THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 19, Issue of May 7, pp. 19502–19511, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Mechanical Compression of Cartilage Explants Induces Multiple Time-dependent Gene Expression Patterns and Involves Intracellular Calcium and Cyclic AMP*[S]

Received for publication, January 14, 2004, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 11, 2004, DOI 10.1074/jbc.M400437200

Jonathan B. Fitzgerald‡, Moonsoo Jin§¶, Delphine Dean‖, David J. Wood, Ming H. Zheng**, and Alan J. Grodzinsky‡§‖‡‡**

*From the ‡Biological Engineering Division, §Center for Biomedical Engineering, and ‖Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the **Department of Orthopaedic Surgery, QEII Medical Centre, University of Western Australia, Nedlands, Western Australia 6009, Australia*

Chondrocytes are influenced by mechanical forces to remodel cartilage extracellular matrix. Previous studies have demonstrated the effects of mechanical forces on changes in biosynthesis and mRNA levels of particular extracellular matrix molecules, and have identified certain signaling pathways that may be involved. However, the broad extent and kinetics of mechano-regulation of gene transcription has not been studied in depth. We applied static compressive strains to bovine cartilage explants for periods between 1 and 24 h and measured the response of 28 genes using real time PCR. Compression time courses were also performed in the presence of an intracellular calcium chelator or an inhibitor of cyclic AMP-activated protein kinase A. Cluster analysis of the data revealed four main expression patterns: two groups containing either transiently up-regulated or duration-enhanced expression profiles could each be subdivided into genes that did or did not require intracellular calcium release and cyclic AMP-activated protein kinase A for their mechano-regulation. Transcription levels for aggrecan, type II collagen, and link protein were up-regulated ~2–3-fold during the first 8 h of 50% compression and subsequently down-regulated to levels below that of free-swelling controls by 24 h. Transcription levels of matrix metalloproteinases-3, -9, and -13, aggrecanase-1, and the matrix protease regulator cyclooxygenase-2 increased with the duration of 50% compression 2–16-fold by 24 h. Thus, transcription of proteins involved in matrix remodeling and catabolism dominated over anabolic matrix proteins as the duration of static compression increased. Immediate early genes c-*fos* and c-*jun* were dramatically up-regulated 6–30-fold, respectively, during the first 8 h of 50% compression and remained up-regulated after 24 h.

Articular cartilage is responsible for the smooth articulation of synovial joints during locomotion. Chondrocytes within cartilage constantly remodel the extracellular matrix (ECM)[1] of the tissue throughout life. The major load-bearing constituents of the ECM are type II collagen and aggregates of the proteoglycan, aggrecan, which provide the tensile and compressive stiffness of the tissue, respectively. Also present in the ECM are families of matrix proteinases, tissue inhibitors of matrix metalloproteinases (TIMPs), growth factors, and cytokines that together regulate ECM remodeling and turnover in health and disease (1). It is known that mechanical exercise of the knee joint *in vivo* increases the density of aggrecan in cartilage (2), whereas knee joint inactivity results in decreased aggrecan deposition (3, 4). Traumatic injury to cartilage diminishes mechanical strength and leads to excessive catabolism of the ECM, increasing the risk of osteoarthritis later in life (5).

A number of model systems have been developed to simulate various aspects of the mechanical loading forces experienced by articular cartilage *in vivo*. Compressive and shear forces have been applied to cartilage explants and chondrocyte cultures *in vitro* to examine the transduction of mechanical signals into biological responses. Application of 50% static compression to cartilage explants decreased synthesis of type II collagen and proteoglycans (PG) within the first 1–2 h of loading, and synthesis remained suppressed throughout a 24-h loading period (6–8). Dynamic compression (8, 9) and shear (10) increased type II collagen and PG synthesis at low amplitudes and frequencies (1–5% strain, 0.01–1 Hz); however, compression also increased the activation of MMP-2 and MMP-9 (11). Injurious compression of cartilage explants results in increased PG loss to medium and damage to the collagen network (12, 13). Currently, many cartilage tissue engineering strategies employ some form of mechanical stimulation to enhance matrix production by chondrocytes during culture (14–18).

Recent studies of mechano-regulation of chondrocyte gene expression showed that application of 25–50% static compression to bovine cartilage explants caused a transient increase in expression of aggrecan (19, 20) and type II collagen (19) mRNA levels during the first 4 h of loading (>1.5-fold), followed by a

* This work was supported by National Institutes of Health Grant AR33236 (to A. J. G.), National Health and Medical Research Council of Australia Grant 254742 (to D. J. W. and M. H. Z.), a Whitaker Foundation graduate fellowship (to D. D.), and a University of Western Australia Hackett Scholarship (to J. B. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Appendix and Figs. A1–A5.

¶ Present address: The CBR Institute for Biomedical Research, Inc., Harvard Medical School, Boston, MA 02115.

‡‡ To whom correspondence should be addressed: Biological Engineering Division, Massachusetts Institute of Technology, NE47-377, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-253-4969; Fax: 617-258-5239; E-mail: alg@mit.edu.

[1] The abbreviations used are: ECM, extracellular matrix; TIMP, tissue inhibitor of matrix protease; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; AP-1, activating protein-1; BAPTA-AM, bis-(aminophenoxy)ethane-tetraacetic acid acetoxymethyl; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; HSP70, heat shock protein-70; COX-2, cyclooxygenase-2; IL-1β, interleukin-1 beta; TNFα, tumor necrosis factor α; PIS, pressure-induced strain; PGs, proteoglycans.

This paper is available on line at http://www.jbc.org

decrease in expression to levels below non-loaded controls by 24 h. Intermittent hydrostatic pressure applied to human chondrocytes in monolayer culture at a frequency of 1 Hz (4 h/day for 4 days) increased aggrecan and type II collagen gene and protein expression (>1.4-fold) (21); intermittent hydrostatic pressure did not deform the chondrocytes. Millward-Sadler *et al.* (22) applied hydrostatic pressure to chondrocyte monolayers at 0.33 Hz for 20 min in a manner that induced strain on the culture dish and plated cells (pressure-induced strain (PIS)). They observed an increase in aggrecan mRNA and a decrease in MMP-3 mRNA within 1 h following stimulation, with a return to base-line levels by 24 h. In a single experiment applying intermittent hydrostatic pressure to human chondrosarcoma cells, changes in the expression of 51 genes were measured by cDNA array technology without widespread change in RNA stability (23), indicating that many genes may be influenced by mechanical stimuli.

Studies have also focused on cellular mechanotransduction events that may initiate changes in gene expression. Application of intermittent PIS to chondrocytes induced $\alpha_5\beta_1$ integrin activation of interleukin-4, which caused cell hyperpolarization via intracellular calcium release (24). Inhibition of interleukin-4 suppressed the up-regulation of aggrecan gene expression observed due to PIS (22). Intracellular calcium release, cAMP, and the phospholipase C pathway have been implicated for aggrecan gene up-regulation in response to static compression in cartilage explants (25). Static compression also increased ERK1/2 phosphorylation within minutes of application, with sustained increases during 24 h of compression (26). Although such signaling pathways have been identified in the mechanical regulation of aggrecan gene expression, less is known about chondrocyte gene expression patterns of other ECM-related molecules or whether common upstream signaling pathways are responsible for their regulation.

Given these observations regarding the sensitivity of chondrocyte biosynthesis to mechanical forces *in vivo* and in the cartilage explant model, we hypothesized that mechanical loading would also induce widespread transcriptional changes, particularly for molecules involved in ECM maintenance. Our objective was to characterize the transcriptional response of chondrocytes from intact cartilage to static compression, focusing on a range of anabolic, catabolic, and signaling genes involved in tissue homeostasis. Temporal expression profiles of 28 genes were measured to compare immediate and long term changes in response to sustained compression. Molecular inhibitors of intracellular calcium, cAMP and AP-1, were used to identify possible upstream signaling pathways involved in the mechanotransduction of the genes studied here. Clustering analysis (27, 28) and principal component analysis (29, 30) were used to elucidate the main expression trends and to highlight genes that appeared to be co-regulated by mechanical compression. These computational techniques can help to classify groups of genes with common upstream signaling pathways and may help to predict certain cell behavior (28, 30). We found that both anabolic and catabolic genes were induced by static compression, but with contrasting expression patterns. Intracellular calcium and cAMP were found to play fundamental roles in the mechanical regulation of gene transcription.

