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Procollagen mRNA metabolism during the fibroblast cell cycle and its synthesis in transformed cells

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ABSTRACT

Procollagen mRNA was isolated from mouse embryos and used for the synthesis of a highly labelled cDNA probe complementary to collagen mRNA. This probe was used for the investigation of procollagen mRNA metabolism during the cell cycle of 3T6 mouse embryo fibroblasts in culture. Titration hybridization experiments revealed that procollagen mRNA was present throughout the cell cycle following stimulation of confluent monolayers. Procollagen mRNA levels of sparse cultures appeared similar to those of unstimulated monolayers. The fluctuating levels of collagen synthesis during the cell cycle can be ascribed to changes in the amount of collagen mRNA present. In mouse sarcoma virus transformed 3T3 cells only 20-30% of the amount of procollagen mRNA in 3T3 cells is present indicating that the decline in collagen synthesis is due to mRNA availability.

INTRODUCTION

It has been shown that during the cell cycle wide fluctuations in cellular specific mRNA content may occur. Thus Stein et al. (1) have shown that histone mRNA in Hela cells was present in polyribosomes of S but not G_1 phases of the cell cycle. Further work by the same authors (2, 3, 4, 5) suggested that regulation resided at the transcriptional level. In a mouse embryo fibroblast cell line (3T6) we have found evidence for both major transcriptional and minor post-transcriptional control with respect to histone mRNA (6). It was of interest to compare changes in content of a specific mRNA coding for a differentiated cell product, collagen, in the same cell line.

We have shown that collagen synthesis in fibroblasts decreases shortly after stimulation of confluent monolayers to proliferate (7). Collagen biosynthesis could be controlled at various stages. The messenger for the large structural collagen molecule is synthesized in the form of procollagen mRNA, which upon translation yields procollagen which in turn, is subjected to several specific post-translational modifications within the cell as well as in the extracellular environment (8). Intracellular modifications such as

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hydroxylation, glucosylation, etc., already commence whilst the molecule is being translated on the polyribosomes, followed by chain association, disulphide bridge and triple helix formation and secretion to the extracellular environment. In the extracellular environment extension peptides at both the amino and carboxyl termini are excised, followed by aggregation into fibrils and by crosslinking (9). Collagen biosynthesis could therefore be regulated at transcriptional, post-transcriptional and translational levels.

A study of procollagen mRNA content at different stages of the fibroblast cell cycle would therefore provide an interesting system to study the molecular processes controlling gene expression during the fibroblast cell cycle.

MATERIALS AND METHODS

Materials

AMV reverse transcriptase was supplied by Dr J.W. Beard (Life Sciences Inc., Florida, USA); 3T6 and 3T3 cells were from Flow Laboratories; all radioactive chemicals from the Radiochemical Centre (Amersham); oligo d(T) 12-18 and oligo d(T) cellulose from Collaborative Research; actinomycin from P-L Biochemicals; dATP, dGTP and dTTP from Boehringer Mannheim and collagenase CLSPA from Worthington. MSV 3T3 cells were a gift from Dr E.L. Wilson (Clinical Science and Immunology, University of Cape Town). Mice were strain ICR/HA supplied by the National Cancer Association Laboratory in Johannesburg. Foetal calf serum (FCS) and HANKS minimal essential medium were obtained from Gibco Biocult.

Isolation and characterization of procollagen mRNA

Three week old mouse embryos were harvested, the gut removed, and polysomes prepared as previously described (6, 10). Polysomal RNA was fractionated on 15-30% sucrose gradients and the 26-30S RNA purified by two successive sucrose density gradient centrifugations. Poly (A) containing RNA was obtained by oligo d(T) cellulose chromatography and desalted on a Sephadex G-50 column. The 26-30S poly $(A)^{+}$RNA was translated in a wheat germ cell free extract as described by Marcu and Duddock (11) and modified by Haralson et al., (12) using (^{3}H)-proline as the labelled precursor. The translation products were analysed on Whatman CM-52 columns both before and after collagenase digestion, as described by Narayanan et al., (13). Samples were heated to 43°C, applied to a CM-52 column (0,9 x 9 cm) maintained at 43°C and eluted with a linear gradient of 0-200 mM NaCl in 40 mM sodium acetate pH 4,8, 4 M urea at 43°C. 2 ml fractions were collected and 100 µl

aliquots counted in 0,5% PPO, 10% BBS-3 (Beckman) in toluene. Mouse skin collagen was isolated from new born mice as described by Piez (14) and used as carrier.

