Biochem. J. (1986) 239, 179–183 (Printed in Great Britain)

Regulation of collagen production and collagen mRNA amounts in fibroblasts in response to culture conditions

Susan R. QUINONES, Donald S. NEBLOCK* and Richard A. BERG†

Department of Biochemistry, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, NJ 08854, U.S.A.

Collagen synthesis and mRNA amounts for the α1 and α2 polypeptide chains of Type I collagen were measured in embryonic-chick tendons and in tendon cells both in suspension and in primary cultures. The percentage of protein production represented by collagen in suspension-cultured cells was initially the same as in the intact tendon; however, on an hourly basis, there was actually a steady decline in collagen production by suspended cells. Collagen production in primary cultures of chick tendon fibroblasts was decreased when compared with intact tendon, even though ascorbate-supplemented primary cultures were able to maintain higher rates of collagen production than were non-supplemented cultures. The amounts of mRNA for α1(I) and α2(I) polypeptide chains of collagen responded in similar fashions to different culture conditions and were compared with the amounts of mRNA for β-actin. In primary cultures the available α1 and α2 collagen mRNAs support proportionately higher collagen production than in the intact tendon. However, the ratio of α1/α2 mRNA and polypeptide-chain synthesis did not remain 2:1, but increased with the concomitant production of Type I trimers composed of three α1 chains. Removal of fibroblasts from their environment *in vivo* appears to alter the amounts of mRNA for α1 and α2 chains and to alter the utilization of those mRNAs for polypeptide synthesis.

INTRODUCTION

The collagen family consists of related proteins which are integral structural components of the extracellular matrix [1]. The most prevalent member is type I collagen, principally located in bone, skin, lung and tendon. Type I collagen is a trimer of two α1(I) and one α2(I) polypeptide chains. These chains are initially synthesized as the precursors proα1(I) and proα2(I) and, after a series of post-translational modifications, are assembled and folded into a triple-helical conformation [2, 3]. There is substantial evidence that control can be exerted at a number of points along the path of collagen synthesis, as is the case for other eukaryotic gene products [4].

We have compared collagen production and mRNA amounts for the α1 and α2 polypeptide chains in intact embryonic-chick tendons in organ culture with those of freshly isolated chick tendon cells in both suspension (before attachment) and in primary culture (after attachment to plastic) in order to determine the effects of the extracellular environment on Type I collagen production. Perturbation of the normal extracellular environment as well as variations in culture conditions result in dramatic changes in collagen production and in amounts of collagen α1- and α2-chain mRNA.

MATERIALS AND METHODS

Materials

Fetal bovine serum, penicillin, streptomycin, trypsin, Dulbecco's modified Eagle medium and minimum essential medium were all obtained from Gibco. Sodium ascorbate and crude bacterial collagenase were purchased from Sigma and Worthington Biochemical Corp. respectively. Bovine serum albumin, calf thymus DNA, diphenylamine, Ficoll and polyvinylpyrrolidone were purchased from Sigma. Glyoxal and orcinol were from Fisher Scientific. DNA polymerase I, formamide, guanidine hydrochloride and yeast tRNA were obtained from Bethesda Research Laboratories. Deoxyribonuclease was from Boehringer Mannheim, dimethyl sulphoxide was from J. T. Baker, and [α-^{32}P]dCTP (800 Ci/mmol) was from Amersham. L-[U-^{14}C]Proline (273 mCi/mmol) was purchased from New England Nuclear, and purified bacterial collagenase was from Advanced Biofactures.

Tendon organ cultures, suspension cultures and primary cultures of freshly isolated matrix-free tendon cells

Tendons were dissected from 17-day embryonic chicks and either incubated directly or prepared for suspension culture as matrix-free cells. Whole-tendon organ cultures were performed by placing two or three whole washed tendons into 1 ml of modified Krebs II [5] medium containing 0.2% glucose, 2% fetal bovine serum and sodium ascorbate (10 μg/ml). Matrix-free cells were prepared from the tendons by digestion with trypsin and crude bacterial collagenase by the method of Dehm & Prockop [5] as modified by Kao *et al.* [6]. The cells were incubated for up to 6 h at a concentration of 7.5×10^6/ml.

For primary culture the cells were prepared as above but under sterile conditions. The cells were then inoculated in 75 cm^2 flasks at a concentration of 3.0×10^6 cells/flask in a total of 20 ml of Dulbecco's modified Eagle medium containing 5% fetal bovine serum,

* Present address: Cistron Technology Inc., 10 Bloomfield Avenue, Pine Brook, NJ 07058, U.S.A.

