A Remote Upstream Element Regulates Tissue-specific Expression of the Rat Aggrecan Gene*

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The regulation of chondrogenesis and of the genes expressed as markers of chondrocyte differentiation is poorly understood. The hyaluronan-binding proteoglycan aggrecan is an essential and specific component of cartilage, but the aggrecan proximal promoter is expressed in an unregulated fashion in vitro. DNA comprising the rat aggrecan gene (83 kb including the 30-kb first intron) was surveyed for active elements, which would impart selective expression to the aggrecan promoter in transfection assays in vitro. A 4.7-kb DNA fragment (P3) with cell-specific enhancer activity was discovered ~12 kb upstream of the transcription start site; this active DNA fragment is position- and orientation-independent, and strongly stimulates aggrecan promoter expression in chondrocytes, while weakly suppressing transcription in fibroblasts. Most of this activity has been localized to P3-7, a 2.3-kb internal fragment of P3. Another enhancer element (A23), which is not tissue-specific, was discovered about 70 kb downstream of the transcription start site. Several lines of transgenic mice were created using combinations of these DNA elements to drive the lacZ reporter gene. Neither a short (900 bp) nor a long (3.7 kb) promoter alone showed detectable expression in 14.5-day embryos, whereas placing the P3 tissue-specific enhancer together with P0 gave strong expression restricted to embryonic cartilage of transgenic mice. The A23 downstream enhancer in conjunction with P0 did not confer expression. This is the first report of a gene control region which confers authentic tissue-specific regulation of aggrecan in vitro or in vivo and should greatly facilitate understanding the coordinate regulation of chondrocytic genes.

Skeletogenesis proceeds through a complex process involving patterning signals, cell differentiation, and growth (1). Most of the skeletal elements including those of the limbs, as well as many parts of the craniofacial and axial skeleton, develop through the specialized process of endochondral ossification, in which a cartilaginous rudiment is first formed, then converted to bone. Central to this process is the formation of cartilage, which occurs in defined locations where mesenchymal cells condense and differentiate into chondrocytes. These cartilage rudiments then proceed in a carefully orchestrated sequence of growth, maturation, and ossification to produce the bony skeleton.

This developmental system has been well studied, and a large number of its controlling genes and circuits have been identified (2, 3). Attention has been focused particularly on the patterning and growth phases of the process. Important patterning genes include the hox genes (4, 5), sonic hedgehog (6, 7), retinoid receptors (8), unt genes (9, 10), fgf and fgf-r genes (11, 12), and bmps and their receptors (13). The growth and final differentiation of cartilage into bone is controlled in part by interactions between the Indian hedgehog and parathyroid hormone-related peptide signaling pathways in the growth plate (14), and the induction of the bone- and cartilage-specific transcription factor Cbfal (15–18). Despite these advances, the central event of endochondral bone formation, overt chondrogenesis from mesenchymal precursor cells, remains poorly understood. Currently the best candidate for a general chondrogenic transcription factor is Sox9 (19), which is implicated in the control of several cartilage-specific genes (20–24).

One of the major cartilage-specific genes encodes the proteoglycan aggrecan (25), an essential gene for normal cartilage development and function (26). The aggrecan gene product is a major component of the extracellular matrix of cartilage and is largely responsible for the unique properties of this tissue (27). Expression of aggrecan is detected as an early event in chondrocyte differentiation, and its high level expression is restricted to cartilage (28, 29). The aggrecan gene has been characterized in several species including rat (30), human (31, 32), mouse (33), and chicken (34), but investigation of transcriptional regulatory mechanisms for this gene remains preliminary. The basal promoter was defined by mapping the transcriptional start site and shown to be active in various cell types (30). Subsequently, promoter activity has been shown to be influenced by first exon sequences (35), biomechanical stimuli (36), sox9 (37), and a region that inhibits expression in chondrocytes (38, 39). Regulatory elements directing expression specifically to cartilage have not been described, however. We have examined remote regions of the aggrecan gene for elements that may confer appropriate tissue-specific expression on the basal promoter in cell-transfection assays of reporter constructs. We report here the identification of two elements that modify the activity of the basal promoter and upstream promoter sequences in vitro, and preliminary evidence that one of the elements confers cartilage-directed expression of the aggrecan gene in vivo.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids—The family of pCAT plasmids (Promega) was used as the basis of most constructs. The B18 promoter construct

* This work was supported by a grant from Shriners of North America. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF468019.

