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Altered β-Actin Gene Expression in Phorbol Myristate Acetate-Treated Chondrocytes and Fibroblasts.

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Phorbol-12-myristate-13-acetate (PMA), a potent tumor promoter, was shown to have opposite effects on the cellular morphology and steady-state levels of β-actin mRNA in embryonic chicken muscle fibroblasts and sternal chondrocytes. When fibroblasts were treated with PMA, they formed foci of densely packed cells, ceased to adhere to culture plates, and had significantly reduced levels of β-actin mRNA and protein. Conversely, when treated with PMA, floating chondrocytes attached to culture dishes, spread out, and began to accumulate high levels of β-actin mRNA and proteins. In the sternal chondrocytes the stimulation of the β-actin mRNA production was accompanied by increased steady-state levels of fibronectin mRNAs and protein. These alterations were concomitant with a fivefold reduction in type II collagen mRNA and a cessation in its protein production. After fibronectin and actin mRNAs and proteins reached their maximal levels, type I collagen mRNA and protein synthesis were turned on. Removal of PMA resulted in reduced β-actin mRNA levels in chondrocytes and in a further alteration in the cell morphology. These observed correlations between changes in cell adhesion and morphology and β-actin expression suggest that the effect of PMA on cell shape and adhesion may result in changes in the microfilament organization of the cytoskeleton which ultimately lead to changes in the extracellular matrix produced by the cells.

Both the differentiated and transformed state of cells are uniquely defined by cellular morphology, determined in part by the cytoskeletal organization. Microfilament bundles, an integral component of the cytoskeleton, confer anchorage dependence on cells for normal growth and are responsible for the flattened appearance of most cells. In addition, they may provide communication between the cytoskeleton and fibronectin in the extracellular matrix (21, 34, 45, 46). In contrast, transformed cells lose their anchorage dependence, contact inhibition, and are unable to produce a normal extracellular matrix (1–3, 38, 40). Microfilaments are believed to be composed of two distinct actin isotypes, β and γ-actin, whose protein and DNA sequences have been shown to be highly conserved in evolution (23, 35). Because the amino acid sequence has been so highly conserved, however, monoclonal antibodies cannot distinguish the different actin isotypes, and hence, their precise location or function in the cytoskeleton has not been determined.

Phorbol esters are potent tumor promoters in vivo (11). Unlike initiating carcinogens, however, they cannot induce transformation by themselves, and their effects can be reversed if exposure is sufficiently brief (11, 29, 36). Tumor promoters, like tumor viruses, adversely affect the expression of differentiated phenotypes of many different cells in vitro and have profound effects on normal cell morphology (10, 13, 29, 37). While examining the effect of phorbol-12-myristate-13-acetate (PMA) on collagen gene expression in differentiated chicken sternal chondrocytes and in fibroblasts derived from embryonic chicken myoblast preplates, we observed dramatic but opposing changes in β-actin gene expression in these two differentiated cell types. We report here the temporal events of PMA-induced changes in cell morphology, β-actin expression, and the composition of the extracellular matrix. A comparison of these results with control and PMA-treated fibroblasts provided an interesting contrast in cell-type response.

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MATERIALS AND METHODS

Cell cultures. All tissue culture supplies were from GIBCO Laboratories, and all tissue culture media were supplemented (per ml) with 100 U of penicillin, 100 U of streptomycin, and 200 μg of para-amino-benzoic acid as fungicide. Fibroblasts were cultured from the preplates of 11-day-old chicken embryo leg myoblast cultures which were prepared by the methods of Caplan (8) and Yaffe (48). Cultures were initially maintained in minimum essential medium–10% horse serum. After 7 days the cells were subcultured into Dulbecco modified Eagle medium–10% fetal bovine serum and maintained another week before use in experiments. All fibroblast experiments were carried out on second subcultivation cells which were plated at a cell density of 2.6×10^4 cells per mm^2.

Sternal chondrocytes were prepared from 16-day-old embryonic chickens by a modification of the method of von der Mark and von der Mark (44). Cells were initially cultured in minimum essential medium–10% fetal bovine serum at a cell density of 2.6×10^4 cells per mm^2. After 7 days only the floating cells were collected by centrifugation, briefly digested with trypsin and plated at a cell density of 2.6×10^4 cells per mm^2 in 10% fetal bovine serum–Dulbecco modified Eagle medium for 72 h. The floating cells were again collected by centrifugation, and these cells were replated at 2.6×10^4 cells per mm^2. After this selection procedure, greater than 95% of these cells remained as floaters for the duration of the experimental periods described. In experiments in which PMA (Sigma Chemical Co.) was used, a concentration of 50 ng/ml was added to the cultures on day 0, 2, 3, 5, 7, 9, and 11. 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. Fibroblasts were maintained with the

same schedule and dosage of PMA as the chondrocytes and were harvested at day 4.

