REFERENCES
Avery, L. (1982) *J. Chem. Phys. 76*, 3242-3248.
Bujalowski, W., Graeser, E., McLaughlin, L. W., & Porschke, D. (1986) *Biochemistry* (preceding paper in this issue).
Clore, G. M., Gronenborn, A. M., & McLaughlin, L. W. (1984) *J. Mol. Biol. 174*, 163-173.
Eigen, M., & DeMaeyer, L. (1963) *Tech. Org. Chem. 8*, 895-1054.
Eisinger, J., Feuer, B., & Yamane, T. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 638-644.
Gassen, H. G. (1980) *Prog. Nucleic Acid Res. Mol. Biol. 24*, 57-86.
Geerdes, H. A. M., Van Boom, J. H., & Hilbers, C. W. (1980) *J. Mol. Biol. 142*, 219-230.
Grosjean, H., Söll, D., & Crothers, D. M. (1976) *J. Mol. Biol. 103*, 499-519.
Högenauer, G. (1970) *Eur. J. Biochem. 12*, 527-532.
Labuda, D., & Porschke, D. (1980) *Biochemistry 19*, 3799-3805.
Labuda, D., & Porschke, D. (1983) *J. Mol. Biol. 167*, 205-209.
Labuda, D., Grosjean, H., Striker, G., & Porschke, D. (1982) *Biochim. Biophys. Acta 698*, 230-236.
Labuda, D., Striker, G., & Porschke, D. (1984) *J. Mol. Biol. 174*, 587-604.
Möller, A., Wild, U., Riesner, D., & Gassen, H. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3266-3270.
Porschke, D. (1977) *Mol. Biol., Biochem. Biophys. 24*, 191-218.
Porschke, D., & Labuda, D. (1982) *Biochemistry 21*, 53-56.
Rigler, R., Claesens, F., McLaughlin, L. W., Kaun, E., & Bäumert, H. (1985) *NATO ASI Ser., Ser. A 74*, 0000.
Rose, S. J., III, Lowary, P. T., & Uhlenbeck, O. C. (1983) *J. Mol. Biol. 167*, 103-117.
Schwarz, U., Menzel, H. M., & Gassen, H. G. (1976) *Biochemistry 15*, 2484-2490.
Uhlenbeck, O. C., Baller, T., & Doty, P. (1970) *Nature (London) 225*, 508-512.
Yoon, K., Turner, D. H., & Tinoco, I., Jr. (1975) *J. Mol. Biol. 99*, 507-518.

Half-Life of Synovial Cell Collagenase mRNA Is Modulated by Phorbol Myristate Acetate but Not by *all-trans*-Retinoic Acid or Dexamethasone†

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ABSTRACT: As part of our studies on the mechanisms controlling the synthesis of the neutral proteinase collagenase by rabbit synovial cells, we used a cDNA clone to measure total collagenase mRNA levels and to determine mRNA half-life. Phorbol myristate acetate was used to induce collagenase synthesis while *all-trans*-retinoic acid and dexamethasone were used to inhibit it. Cells stimulated with phorbol myristate acetate contained substantial amounts of collagenase mRNA, but cells treated with *all-trans*-retinoic acid or dexamethasone contained decreased amounts of collagenase mRNA which correlated well with levels of collagenase protein. Studies on mRNA half-life showed that the $t_{1/2}$ for total poly(A+) RNA was about 25 h, while that of collagenase varied from as short as 12 h to as long as 36 h. The half-life was not affected by treatment with *all-trans*-retinoic acid or dexamethasone but was affected by the level of induction of collagenase mRNA: the greater the amount of collagenase mRNA induced, the longer the $t_{1/2}$. We conclude that (1) our data are consistent with the hypothesis that retinoic acid and dexamethasone act at the level of transcription to decrease collagenase production and (2) the increased level of collagenase mRNA resulting from stimulation with phorbol esters is, in part, due to increased stability of the induced collagenase mRNA.

The metalloproteinase collagenase (EC 3.4.24.7) is the only enzyme capable of initiating breakdown of the interstitial collagens, types I, II, and III, at a neutral pH [for reviews, see Harris et al., (1984) and Harris (1985)]. Nowhere is the result of the action of collagenase more apparent than in the connective tissue disease rheumatoid arthritis, where production of collagenase by the synovial cells that line the joint culminates in extensive joint destruction and crippling (Harris, 1985). Previous work from this and other laboratories has, therefore, been concerned with mechanisms regulating the induction and inhibition of collagenase synthesis in synovial cells. Using a model system of monolayer cultures of rabbit synovial fibroblasts, we have shown that collagenase synthesis can be stimulated with a variety of agents, such as poly(ethylene glycol) which causes giant cell formation, phorbol myristate acetate (PMA), crystals of monosodium urate monohydrate, or the monocyte/macrophage product interleukin 1 (Brinckerhoff & Harris, 1978; Brinckerhoff et al., 1979; McMillan et al., 1981; Mizell et al., 1981; Dayer et al., 1986).

Addition of a collagenase inducer results in an increase in the level of collagenase mRNA within the cell by 5 h, measured by cell-free translation and Northern blot hybridization

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with a 650 base pair (bp) cDNA clone for rabbit synovial cell collagenase (Brinckerhoff et al., 1982; Gross et al., 1984). This is accompanied by an increase in collagenase protein, detectable in culture medium by 10 h. Thus, as collagenase synthesis is stimulated, the enzyme is rapidly secreted from the cell (Valle & Bauer, 1979; Nagase et al., 1983). In addition, collagenase production can be antagonized. Treatment of cell cultures with glucocorticoids, such as dexamethasone, or the vitamin A analogue *all-trans*-retinoic acid and the synthetic retinoids (Brinckerhoff et al., 1980, 1981; Brinckerhoff & Harris, 1981) decreases collagenase synthesis. However, the mechanism of suppression is unclear, although regulation at a transcriptional level is suspected (Brinckerhoff & Harris, 1981; Brinckerhoff et al., 1981).