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Received for publication, October 4, 2001, and in revised form, January 25, 2002
Published, JBC Papers in Press, February 7, 2002, DOI 10.1074/jbc.M109627200

This paper is available online at http://www.jbc.org
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Restrictions of subcloned genomic DNA were rendered blunt-ended by Klenow or T4 DNA polymerase treatment, ligated to BamHI linkers, and cloned into the unique BamHI site in the pCAT-basic vector portion of B18. The P3 5-kb EcoRI fragment from approximately –12 kb to 7 kb contained an intact internal XhoI site to allow generation of unidirectional EcoHI deletions of ~500 bp. EcoHI-digested DNA was blunt-ended with mung bean nuclelease, ligated to HindIII linkers, and cloned into the HindIII site upstream of P0 in pCAT-basic. A23 is a BamHI/EcoRI fragment located at ~68 kb; it was end-filled with Klenow, ligated to BamHI linkers, and cloned into the BamHI site of pCAT-B18, pCAT-P0, and pCAT-SV promoter. The subfragments of the A23 fragment were generated by restriction digestion with SphI and EcoRV, filled-in with Klenow, ligated to BamHI linkers, and cloned into the BamHI site of B18 or P0. pGL3 constructs (for dual-luciferase assays) and pGAL constructs (for transgenic mice) were produced by first assembling the DNA insert fragments in pBSK (Stratagene). B18 or P0 HindIII promoter fragments were placed into the Clal site of pBSK by blunt-end ligation following Klenow fill-in of the insert and vector. The 5-kb P3 EcoRI fragment was cloned into the EcoRI site, and the 2.8-kb A23 BamHI fragment was cloned into the BamHI site of the vector. The assembled fragments could be excised intact from the vector with NotI and XhoI. For cloning into pGL3, the first fragment was modified by conversion of the Mini I site to a NotI site by end-filling using Klenow, followed by NotI linker ligation. Then the NotI-XhoI fragment from pBSK containing combinations of B18 or P0 and P3 or A23 were ligated into the NotI-XhoI sites in pGL3. For cloning into pGAL (CLONTECH), the first fragment was modified by conversion of the Smal site to NotI by linker ligation, and conversion of the Sall site to SfiI by end-filling with Klenow and linker ligation. The NotI- XhoI fragments from pBSK were then ligated into the NotI-XhoI site in the modified pGAL. Plasmid DNA was prepared using Qiagen kits and quantitated by densitometry relative to markers on agarose electrophoretic gels.

Transfections and Assays of Transcriptional Activity—15-day white leghorn chicken embryos (Spasfas) were sacrificed, and sterna and skin were dissected out as a source for chondrocytes and fibroblasts. Tissues were placed into sterile 0.2% collagenase (Worthington, class 2) in F-12 medium (Invitrogen) and incubated ~3 h with stirring at 37 °C, 5% CO2 until single-cell suspension was obtained. Cells were washed, sedimented, and resuspended in F-12 medium containing 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Sigma). 4 × 103 cells were plated in 60-mm Primaria cell culture dishes (Falcon) and incubated at 37 °C in a humidified, 5% CO2 chamber. Cells were grown 24 h, then transfected with 2 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid, 0.1 μg of SV40-luciferase reporter plasmid (pGL3, Promega), 16 μl of Superfect (Qiagen), 1 ml of F-12 containing 10% serum. DNA complexes were removed after 12 h by rinsing with PBS1 and cells were incubated an additional 36 h in F-12, 10% serum, 1% penicillin-streptomycin. Cells were harvested by scraping after rinsing with 0.15 M sodium chloride, 0.012 M sodium/potassium phosphate, pH 7.4, lysed by sonication at 4 °C in 200 μl of 0.25 M Tris-HCl, pH 8, centrifuged 5 min, and a 10-μl aliquot of the supernatant was assayed for luciferase activity using a luminoimeter (Turner) and a reagent kit, as described by the manufacturer (Promega). The remaining extract was heated at 65 °C, 15 min, and varying amounts assayed for CAT activity using thin-layer chromatography on silica gel plates (Alttech) and the n-butyryl CoA reagent kit (Promega), essentially as described by the manufacturer, with the exception that more than 0.8 μl was assayed for no more than 30% conversion of [14C]chloramphenicol (PerkinElmer Life Sciences) to the butyrylated form would be achieved in the standard assay. Chromatograms were detected and quantitated using a Storm 840 PhosphorImager (Molecular Dynamics) and ImageQuant software. Data were expressed as percentage of modified substrate per total extract volume, normalized to the luciferase activity. Each data point was performed in duplicate transfections per experiment, and a minimum of three experiments (each in duplicate) was averaged for each data set. In all cases, standard errors of the mean were calculated. Data were also subjected to statistical analysis using either a two-tailed t test or Dunnett’s test for multiple comparisons to a single control (40), where appropriate. Reported data all reached significance levels of at least p < 0.05, where a difference from the control was concluded.