Protein pulse-labeling and analysis. For each experimental point two 100-mm^2 culture dishes were used. One day before labeling, the cell cultures were placed in 10 ml of fresh medium, and on the day of the pulse they were preincubated for 2 h in serum-free leucine minus medium. Cell labeling was for either 1 or 24 h at 37°C with 50 μCi of [^{3}H]leucine per ml (120 mCi/mM; Amersham-Searle Corp.) in 5 ml of fresh serum-free leucine minus medium. After the pulse period, total proteins were extracted from either the cell layers or the floating sternal chondrocytes, which were collected by centrifugation as previously reported (17). Incorporated counts were determined by scintillation counting and normalized to DNA for each sample. For one-dimensional protein analysis, 50,000 cpm of nondialyzable counts from the 1-h pulse-labelings were loaded for the control samples. All other samples were adjusted so that the counts per minute of labeled protein loaded was for a comparable amount of DNA. The samples were electrophoresed on a 30-cm, 3 to 12.5% polyacrylamide gel (24). Fluorography was carried out by the methods of Bonner and Laskey (7) and Laskey and Mills (26). Quantification was done on fluorograms of one-dimensional gels by scanning densitometry. An average of at least two gels from two separate experiments was used for each determination.

Two-dimensional isoelectric focusing was carried out by the method of O'Farrel (33) as modified by Izant and Lazarides (22). In these experiments 100,000 cpm of the 24-h pulse were loaded for the control samples, and the remaining samples were adjusted as described for one-dimensional polyacrylamide gel analysis. All samples were applied to the first dimension with 30 μg of purified chicken actin (Sigma Chemical Co.). The second dimension was run on a 7.5 to 15% continuous gradient sodium dodecyl sulfate-polyacrylamide gel. After Coomassie staining, fluorography was carried out as described above. So that the radiolabeled actin isotypes could be accurately identified relative to the stained standard, the dried gel was keyed with radioactive ink at the four corners before being exposed to the film.

Nucleic acid extraction and RNA hybridization. Nucleic acids were extracted as previously described (16), and RNA was fractionated on a 1.4 or 0.8% formaldehyde agarose gel (27) depending on the size of the RNA which was being examined. RNAs were blotted onto either Gene Screen (New England Nuclear Corp.) by the transfer conditions of the manufacturer or nitrocellulose as previously reported (17). Whole inserts were removed from their cDNAs with the appropriate restriction enzymes and nick translation, prehybridization, and hybridization conditions were as previously described (17). Hybridization with the 3′ untranslated chicken β-actin cDNA (pA2) (9) was carried out at 45°C, and hybridization with the human protein-coding sequence actin cDNA (pHF-1) (35) was carried out at 37°C. Fibronectin mRNA hybridizations were carried out at 42°C with nick translations of the genomic clone FC36 (20). Relative mRNA levels were determined by scanning densitometry of underexposed autoradiograms of Northern blots.

Immunofluorescence of collagen types I and II. In situ localization of collagens was monitored by indirect immunofluorescence with either collagen type I or II antiserum (28). Cells were fixed and reacted with these antibodies, followed by second antibody staining with rhodamine-conjugated anti-rabbit antiserum (Cappel-Worthington) as described by Hanahan et al. (19) for indirect immunofluorescence of simian virus 40 T-antigen. Immunofluorescence was observed on a Zeiss microscropy system, and photography was performed with Kodak 160 Ektochrome tungsten film. Microphotographs were magnified at ×400.

RESULTS

Relationship of chondrocyte and fibroblast morphology to β-actin gene expression. Normal fibroblasts were anchorage dependent, grew as very elongated cells at confluency, and showed contact inhibition (Fig. 1A). Conversely, chondrocytes were spherical and grew in suspension as was illustrated by their birefringent halo (Fig. 1B). PMA-treated fibroblasts lost their normal growth pattern, showed no contact inhibition and in many areas of the tissue culture dishes formed foci of clumped cells (Fig. 1C). When chondrocytes were cultured in the presence of PMA the cells behaved in an opposite fashion to fibroblasts. They attached to the culture dishes and flattened out, but they displayed no discernable growth pattern or contact inhibition (Fig. 1D). When PMA was withdrawn from the chondrocytes, the cells continued to adhere to the culture dishes, but they took on a polygonal appearance identical to differentiated vertebral chondrocytes (Fig. 1E). However, about 30% of these cells showed an elongated and fibroblastic appearance.

The morphological changes can be correlated with the levels of β-and γ-actin mRNAs in embryonic chicken fibroblasts and chondrocytes. The relative amounts of β-actin and total actin mRNAs in total RNA isolated from fibroblasts are shown in Fig. 2. The cDNA complementary to the 3′ untranslated sequence of chicken β-actin mRNA, a sequence not conserved between isotypes and previously shown to be isotype specific in its hybridization (9), was used to hybridize to fibroblast RNA (Fig. 2). A single major band of hybridization is seen in the RNA obtained both from control fibroblasts and from leg muscles. When the same blot was rehybridized with the cDNA (PHF-1) containing the conserved actin protein-coding sequence, a slightly larger as well as several smaller mRNA species were visible in the muscle RNA (Fig. 2). These were identified as γ-actin and the cardiac and skeletal α-actin mRNAs, based on their respective molecular weights (31, 35). Only one band, corresponding to β-actin mRNA hybridized strongly in total RNA isolated from fibroblasts. This experiment showed the specificity of the β-actin cDNA probe as well as the ability of the total actin probe to cross-hybridize, thus making it possible to demonstrate that fibroblasts isolated from embryonic chicken muscle contain predominantly β-actin mRNA.

