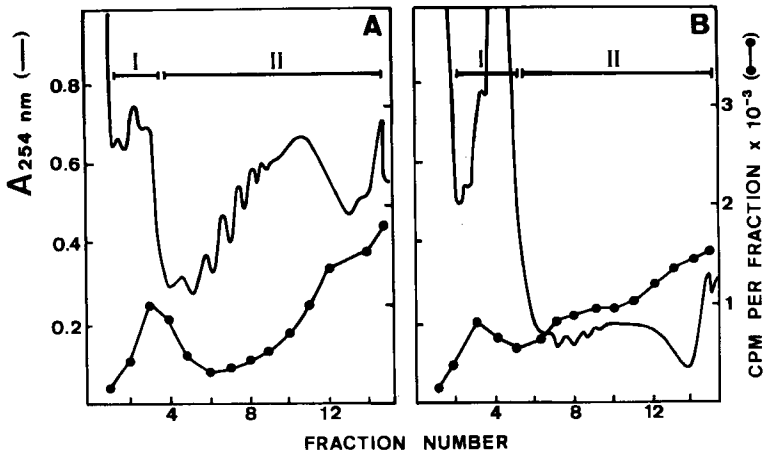

Fig. 6. *Polysome profiles and distribution of α1(I) mRNA in log-phase (A) and confluent cells (B).* Cytoplasmic RNA from about 2.5×10^7 cells was loaded onto each sucrose gradient. α1(I) mRNA (●——●) levels were quantified by a solution hybridization assay using equal amounts of fractions in (A) and (B). The bars mark the pooled mRNP particles (I) and polysome fractions (II)

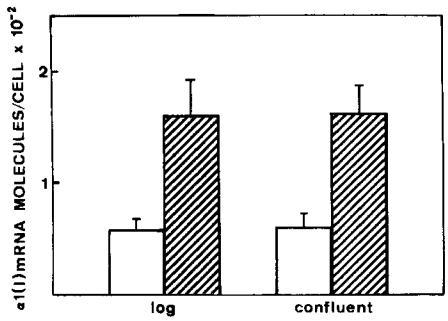

Fig. 7. *Level of α1(I) mRNA molecules in the mRNP (□) and polysome fractions (▨) of log-phase and confluent cells.* Error bars (SE) are given for six experiments

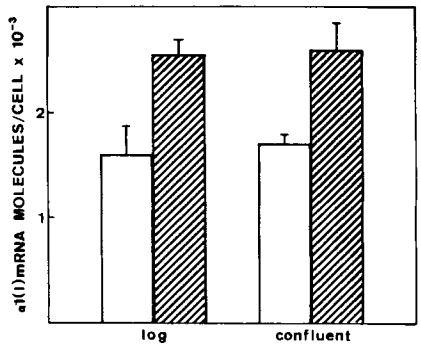

Fig. 9. *Levels of α1(I) mRNA in the cytosol (□) and in the cytoskeletal (▨) fractions of log-phase and confluent cells.* The SE is given for four experiments

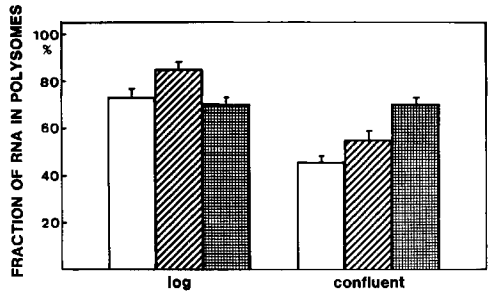

Fig. 8. *Proportion of different RNA fractions in the polysome pool of log-phase and confluent cells.* (□) Total RNA; (▨) poly(A)-rich RNA; (▦) α1(I) mRNA. For total RNA 100% equals the sum of the absorbance of the mRNP and polysome fractions. The bars show the SE of six experiments

they represent a relatively simple model system in which to examine mechanisms that operate intracellularly, particularly transcriptional and translational controls. However, in order for such experiments to be readily interpretable it is necessary to know whether the proliferative state of the cells and the resulting degree of confluence of the culture influence the processes of transcription and translation for collagen. This is especially true since it is known that there is translational control of synthesis of many intracellular proteins as cells become confluent and reduce their rate of growth [9]. This control may result from the need for actively translated mRNA in polysomes to be associated with a cytoskeletal framework [10, 12] and the tendency for such assemblies to be organized differently in rapidly dividing cells [13].