Alternatively, dual luciferase assays were performed following transfection with the experimental pGL3 constructs (2 μg) and an internal control of pRL-SV40-Renilla (0.025 μg). Transfections were performed using FuGENE (Roche Molecular Biochemicals) according to the manufacturer’s protocol, with the following specifications. A 2:1 v/w ratio of FuGENE to DNA was used, transfection was performed overnight in the absence of antibiotic, but in the presence of serum, and the DNA-FuGENE complexes were removed the following morning, after which the rinsed cells were grown an additional 24 h in medium with serum and antibiotic. Cells were harvested by rinsing, scraping, and sedimenting in PBS, lysed in 200 μl of passive lysis buffer (Promega), and assayed for firefly and Renilla luciferase activity on a Turner luminometer using the dual luciferase kit (Promega).

Luciferase Assays and Analysis of Transgenic Mice—Plasmid DNA for pGAL constructs was purified using Qiagen columns, cut with NotI/SalI to remove vector DNA, and the target DNA fragments were gel-purified using Strataprep columns (Stratagene). Microinjection of embryos and transgenic mouse production was performed by the NICH Transgenic Mouse Development Facility, University of Alabama at Birmingham (Dr. Carl Pinkert, Director). Founder mice were produced as B6×C3F1 hybrids, and subsequent breedings were maintained in this strain. Transgenic mice were identified by a PCR assay using primers within the lacZ gene. The primer sequences were ggtcactcggctgttgg and cagcttaacccagcgtag; conditions for the reaction included 100 ng mouse tail genomic DNA, 2 mM MgCl2, 0.2 μM primers, 0.2 μM dNTPs, 29 cycles with annealing temperature of 56 °C. Genomic DNA was prepared from the DNeasy Tissue kit (Qiagen). Embryos were sacrificed at 14.5 dpc, and processed for whole-mount histochemical detection of lacZ as described (43). Briefly, embryos were rinsed in cold PBS and fixed for 20 min at 4 °C in 4% paraformaldehyde/PBS, with gentle agitation. Embryos were then rinsed again with PBS and placed in X-gal stain (1 mg/ml X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl2, in PBS) overnight at 30 °C. Following staining, embryos were rinsed in PBS and stored in 30% sucrose at 4 °C. In some cases, stained embryos treated as above were embedded in Cryomatrix medium (Shandon), and 10-μm sections were produced on a cryotome. Sections were stained for 5 min with 1% alcian blue, 0.1 M HCl to highlight cartilage matrix; the X-gal staining could be distinguished as dark blue intracellular stain superimposed on the lighter alcin blue. Alternatively, sections were stained briefly in 2% Alcian Red S, pH 4.2, to identify regions of calcification.