As part of our studies on mechanisms controlling both the induction and inhibition of collagenase synthesis in synovial cells, here we have measured the effect of an inducer of collagenase, PMA, and inhibitors of collagenase, dexamethasone and retinoic acid, on the half-life of collagenase mRNA. Using a collagenase cDNA as a probe, we show that dexamethasone and retinoic acid decrease the amount of collagenase mRNA and protein synthesized by the cells but do not affect the $t_{1/2}$ for collagenase. In contrast, PMA does influence the half-life: induction of greater amounts of collagenase mRNA is associated with a longer $t_{1/2}$.

Materials and Methods

Cell Culture. Monolayer cultures of rabbit synovial fibroblasts were established from synovium taken from the knee joints of 4–6-week-old New Zealand white rabbits (Snelling Rabbitry, Springfield, VT) as previously described (Dayer et al., 1976). Synovium was dissociated into a single-cell suspension by treatment with bacterial collagenase (4 mg/mL; Worthington, Freehold, NJ). Cells were plated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) in 20% fetal calf serium (FCS; Gibco) in 60-mm diameter culture dishes. Cells became confluent after 5–7 days and were passaged into 100-mm diameter culture dishes in 10% FCS. For experiments, cells at passage three or four were split 1:3 into 150-mm diameter culture dishes, grown to confluence, and then washed 3 times with Hank's balanced salt solution (HBSS; Gibco) and placed in serum-free medium containing 0.2% lactalbumin hydrolysate and PMA (10^{-8} M).

Pulse-Labeling and Chase. At the start of the pulse, PMA (Consolidated Midland, Brewster, NY) was added to confluent cultures in serum-free medium at a final concentration of 10^{-8} M. Four hours into the pulse, when collagenase mRNA levels began to increase (Gross et al., 1984), [^{3}H]uridine (40 μCi/mL; 25 μM; Amersham, Chicago, IL) was added to cultures for an additional 18 h. To be certain that the cells were induced, an aliquot of culture medium was then tested for collagenase activity by activating latent collagenase with aminophenylmercuric acetate (Vater et al., 1983) and by visualizing the disappearance of a collagen fibril (Harris et al., 1969). Concomitantly, the chase was begun by washing the cells quickly 3 times in Hank's balanced salt solution and transferring the cells to medium without radioactivity, but with 5 mM uridine and 2.5 mM cytidine; the chase consisted of two parts, a "prechase" to deplete the [^{3}H]UTP pool (see Results) and a "chase" during which the $t_{1/2}$ of mRNA was measured. Depending upon the experiment, PMA was either continued or discontinued during the chase, and *all-trans*-retinoic acid (10^{-6} M) or dexamethasone (10^{-7} M) was added. At selected intervals, cultures were terminated, and RNA was extracted.

Harvesting of RNA and Isolation of Poly(A+) RNA. Culture medium was removed, and the cells were scraped from dishes with a rubber policeman and pelleted. Pellets were resuspended in 2 volumes of 6 M guanidine hydrochloride (Gdn·HCl) and centrifuged through a 1.5-mL cushion of 5.7 M CsCl (Chirgwin et al., 1979). The RNA was phenol extracted and quantitated by the A_{260}/A_{280} optical density and by dot quantitation (Sharp, 1984). The specific activity (dpm per microgram of RNA) was determined. Poly(A+) RNA was obtained by passage over an oligo(dT)–cellulose column (Aviv & Leder, 1972; Brinckerhoff et al., 1982; Gross et al., 1984).

Measurement of [^{3}H]UTP by Fast-Performance Liquid Chromatography. At selected intervals during the chase period, medium from cells in 35-mm 6-well cluster dishes (approximately 2×10^5 cells/well) was removed, and the cells were rinsed 3 times with calcium/magnesium-free phosphate-buffered saline after which 2 mL of ice-cold 0.5 M perchloric acid (PCA) was added to the culture dish and collected (Garrett & Santi, 1979). This was repeated 1 time. Samples were kept at 4 °C and centrifuged to pellet particulate matter. The acid-soluble supernatant was neutralized with 5 M KOH, left on ice for 30 min, centrifuged, and filtered through a 0.22-μM Millex-GS filter unit (Millipore) in preparation for separation by fast-performance liquid chromatography (FPLC). To determine radioactivity in the acid-soluble pool, aliquot portions of the filtered material were counted in a Beckman liquid scintillation counter.

For FPLC, 0.5-mL samples of the PCA-soluble fraction derived from cells were applied to a polyanion S1 HR 5/5 anion-exchange column (Pharmacia, Piscataway, NJ) in 10 mM phosphate buffer, pH 7.0. Nucleotides were eluted with a 10–850 mM $KH_2PO_4/K_2HPO_4 \cdot 3H_2O$, pH 7.0, gradient. The column eluate was monitored at 254 nm, and the positions of UMP, UDP, and UTP in the samples were determined by comparison to the retention time of standards (0.5 mL of 0.5 μM each).

Quantitation of RNA by Dot Blot Hybridizations and Determination of the $t_{1/2}$ for Collagenase. All samples of RNA were assayed in triplicate. To measure the decay of [^{3}H]uridine collagenase mRNA, [^{3}H]mRNA was hybridized to 4 μg of collagenase cDNA that had been denatured by boiling in 2 M NaCl and 0.2 M NH_4OH and then quick-cooled. The cDNA was spotted onto nitrocellulose prewet with 20 × SSC and baked for 2 h at 80 °C. We have determined that 4 μg of plasmid DNA contains at least a 10-fold excess of collagenase sequences relative to the added mRNA. As a control, filters spotted with pBR322 and blank filters were used. Filters were prewashed for 30 min with 0.3 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, and 0.1% sodium dodecyl sulfate (SDS) and prehybridized for 4 h at 42 °C in 50% formamide, 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4) 4 × SSPE, 100 μg/mL poly(A+) RNA, 100 μg/mL yeast tRNA, and 5 × Denhardt's. The mRNA samples (2 μg) were then hybridized in this same solution but with 1 × Denhardt's for 48 h at 42 °C. The filters were washed 3 times for 1 h each in 0.3 M NaCl, 2 mM EDTA, 10 mM Tris, pH 7.5, and 0.1% SDS. Radioactivity was then eluted with 0.04 M NaOH, neutralized with acetic acid, and quantitated by counting in PCS II scintillation fluid in a Beckman liquid scintillation counter.