EXPERIMENTAL PROCEDURES

Cartilage Extraction and Mechanical Loading—Articular cartilage disks (3 mm diameter, 1 mm thick) were obtained from the middle zone of the patello-femoral groove of 1–4-week-old calves as described previously (8). Disks were washed with phosphate-buffered saline and maintained in low glucose Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 20 μg/ml ascorbate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Disks were allowed to equilibrate for 2–5 days before loading, with media changes every 2nd day and 12 h before loading. Anatomically matched disks (6 disks per time point per loading treatment) were transferred into polysulfone loading chambers (8), slowly compressed over an ~3-min period to 25 or 50% of cut thickness, and maintained at these static strain levels for 1, 2, 4, 8, or 24 h (25% strain, $n = 4$; 50% strain, $n = 11$), with disks kept in free-swelling conditions as controls (see Fig. 1, *A* and *B*). Upon completion of loading, disks were stored at −80 °C in RNA-*later* solution (Qiagen).

Inhibitor Studies—To investigate the role of intracellular signaling pathways during static compression, molecular inhibitors were added to media prior to application of 50% compression (Fig. 1, *C* and *D*). Cartilage disks were pre-incubated for 1 h with 10 μM BAPTA-AM, a chelator of intracellular calcium (AG Scientific, Inc.), or 50 μM (R_p)-cAMP, an inhibitor of cAMP-activated protein kinase A (Sigma), which have been previously shown to completely inhibit mechanically induced aggrecan gene regulation (20, 25). For each time point six untreated and six treated disks were placed into a 12-well polysulfone chamber to allow identical compression ($n = 4$–5). In separate experiments, paclitaxel, an inhibitor of transcription factor AP-1 binding to DNA (10 μM, Sigma), was added 24 h prior to 2 h of 50% static compression ($n = 5$), at concentrations shown previously to inhibit AP-1 activity in bovine chondrocytes (31). To assess RNA stability, disks were either maintained in free-swelling conditions or loaded for 2 h at 50% strain and then released to free-swell for up to 6 h before being stored at −80 °C ($n = 2$). Actinomycin D was added to media immediately upon completion of loading (Fig. 1*E*) at concentrations shown previously to inhibit transcription in bovine chondrocytes and cartilage explants (30 μM, Sigma) (23).

RNA Extraction, Primer Calibration, and Real Time PCR—For each time point and loading treatment, six cartilage disks were pulverized using liquid nitrogen-cooled mortar and pestles and homogenized in QIAshredder tubes (Qiagen) spun at 10,000 rpm for 2 min. RNA was then extracted from the clear supernatant by using the Qiagen RNeasy mini kit protocol with the DNase digest (Qiagen). RNA was stored in 40 μl of RNase-free water at −80 °C until reverse transcription was performed using Applied Biosystems Reagents. Real time PCR was performed on a 384-well/plate ABI7900HT machine (2 min at 50 °C, 10 min at 95 °C and 50 cycles of 15 s at 94 °C and 1 min at 60 °C) by using SybrGreen MasterMix (Applied Biosystems). The SybrGreen Master mix and H_2O were combined, and aliquots were dispersed into cDNA-containing tubes. A multipipette was used to distribute 9-μl aliquots into a 384-well plate, followed by 1 μl of 10 μM forward and reverse primer mix. Free-swelling controls and inhibitor-treated samples were always run on the same plate as untreated compressed samples. A total of 28 primer pairs for the 28 genes listed in Table I were designed with amplification product length of 85–130 bp and annealing temperature of ~60 °C. All primers were tested to produce proportional changes in threshold cycle with varying starting cDNA quantity. Measured threshold cycles (C_T) were converted to relative copy numbers using primer-specific standard curves.

Data Normalization and Statistical Analyses—For each experiment and every loading condition, gene expression levels were normalized by the average levels of mean-centered housekeeping genes 18 S and G3PDH. The time course of each gene was then normalized by the corresponding free-swelling expression level. For example, the BAPTA-AM-treated time courses were normalized by BAPTA-AM-treated free-swelling controls after normalizing by housekeeping genes. Expression levels further than three standard deviations from the mean were considered outliers and removed. Gene expression levels induced by compression were compared with free-swelling expression levels using two-tailed Student's t tests. Comparison between compression time courses with and without the presence of inhibitors was performed using comparison of means two-tailed t tests, with Welch corrected degrees of freedom for unequal variance. Games-Howell corrected standard deviations were used to account for unequal sample sizes, as F tests had revealed significant differences between untreated and treated gene expression level variances (data not shown). Skew and Kurtosis criteria were used to confirm that the data were normally distributed. A p value of less than or equal to 0.05 was used to assess statistical significance.

Clustering Analysis—To analyze further the expression patterns and pathways activated by compression, the expression levels for each gene from the 50% compression, BAPTA-AM-treated, and (R_p)-cAMP-treated time courses were combined into 15-point expression vectors (23 genes had complete time courses). These expression vectors were then standardized to have equal variance, to emphasize expression *patterns* rather than amplitudes, and were then iteratively clustered using two

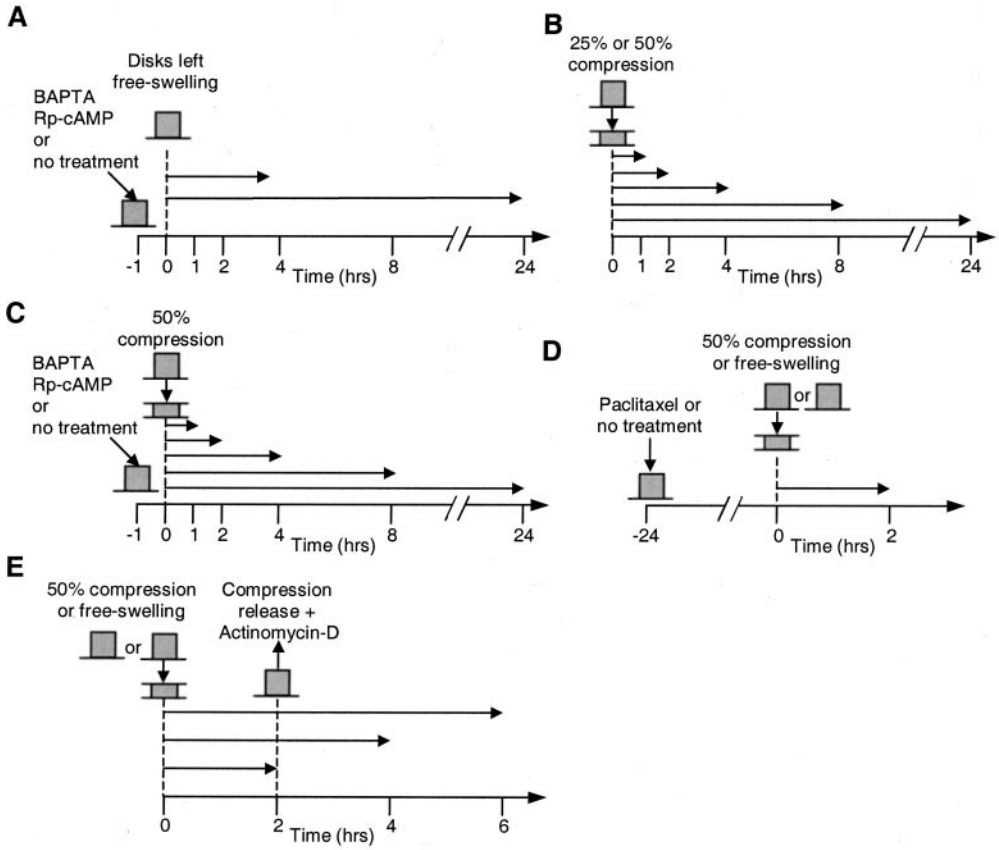

FIG. 1. **Protocols for mechanical compression of cartilage explants with and without inhibitor treatment.** *A*, free-swelling cartilage disks incubated for 4 or 24 h with or without inhibitor treatment were used as controls for compression time series. *B*, continuous 25 or 50% compression was applied for 1–24 h. *C*, no treatment, 10 μM BAPTA-AM or 50 μM (R_p)-cAMP was added to media 1 h before commencement of a 50% compression time series, with controls shown in *A*. *D*, 10 μM paclitaxel was added 24 h before application of 2 h of 50% compression or a 2-h extended period of free-swelling. *E*, cartilage discs were subject to 50% compression or kept free-swelling for 2 h. Compression was released; 30 μM Actinomycin D was added to media, and disks were kept free-swelling for up to 4 h. Six cartilage disks were used for every time point of each condition for all repeated experiments.

