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Collagen Expression in Embryonic Chicken Chondrocytes Treated with Phorbol Myristate Acetate†

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Growth of embryonic chicken sternal chondrocytes in the presence of phorbol-12-myristate-13-acetate (PMA), a potent tumor promoter, resulted in a dramatic morphological change from spherical floating cells to adherent fibroblastic cells. This morphological change was accompanied by a quantitative switch from synthesis of cartilage-specific type II procollagen to type I procollagen. Type II procollagen mRNA levels decreased 10-fold in PMA-treated cells. Activation of type I collagen genes led to the accumulation of type I procollagen mRNA levels comparable to those of type II mRNA in these cells. However, only type I procollagen mRNA was translated. In addition to gene activation, unprocessed pro α1(I) transcripts present at low levels in control chondrocytes were processed to mature mRNA species. Redifferentiation of PMA-treated chondrocytes was possible if cells were removed from PMA after the morphological change and cessation of type II procollagen synthesis but before detectable amounts of type I procollagen were synthesized. Production of type I collagen thus marks a late phase of chondrocyte "dedifferentiation" from which reversion is no longer possible. Redifferentiated cell populations contained 24-fold more pro α1(II) collagen mRNA than pro α1(I) collagen mRNA, but the rates of procollagen synthesis were comparable. This suggests that the PMA-mediated dedifferentiation of chondrocytes as well as their redifferentiation is under both transcriptional and posttranscriptional regulation.

In vivo and in vitro, differentiated chondrocytes secrete and are imbedded in an extracellular matrix composed of type II collagen (16, 36, 44, 50), several minor collagens specific to hyaline cartilage (33), and chondrocyte-specific type IV sulfated proteoglycans (14, 20). The latter can be identified because they stain with toluidine blue (13). Chondrocytes in culture are either spherical and remain in suspension or attach to the culture dish and assume a characteristic polygonal morphology (50). When subcultured in monolayer, the cells gradually assume a more fibroblast-like morphology. Morphological changes are followed by a decrease in the expression of the chondrocyte matrix components, including type II collagen and type IV sulfated proteoglycans. Concomitantly, low levels of type I collagen and fibronectin, characteristic of the extracellular matrix of fibroblasts in skin, bone, and tendons are produced. These changes are not the result of overgrowth by contaminating fibroblasts since the switch from type II to type I collagen production can be observed in the progeny of a cloned chondrocyte (10, 15, 34). The failure to continue to produce the differentiated phenotype led to the characterization of this switch as "dedifferentiation" (22). This is not meant to imply that the cells had returned to an uncommitted or multipotent state, but rather that adverse environmental conditions have interrupted the expression of the differentiated phenotype, somewhat like the heat shock effect. As a result, the cells either revert to an earlier stage in the differentiation pathway when prechondrogenic cells produce type I collagen (29, 50) or they produce a maintenance phenotype (7).

The dedifferentiation of chondrocytes grown in monolayer subculture requires four or five subcultures, each of which takes about 7 days (6). A similar effect can be achieved in days instead of weeks if the cells are exposed to bromodeoxyuridine (1, 35, 40, 44), transformed with Rous sarcoma virus (3, 38), or exposed to the potent tumor promoter phorbol 12-myristate-13-acetate (PMA) (30, 39). In all cases, the chondrocyte morphology changes to a fibroblast-like morphology, and in most cases the chondrocyte extracellular matrix is replaced by a fibroblast extracellular matrix that includes type I collagen and fibronectin (15). Although the effect of bromodeoxyuridine appears to be irreversible (40), the effect of viral transformation (3, 38) and exposure to PMA (30, 39) have been shown to be reversible. Moreover, in at least one case, the effect of serial subculture in monolayer has also been reversed (8).

To gain some insight into how this reversible switch is regulated, we examined the levels of type II and type I collagen mRNAs and proteins in sternal chondrocytes isolated from 16-day-old chicken embryos and in chondrocytes treated with PMA for 4 or 12 days and in cells removed from PMA after 4 days and then grown in normal media for 8 days (revertant cells). We report here that the switch from type II to type I procollagen expression was quantitative and was accompanied by decreased levels of type II procollagen mRNA and the appearance of comparatively low levels of type I procollagen mRNA. The levels of pro α1(II) and pro α1(I) collagen mRNA were the same in chondrocytes grown in PMA for 12 days; however, no pro α1(II) collagen synthesis was detected in these cells.

MATERIALS AND METHODS

Sternal chondrocyte cell cultures. Sternal chondrocytes were prepared from 16-day-old chicken embryos by the method of von der Mark and von der Mark (51), plated at 2.6×10^4 cells per mm^2 in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (FBS; (Flow Laboratories), and incubated at 37°C in a CO_2 incubator. The cells were fed on days 3 and 5 by removal of the floating chondrocytes from the media by

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† Dedicated to Paul Doty on the occasion of his 65th birthday.

centrifugation at 2,500 rpm for 10 min and replating on new dishes in fresh Eagle minimal essential medium supplemented with 10% FBS. On day 7, floating chondrocytes were collected by centrifugation, washed once in phosphate-buffered saline, treated with 0.25% trypsin (GIBCO) and 2 mg of bacterial collagenase per ml (Boehringer Mannheim) for 20 min at 37°C. Calf serum was added to inactivate trypsin, the cells were washed once in PBS and plated in Dulbecco modified essential medium (GIBCO) supplemented with 10% FBS at a density of 2.6×10^4 cells per mm^2. After 72 h, greater than 95% of the cells remained floating. The cells were harvested by centrifugation and replated at 2.6×10^4 cells per mm^2 in Dulbecco modified essential medium supplemented with 10% FBS for tumor promoter experiments. Initially, PMA (Sigma Chemical Co.) was added at concentrations of 10 to 100 ng/ml on day 0, 2, and 3, and the cells were fed before each addition of PMA. A concentration of 50 ng/ml was found to be the lowest amount of PMA which brought about morphological change without resulting in extensive cell death. Therefore, 50 ng/ml was added to the cells on day 0, 2, 3, 5, 7, 9, and 11, and the cells were fed before each addition of PMA. Revertant cells were withdrawn from PMA on day 5 and fed according to the same schedule as PMA-treated cells. mRNA was extracted and pulse-labeled protein was prepared on day 4 and on day 12 for control, PMA treated, and revertant cells.

