Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation rates of procollagen mRNA

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Received 2 September 1983; Revised and Accepted 6 February 1984

ABSTRACT

Procollagen $\alpha 2$ (I) mRNA can be induced $\approx$ 6-fold in primary avian tendon (PAT) cells on addition of ascorbate to the culture medium. Previously, we have shown that the induction is linear after a 12 h lag and requires a total of 60–72 h to achieve maximum levels. We have now investigated in more detail the changes that have occurred in the metabolism of procollagen mRNA in fully induced cells to account for the observed induction. Ascorbate was found to triple the rate of procollagen gene transcription. In addition, there was a stabilization of the mRNA causing the half-life to increase from 10.5 h to 20 h. The increased stability of the procollagen mRNA, however, did not correlate with its ability to bind to oligo (dT)-cellulose. Since a 3-fold change in transcription rates and a 2-fold increase in half-life would account for the 6-fold overall increase in procollagen mRNA levels, we conclude that these are the primary alterations caused by ascorbate addition that give rise to the specific increase in procollagen mRNA.

INTRODUCTION

In eucaryotic cells the control of specific mRNA levels has an important (1), although not necessarily rate-limiting (2–4), role in the regulation of protein production. Many steps in RNA metabolism could play an active part in regulating the final pool size of a specific mRNA within the cytoplasm. Even under a narrowly defined set of conditions, it can be relatively difficult to obtain a clear indication of the control processes that account for this quantitative regulation.

Primary avian tendon (PAT) cells are an ideal system for this type of study. Upon ascorbate addition to the medium the level of procollagen mRNA in the cell increases $\approx$ 6-fold (30). Previous analysis of the kinetics of the process reveal a 12 h lag followed by a 48–60 h linear increase to maximum levels (2). At full induction, the procollagen mRNA pool size is sufficient for half of the total protein synthesis of the cell to be devoted to procollagen (2,5). Therefore, both the level of procollagen mRNA within the cell and the relative change after ascorbate addition are sufficiently large

to allow one to determine the steps which were altered. In addition, the ascorbate effect is highly specific; there is no change in the growth rate of the cell or the rate of noncollagen protein synthesis (5,6).

In this paper we have analyzed for differences in transcription and degradation rates for procollagen mRNA in PAT cells fully induced with ascorbate and in uninduced PAT controls. The observed increase in procollagen mRNA levels in induced PAT cells can be attributed to a combined change in both transcription and degradation rates.

METHODS

Cell culture

PAT cells were isolated from 16-day chicken embryos by a modification (5) of the Dehm and Prockop (7) procedure and were grown in F12 medium (8) with 0.2% fetal calf serum in 25-cm² flasks as described previously (6,9). Fully induced PAT cells were achieved by adding ascorbate (50 µg/ml) to the medium for more than 60 h prior to the beginning of the experiment (2).

Transcription Rates

PAT cells were labeled with [5-³H]uridine (Amersham) 250 µCi/2 ml medium per flask. At various times up to 65 minutes 2 flasks were removed from the incubator, RNA was extracted and incorporation of the label into procollagen mRNA was determined as described below.

Pulse-Chase Experiments

PAT cells were labeled with [³H]uridine (Amersham) 50 µCi/10 ml medium per flask with 48 h (with the medium changed after 24 h). The cells were then washed with medium (time 0), and reincubated with medium containing 5 mM cold uridine and 2 mM cold cytidine (27). At various times during the chase RNA was extracted from 2 sets of 2 flasks and the level of labeled uridine remaining in procollagen mRNA was determined as described below.

RNA Extraction

Cells were lysed with 0.5% sodium dodecyl sulfate (SDS) in buffer (0.05 M sodium acetate, 0.01 M Na₂ EDTA, pH 5.1) and phenol/chloroform extracted as previously described (2,10).