† To whom correspondence and requests for reprints should be sent.

100 units of penicillin/ml and 100 μg of streptomycin/ml. In some experiments, 1.0×10^6 cells were seeded in 6 cm-diam. culture dishes containing 6 ml of growth medium. Microscopic examination of the cultures was made daily and the media were changed every second day. Ascorbate-treated cultures had ascorbate (10 μg/ml) added daily.

Protein synthesis and processing of samples

The synthesis of collagenous and non-collagenous peptides in each culture system was determined by the incorporation of [^{14}C]proline. In tendon organ cultures, the concentration of [^{14}C]proline was 5 μCi/ml. Freshly isolated tendon cells were labelled with 1 μCi of [^{14}C]proline/ml. Whole tendons and freshly isolated cells were preincubated at 37 °C for 0.5 h and labelled for 5.5 h. In some experiments freshly isolated cells were labelled for 1 h periods. Primary cultures of the tendon fibroblasts were labelled with 2 μCi of [^{14}C]proline/ml on day 4 and day 7. Primary cultures were scraped into plastic culture tubes at the completion of labelling. Culture tubes were placed in a 100 °C water bath for 10 min to halt synthetic and proteolytic processing in the cultures. Each sample was sonicated with a microprobe sonicator (Kontes Instruments) to ensure the disruption of the cells.

Samples were dialysed against several changes of deionized water at 4 °C for no more than 48 h, until less than 100 c.p.m. remained in the dialysis residue. The latter material was then freeze-dried, and the amount of [^{14}C]proline present in collagenous and non-collagenous peptides was determined as described [7–9].

The ratio of α1 to α2 collagen polypeptides was measured after incubation of 4-day cultures with [^{14}C]proline for 60 min as above to label newly synthesized collagen. The culture media were separated from the cell layers, which were scraped in 10 mM-HCl. To allow us to measure Type III collagen, both cells and media were dialysed against 10 mM-HCl, and digested with 1 mg of pepsin/ml at 4 °C for 24 h as described [6]. The pepsin-resistant collagen polypeptides were dialysed against 0.1 M-Tris/HCl buffer, pH 8.0, treated with SDS and run on polyacrylamide slab gels in SDS with delayed reduction [10] to avoid contamination of α1(I) chains with any α1(III) chain that may be present. After fluorography, the ^{14}C-labelled α1 and α2 chains were scanned and the density of each band was quantified by planimetry.

RNA extraction and determination of RNA and DNA contents

Total RNA was extracted from whole tendons or fibroblasts in buffered guanidine hydrochloride. Freshly isolated tendons were homogenized briefly in 9 vol. of 10 mM-sodium acetate, pH 5.5, containing 8 M-guanidine hydrochloride and 500 μM-dithiothreitol. Fibroblasts were also suspended in 9 vol. of the same buffer; however, homogenization was unnecessary. Samples were removed at this point for RNA and DNA determinations, and RNA was isolated from the remainder essentially as described [11, 12].

RNA and DNA were precipitated in cold 0.5 M-$HClO_4$. The RNA in the precipitate was hydrolysed in NaOH and measured colorimetrically with orcinol [13]. Known concentrations of yeast tRNA were simultaneously processed for a standard curve. Hydrolysis in 0.5 M-$HClO_4$ preceded measurement of DNA content by the diphenylamine method [14]. Calf thymus DNA was used for the construction of a standard curve.

Preparation of cDNA probes

Plasmids pGV1265 and pYN535, containing nearly full-length cDNAs to α1(I) and α2(I) chick collagen mRNA respectively, were supplied by Dr. Bjorn Olsen (UMDNJ–Rutgers Medical School). A β-actin cDNA clone (pA2) was provided by Dr. Marc W. Kirschner [15]. Large batches of each plasmid were isolated by the procedure of Ish-Horowicz & Burke [16]. The plasmids were nick-translated by using [α-^{32}P]dCTP, deoxyribonuclease and DNA polymerase I to a specific radioactivity of 0.5×10^8–2.0×10^8 c.p.m./μg, essentially as described by Rigby *et al.* [17].