Staining and photography. Chondrocytes were stained for metachromatic matrix production with toluidine blue (10). Stained dishes were overlaid with 80% glycerol and immediately photographed under phase contrast at 160× and 400×, using Kodak color photomicrography 2483 film or under phase contrast at 225× using Polaroid 5200 black-and-white film.

Protein pulse-labeling and analysis. For each time point two 100-mm dishes were washed twice with 5 ml of phosphate-buffered saline incubated in Eagle minimal essential medium–1% amino acids without proline for 2 h, followed by pulse-labeling for 30 min with [2,3,4,5-^{3}H]proline (102 Ci/mmol; Amersham). The total cell layer was extracted by the method of Beldekas et al. (5) for proline pulse-labeled cultures. Organ cultures were labeled and the procollagen chains were collected from the media by the method of Gonnerman et al. (21). Incorporated counts were normalized to DNA for each sample. A total of 50,000 cpm were loaded for the control chondrocytes, and the remaining samples were adjusted so that the protein loaded corresponded to that synthesized by a constant number of cells. The samples were electrophoresed on 3 to 10% continuous gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (24). Fluorography was carried out by the method of Bonner and Laskey (9) and Laskey and Mills (25). The gels were dried and exposed for 7 days at −50°C.

In situ CNBr reactions were performed on Coomassie blue-stained gel slices. A total of 150,000 cpm of each sample was loaded onto 5 to 10% continuous gradient SDS-polyacrylamide gels. Bands corresponding to pro α1(I) collagen from calvaria organ culture, pro α1(I) collagen from chondrocytes grown in the presence of PMA for 12 days, pro α1(II) collagen from sternal organ culture, and pro α1(II) collagen from control chondrocytes were treated with CNBr as described in Gerstenfeld et al. (17). The products were run in a second dimension on 7 to 15% gradient polyacrylamide gels. Fluorography was carried out as described above.

Proline pulse-labeled protein (50,000 cpm) was digested with collagenase, and the amount of collagenase-sensitive material was determined by the method of Schwarz et al. (45).

RNA extraction. Total RNA was extracted from sternal chondrocytes or on the day described above by using a modification of the phenol-proteinase K method (41). Cells, on ice, were washed twice with 5 ml of ice-cold phosphate-buffered saline and scraped off dishes in 2 ml of phosphate-buffered saline with a rubber policeman. After collection by centrifugation, the cells were suspended in 10 ml of 10 mM Tris (pH 7.5)–5 mM EDTA–1% SDS and passed through a 23-gauge needle three times to shear the DNA. Proteinase K was added to 100 μg/ml, and the suspension was then incubated for 45 min at 50°C. The nucleic acid mixture was extracted three times with an equal volume of phenol-chloroform (50:50) saturated with 100 mM Tris (pH 7.5)–10 mM EDTA, adjusted to 0.1 M NaCl, and precipitated overnight at −20°C after the addition of 2 volumes of ethanol. Total nucleic acid was recovered by centrifugation at 8,500 × *g* for 30 min, washed in 70% ethanol, dried, and reprecipitated. Nucleic acid samples were suspended in 6 M GuHCl at 400 μg/ml, followed by addition of 0.5 volume of ethanol, and precipitated at −20°C for not more than 12 h. Total RNA was recovered by centrifugation at 8,500 × *g*, washed with 70% ethanol, and reprecipitated. All manipulations were carried out at 4°C. RNA samples were analyzed on 0.8% agarose–2.2 M formaldehyde gels to determine that the RNA was intact and free of DNA. Gerstenfeld et al. (18) have shown that quantitative separation of RNA from DNA can be achieved by using this modification of the original GuHCl RNA isolation procedure (12). We found this method to give greater yields of intact mRNA despite previous reports which suggested that the GuSCN method (12) was more efficient for extraction of RNA from chondrocytes (31). Polyadenylated mRNA was prepared by the method of Tate et al. (47).

Northern blot hybridizations. RNA was electrophoresed on 25-cm 0.8% agarose gels containing 2.2 M formaldehyde by the method of Lehrach et al. (26). This allows resolution of the multiple species unique to each collagen-type mRNA. Transfer of RNA onto nitrocellulose was carried out by the method of Thomas (48). Nick-translated probes with a specific activity of 10^8 cpm/μg were hybridized in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 48°C for 12 h and washed at 54°C in 2× SSC–0.1% SDS for 2 h, followed by 0.2× SSC–0.1% SDS for 1 h. Blots were exposed with an intensifying screen at −50°C. The cDNA clones pCg45 (28), pCg54 (27), and pCs2 (53) were used as probes for pro α2(I), pro α1(I), and pro α1(II) collagen mRNAs, respectively. A 600-base-pair cDNA clone, pMF1A8, was also used as a probe for pro α1(I) mRNA. This clone corresponds to the amino propeptide, the signal peptide, and the 5′-untranslated region of the pro α1(I) mRNA, as determined by DNA sequencing (M. H. Finer, unpublished data). pMF1A8 and pCg54 hybridized to identical mRNA species in RNA isolated from calvaria. Autoradiograms were scanned, and the area under the peaks were integrated by using a soft laser scanning densitometer-integrator.