TABLE I
Genes examined using real time PCR, categorized by function

Primers were designed using Primer Express software (Applied Biosystems) and Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi) to have optimum annealing temperatures of 60 °C and PCR product length between 85 and 130 bp. Serial dilution standard curves comparing threshold cycle, C_T, and relative starting cDNA quantity, were created for all primers and had average correlations of 0.98, and slopes close to unity.

Matrix proteins	Matrix proteases	Protease inhibitors	Transcription factors	Cytokines/growth factors	Intracellular signaling	Reference genes	Housekeeping
Aggrecan	MMP1	TIMP1	Sox9	IL-1β	COX-2	HSP70	18S
Type I collagen	MMP3	TIMP2	c-Fos	TNFα	MAPk1	Ribosomal-6P	G3PDH
Type II collagen	MMP9	TIMP3	c-Jun	TGFβ	NOS2		
Type X collagen	MMP13			IGF1			
Fibromodulin	ADAMTS4						
Fibronectin	ADAMTS5						
Link protein							

different k means clustering techniques (see Appendix in the Supplemental Material). First, principal component analysis (29, 30) was used to determine the principal components of the data matrix composed of the genes and time points. Each standardized expression vector was then projected onto the three main principal components, and the resulting three-dimensional coordinates were k means clustered using a Euclidean distance metric. The second approach was to cluster the standardized expression vectors directly using correlation as a metric (27, 28). The main expression trends were then identified from comparison of the two techniques. Projection coordinates for each group centroid were calculated by averaging the projection coordinates of each gene within a group. Centroid vectors were formed by adding the three main principal components weighted by the centroid projection coordinates. Centroid variances were calculated, and the Euclidean distance between the projection coordinates of centroids was used to perform comparison of means Student's t tests to assess the distinctiveness of expression trends (for details see Appendix in the Supplemental Material).

RESULTS

Effect of Static Compression on Gene Expression—Static compression was applied for 1–8 h at 25% strain and 1–24 h at 50% strain, and expression levels of 28 ECM maintenance genes were monitored. For ease of presentation and discussion, selected results for specific genes are reported in Figs. 2–4; the complete results for all 28 genes in this study are given in the Appendix Figs. A1 and A2, in the Supplemental Material. Consistent with previous findings, aggrecan (19, 20) and type II collagen (19) were transiently up-regulated in a strain-dependent manner during the first 8 h of compression (up to 2.5-fold)

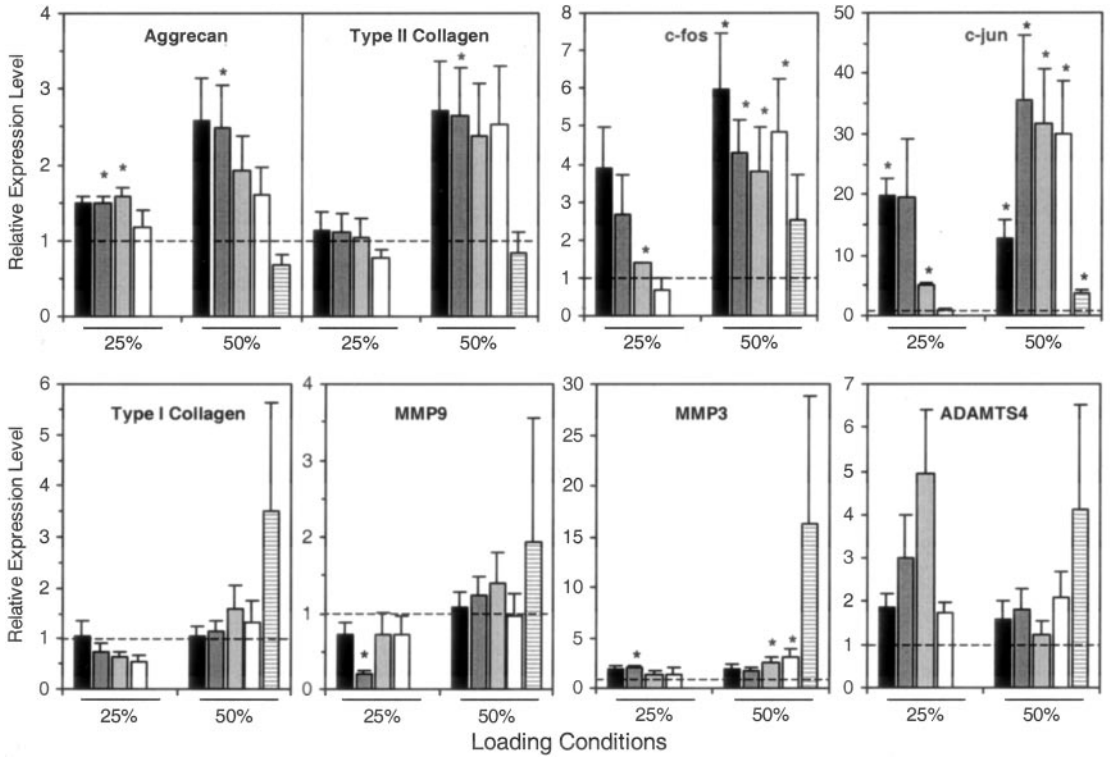

FIG. 2. **Selected gene expression levels induced by static compression, measured by real time PCR.** 25% = 1–8 h of 25% static compression (n = 4). 50% = 1–24 h of 50% static compression (1–8 h, n = 11 and 24 h, n = 4). Expression levels were normalized by 18 S and G3PDH housekeeping genes and divided by free-swelling expression levels. Mean ± S.E. *, $p < 0.05$ compared with free-swell control using Student's two-tailed t test. ■, 1 h; ▩, 2 h; □, 4 h; □, 8 h; ▤, 24 h, relative free-swelling expression level = 1.

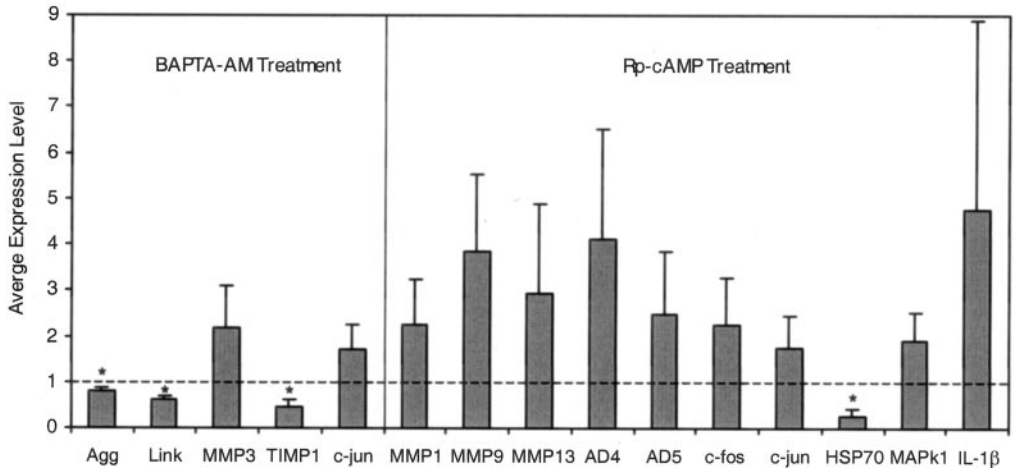

FIG. 3. **Expression levels of genes affected by the addition of BAPTA-AM or (R_p)-cAMP to media under free-swelling conditions.** Six cartilage disks were incubated in media with or without the addition of 10 μM BAPTA-AM or 50 μM (R_p)-cAMP for 4 or 24 h. Most of the 28 genes examined were unaffected by the addition of either inhibitor under free-swell conditions, and only genes for which the inhibitor-treated expression level changed by >70% or were statistically significantly affected are shown. Mean ± S.E. (n = 3–6). *, $p < 0.05$ Student's t test comparing inhibitor-treated expression to the untreated relative expression level = 1. *Agg*, aggrecan; *AD4*, ADAMTS4; *AD5*, ADAMTS5.

