Microfilament Modification by Dihydrocytochalasin B Causes Retinoic Acid–modulated Chondrocytes to Reexpress the Differentiated Collagen Phenotype without a Change in Shape

Paul D. Benya, Peter D. Brown, and Silvia R. Padilla

Bone and Connective Tissue Research Program, Department of Orthopedics, Orthopedic Hospital, University of Southern California, Los Angeles, California 90007-2697

Abstract. Primary monolayers of rabbit articular chondrocytes synthesize high levels of type II collagen and proteoglycan. This capacity was used as a marker for the expression of the differentiated phenotype. Such cells were treated with 1 μg/ml retinoic acid (RA) for 10 d to produce a modulated collagen phenotype devoid of type II and consisting of predominantly type I trimer and type III collagen. After transfer to secondary culture in the presence of RA, the stability of the RA-modulated phenotype was investigated by culture in the absence of RA. Little reexpression of type II collagen synthesis occurred in this period unless cultures were treated with 3×10^{-6} M dihydrocytochalasin B to modify microfilament structures. Reexpression of the differentiated phenotype began between days 6–8 and was essentially complete by day 14. Substantial reexpression occurred by day 8 without a detectable increase in cell rounding. Colony formation, characteristic of primary chondrocytes, was infrequent even after reexpression was complete. These data suggest that the integrity of microfilament cytoskeletal structures can be a source of regulatory signals that mechanistically appear to be more proximal to phenotypic change than the overt changes in cell shape that accompany reexpression of subculture-modulated chondrocytes in agarose culture (Benya, P. D., and J. D. Shaffer, 1982. *Cell.* 30:215–224).

CHONDROCYTE differentiated functions include the synthesis, deposition, and maintenance of an extracellular matrix composed of cartilage-specific collagens and proteoglycans. Analysis of the transitions in synthesis of these macromolecules to other genetically distinct collagens and proteoglycans has demonstrated the phenotypic flexibility of chondrocytes in culture (7). Modulation of the differentiated phenotype occurs after subculture (13), growth to senescence (32), and treatment with retinoic acid (RA)[1] (8, 51), 5-bromo-2′-deoxyuridine (BrdU) (33, 42), PMA (18), and temperature-sensitive virus (1, 3). In several of these cases modulation can be reversed (1, 10, 18). Using rabbit articular chondrocytes, we have previously demonstrated essentially complete reexpression of the differentiated collagen phenotype when subculture-modulated cells respond to an overt change in shape, from spread/attached to spherical, that accompanies the transition from monolayer to agarose gel culture (10). Thus, chondrocytes (10, 52), their mesenchymal precursors (41), and other shape-responsive expression systems, i.e., preadipocytes (46), synovial fibroblasts (2, 48), and mammary epithelial cells (21), may share similar regulatory mechanisms.

To investigate the possible role of modifications in microfilament architecture as a mediator in shape-dependent reexpression of chondrocytes, we have tried to separate these events and identify microfilament modification as an independent signal for reexpression. Dihydrocytochalasin B (DHCB) was used as a specific perturbant of microfilament organization (4) at a dose previously determined to minimize cell rounding while permitting reexpression of subculture-modulated chondrocytes (11). RA-modulated chondrocytes were used as an experimental system because they produce no type II collagen, exhibit a different modulated phenotype than subculture-modulated cells, and are flattened and tightly adherent (8).

We report here that RA-modulated chondrocytes exhibit nearly complete reexpression of the differentiated collagen phenotype only after removal of RA and culture in the presence of DHCB for 14 d. During the first 8 d of microfilament modification (14) ~50% reexpression occurs without apparent cell rounding. This suggests that microfilament modification can be a sufficient signal for phenotypic change and may mediate the effects of alterations in cell shape. In monolayer culture, however, this signal does not induce type II collagen synthesis in all cells as determined by collagen-type specific immunofluorescence. We also report that reexpression in this system requires the presence of 10% FBS; 1 or 2% serum will not support this transition.

Address reprint requests to Paul D. Benya, Bone and Connective Tissue Research Program, Orthopedic Hospital, 2400 S. Flower St., Los Angeles, CA 90007-2697. Peter D. Brown's current address is Laboratory of Chemoprevention, National Cancer Institute, Bldg. 41, Rm. C629, Bethesda, MD 20892.

1. *Abbreviations used in this paper*: BrdU, 5-bromo-2′-deoxyuridine; DHCB, dihydrocytochalasin B; RA, retinoic acid.

Materials and Methods

Cell Culture

Chondrocyte monolayer cultures were established (12, 13) from the articular cartilage of the humeral head of 8-wk-old New Zealand white rabbits. Cells were plated at 6.0×10^4 cells/cm^2. The growth medium was DME, high-glucose modification (Gibco, Santa Clara, CA), containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) and 50 μg/ml gentamycin. Treatment with RA was initiated on the fourth day of culture. All-trans RA (Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethanol at 1.0 mg/ml and held as a stock solution at −20°C in the dark. This solution was diluted with growth medium each day before the cultures were fed. Control and treated cultures were fed every other day after the fourth day. After 10 d of RA treatment, RA-modulated cells were transferred to secondary culture and plated in the presence of RA at 2.8×10^4 cells per cm^2. When confluent cultures were obtained after 4 d, RA was removed to begin the reexpression period. During this time cultures were maintained in 10%, or 1% or 2% serum, in the absence or presence of 3×10^{-6} M DHCB (Sigma Chemical Co.). Cultures maintained in 1% serum were treated with 10% serum for 1 d before the labeling period to equilibrate the levels of protein synthesis with those in cultures maintained in 10% serum.

DNA and Proteoglycan Synthesis

Proteoglycan synthesis and DNA content were measured in aliquots of pronase digests of combined medium and cell layers as cetyltrimethylammonium bromide-precipitable $^{35}SO_4$ and diaminobenzoic acid-derived DNA fluorescence, respectively (8).

Collagen Synthesis

Duplicate cultures were labeled for 24 h with L-[5-^{3}H]-proline (20–50 Ci/mmol, 50 μCi/ml; Amersham Corp., Arlington Heights, IL) in growth medium supplemented with β-amino-proprionitrile (62.5 μg/ml) and ascorbate (50 μg/ml). Whole cultures (medium and cell layer) were stored frozen, thawed to 4°C, adjusted to 0.5 M acetic acid and treated with 1 mg/ml pepsin (PM grade; Cooper Biomedical, Malvern, PA) for 24 h at 4°C. Tris base was added to 0.1 M and the pH was adjusted with NaOH to 8.0 to inactivate the pepsin. After adjustment to 200 μg/ml carrier collagen, 0.5 M NaCl, and 2 M urea, samples were extracted for 1 h at 4°C, removed from the culture flasks, and dialyzed against 1 M NaCl, 50 mM Tris, pH 7.5 to remove unincorporated label. Cellular debris was removed by centrifugation and the supernatants were dialyzed overnight against 0.4 M NaCl, 50 mM Tris, pH 7.5, before collagen precipitation by addition of an equal volume of 20% polyethylene glycol 8000 (J. T. Baker) and overnight incubation at 4°C (modified from reference 40). Collagen precipitates were dissolved in 0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, and dialyzed against 0.5 M acetic acid before counting to determine total collagen synthesis and analysis by SDS-PAGE.