RESULTS

Effect of PMA on morphology and proliferation. To study chondrocyte dedifferentiation it is essential to obtain pure populations of chondrocytes free of contaminating fibroblasts. Therefore chondrocytes from 16-day-old chicken embryo sterna were plated in Eagle minimal essential medium as described above. After 6 days, floating cells were

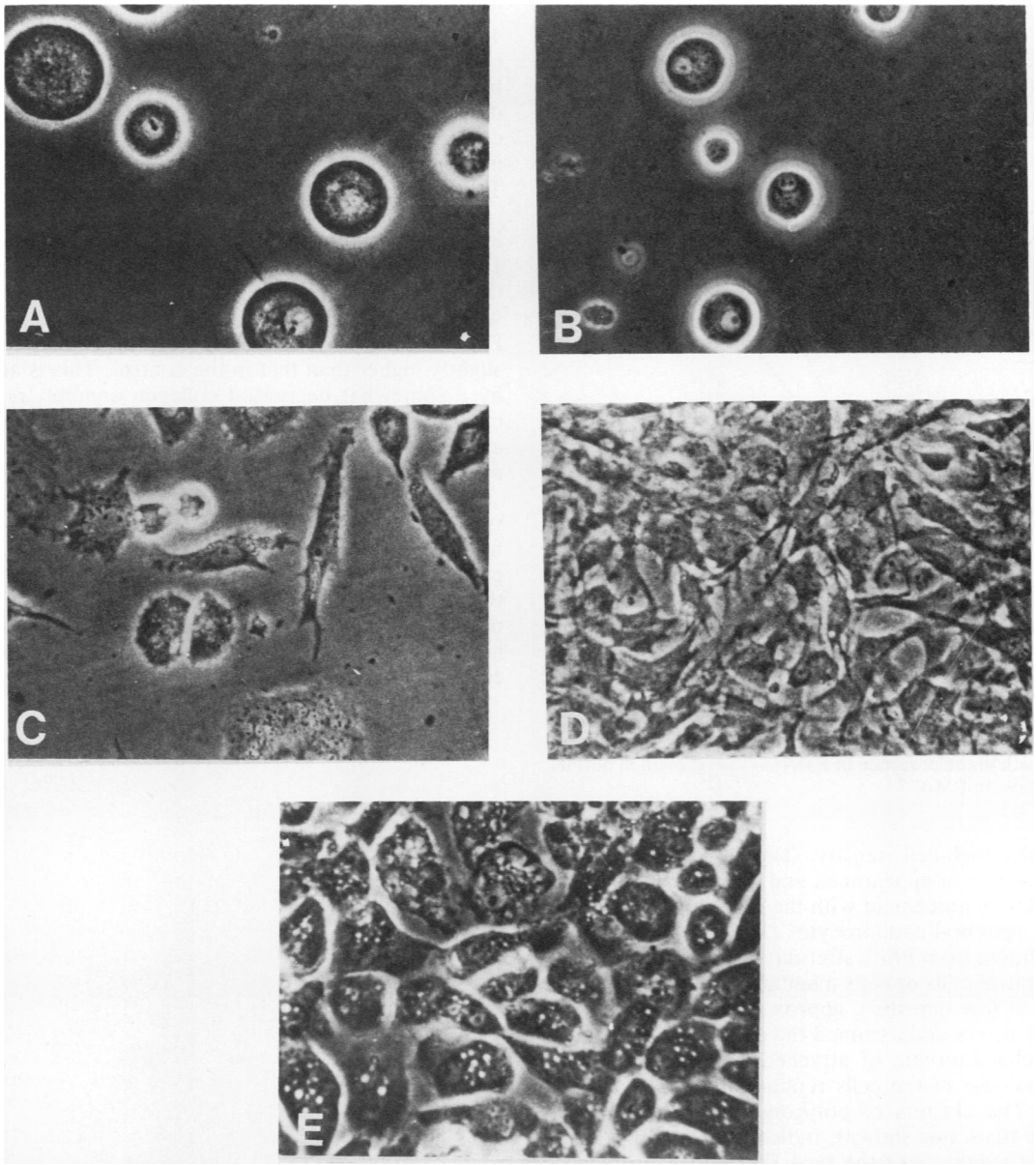

FIG. 1. Morphology of control and PMA-treated chondrocytes. Sternal chondrocytes after 4 (A) and 12 (B) days in culture. Chondrocytes grown in the presence of PMA for 4 (C) and 12 (D) days and withdrawn from PMA after 4 days and cultured for an additional 8 days in normal media (revertant cells) (E). Cells were photographed at 225× under phase contrast.

harvested free of fibroblasts which had attached to the dish. The cells were then plated at 2.6×10^4 cells per mm^2 in Dulbecco modified essential medium plus 10% FBS. When viewed by phase-contrast microscopy, the cells appeared to be spherical with a birefringant halo, indicating that they were floating (Fig. 1A). The cells were imbedded within a fibrillar matrix, which stains with toluidine blue (data not shown). These cells underwent about two doublings during the first 8 days. Thereafter no further growth was detected (see Fig. 2). After 12 days in culture, 95% of the chondrocytes remained in suspension (Fig. 1B) and in a matrix which stained with toluidine blue. The absence of growth after day 8 as well as the morphology of the cells and the staining suggest that these cells were free of fibroblast contamination.

When chondrocytes were treated with PMA, there was a dramatic change in morphology. One day after PMA addition, 40% of the chondrocytes had adhered to the culture dish, and after 2 days in PMA, greater than 95% of the cells adhered to the culture dish. After an additional 2 days in PMA, the attached cells had taken on bizarre, unusual morphologies (Fig. 1C). Since these cells failed to stain with toluidine blue (data not shown), they were no longer producing the chondrocyte-specific type IV sulfated proteoglycan. After 4 days of growth in PMA, floating chondrocytes became irreversibly attached.