RNA gel electrophoresis and 'Northern' analysis

'Northern' blots were prepared as described by Thomas [18]. Total RNA was glyoxylated in 10 mM-sodium phosphate, pH 7.0, containing 50% (v/v) dimethyl sulphoxide and 1 M-glyoxal for 60 min at 50 °C [19]. The RNA was electrophoresed through 1.0% agarose at 20–30V overnight with continuous buffer recirculation. After transfer to nitrocellulose, prehybridization and hybridization were carried out as described [18]. The ^{32}P-labelled nick-translated probes were added to a final concentration of 2.0×10^5–3.0×10^5 c.p.m./ml and allowed to hybridize for 24–48 h. After fluorography, band intensities were measured by densitometry.

RESULTS

Collagen production and non-collagen protein synthesis

Fibroblasts were isolated from 17-day-embryonic chick tendons and placed in either suspension or primary culture. Fresh cells in suspension culture for 6 h exhibited similar collagen-producing capabilities to intact tendons in organ culture. However, when shorter labelling periods of 1 h duration were used, it was found that the fibroblasts did not produce collagen at a constant rate throughout the 6 h of incubation (Fig. 1). From the second hour until the sixth hour, collagen production decreased by more than 30%. Non-collagen protein synthesis did not change during the same 6 h incubation.

Ascorbate has been shown to be a modulator of collagen production in cultured cells [20], and acts at several steps in collagen synthesis, including serving as a cofactor for the hydroxylation of prolyl residues [3] and serving to increase the synthesis and stability of α2-chain mRNA [21]. To compare the effects of ascorbate on collagen production and amounts of mRNA for both α1 and α2 chains in primary cell cultures, the cells were maintained for up to 7 days in either the presence or the absence of ascorbate. The cells were in the late-exponential phase of growth at 4 days and were confluent by 7 days. The population doubling time of 15–18 h was unaffected by ascorbate, as determined by cell number and DNA content (results not shown). As previously reported, ascorbate greatly influenced the percentage of total protein synthesis represented by collagen at both 4 days and 7 days of primary culture [22, 23]. Ascorbate-treated confluent cultures supported an extent of collagen production that was 6 times that found in non-ascorbate-

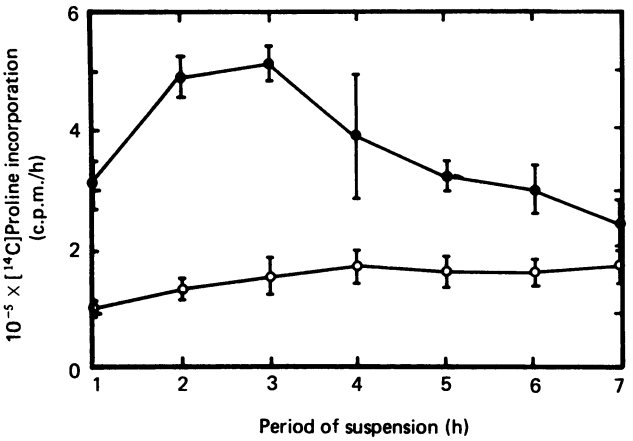

Fig. 1. Hourly collagen production and total protein synthesis in suspended fibroblasts

Tendon fibroblasts were labelled for 1 h periods with [^{14}C]proline. The incorporation of label into collagenase-sensitive (●) and -insensitive (○) protein was measured. Results are means ± S.E.M.

Table 1. Comparison of collagen production in fibroblasts maintained under various conditions

Chick tendon fibroblasts were labelled with [^{14}C]proline for 6 h and treated as described in the Materials and methods section. Values are means ± S.E.M. ($n = 6$).

Cell source	Collagen (%)	$10^{-5} \times$ Non-collagen protein (c.p.m.)
Intact tendon	27.4 ± 1.3	
Suspension culture	29.8 ± 1.3	
Primary culture		
4 day, grown with ascorbate	19.1 ± 0.4	9.49 ± 0.32
4 day, grown without ascorbate	5.1 ± 1.0	9.16 ± 0.46
7 day, grown with ascorbate	10.6 ± 1.7	8.17 ± 0.56
7 day, grown without ascorbate	1.7 ± 0.2	6.68 ± 0.17

treated confluent cells (Table 1). Non-collagen protein synthesis was significantly enhanced in the presence of ascorbate in 7-day cultures ($P < 0.05$), but was not affected by ascorbate in 4-day cultures. The percentage of protein synthesis represented by collagen in primary cultures was always less than that found in the intact tendons (Table 1).