SDS-PAGE

We fractionated lyophilized samples of purified radioactive collagen in composite linear-gradient acrylamide slab gels using the SDS sample and gel buffers described by Laemmli (31). Composite gels were made from a stock solution containing 30% acrylamide, 0.8% bis, by combining 1 vol of 20% acrylamide in a closed mixing outlet chamber, 1.5 vol of 3% acrylamide in an open supply chamber, and using the entire contents of both chambers for each slab gel. Samples were electrophoresed, fixed in 20% TCA, and processed for fluorography as previously described (9).

2-D CNBr Peptide Mapping

Purified collagen was precipitated from 0.5 M acetic acid with 2.5 M NaCl to remove polyethylene glycol and dialyzed against 0.5 M acetic acid before lyophilization. Samples were suspended in 500 μl of 25% β-mercaptoethanol, 0.2 M ammonium bicarbonate, pH 7.8, adjusted with acetic acid, and incubated overnight at 60°C (5). This procedure completely reduced the oxidized methionines caused by contaminants in the polyethylene glycol, as verified by the expected low level of peptides incompletely cleaved by CNBr (see Fig. 5). CNBr cleavage (9) was followed by two-dimensional (2-D) CNBr peptide mapping which consisted of nonequilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE using Tris-borate buffers in the second dimension (6). Peptide spots in 2-D maps were quantitated in the linear range as total integrated optical density using the Visage machine vision image analyzer (Bio Image Corp., Ann Arbor, MI), which has been previously described (37) and utilized for 2-D mapping analysis (43). The resultant data were used to calculate the synthesis per microgram DNA of collagen types I, II, and III as described in the legend to Fig. 5.

Collagen Immunofluorescence

Cells were cultured in chamber slides (14), incubated at 37°C with 1 μM monensin (Sigma Chemical Co.) for 6 h to enhance the intracellular accumulation of collagen (36), washed with PBS, and fixed in 3.7% paraformaldehyde in PBS at 37°C for 10 min. After methanol treatment at −20°C for 6 min and air drying, slides were incubated with PBS before treatment with hyaluronidase (Cooper Biomedical) 1,000 U/mg, 1 mg/ml in PBS, for 40 min at 37°C. Type II collagen-specific mouse mAb (clone C2) was generously provided by K. Rubin and has been previously characterized (25). Cells were incubated for 1 h at 37°C with ascites fluid diluted 1:50 with 1 mg/ml BSA in PBS. This was followed by washing, incubation with biotinylated second antibody, and detection with Texas Red–labeled streptavidin as described (14).

Results

Morphology

After 4 d of secondary culture in the presence of RA, confluent RA-modulated chondrocytes were evaluated for their capacity to reexpress the differentiated phenotype during a 21-d reexpression period in the absence of RA. Cultures were maintained in medium that contained 10% FBS without or with DHCB. The dose of DHCB, 3×10^{-6} M, was chosen because it maximized reexpression and minimized cell rounding in subculture-modulated chondrocytes (11). Representative phase contrast micrographs of RA-modulated chondrocytes during the reexpression period (Fig. 1) demonstrate that DHCB did not alter the degree of cellular rounding during the first 8 d of exposure and only slightly modulated the shape of the cellular footprint to a more elongate form (Fig. 1, *a* and *b*), less fibroblastic than that of subculture-modulated chondrocytes (13). Substantial reexpression of the collagen phenotype occurred in this 8-d period (see next section). The morphology of nearly all DHCB-treated cells was maintained at 14 d when collagen phenotypic reexpression was essentially complete. Colony formation, characteristic of differentiated primary chondrocyte cultures, was only occasionally observed at this time (Fig. 1 *c*), but increased slightly by 21 d of treatment (Fig. 1 *d*).

Proteoglycan and Collagen Synthesis

During the reexpression period these confluent cultures exhibited only small changes in DNA content (Fig. 2). The gradual increase observed in untreated cultures was arrested in DHCB-treated cultures after 8 d. The maintenance of DNA content over the extended exposure to DHCB verifies the utility of the dose and supports the continued adherence of spread cells presented in Fig. 1.

At the beginning of the reexpression period proteoglycan synthesis per microgram DNA by cells in RA remained at the same low level as RA-treated cells in primary culture (Fig. 2 and unpublished data). The eightfold greater synthesis observed in primary control cells (8) was reduced to threefold in secondary control cells (Fig. 2 and unpublished data) due to the decline in proteoglycan synthesis that accompanies subculture-dependent modulation of the chondrocyte