Synthesis of cDNA

DNA complementary to procollagen mRNA was synthesized using AMV reverse transcriptase as previously described for histone mRNA (15), and analysed on 15-30% alkaline sucrose gradients.

Hybridization reactions

RNA:cDNA hybridizations were done as described by Harrison et al., (16) in 2 or 5 µl volumes. Hybrids were analysed by S_1 nuclease digestion (17) and computer plot using the program of least squares analysis of Pearson et al., (18).

Cell cultures and RNA extraction

Cells were grown to confluency in Hanks minimal essential medium containing 0,5% FCS and stimulated to proliferate by the addition of fresh medium containing 10% FCS. Nuclei and cytoplasm were prepared, extracted with phenol/chloroform and the RNA purified with DNase as previously described (19). Autoclaved or diethylpyrocarbonate treated glassware and solutions were used. In sparse cultures the cells were harvested before reaching confluency. The S phase corresponds to the period between 12 and 26 hours after stimulation, early G_1 and late G_1 to 1 hour and 8 hours respectively after stimulation (6).

RESULTS

Isolation and characterization of procollagen mRNA

Procollagen mRNA was isolated from those polyribosomes which can be pelleted by low speed centrifugation. Successive sucrose density gradient centrifugation of the 26-30S polyribosomal RNA showed that this RNA preparation was homogenous. Translation of the 26-30S poly(A) containing RNA in wheat germ cell free extract revealed that this RNA preparation stimulated the incorporation of (^{3}H)-proline into TCA precipitable material in a concentration dependent manner (Figure 1).

In order to analyse the translation products, one half of the incubate was digested with 10 µg/ml collagenase for 2 hours (at 25°C in order to reduce non specific proteolytic activity (12)). Analysis of these translation products on CM-cellulose columns indicated that the major translation product is collagen (83%), and that a minor fraction was not attributable to collagen. Digestion with collagenase confirmed that the radioactivity was

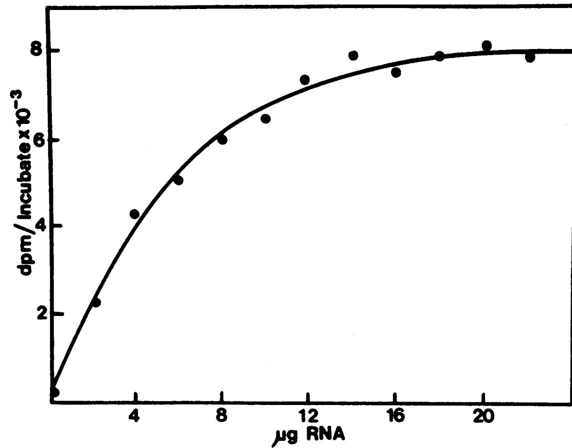

Figure 1 Translation of 26-30S RNA in a wheat germ cell free extract. Increasing amounts of RNA were added to a constant amount of cell free extract and incorporation of (^{3}H)-proline into TCA precipitable material was determined.

incorporated into collagen (Figure 2).

Synthesis of cDNA

The procollagen mRNA served as an excellent template for reverse trans-

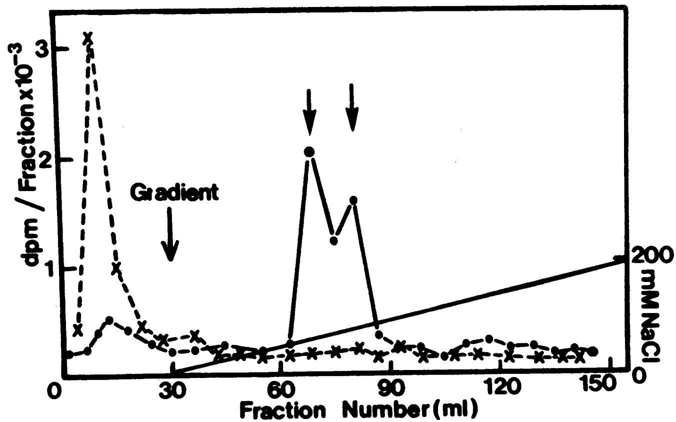

Figure 2 Procollagen mRNA was translated in a wheat germ cell free extract, and the translation products analysed on Whatman CM-52 cellulose. Samples were analysed either before (● - ●) or after digestion with collagenase (x - x). The arrows indicate the position of elution of carrier mouse collagen.

criptase and gave a yield of 10-15% of the input RNA. Analysis of this cDNA on 15-30% alkaline sucrose gradients revealed a cDNA with sedimentation coefficient of 7S; it's specific activity was found to be 4 x 10^7 dpm/µg DNA.