RESULTS

The rat aggreccan gene has been previously characterized, and the start of transcription determined (30). The first exon is large (384 bp), is entirely untranslated sequence, and is separated from the second exon where translation begins by a very large first intron (30 kb). A fragment of the aggreccan gene consisting of the region –640 to +280 has been previously shown to provide basal promoter activity in cell transcription assays (30). This DNA, designated B18, in a CAT reporter vector, had the capacity to respond to the SV40 enhancer and was completely inactive in the reverse orientation, but did not direct preferential expression to chondrocytes as compared with fibroblasts in culture (Figs. 2 and 3). An extended promoter construct, −3400 to +280, designated P0, showed similar...
lar properties of unregulated expression in different cell types (Fig. 2), indicating that tissue-specific regulatory elements must reside at remote locations in the aggrecan gene. A search for these tissue-specific regulatory elements within the 83-kb rat aggrecan gene was conducted by cloning DNA fragments of the gene into the B18 basal promoter reporter construct and using cell transfection assays to test for specific expression in chondrocytes and fibroblasts. A summary of the DNA fragments tested in this way is shown (Fig. 1). These assays were performed using primary cultures of chick embryo chondrocytes and fibroblasts, and CAT as a reporter gene. Most of these DNA fragments showed no reproducible effects on transcription (data not shown), with two exceptions: an ~5-kb fragment called P3, from the 5' end of the cloned gene, 12 kb upstream of the transcription start site; and a 2.8-kb fragment from the 3' end of the cloned gene called A23, roughly 70 kb downstream of the transcription start site. The 5-kb P3 fragment modulated the activity of the B18 proximal promoter in a tissue-specific manner, down-regulating transcription in fibroblasts, while enhancing transcription in chondrocytes (Fig. 2). The A23 fragment, in contrast, stimulated transcription from the B18 promoter with similar activity in both fibroblasts and chondrocytes (Fig. 3). In these experiments, the A23 fragment was active in both the forward and reverse orientation relative to the promoter, at least in chondrocytes. Fig. 2 also demonstrates that the P0 long promoter was significantly less active than the B18 short promoter in fibroblasts.

Localization of the Active Region of A23—To localize the active region of this 2.8-kb piece of DNA, the A23 fragment was separated into three contiguous restriction fragments of 0.9, 1.2, and 0.7 kb (Fig. 1), and each was tested for activity with the B18 promoter. Only the 0.7-kb fragment showed a significant difference in expression level from the promoter alone, showing most, if not all, of the enhancer activity of the full-length A23 fragment in both cell types (Fig. 3).

Promoter Specificity of the A23 Enhancer—To test whether the A23 sequence is a general enhancer, or specific for the aggrecan promoter, the 2.8-kb fragment was cloned into a reporter construct in which the CAT gene is driven by the SV40 promoter, and which is dependent upon an enhancer for expression. Both forward and reverse orientations of A23 were tested. The A23 element exerted a modest 3–4-fold stimulation of the SV40 promoter in primary chick embryo cells (data not shown), similar in magnitude to its effect on the aggrecan promoter (Fig. 3). The SV40 enhancer, in comparison, strongly
enhanced both the SV40 promoter and the B18 aggrecan promoter in chick embryo fibroblasts and chondrocytes (30).

Activity in Cell Lines and with Other Assay Systems—To extend the observations seen with the chick cells, a number of rodent cell lines were also analyzed. The LTC cell lines were derived from the Swarm rat chondrosarcoma by adaptation to culture and have been used to analyze type II collagen transcription (41). The IRC line was created from rat embryo costal chondrocytes by infection with \textit{myc} and \textit{raf} oncogenes (42). A rat embryo fibroblast line and NIH/3T3 cells were used as negative controls. Optimization of transfection of these cell lines showed highest efficiency with the reagent FuGENE 6 (Roche Molecular Biochemicals), so this method was adopted. Additionally, several constructs had meanwhile been produced using the pGL3 luciferase reporter vector, thus allowing analysis of transfection results by the dual-luciferase assay for some cases. The combination of these changes showed P3 and A23 effects of greater magnitude than seen with the chick cells/CAT assay methods, though largely consistent with the previous results (Figs. 4–6). The B18 and P0 promoters alone were relatively inactive in the chondrocyte cells, but were greatly stimulated by the P3 element, by ~40-fold in LTC cells (Fig. 4); in contrast, in fibroblasts the solitary B18 promoter was quite active, but was strongly inhibited by P3. P0 was considerably less active than B18 in fibroblasts, but was further inhibited by P3, in the REF cells at least. P3 stimulated both B18 and P0 by similar factors, although this level is higher in LTC cells than IRC.

The behavior of the A23 enhancer was similarly examined in these cell lines, as shown in Fig. 5. This enhancer was strongly active in both chondrocytic and non-chondrocytic cell lines, regardless of whether the short B18 or long P0 promoter was used. The degree of enhancement was highest in chondrocytes using the P0 promoter (30-fold) and lowest in fibroblasts using the B18 promoter (7–15-fold).