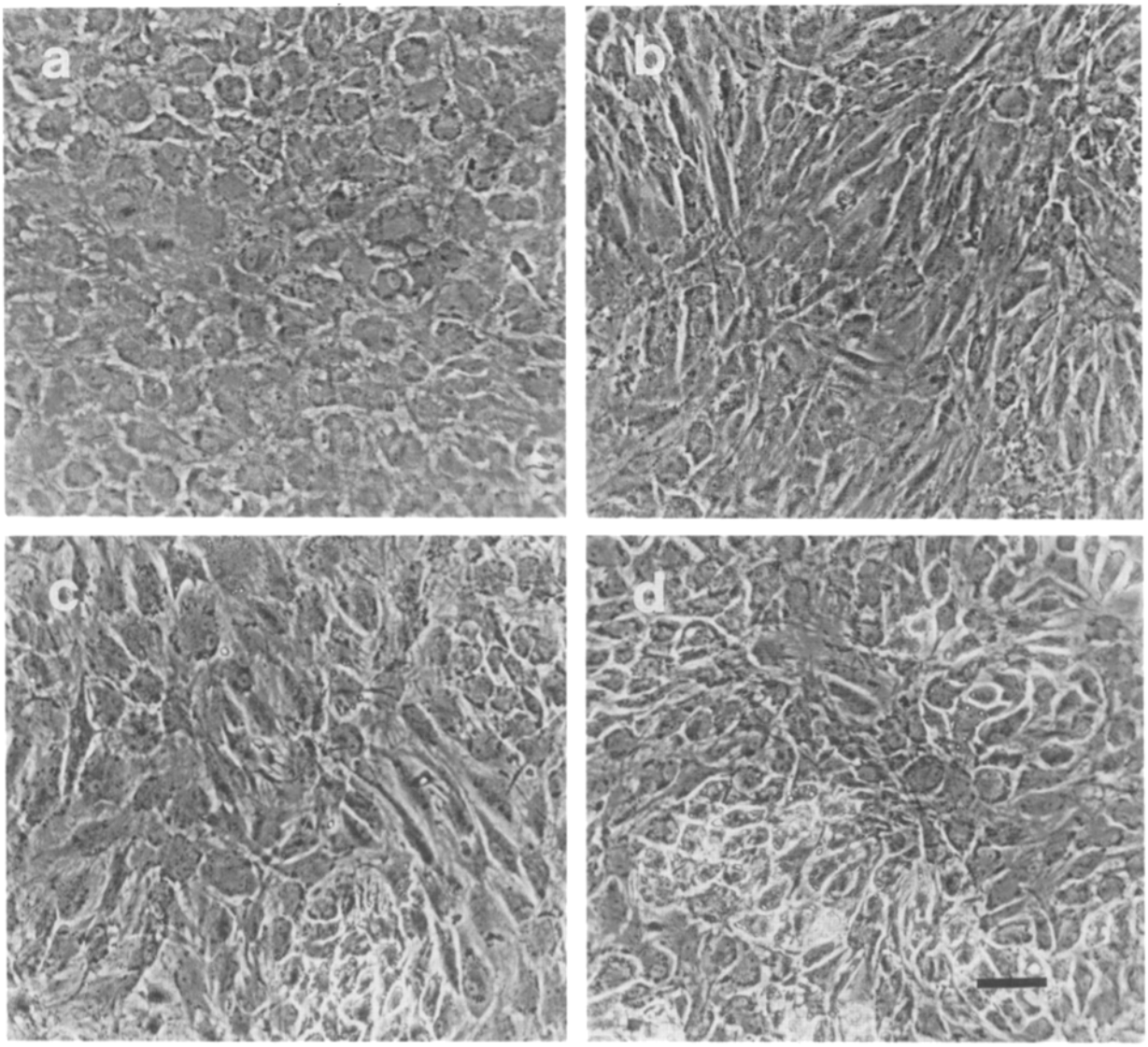

Figure 1. Phase contrast micrographs of RA-modulated chondrocytes in secondary culture at various times after removal of RA. After 8 d without DHCB (*a*); paired 8-d culture treated with 3×10^{-6} M DHCB (*b*); after 14 (*c*) and 21 (*d*) days of DHCB treatment. The field in (*d*) was not representative but was chosen to demonstrate colony formation, which was infrequent. Bar, 100 μm.

phenotype (10). After removal of RA, DHCB-treated cells exhibited a steady increase in proteoglycan synthesis, first noticeable after 4 d and reaching a maximum after 14 d of 2.5-fold above that of cells cultured in the absence of DHCB. The final level was above that exhibited by control chondrocytes at the beginning (Fig. 2) and the end (unpublished data) of the reexpression period. This suggests a quantitative return of this characteristic of differentiated chondrocytes.

Collagen synthesis per microgram DNA in RA-modulated cells at the beginning of the reexpression period was only slightly less than that in untreated control cells (Fig. 3). The eightfold lower rate observed in such cells in primary culture (8) was not retained due to the subculture-dependent reduction in type II collagen synthesis by control chondrocytes (13) and the fact that secondary RA-modulated chondrocytes produce collagen approximately fivefold faster than primary chondrocytes treated with RA for 10 d (unpublished data). The stimulated synthesis in RA-treated secondary cells reflects the recovery of enhanced type III synthesis (8) producing the high type III:I-trimer ratios seen in Figs. 4 and 6 at the beginning of the reexpression period. When RA was removed and cells were cultured without DHCB, collagen synthesis was maintained until day 4 and then declined, reaching a minimum 10-fold less than the initial value at 21 d. DHCB-treated cells exhibited a marked decrease in synthesis after 2 and 4 d and then nearly regained the initial rate by 14 d. Collagen synthesis in DHCB-treated cultures was greater than in untreated cultures beginning on day 6 and reached a maximum of eightfold greater than such cultures by 21 d. After 14 d of reexpression, synthesis in DHCB-treated cultures was slightly less than in control cultures (never treated wth RA) at the beginning of reexpression (Fig. 3) but equal to or greater than control cultures at 14 d (two separate studies).

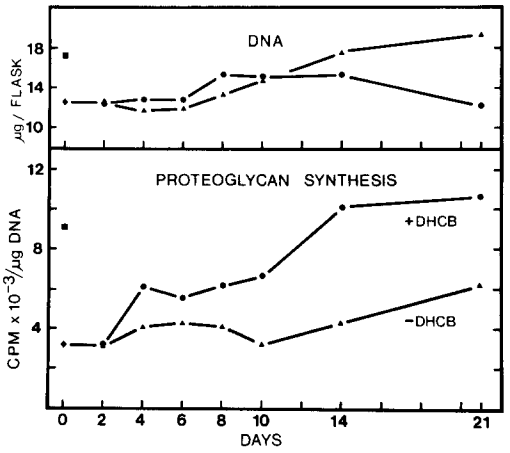

Figure 2. DNA content and proteoglycan synthesis during the 21-d reexpression period that followed removal of RA. Synthesis/DNA content of RA-modulated cells in the presence of RA at the beginning of the reexpression period (*diamond*); of secondary control cells never treated with RA (*square*); and of RA-modulated cells cultured in the absence of RA without (*triangles*) and with (*circles*) DHCB treatment. Data represent the average of duplicate cultures. The mean of the ranges for the DNA data is ±7%, and for the proteoglycan data, ±18%.

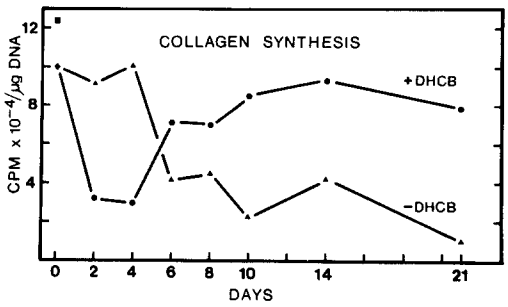

Figure 3. Collagen synthesis during the reexpression period. Data are identified as in Fig. 2 and represent the average of duplicate cultures. The mean of the ranges is ±11%.

Collagen Phenotype

A proportional analysis of the change in collagen phenotype during the RA-free reexpression period is presented in Fig. 4 based on separation of intact collagen chains by SDS-PAGE. Variation in the ratio of type III collagen to α1-chains occurred during the first 6 d in both untreated and DHCB-treated cultures. The initiation of type II synthesis was first detected on day 8 in DHCB-treated cultures. This is most clearly seen in the underexposed inset, where the more abundant α1(II)-chains migrate slightly faster than the α1(I)-chains of type I trimer. At later times the abundance of α1(II)-chains obscures this separation. By 21 d DHCB-treated cultures exhibited a phenotype more differentiated than control secondary chondrocytes as indicated by the complete absence of α2(I) chains.

To provide a detailed quantitative time-course for reexpression based on the synthesis per microgram DNA of collagen types I trimer, II, and III, and to significantly enhance the reliability and detection sensitivity for the initiation of type II synthesis, we cleaved each of the collagen samples with CNBr, and the resultant peptides were mapped in two dimensions and quantitated by digital imaging. A map from DHCB-treated cultures after 8 d of reexpression is presented in Fig. 5. It represents the midpoint of the phenotypic transition of type II collagen, contains peptides from each of the collagen types, and provides an example of the calculations used (see Fig. 5, legend) to obtain the time-course data presented in Figure 6.

In this culture system enhanced synthesis of type III collagen is the major additive change that characterizes the RA-modulated collagen phenotype. In both untreated and DHCB-treated cultures type III synthesis dropped sharply (approximately eightfold) during the first two days after removal of RA (Fig. 6). DHCB-treated cultures maintained this unstimulated level until day 6 and then exhibited a gradual decline. Untreated cultures showed a transient stimulation of type III synthesis on day 4 and then essentially followed the levels of synthesis in DHCB-treated cultures after day 6. The initial level of type I trimer synthesis was maintained by DHCB-treated cultures until day 8 and then dropped more than fourfold by day 10. In contrast, type I trimer synthesis in untreated cultures abruptly increased fourfold by day 2, declined to about the initial level by day 6, and then remained at this level. Thus, once established by RA treatment in primary culture, type I trimer synthesis can be maintained in the absence of RA. Because the transient stimulation immediately followed the decline in type III synthesis, it may represent a rebound reutilization of the translational apparatus. Regardless of the cause, the stimulation was completely blocked by DHCB-dependent microfilament modification. Collectively these results define the first phase of reexpression, from day 0 to day 6, principally involved with the loss of stimulated, and subsequently RA-dependent, aspects of the RA-modulated collagen phenotype.