The levels of β-actin mRNA were greatly reduced in total RNA isolated from embryonic chicken fibroblasts treated for 4 days with PMA (Fig. 2). However they were not replaced with γ-actin mRNAs. In contrast, there were only very low levels of β-actin mRNAs in RNA isolated from normal embryonic chicken sternal chondrocytes, and very long exposures of the hybridizations were required to detect it (Fig. 3). Within 2 days after PMA treatment of sternal chondrocytes a 5-fold increase in β-actin mRNAs was observed, and after 4 days the near maximal 10-fold increase was obtained (See Fig. 6). After PMA was withdrawn from the chondrocytes, they remained attached to the culture dishes and exhibited an altered polygonal morphology characteristic of vertebral chondrocytes, but β-actin mRNA levels were again greatly reduced (lanes denoted as reversion in Fig. 3).

The increased β-actin mRNA accumulation paralleled but did not precede the morphological changes of these cells since, by day 2 of PMA addition, 95% of the cells had adhered to the culture dishes. The increased β-actin is not

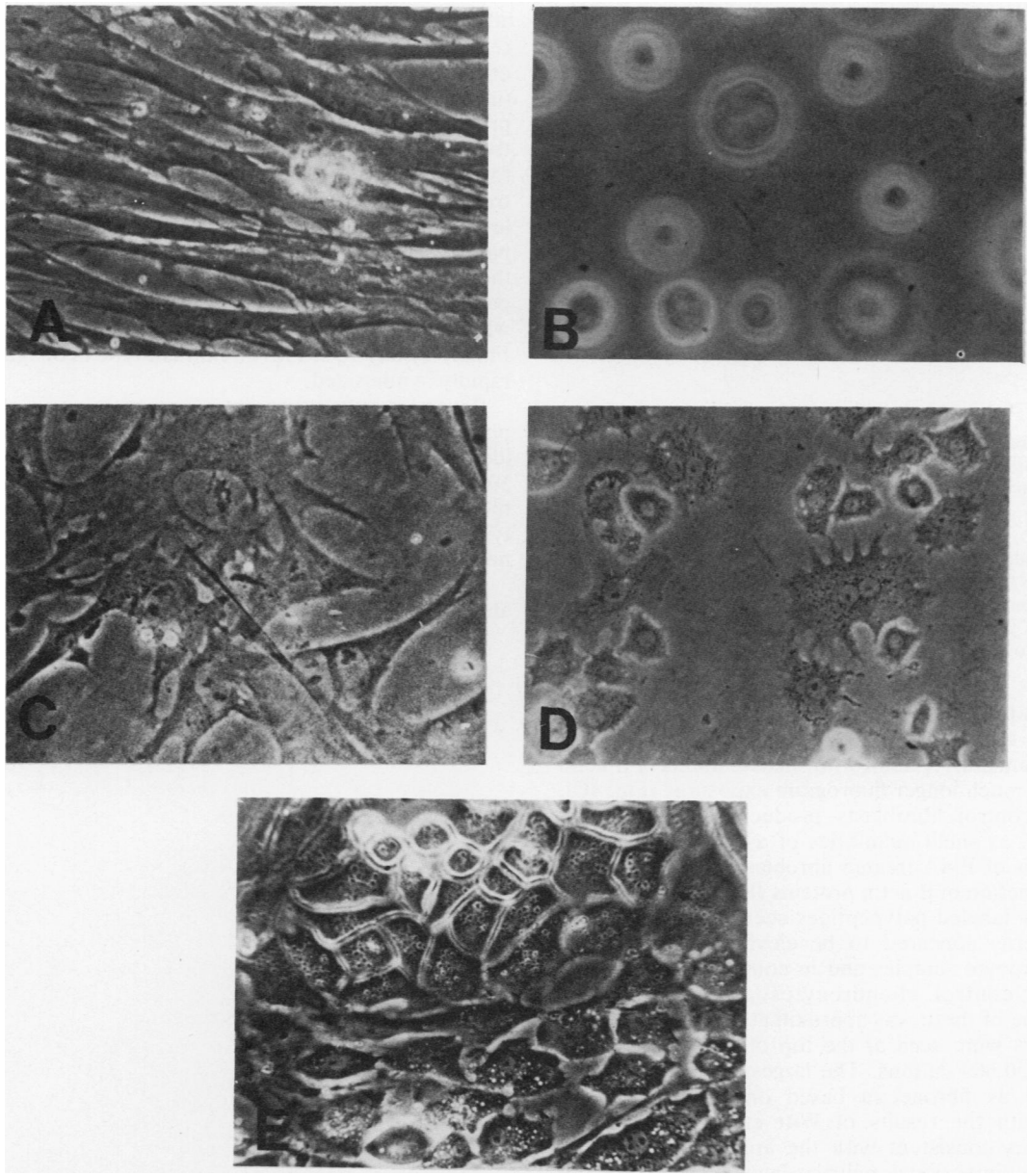


FIG. 1. Effect of PMA on fibroblast and chondrocyte morphology. (A) Normal fibroblasts after 4 days in culture. (B) Normal chondrocytes after 4 days in culture. (C) Fibroblasts after 4 days of PMA treatment. (D) Chondrocytes after 4 days of PMA treatment. (E) Chondrocytes grown for 4 days in the presence of PMA and then withdrawn and grown an additional 8 days (revertant cells). All photographs are magnified at ×225.

related to cell proliferation since there was no cell proliferation during the first 4 days of PMA treatment. Sternal chondrocytes do contain relatively high levels of γ-actin mRNA, however, since control chondrocyte mRNA had very strong hybridization to the total actin probe (data not shown). The levels of γ-actin mRNA may be determined from taking the difference between the total actin mRNA quantities and the β-actin mRNA levels (see Fig. 6). Such an analysis shows that the γ-actin levels remained at about 15 to 30% of the total actin mRNA levels of the cells at all of the experimental points examined.