Chondrocytes were subsequently cultured for an additional 8 days in either PMA or normal media (revertant cells). Cells maintained in PMA initially grew much more rapidly than did control cells. Between days 8 and 12, the growth rate slowed (Fig. 2), either because of insufficient nutrients or because the pH of the culture media was

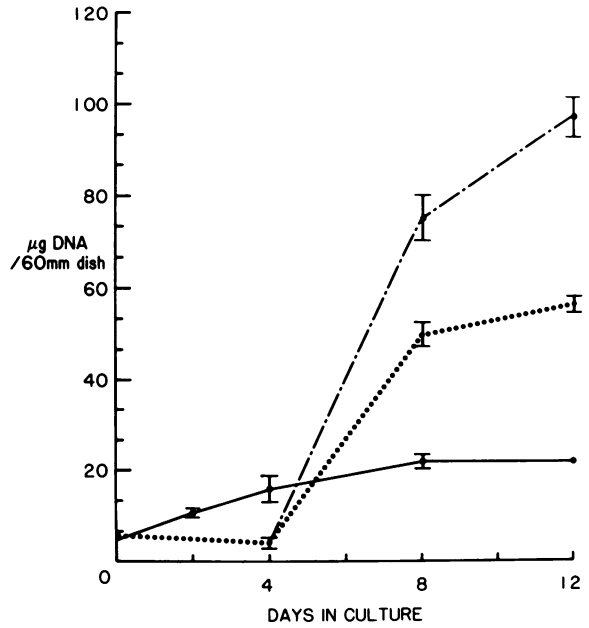

FIG. 2. Growth of control, PMA-treated, and revertant chondrocytes. DNA content per 60-mm^2 dish was determined for each sample by measuring the concentration of total nucleic acids and total RNA, isolated as described in the text, by UV absorbance at 260 nm. DNA content, determined by RNase treatment of total nucleic acid samples, differed by only 10%. Symbols: ——, control; · · · ·, growth in the presence of PMA; — · —, growth in normal media after 4 days in PMA.

lowered by the high-cell density. The PMA-treated cells were fibroblast-like in appearance and had no contact inhibition (Fig. 1D), in agreement with the results obtained with PMA-treated vertebral chondrocytes (30).

Cells withdrawn from PMA after day 4 grew more rapidly than either control cells or cells maintained in PMA (Fig. 2). When plated at low densities, approximately 70% of these cells formed clusters and assumed the epitheloid, polygonal morphology characteristic of attached chondrocytes (Fig. 1E). The remainder of the cells retained the fibroblast-like morphology. The clusters of polygonal cells stained with toluidine blue (data not shown), indicating that these cells had reinitiated synthesis of the type IV sulfated proteoglycan matrix characteristic of differentiated chondrocytes. However, the cells grew much more rapidly than control cells and were not fully contact inhibited. Even though some of the cells had reexpressed the differentiated phenotype, others had been irreversibly altered by PMA treatment.

Effect of PMA on procollagen and total protein synthesis. Having confirmed the partly reversible morphological changes and suppression of type IV sulfated proteoglycans caused by PMA reported previously (30, 39), we next examined the effect of PMA on procollagen and total protein synthesis. Chondrocytes were labeled for 30 min with [^{3}H]proline. Since the pro α1(II) and pro α1(I) collagen chains normally comigrate on SDS-polyacrylamide gels, whereas the pro α1(II) chain migrates faster than the pro α1(I) chain when these chains are underhydroxylated (11), chondrocytes were cultured in the absence of ascorbate.

The results obtained with control chondrocytes, chondrocytes grown in PMA for 4 days and for 12 days, and revertant cells are shown in Fig. 3 and summarized in Table 1. The identity of pro α1(I), and pro α1(II) collagen chains was verified by in situ CNBr peptide mapping shown in Fig. 4. Although control chondrocytes synthesize only type II collagen, cells exposed to PMA for 4 days synthesized barely detectable amounts of type II collagen, which can only be seen on longer exposures (data not shown). This is consistent with the very low fraction of the total proline incorporation which is collagenase sensitive as shown in Table 1.

Proline incorporation per microgram of DNA after 4 days in PMA was almost four times that of the control cells (Table 1). The metabolic activity of the PMA-treated cells was therefore maximal in these cells, and the collagen synthesis was lowest. Further growth in the presence or absence of PMA results in total protein synthesis per cell which is slightly higher than that in the control. This is accompanied by a somewhat decreased collagen content, resulting in a rate of collagen synthesis per cell that is the same in control and cells treated with PMA for 12 days and slightly higher in revertant cells.

Cells grown in PMA for 12 days synthesized high levels of type I procollagen (Fig. 3, land 4). From densitometer tracings of this fluorogram, we determined that the ratio of pro α1 to pro α2 collagen chains was 2.5:1, which is higher than the ratio of 2:1 found in normal fibroblasts. Presumably the extra pro α1(I) chains are derived from the homotrimer [α1(I)]. Such trimers were previously detected in embryonic chicken chondrocytes treated with bromodeoxyuridine (35,

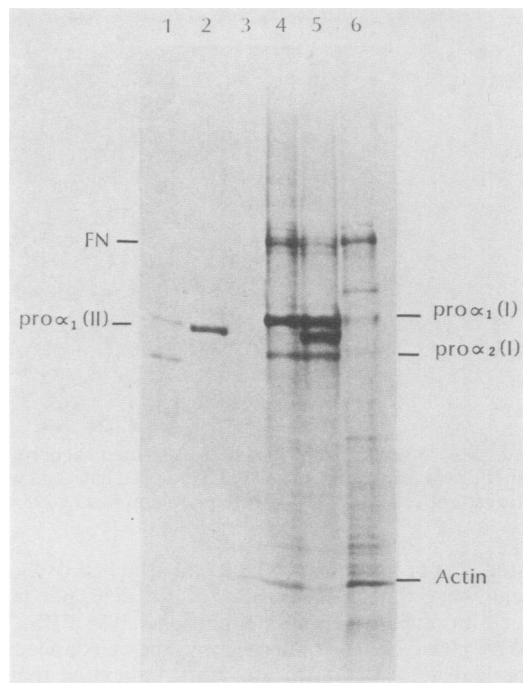

FIG. 3. SDS-polyacrylamide gel electrophoresis of [^{3}H]proline pulse-labeled protein. Cell cultures were pulse-labeled with [^{3}H]proline for 30 min and extracted as described in the text. Protein was electrophoresed on 3 to 10% SDS-polyacrylamide gradient gels. The samples were adjusted such that the amount of counts loaded represented an amount of DNA equal to the amount of DNA present in 5×10^4 cpm of control chondrocytes. Lanes: 1, sternal organ culture; 2, control chondrocytes; 3, chondrocytes cultured in the presence of PMA for 4 days, 4, chondrocytes cultured in the presence of PMA for 12 days; 5, revertant cells; 5, chicken embryo fibroblasts. FN, Fibronectin; pro α1(II), pro α1(II) collagen; pro α1(I), pro α1(I) collagen; pro α2(I), pro α2(I) collagen.