The second phase of reexpression, after day 6, was defined by the reinitiation of differentiated characteristics originally suppressed by RA, namely type II collagen synthesis. Such synthesis was substantial when first detected in DHCB-treated cultures on day 6 and maximal on day 14. The dependence of reexpression on microfilament modification was demonstrated by the fact that type II synthesis in DHCB-treated cultures was 8- and 10-fold greater than in untreated cultures at these times. DHCB treatment was also responsible for the decline in type I-trimer synthesis which occurred after day 8, well after the reinitiation of type II synthesis. This and the late slow decline in type III synthesis were required to establish the final collagen phenotype on day 14, which was essentially identical to that expressed by primary differentiated chondrocytes. The essential features of DHCB-dependent reexpression in this system have been replicated in three separate cell strains. These features include the decline in collagen types III and I trimer, the substantial return of type II collagen synthesis by 1 wk without cell rounding, and the final high proportion of type II collagen synthesis after 14 d.

Serum Is Required for Reexpression

In parallel experiments, RA-modulated secondary chondrocytes were tested for DHCB-induced reexpression of the differentiated collagen phenotype in low serum concentrations (1 and 2%) or 10% serum. The collagen phenotype was

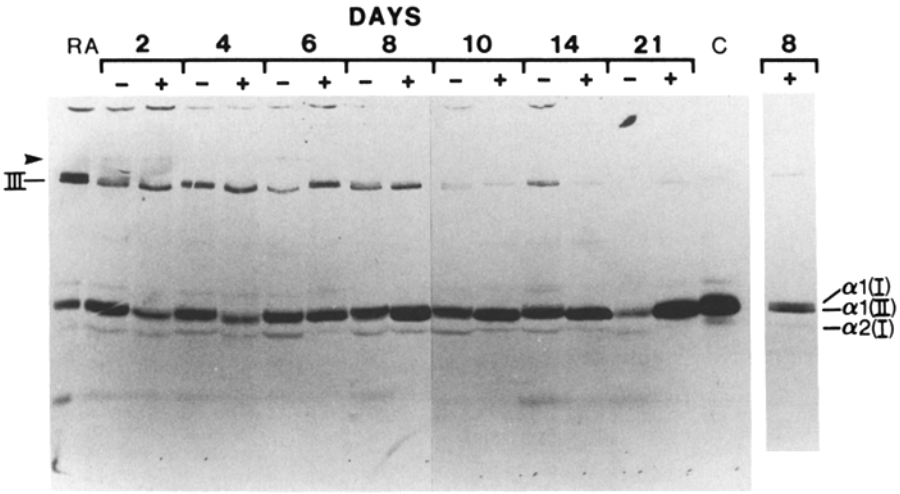

Figure 4. Proportional analysis of the collagen phenotype using SDS-PAGE of collagen chains. Approximately equivalent amounts of radioactivity were loaded in most lanes. (*RA*) Collagens from RA-modulated cultures labeled in the presence of RA at the beginning of the reexpression period. (*C*) Collagens from control secondary cultures never exposed to RA. The remaining lanes contain analyses from RA-modulated cells, cultured for various times (indicated in days) in the absence of RA and the absence (−) or presence (+) of DHCB. (*Arrowhead*) Origin of the separating gel. Right inset allows visualization of the separation of α1(I) and α1(II) chains and contains a lower fluorographic exposure of the same 8-d +DHCB sample presented in the time course region of the gel.

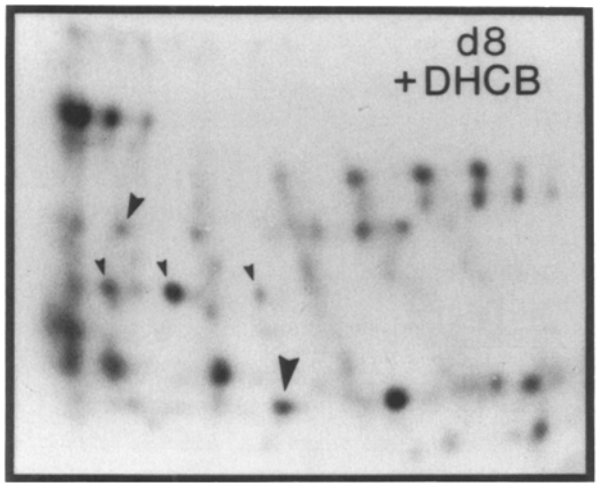

Figure 5. 2-D CNBr peptide map of purified radioactive collagen from RA-modulated chondrocytes after 8 d of reexpression in the presence of DHCB and the absence of RA. Peptides from collagen types, I trimer, II, and III are represented. The indicated peptides were quantitated by digital imaging in this map and maps from cultures treated with and without DHCB during the first 14 d of reexpression described in Figs. 1–4. The percent distribution of these collagens was determined from a single fluorographic exposure of each map with the intensity of each indicated peptide within the measurable range of OD. These peptides were chosen because they were well resolved in all mixtures. The integrated OD for the indicated charge isomers of α1(I)-CB6 (*small arrowheads*), α1(II)-CB9,7 (*large arrowhead*), and α1(III)-CB5 (*medium arrowhead*), were used to calculate total OD for these CNBr peptides by comparison with standard maps. When the range of isomer intensities permitted, alternate isomers or the sum of all isomers were used to verify the calculation; these values were within 10% of those from the standard calculation. Quantitation of standard maps indicated that CB6 represented 24% of the total peptide OD of α1(I); CB9,7 represented 16.5% of α1(II); and CB5 represented 32% of α1(III). OD of each type of α-chain was summed, the percent distribution was calculated, and the synthesis per microgram DNA of each collagen type was obtained by multiplying the decimal equivalent of these values by total collagen synthesized per microgram DNA. The resultant data are presented in Fig. 6.

determined by 2-D CNBr peptide mapping (6, 8, 10) to provide multiple peptide markers for the phenotypic transition to type II synthesis (Fig. 7). Cells labeled in RA at the beginning of the reexpression period contained no detectable type II collagen, as indicated by the absence of α1(II)CB10,5 and the other type II peptides (Fig. 7 *a*, *arrowheads*). Type III collagen peptides (*brackets*) predominated over α1(I) peptides (*parentheses*). After 14 d in medium containing 1% serum, DHCB, and no RA, the phenotype was essentially unchanged except for traces of type II and α2(I) (Fig. 7 *b*). Similar results were obtained with 2% serum (unpublished data). In contrast, when 10% serum was used during the reexpression period, type II collagen peptides were clearly dominant, α1(I) peptides sharply reduced, type III peptides minimal, and α2(I) peptides were essentially undetectable (Fig. 7 *c*). This pattern is the same as that for secondary control chondrocytes at the beginning of the reexpression period (Fig. 7 *d*). These results verify the endpoint of reexpression described in Fig. 6 in a different cell strain and demonstrate the absolute requirement for serum during DHCB-induced reexpression.