Procollagen mRNA assay

Cells were labeled with [³H]uridine and RNA was extracted at various times as described above or in the figure legends. The total RNA was hybridized to a filter (1.5 x 1.5 cm) with 1 µg (2 µl) of the plasmid pCg45 (11) spotted on each of the four corners (12). Before spotting the plasmid DNA had been nicked and denatured by boiling in 0.1 M NaOH for 3 min and then

partly neutralized by making 1 M in ammonium acetate. After spotting with plasmid DNA the filters were baked in a vacuum oven (2 h, 80°C), prehybridized (13) in 1 ml (50% formamide, 5 x SSC [0.15 M NaCl/0.015 M trisodium citrate], 0.05 M sodium phosphate, 125 µg/ml yeast RNA, 125 µg/ml poly A and 0.02% (bovine serum albumin, Ficoll, and polyvinylpyrrolidone) for 4–8 h at 42°C in a siliconized scintillation vial. The hybridization was carried out (12,14) in 1 ml (70% formamide, 0.1 M Pipes [piperazine-N,N'-bis (2-ethanesulfonic acid)] pH 6.4; 0.4 M NaCl, 125 µg/ml yeast RNA and 125 µg/ml poly A) for 48 h at 42°C. As a control for non-specific binding, a filter containing *E. coli* DNA, treated identically to filters containing the plasmid, was added to each vial and simultaneously hybridized.

At the end of the hybridization two washing procedures were used to reduce non-specific binding. In the procollagen mRNA half-life (pulse-chase) experiments the level of procollagen mRNA was sufficiently high that the washing procedures described by Thomas (13) could be used. In measuring transcription rates, the more stringent washing conditions of McKnight and Palmiter (15) had to be employed in order to detect the small signal from the high background levels. Basically, this procedure employed RNase to remove [³H]RNA which was not protected in a RNA/DNA hybrid. The [³H]RNA remaining bound to the filters was released with 0.04 M NaOH (0.75 ml), neutralized with 0.1 N acetic acid (0.3 ml), and then counted in a scintillation counter with 5 ml Atomlite (New England Nuclear).

Binding to Oligo (dT)-Cellulose

PAT cells from 30 flasks fully induced with ascorbate and 30 flasks from scorbutic controls were labeled for 2 h with [³H]uridine (15 µCi/2 ml). The RNA was extracted from both sets and the specific activity of the RNA was determined.

The chromatography procedure was a slight modification of the Aviv and Leder procedure (16). Approximately 0.5 mg of total RNA was loaded on a column (0.5 cm in diameter) containing 0.25 g (dry weight) of oligo (dT)-cellulose (Collaborative Research, Type 2) in binding buffer (10 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS). Twenty ml of flow-through was collected. The column was then washed with 10 ml of an intermediate salt buffer (10 mM Tris-Cl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% SDS). The relative levels of procollagen mRNA and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA in each fraction was determined by using appropriate nick-translated cDNA probes (pCg-45 [11], p13 [17]) and hybridizing to RNA from various fractions by methods already described (2).

Figure 1. Incorporation of [^3H]uridine into procollagen ($\alpha 2$) mRNA in cells fully induced with ascorbate (●) and uninduced (▲) PAT cells as detected by hybridization of 30 μg of total RNA to a specific procollagen cDNA clone (pCg45). The labeling of the cells, the RNA extraction and the hybridization conditions are described in the Methods section. In the ascorbate induced case, the counts hybridized were corrected for the lower specific activity of the RNA obtained from these cells (see text). For each time point the ratio of specific activities (cpm/ μg) of total RNA (uninduced/induced, Fig. 2) was used to adjust the actual number of counts hybridized. The data (Figs. 2, 3, 4) are all in agreement that at a steady state, the specific activity of the RNA produced in uninduced cells is $\sim 1.5\text{X}$ that from induced cells. The slopes of the lines were determined by using linear regression analysis for the last six time points. Values below 1 cpm/ μg were considered below the accurately detectable threshold.

RESULTS

Transcription Rates

PAT cells grown to a confluent monolayer in the presence of ascorbate accumulate 6-fold more procollagen mRNA than do similar scorbutic cultures. The simplest explanation to account for this change would be an increase in procollagen transcription rates. To test this, we assayed both ascorbate induced (cells grown in the presence of ascorbate for >60 h) and scorbutic PAT cells for the short-term accumulation of [^3H]uridine into RNA that would hybridize to a cDNA clone (pCg45) for procollagen $\alpha 2$ (I) mRNA (11). In both cases, PAT cells incorporated [^3H]-uridine into procollagen mRNA sequences at

Figure 2. The increase in specific activity (cpm/μg) of total RNA with time in ascorbate induced (●) and uninduced (▲) PAT cells. The labeling of the cells and the extraction of the RNA are described in the Methods section. The labeled uridine was added first to the induced flasks and this accounts for the slightly higher than expected specific activities (when compared to uninduced cells) at early time points.

a linear rate (Fig. 1). However the rate for the induced PAT cell was approximately 3 times greater. Therefore, part of the increase in procollagen mRNA accumulation could be accounted for by an increase in transcription of the procollagen gene.