TABLE 1. Total protein and procollagen synthesis in PMA-treated chondrocytes

Cell type	Proline incorporated (cpm)/μg of DNA	Proline incorporated (cpm) into collagen/μg of DNA[a]	% Collagen	Proline incorporated (cpm) into pro α chains/μg of DNA[b]		
				pro α1(II)	pro α1(I)	pro α2(I)
Control, 4 days	55,000	6,600	12 ± 1.3	28 ± 7	—[c]	—
PMA treated						
4 days	200,000	1,200	0.6 ± 0.1	—	—	—
12 days	79,000	6,600	8 ± 1.2	—	28 ± 7	11 ± 3
Revertant	85,000	7,500	9 ± 0.6	32 ± 8	28 ± 7	16 ± 4

[a] Average of two experiments, calculated from percent proline incorporated which is collagenase digestible, as described by Schwarz et al., (45).
[b] Areas of the peaks taken from densitometer scans of fluorographs of SDS-polyacrylamide gels of pulse-labeled proteins. Labeled protein proportional to DNA content per dish was applied per lane.
[c] —, Not detectable.

40) and in rabbit articular chondrocytes serially subcultured in monolayer cultures (6). The total incorporation of [^{3}H]proline into pro α1(I) and pro α2(I) collagen chains in chondrocytes continuously treated with PMA is about 40% higher than the incorporation of [^{3}H]proline into pro α1(II) collagen chains in control chondrocytes. This suggests that the switch from type II to type I production may have been quantitative. We do not know, however, whether the amount of incorporation is a true measure of increased rate of synthesis, or reflects some pleiotropic effect of PMA such as more rapid uptake of labeled proline by the PMA-treated cells.

In addition to the two type I collagen bands, a very prominent band corresponding to the fibronectin doublet was readily visible in pulse-labeled protein in cells treated with PMA for 12 days. This protein was normally associated with the extracellular matrix of fibroblasts and was also highly labeled in the control fibroblasts (Fig. 3, lane 6).

Revertant chondrocytes reinitiated the synthesis of pro α1(II) collagen chains at about the same rate as control chondrocytes (Fig. 3, land 5). Rather surprisingly, these revertant cells synthesized pro α1(I) and pro α2(I) chains at about the same rate as chondrocytes treated with PMA for 12 days in which the switch from type II to type I production is complete. Fibronectin is also produced although at lower rates. The persistence of type I collagen and fibronectin synthesis in revertant cells is consistent with the failure of about one-third of the cells to stain with toluidine blue and with the persistence of fibroblast-like morphology of these cells. These observations were confirmed by reacting the revertant cells with antibodies to either type I or type II collagen, followed by rhodamine-conjugated anti-rabbit immunoglobulin G. Most of the polygonal cells reacted with type II antibodies, and the elongated cells surrounding them reacted with type I antibodies (19).

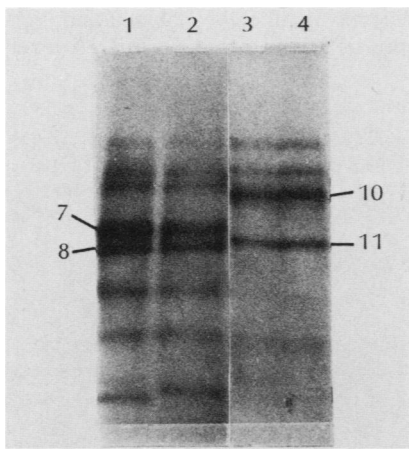

FIG. 4. Cyanogen bromide peptide analysis of isolated procollagen chains from PMA-treated chondrocytes. Gel slices from preparative SDS-polyacrylamide gels containing the collagen species to be analyzed were treated in situ with CNBr, followed by electrophoresis in a second dimension on 7 to 15% SDS-polyacrylamide gradient gels. Lanes: 1, pro α1(I) collagen isolated from calvaria organ culture; (2), pro α1(I) collagen isolated from chondrocytes grown in the presence of PMA for 12 days; 3, pro α1(II) collagen isolated from sternal organ culture; 4, pro α1(II) collagen isolated from control chondrocytes. 7 and 8, CB7 and CB8, CNBr peptides unique to pro α1(I) collagen; 10 and 11, CB10 and CB11, CNBr peptides unique to pro α1(II) collagen.

Procollagen synthesis is under both transcriptional and posttranscriptional control. To determine whether changes in type II and type I procollagen synthesis observed in PMA-treated chondrocytes reflected comparable changes in the steady-state levels of the three procollagen mRNAs, total RNA from control chondrocytes, PMA-treated chondrocytes, and revertant cells were analyzed by Northern blot hybridization. The 1,200-base-pair pro α1(II) collagen cDNA clone, pCs2 (53), the 1,100-base-pair pro α1(I) collagen cDNA clone, pCg54 (28), and the 2,700-base-pair pro α2(I) collagen cDNA clone, pCg45 (27) were used as probes. Since the ratio of total RNA/DNA was determined in control, PMA-treated, and revertant chondrocytes and found to be equal to 2 ± 0.2 in each of these cells, changes in intensity of hybridization are proportional to the changes in the mRNA levels per cell.