Localization of Tissue-specific Enhancer Activity within P3—The 4.6-kb P3 fragment was subdivided by digestion with restriction enzymes into three contiguous fragments, of 1.3, 2.2, and 1.1 kb, designated P3–6, P3–7, and P3–4. These fragments were subcloned into the \textit{HindIII} site upstream of the promoter.
in the P0 extended promoter construct. This configuration allows investigation of possible interaction of P3 subfragments with upstream promoter sequences. These constructs were tested in transfection assays in NIH/3T3 and LTC cells (Fig. 6). In this set of experiments, there was a significant decrease of expression from P0 in NIH/3T3 fibroblasts when full-length P3 was included, but also for P3–7 and, to a lesser extent, P3–4. The LTC chondrocytes showed a strong enhancement by P3 of P0, and only P3–7 showed a similar activity in these cells; there was no enhancement of P0 by P3–6 or P3–4. The magnitude of the enhancement by P3–7 appeared less than that of full-length P3, although this was not evaluated statistically. There may thus be some ancillary sequence that modulates the effect of P3–7 outside of the arbitrarily chosen boundaries of this fragment. Similar results were obtained using the primary chick cells and the CAT reporter vectors, and in those experiments the activity of P3–7 was seen in both forward and reverse orientations (data not shown).

Analysis of Promoter Activity in Transgenic Mice—To test whether these active enhancer elements from the aggrecan gene would have regulatory function in vivo, constructs were prepared in which the B18 and P0 promoters were used to drive expression of the lacZ reporter gene, in the presence or absence of either the P3 or A23 enhancers (Fig. 7). These four constructs were used to produce transgenic mouse lines: B18-lacZ (three founders), P0-lacZ (five founders), P0-P3-lacZ (seven founders), and P0-A23-lacZ (five founders). These founders have been bred with wild type females of the same hybrid B6SJL background. Embryos at 14.5 dpc from these breedings were evaluated for lacZ staining by whole-mount X-gal staining. No lacZ expression was observed for any of the transgenic offspring of
the B18, P0, or P0-A23 lines, whereas four of the seven founders for P0-P3 produced litters in which the transgenic embryos showed identical patterns of specific staining in cartilage. Representative 14.5 dpc embryos for the B18, P0-P3, and P0-A23 lines are shown stained with X-gal as Fig. 8. At this stage the P0-P3 transgenic embryo showed staining for lacZ expression in developing cartilages of the long bones of the fore and hind limbs, the ribs, the scapula, and some craniofacial cartilages. No staining was seen in the vertebrae or the digits at this stage. Neither the B18 short promoter (Fig. 8C) nor the P0 long promoter with (Fig. 8D) or without (data not shown) the A23 enhancer directed any specific staining for lacZ. The same staining pattern was seen for all transgenic littermates for four of seven P0-P3 founder lines, whereas the non-transgenic littermates showed no staining (Fig. 8B). Histological sections of the X-gal-stained P0-P3 embryos showed that the lacZ expression occurred exclusively in cartilage, which also stained with alciain blue (Fig. 8E), but was excluded from areas staining with Alizarin Red (Fig. 8F), which denoted areas undergoing calcification.

**DISCUSSION**

The aggrecan proximal promoter as first reported gave slightly preferential expression in chondrocytes over fibroblasts (30); subsequent experiments did not support this initial observation and indicated that there must be additional regulatory elements elsewhere in the gene required to confer appropriate expression on the aggrecan promoter. The search for these regulatory elements focused on DNA fragments up to 12 kb upstream of the transcription start site and throughout the >30-kb first intron, but also included flanking sequences at the 3’ end of the gene. These DNA fragments, summarized in Fig. 1, were tested in transfection experiments in reporter constructs with the proximal B18 promoter, but also with larger promoter fragments, and in various combinations with each other. The B18 promoter includes a large portion of the first exon.