To determine whether the mRNA levels reflected the amounts of actin protein synthesis, cell cultures were pulse-labeled for either 1 or 24 h with [^{3}H]leucine. Total actin synthesis was initially assessed for the 1-h pulse-labeling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) and quantified by scanning densitometry (see Fig. 6). The various actin isotypes were resolved by two-dimensional gel electrophoresis of the 24-h-labeled samples. Each radiolabeled sample was analyzed by isoelectric focusing in the presence of 30 μg of unlabeled purified chicken actins. In this fashion, the major actin isotypes could first be identified by Coomassie staining. The subsequent fluorograms of the labeled samples were then identified relative to the stained standard. Three distinct isoelectric variants could be resolved in the Coomassie-stained standard and were identified as γ, β, and α in the direction of the isoelectric focusing. This identification is completely consistent with many previous reports (15, 43, 47).

Control chondrocytes synthesized predominantly γ-actin,

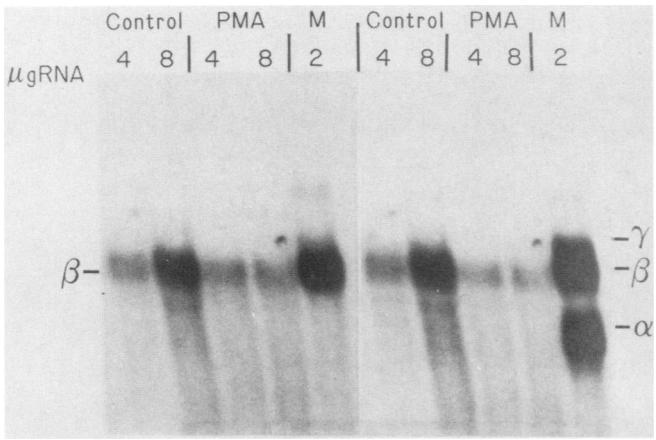


FIG. 2. Northern blot analysis of steady-state actin mRNA levels in control fibroblast or PMA-treated cells. β-actin mRNAs were identified by hybridization of total RNA from normal or cells treated with PMA for 4 days to PA2 (left panel), and total actin mRNAs were identified by hybridization of the same blot with pHF-1 (right panel). The amount (micrograms) of RNA applied to the gel and the source of the RNA sample are denoted in the figure. M, Polyadenylated mRNA from 17-day-old chicken embryo legs. Positions of migration of γ-, β-, and α-actin MRNAs are indicated. Exposure time of both autoradiograms was 2 days.

whereas PMA-treated cells synthesized both cytoskeletal variants (Fig. 4A and B). Revertant chondrocytes again showed predominantly γ-actin with small amounts of β-actin observable on much longer fluorogram exposures (Fig. 4C). In contrast, control fibroblasts produced high levels of β-actin as well as small quanitities of α-actin (Fig. 4D). A similar analysis of PMA-treated fibroblasts demonstrated a significant reduction of β-actin proteins (Figure 4E). Several other intensely labeled polypeptides seen in the isoelectric focusing patterns appeared to be elevated in the PMA-treated chondrocyte samples and in control fibroblasts but decreased in control chondrocytes and PMA-treated fibroblasts. One of these was approximately 50,000 daltons, and two others were seen at the top of the gel and were greater than 150,000 daltons. The largest of these proteins was identified as fibronectin based on its size and by comparison with the results of Witt et al. (47), and its appearance was consistent with the appearance of fibronectin on one-dimensional polyacrylamide gels (data not shown).

Alterations in β-actin gene expression may be related to extracellular matrix gene expression. Because of the possible interaction between microfilaments and fibronectin (21), we also examined the levels of fibronectin mRNAs and protein synthesis during PMA treatment of chondrocytes. Analysis of newly synthesized protein in 1-h pulse-labeling of cells demonstrated that very small amounts of fibronectin proteins may first be detected by day 4 of PMA treatment and increased to maximal levels by day 12. In revertant cells intermediate levels were detected. Analysis of the fibronectin mRNA levels is shown in Fig. 5. The induction of fibronectin mRNAs was very similar to that of β-actin mRNAs. However, full expression of the protein was not seen until PMA treatment for 12 days.

A compilation of the data obtained from the chondrocytes for actin and fibronectin is presented in Fig. 6, and it was compared to the data obtained for type I and type II collagen (14). Such a comparison revealed several interesting correlations. The actin, fibronectin, and type I collagen mRNAs all appeared earlier than did the proteins. This probably reflects two factors: (i) the difference between the sensitivity of Northern blot hybridizations and in vivo cell labeling and (ii) the lag between mRNA synthesis and its utilization for protein synthesis. The second aspect of this comparison is the temporal sequence of the mRNA and protein synthesis for these three proteins. During the first 4 days of PMA treatment, collagen type II was reduced to barely detectable levels and its mRNA was reduced fivefold. This was accompanied by a rapid change in cell morphology which preceded the appearance of β-actin and fibronectin mRNAs. These proteins began to appear in detectable levels by day 4, at which time type I collagen mRNAs were first detected. Finally by day 12 of PMA treatment type I collagen was very rapidly synthesized.