Measurement of mRNA amounts

In order to further delineate the controls that might be involved with the changes in collagen production observed here, amounts of mRNA for both the α1 chain and α2 chains were examined under the same conditions as for the protein synthesis. Total RNA was prepared and hybridized under stringent conditions to chimeric plasmids containing cDNAs specific for chick α1(I) collagen and α2(I) collagen. As a precaution against the possibility that any changes in collagen mRNA might represent generalized effects on total mRNA amounts,

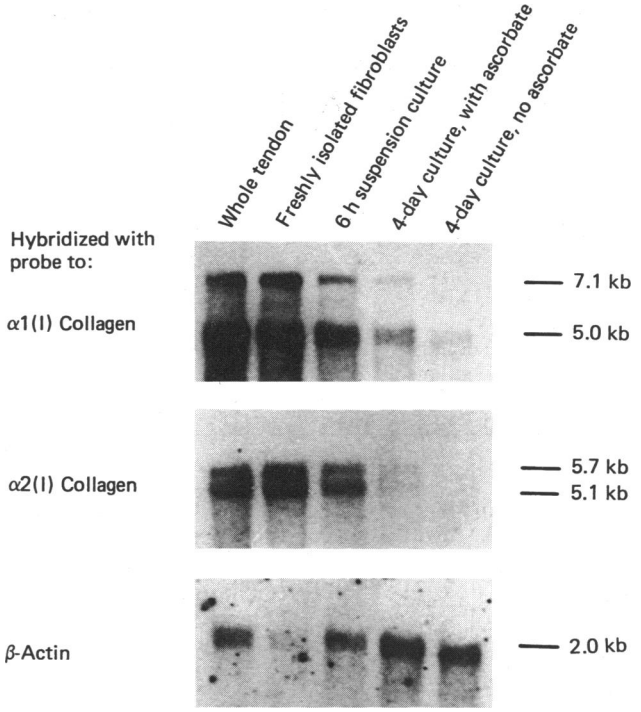

Fig. 2. Northern blots of total RNA hybridized to probes for chick proα1(I) and proα2(I) collagen and for chick β-actin mRNA

Total RNA (5 μg/lane) from each sample was denatured and electrophoresed. Hybridizations were performed as described in the Materials and methods section. Pictured is same Northern blot separately hybridized to cDNA for the RNA types designated. In each autoradiogram, all hybridizing RNA species are shown. The approximate sizes of the RNAs [33] are indicated at right (kb, kilobases).

the mRNA for an abundant intracellular protein, β-actin, was also measured. The results of the Northern-blot analyses are shown in Fig. 2 and summarized in Table 2. The relative amounts of the collagen α1-chain and α2-chain mRNAs responded in similar fashions to the different culture conditions, but in marked contrast with β-actin mRNA. The isolation of fibroblasts from intact tendon caused a dramatic loss of actin mRNA; however, after 6 h in suspension, there was significant recovery, approaching that found in the tendons. The immediate loss of actin mRNA after fibroblast liberation was probably not due to damage during the isolation procedure, because neither the amounts of the collagen αl- or α2-chain mRNAs nor that of total RNA changed significantly. As the proportion of actin mRNA increased during suspension culture, the collagen α1- and α2-chain mRNAs both decreased. In primary cultures, the relative collagen mRNA amounts were even more depressed than in suspension cultures, whereas the proportion of actin mRNA was greatly elevated. Although the amounts of both collagen α1- and α2-chain mRNAs in ascorbate-treated cultures were higher than in cultures grown without ascorbate, as expected on the basis of previous results for α2-chain mRNA amounts [21], ascorbate had no significant effect on the β-actin mRNA.

The data presented above pertain to the changes in the

Table 2. RNA/DNA ratios and comparison of mRNA amounts as a function of culture condition

Relative mRNA amounts were determined by microdensitometric scanning of autoradiograms, followed by measurement of peak area. The values were corrected for the total amount of RNA per cell for the comparison of absolute mRNA amounts above. Tendon mRNA was arbitrarily assigned a value of 1 and all other mRNA amounts were expressed relative to this.

	Intact tendons	Freshly isolated fibroblasts	6 h suspension culture	4-day primary culture	
				+Ascorbate	−Ascorbate
RNA/DNA	1.25±0.08	1.13±0.07	0.73±0.01	3.40±0.07	4.04±0.27
mRNA					
α1(I) collagen	1	0.81±0.05	0.30±0.06	0.71±0.17	0.33±0.03
α2(I) collagen	1	0.76±0.08	0.35±0.02	0.17±0.01	0.08±0.01
β-Actin	1	0.25±0.06	0.50±0.09	6.09±1.83	4.46±0.63

proportions of the specific mRNAs to constant amounts of total RNA. However, the amount of total RNA per cell does not remain constant under the various culture conditions (Table 2). Therefore, changes in the relative amount of collagen α1- or α2-chain mRNAs or actin mRNA were corrected for the amount of total RNA per cell (Table 2).