Two points about the data shown in Fig. 1 deserve comment. The linearity observed in the accumulation of procollagen mRNA results from the use of a cDNA clone and the application of stringent washing conditions, using RNAse to digest away RNA not protected in a RNA/DNA hybrid. The excision and presumed degradation of the large number of intervening sequences occurring in the procollagen gene (18) are, therefore, not observed. Total RNA accumulation, in contrast, shows a biphasic pattern of accumulation (Fig. 2) reflecting rapid degradation of much of the RNA in the nucleus (19). In addition, the curve for the accumulation of newly synthesized total RNA appears to reach a higher maximum in uninduced cells. The simplest explanation for this is that the level of unlabeled uridine synthesized intracellularly is greater in PAT cells induced with ascorbate (29). Further evidence for this comes from long-term labeling experiments (48 h) where labeling of the RNA within the cell with [³H]-uridine approaches equilibrium (Fig. 3). The specific activity of the RNA in induced cells was 2/3 that of uninduced cells (see also Fig. 4A), and the data obtained for the rate of synthesis of procollagen mRNA in ascorbate induced PAT cells was corrected to equalize for changes in specific activity (see legend to Fig. 1).

Figure 3. The increase in specific activity (cpm/ μ g) of total RNA with time in ascorbate induced (●) and uninduced (▲) PAT cells. Cells were labeled with 30 μ Ci [3 H]uridine/10 ml medium and the medium was changed at 24 h. The RNA extraction conditions are described in the Methods section.

Degradation Rates

Changes in specific degradation rates have been shown to be an important part of the regulation of mRNA levels in other cell systems (15,20,21,29,31). Therefore, in ascorbate induction of PAT cells part of the increase in procollagen mRNA levels could be due to an increase in the half-life of procollagen mRNA.

To examine the half-life of procollagen mRNA, we used a standard pulse-chase protocol as described in the Methods section. The success of the chase can be seen in Fig. 4A where the specific activity of total RNA was plotted versus time. PAT cells, with and without ascorbate, showed a continuous decay in specific activity of the RNA with a half-life of approximately 32 h. This method, with time points taken from cells grown in individual flasks, is not sensitive enough to detect minor components with short half-lives. The decrease in specific activity with time that we observed was consistent with the expected long half-life of ribosomal RNA (22). When these same RNA samples from the chase period were hybridized to an excess of a cDNA clone for procollagen mRNA (pCg45), we observed that the degradation rate of procollagen α 2 (I) mRNA was dependent on the presence of ascorbate (Fig. 4B). The half-life for procollagen mRNA (from two experiments) in induced cells was twice as long (20 ± 1.4 h) as that in uninduced cells (10.5 ± 0.7 h).

Figure 4. Loss of incorporated [³H]uridine in a pulse-chase experiment during the chase period in both ascorbate induced (●) and scorbutic (▲) PAT cells. The experimental protocol is described in the Methods section. (A) The decrease with time in specific activity (cpm/µg) of total RNA. (B) The decrease with time of the counts incorporated into 20 µg of total RNA hybridizing to a specific procollagen cDNA clone (pCg45).

Binding to Oligo (dT)-Cellulose

At this time there is no clear explanation for the ability of ascorbate to stabilize procollagen mRNA. One simple possibility with support in the literature (23) is that ascorbate induction might affect the length of the poly A tail appended to the mRNA.

This explanation for the increased stability of procollagen mRNA was tested by determining whether procollagen mRNA extracted from ascorbate induced PAT cells would bind better than that from scorbutic cells to an oligo (dT)-cellulose column. As a control, we also determined the ability of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA from ascorbate induced

Table 1

Ability of oligo (dT)-cellulose chromatography to selectively enrich procollagen and GAPDH mRNA.