Revertant cells represent a unique mixture of cell morphologies, in which 70% of the cells took on an appearance identical to vertebral chondrocytes (29), whereas the other 30% of these cells were elongated and fibroblastic. It is therefore interesting to note that these cells produced both type I and type II collagen and contained intermediate fibronectin and actin levels.

Is cell shape related to collagen phenotype? The intermediate levels of β-actin and fibronectin and the coexpression of

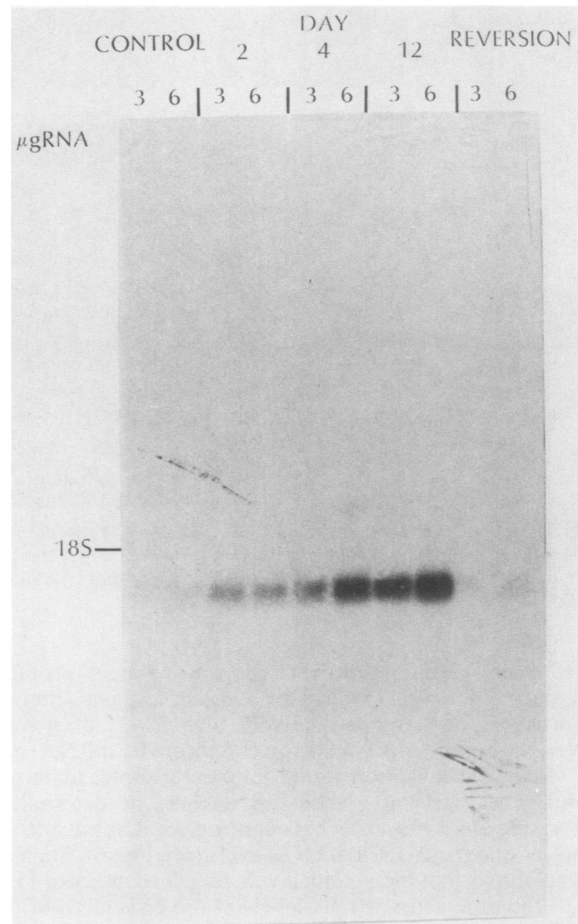
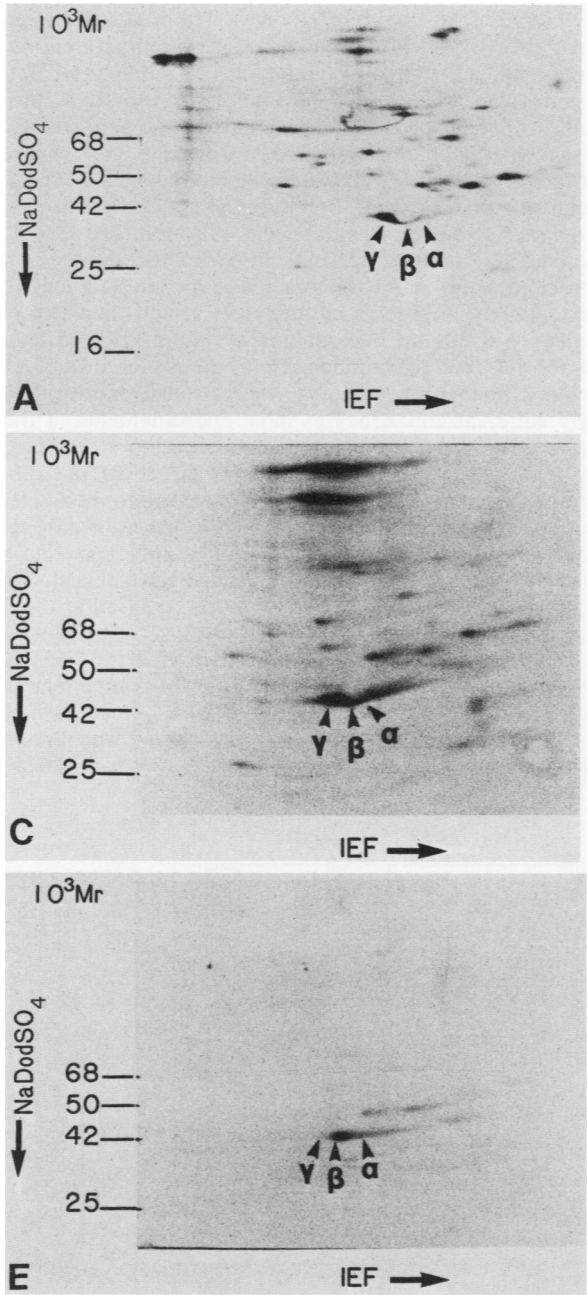


FIG. 3. Northern blot analysis of the steady-state β-actin mRNAs in sternal chondrocytes at various times after PMA addition to the cultures. Micrograms of total RNA applied to the gel and RNA sample are denoted in the figure. Exposure time of the autoradiogram was 2 days.