The upstream P3 element in the context of the short promoter B18 exhibited both positive regulation (in chondrocytes) and negative regulation (in fibroblasts) (Figs. 2 and 4). The inhibitory activity of P3 in fibroblasts was more pronounced with the short promoter B18 than with P0, because the upstream promoter sequences in P0 itself inhibited expression in fibroblasts.
fibroblasts relative to B18 (Figs. 2, 4, and 5). Nevertheless, there was an additional inhibitory activity of P3 exerted upon P0 in fibroblasts, seen in Fig. 4 (REF cells) and Fig. 6 (NIH/3T3 cells). The stimulatory effect in chondrocytes of P3 is roughly the same on B18 or P0, ~40-fold in LTC cells, and 20-fold in IRC cells (Fig. 4). When P3 was subdivided into fragments 6, 7, 4, only P3–7 exhibited both the inhibitory properties in fibroblasts and the stimulatory activity in chondrocytes of the parent molecule (Fig. 6). Both activities were damped relative to full-length P3, however, suggesting that portions of P3–6 and/or P3–4 may also contribute to full activity. In fact, P3–4 did show some inhibitory activity in fibroblasts, but no enhancement in chondrocytes (Fig. 6). Consistent with the importance of P3–7 is the fact that both P3–6 and P3–4 contain considerable amounts of repetitive sequence, whereas P3–7 has a high degree of informational content (P3 sequence has been submitted to GenBank\(^{28}\), under accession no. AF468019).

The remote downstream A23 element is also an enhancer, displaying position and orientation independence. A23 has been shown to be active on the heterologous SV40 promoter (data not shown). A 0.7-kb fragment of A23 appeared to contain most of the enhancing activity (Fig. 3). The full-length 2.8-kb fragment enhanced expression of the B18 promoter by ~3–4-fold in both primary chick embryo fibroblasts and chondrocytes (Fig. 3), and thus was lacking in tissue-specific regulatory activity with this promoter. This lack of tissue specificity of A23 was also seen in rodent cells (Fig. 5), although in this case the degree of enhancement appeared somewhat higher in chondrocytes (30-fold) than in fibroblasts (7–15-fold).

Despite these indications that both P3 and A23 separately have the ability to modulate expression of the P0 promoter, when the active subregions of these two DNAs (P3/7 and A23/0.7) were placed together in a P0 reporter construct, nonspecific enhancement of expression was observed (data not shown). The nonspecific enhancing activity of A23 appears to have overruled any tissue-specific regulation by P3, at least in the in vitro system. These results suggest that either these two enhancer elements do not function together in vivo to confer tissue-specific expression of aggrecan or that there are still other elements required in combination with those here described to achieve full regulated expression of this gene.

These studies have been performed in both primary chick cells and rodent cell lines. In the initial work, the survey of the rat aggrecan gene for regulatory elements was performed using the chick system, and some experiments, e.g. the testing of the A23 fragments, have only been performed in the chick cells. It is important to test primary cells because they may be closer to the in vivo condition than cells adapted for long term culture. Cell lines are more convenient, however, and, in addition, it was important to test the rat promoter in rodent cells, even though primary chick embryo cells had been used successfully to characterize the rat type II collagen promoter/enhancer (44). Pure rodent primary chondrocytes are difficult to obtain, so two rat chondrocyte cell lines were tested for this purpose: LTC (41) and IRC (42). Both lines actively express aggrecan (45), although the IRC cells require growth in suspension culture. Negative cell types (not expressing aggrecan) were mouse and rat fibroblast cell lines (NIH/3T3 and REF).

Results using the luciferase vectors and the rodent cell lines confirmed and extended the findings from the chick cell/CAT assay experiments. P3 silenced B18 and to a lesser extent P0 in fibroblasts, while strongly enhancing both B18 and P0 in chon-
drocytes (Figs. 4 and 6). A23 strongly enhanced both B18 and P0 in fibroblasts and chondrocytes (Fig. 5). In general, the magnitude of the P3 and A23 effects were greater in the experiments using the rodent cell lines as compared with those using the primary chick cells. In addition, in the rodent cell lines, the level of expression of the promoter constructs was much higher in fibroblasts than in chondrocytes; this difference was much greater than was seen in the primary chicken cells. There are methodological differences between these sets of experiments (methods of transfection and reporter gene analysis), as well as the difference in cells (chick versus rodent, primary versus immortalized), and any combination of these variables may have influenced the magnitude of the results. However, the chicken aggrecan promoter shows no similarity to those of the rat, human, and mouse (38), so it is possible that a species difference between the test gene and the host cells diffused the results. The overall conclusion from both approaches, however, is that P3 exhibits chondrocyte-specific enhancement of either the short or long promoter, whereas A23 clearly enhances both promoters, but not in a cell-specific manner.