Although the α1(I) and α2(I) collagen mRNAs decreased co-ordinately in suspension culture in 6 h to 30–35% of what was found in intact tendons, in primary cultures there was a disproportionate decrease in the amounts of mRNA for α1 and α2 chains. In ascorbate-treated cultures, the cells contained approx. 71% of the α1(I)-chain mRNA of tendons, but only 17% of the tendon α2(I)-chain mRNA. The similar occurrence in cultures grown in the absence of ascorbate indicates that ascorbate is not involved in the process. The reasons for this phenomenon are unknown, but further experiments were performed to determine if this was reflected in the amounts of the newly synthesized α1 and α2 collagen polypeptide chains. Primary cultures of chick tendon cells grown for 4 days were labelled for 1 h in the presence of [^{14}C]proline. The collagen was extracted from the cultures and incubated with pepsin to obtain pepsin-resistant collagen synthesized in primary cultures. The ratio of α1 to α2 chains was measured by separating them by SDS/polyacrylamide-gel electrophoresis followed by fluorography. The results indicated that the ratio of α1 to α2 collagen polypeptide chains in cells was 4.3±0.23 and in medium was 3.9±0.16, which was similar to the ratio of α1 to α2 mRNA amounts (Table 2). Since $(\alpha 1)_2,\alpha 2$ and $(\alpha 1)_3$ are the only stable pepsin-resistant assemblies produced from α1 and α2 chains, the data indicate that type I trimer was produced in addition to normal type I collagen in response to the disproportionate increase in α1-chain mRNA.

DISCUSSION

Although the exact relationship between the extracellular environment and collagen production has not been established, it is clear that fibroblasts are dependent on attachment and cell shape for macromolecular metabolism and cell growth [24, 25]. Protein synthesis has been shown to decline gradually in some cells after 18 h in suspension, even though mRNA amounts remain constant [26]. The availability of translatable mRNA is the controlling factor limiting protein synthesis in 3T6 fibroblasts [27]. Chick tendon fibroblasts examined here behaved similarly to 3T6 cells in that their protein-synthesis rates were decreased as the cells were incubated in suspension, but recovered after the cells attached to a solid substrate in primary cultures.

Ascorbate is an essential cofactor in proline hydroxylation [3] and stimulates collagen polypeptide synthesis through increased transcription and stability of collagen α2-chain mRNA [21, 28]. The data here support the findings originally reported [21] that implicate ascorbate in specifically increasing collagen α2-chain mRNA and extend them to also include α1-chain mRNA.

Many type-I-collagen-synthesizing systems have been studied where the ratios of α1- to α2-chain mRNA and of α1 and α2 polypeptide chains are 2:1 [6, 29–31]. The data presented here indicate that co-ordinated regulation of α1 and α2 chains is not always maintained. The ratio of α1(I) mRNA to α2(I) mRNA is 4 times greater in primary culture cells than in tendons or freshly suspended cells. The ratio of α1 mRNA to α2 mRNA was greater than 2:1, and was correlated with an increased ratio of α1 to α2 chains in pepsin-resistant collagen that reflected the presence of type I trimers. Evidence exists for uncoupled co-ordination of α1- and α2-chain synthesis in other systems. In de-differentiating chondrocytes, α1(I) mRNA is detectable several days before α2(I) mRNA [32]. There is a lag in time before each mRNA becomes translatable, and α1(I) mRNA is translated well before that of α2(I) mRNA. Chick embryos contain detectable amounts of α2(I) mRNA at 24 h of development, but α1(I) mRNA is measurable only after 48 h [33].

It is apparent that regulation of collagen formation in cultured cells is different from regulation of collagen formation *in vivo*. The evidence presented here suggests that care must be taken when extrapolating primary culture data to explain phenomena *in vivo*. Study of collagen production *in vitro* is, however, a powerful tool for the investigation of the cell's ability to adapt to external conditions and to discover potential regulation points.