The half-life for collagenase was determined from the data points from each experiment. The best fitting exponential curve was obtained by using the method of least squares on log-transformed data (Kleinbaum & Kupper, 1978). The coefficient of determination and the slope of the regression

Table I: Time Course of Labeling of RNA with [^{3}H]Uridine[a]

total hours of PMA	hours pulse-labeled	dpm/μg of RNA	dpm/μg of poly(A+)	dpm of hybridizable collagenase mRNA/μg of poly(A+)
12	4	8.1×10^3	4.1×10^3 (2:1)[b]	75 (1.8)%[c]
20	12	1.8×10^5	1.0×10^5 (2:1)	1252 (1.2%)
24	16	3.4×10^5	0.8×10^5 (4:1)	1555 (2.0%)

[a] PMA (10^{-8} M) was added to confluent monolayers of rabbit synovial fibroblasts. Eight hours later, [^{3}H]uridine (40 μCi/mL; 25 μM) was added. After 4, 12, or 16 h of exposure to [^{3}H]uridine, selected cultures were terminated, and the dpm per microgram of total RNA, the dpm per microgram of poly(A+) RNA, and the dpm of hybridizable collagenase RNA per microgram of poly(A+) were determined. [b] Ratio of specific activities of total RNA to poly(A+) RNA. [c] Percentage of poly(A+) RNA.

line were calculated by using standard methods (Rosner, 1982). The half-life was determined from the slope of the regression line (Franklin & Newman, 1973).

To measure total collagenase mRNA, slot blot hybridizations were performed. Samples of poly(A+) RNA at 0.33, 0.67, and 1 μg or at 2 μg from each experimental time point were denatured at 65 °C in 6.15 M formaldehyde and 10 × SSC and were spotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) that had been prewet with 10 × SCC and baked at 80 °C for 2 h in a vacuum oven. The blots were prewet and prehybridized for 6 h at 42 °C. Prehybridization conditions were 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS, and 200 μg/mL denatured salmon sperm DNA. Following prehybridization, probe cDNA was added in excess, and hybridization was allowed to proceed for 36 h at 42 °C. Filters were then washed 3 times for 10 min in 20 × SSC and 0.1% SDS at room temperature and 1 time for 30 min in 1 × SSC and 0.1% SDS at 65 °C. The blots were then autoradiographed. Quantitation was achieved by liquid scintillation counting of the filters.

Preparation of Plasmid DNA and Radiolabeling with ^{32}P. Plasmid DNA containing a cDNA insert for collagenase was prepared by standard methods (Maniatis et al., 1982). The insert was excised from its vector (pBR322) by digestion with *Pst*I (Gross et al., 1984), isolated by electrophoresis through a 1% agarose gel, and radiolabeled with ^{32}P either by the oligo-labeling method of Feinberg and Vogelstein (1983, 1984) or by nick translation (Maniatis et al., 1982).

Fibril Assay for Collagenase. To quantitate collagenase activity in culture medium, latent collagenase was activated with trypsin (Werb et al., 1977; Brinckerhoff & Harris, 1978) and then assayed with fibrils of reconstituted collagen labeled with ^{14}C (Gisslow & McBride, 1975) in a standard fibril assay (Harris et al., 1969). One unit of collagenase degraded 1 μg of collagen per hour per milliliter of culture medium at 37 °C.

Results

Effect of all-trans-Retinoic Acid and Dexamethasone on mRNA Levels for Collagenase. We first determined the effect of *all-trans*-retinoic acid and dexamethasone on the levels of hybridizable collagenase mRNA in rabbit synovial fibroblasts. For these experiments, populations of cells were induced with PMA, and at time zero, the inducer was removed, and cultures were treated with either retinoic acid (10^{-6} M) or dexamethasone (10^{-7} M). At intervals, the amount of mRNA in the cell was measured by hybridization to a cDNA clone for collagenase, and collagenase activity in the culture medium was determined. Figure 1 shows that a 60-h treatment with either retinoic acid or dexamethasone greatly reduced the amount of mRNA hybridizing to the cDNA clone for collagenase and that this decrease was paralleled by a decrease in collagenolytic protein in the culture medium. The data obtained by treatment with the retinoid or the steroid are nearly identical and suggest that, similar to steroids (Baxter & Funder, 1979), retinoids may be acting transcriptionally to decrease collagenase synthesis. However, the possibility of glucocorticoid and retinoid effects on mRNA half-life must also be considered (Guyette et al., 1979; Chomczynski et al., 1986).