Hybridization and Characterization of procollagen cDNA

The kinetics of hybridization (Figure 3)shows that the reaction proceeded with a $Rot_{\frac{1}{2}}$ of 0,038 moles.sec/litre. On titration of this cDNA against increasing amounts of collagen mRNA, the cDNA became saturated at a RNA:cDNA ratio of approximately 20:1. When the cDNA was titrated against mouse liver or red blood cell RNA, a maximum of 10% of the cDNA became hybridized, whereas it hybridized to 70% with procollagen mRNA (Figure 4).

Using a globin cDNA:mRNA system as a kinetic standard, the complexity of the procollagen mRNA can be calculated. Mouse globin mRNA with a total sequence complexity of 1 200 nucleotides hybridized under similar conditions with a $Rot_{\frac{1}{2}}$ of 0,006 moles.sec/litre (19,20). These data were used to calculate the sequence complexity of procollagen mRNA, and a total sequence complexity of 11 500 nucleotides was obtained.

Assuming the nucleotide length of 4 500 for each of the procollagen

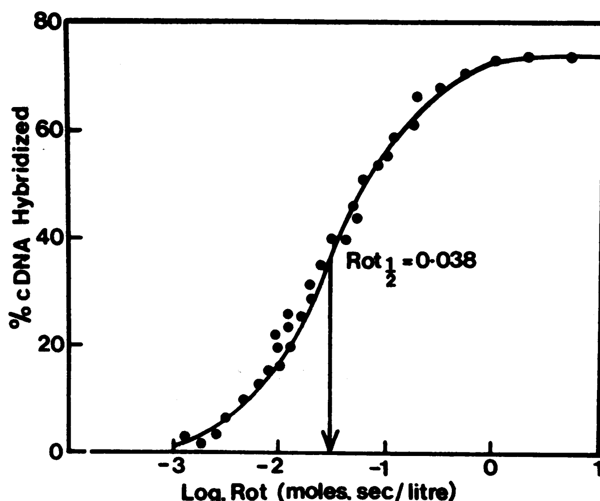

Figure 3 Kinetics of hybridization of (^{3}H)-cDNA to procollagen mRNA. Hybridization reaction mixtures contained 0,1 ng of cDNA in 5 µl of hybridization buffer. The mRNA concentration varied between 0,5 - 50 µg/ml and the time of incubation between 0,5 and 36 hours.

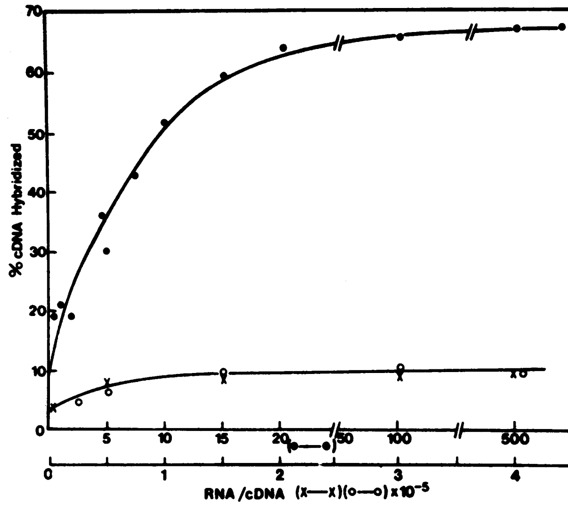

Figure 4 Titration of (^{3}H)-cDNA to procollagen mRNA. Reaction mixtures containing 0,1 ng cDNA were incubated to a Cot of 0,4 moles.sec/litre with increasing concentrations of RNA. (● - ●) collagen mRNA; (O - O) mouse liver RNA; (x - x) mouse red blood cell RNA.

mRNA's, these data suggest that 2-3 different collagen messengers are present. Although at least 5 different collagen α chains exist (21) not all types may be present in the embryo. Alternatively, it is possible that 2 or more of the procollagen mRNA's have identical or nearly identical sequences at their 3'OH termini, in which circumstances this probe would be unable to distinguish between the individual procollagen messengers. This is feasible since the cDNA probe is only 620 nucleotides long as calculated from the sedimentation coefficient, using the equation of Studier (26). This corresponds to a length of only $^1/_7$ of the mRNA molecule.