A preliminary report of this work was presented at the Annual Meeting of the American Society of Biological Chemists in San Francisco, June 1983. This work is submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy (S. R. Q.) from Rutgers University and UMDNJ–Rutgers Medical School. We gratefully acknowledge the gifts of the plasmid pA2 containing a cDNA for β-actin from Dr. Marc Kirschner, Department of Biochemistry and Biophysics,

University of California Medical School, San Francisco, and the plasmids pGV1265 and pYN535 containing cDNAs for the chick α1(I) and α2(I) chains of collagen from Dr. Bjorn R. Olsen, UMDNJ–Rutgers Medical School. We express our appreciation to Mrs. Moira Schneider for her invaluable assistance with typing this manuscript. This work was supported in part by N.I.H. grants AM 31839 and HL 07467.

REFERENCES

1. Bornstein, P. & Sage, H. (1980) Annu. Rev. Biochem. **49**, 957–1003
2. Fessler, J. H. & Fessler, L. I. (1978) Annu. Rev. Biochem. **47**, 129–162
3. Kivirikko, K. I. & Myllyla, R. (1984) in Extracellular Matrix Biochemistry (Piez, K. A. & Reddi, A. U., eds.), pp. 83–118, Elsevier, New York
4. Darnell, J. E., Jr. (1982) Nature (London) **297**, 365–371
5. Dehm, P. & Prockop, D. J. (1971) Biochim. Biophys. Acta **240**, 358–369
6. Kao, W. W.-Y., Berg, R. A. & Prockop, D. J. (1977) J. Biol. Chem. **252**, 8391–8397
7. Neblock, D. S. & Berg, R. A. (1984) Arch. Biochem. Biophys. **233**, 338–344
8. Peterkofsky, B. & Diegelmann, R. (1971) Biochemistry **10**, 988–994
9. Breul, S. D., Bradley, K. H., Hance, A. J., Schafer, M. P., Berg, R. A. & Crystal, R. G. (1980) J. Biol. Chem. **255**, 5250–5260
10. Sykes, B., Puddle, B., Francis, M. & Smith, R. (1976) Biochem. Biophys. Res. Commun. **72**, 1472–1480
11. Tolstoshev, P., Berg, R. A., Rennard, S. I., Bradley, K. H., Trapnell, B. C. & Crystal, R. G. (1981) J. Biol. Chem. **256**, 3135–3140
12. Strohman, R. C., Moos, P. S., Micou-Eastwood, J., Spector, D., Przybyla, A. & Paterson, B. (1977) Cell **10**, 265–275
13. Merchant, D. J., Kahn, R. H. & Murphy, W. H. (1964) Handbook of Cell and Organ Culture, pp. 165–166
14. Burton, K. (1955) Biochem. J. **62**, 315–323
15. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) Cell **20**, 95–105
16. Ish-Horowicz, D. & Burke, J. F. (1981) Nucleic Acids Res. **9**, 2989–2998
17. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1978) J. Mol. Biol. **113**, 237–251
18. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
19. McMaster, G. K. & Carmichael, G. G. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 4835–4838
20. Murad, S., Grove, D., Lindberg, K. A., Reynolds, G., Sivarajah, A. & Pinnell, S. R. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2879–2882
21. Lyons, B. L. & Schwarz, R. I. (1984) Nucleic Acids Res. **12**, 2569–2579
22. Schwarz, R. I. & Bissell, M. J. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 4453–4457
23. Schwarz, R. I., Farson, D. A. & Bissell, M. J. (1979) In Vitro **15**, 941–948
24. Folkman, J. & Moscona, A. (1978) Nature (London) **273**, 345–349
25. Ben-Ze'ev, A., Farmer, S. R. & Penman, S. (1980) Cell **21**, 365–372
26. Benecke, B.-J., Ben-Ze'ev, A. & Penman, S. (1978) Cell **14**, 931–939
27. Farmer, S. R., Ben-Ze'ev, A., Benecke, B.-J. & Penman, S. (1978) Cell **15**, 627–637
28. Tajima, S. & Pinnell, S. R. (1982) Biochem. Biophys. Res. Commun. **106**, 632–637
29. Vuust, J. (1975) Eur. J. Biochem. **60**, 41–50
30. Vuust, J., Sobel, M. E. & Martin, G. R. (1983) Collagen Relat. Res. **3**, 70
31. de Wet, W. J., Chu, M.-L. & Prockop, D. J. (1983) J. Biol. Chem. **258**, 14385–14389
32. Duchene, M., Sobel, M. E. & Muller, P. K. (1982) Exp. Cell Res. **142**, 317–324
33. Merlino, G. T., McKeon, C., de Crombrugghe, B. & Pastan, I. (1983) J. Biol Chem. **258**, 10041–10048

Received 4 February 1986/28 March 1986; accepted 16 June 1986