before decreasing below free-swelling expression levels by 24 h of 50% compression; link protein was similarly affected (Fig. 2). In contrast, type I collagen was down-regulated in response to 25% compression and increasingly up-regulated during 50% compression, greater than 3-fold after 24 h (Fig. 2); however, comparison of C_T values showed that absolute mRNA abundance of type I collagen was 2 orders of magnitude lower than type II collagen (data not shown). MMP-3 and ADAMTS4 were increasingly up-regulated with 50% compression duration up to 16- and 4-fold, respectively (Fig. 2), and TNFα, IL-1β, and COX-2 were similarly affected (Fig. A2, Supplemental Material). MMP-9 and -13 were up-regulated 2- and 8.6-fold by 24 h of 50% compression, whereas MMP-1 and ADAMTS5 were down-regulated by 30% (Fig. A1, Supplemental Material). Transcription factor Sox9 was up-regulated more by 25% compression (up to 1.7-fold) than 50% compression; in both cases the effect was transient lasting 4 h or less (Fig. A2, Supplemental Material). c-Fos and c-Jun showed marked up-regulation during the 25% compression time course as well as peaked up-regulation (6- and 35-fold, respectively) during the 50% compression time course (Fig. 2). The TIMPs were generally down-regulated by 50% compression with up-regulation only occurring at initial time points (Fig. A2, Supplemental Material). Fibromodulin, fibronectin, and ribosomal 6-phosphate were in general unaffected by static compression (Figs. A1 and A2, Supplemental Material). HSP70 was slightly up-regulated

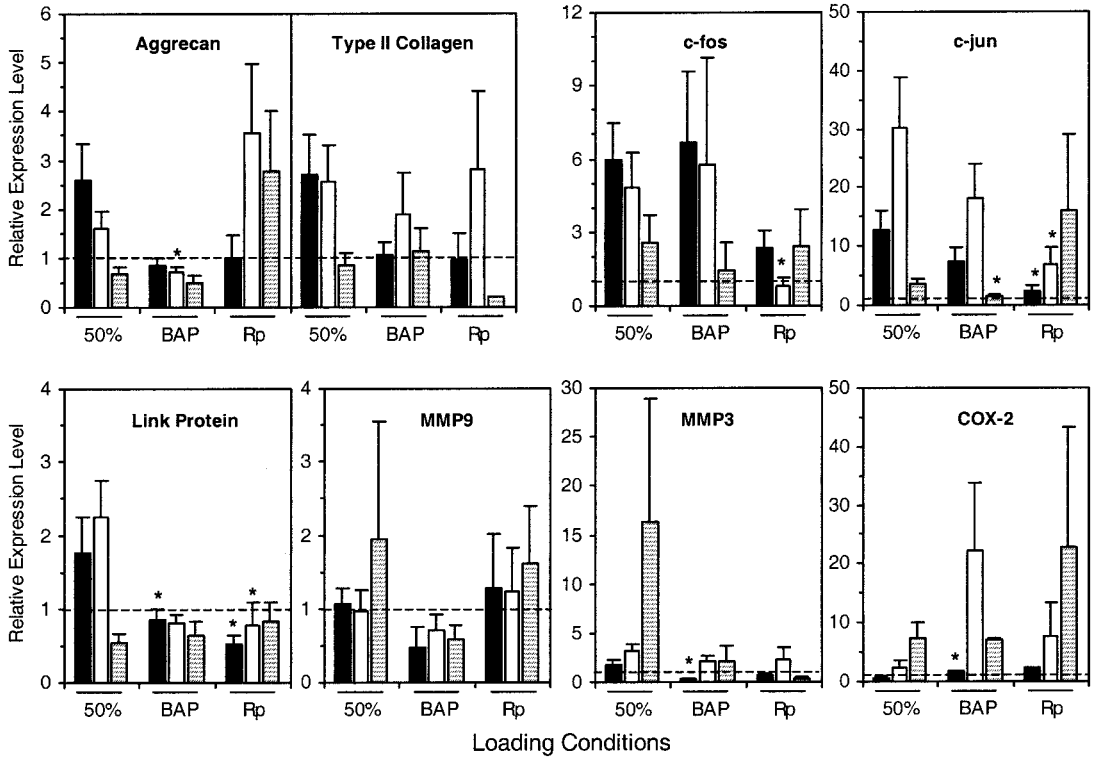

FIG. 4. **Effect of BAPTA-AM and (R_p)-cAMP pretreatment on gene expression levels induced by 50% static compression for a selection of genes.** *50%*, untreated 50% compression time course (1–8 h, $n = 11$ and 24 h, $n = 4$); *BAP*, BAPTA-treated 50% compression time course ($n = 4$); *Rp*, (R_p)-cAMP ($n = 3$) treated 50% compression time course. Expression levels were normalized using 18 S and G3PDH housekeeping genes and divided by the appropriately treated free-swelling expression levels. Mean ± S.E. *, $p < 0.05$ compared with untreated 50% compression time course, using Welch and Games-Howell corrected, comparison of means, two-tailed t tests. ■, 1 h; □, 8 h; ▤, 24 h, relative free-swelling expression level = 1.

throughout the 50% compression time course (Fig. A2, Supplemental Material). Insulin-like growth factor-1 and nitric-oxide synthase-2 were highly up-regulated at certain time points, but their expression levels were scarcely detectable even with real time PCR (Fig. A2, Supplemental Material).

Effect of BAPTA-AM Treatment—To determine whether intracellular calcium release was a prevalent step in the mechanotransduction pathway, 10 μM BAPTA-AM was added to the medium 1 h before application of the 50% compression time course (Fig. 1*C*). The addition of BAPTA-AM did not appear to affect free-swell gene expression levels; however, genes that did show a greater than 70% or significant change are summarized in Fig. 3. Aggrecan, link protein, and TIMP1 free-swelling expression levels were significantly down-regulated by 20, 38, and 54%, respectively. In general BAPTA-AM treatment suppressed the regulation induced by 50% compression (Fig. 4 and Figs. A1 and A2, Supplemental Material). In particular, aggrecan, link protein, and fibromodulin expression remained close to free-swelling expression levels throughout the BAPTA-AM-treated time course, whereas type II collagen was partially suppressed (Fig. 4 and Fig. A1, Supplemental Material). The expression pattern of c-Fos was unaffected by the presence of BAPTA-AM; however, c-Jun levels, although up-regulated compared with free-swelling controls, were reduced by approximately half compared with the untreated 50% compression time course (Fig. 4). MMP-1, -9, and -13 were down-regulated below free-swelling controls throughout the BAPTA-AM-treated time course, and the up-regulation of MMP-3 was largely suppressed, particularly at 24 h (Fig. 4 and Fig. A1, Supplemental Material). In contrast, ADAMTS4 was mainly unaffected by the presence of BAPTA-AM during 50% static compression, and HSP70 was actually increased (Figs. A1 and A2, Supplemental Material). IL-1β was suppressed below free-swelling expression levels; however, COX-2 remained up-regulated (Fig. A2, Supplemental Material).

Effect of (R_p)-cAMP Treatment—To determine whether cAMP activation of protein kinase A was a prevalent step in the mechanotransduction pathway, (R_p)-cAMP was added 1 h prior to application of the 50% compression time course (Fig. 1*C*). (R_p)-cAMP had a more pronounced effect on free-swelling expression levels than BAPTA-AM, although it still only affected a subset of genes (Fig. 3). Notably, HSP70 was suppressed by 70%, and IL-1β was up-regulated 4.7-fold, and MMP 1, -9, and -13, and ADAMTS4,5 were up-regulated >2-fold. In general, (R_p)-cAMP suppressed gene induction by compression similar to BAPTA-AM but also enhanced the regulation of a number of genes (Fig. 4, and Figs. A1 and A2, Supplemental Material). The initial time points of aggrecan and type II collagen were suppressed by (R_p)-cAMP; however, after 8 h of loading, the expression levels of both genes were higher than corresponding untreated levels, and also after 24 h for aggrecan (Fig. 4). Although c-Jun was still up-regulated in the presence of (R_p)-cAMP, overall expression levels were much lower than in response to BAPTA-AM treatment or 50% compression alone and increased with compression duration (Fig. 4). In contrast to the BAPTA-AM-treated time course, the up-regulation of c-Fos was mostly suppressed during the first 8 h of (R_p)-cAMP-treated compression (Fig. 4). Type I collagen and MMP expression were suppressed even below free-swelling expression levels for most time points, particularly MMP-3, which was reduced to 0.30-fold after 24 h of compression (Fig. 4). In contrast, ADAMTS4 remained up-regulated after 24 h, and COX-2, HSP70, and TIMP3 gene expression levels were higher with (R_p)-cAMP treatment during compression, peaking after 24 h at 23-, 10.5-, and 4-fold, respectively (Figs. A1 and A2, Supplemental Mate-