Procollagen mRNA determination during the cell cycle

Nuclear and cytoplasmic RNAs were extracted from cells at various time intervals after stimulation to proliferate as previously described (6). Procollagen cDNA was titrated against increasing amounts of 3T6 nuclear and cytoplasmic RNA to a final RNA:cDNA ratio of up to 10^5:1 (Figure 5).

From these data, the amount of collagen mRNA in each cellular fraction can be calculated, as shown in Figure 6. These results show that S phase nuclear and cytoplasmic RNA contain less collagen mRNA than RNA from log phase non-confluent cells or unstimulated monolayers. The nuclear as well

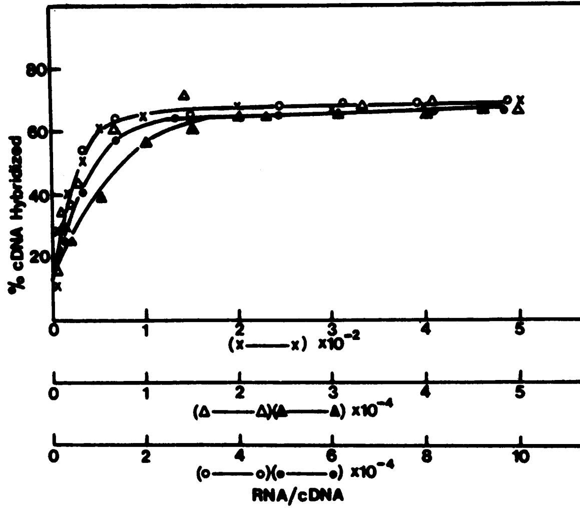

Figure 5 Titration of (^{3}H)-procollagen cDNA to 3T6 RNA isolated from various stages of the cell cycle. Hybridizations were done as described in the legend to Figure 4. (x - x) procollagen mRNA; (● - ●) S phase nuclear RNA; (O - O) S phase cytoplasmic RNA; (▲ - ▲) G_1 phase cytoplasmic RNA. (Δ - Δ) G_1 phase nuclear RNA.

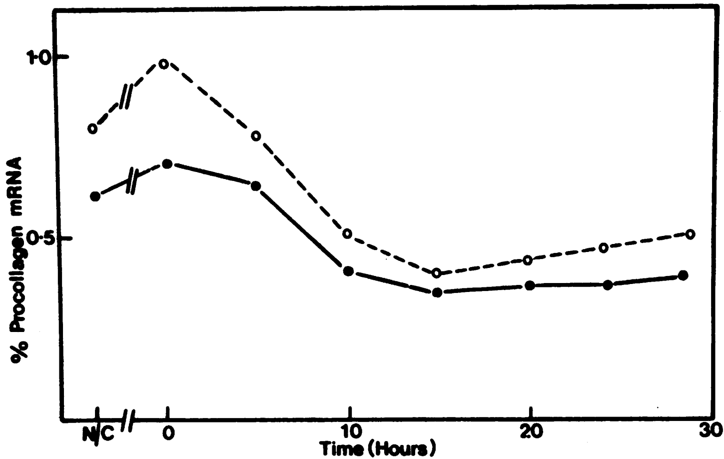

Figure 6 The distribution of procollagen mRNA in 3T6 cell nuclei and cytoplasm at various stages after stimulation of confluent monolayers to proliferate. The percentages are expressed relative to nuclear and cytoplasmic RNA respectively. N/C indicates RNA isolated from non-confluent cells. (O - O) cytoplasmic procollagen mRNA; (● - ●) nuclear procollagen mRNA

as cytoplasmic appearance of procollagen mRNA follow closely the pattern of collagen synthesis in these cells (7). Collagen synthesis therefore appears to be controlled primarily by the level of procollagen mRNA in the cytoplasm which could be the result of control at either the transcriptional and/or the post transcriptional level.