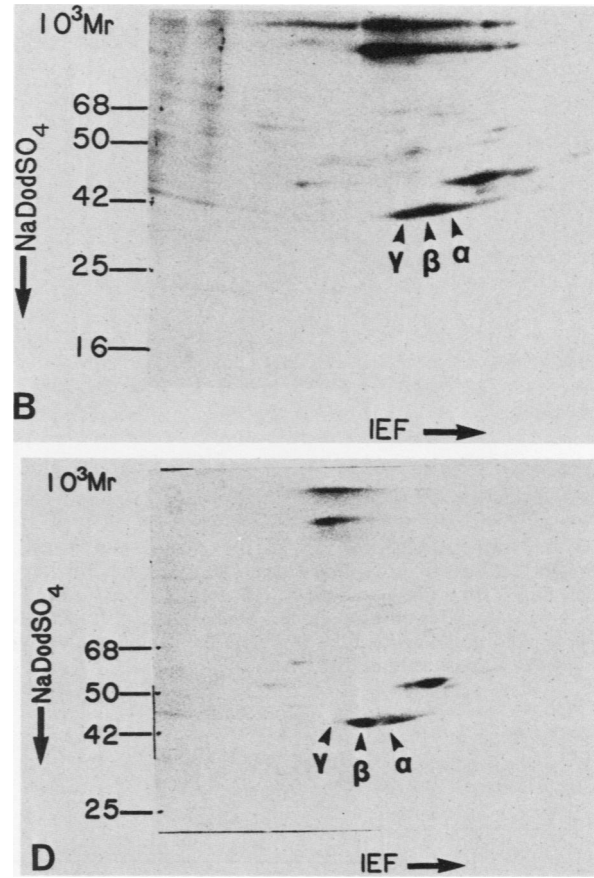


FIG. 4. Two-dimensional protein analysis of actin isotypes from control and PMA treated chondrocytes and fibroblasts. (A) Fluorogram of the tritiated proteins isolated from control chondrocytes. (B) Fluorogram of the tritiated proteins isolated from chondrocytes treated for 12 days with PMA. (C) Fluorogram of the tritiated proteins isolated from revertant chondrocytes. (D) Fluorogram of the tritiated proteins isolated from control fibroblasts. (E) Fluorogram of the tritiated proteins isolated from PMA-treated fibroblasts. All fluorograms were exposed for 5 days. IEF, Isoelectric focusing.

both collagen types in the revertant cultures suggested that they contained two discrete populations of cells. To determine whether the two cell populations were biochemically unique and whether there was a relationship between cell shape and collagen phenotype, the cells were reacted with either type I or type II collagen antibodies. They were then counter-reacted with a rhodamine-conjugated second antibody and examined by fluorescence microscopy. The results of this experiment are shown in Fig. 7. The cells were reacted with type II antibodies (Fig. 7A), and the accompanying phase contrast micrographs of the same field are shown in Fig. 7B. Only the polygonal or cells appearing like normal chondrocytes reacted with the type II collagen antibody. In contrast, Fig. 7C and D show a field which was reacted with type I collagen antibody and its accompanying light field. Predominantly the fibroblastic cells reacted with this antibody, although some polygonal cells also reacted. These results demonstrate that there is a significant but not exclusive relationship between cell shape and collagen phenotype.

DISCUSSION

By comparing the effect of PMA on actin expression in chondrocytes and fibroblasts, we obtained the first evidence of differential patterns of expression of γ and β-actin isotypes which appears to be related to cell shape. Fibroblasts derived from myoblast preplates adhered to culture dishes and almost exclusively expressed β-actin, whereas floating sternal chondrocytes predominantly expressed γ-actin. Since fibroblasts have stress fibers (34, 37) and floating sternal chondrocytes do not (30), our results would indicate that some actin microfilaments are composed of primarily β-actin.

PMA-treated fibroblasts have decreased cell adhesion, exhibit a dramatic decrease in their steady-stage mRNA levels of β-actin gene expression, and have reduced type I collagen protein synthesis and steady-state mRNA levels (6;

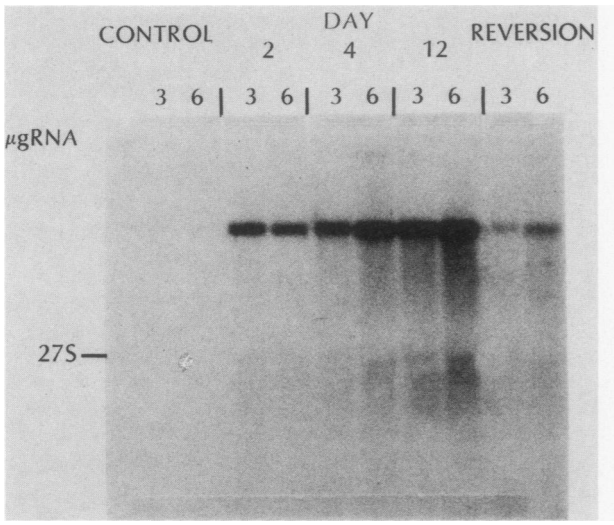


FIG. 5. Northern blot analysis of the steady-state levels of fibronectin mRNAs in total RNA from sternal chondrocytes at various times after PMA treatment. The blot was hybridized to FC36, a genomic fibronectin clone. Micrograms of total RNA sample applied to the gel and the source of the sample are denoted in the figure. Exposure time was 1 day.