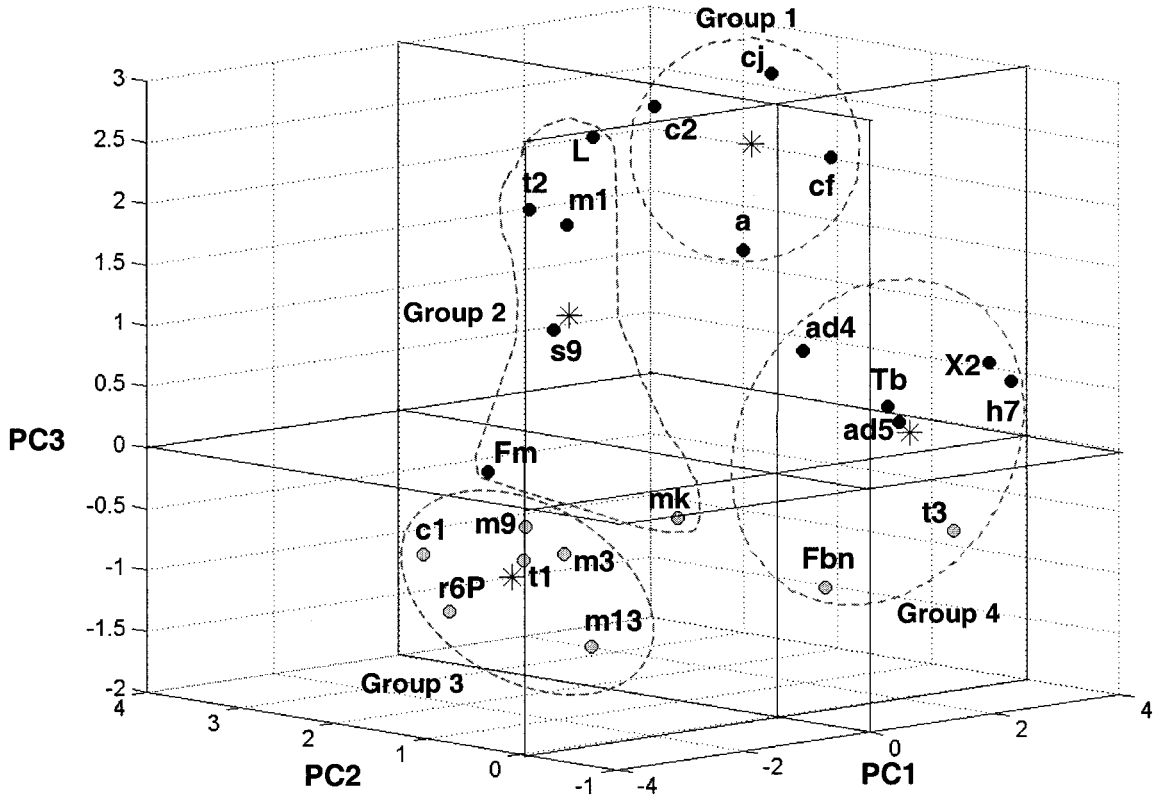

FIG. 5. **Projection plot of the genes represented by the three main principal components.** The standardized gene expression vectors were projected onto the three main principal components found using principal component analysis, with groupings found using *k* means clustering. ● indicates PC3 projection coordinate of gene is >0; ◍ indicates PC3 coordinate < 0. * marks the projection coordinates of the four cluster centroids. Abbreviations used are as follows: *a*, aggrecan; *c1*, type I collagen; *c2*, type II collagen; *L*, link protein; *Fm*, fibromodulin; *Fbn*, fibronectin; *ad4*, ADAMTS4; *ad5*, ADAMTS5; *m1*, MMP-1; *m3*, MMP-3; *m9*, MMP-9; *m13*, MMP-13; *t1*, TIMP1; *t2*, TIMP2; *t3*, TIMP3; *s9*, Sox9; *cf*, c-Fos; *cj*, c-Jun; *r6p*, ribosomal 6-phosphate; *mk*, MAPk1; *Tb*, TGFβ; *h7*, HSP70; *X2*, COX-2.

rial). Notably, Sox9 was down-regulated to 0.06-fold after 24 h (Fig. A2, Supplemental Material).

Main Expression Trends Induced by Static Compression—Principal component analysis revealed three main eigenvectors (principal components) that accounted for 60% of the variance in the data. The coordinates of each standardized gene expression vector when projected onto the three main principal components are shown in Fig. 5. Visual examination of the projection plot and varying the number of groups while clustering revealed that the genes were best divided using 4 groups. *k* means clustering of the projection coordinates using Euclidean distance produced four clusters with 4–7 genes each, shown in Fig. 5 and Table II. *k* means clustering using a correlation metric and the standardized gene expression vectors produced almost identical results, with only aggrecan and ADAMTS4 swapping groups, indicating that the groupings were very robust. The optimal groupings produced by either method were significantly better than randomly assigning genes to groups ($p \ll 0.001$, see Appendix in the Supplemental Material) and examination of the top five groupings showed only 1–2 gene placement variations from the optimal solutions produced by either clustering method. Comparing inter-centroid distances using comparison of means Student's *t* tests revealed that the four clusters were significantly separated and distinct (Table III).

The centroids of the four main expression patterns induced by compression with and without the presence of inhibitors are shown in Fig. 6. Group 1 paired aggrecan and type II collagen, which was expected from previous experiments, along with the AP-1-binding protein elements c-Fos and c-Jun. Both Centroids 1 and 2 transiently increased during the first 4–8 h of static compression followed by a decrease toward free-swelling expression levels by 24 h (Fig. 6). However, Centroid 2 was suppressed by the addition of either BAPTA-AM or (R_p)-cAMP, whereas Centroid 1 was only partially suppressed by BAPTA, and only suppressed at initial time points by (R_p)-cAMP (Fig. 6). Group 3 contained mainly matrix metalloproteinases and type I collagen and exhibited a 50% compression-induced up-regulation that peaked after 24 h (Fig. 6). Group 4 had a similar pattern during 50% compression with a less pronounced peak at 24 h. However, in contrast to Centroid 3, which was almost completely suppressed by either BAPTA-AM or (R_p)-cAMP, Centroid 4 mechano-induction was enhanced by BAPTA-AM and even more so by (R_p)-cAMP.

Effect of Paclitaxel Treatment—To determine whether the up-regulation of c-Fos and c-Jun by compression, which may increase AP-1 signaling, was involved in regulating gene transcription during mechanical loading, paclitaxel was added 24 h before 50% compression (Fig. 1*D*). Genes in Group 1 remained up-regulated after 2 h of compression with paclitaxel present, and free-swelling controls remained unaffected (Fig. 7). Sox9 levels were reduced below controls, and c-Fos up-regulation was suppressed by 50% (Fig. 7), although c-Jun expression was unaffected. MMP-3 expression was reduced below free-swelling expression levels; MMP-13 was up-regulated by >2-fold, and COX-2 remained up-regulated by 4-fold during paclitaxel-treated compression (Fig. 7). MMP-1 and MMP-9 remained up-regulated to the same extent with paclitaxel present; however, TIMP expression was further increased ~1.5-fold and type I collagen was further doubled by the presence of paclitaxel during compression (data not shown).

RNA Stability—To determine whether the changes in mRNA expression were due to changes in transcription or changes in RNA half-life, actinomycin D was added upon release of com-

TABLE II
Optimal groupings found by gene clustering

The 50% compression time course and BAPTA-AM-treated and (R_p) cAMP-treated time courses were combined into gene expression vectors. Groupings found by k means clustering the standardized gene expression vectors projected onto the three main principal components. Centroid coordinates represent the location of the average of all genes within a group projected onto the three main principal components.

Group	Grouped genes	Centroid coordinates (PC1, PC2, PC3)
1	Aggrecan, Type II Collagen, c-Fos, c-Jun	(1.45, 1.21, 2.45)
2	Link protein, MMP1, TIMP2, Sox9, Fibromodulin, MAPk1	(−2.11, 0.77, 1.35)
3	Type I Collagen, MMP3, MMP9, MMP13, TIMP1, Ribosomal 6-P	(−0.12, 2.71, −1.19)
4	ADAMTS4, ADAMTS5, TIMP3, Fibronectin, HSP70, TGFβ, COX-2	(2.42, 0.18, 0.1)

TABLE III
p values produced by comparing centroid locations

The Euclidean distance between the projection coordinates of pairs of centroids was used in comparison of means Student's t tests. The number of genes within each group represented the degrees of freedom. Intra-centroid variance was calculated using Equation A6 of the Supplemental Material.

p values for centroid separation	Centroid 1	Centroid 2	Centroid 3
Centroid 2	1.3×10^{-3}		
Centroid 3	2.8×10^{-4}	4.5×10^{-4}	
Centroid 4	4.8×10^{-3}	4.7×10^{-5}	1.7×10^{-4}

pression, and gene expression was monitored for up to 6 h post-compression (Fig. 1*E*). Individual and group expression trends were examined for the genes listed in Table II. However, transcript half-lives exceeded the 6-h time point, and no clear differences between compressed and free-swelling conditions could be determined (data not shown). Therefore, 2 h of 50% static compression did not appear to modify RNA stability over the time period examined.