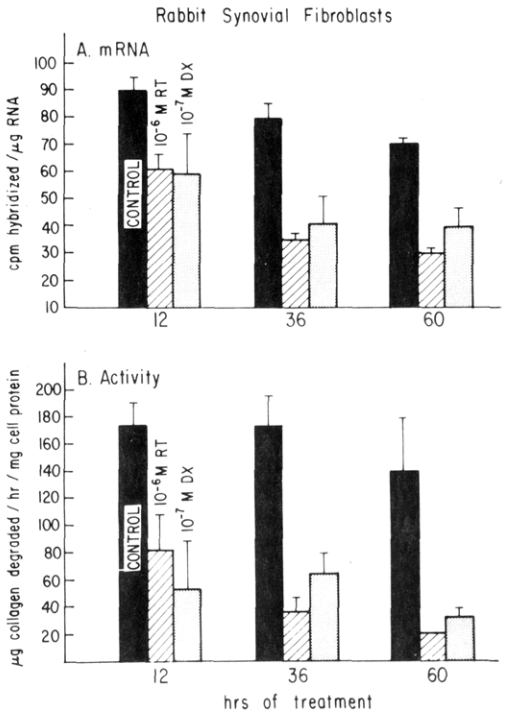

FIGURE 1: Effect of *all-trans*-retinoic acid and dexamethasone on levels of collagenase mRNA and protein. Confluent cultures of rabbit synovial fibroblasts in serum-free medium were stimulated to produce collagenase by 48 h of treatment with PMA (10^{-8} M). PMA was removed from all cultures, and medium was replaced; selected cultures received *all-trans*-retinoic acid (10^{-6} M) or dexamethasone (10^{-7} M). At intervals, cultures were terminated by harvesting medium and RNA, and medium and drugs were replaced on remaining cultures. Collagenase mRNA was measured by dot blot analysis with 1 and 2 μg of RNA spotted onto nitrocellulose filters. The filters were hybridized with a cDNA clone for collagenase radiolabeled by nick translation with ^{32}P. The amount of hybridization was quantified by excising the dots and counting them in PCS liquid scintillation fluid. Collagenase activity in the medium taken from these same cultures was determined in a standard fibril assay with radiolabeled collagen.

Time Course of Labeling of RNA with [^3H]Uridine for Pulse–Chase Experiments. To measure the efficacy of the pulse, we monitored the incorporation of [^{3}H]uridine into whole cell RNA, into poly(A+), and into mRNA specific for collagenase. Cells were treated with PMA for 8 h, a time sufficient to induce transcription of collagenase mRNA (Gross et al., 1984), and then the cells were pulsed with the [^{3}H]-uridine for varying periods of time. Table I shows that after 4 h of labeling, the specific activity of the poly(A+) RNA, measured as dpm per microgram, is half that of total RNA, confirming dogma that mRNA labels preferentially over ri-

Table II: Decay of [^{3}H]Uridine in the Acid-Soluble Pool of Rabbit Synovial Fibroblasts[a]

hours of chase	dpm of [^{3}H]uridine/2 × 10^5 cells	% decrease
0	3.3 × 10^6	
4	2.3 × 10^6	30
12	2.1 × 10^6	36
24	1.2 × 10^6	64
48	0.4 × 10^6	88

[a] Confluent cultures of rabbit synovial fibroblasts in serum-free medium were pulse-labeled for 18 with [^{3}H]uridine and PMA (10^{-8} M). Radioactivity was then removed, and the cells were placed in chase medium. At selected intervals, duplicate cultures were terminated for the amount of radioactivity in the PCA-soluble fraction.

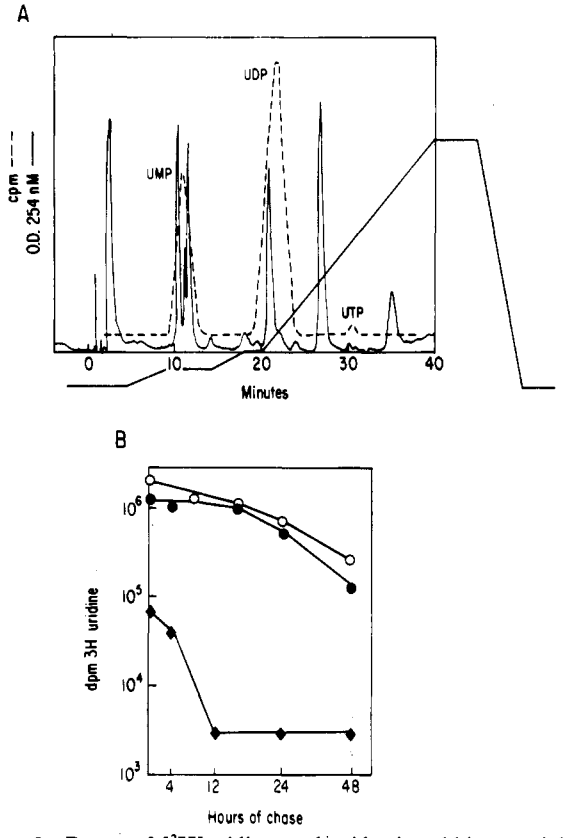

FIGURE 2: Decay of [^{3}H]uridine nucleotides in rabbit synovial fibroblasts. The PCA-soluble fraction, extracted as described under Materials and Methods, was fractionated by FPLC. Five hundred microliter samples were applied to a polyanion S1 HR 5/5 anion-exchange column in 10 mM phosphate buffer. Nucleotides were eluted with a 10–850 mM phosphate gradient, and the amount of radioactivity in each peak was determined. Solid line rising to the right indicates the salt gradient (Figure 2A). (A) FPLC profile of nucleotides at start of the chase. (B) Decay of ^{3}H nucleotides: UMP (●); UDP (○); UTP (♦).

bosomal RNA. After 16 h in [^{3}H]uridine, the pool of ribosomal RNA has become more fully labeled, and the specific activity of poly(A+) RNA is only one-fourth that of total RNA.

Note that in this experiment, after a 12-h exposure to PMA, approximately 2% of the poly(A+) RNA represents collagenase mRNA. Note, too, however, that 16 h of labeling with [^{3}H]uridine is needed in order to achieve a specific activity sufficient to sensitively measure the decay of collagenase mRNA. Results of eight other experiments showed that the amount of collagenase mRNA relative to the total poly(A+) RNA varied from 1% to 12%, depending upon the experiment (data not shown).