L. C. Gerstenfeld, unpublished data). The role of β-actin in normal growth control, cell adhesion, and contact inhibition is clearly implicated in a clonal neoplastic cell line (HUT 14) derived from chemically mutagenized human diploid fibroblasts. These cells exhibit a single amino acid substitution in their β-actin. A subclone of the original line exhibited a progressive increase in tumorigenicity, had a second undefined mutation in its β-actin gene product, and interacted with fibronectin in an abnormal fashion (25, 42, 43).

Conversely, floating sternal chondrocytes treated with PMA will cease to make their cartilage-specific extracellular matrix, flatten, adhere to culture dishes, and then express high levels of β-actin. Microfilament organization in chondrocytes has been examined morphologically during cell spreading and Rous sarcoma virus transformation (30). Newly adherent chondrocytes have microfilaments at their peripheral adherent surface and then develop stress fibers when they flatten. After Rous sarcoma infection, the chondrocytes reorganize their actin filaments into distinctive structures, or "actin flowers," which are on their adherent surface and appear to accumulate vinculin. The role of microfilaments in dedifferentiation has been suggested by Benya and Shaffer (4, 5). They found that adult rabbit articular chondrocytes dedifferentiated by serial subcultivation in monolayers can be redifferentiated if the cells are grown in agarose suspension or if actin microfilament polymerization is blocked by treatment with cytochalasin B. The latter result is of particular interest since it was recently shown that β-actin is the specific target of cytochalasin B

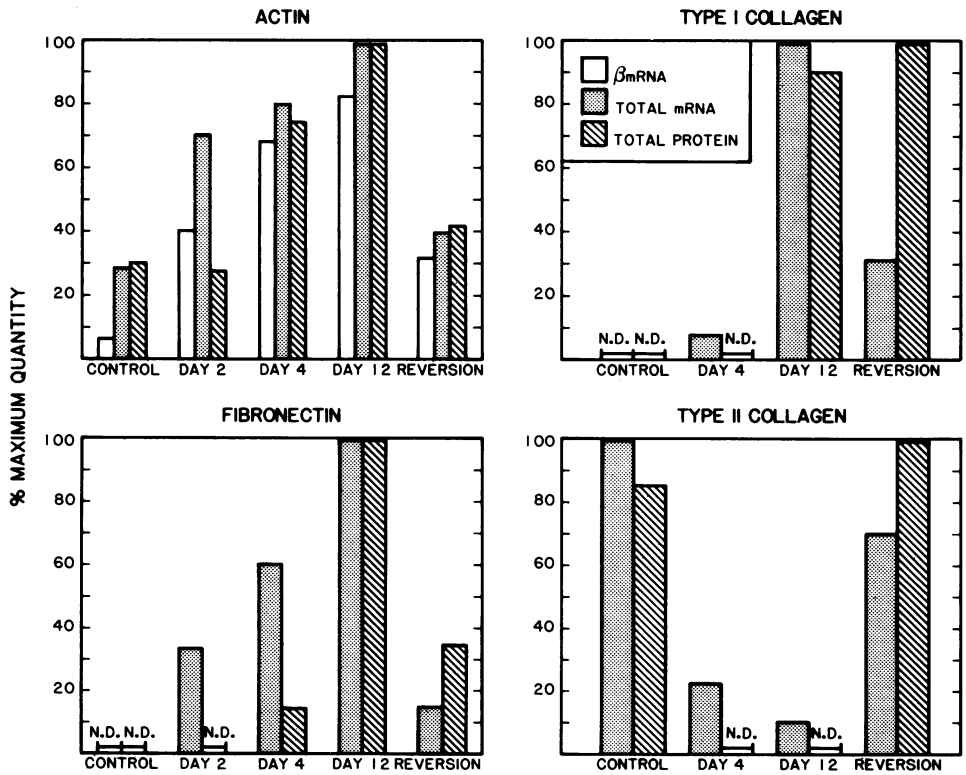


FIG. 6. Comparative analysis of the relative mRNA and protein levels for fibronectin, β and total actins, and types I and II collagens in control and PMA-treated chondrocytes. Relative protein and mRNA levels were determined as the percentage of the maximal quantity determined for collagen fibronectin and total actin. β-actin mRNA was expressed as a percentage of the total actin mRNA hybridization. The latter was determined by normalizing the hybridization of the two actin probes with respect to probe length, specific activity, and autoradiographic exposure time. It was assumed that the actin probes hybridize with equal efficiency under the conditions used. The values for collagen types I and II were derived from the data of Finer et al. (14). N.D., Not detected.