RNA extracted from control chondrocytes contained a single 5.0-kilobase (kb) species which hybridized to pCs2, and corresponds to pro α1(II) collagen mRNA (Fig. 5A). When the same RNA was probed with pCg45, the pro α2(I) collagen cDNA clone, no hybridization was detected (Fig. 6A). RNA extracted from chondrocytes treated with PMA for 4 or for 12 days contained significantly lower amounts of the 5.0-kb pro α1(II) collagen mRNA species (Fig. 5A and B), whereas increasing amounts of the characteristic 4.6- and 5.0-kb pro α2(I) collagen mRNAs could be detected (Fig. 6A and 6B). The relatively low levels of both the pro α1(II) and pro α2(I) collagen mRNAs in cells treated with PMA for 4 days were consistent with the very low levels of type II and the absence of detectable type I procollagen chain synthesis in these cells. The even lower levels of the pro α1(II) and relatively high levels of pro α2(I) collagen mRNA in cells treated with PMA for 12 days were also consistent with the absence of pro α1(II) and the high rate of pro α2(I) collagen synthesis in these cells. Finally, RNA

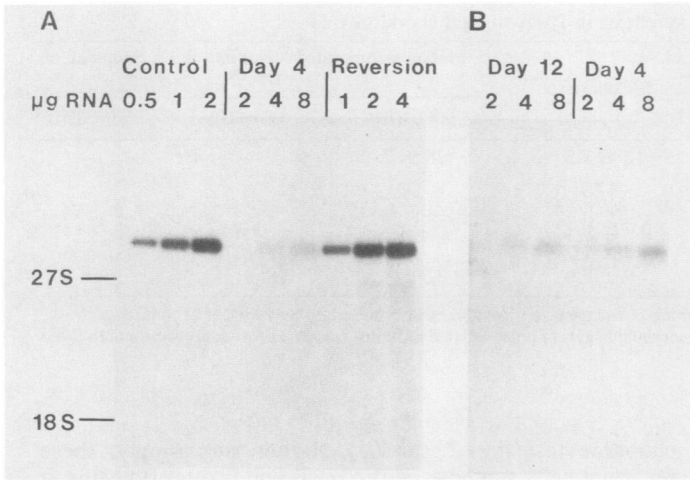

FIG. 5. Steady-state levels of pro α1(II) collagen mRNA in PMA-treated chondrocytes. (A) Hybridization of pCs2 to Northern blots of total RNA. 0.5, 1.0, and 2.0 μg from control chondrocytes (control). 2, 4, and 8 μg from chondrocytes grown in the presence of PMA for 4 days (day 4). 1, 2, and 4 μg from revertant cells exposed for 1 h. (B) 2, 4 and 8 μg from chondrocytes grown in the presence of PMA for 4 days (day 4) and 12 days (day 12) hybridized to pCs2 and exposed for 8.5 h.

extracted from revertant cells contained more than 50% of the control level of pro α(II) mRNA, but the pro α2(I) mRNA level was significantly lower than in cells treated with PMA for 12 days. The type II mRNA level is consistent with the type II protein synthesis rates in revertant cells. The lower pro α2(I) mRNA level was somewhat surprising, however, since the rate of pro α2(I) chain synthesis was very similar in revertant cells and in cells treated with PMA for 12 days.

The Northern blot hybridizations to the pro α1(I) collagen cDNA, pCg54, were similar to those obtained with the pro α2(I) cDNA probe, suggesting that the type I mRNAs may be coordinately expressed after PMA treatment of chondrocytes. There were, however, some significant differences. First, significant hybridization could be detected in RNA isolated from control chondrocytes (Fig. 7A and B). The hybridization was not to the characteristic 4.7- and 4.9-kb pro α1(I) doublet (18) but rather to a single 5.0-kb RNA species (Fig. 7B). This species is probably not pro α1(I) collagen mRNA since it did not hybridize to a cDNA clone, pMF1A8, containing the 5′ end of the pro α1(I) mRNA which encodes the N-terminal propeptide, the signal peptide, and the 5′-untranslated region of pro α1(I) collagen as determined by DNA sequence analysis (M. H. Finer, unpublished data). Therefore the aberrant hybridization is probably cross-hybridization to pro α1(II) collagen mRNA which is present in high concentration in RNA isolated from control chondrocytes (see below).

Three additional high-molecular-weight bands, all greater than 10 kb, hybridize to both pCg54 and pMF1A8 (Fig. 7B and 8). These high-molecular-weight species appeared to be authentic pro α1(I) collagen transcripts which were not polyadenylated and probably only partly processed. These high-molecular-weight species were not detectable in chondrocytes treated with PMA for 4 days, in revertant cells, or in cells treated with PMA for 12 days, but the characteristic 4.7- and 4.9-kb as well as the 6.1-kb pro α1(I) species were found in increasing amounts (Fig. 7A and B).

To compare the absolute levels of the three procollagen mRNAs at various times, the extent of hybridization was corrected for the size of the probe, its specific activity, the exposure time, and the fraction of the labeled probe which reannealed to its template. The corrected levels of three mRNAs (Table 2), were surprising in that the level of pro α1(II) collagen mRNA in control chondrocytes was 10 times higher than the level of pro α1(I) in cells treated with PMA for 12 days, whereas the rate of synthesis of pro α1(II) and pro α1(I) collagen chains were identical in the control and PMA-treated cells. Moreover, the pro α1(II) mRNA levels in the PMA-treated cells were essentially the same as the pro α1(I) mRNA levels, but the former mRNAs did not produce detectable levels of pro α1(II) chains. Finally, in revertant cells, in which the synthetic rates of the pro α1(II) and pro α1(I) collagen chains are very similar, the pro α1(II) mRNA levels are 24 times that of the pro α1(I) mRNA.