DISCUSSION

In this study, we demonstrated that most genes investigated responded to static compression, with expression profiles that were both strain- and time-dependent. The effects of static compression on transcription evolved with time, and after 24 h the transcription of proteases and signaling molecules dominated over matrix molecules and early response genes. Cluster analysis revealed four group expression patterns induced by compression, with two groups requiring intracellular calcium and cAMP as common upstream mechano-regulators.

To further interpret the changes in gene expression in response to compression, we can compare our results to the kinetics of the intratissue mechanical forces and flows caused by compression. During joint loading *in vivo*, cartilage experiences a complex mixture of compressive and shear deformation having both static and dynamic components. For example, *in vivo* joint loading can result in high peak mechanical stresses (15–20 MPa) that occur over very short durations (<1 s) causing cartilage compressive strains of only 1–3% (32). In contrast, sustained (static) physiological stresses applied to knee joints for 5–30 min can result in compressive strains in certain knee cartilages as high as 40–45% (33). In the present study, application of a slow compression over ~3 min to a final strain of 25 or 50% causes an initial transient intratissue pressurization and fluid flow within the matrix immediately following compression and during a 15–30-min period of stress relaxation (8, 34). After stress relaxation has ended, fluid flow ceases, and intratissue pressure returns to zero (*i.e.* that of the medium) as the new equilibrium compressed state of the tissue is reached. Thus, the initial compression transient has certain physical attributes of slow dynamic compression, whereas the final compressed state mimics the static component of *in vivo* compression. Therefore, our objective was to explore the kinetics of changes in gene expression to both the initial transient loading and final static loading phases.

Static compression has been shown previously to decrease PG and type II collagen synthesis within 1–2 h (8, 35). In our experiments, 25% compression transiently up-regulated aggrecan gene expression and did not alter type II collagen during 8 h of loading (Fig. 2). Consistent with previous studies that used Northern analyses (19), 50% compression caused transient up-regulation of both aggrecan and type II collagen during the first 8 h and a subsequent decrease in expression below free-swelling levels by 24 h (Fig. 2). Thus, the temporal kinetics of transcriptional and biosynthetic responses to loading are considerably different, although they converge by 24 h after application of static compression. Therefore, the initial transient up-regulation of aggrecan and type II collagen genes may be more sensitive to the dynamic components of the applied compression. Recent experiments have shown that ERK1/2 and p38 phosphorylation levels peak within 10 min of static compression, but only ERK1/2P levels remained up-regulated after 24 h (26). It was suggested that such an initial transient response was due to the dynamic components of static compression, consistent with the results of Li *et al.* (36), which may similarly explain the transient transcriptional up-regulation of matrix proteins observed here. In addition, loading may affect the apparatus for transcription and translation differently. Studies (37) have shown that high pressure can cause changes in cell morphology and disorganization of the Golgi and microtubules in chondrocytes. Compression of cartilage explants also reduces cell volume and the volumes of several intracellular organelles; however, the volume of the Golgi remains unaffected by static compression of up to 50% strain (38). Thus, the synthesis of proteins that require significant post-translational modification may be affected by compression differently than transcription.

Dynamic compression, a known stimulator of matrix protein synthesis, was also found to induce MMP-2 and -9 gene expression and activity (11). In our study, matrix protease gene expression followed a common trend of increasing up-regulation with 50% compression duration (Fig. 2 and Fig. A1, Supplemental Material). COX-2 and IL-1β were up-regulated in a similar pattern to the matrix proteases, and TNFα was highly up-regulated after 24 h (Fig. A2, Supplemental Material). IL-1β is a known modulator of COX-2 gene and protein expression (39), and IL-1β, TNFα, and COX-2 are known to regulate the gene and protein expression of matrix proteases (40–45). Hence, the regulation profile of the MMPs may follow the regulation of IL-1β, TNFα, and COX-2. IL-1β did not increase above control levels until after 8 h of compression, and a similar delay of 3 h was seen during 50% loading experiments by another group (46). Furthermore, blocking IL-1β signaling during compression suppressed the expected decrease in PG synthesis but only at longer time points (6 h) (46), confirming the involvement of IL-1β signaling during prolonged periods of static compression.

The early up-regulation of transcription factors c-Fos and

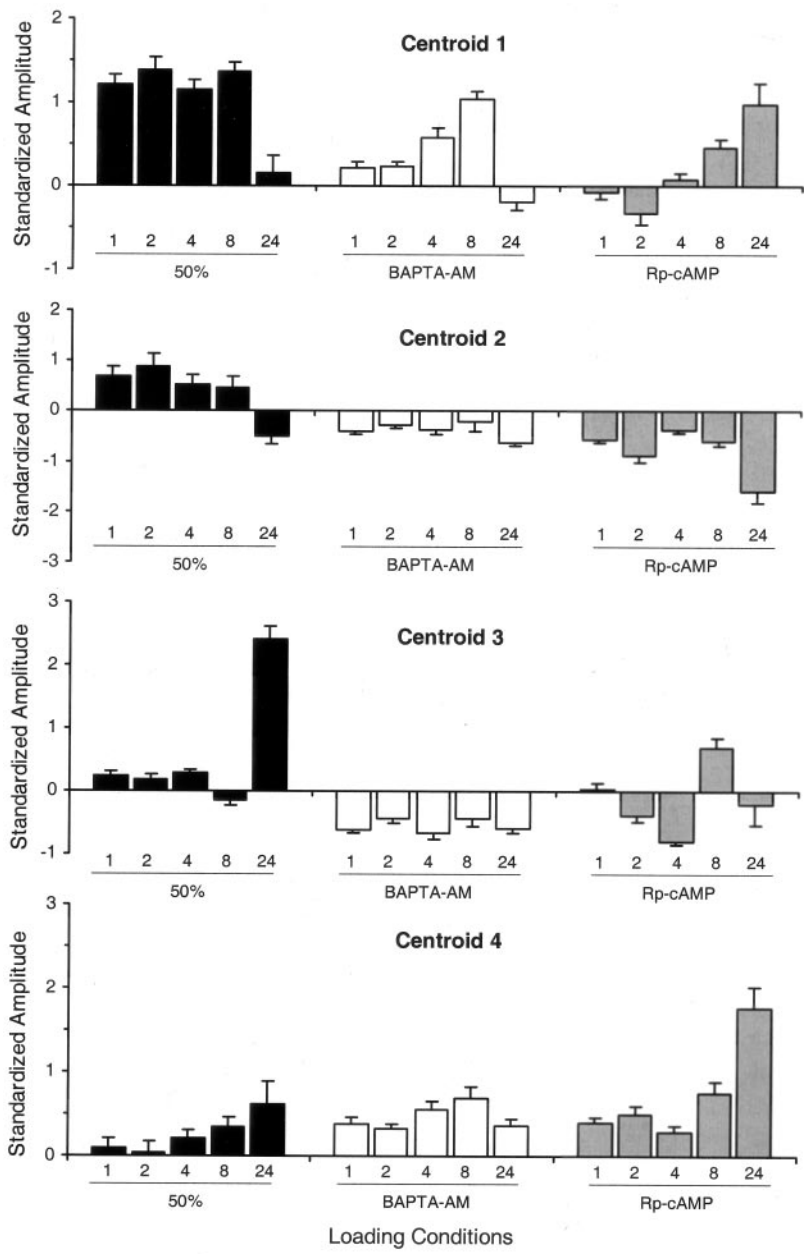

FIG. 6. **Four main expression trends induced by 1–24 h of 50% static compression with and without the presence of BAPTA-AM or (R_p)-cAMP.** Centroid vectors were calculated from the average projection coordinates of genes within each group, re-constructed using the three main principal components. Optimal groups were found using k means clustering of gene projection coordinates using Euclidean distance. Standardized free-swelling expression level = 0. Mean ± S.E. Standardized amplitudes represent relative changes from control level within an expression vector. For example, the 24-h time point in the 50% compression time course of Centroid 4 is up-regulated roughly twice as much as the corresponding 8-h time point.