Type II Collagen Immunofluorescence

We determined the population of chondrocytes capable of producing type II collagen using a mAb specific for this collagen (25). Approximately 70% of the chondrocytes in primary monolayer culture for 14 d exhibited characteristic vesicular fluorescence (Fig. 8 *a*). In contrast, no intracellular fluorescence was detected in paired RA-modulated chondrocytes after 10 d of treatment (Fig. 8 *b*). However, traces of extracellular fluorescence were present, due to type II deposition before RA treatment. Such uniformly negative cells were transferred to secondary culture in the presence of RA and released from RA exposure for 14 d in the absence (Fig. 8 *c*) or presence (Fig. 8 *d*) of DHCB. Only an occasional type II collagen-positive cell was detected in the absence of DHCB, whereas ~50% of the cells were positive in its presence. These results have been replicated in two separate cell strains and indicate that the changes in type II expression de-

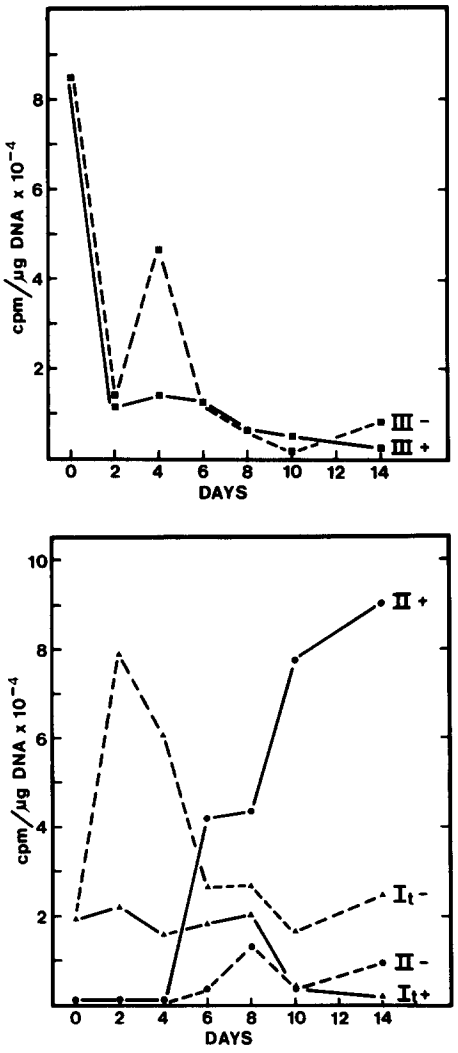

Figure 6. Time course of reexpression of the differentiated phenotype by RA-modulated chondrocytes in secondary culture as measured by changes in the synthesis of collagen types I trimer (*triangles*), II (*circles*), and III (*squares*). Data were obtained from the same cultures described in Figs. 1–5. Reexpression was in the absence of RA and the presence (*solid lines*) or absence (*dashed lines*) of the microfilament-modifying drug, DHCB. The differentiated state is characterized by high levels of type II synthesis (*circles*). These data were obtained using the calculations described in the legend to Fig. 5.

tected biochemically result from the activity of many, but not all, of the cells in these cultures.

Discussion

We have previously shown that rabbit articular chondrocytes are modulated away from the differentiated phenotype by subculture (13) and induced to reexpress the differentiated phenotype by the transition from a spread morphology in monolayer culture to a spherical morphology in agarose culture (10). This shape-dependent reversible modulation system shares with mammary epithelia (21), synovial cells (2, 48), and preadipocytes (46) the characteristic that change away from a flattened morphology leads to both increased production and a shift toward a more differentiated pheno-

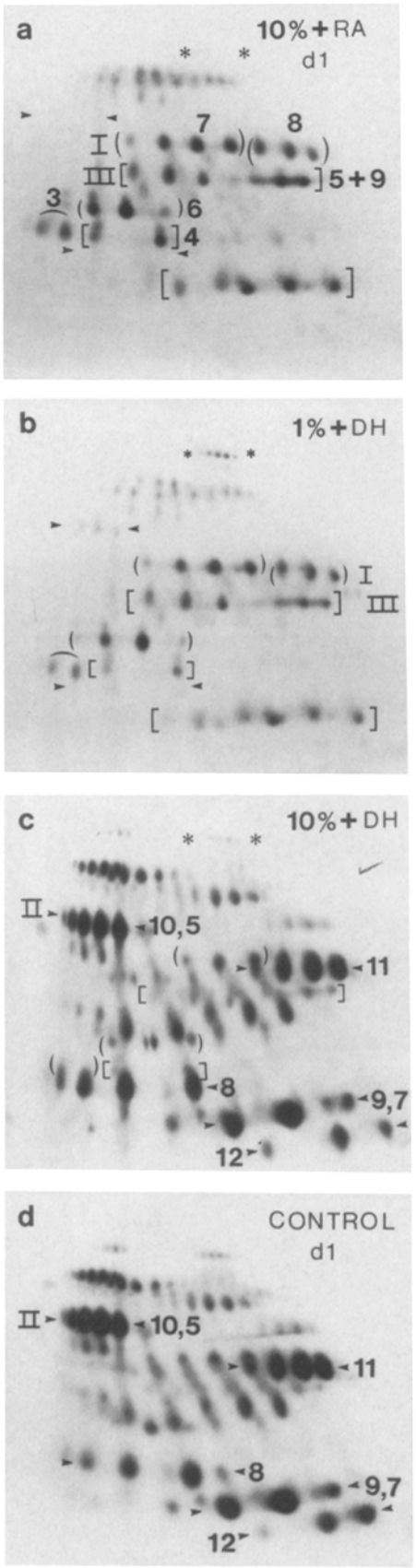

Figure 7. Two-dimensional CNBr peptide maps of collage cultures of RA-modulated cells (*a*) labeled at the beginnir reexpression period in the presence of RA (10% +RA), (*b*) exposed to DHCB in 1% serum for 14 d (1% +DH), and (*c*)