The amount of procollagen mRNA in 3T3 cells was found to be approximately 51% of that present in 3T6 cells (Table 1) which may be related to the observation that 3T3 cells have a lower collagen content than 3T6 cells. Titration of procollagen cDNA against MSV 3T3 nuclear and cytoplasmic RNA showed that these cells contain 20-30% of the procollagen mRNA content present in 3T3 cells.

Table 1 shows the distribution of procollagen mRNA in the nucleus and cytoplasm of the three cell types. In MSV 3T3 cells procollagen mRNA sequences comprise approximately 0.1% of the nuclear as well as the cytoplasmic RNA. These results indicate that the decreased level of procollagen protein synthesis upon viral transformation may be due to decreased levels of procollagen mRNA in the cytoplasm and not due to competition of viral mRNA for the protein synthetic apparatus.

DISCUSSION

In probing for specific mRNA sequences by in vitro translation in a cell free extract, the disadvantage is that the mRNA may be present in an untranslatable form. In our studies, we therefore used a highly labelled cDNA probe to search for the presence of procollagen mRNA sequences. This cDNA was

Table 1 Distribution of procollagen mRNA in the nucleus and cytoplasm of 3T6, 3T3 and MSV 3T3 cells. G_1 (8 hour) indicates that the cells were harvested 8 hours after stimulation, whilst G_0 cultures were harvested 48 hours after reaching confluency in medium containing 0,5% foetal calf serum.

Cell Type	Phase	NUCLEAR RNA			CYTOPLASMIC RNA		
		μg total RNA	μg pro-collagen mRNA	% pro-collagen mRNA	μg total RNA	μg pro-collagen mRNA	% pro-collagen mRNA
3T6	S	228	0,68	0,30	1030	4,12	0,4
	G_1 (8 hr)	160	0,64	0,40	490	2,45	0,5
	Resting	200	1,40	0,70	700	7,00	1,0
3T3	Resting	150	0,54	0,36	480	2,4	0,5
MSV 3T3	Confluent	300	0,21-0,30	0,07-0,10	1210	1,09-1,45	0,09-0,12

prepared from 26-30S polyribosomal poly (A) containing RNA which has previosly been shown to have collagen synthetic activity (22, 23). The specificity of this cDNA probe was demonstrated by its failure to hybridize significantly to mouse liver or red blood cell RNA. On titration of the cDNA against collagen mRNA, the cDNA became saturated at a RNA:cDNA ratio of approximately 20:1, which could be due to the short length (620 nucleotides) of the cDNA molecule. However, such a short cDNA probe was also used by Frischauf et al., (24) for the detection of procollagen genes. Since this cDNA probe was to be used to determine relative procollagen mRNA concentrations, it was considered to be adequate for this purpose.

That procollagen gene expression is controlled at the transcriptional level in chicken embryo fibroblasts and in mouse sarcoma virus transformed chicken embryo fibroblasts, has been reported by Rowe et al., (23) using such a cDNA probe, and by Howard et al., (25) using procollagen cDNA restriction fragments. Whether a minor post transcriptional mechanism such as that described for the histone gene (6), is also operative here, in conjunction with a major transcriptional control mechanism, is not certain. The finding that procollagen mRNA is present throughout the cell cycle agrees well with previous studies on the synthesis of collagen throughout the cell cycle (7, 27).

These studies do not rule out the possibility that procollagen mRNA is being synthesized at the same rate throughout the cell cycle, but that some or more procollagen mRNA is degraded immediately after transcription in S phase cells. This uncertainty could be resolved by the in vitro transcription of chromatin isolated from G_1, S, and G_2 phases of the cell cycle under conditions in which the RNase activity or rate of degradation of procollagen mRNA has been predetermined.

Cell contact recognized by cell surface receptors could affect the synthesis of proteins involved in cell attachment and possibly cell recognition (28). Sparse (nonconfluent) cultures of logarithmically growing 3T6 cells which would have a higher proportion of cells in late G_1 and S phases than resting confluent cells, have procollagen mRNA levels which are similar to these rather than to the partially synchronized cells in late G_1 and S phase (Fig.6). This result suggests that cell contact in the latter may depress collagen gene expression.

These results, together with our previous finding that histone mRNA is synthesized predominantly during the S phase of the cell cycle in these fibroblasts (6), show that the temporal appearance of different messengers

is subject to specific control during the mouse fibroblast cell cycle.

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