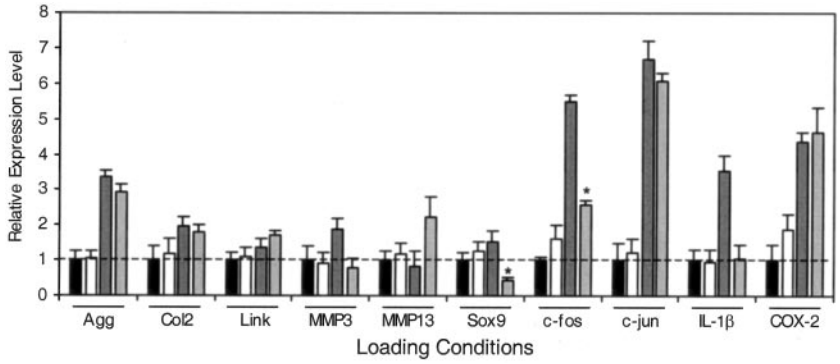

FIG. 7. **Effect of paclitaxel on gene expression changes induced by 2 h of 50% compression.** Paclitaxel was added 24 h before loading. Gene expression levels were normalized by housekeeping genes; repeated experiments were averaged and then scaled by free-swelling expression levels. ■, free-swell; □, free-swell + 10 μM paclitaxel; ▩, 2 h at 50% compression; ▢, 2 h at 50% compression + 10 μM paclitaxel. Mean ± S.E. *, $p < 0.05$ using Games-Howell corrected comparison of means, t test ($n = 5$). Abbreviations: *Agg*, aggrecan; *Col2*, type II collagen; *Link*, link protein.

c-Jun (Fig. 2), which form the AP-1-binding protein, may be another signal for compression-induced matrix remodeling or catabolism. Increased AP-1 activity is a precursor to the IL-1β induction of matrix proteases, which can take up to several hours (41, 43, 47). ERK1/2, p38, and c-Jun N-terminal kinase phosphorylation is also up-regulated as early as 10–60 min after compression (26), indicating activation of mitogen-activated protein kinase pathways possibly responsible for c-Fos and c-Jun up-regulation. Therefore, the present study provides evidence for the temporal up-regulation of transcription of matrix proteases in response to static compression.

ECM gene expression was unaffected by the presence of the

AP-1 inhibitor, paclitaxel, during 2 h of 50% compression (Fig. 7), demonstrating that AP-1 activation was not responsible for the early up-regulation of aggrecan and type II collagen (Fig. 2). Another role for the pronounced up-regulation of c-Fos and c-Jun is suggested by studies in which chondrocytes were transfected with c-Fos, causing a decrease in PG synthesis (48) similar to static compression (6, 8). COX-2, which was up-regulated in response to 50% compression (Fig. A2, Supplemental Material) and by PIS (49), is known to cause PG destruction via prostaglandin E_2 (50). Most interesting, in chondrocyte cell lines, prostaglandin E_2 regulated cAMP and intracellular calcium pathways (51). Thus, the anti-anabolic effects of static compression may be mediated in part by mechanisms dependent on short term c-Fos/c-Jun up-regulation and long term COX-2 up-regulation.

When the 50% compression data were formed into gene expression vectors and clustered, two main untreated 50% compression expression profiles were found. Groups 1 and 2 (Table II) were characterized by a transient 4–8-h up-regulation followed by a decline toward free-swell levels after 24 h (Fig. 6), whereas Groups 3 and 4 showed increased up-regulation with compression duration (Fig. 6). Each group behaved distinctly in response to BAPTA-AM or (R_p)-cAMP, further dividing the transcriptional responses induced by static compression into a total of four groups. Chelation of intracellular calcium using BAPTA-AM suppressed aggrecan gene up-regulation in response to compression (Fig. 4) similar to previous findings (25). Calcium-dependent K^+ channels have been implicated in the mechanotransduction pathway of isolated chondrocytes (24), suggesting intracellular calcium release is an initial event in mechanotransduction. We found that the presence of BAPTA-AM during mechanical loading suppressed the up-regulation of many genes, including aggrecan, type II collagen, link protein, c-Jun, and many MMPs (Fig. 4, and Figs. A1 and A2, Supplemental Material). In particular Centroids 2 and 3 were completely suppressed when BAPTA-AM was present during loading; Centroid 1 was partially suppressed, and Centroid 4 was mainly unaffected. The selective suppression by BAPTA-AM supports the idea that intracellular calcium is a common but not complete upstream signaling event controlling the mechano-regulation of anabolic, catabolic, and anti-catabolic genes. Most interesting, expression of stress protein HSP70 during compression was significantly greater when in the presence of BAPTA-AM (up to 3.8-fold) (Fig. A2, Supplemental Material). Hence, intracellular calcium release may also be required to elicit the stress-protective response seen in chondrocytes during loading (52). IL-1β was down-regulated below free-swelling controls, and c-Jun expression was significantly suppressed during BAPTA-AM-treated compression, which may explain the down-regulation and suppression of matrix proteases, even though COX-2 expression remained up-regulated (Fig. A2, Supplemental Material).

The inhibition of cAMP-activated protein kinase A during 50% compression prevented aggrecan gene up-regulation, although only during the first 4 h of loading (Fig. 4), consistent with previous findings (20). Cluster analysis revealed that the effect of (R_p)-cAMP was distinct from that of intracellular calcium chelation. The presence of (R_p)-cAMP during loading suppressed Centroids 2 and 3 similar to BAPTA-AM; however, the up-regulation of Centroid 4 was enhanced at later time points. In contrast to BAPTA-AM treatment, the early up-regulation of both c-Fos and c-Jun was suppressed during (R_p)-cAMP-treated compression (Fig. 4) which might prevent the AP-1 signaling necessary for MMP up-regulation. The dominant change in Centroid 1 in response to (R_p)-cAMP treatment during loading was a shift from a transient initial up-regulation to increasing up-regulation during longer periods. These results confirm cAMP activation as a prevalent upstream component of the intracellular mechanotransduction pathway. Both intracellular calcium and cAMP were necessary for Group 2 and 3 mechano-induction, suggesting that common downstream mechanisms may be involved. Suppression of c-Fos by only (R_p)-cAMP, and the differing regulation of Groups 1 and 4 by the two inhibitors, suggests that cAMP is not simply downstream of intracellular calcium as was previously proposed (25). The failure of either BAPTA-AM or (R_p)-cAMP to suppress the mechano-induction of Group 4 genes suggests that additional upstream signaling mechanisms exist.

The widespread impact of mechanical loading on the transcription of genes involved in ECM maintenance has been demonstrated in this study. It is possible to speculate that gene mechano-regulation may play a role in maintaining a healthy cartilage ECM throughout life and that the inferior cartilage phenotype developed during osteoarthritis may include improper gene mechano-regulation. Ongoing studies are examining gene mechano-regulation in response to the dynamic compression and shear components of *in vivo* mechanical loading. The exact order in which intracellular pathways are activated and the role of the transcribed signaling molecules cannot be directly inferred from the inhibitor studies and gene expression data presented here. Further studies are required to determine what factors are responsible for the divergent anabolic and catabolic temporal expression profiles. Four main expression patterns were identified in response to static compression and could represent genes co-regulated by intracellular calcium and/or cAMP. The dramatic up-regulation of c-Fos and COX-2 by static compression suggests a possible role in mechanically mediated cartilage remodeling and/or degradation, and it will be worthwhile to examine further these molecules in the presence of injurious mechanical compression of cartilage.

Acknowledgments—We thank Dr. Steven Wilton, Rachel Duff, and the Australian Neuromuscular Research Institute for access to the ABI7900HT real time PCR machine.