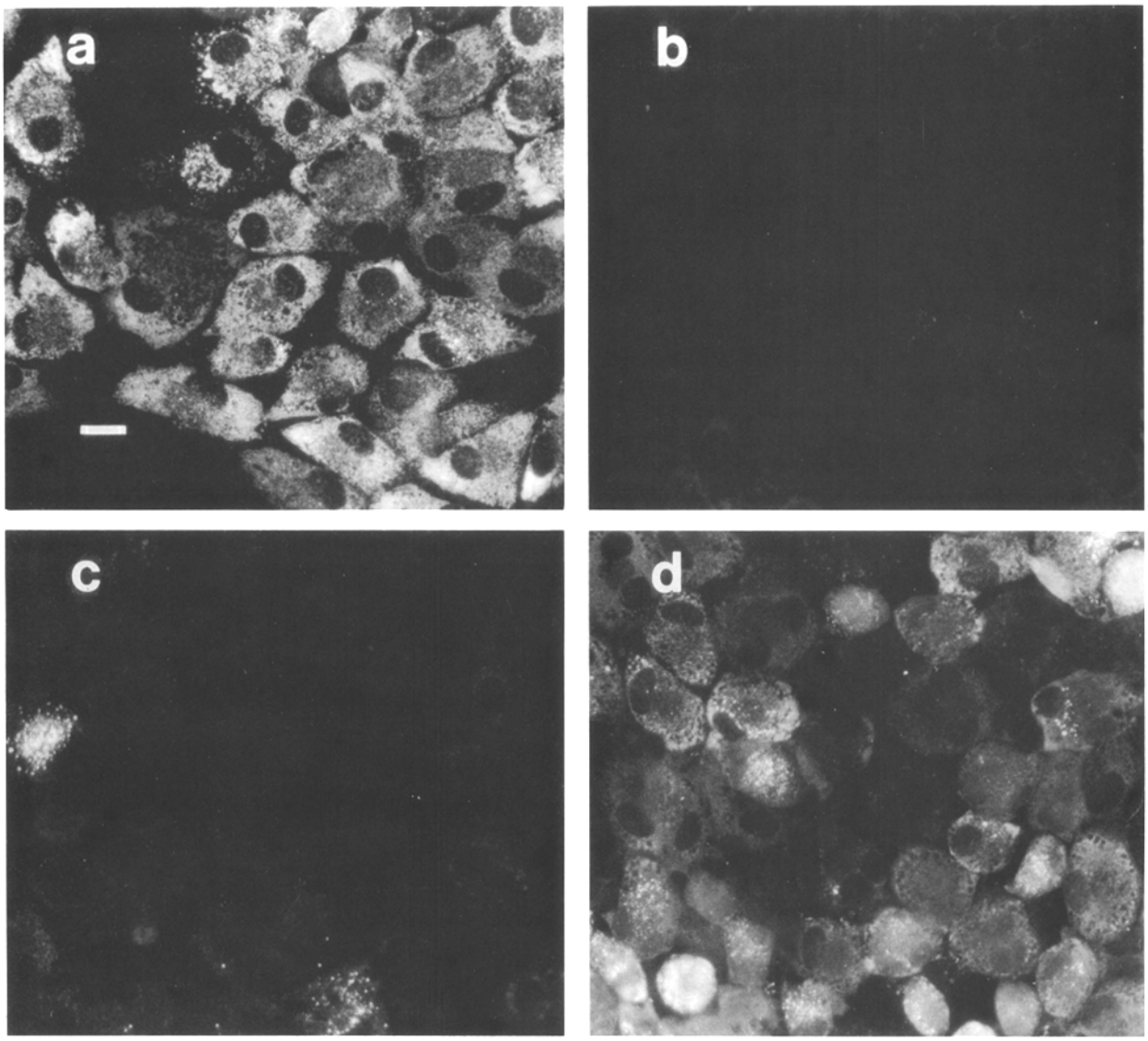

Figure 8. Type II collagen-specific immunofluorescence in chondrocytes after 14 d in primary culture (*a* and *b*) and after reexpression for 14 d in secondary culture in the absence of RA (*c* and *d*). Cells in (*a*) are controls; cells in (*b*) were treated with 1 μg/ml RA between day 4 and 14 of primary culture. Reexpression (*c* and *d*) was in the absence (*c*) or the presence (*d*) of DHCB. Identical exposures and developing times were used in all cases. Bar, 20 μm.

type. The positive response appears to be correlated with the in vivo expression of differentiated functions where the cell exhibits a three-dimensional morphology and may be mechanistically related to the capacity of transformed cells to survive in soft agar. In contrast, anchorage-dependent spread cells, such as 3T3 and other fibroblast lines, respond to such a change with a nearly universal suppression of cellular functions (50).

In the present report we have investigated the idea that transduction of shape information to the genome might be mediated by the organizational state of microfilament structures, and more precisely, whether microfilament modification can signal phenotypic change without the involvement of alterations in cell shape (rounding). We have used DHCB as a specific perturbant of microfilament architecture (4, 14) because it does not alter glucose transport (4) and is well tolerated by chondrocytes and their modulated progeny (11). Both RA-modulated chondrocytes and subculture-modulated chondrocytes (where RA effects are not involved) respond to microfilament modification by DHCB with reexpression of the differentiated phenotype (7, 11). However, these results do not necessarily implicate microfilament signaling, because a previous use of DHCB and other analogues of cytochalasin B has been the stimulation of rounded or spheri-

exposed to DHCB in 10% serum for 14 d (10% +DH). The map from a paired control culture (never treated with RA) labeled at the beginning of reexpression is presented (*d*). These fluorographs were intentionally overexposed to enhance detection of minor peptides. Consequently, the proportion of any minor component appears greater than it is in actuality. The quantitative assessment of properly exposed fluorographs in Fig. 6 more accurately reflects the composition of the reexpressed phenotype. The CNBr peptides of α1(II) are indicated by arrowheads, α1(I) by parentheses, α1(III) by brackets, and α2(I) by asterisks.

cal morphology. This is exemplified by cytochalasin D–induced chondrogenesis in low-density chick limb mesenchymal cells (52). Similarly, synovial fibroblasts show a positive correlation between the degree of cell rounding caused by different doses of cytochalasin B or other agents and the extent of collagenase induction (2).

In the present experiments we have essentially eliminated cell rounding by using a low dose of DHCB (3×10^{-6} M) and by using RA-modulated cells which are tightly adherent, possibly due to increased cellular binding of fibronectin (22). The employed dose is threefold less than required for initiation of rounding and ninefold less than required for complete arborization of subculture-modulated chondrocytes (manuscript in preparation). In the present experimental system DHCB did not eliminate stress fibers but caused substantial modification of microfilament architecture (14). Thus, both microfilament-containing and microfilament-free membrane attachment plaques may still stabilize the spread morphology through interactions between the extracellular matrix (fibronectin [17, 45], chondronectin [23], collagen [47]) and linking proteins, such as fibronectin receptor complex (24, 27), collagen-binding proteins (34, 35), connectin (15), vinculin (17), and talin (27).

In this environment of a stable spread morphology reexpression proceeds in two phases. Chondrocytes first respond to the absence of RA. This results in the rapid DHCB-independent decline of type III synthesis, which was enhanced but not induced by RA treatment in primary culture (8). A compensatory coincident stimulation of type I trimer synthesis also transiently occurs in untreated cells. This is blocked in DHCB-treated cells, possibly as a result of the changes in microfilament architecture that occur at this time (14) and their potential effects on the translational apparatus (16, 28). These adjustments are complete by day 6. The second phase requires DHCB-dependent microfilament modification, consists of reexpression of the differentiated functions originally suppressed by RA, and partially overlaps the first phase. Enhancement of proteoglycan synthesis is first detected on day 4 and precedes the reinitiation of type II synthesis that is detected on day 6. The early response of proteoglycan synthesis is consistent with its early suppression and greater sensitivity to RA in primary culture (8) and may reflect the operation of regulatory mechanisms that supplement or are different from those controlling the collagen phenotype. Essentially complete reexpression of the differentiated collagen phenotype occurs by day 14. Synthesis of type II collagen at this time is nearly identical to that of control secondary chondrocytes. The transitions of type II collagen and type I trimer are not coincident during this phase, because the suppression of type I-trimer synthesis only begins on day 10. Because of the absence of sensitive, specific antibodies to type I trimer, it has not been possible to determine if the same cell synthesizes these two collagens, and whether such synthesis is simultaneous or temporally separated (previously shown for collagen types I and II [18, 19, 49]).