Decay of [^{3}H]Uridine in the Perchloric Acid Soluble Pool in Rabbit Synovial Fibroblasts during the Chase Period. To determine the efficacy of the chase, we (1) measured the turnover of total [^{3}H]uridine in the PCA-soluble pool and (2) fractionated the [^{3}H]uridine in the PCA-soluble pool by FPLC. After 24 h of chase, the total [^{3}H]uridine in the soluble pool had decreased by 64% and after 48 h, by 88% (Table II). Similarly, the specific activity of [^{3}H]uridine in total RNA within the cell, measured as cpm per microgram of RNA, declined very slowly, with only a 40% decrease after 60 h (data not shown).

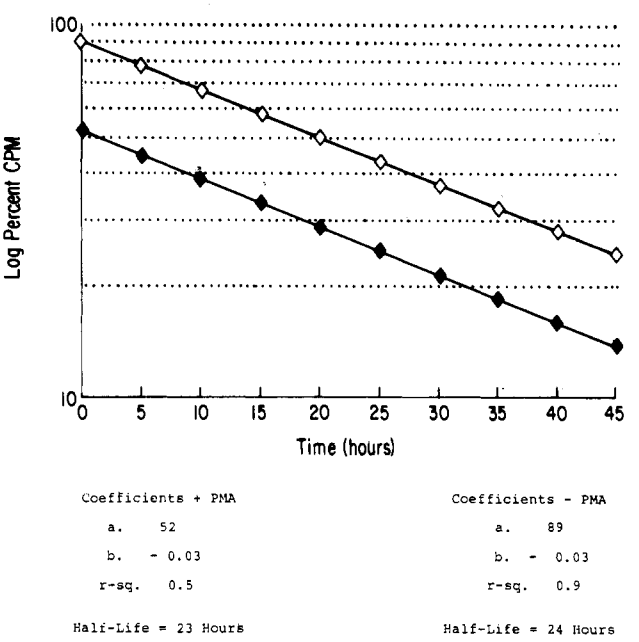

FIGURE 3: Decay of poly(A+) RNA in rabbit synovial fibroblasts. Confluent monolayers of rabbit synovial fibroblasts in 150-mm diameter culture dishes were pulse-labeled in serum-free medium for 18 h with PMA (10^{-8} M). Radioactivity was then removed, and the cells were placed in chase medium for the "prechase" and "chase" (see Materials and Methods). Half of the cultures received PMA, and half did not. At each time pont, dishes were terminated, and the amount of [^{3}H]mRNA at each point was measured in triplicate; (♦) cpm poly(A+) from cells treated with PMA during the chase periods; (◇) cpm poly(A+) from cells without PMA during the chase.

Fractionating the soluble pool by FPLC allowed measurement of the [^{3}H]UTP pool at different times during the chase. The data are presented in Figure 2 and show, in agreement with results of other investigators (Brock & Shapiro, 1983a,b), that (a) the UTP pool comprised only a small fraction of the acid-soluble pool and (b) the radioactivity in the UTP pool decayed at a faster rate than that in either the UMP or the UDP pools. By 12 h, only 6% of the UTP pool contained radiolabeled material. Thus, we calculated the $t_{1/2}$ for the decay of the UTP pool to be about 4–5 h. By incubating the cells in nonradiolabeled medium for 8–10 h as a "prechase" that preceded the start of the actual chase period, the pool of [^{3}H]UTP that could potentially be incorporated into mRNA was depleted. This assured us that incorporation of radiolabeled material would not occur during the chase period.

Decay of Poly(A+) RNA, Decay of Collagenase mRNA, and Effect of all-trans-Retinoic Acid and Dexamethasone. The presence of PMA did not influence the half-life of the total population of mRNAs (Figure 3). Results of eight experiments indicate that the $t_{1/2}$ of the poly(A+) RNA is 25 ± 4 h.

To measure the half-life of collagenase mRNA, we tested whether half-life was affected by the presence of PMA, *all-trans*-retinoic acid, or dexamethasone during the chase. In all experiments, collagenase [^{3}H]mRNA was induced by PMA in the presence of [^{3}H]uridine. For the prechase and chase

periods, the [^{3}H]uridine was removed, and *all-trans*-retinoic acid (10^{-6} M) or dexamethasone (10^{-7} M) was added to cultures, either with PMA (experiment 1) or without it (experiment 2). At intervals during the chase, cultures from each experiment were terminated, and the amount of [^{3}H]mRNA hybridizing to collagenase cDNA spotted onto nitrocellulose filters was measured. As a control for the decrease in total collagenase mRNA in cells treated with retinoic acid or dexamethasone, poly(A+) RNA from experiments 1 and 2 was spotted onto nitrocellulose filters and hybridized with ^{32}P-labeled cDNA, as described in Figure 1. RNA from both experiments was tested simultaneously in one hybridization with [^{32}P]cDNA. Results showed that, similar to data shown in Figure 1, 24 h of treatment with retinoic acid or dexamethasone decreased the amount of total collagenase mRNA within the cells by 30–50% (data not shown).

The results also showed, however, that at time zero, the level of total collagenase mRNA (plotted on a log scale) in experiment 1 was nearly twice that of experiment 2 (Figure 4A). Further, total collagenase mRNA levels in experiment 1 remained high during the chase period in the presence of PMA, while those in experiment 2 declined rapidly during a chase without PMA. Reasons for the different levels of collagenase mRNA at time zero are unclear, although possible explanations are considered under Discussion.

Measurements of the decay of [^{3}H]collagenase mRNA revealed that the half-life of [^{3}H]collagenase mRNA was approximately 35 h in the cells in experiment 1 (Figure 4B) while it was only 12 h in the cells in experiment 2 (Figure 4C). In neither case, however, was the $t_{1/2}$ affected by retinoic acid or dexamethasone. Thus, we conclude that neither retinoic acid nor dexamethasone affected the half-life of collagenase mRNA. Half-life, however, was directly correlated with the level of collagenase mRNA induced in the cells: the greater the level of induction, the longer the half-life of the mRNA. Five other experiments gave similar results.