DISCUSSION

After confirming the dramatic changes in the morphology and in the composition of the extracellular matrix that occur after PMA treatment of chondrocytes reported previously (29, 38), we report here the first qualitative and quantitative determination of the changes in the rate of synthesis of type II and type I procollagen in embryonic chicken chondrocytes before and after PMA treatment as well as in revertant cells. We also made the first quantitative determination of type II and type I procollagen mRNA levels in these cells. Control chondrocytes exhibited high rates of type II chain synthesis but no detectable type I chain synthesis. Chondrocytes treated with PMA for 12 days, on the other hand, synthesized type I chains at rates comparable to type II chain synthesis in the control cells while making no type II chains. Type II chain synthesis was, in fact, barely detectable in cells grown in PMA for 4 days before any type I chain synthesis was detectable. Although these changes in collagen production qualitatively reflect changes in procollagen mRNA levels, they do not do so quantitatively. Thus, the pro α1(II) collagen mRNA levels are higher than the pro α1(I) mRNA levels in all cells analyzed and higher than the sum of the pro α1(I) and pro α2(I) mRNA levels in all but

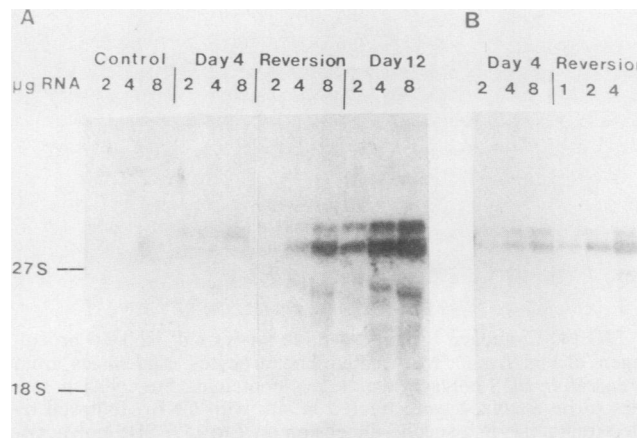

FIG. 6. Steady-state levels of pro α2(I) collagen mRNA in PMA-treated chondrocytes. (A) Hybridization of pCg45 to Northern blots containing 2, 4, and 8 μg of total RNA. Control, day 4, day 12, and revertant cells identified as described in the legend to Fig. 1 exposed for 8 h. (B) 2, 4, and 8 μg of total RNA from day 4 and 1, 2, and 4 μg of total RNA from revertant cells were hybridized to pCg45, and the blot was exposed for 24 h.

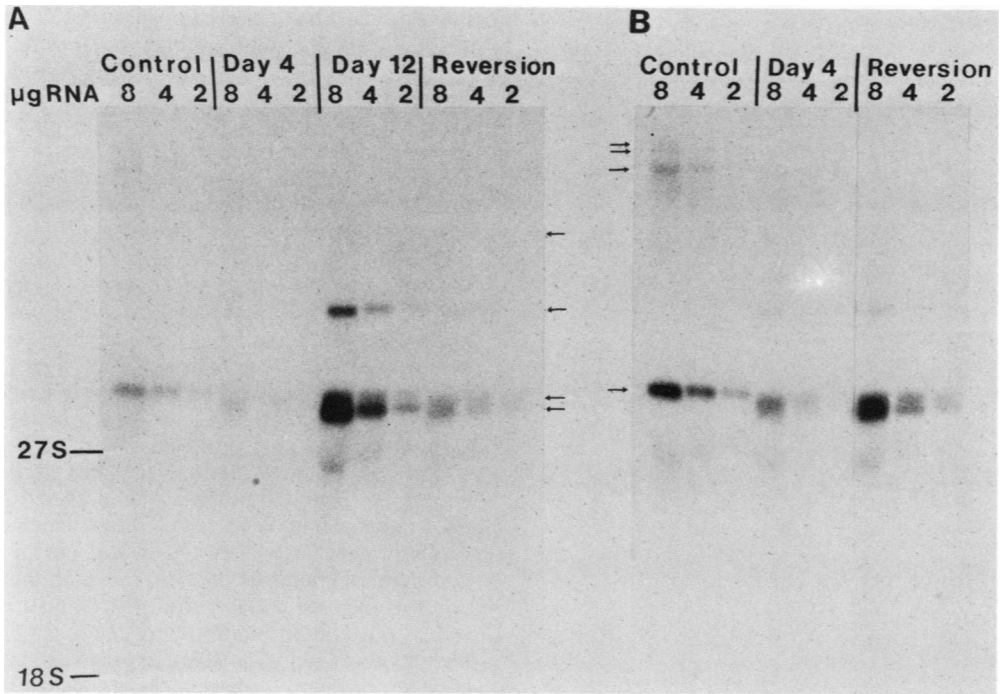

FIG. 7. Steady-state levels of pro α1(I) collagen mRNA in PMA-treated chondrocytes. Hybridization of pCg54 to Northern blots containing 8, 4, and 2 μg of total RNA. (A) Control, day 4, day 12, and revertant cells identified as described in the legend to Fig. 5 hybridized to pCg54 and exposed for 3 days. (B) Control, day 4, and reversion lanes from the same blot exposed for 6 days.

cells treated with PMA for 12 days. The most dramatic difference was found in the revertant cells. The pro α1(II) collagen mRNA level was 24 times higher than the pro α1(I) mRNA level, although the rate of pro α1(II) chain synthesis was about the same as the rate of pro α1(I) chain synthesis.