	Recovery of mRNA in Bound Fraction	
	Minus Ascorbate	Plus Ascorbate
Procollagen	34%	25%
GAPDH	65%	38%

The RNA from ascorbate induced and uninduced PAT cells before and after chromatography on oligo (dT)-cellulose was assayed for levels of procollagen $\alpha 2$ (I) mRNA and GAPDH mRNA using a dot hybridization method (2). In the unfractionated RNA the relative amount of procollagen mRNA increased 5.4 fold (2) while the relative amount of GAPDH mRNA decreased by half after stimulation of the cells with ascorbate. The bound fraction was 2.9% of the total input RNA in the minus ascorbate case and 4.7% in the plus ascorbate case.

and uninduced cells to bind to oligo (dT)-cellulose. The data presented in Table I shows that procollagen mRNA did not bind well to oligo (dT)-cellulose columns (28). But, more importantly, the overall recovery in the bound fraction was greater from scorbutic PAT cells. Interestingly, the GAPDH mRNA from scorbutic cells bound significantly better to the column. Assuming that oligo (dT)-cellulose chromatography accurately reflects poly A tail length, then there does not appear to be a correlation between length of the poly A tail and the stability of procollagen mRNA.

DISCUSSION

The 6-fold increase in procollagen $\alpha 2$ (I) mRNA which results from ascorbate induction can be accounted for by a 3-fold increase in transcription rates and by 2-fold increase in mRNA half-life from 10.5 to 20 h. Ascorbate induction of procollagen mRNA is not, therefore, a simple one step event. Moreover, at this time, we cannot rule out the possibility that other steps in the pathway (such as nuclear processing or secretion from the nucleus) could be making a small contribution to the increase seen in procollagen mRNA.

This observation that mRNA species are controlled by changes in both transcription and degradation rates has been made with several other cell

systems (4,20,21,29). While many reasons could be advanced for multifaceted control of mRNA levels, in the specific case of procollagen mRNA and tendon cells, a rationale can be formulated as follows: In tendon tissue there is a need to control over the course of development the level of procollagen expression from less than 1% to about 50% of total protein synthesis (9). Whether a commensurate change to high mRNA levels could be achieved by simply altering transcription rates is unclear given the limitation that the procollagen gene ($\alpha 2$ [I]) is present as a single copy (24,25). In these cases the fraction of specific RNA production to total transcription is extremely small, around 5×10^{-5} to 5×10^{-6} (1). In the case of the procollagen $\alpha 2$ (I) gene, our results are in line with expectation considering the large size of the gene. Approximately 3×10^{-5} of the total RNA production hybridized to the pCg45 probe. The fact that this probe only recognizes a fifteenth of the genomic sequence (11) means that approximately 5×10^{-4} of the RNA transcribed was procollagen $\alpha 2$ (I) specific in the induced case. Since the procollagen $\alpha 2$ (I) gene is large (18), the transcription levels on a molar basis does not appear to be unusually high. To account for the abundant levels of procollagen mRNA in the cytoplasm one needs an additional mechanism. One hypothesis that has been proposed (31) is that the stability of the mRNA in the cytoplasm is critical because eucaryotic mRNA can vary widely with regard to this parameter. In particular, since the average mRNA half-life has been estimated in other cell culture systems to be considerably less than 3 h (26), the long-half of procollagen mRNA in the ascorbate induced PAT cell has the apparent affect of raising the ultimate steady-state level by approximately an order of magnitude. A reasonable model is that a wide range of control in the production of mRNA from a single copy gene can most readily be brought about by the regulation of both transcription and degradation rates.

This raises the more complex question of how ascorbate can coordinately control two seemingly unrelated steps occurring in different compartments of the cell - mRNA transcription in the nucleus, and mRNA degradation in the cytoplasm. With regard to specific degradation rates, it will be necessary to determine whether stabilization requires a modification in the mRNA molecule itself (such as polyadenylation or capping of the 5' end [1]) or a change in another step in the pathway (for example, whether increased translation rates can stabilize the mRNA). Similarly, do the increases observed in transcription rate suggest that a specific inducer may enter the nucleus (by analogy to certain hormones [15]); alternatively, transcription rates may be controlled by a feedback of intermediates in the pathway. Elucidation of

the mechanisms by which mRNA levels are controlled is critical for a better understanding of how cells regulate specific functions.

ACKNOWLEDGMENT

This work was supported in part by a Public Health Service grant, CA 31767, from the National Institutes of Health, and in part by the Department of Energy under Contract No. DE-AC03-76SF00078.

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