Discussion

In this paper, we show that the half-life of collagenase mRNA is correlated with the amount of collagenase mRNA that is induced in the cells in response to treatment with phorbol myristate acetate. Addition of phorbol esters both induces the synthesis of collagenase mRNA and prolongs its half-life. Higher levels of induction are associated with a longer $t_{1/2}$. We also show that addition of *all-trans*-retinoic acid or dexamethasone to collagenase-producing cultures decreases the amount of collagenase mRNA in the cells without affecting collagenase mRNA half-life.

The mechanism by which glucocorticoids and retinoids inhibit collagenase synthesis is of some interest. Both glucocorticoids (Baxter & Funder, 1979) and, more recently, retinoids (Roberts & Sporn, 1984) have been shown to act at the level of transcription. Our data showing the ability of retinoic acid and dexamethasone to decrease collagenase mRNA levels in the cell are consistent with the hypothesis that these compounds act transcriptionally to decrease collagenase synthesis but do not affect existing mRNA. However, in other systems, such as the effects of hydrocortisone on casein mRNA (Guyette et al., 1979; Chomczynski et al., 1986) or estrogens on ovalbumin mRNA (McKnight & Palmiter, 1979), steroids influenced mRNA half-life, and this was a possibility in our system too.

In studying the $t_{1/2}$ of synovial cell mRNAs, it became apparent that the turnover of the UTP pool and ribosomal RNA in confluent cultures of resting fibroblasts was slow (Tables I and II, Figure 2)(Abelson et al., 1974; Lyons & Schwarz, 1984). It was also apparent that an overnight pulse with [^{3}H]uridine was needed in order to achieve a sufficient specific activity of [^{3}H]collagenase mRNA. Thus, it was necessary to monitor the decay of the [^{3}H]UTP pool in the

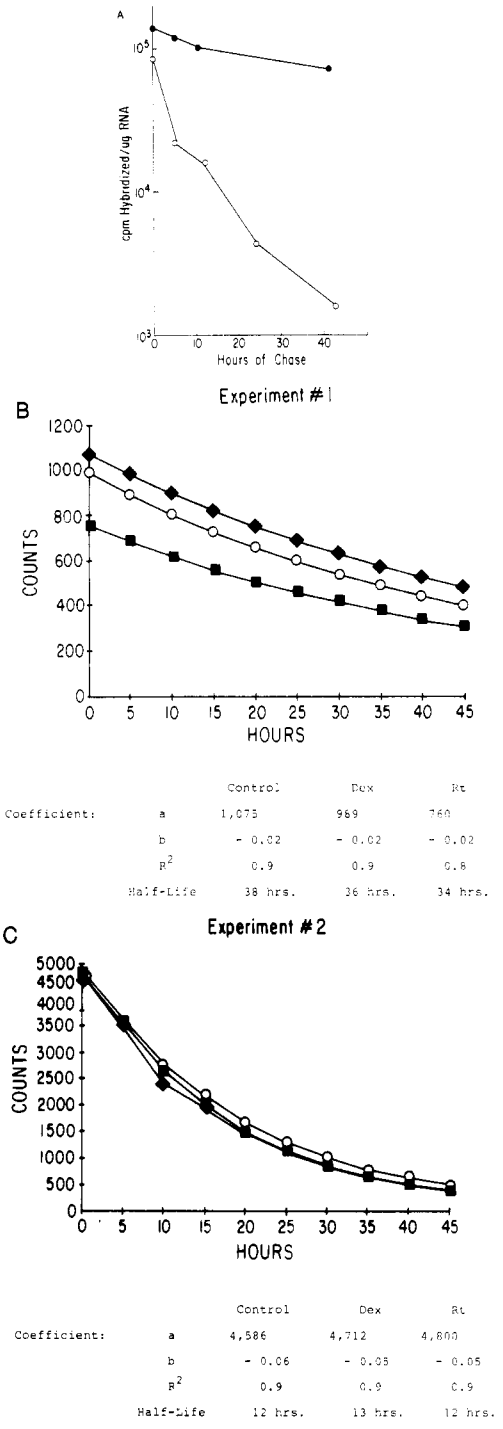

FIGURE 4: Effect of PMA, *all-trans*-retinoic acid, and dexamethasone on the half-life of collagenase mRNA. Confluent cultures of rabbit synovial fibroblasts were pulse-labeled with [^{3}H]uridine and PMA for 18 h. The cells were then placed in chase medium for an 8-h prechase to deplete the pool of [^{3}H]UTP. Following the prechase, the chase period was begun by replacing the medium and adding *all-trans*-retinoic acid (10^{-6} M) or dexamethasone (10^{-7} M) to selected cultures. In experiment 1, PMA was present throughout the pulse, prechase, and chase periods; in experiment 2, PMA was removed after the 18-h pulse. At various times during the chase, cultures were terminated, and the amounts of total collagenase mRNA and [^{3}H]collagenase mRNA were measured in triplicate. (A) Total collagenase mRNA: experiment 1 (●); experiment 2 (○). (B and C) [^{3}H]Uridine collagenase mRNA: control (♦); dexamethasone treated (○); retinoic acid treated (■).

cells to assure that no incorporation of [^{3}H]UTP occurred during the chase. In light of our findings that the $t_{1/2}$ for collagenase could vary from one experiment to another, it was important to know that the chase was effective and that we had successfully controlled for this variable.

It was also important to note that despite the differential effect of PMA on the half-life of collagenase mRNA, the half-life of total poly(A+) RNA in synovial cells was not altered. A similar finding was noted by Krowczynska et al. (1985) in their studies on the $t_{1/2}$ for tubulin and actin mRNAs in leukemia cells treated with dimethyl sulfoxide: even though treatment differentially affected the half-life of tubulin and actin mRNAs, it did not affect the decay curve for total poly(A+) RNA.