REFERENCES

1. Hascall, V. C., and Kuettner, K. E. (eds) (2002) *The Many Faces of Osteoarthritis*, Birkhauser Verlag, Basel
2. Saamamen, A. M., Kiviranta, I., Jurvelin, J., Helminen, H. J., and Tammi, M. (1994) *Connect. Tissue Res.* **30,** 191–201
3. Behrens, F., Kraft, E. L., and Oegema, T. R., Jr. (1989) *J. Orthop. Res.* **7,** 335–343
4. Slowman, S. D., and Brandt, K. D. (1986) *Arthritis Rheum.* **29,** 88–94
5. Lohmander, L. S., Hoerrner, L. A., Dahlberg, L., Roos, H., Bjornsson, S., and Lark, M. W. (1993) *J. Rheumatol.* **20,** 1362–1368
6. Guilak, F., Meyer, B. C., Ratcliffe, A., and Mow, V. C. (1994) *Osteoarthr. Cartilage* **2,** 91–101
7. Kim, Y. J., Grodzinsky, A. J., and Plaas, A. H. (1996) *Arch. Biochem. Biophys.* **328,** 331–340
8. Sah, R. L. Y., Kim, Y. J., Doong, J. Y. H., Grodzinsky, A. J., Plaas, A. H. K., and Sandy, J. D. (1989) *J. Orthop. Res.* **7,** 619–636
9. Bonassar, L. J., Grodzinsky, A. J., Frank, E. H., Davila, S. G., Bhaktav, N. R., and Trippel, S. B. (2001) *J. Orthop. Res.* **19,** 11–17
10. Jin, M., Frank, E. H., Quinn, T. M., Hunziker, E. B., and Grodzinsky, A. J. (2001) *Arch. Biochem. Biophys.* **395,** 41–48
11. Blain, E. J., Gilbert, S. J., Wardale, R. J., Capper, S. J., Mason, D. J., and Duance, V. C. (2001) *Arch. Biochem. Biophys.* **396,** 49–55
12. Thibault, M., Poole, A. R., and Buschmann, M. D. (2002) *J. Orthop. Res.* **20,** 1265–1273
13. Patwari, P., Cook, M. N., DiMicco, M. A., Blake, S. M., James, I. E., Kumar, S., Cole, A. A., Lark, M. W., and Grodzinsky, A. J. (2003) *Arthritis Rheum.* **48,** 1292–1301
14. Davisson, T., Kunig, S., Chen, A., Sah, R., and Ratcliffe, A. (2002) *J. Orthop. Res.* **20,** 842–848
15. Hunter, C. J., Imler, S. M., Malaviya, P., Nerem, R. M., and Levenston, M. E. (2002) *Biomaterials* **23,** 1249–1259
16. Mauck, R. L., Soltz, M. A., Wang, C. C., Wong, D. D., Chao, P. H., Valhmu, W. B., Hung, C. T., and Ateshian, G. A. (2000) *J. Biomech. Eng.* **122,** 252–260
17. Mizuno, S., Tateishi, T., Ushida, T., and Glowacki, J. (2002) *J. Cell. Physiol.* **193,** 319–327
18. Kisiday, J. D., Jin, M., DiMicco, M. A., Kurz, B., and Grodzinsky, A. J. (2004) *J. Biomech.* **37,** 595–604
19. Ragan, P. M., Badger, A. M., Cook, M., Chin, V. I., Gowen, M., Grodzinsky, A. J., and Lark, M. W. (1999) *J. Orthop. Res.* **17,** 836–842

20. Valhmu, W. B., Stazzone, E. J., Bachrach, N. M., Saed-Nejad, F., Fischer, S. G., Mow, V. C., and Ratcliffe, A. (1998) *Arch. Biochem. Biophys.* **353,** 29–36
21. Ikenoue, T., Trindade, M. C., Lee, M. S., Lin, E. Y., Schurman, D. J., Goodman, S. B., and Smith, R. L. (2003) *J. Orthop. Res.* **21,** 110–116
22. Millward-Sadler, S. J., Wright, M. O., Davies, L. W., Nuki, G., and Salter, D. M. (2000) *Arthritis Rheum.* **43,** 2091–2099
23. Sironen, R. K., Karjalainen, H. M., Torronen, K., Elo, M. A., Kaarniranta, K., Takigawa, M., Helminen, H. J., and Lammi, M. J. (2002) *Biorheology* **39,** 111–117
24. Millward-Sadler, S. J., Wright, M. O., Lee, H., Nishida, K., Caldwell, H., Nuki, G., and Salter, D. M. (1999) *J. Cell Biol.* **145,** 183–189
25. Valhmu, W. B., and Raia, F. J. (2002) *Biochem. J.* **361,** 689–696
26. Fanning, P. J., Emkey, G., Smith, R. J., Grodzinsky, A. J., Szasz, N., and Trippel, S. B. (2003) *J. Biol. Chem.* **278,** 50940–50948
27. Dougherty, E. R., Barrera, J., Brun, M., Kim, S., Cesar, R. M., Chen, Y. D., Bittner, M., and Trent, J. M. (2002) *J. Comput. Biol.* **9,** 105–126
28. Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14863–14868
29. Alter, O., Brown, P. O., and Botstein, D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10101–10106
30. Holter, N. S., Mitra, M., Maritan, A., Cieplak, M., Banavar, J. R., and Fedoroff, N. V. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8409–8414
31. Hui, A., Min, W. X., Tang, J., and Cruz, T. F. (1998) *Arthritis Rheum.* **41,** 869–876
32. Hodge, W. A., Fijan, R. S., Carlson, K. L., Burgess, R. G., Harris, W. H., and Mann, R. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2879–2883
33. Herberhold, C., Faber, S., Stammberger, T., Steinlechner, M., Putz, R., Englmeier, K. H., Reiser, M., and Eckstein, F. (1999) *J. Biomech.* **32,** 1287–1295
34. Kim, Y. J., Sah, R. L., Grodzinsky, A. J., Plaas, A. H., and Sandy, J. D. (1994) *Arch. Biochem. Biophys.* **311,** 1–12
35. Bonassar, L. J., Grodzinsky, A. J., Srinivasan, A., Davila, S. G., and Trippel, S. B. (2000) *Arch. Biochem. Biophys.* **379,** 57–63
36. Li, K. W., Wang, A. S., and Sah, R. L. (2003) *Arthritis Rheum.* **48,** 689–699
37. Parkkinen, J. J., Lammi, M. J., Pelttari, A., Helminen, H. J., Tammi, M., and Virtanen, I. (1993) *Ann. Rheum. Dis.* **52,** 192–198
38. Grodzinsky, A. J., Levenston, M. E., Jin, M., and Frank, E. H. (2000) *Annu. Rev. Biomed. Eng.* **2,** 691–713
39. Geng, Y., Blanco, F. J., Cornelisson, M., and Lotz, M. (1995) *J. Immunol.* **155,** 796–801
40. Tsuzaki, M., Guyton, G., Garrett, W., Archambault, J. M., Herzog, W., Almekinders, L., Bynum, D., Yang, X., and Banes, A. J. (2003) *J. Orthop. Res.* **21,** 256–264
41. Liacini, A., Sylvester, J., Li, W. Q., and Zafarullah, M. (2002) *Matrix Biol.* **21,** 251–262
42. Vincenti, M. P., Coon, C. I., Lee, O., and Brinckerhoff, C. E. (1994) *Nucleic Acids Res.* **22,** 4818–4827
43. Vincenti, M. P., and Brinckerhoff, C. E. (2002) *Arthritis Res.* **4,** 157–164
44. Hui, W., Rowan, A. D., and Cawston, T. (2001) *Cytokine* **16,** 31–35
45. Liacini, A., Sylvester, J., Li, W. Q., Huang, W., Dehnade, F., Ahmad, M., and Zafarullah, M. (2003) *Exp. Cell Res.* **288,** 208–217
46. Murata, M., Bonassar, L. J., Wright, M., Mankin, H. J., and Towle, C. A. (2003) *Arch. Biochem. Biophys.* **413,** 229–235
47. Vincenti, M. P., and Brinckerhoff, C. E. (2001) *Arthritis Res.* **3,** 381–388
48. Tsuji, M., Funahashi, S., Takigawa, M., Seiki, M., Fujii, K., and Yoshida, T. (1996) *FEBS Lett.* **381,** 222–226
49. Fermor, B., Weinberg, J. B., Pisetsky, D. S., Misukonis, M. A., Fink, C., and Guilak, F. (2002) *Osteoarthr. Cartilage* **10,** 792–798
50. Hardy, M. M., Seibert, K., Manning, P. T., Currie, M. G., Woerner, B. M., Edwards, D., Koki, A., and Tripp, C. S. (2002) *Arthritis Rheum.* **46,** 1789–1803
51. Lowe, G. N., Fu, Y. H., McDougall, S., Polendo, R., Williams, A., Benya, P. D., and Hahn, T. J. (1996) *Endocrinology* **137,** 2208–2216
52. Kaarniranta, K., Holmberg, C. I., Lammi, M. J., Eriksson, J. E., Sistonen, L., and Helminen, H. J. (2001) *Osteoarthr. Cartilage* **9,** 7–13
53. Armitage, P., Berry, G., and Matthews, J. N. S. (2002) *Statistical Methods in Medical Research*, 4th Ed., pp. 455–463, Blackwell Science Ltd., Oxford
54. Yeung, K. Y., and Ruzzo, W. L. (2001) *Bioinformatics* **17,** 763–774