Since the apparent translation efficiency of the type II mRNA is comparable in control and revertant cells, its value cannot be attributed to some pleiotropic effect of PMA. This argument cannot be extended to explain the apparently much more efficient translation of type I procollagen mRNAs in cells treated with PMA for 12 days and revertant cells. It is possible that those chondrocytes which express type I collagen were irreversibly altered by PMA, so that either the uptake of labeled proline was greatly increased, the translation apparatus became more efficient, or chains were secreted more rapidly and thus stabilized. Hence, the observed translation efficiency may not be an inherent property of the mRNAs but rather PMA-induced artifacts. There are two reasons why this may not be the case, however. First, RNA isolated from embryonic chicken sterna has been shown to contain both type II and type I procollagen mRNA. When translated in reticulocyte lysates, about equal amounts of type II and type I procollagen chains are produced (31, 37), although the ratio of type II to type I mRNA is about 10/1 (F. Fuller, Ph.D. thesis, Harvard University, Cambridge, Mass., 1981). This suggests a more efficient utilization of type I mRNAs in vitro, in which uptake of labeled amino acid, pool size, and translation apparatus are identical. Second, the 5'-untranslated region of the rat pro α1(II) collagen mRNA sequence has recently been determined by DNA sequence analysis of a cDNA clone (23). The former is quite different from the 5'-untranslated region of the chick pro α1(I), pro α2(I), and pro α1(III) collagen mRNAs which have a highly conserved sequence preceding and following the initiating AUG (52). There was 83 and 85% homology between the pro α1(I) collagen mRNA and the pro α2(I) and pro α1(III) collagen mRNAs, respectively, in a 48-base region which is not found in the pro α1(II) mRNA. The sequence differences in this region might be responsible for the difference in the apparent translation efficiency of type I and type II procollagen mRNAs. This suggests that the cessation of type II collagen expression may be regulated both by reducing the mRNA level and by inefficient utilization of this mRNA or translational regulation.

TABLE 2. Procollagen mRNAs levels in PMA-treated chondrocytes

Cell type	Procollagen mRNA level[a]		
	pro α1(II)	pro α1(I)	pro α2(I)
Control, 4 days	68	—[b]	—
PMA treated			
4 days	13.6	0.44	0.13
12 days	6.8	6.4	3.4
Revertant cells	48	2.0	1.0

[a] From densitometer tracings, corrected for probe size, specific activity, exposure time, and fraction of counts per minute that reanneal to template, with the area of pro α2(I) in revertant cells set at 1.0 arbitrary unit.
[b] —, Not detectable.

Translational control has been suggested in other collagen-producing cells. During sheep embryogenesis, the levels of type I collagen expression decrease sharply with no change in type I mRNA levels (49). In primary avian tendon cells, ascorbate induction results in an increase in the rate of type I procollagen synthesis before an increase in mRNA levels can be detected (42). Conversely, reduction in type I procollagen synthesis in Rous sarcoma-transformed chicken embryo fibroblasts is accompanied by reduced type I mRNA

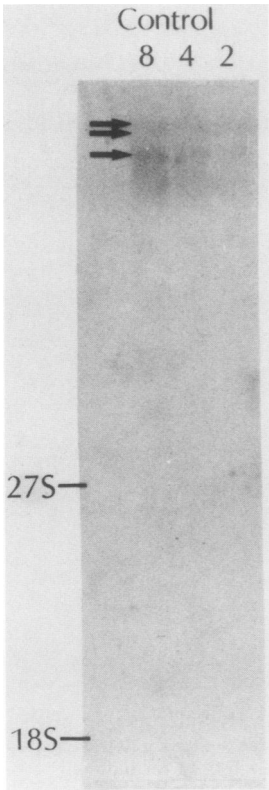

FIG. 8. Hybridization of pMF1A8 to control chondrocyte mRNA. pMF1A8 was hybridized to 8, 4, and 2 μg of total RNA from control chondrocytes as described in the text and exposed for 6 days.

levels (2, 43). The most striking example of translational control in noncollagen-producing cells have been reported for *Drosophila melanogaster* (46) and chick reticulocyte (4) cells after heat shock. In *Drosophila* cells, the switch in expression from normal embryonic proteins to heat shock proteins is achieved by the activation of transcription of heat shock genes and inactivation of translation of normal embryonic mRNAs (46), whereas in chick reticulocytes, there is no change in mRNA levels but a decreased utilization of α- and β-globin and other normal reticulocyte mRNAs (4).

Although no transcription studies have been carried out, the absence of detectable pro α2(I) collagen mRNA in control chondrocytes and its presence in PMA-treated cells is consistent with transcriptional activation of the pro α2(I) collagen gene after PMA treatment. However, the appearance of pro α1(I) collagen mRNA after PMA treatment may result from both gene activation and processing of existent transcripts, since RNAs larger than 10 kb which hybridize to cDNA clones complementary to the 5′ and 3′ ends of pro α1(I) collagen mRNA were detected in control cells. We have not determined whether the changes in pro α1(II) mRNA levels we observed are the result of changes in the rate of transcription or in mRNA stability, or in both. In primary avian tendon cells, changes in type I procollagen mRNA levels reflect changes in both mRNA stability and in transcription rates (32).

Although we demonstrated partial reversibility of PMA effects, we also found that not all of the cells revert to expressing the differentiated phenotype or return to the typical chondrocyte morphology. Moreover, even the cells that do revert have altered properties. They are no longer in suspension and exhibit limited contact inhibition.

In evaluating both the phenotypic switch in gene expression caused by growth in PMA and its partial reversal it is important to point out that the cells which revert to type II expression are probably reverting from not expressing type II rather than from expressing type I procollagen. The only time at which the PMA effect is reversible is up to 4 days (30), and no type I procollagen synthesis is detectable in these cells. Once type I collagen synthesis can be detected, the cells can no longer be reverted. Hence, the cells can undergo morphological changes and loss of type II procollagen and type IV sulfated proteoglycan expression and still be reverted, but once high levels of type I procollagen are synthesized, this expression can no longer be reversed. Complete reversion can, however, be achieved in Rous sarcoma virus-transformed chick vertebral chondrocytes in which type I procollagen mRNAs but no type I protein are expressed (2) and in rabbit articular chondrocytes which are aged in serial monolayer subcultures which express only low levels of type I collagen (7, 8), but not in bromodeoxyuridine-treated chondrocytes (40) in which the total collagen in control and bromodeoxyuridine-treated cells is about the same (33). The correlation between loss of reversibility and high levels of type I collagen expression suggests that type I collagen production marks a late stage in the dedifferentiation of chondrocytes, a stage from which redifferentiation is no longer possible.

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