The finding that the half-life of synovial cell collagenase mRNA can be directly correlated with the level of mRNA induction also agrees with results of other investigators who have described stabilization of newly transcribed mRNA as one control mechanism for the accumulation of RNA in numerous other systems. These include type 1 collagen mRNA induced in tendon fibroblasts by ascorbate (Lyons & Schwarz, 1984), insulin mRNA induced in insulinoma cells with glucose (Welsh et al., 1985), casein mRNA induced with prolactin in mammary gland organ culture (Guyette et al., 1979; Chomczynski et al., 1986), and malic enzyme mRNA induced by feeding starving ducklings (Goldman et al., 1985). In our work, we have long noted that the amount of collagenase that can be induced in synovial cells varies considerably among cell populations (Brinckerhoff & Harris, 1978; Brinckerhoff et al., 1979, 1982, 1985; McMillan et al., 1981). In this paper, this variability is evident by the difference in the amount of collagenase mRNA present at time zero in two experiments (Figure 4A). Despite the fact that the cultures are established from rabbits of the same age and the fact that the cells used for experiments are at the same passage number and are under the same culture conditions, they are somewhat heterogeneous in that they are derived from an out-bred strain of rabbits. Furthermore, this variability in inducibility holds true whether the cells are stimulated with poly(ethylene glycol), PMA, or crystals of monosodium urate monohydrate. These previous data and the data presented in this study all suggest that the variability in collagenase levels is probably due to factors inherent within the cells that might influence their ability to respond to inductive stimuli.

As has been discussed (Guyette et al., 1979), the level of particular species of mRNA within a cell is governed by numerous mechanisms, including transcription, processing, and degradation of the mRNA. However, more recently it has been suggested that there may be specific intracellular factors, such as a putative repressor protein controlling transcription of interleukin 2 mRNA (Efrat & Kaemper, 1984) or signals mediated by insulin and thyroid hormones (Goldman et al., 1985), that contribute to the regulation of mRNA levels. In addition, many have suggested that the 5′ or 3′ untranslated regions of mRNAs may contain sequences that are important in controlling mRNA stability. For example, Morris et al. (1986) showed that the first 20 nucleotides of the leader were sufficient to couple histone mRNA stability with DNA replication. The authors suggest that structural determinants of mRNA may be important regulators of any gene that is expressed transiently during the cell cycle or in response to a metabolic signal.

PMA is known to induce several proteins in synovial cells, among them plasminogen activator (Brinckerhoff et al., 1981) and proactivator (Vater et al., 1983), a neutral proteinase that is essential for the activation of latent collagenase and that appears to be coregulated with collagenase. Activator is induced by phorbol esters along with collagenase and suppressed by retinoic acid (Brinckerhoff et al., 1986). Plasminogen activator, on the other hand, although similarly induced by phorbol esters, is not suppressed by retinoic acid, suggesting disparate paths of regulation for these proteinases (Brinckerhoff et al., 1981). Recent experiments have resulted in the isolation and characterization of genomic clones for synovial cell collagenase and in cDNA clones for proactivator (Fini et al., 1986; Brinckerhoff et al., 1986). Ultimately, we may isolate and characterize a family of genes that are linked by their ability to be induced by agents such as phorbol esters and interleukin 1 that "turn on" expression of the collagenase gene and by agents such as retinoids and glucocorticoids that "turn off" the collagenase gene.

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Registry No. *all-trans*-Retinoic acid, 302-79-4; dexamethasone, 50-02-2; phorbol myristate acetate, 16561-29-8; collagenase, 9001-12-1.

REFERENCES

Abelson, H. T., Johnson, L. F., Penman, S., & Green, H. (1974) *Cell (Cambridge, Mass.) 1*, 161–165.

Aviv, H., & leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1422.

Baxter, J. D., & Funder, J. W. (1979) *N. Engl. J. Med. 301*, 1149–1161.

Brinckerhoff, C. E., & Harris, E. D., Jr. (1978) *Arthritis Rheum. 21*, 745–753.

Brinckerhoff, C. E., & Harris, E. D., Jr. (1981) *Biochim. Biophys. Acta 677*, 424–432.

Brinckerhoff, C. E., McMillan, R. M., Fahey, J. V., & Harris, E. D., Jr. (1979) *Arthritis Rheum. 22*, 1109–1116.

Brinckerhoff, C. E., McMillan, R. M., Dayer, J.-M., & Harris, E. D., Jr. (1980) *N. Engl. J. Med. 303*, 432–435.

Brinckerhoff, C. E., Nagase, H., Nagel, J. E., & Harris, E. D., Jr. (1981) *J. Am. Acad. Dermatol. 6*, 591–602.

Brinckerhoff, C. E., Gross, R. H., Nagase, H., Sheldon, L. A., Jackson, R. C., & Harris, E. D., Jr. (1982) *Biochemistry 21*, 2674–2679.

Brinckerhoff, C. E., Benoit, M. C., & Culp, W. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1916–1920.

Brinckerhoff, C. E., Fini, M. E., Plucinska, I. M., Ruby, P. L., Borges, K. A., & Karmilowicz, M. J. (1986) *UCLA Symposium on Cartilage and Bone Matrix* (in press).

Brock, M. L., & Shapiro, D. J. (1983a) *J. Biol. Chem. 258*, 5449–5455.

Brock, M. L., & Shapiro, D. J. (1983b) *Cell (Cambridge, Mass.) 34*, 207–214.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chomczynski, P., Gasba, P., & Topper, Y. J. (1986) *Biochem. Biophys. Res. Commun. 134*, 812–818.

Dayer, J.-M., Krane, S. M., Russell, R. G. G., & Robinson, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 945–949.