Cell culture transfection experiments are prone to experimental difficulties, especially when attempting to compare activities in different cell types; these approaches can also never replicate complex developmental and tissue-specific signals found in vivo. A more definitive test of promoter specificity is to express a reporter gene in vivo using transgenic methods. As practical considerations limited the number of constructs that could be tested in this way, the four shown in Fig. 7 were prepared, based on the in vitro transfection data: B18, P0, P0-P3, and P0-A23, all driving the lacZ reporter gene. Five founders of each construct were obtained, except for P0-P3, which yielded seven founders. Two of the B18 founders were either lost or failed to breed, leaving only three for that construct. Preliminary evaluation of litters from each of these founders has been conducted, using whole-mount staining with X-gal at embryonic age 14.5 dpc to roughly evaluate transgene expression. A more in-depth developmental analysis of these lines is currently under way to evaluate the detailed anatomical and embryological features of this expression in relation to the endogenous expression of aggrecan and other cartilage-expressed genes; this examination will be reported as another study. The absence of lacZ staining in embryos transgenic for B18, P0, and P0-A23 constructs confirms the lack of specific expression from the two promoters seen in vitro and rules out a primary role for A23 in providing tissue-specific expression of aggrecan. Four of seven P0-P3 transgenic lines showed identical expression of the transgene in developing cartilages of long bones, ribs, and scapulae, as well as in nasal cartilage (Fig. 8); two other lines did not stain, and the last line never bred. The observation of this expression pattern in four of seven lines rules out the possibility of insertionional position artifacts.

These results clearly confirm a major role for P3 in the tissue-specific regulation of aggrecan, because only cartilage expression is observed. There are some indications, however, that P3 may not be sufficient for all aspects of aggrecan regulation. It is surprising that no expression was seen in vertebral bodies at this stage of development, and expression in distal cartilage of the digits would also be expected, based on a comparison to the expression pattern driven by the type II collagen p300/63020 promoter/enhancer in transgenic mice at this stage (46). There are a number of possible explanations for these surprising observations. It has been suggested that there would be differences in developmental expression between type II collagen and aggrecan (29). Even where these genes are coexpressed, there are differences of at least 5-fold in the steady-state levels of their mRNAs (45), adding the complica-

tion of differences in detection thresholds. RNA levels may not directly reflect transcriptional activity; thus, a comparison of transcriptional activities of these two genes during development would be informative. The staining with alizarin red does not overlap with the expression of the lacZ reporter (Fig. 5F), but there may be increased expression of aggrecan in regions of cartilage near or associated with areas of mineralization. Further studies will determine the extent to which P3 confers the full range of expression of aggrecan in vivo, or whether its activity represents a specialized subprogram for this gene’s expression. In the latter case, there will be the need to identify additional areas of the gene, which can supplement the function of P3. It is possible that A23 will yet be shown to play a role in vivo; alternatively, A23 may be found to be part of a neighboring gene. P3 has been confirmed to be an important part of the aggrecan regulatory apparatus, however, and is the first such controlling region yet described that regulates tissue and developmental expression of this important gene.

Despite a great deal of interest, there still is relatively little known about transcriptional regulation of genes expressed as specific products of differentiated chondrocytes. The type II (20, 21, 46, 47) and type XI (22, 24) collagen genes appear to be regulated by similar elements, which bind to members of the Sox9 family of transcription factors. The gene known as CD-RAP is also regulated by Sox9 (23). Targeted deletion of Sox9 has been shown to disrupt cartilage formation (19). Aggrecan gene expression has been reported to respond to Sox9 in cultured cells transfected with a mouse aggrecan promoter not containing the P3 element reported here (37). The 5-kb P3 element shows no overt similarity to the type II collagen enhancer sequence (48), but does contain consensus HMG-boxes within the P3–7 subfragment (GenBank® AF468019). It will be important to test such elements functionally, and otherwise identify sequences in P3 responsible for the tissue-specific enhancing and repressing activities, so that the mechanism of aggrecan regulation can be compared with that of other cartilage-specific genes, and contribute to the search for general regulators of chondrogenesis.

Acknowledgments—We thank Dr. James Kimura for providing the LTC rat chondrosarcoma cells and Jill Woods and Thad Mick for additional technical support.

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J. Biol. Chem. 2002, 277:13989-13997.
doi: 10.1074/jbc.M109627200 originally published online February 7, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M109627200

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