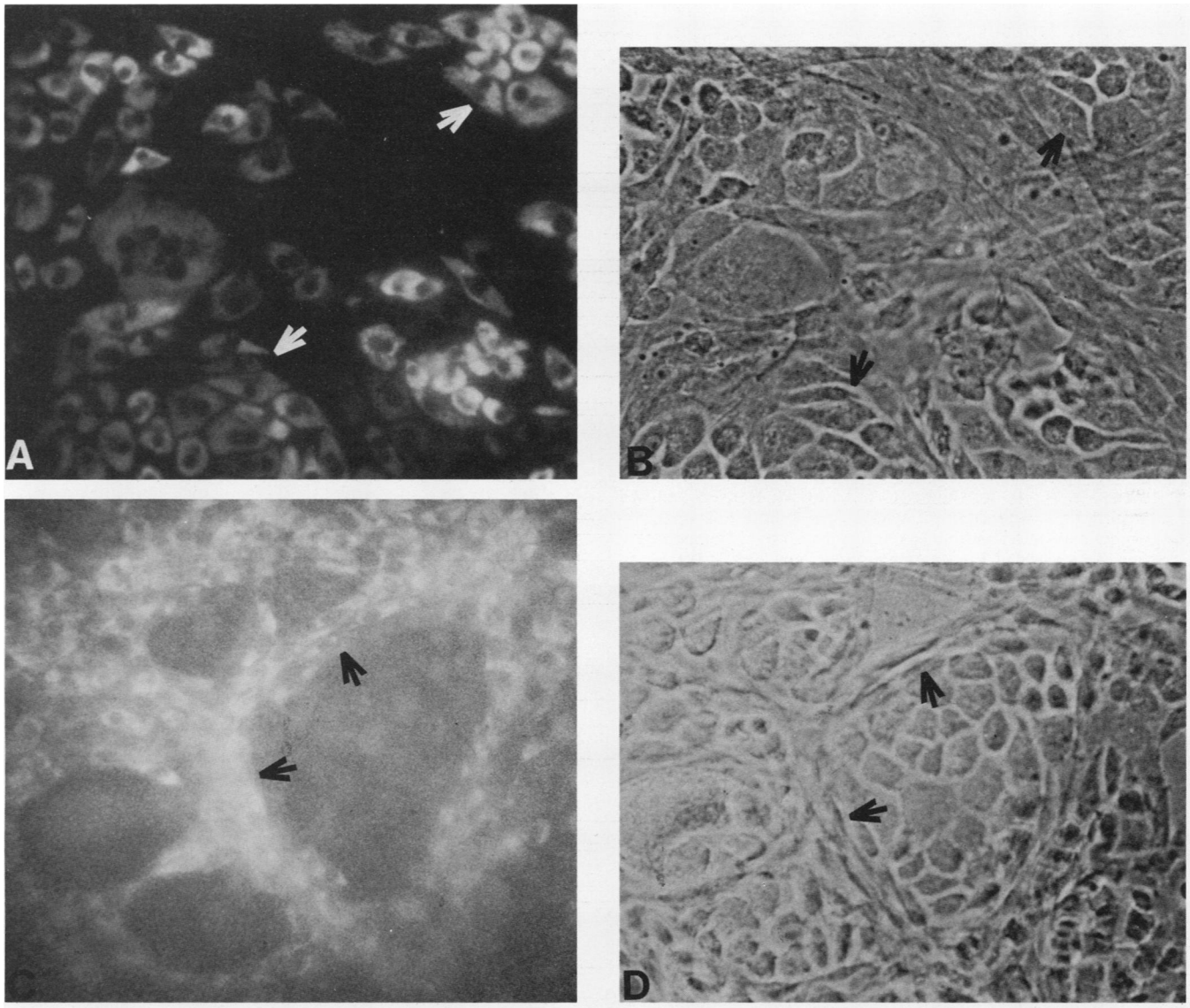


FIG. 7. Immunofluorescence of types I and II collagen in revertant chondrocyte cultures. (A) Type II immunofluorescense displayed only by the polygonal shaped cells (arrows). (B) Light field of the same area of the cells shown in panel A. (C) Type I immunofluorescence displayed by the predominantly fibroblastic shaped cells (arrows). (D) Light field of the same area of cells shown in panel C. Compare the positions of the arrows between panels A and B and between panels C and D.

(41). In contrast, a methylcellulose suspension has been used to disrupt the differentiated phenotype of mouse 3T3 fibroblasts. In this case actin protein synthesis was reduced twofold with no change in actin mRNA levels (12). Our results with PMA-treated fibroblasts suggest both pretranslational and translational regulation, in which β-actin mRNA levels are reduced but the mRNAs are apparently more efficiently translated.

By examining the temporal events during chondrocyte growth in PMA, the first changes which we detected were morphological. Suprisingly, these changes occurred several days before the expression of either the β-actin or fibronectin. Therefore, either the activation of these genes or the stabilization of their mRNAs and subsequent expression probably represents an intermediate step in a series of events which PMA has mediated. Indeed, the immediate action of PMA is the activation of C-protein kinase, which has been shown to phosphorylate a wide variety of proteins (18, 32). Recently Fey and Penman (13) have demonstrated that the disorganization of intermediate filaments is the earliest event which may be related to altered cell morphology in epithelial cells, and this is independent of protein synthesis. Thus, the disruption of the intermediate filaments may be responsible for the initial morphological changes observed and the loss of contact inhibition which has also been suggested as an effect of PMA (32). The onset of type I collagen synthesis, which is a very late event in the PMA-treated cells, may require the prior production of β-actin and fibronectin. The interaction of the extracellular matrix and the intracellular microfilaments has been observed indirectly as contiguous regions of immunofluoroscence between anti-actin and anti-fibronectin at the cell membranes (21). Our results would suggest that this interaction is between β-actin and fibronectin and that it is the

first event in the formation of a fibroblastic extracellular matrix of PMA-treated chondrocytes. Conversely, the loss of a fibroblastic matrix in PMA-treated fibroblast results in a concomitant decrease in β-actin and fibronectin expression.

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