Dayer, J.-M., Rochemonteix, B. de, Burrus, B., Demezuk, S., & Dinarello, C. A. (1986) *J. Clin. Invest. 77*, 645–648.

Efrat, S., & Kaemper, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2601–2605.

Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6–13.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 137*, 266–267.

Fini, M. E., Gross, R. H., & Brinckerhoff, C. E. (1986) *Arthritis Rheum.* (in press).
Franklin, D. A., & Newman, G. B. (1973) *A Guide to Medical Mathematics*, pp 110–112, Blackwell Scientific Publications, London.
Garrett, C., & Santi, D. V. (1979) *Anal. Biochem. 99*, 268–273.
Gisslow, M. T., & McBride, B. C. (1975) *Anal. Biochem. 68*, 70–78.
Goldman, M. J., Back, D. W., & Goodridge, A. G. (1985) *J. Biol. Chem. 260*, 4404–4408.
Gross, R. H., Sheldon, L. A., Fletcher, C. F., & Brinckerhoff, C. E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1981–1985.
Guyette, W. A., Matusik, R. J., & Rosen, J. M. (1979) *Cell (Cambridge, Mass.) 17*, 1013–1023.
Harris, E. D., Jr. (1985) in *Textbook of Rheumatology* (Kelley, W. N., Harris, E. D., Jr., Ruddy, S., & Sledge, C. B., Eds.) pp 886–914, W. B. Saunders, Philadelphia, PA.
Harris, E. D., Jr., DiBona, D. R., & Krane, S. M. (1969) *J. Clin. Invest. 48*, 2104–2113.
Harris, E. D., Jr., Welgus, H. B., & Krane, S. M. (1984) *Collagen Relat. Res. 4*, 493–512.
Kleinbaum, D., & Kupper, L. (1978) in *Applied Regression Analysis and Other Multivariable Methods*, pp 50–51, Duxbury Press, Boston.
Krowczynska, A., Yenofsky, R., & Brawerman, G. (1985) *J. Mol. Biol. 181*, 231–239.
Lyons, B. L., & Schwarz, R. L. (1984) *Nucleic Acids Res. 12*, 2569–2579.
Maniatis, R., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McKnight, G. S., & Palmiter, R. D. (1979) *J. Biol. Chem. 254*, 9050–9058.
McMillan, R. M., Vater, C. A., Hasselbacher, P., Hahn, J. L., & Harris, E. D., Jr. (1981) *J. Pharm. Pharmacol. 33*, 382–383.
Mizel, S. B., Dayer, J.-M., Krane, S. M., & Mergenhagen, S. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2471–2477.
Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J., & Stein, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 981–985.
Nagase, H., Brinckerhoff, C. E., Vater, C. A., & Harris, E. D., Jr. (1983) *Biochem. J. 214*, 281–288.
Roberts, A. B., & Sporn, M. B. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, DeW. S., Eds.) pp 210–286, Academic Press, New York.
Rosner, B. (1982) in *Fundamentals of Biostatistics*, pp 349–450, Duxbury Press, Boston.
Sharp, Z. D. (1985) *BioTechniques 2*, 194.
Valle, K. J., & Bauer, E. A. (1979) *J. Biol. Chem. 254*, 10115–10122.
Vater, C. A., Nagase, H., & Harris, E. D., Jr. (1983) *J. Biol. Chem. 258*, 9374–9382.
Welsh, M., Neilsen, D. A., MacKrell, A. J., & Steiner, D. F. (1985) *J. Biol. Chem. 260*, 13590–13594.
Werb, Z., Mainardi, C. L., Vater, C. A., & Harris, E. D., Jr. (1977) *N. Engl. J. Med. 296*, 1017–1022.

Treatment of Chinese Hamster Ovary Cells with the Transcriptional Inhibitor Actinomycin D Inhibits Binding of Messenger RNA to Ribosomes†

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ABSTRACT: Inhibitors of RNA synthesis such as actinomycin D, MPB, and cordycepin progressively inhibit the initiation of protein synthesis in intact, nucleated mammalian cells. This inhibition is not dependent on the levels of mRNA, ribosomes, or tRNA. Lysates prepared from CHO cells treated with actinomycin D do not incorporate labeled globin mRNA or ovalbumin mRNA into 80S initiation complexes at the rates of untreated control extract. The ability of the extracts to produce and accumulate 48S preinitiation complexes was assessed using the 60S subunit joining inhibitors edeine and 5′-guanylyl imidodiphosphate. Control extracts were able to accumulate both the 48S preinitiation complexes and the migration-related intermediates in the presence of both inhibitors. However, lysates derived from CHO cells treated with actinomycin D were unable to produce these complexes. This was also true at low temperature, a condition that does not inhibit mRNA binding but prevents migration of the 43S complex along the mRNA. Mixing experiments with extracts from untreated control or AMD-treated CHO cells provided no evidence for a translational inhibitor. Thus, our data are consistent with the hypothesis that treatment of whole cells with actinomycin D inhibits protein synthesis initiation at the level of mRNA binding and not at migration or 60S subunit joining.

A number of investigators have determined that the rate and extent of protein synthesis in nucleated eukaryotic cells is dramatically inhibited by treatment with transcriptional inhibitors such as actinomycin D (AMD),[1] MPB,[1] and cordycepin. The inhibition has been observed in numerous cell types including HeLa cells (Singer & Penman, 1972, 1973),

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[1] Abbreviations: MPB, 2-mercapto-1-(α-4-pyridylethyl)benzimidazole; AMD, actinomycin D; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; eIF, eukaryotic initiation factor; $Gpp(NH_2)p$, 5′-guanylyl imidodiphosphate; pCp, cytidine 5′,3′-bisphosphate; m^7GTP, 7-methylguanosine triphosphate; CHO, Chinese hamster ovary.

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