When reexpression in the presence of DHCB is essentially complete (14 d) the synthesis of type II collagen is restricted to ~50% of the cells, based on type II collagen-specific immunofluorescence. These cells are predominantly well-spread and need not be involved in colony formation. In the absence of DHCB, little type II synthesis is detected biochemically. This is due to the presence of an occasional producing cell rather than a low level of fluorescence in all of the larger population of cells capable of type II synthesis. The initial proportion of producing cells in primary monolayer culture is slightly smaller than the 80% reported for freshly isolated articular chondrocytes after overnight suspension culture (53). The difference may be due to the effects of monolayer culture and may be enhanced by secondary culture. It remains to be determined if the proportion of producing cells following reexpression in monolayer culture can be increased by transition to spherical shape in agarose culture. Although the synthetic history of a single cell cannot be determined by intracellular immunofluorescence to demonstrate phenotypic switching, it is important that the progeny of a single cloned chondrocyte cease producing type II collagen and begin producing type I and type I trimer (32).

It is likely that reinitiation of type II collagen synthesis results predominantly from regulation at the level of transcription, although run-on transcription data are not available. Pawlowski et al. (38) have shown that suppression of type II synthesis in response to BrdU results from a parallel reduction of both nuclear and cytoplasmic type II procollagen mRNA. Finer et al. (18) demonstrated a reversible fivefold reduction in equilibrium levels of type II procollagen mRNA associated with the treatment and removal of PMA. Posttranscriptional events may also play a role, because they cause substantial changes in the synthesis of type I collagen (39) and other proteins (29). However, in chondrocytes it is more likely that such events will influence the regulation of collagen type I and I trimer. Finer et al. (18) have demonstrated that the translational efficiency of type I procollagen mRNA is altered in PMA-treated chondrocytes and is 24-fold greater than that of type II procollagen mRNA after removal of PMA. In addition, partially processed (18) and untranslated mRNA for pro α1(I) (1, 3) and pro α2(I) (20) have been detected in vertebral and virally transformed chondrocytes. It is not clear whether similar mechanisms account for the absence of α2(I) synthesis during RA treatment of rabbit articular chondrocytes (8), when similar treatment of chicken sternal chondrocytes results in the synthesis of heterotrimeric type I collagen (51).

The transitions in synthesis of collagen types I, I trimer, and II are maximal within the time frame of 7–14 d after the initial stimulus for phenotypic change in the processes of modulation by RA (8), subsequent DHCB-dependent reexpression, and shape-dependent reexpression after modulation by subculture (10). In comparison with the rapid changes in type III synthesis stimulated by treatment (8) or removal of RA, and the early modifications in microfilament architecture produced by DHCB (14), modulation of the differentiated collagen phenotype is delayed in onset and develops slowly. This suggests the operation of complex regulatory mechanisms and/or slow changes in chromatin structure.

The present results (and those in references 7 and 11) suggest that some change in microfilament organization can initiate a signal that is capable of altering the phenotype without mediation by events that only result from cell rounding. Similarly, Unemori and Werb (48) have shown in synovial fibroblasts that the transition to spherical shape does not necessarily mimic the microfilament modifying effects of cytochalasin B, which are capable of inducing collagenase production. Effective microfilament modification may alter

the interaction between actin and membrane-associated proteins to modulate membrane-based signaling. This is suggested by the cytochalasin D-dependent reversal of fibronectin-mediated inhibition of chondrogenesis (52) and the assumed membrane origin of protein kinase C-mediated, PMA-dependent modulation of the chondrocyte phenotype (18). The emphasis on microfilament modification during reexpression is not in conflict with the results of Horton and Hassel (26), which demonstrate that RA can modulate spherical chondrocytes. A spread morphology is thus not a necessary intermediate for RA-dependent change, in contrast to our initial expectations (8). With regard to reexpression, the present study suggests that an important aspect of the RA-signaling mechanism is stabilized by monolayer culture so that simple removal of RA does not result in reexpression.

Reexpression also exhibits an absolute requirement for 10% FCS. The concentration of factor(s) in 1 or 2% serum is not sufficient to permit reexpression even though DHCB-dependent modification of microfilaments is essentially identical in both 10 and 2% serum (14). The mechanism of this synergism is not presently known but similar effects are seen during chondrogenesis (41). Limb mesenchyme requires a prior commitment step before cytochalasin D can induce chondrogenesis (52). This process has been clarified by studies with purified cartilage-inducing factor A/transforming growth factor β (41, 44). Rat muscle mesenchymal cells can be committed to chondrogenesis by this factor alone but do not express the phenotype until stimulated by shape-change or microfilament modification with DHCB. The use of multiple factors may bypass the requirement for overt shape change (30). It is not yet clear whether cartilage-inducing factor A and the factor required for reexpression are identical.

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References

1. Adams, S. L., D. Boettiger, R. J. Focht, H. Holtzer, and M. Pacifici. 1982. Regulation of the synthesis of extracellular matrix components in chondroblasts transformed by a temperature-sensitive mutant of rous sarcoma virus. *Cell.* 30:373–384.
2. Aggeler, J., S. M. Frisch, and Z. Werb. 1984. Changes in cell shape correlate with collagenase gene expression in rabbit synovial fibroblasts. *J. Cell Biol.* 98:1662–1671.
3. Allebach, E. S., D. Boettiger, M. Pacifici, and S. L. Adams. 1985. Control of types I and II collagen and fibronectin gene expression in chondrocytes delineated by viral transformation. *Mol. Cell. Biol.* 5:1002–1008.
4. Atlas, S. J., and S. Lin. 1978. Dihydrocytochalasin B. Biological effects and binding to 3T3 cells. *J. Cell Biol.* 76:360–370.
5. Babel, W., and R. W. Glanville. 1984. Structure of human-basement-membrane (type IV) collagen. Complete amino-acid sequence of a 914-residue-long pepsin fragment from the alpha 1(IV) chain. *Eur. J. Biochem.* 143:545–556.
6. Benya, P. D. 1981. Two-dimensional CNBr peptide patterns of collagen types I, II, and III. *Collagen Relat. Res.* 1:17–20.
7. Benya, P. D., and P. D. Brown. 1986. Modulation of the chondrocyte phenotype in vitro. *In* Articular Cartilage Biochemistry. K. E. Kuettner and R. Schleyerbach, editors. Raven Press, New York. 219–233.
8. Benya, P. D., and S. R. Padilla. 1986*a*. Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen. The modulated phenotype differs from that produced by subculture. *Dev. Biol.* 118:296–305.
9. Benya, P. D., and S. R. Padilla. 1986*b*. Isolation and characterization of type VIII collagen synthesized by cultured rabbit corneal endothelial cells. A conventional structure replaces the interrupted-helix model. *J. Biol. Chem.* 261:4160–4169.
10. Benya, P. D., and J. D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 30:215–224.
11. Benya, P. D., and J. D. Shaffer. 1983. Microfilament involvement in the reexpression of the differentiated chondrocyte collagen phenotype. *Orthop. Trans.* 7:263.
12. Benya, P. D., S. Padilla, and M. E. Nimni. 1977. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verification by cyanogen bromide peptide analysis. *Biochemistry.* 16:865–872.
13. Benya, P. D., S. Padilla, and M. E. Nimni. 1978. Independent regulation of collagen types of chondrocytes during the loss of differentiated function in culture. *Cell.* 15:1313–1321.
14. Brown, P. D., and P. D. Benya. 1986. Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced reexpression. *J. Cell Biol.* 106:171–179.
15. Brown, S. S., H. L. Malinoff, and M. S. Wicha. 1983. Connectin: cell surface protein that binds both laminin and actin. *Proc. Natl. Acad. Sci. USA.* 80:5927–5930.
16. Cervera, M., G. Dreyfuss, and S. Penman. 1981. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. *Cell.* 23:113–120.
17. Chen, W. T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205–222.
18. Finer, M., L. Gerstenfeld, D. Young, P. Doty, and H. Boedtker. 1985. Collagen expression in embryonic chicken chondrocytes treated with phorbol myristate acetate. *Mol. Cell. Biol.* 5:1415–1424.
19. Gerstenfeld, L. C., M. H. Finer, and H. Boedtker. 1985. Altered beta-actin gene expression in phorbol myristate acetate treated chondrocytes and fibroblasts. *Mol. Cell. Biol.* 5:1425–1433.
20. Gionti, E., O. Capasso, and R. Cancedda. 1983. The culture of chick embryo chondrocytes and the control of their differentiated functions *in vitro*. *J. Biol. Chem.* 258:7190–7194.
21. Haeuptle, M. T., Y. L. M. Suard, E. Bogenmann, H. Reggio, L. Racine, and J. P. Kraehenbuhl. 1983. Effect of cell shape change on the function and differentiation of rabbit mammary cells in culture. *J. Cell Biol.* 96: 1425–1434.
22. Hassell, J. R., J. P. Pennypacker, H. K. Kleinman, R. M. Pratt, and K. M. Yamada. 1979. Enhanced cellular fibronectin accumulation in chondrocytes treated with vitamin A. *Cell.* 17:821–826.
23. Hewitt, A. T., H. K. Kleinman, J. P. Pennypacker, and G. R. Martin. 1980. Identification of an adhesion factor for chondrocytes. *Proc. Natl. Acad. Sci. USA.* 77:385–388.
24. Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 83: 6470–6474.
25. Holmdahl, R., K. Rubin, L. Klareskog, E. Larsson, and H. Wigzell. 1986. Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II antibodies. *Arthritis Rheum.* 29:400–410.
26. Horton, W., and J. R. Hassel. 1986. Independence of cell shape and loss of cartilage matrix production during retinoic acid treatment of cultured chondrocytes. *Dev. Biol.* 115:392–397.
27. Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531–533.
28. Howe, J. G., and J. W. B. Hershey. 1984. Translational initiation factor and ribosome association with the cytoskeletal framework fraction from HeLa cells. *Cell.* 37:85–93.
29. Jefferson, D. M., D. F. Clayton, J. E. Darnell, Jr., and L. M. Reid. 1984. Posttranscriptional modulation of gene expression in cultured rat hepatocytes. *Mol. Cell. Biol.* 4:1929–1934.
30. Koskinen, K. P., Y. S. Kanwar, B. Sires, and A. Veis. 1985. An electron microscopic demonstration of induction of chondrogenesis in neonatal rat muscle outgrowth cells in monolayer cultures. *Connect. Tissue Res.* 14: 141–158.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
32. Mayne, R., M. S. Vail, P. M. Mayne, and E. J. Miller. 1976. Changes in the type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. *Proc. Natl. Acad. Sci. USA.* 73: 1674–1678.
33. Mayne, R., M. S. Vail, and E. J. Miller. 1975. Analysis of changes in col-