We have developed a solution hybridization assay for quantification of type I collagen mRNA in cultured fibroblasts. The technique is based on the use of RNA polymerases to transcribe cDNAs cloned into plasmids containing highly specific promoters for these polymerases [7]. Radiolabeled antisense RNAs were used as probes and sense RNAs were used to standardize the assay. This technique offers a number of advantages over the use of nick-translated DNA. The probe is easier to prepare and more sensitive. Since RNA · RNA duplexes are more stable than RNA · DNA hybrids, hybridization can be performed under conditions of higher stringency and backgrounds can, therefore, be lowered. Hybridization in solution is superior to hybridization of mRNA bound to nictrocellulose membranes or in agarose, since accurate quantification is difficult with the latter techniques.

We have found that sparsely cultured, actively dividing HFL-1 fibroblasts and resting, confluent cells contained the same number of α1(I) and α2(I) collagen mRNA molecules per cell (Fig. 3) and that the ratio of α1(I) to α2(I) mRNA was around 2 as previously determined by other workers [14, 15]. The total cellular RNA content and poly(A)-rich RNA were also the same in the two growth states (Fig. 4).

In contrast to total protein synthesis, which is reduced as HFL-1 fibroblasts achieve a quiescent growth state, collagen production remains unchanged [2, 3]. Although Aumailley et al. [16] reported that proliferating human skin fibroblasts synthesized more radiolabeled peptide-bound hydroxyproline per cell than did confluent cultures, their data were not corrected for the specific activity of intracellular proline and it has been shown, in HFL-1 cells, that proline-specific activity is higher in proliferating than in confluent cells [2]. A disparity, therefore, exists in view of the good correlation between collagen mRNA levels and collagen synthesis on the one hand and the lack of correlation between total poly(A)-rich RNA and protein synthesis on the other. In order to examine this apparent difference in translational control, the distribution of type I collagen mRNA and total poly(A)-rich mRNA in polysomes and in non-polysomal mRNP particles was studied. As cells become confluent and quiescent the fractions of total RNA and poly(A)-rich RNA in polysomes decrease and those in mRNA particles increase (Figs 6 and 8) but the distribution of type I collagen mRNA remains unchanged (Fig. 7). These findings suggest that translation of collagen mRNA is less subject to the type of control exerted on most mRNAs for intracellular proteins; the latter appears to result from a functional compartmentation of mRNA in the mRNP pools as cells become confluent. It is known that this shift of mRNA from polysomes to mRNP particles represents an important translational control mechanism [17, 18].

It has been shown in a number of systems that actively translated polysomal mRNA is associated with the cytoskeletal framework whereas non-translated mRNP particles are found in a supernatant fraction [10–12]. It was therefore possible that our comparison of polysomal collagen mRNA levels in growing and quiescent fibroblasts may have failed to detect a difference because our extraction procedure for polysomal mRNA did not efficiently release polysomes bound to the cytoskeleton. However, as shown in Fig. 9, α1(I) mRNA levels did not differ in cytoskeletal fractions prepared from logarithmically growing and quiescent cells. We therefore conclude that the mRNA levels, determined in polysomal fractions, accurately reflect the translatable mRNA pools present in these fibroblasts.

Tolstoshev et al. [3], in a careful study using HFL-1 fibroblasts, reported that confluent cells contained approximately twice as many type I mRNA molecules as did logarithmically growing fibroblasts. These workers used a partially purified single-stranded cDNA probe, prepared from enriched sheep tendon poly(A)-rich mRNA, which contained presumably sequences complementary to both α1(I) and α2(I) mRNA. We do not have any adequate explanation for this discrepancy but suggest that the use of well-characterized, human RNA probes offers advantages in specificity and in sensitivity, which make our determinations less subject to error.

The results described in this study indicate that fibroblasts in culture may be even more suitable than previously thought for the investigation of factors and mechanisms that regulate collagen production since growth rate and cell-cell interactions do not seem to play a major role in changing mRNA levels or rates of synthesis. It is clear that regulatory influences can be brought to bear on collagen synthesis by fibroblasts *in vitro*. Transformation by Rous sarcoma virus leads to a reduction in collagen synthesis and in mRNA levels [19–21] and this change occurs as a result of a decrease in the rate of transcription of collagen genes [22, 23]. Transcriptional control of collagen synthesis by fibroblasts has also been observed upon addition of gamma interferon [24, 25] and a factor isolated from thioacetamide-induced fibrotic rat liver [26]. In the latter case, stimulation of collagen synthesis and an increase in mRNA levels were observed.

Translational control of collagen synthesis in fibroblast cultures by a fragment derived from the NH_2-terminal non-triple-helical region of the pro α1(I) chain has also been reported [27, 28], but the mechanism of action of this fragment remains to be established. If, as suggested by the present study, collagen mRNA is able to escape the translational control imposed upon most mRNAs in confluent cells, this property may reflect an unusual structural characteristic of this mRNA, which may well deserve additional study.

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