lagen biosynthesis that occur when chick chondrocytes are grown in 5-bromo-2'-deoxyuridine. *Proc. Natl. Acad. Sci. USA.* 72:4511–4515.
34. Mollenhauer, J., and K. von der Mark. 1983. Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:45–50.
35. Nagata, K., and M. Yamada. 1986. Phosphorylation and transformation sensitivity of a major collagen-binding protein of fibroblasts. *J. Biol. Chem.* 261:7531–7536.
36. Nishimoto, S. K., T. Kajiwara, P. W. Ledger, and M. L. Tanzer. 1982. Effects of the ionophore monensin on type II collagen and proteoglycan synthesis and secretion by cultured chondrocytes. *J. Biol. Chem.* 257: 11712–11716.
37. Parker, W. R., J. R. Hartman, and W. M. Jaynes. 1983. Bioimage system: data aquisition and analysis of 2-dimensional gel electrophoretograms. *Proc. Natl. Computer Graphics Assoc. Conf.* 136–143.
38. Pawlowski, P. J., G. T. Brierley, and L. N. Lukens. 1981. Changes in the type II and type I collagen messenger RNA population during growth of chondrocytes in 5-bromo-2-deoxyuridine. *J. Biol. Chem.* 256:7695–7698.
39. Raghow, R., D. Gossage, and A. H. Kang. 1986. Pretranslational regulation of type I collagen, fibronectin, and a 50-kilodalton noncollagenous extracellular protein by dexamethasone in rat fibroblasts. *J. Biol. Chem.* 261:4677–4684.
40. Ramshaw, J. A. M., J. F. Bateman, and W. G. Cole. 1984. Precipitation of collagens by polyethylene glycols. *Anal. Biochem.* 141:361–365.
41. Rosen, D. M., S. A. Stempien, A. Y. Thompson, J. E. Brennan, L. R. Ellingsworth, and S. M. Seyedin. 1986. Differentiation of rat mesenchymal cells by cartilage-inducing factor. Enhanced phenotypic expression by dihydrocytochalasin B. *Exp. Cell Res.* 165:127–138.
42. Saxe, S. A., L. N. Lukens, and P. L. Pawlowski. 1985. Changes in the nuclear and cytoplasmic levels of type I and type II collagen RNAs during growth of chondrocytes in 5-bromo-2'-deoxyuridine. *J. Biol. Chem.* 260:3812–3819.
43. Schoenle, E. J., L. D. Adams, and D. W. Sammons. 1984. Insulin-induced rapid decrease of a major protein in fat cell plasma membranes. *J. Biol. Chem.* 259:12112–12116.
44. Seyedin, S. M., A. Y. Thompson, H. Bentz, D. M. Rosen, J. M. McPherson, A. Conti, N. R. Siegel, and K. A. Piez. 1986. Cartilage-inducing factor A. Apparent identity to transforming growth factor beta. *J. Biol. Chem.* 261:5693–5695.
45. Singer, I. I., and P. R. Paradiso. 1981. A transmembrane relationship between fibronectin and vinculin (130 kd protein): serum modulation in normal and transformed hamster fibroblasts. *Cell.* 24:481–492.
46. Spiegelman, B. M., and C. A. Ginty. 1983. Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell.* 35:657–666.
47. Tomasek, J. J., E. D. Hay, and K. Fujiwara. 1982. Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts: distribution of actin, alpha-actinin, and myosin. *Dev. Biol.* 92:107–122.
48. Unemori, E. N., and Z. Werb. 1986. Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. *J. Cell Biol.* 103:1021–1031.
49. von der Mark, K., V. Gauss, H. von der Mark, and P. Muller. 1977. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. *Nature (Lond.).* 267: 531–532.
50. Wittelsberger, S. C., K. Kleene, and S. Penman. 1981. Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype. *Cell.* 24:859–866.
51. Yasui, N., P. D. Benya, and M. E. Nimni. 1986. Coordinate regulation of type IX and type II collagen synthesis during growth of chick chondrocytes in retinoic acid or 5-bromo-2'-deoxyuridine. *J. Biol. Chem.* 261: 7997–8001.
52. Zanetti, N. C., and M. Solursh. 1984. Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. *J. Cell Biol.* 99:115–123.
53. Zanetti, M., A. Ratcliffe, and F. M. Watt. 1985. Two subpopulations of differentiated chondrocytes identified with a monoclonal antibody to keratan sulfate. *J. Cell Biol.* 101:53–59.